Mechanistic Studies of the Phenobarbital-Type Induction of Cytochromes P450: Role of AMP-Activated Protein Kinase

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Prof. Dr. Hans-Peter Hauri

A mio nonno e a Markus

"The important thing in science is not so much to obtain new facts,
as to discover new ways of thinking about them."
Sir William Bragg (1862 – 1942)

"When I'm working on a problem, I never think about beauty.
I think only how to solve the problem.
But when I have finished, if the solution is not beautiful, I know it is wrong."
R. Buckminster Fuller (1895 - 1983)

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Abstract

Inside the liver cells there are sophisticated mechanisms that have evolved over millions of years to metabolize toxic substances, many of which are fat-soluble compounds making them difficult for the body to excrete. Cytochromes P450 (CYPs) are drug-metabolizing enzymes predominantly expressed in the liver that catalyze the first step in the metabolism of lipophilic compounds into water-soluble and thus excretable compounds. Transcriptional activation of CYPs and other drug-metabolizing enzymes by drugs and xenobiotics in the liver is mediated by the mammalian constitutive androstane receptor (CAR) and pregnanes X receptor (PXR), whereas the chicken xenobiotic receptor (CXR) mediates drug responses in chicken hepatocytes. This phenomenon, which is called drug induction, is fully reversible and dose-dependent. By increasing the capability for metabolic detoxification and elimination, induction of CYPs is an integral part of the defense mechanism against xenochemical insult. However, it also has important negative clinical consequences, such as altered pharmacokinetics of drugs and carcinogens, drug-drug interactions, and changes in the metabolism of steroids, vitamin D, and other endogenous compounds. For these reasons, it is of great importance to understand the molecular mechanisms leading to drug induced gene expression of hepatic drug-metabolizing enzymes.

In this thesis the role of the AMP-activated protein kinase (AMPK) in the phenobarbital-mediated transcriptional regulation of CYPs was studied in hepatoma cells (chicken and human), and in hepatocytes (human and mouse). We show that AMPK activation by phenobarbital occurs via increased reactive oxygen species (ROS) formation in mitochondria and that increased AMPK activity is necessary for drug induction. Furthermore, we demonstrate that phenobarbital-type drugs affect the AMPK upstream kinase LKB1 and the protein kinase C zeta (PKCζ) activities. Importantly, these studies reveal that the signaling cascade involved in drug responses is conserved in evolution from birds to mice and humans. In addition, CXR interacting proteins were identified, which may also bind CAR in mammalian liver.

In conclusion, these findings provide new insights into the so far poorly characterized molecular mechanism by which drugs lead to transcriptional regulation of CYP enzymes.

Introduction

Drug Metabolism in the Liver – The Role of Cytochromes P450

Although the cell membrane is a very efficient barrier against water-soluble toxic compounds, the human body is exposed every day to a huge number of exogenous or endogenous lipophilic toxic substances, which can cross this boundary and thus have to be excreted in order not to harm the organism. Over millions of years, sophisticated mechanisms have evolved in the liver to metabolize toxic substances, biotransformation and transport. One challenge in the detoxification pathway is the hydrophobicity of these compounds, which must be made more polar for an easy excretion from the organisms via body fluids. The liver is the primary detoxification site where fat-soluble substances are rendered water-soluble by several enzymes including cytochromes P450 (CYPs), which catalyze the so-called phase I in the detoxification pathway. In phase II, other enzymes further modify these compounds, which are subsequently transported in phase III for excretion by blood, urine, feces and bile [1].

CYPs are a huge family of heme proteins, which catalyze mono-oxygenase reactions from bacteria to animals. These proteins are located at the endoplasmic reticulum or at the inner mitochondrial membrane, whereas some bacteria have cytoplasmic CYPs forms. Comparison of amino acid sequences of prokaryotic and eukaryotic CYP genes indicates that they have diverged from a single common ancestor. These enzymes are essential to eukaryotic organisms because they are involved in a number of anabolic and catabolic biosynthetic pathways, such as metabolism of cholesterol, steroid hormones, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, and retinoic acids [2-5].

In addition to their role in endogenous metabolism, CYPs play an important role in the metabolism of foreign compounds, such as drugs, pollutants, pesticides and diet components [6]. CYPs involved in the xenobiotic metabolism show broad and overlapping substrate specificities, so that one particular CYP metabolizes a large number of different compounds, and a given compound may be metabolized by several CYPs. Nowadays, organisms have everything they need to deal with toxic compounds probably as result of an evolution started a long time ago when animals were challenged by chemicals, with which plants defended themselves from being eaten.

The Phenomenon of Drug-Mediated Induction of CYPs

Numerous xenobiotics and endobiotic substances induce the expression of drug- and steroid-metabolizing enzymes such as cytochrome P450 in the liver, a phenomenon called induction [4, 6-8]. Altered expression of CYP genes has a major impact on drug effects including drug-drug interactions, drug toxicity, xenobiotic carcinogenicity, and therefore contributes to clinically important interindividual variability in drug responses and hormones disposal [9-13]. For these reasons, although xenobiotic metabolism by CYPs is necessary for organism detoxification, it represents a challenge from the clinical point of view and has to be considered in the treatment of patients.

In vertebrates, drug induction occurs predominantly in the liver, in the intestine and to a lesser extent in other extrahepatic tissues, such as skin, kidney, lung and brain. The phenomenon of drug induction was first observed more than 40 years ago in rats, which became progressively tolerant to treatment with phenobarbital (PB), a sedative and anticonvulsant drug, in long-term studies, due to enhanced metabolism and clearance. In these animals the smooth endoplasmic reticulum of hepatocytes was found to proliferate under PB treatment and the number of CYP enzymes, located on the membrane of smooth endoplasmic reticulum was also increased [14, 15].

Induction most often occurs at the level of transcription and is a rapid, dose-dependent and reversible phenomenon. Many drugs have, similar to the prototypic substance PB, the ability to induce their own and others metabolism by transcriptional activation of CYPs. In addition, inducer compounds can drastically alter the panel of gene expression in hepatocytes giving raise to pleiotropic hepatic responses. For instance, PB was shown to regulate more than 50 genes in chicken embryos [16], and recent gene expression array data in mice indicate more than 100 affected genes after PB treatment [17]. The effects triggered by PB include beside the proliferation of smooth endoplasmic reticulum, also liver weight gain and liver tumor promotion (in rodents) [6].

Drug-metabolizing enzymes mainly belong to the CYP1, CYP2, CYP3 and CYP4 families and their prototypical inducers are categorized in five classes: dioxin for the group of polyaromatic hydrocarbons inducing mainly CYP1As, PB-type inducers have a predominant effect on CYP2Bs, CYP3As and CYP2Cs subfamily genes, dexamethasone or rifampicin involved in CYP2Cs and CYP3As induction, clofibrate acting on CYP4As and ethanol that increases the activity of CYP2Es (reviewed in [18, 19]). PB induction has been reported in different species such as mammals, birds and bacteria, but not in yeast.

The molecular mechanism of hydrocarbon induction of CYP1As by the polyaromatic hydrocarbon receptor (AhR) was elucidated already in the 1980s [20], whereas the scientific community had a hard time to figure out the mechanism of PB-type induction of CYPs due to several experimental limitations. Among them two contributed massively to the poor knowledge in this field; first, PB-type inducers bind with low affinity to their receptors, which made the discovery of the receptors very difficult; second, lack of cell lines, which maintain PB-type responses constrained the use of either whole organs or primary preparation of hepatocytes in these studies, which are both restricted systems in terms of mutagenesis and transfection studies. Continuously dividing cell systems lack this phenomenon due to the dedifferentiation process occurring in these systems. In fact, drug induction and metabolism are a hallmark of highly differentiated, nondividing hepatocytes [21].

To find a system to study the mechanisms underlying drug induction, our group developed the use of a chicken hepatoma cell line, the leghorn male hepatoma (LMH) cell line. Besides heme oxygenase-1 regulation, triglycerides synthesis and lipogenesis comparable to chicken hepatocytes in primary cultures, these cells maintain the PB-type induction of CYPs [22] with all the advantages of continuously dividing cell lines in comparison to primary cultures: they are easy to handle and transfect, stably transfected subclones can be produced and there are no differences from preparation to preparation. When our group started using this system to study the molecular mechanism of drug

induction, several theories pointed to a receptor-dependent mechanism and experimental observations suggested the involvement of protein phosphorylation events (discussed below).

The first insights in the transcriptional regulation of CYPs came from a negative regulated element in the proximal promoter of P450 BM-3 in *Bacillus megaterium* [23, 24]. Sequence homologous of this element, referred to as Barbie box, were also found in mammalian CYPs proximal promoters [25, 26]. Several proteins binding to the proximal promoter were identified and shown to regulate the basal levels of expression and the tissue-specific expression. However, accumulating body of evidence suggested an important role of distal enhancer elements in drug regulation of CYPs, first of all the identification of such a regulatory sequence in the CYP2H1 gene in chicken embryo hepatocytes [27] and later in rat CYP2B2 [28] and mouse Cyp2b10 [29] gene 5'-flanking regions. In the attempt to isolate small and well-defined distal elements in CYP genes that are necessary for drug-mediated induction, the regulatory sequences found in the chicken CYP2H1, rat CYP2B2 and mouse Cyp2b10 were reduced to the minimal length still responsive to drugs. A 163-bp responsive element in the CYP2B2 [30, 31] as well as a 264-bp in CYP2H1 [22] and a 51-bp in the Cyp2b10 [29, 32] were shown to be sufficient to confer PB induction. Analysis of these sequences revealed the presence of putative hexamer half-sites suggesting for the first time that nuclear receptors are involved in drug-regulated expression of CYPs (see next section).

As mentioned earlier, since CYPs are heme-containing proteins, a dramatic increase in CYP proteins due to drug exposure enhances the demand of heme. As δ-aminolevulinic acid synthase 1 (ALAS1) is the rate limiting enzyme of heme biosynthesis and is highly inducible in the liver when heme demand increases, drug induction of CYPs affects the heme biosynthesis [33]. Therefore we characterized the regulatory elements in the *ALAS1* gene that are both responsible for its drug-induced expression of and activated by xenosensing nuclear receptors. Binding sites for CAR, PXR and CXR were discovered in human, mouse and chicken 5'-flanking region of ALAS1 genes [34-36]. Thus, drug-mediated expression of ALAS1 is increased in parallel to the induction of CYPs as a response to an enhanced demand of heme in the liver.

Nuclear Receptors

Cell-cell communication within an organism is necessary in all phases from the embryogenesis to the coordination of all vital functions during the life span. Communication is assured by messenger molecules, which usually bind to membrane receptor or if they are lipophilic and small enough to cross the membrane they interact with members of the family of nuclear receptors, which act as transcription factors. This family is subdivided in three classes, the classical hormones receptors, the sensor receptors and the orphan nuclear receptors [37]. The endogenous ligands for the orphan nuclear receptors are not known, whereas the classical hormones receptors bind molecules such as glucocorticoids, thyroid hormone, estrogen and retinoic acids, and are thus key factors in the endocrine homeostasis maintenance. The third class of sensor receptors bind substances of the metabolic pathways such as fatty acids (peroxisome proliferators activated receptor, PPAR), oxysterols

(liver X receptor, LXR), bile acids (farnesoid X receptor, FXR), or sense drugs and toxic levels of endobiotics (constitutive androstane receptor, CAR and pregnanes X receptor, PXR). These receptors are sensors of the metabolic state and responsible for the metabolic adaptation by responding to incoming dietary signals and metabolites generated in the organism. The hepatocyte nuclear factor 4α (HNF-4α) is also considered a metabolic sensor receptor because of its effects on glucose, fatty acids and cholesterol metabolism; however the question about its endogenous ligands is not completely solved.

The NR family is ancient in origin and was well diversified even before the arthropod/vertebrate split [38]. NRs control the activity of target genes directly by interacting as monomers, homodimers or heterodimers with the distal enhancer elements, located in the target gene flanking region. Response elements (RE) consist of two hexamer core half-sites, with consensus AG$^{G}/_{T}$TCA, which are arranged as direct repeats (DRs), inverted repeats (IRs) or everted repeats (ERs) spaced by different numbers of nucleotides. The NRs that bind as monomer, usually bind to an extended half-site, the extension being rich in A and T [39-41].

The mechanisms by which the nuclear receptors can regulate the transcription of the target gene are currently under intensive investigation. In addition to direct contact to the transcriptional machinery, NRs enhance or inhibit transcription by recruiting coactivator and corepressor proteins. The ligand binding causes a conformational change in the receptor, which leads to the release of corepressors and recruitment of coactivators. The role of the coactivators is to facilitate the communication between NRs, the basal transcriptional machinery and the chromatin environment in the presence of agonists. Because the NRs need to gain access to the DNA, which is wrapped around histones forming nucleosomes, they recruit proteins, which destabilize the chromatin structure by several mechanisms including histone acetylation. On the other hand, in the absence of ligands or in the presence of antagonists, the corepressors recruit the histone deacetylase, which stabilizes the chromatin and inhibits transcription [42-46].

Members of the nuclear receptor superfamily share structural and functional domains such as a highly conserved zinc finger DNA binding domain (DBD or C domain), which allows binding of the receptor to DNA, and a C-terminal ligand binding domain (LBD or E domain), which interacts with the ligand and allows dimerization. Other less conserved regions are the N-terminal region (A/B domain), a hinge region (D domain) between the DBD and the LBD, and a C-terminal region (F domain) in some receptors [47, 48].

The Role of the Xenosensors Pregnane X Receptor and Constitutive Androstane Receptor in Drug Induction

In the last years, unique roles for nuclear receptors in the regulation of CYP enzymes emerged. In particular, two xenosensing receptors were discovered in mammals, CAR and PXR, which regulate the CYP2B and CYP3A genes expression, respectively [49, 50]. PXR and CAR are abundantly expressed in liver and intestine, the places where drug metabolism occurs. They bind to DNA as heterodimers with RXR, which is the heterodimerization partner of several sensor receptors (LXR, FXR, PPAR). In chicken, only one xenosensor is present, the chicken X receptor (CXR), which was characterized in our group.

Pregnane X Receptor

PXR, alternatively called steroid and xenobiotic receptor, was discovered in mice and human by three independent groups in 1998 [51-54]. This receptor is activated by a multitude of very different drugs due to its extraordinarily large and flexible ligand binding pocket. Amino acid sequence comparison of LBD of different PXR orthologs revealed an unusual high divergence [55]. This divergence explains the species differences observed in induction of CYPs by PXR. For example, changing four amino acids of mouse PXR into the corresponding amino acids of the human counterpart led to a typical human activation pattern [56]. Similarly, PXR knockout mice that express the human PXR exhibit a typical human response to different inducers [57]. The flexibility and relative lack of specific binding interactions is supported by the observation that even the potent inducer SR12813 can bind to PXR in three distinct orientations [56]. The analysis of the binding site in PXR target genes indicates that this receptor is rather flexible in its binding specificity. Among others, binding and activation of DR-3, DR-4, ER-6, ER-8 and IR-0 were reported [52, 53, 58, 59]. PXR is found normally in the nucleus, where it is activated by ligand binding [60]. However, PXR was recently proposed to accumulate also in the cytoplasm in complex with Hsp90 and CCRP by overexpression of the latter [61]. The first identified targets of PXR were CYP3A1 and CYP3A4 in rat and human, respectively [53, 54, 57]. Later, other genes were shown to be regulated by PXR, such as CYP2Bs, MDR1, MRP2, OATP2 among others (for a recent review, see [62, 63]).

In PXR knockout mice, induction of Cyp3a11 by PCN, which is a typical PXR inducer, is impaired. However, Cyp3a11 can still be activated by PB via CAR [57, 64], indicating that CAR may cross-regulate Cyp3a11 as suggested by DNA binding and transfection experiments.

Constitutive Androstane Receptor

The PB-responsive unit (PBRU) in the CYP2B genes flanking region is composed of one DR-4 part of which was used in affinity purification for isolation of proteins binding to this sequence and mediating drug induction. This approach led to the observation that murine CAR binds to Cyp2b10 [65]. Like PXR, CAR shows high species-specific divergence in amino acid sequence in the LBD. Moreover, low-affinity ligands are typical for CAR, although its ligand binding pocket is smaller in comparison to PXR.

CAR recognizes preferably DR-4 and DR-5 repeats [66, 67], but ER-6 and ER-8 can serve as CAR binding sites, too [68, 69]. Different activation mechanisms of CAR were reported, which do not require the binding of the inducer to the receptor. Although CAR can be directly activated by TCPOBOP binding, PB and other inducers were never shown to bind to the receptor. In contrast to PXR, CAR is usually located in the cytoplasm and is transferred to the nucleus by ligand binding or by an indirect mechanism, which does not require receptor binding. Initial reports described CAR as a constitutively active receptor [66, 67, 70] since transiently transfection of CAR in HepG2 cells elicited high basal activity of Cyp2b10 or CYP2B6 PBRU in reporter gene assays [69]. In these cells CAR is located already in the nucleus and can be inhibited by certain androstanols, which act as inverse agonists (mouse CAR).

In primary cultures of hepatocytes and in liver, CAR is located in the cytoplasm. PB was shown to activate CAR by an indirect mechanism leading to its cytosolic-nuclear translocation [71]. This process is controlled by protein dephosphorylation events since the protein phosphatase inhibitor okadaic acid inhibits nuclear CAR transfer. In addition, translocation appears to be mediated by a leucine-rich xenobiotics response signal (XRS), which is located in the C-terminal region of CAR [72]. This xenosensor is retained in the cytoplasm under normal conditions in a complex of several proteins of which only a few are known. Heat shock protein 90 (Hsp90) and cytoplasmic CAR retention protein (CCRP) were recently described to interact with CAR in the cytoplasm, whereas protein phosphatase 2A (PP2A) was shown to be recruited by PB treatment [73, 74]. If this cytosolic complex retains CAR in the cytoplasm and/or protects it from degradation is still not known. Experiments using calcium/calmodulin-dependent protein kinase (CaMK) inhibitors revealed that once CAR is in the nucleus, its activity is modulated by protein phosphorylation events [75].

In CAR knockout mice induction of Cyp2b10 by TCPOBOP and PB in the liver was absent establishing a fundamental role for CAR in this response. Furthermore, induction of other CAR target genes was impaired in the liver of knockout mice [17, 76-78]. As previously mentioned, pleiotropic effects in the liver were observed by treatment with PB, such as liver hypertrophy or liver tumor promotion by long-term treatment, both of which are absent in CAR-null mice in comparison to wild-type mice [78-80]. Thus, CAR is not only responsible for the transcriptional upregulation of CYP genes caused by PB, but also for other hepatic responses.

Despite much progress in understanding CAR-dependent hepatic responses, the molecular mechanism of CAR-mediated signal transduction remains enigmatic.

Chicken X Receptor

As mentioned above, the chicken hepatoma cell line LMH uniquely maintains a pleiotropic response to PB. This observation and the fact that the similarity between mammalian and chicken PBRUs was very high [22], prompt our group to clone the chicken orthologs of CAR and PXR. Surprisingly, only one xenosensor, the chicken xenobiotic receptor (CXR) was identified [81, 82], which is equally related to both mammalian CARs and PXRs.

Chicken CXR and mammalian CAR and PXR as well as drug-inducible PBRUs could be freely interchanged in transactivation and electrophoretic mobility shift assay suggesting evolutionary conservation of the fundamental hepatic drug-induction mechanisms from birds to man. In addition, mouse *Cyp2b10*, rat *CYP2B2* and human *CYP2B6* PBRUs could be activated in LMH cells indicating that the proteins involved in the activation of the *CYP2H1* PBRU are identical or similar to proteins binding to the mouse and rat PB-responsive enhancer sequences [83] despite the presence of only one xenosensor in chicken. Thus, LMH cells are the first PB-inducible cell line showing both qualitative and quantitative responses comparable to in vivo findings. Since our recent data show evolutionary conservation of hepatic PB responses, LMH cells are a valuable tool for the study of the regulatory mechanism of PB-type induction. In addition, with the release of the chicken genome in 2004 [84, 85], studies in LMH cells will be facilitated in the future, especially experiments aimed to identify proteins or protein modification by mass spectrometry. From initial comparison with the mouse and human genome, it turned out that the overall pattern of gene order in the human genome is more similar to the chicken than to the mouse and that humans share about 60% of their genes with the chicken, with 75% identity on average.

Phosphorylation/Dephosphorylation Events in Phenobarbital-Mediated Induction of Cytochromes P450

Several experimental observations pointed to a role of phosphorylation and dephosphorylation events in the indirect mechanism of PB induction of CYPs, but the literature in this field is chaotic and sometimes contradictory.

For some years it was assumed that PB induction requires *de novo* protein synthesis. However, evidence accumulated indicated that *de novo* protein synthesis is not required for PB induction and that rather post-transcriptional processes are regulators of PB signaling in hepatocytes, i.e. protein kinase/phosphatase. 2-Aminopurine, a general inhibitor of serine/threonine protein kinases, blocked CYP2B1/2 induction in rat hepatocytes [86], and CYP2H1 and ALAS1 induction in chick embryo hepatocytes by PB [87]. Genistein, a tyrosine protein kinase inhibitor, also fully blocked Cyp2b10 induction by PB in mouse hepatocytes [88]. However, other studies reported no effect of genistein on CYP2H or CYP2B induction by PB [87, 89, 90]. Elevated intracellular levels of cAMP resulted in a dramatic inhibition of PB-mediated induction of CYP2B1, CYP2B2 and CYP3A1 gene expression in primary cultures of rat hepatocytes [91] and of Cyp2b10 in mouse hepatocytes [71], suggesting an involvement of cAMP-dependent protein kinase A (PKA). In addition, elevation of intracellular cAMP by forskolin, an adenylate cyclase activator, led to a dose-dependent repression of PB-inducible gene expression. Consequently, PKA may exert a negative role on CYP2B induction by PB. However, a study by Honkakoski et al. in mouse hepatocytes did not find any effect of forskolin and dibutyryl cAMP on Cyp2b10 inducibility by PB [92]. Moreover, no direct effect of PB on cAMP levels or PKA activity could be detected in mouse and rat hepatocytes [92, 93] and in avian LMH cells [22]. However, data obtained by the use of forskolin have to be handled carefully, because this PKA modulator is a ligand

and activator of human and mouse PXR [94]. Other studies showed inhibition of Cyp2b10 and Cyp3a11 induction by PB in mouse hepatocytes treated with the Ca^{2+}/calmodulin-dependent protein kinase inhibitor KN-62 or intracellular Ca^{2+} chelator BAPTA-AM [71]. In this regard, Yamamoto et al. blocked the TCPOBOB-mediated induction of Cyp2b10 in mouse hepatocytes by KN-62 [95]. Interestingly, this compound did not affect the nuclear translocation of CAR. Alternatively, okadaic acid, a potent inhibitor of serine/threonine phosphatases PP1 and PP2A, also fully prevented PB induction of CYP2B1/2B2 and Cyp2b10 in hepatocytes [96, 97]. These and other experimental observations strengthen the hypothesis that phosphorylation of yet unidentified proteins is involved in the molecular mechanism of PB-mediated regulation of CYPs, even though contradictory studies exist due probably to different experimental conditions, such as the systems employed or the concentration of inhibitors used. Functional studies approaching the role of phosphorylation in PB-mediated induction of CYPs without the use of pharmacological agents could shed more light on this very intricate field.

Crosstalk Between Drug, Lipid and Bile Acid Metabolism

The wide ligand specificity and overlapping DNA binding preferences of CAR and PXR lead to complex cross-regulation of drug-metabolizing enzymes. In addition, other nuclear receptors that are able to bind similar DNA motifs and are expressed in liver, such as VDR, LXR and FXR, may also modulate CAR and PXR target genes activation [98, 99]. Several data from our and other groups clearly show that a complex regulatory network involving crosstalk between nuclear receptors maintains lipid homeostasis, energy metabolism and drug detoxification.

Drug Induction – Lipid Homeostasis

A link between drug induction and lipid homeostasis has been established by several observations in cell culture, animals and patients. Squalestatin 1, an inhibitor of de novo cholesterol biosynthesis, was found to induce CYP2B1/2 in rat liver and primary cultures of hepatocytes [100-102]. Our studies in chicken hepatoma cells showed dose-dependent induction of CYP2H1 and CYP3A37 by squalestatin 1 [103]. Rats fed with high cholesterol diet have a reduced basal and PB-induced CYP2B levels [104]. Furthermore, in obese fa/fa Zucker rats without functional leptin receptor PB induction of CYP2Bs is almost completely lost [105]. Long-term treatment with PB affects lipoprotein levels in rats [106, 107] and changes in plasma and hepatic lipid profiles in patients [108-110]. In fact, beneficial effects of this drug on cholestasis have been observed [111]. In our studies, we could demonstrate that PB lowers hepatic triglycerides by a direct effect of CAR and PXR on Insig-1 expression, a protein with antilipogenic properties (Roth A et al, submitted).

These findings suggest that processes that influence hepatic cholesterol levels, such as defects in cholesterol biosynthesis pathways or hepatic accumulation of lipids, can ultimately alter drug metabolism, affecting the efficiency of drug treatment.

Drug Induction – Bile Acids

Another example of crosstalk between drug metabolism and cholesterol homeostasis is regulation of bile acid levels. A significant part of hepatic cholesterol is metabolized into bile acids, which are then excreted. Bile acids have to be tightly regulated because they can reach toxic levels. FXR is the bile acid receptor and inhibits cholesterol 7α-hydroxylase (CYP7A), the enzyme of the rate limiting step of bile acid synthesis [112, 113]. In addition, it has been shown recently that PXR [114, 115], VDR [116] and even CAR [117, 118] and CXR [119] can sense toxic levels of primary bile acids. The CYP3A family is of particular importance for drug metabolism, because it is estimated that more than 50% of clinically used drugs are at least partly metabolized by the predominant human 3A isoform, CYP3A4, which is induced by several compounds and shows high interindividual expression variability. This has important clinical consequences, affecting the efficacy of drug treatment and causing drug-drug interactions [120-122]. For this reason understanding the regulation of this gene is of great interest. CYP3A can metabolize bile acids and indirect evidence shows that CYP3A induction occurs in response to cholestasis [123]. Since PXR was shown to respond to toxic bile acids levels and CYP3As are target genes of this receptor, it can be hypothesized that PXR regulates CYP3A under cholestasis condition. Interestingly, Cyp3a11 induction by bile acids was reported also in PXR-null mice [124]. Therefore, we studied the regulation of CYP3A4 via the xenobiotic responsive enhancer module (XREM) under cholestatic conditions. In this study we showed that primary bile acids modulate the expression of CYP3A via FXR, thus enhancing bile acids metabolism, in addition to inhibit their biosynthesis by inhibition of *CYP7A* genes (APPENDIX 4 [125]). The human CYP3A4 promoter is a good example for nuclear receptor interplay, because it responds to PXR, CAR and even FXR [125] and VDR [126].

Drug Induction – Liver X Receptor

Another example of regulated crosstalk is the oxysterols receptor LXR, which not only regulates CYP7A genes, but also affects drug-inducible CYPs by binding to the drug-responsive enhancer element in the 5'-flanking region of chicken CYP2H1, and the human CYP2B6 and CYP3A4 [119], respectively. Our studies showed that LXR competes with PXR, CAR and CXR for binding to the DR-4 in the PB-responsive units, thus inhibiting PB-mediated induction of CYPs. We extended these results by in vivo experiments in LXR-null mice [127].

These three examples of crosstalk between drug induction and metabolic homeostasis clearly show that it is impossible to separate one from the other and that studies in this field have to consider the complicate network existing between them. In the next pages, the roles of CAR in the liver beside the regulation of drug metabolism are described.

Diverse Roles of the Constitutive Androstane Receptor

CAR in Xenobiotic Metabolism

CAR coordinates the regulation of multiple genes resulting in a higher metabolic capability of liver cells. As already mentioned, CYP genes were the first discovered targets of CAR. cDNA microarray analysis of wildtype and CAR-null mice revealed that CAR positively regulates not only CYPs (phase I enzymes) but also phase II drug-metabolizing enzymes and drug transporters. By this mean, CAR increases both metabolism and excretion of drugs, and thus plays a central role in the defense against toxic compounds.

The Hepatotoxic Role of CAR - CAR as a Risk Factor

Although induction is advantageous in most instances, it is also associated with hepatotoxic effects, such as metabolic activation of procarcinogens. In fact, CAR may inadvertently activate some compounds to carcinogens or toxic metabolites by promoting their modification. One example of the role of CAR in hepatotoxicity is the metabolism of acetaminophen (APAP) [128]. High levels of APAP activate CAR resulting in activation of CYPs involved in generating the more toxic APAP metabolite, N-acetyl parabenzoquinone imine (NAPQ1). This metabolite can react with cellular proteins causing toxicity. CAR-null mice are relatively resistant to APAP toxicity because no CAR-mediated increase in CYP production occurs, which would metabolize APAP to its toxic metabolite. The hepatotoxic role of CAR should thus not be underestimated.

CAR as a Protective Factor - CAR and Hyperbilirubinemia

Because the enzymes and transporters upregulated by CAR are also involved in the metabolism and secretion of endobiotics, the role of CAR as protective factor is extended to toxic endogenous substances. Bilirubin is the end product of the heme catabolism and is one of the most toxic breakdown product in the body. Hyperbilirubinemia is caused by increased accumulation of bilirubin, which can deposit in the central nervous system causing neurotoxicity and encephalopathy. The *UGT1A1* gene encodes bilirubin UDP-glucuronosyltrasnferase that catalyzes conjugation of bilirubin with glucuronic acid. This conjugation reaction followed by transport by organic anion transporting polypeptide 2 (OATP2) and multidrug resistance-associated protein 2 (MRP2) constitutes the major bilirubin detoxification pathway. For a long time it has been known that PB can decrease elevated bilirubin levels [129]. This is now revealed to be due to the ability of CAR to activate the expression of several components involved in bilirubin clearance [130, 131].

CAR as a Protective Factor - CAR and Cholestasis

One of the most important functions of the liver is bile production. The major products of cholesterol catabolism are bile acids, which help intestinal absorption of lipophilic nutrients by acting as physiological detergents. The level of bile acids is regulated by the balance of synthesis and elimination; cytochrome P450 7A (CYP7A) is the rate-limiting enzyme of bile acid synthesis, while other enzymes and transporters play a major role in the elimination. Elevated bile acids level through

dysregulation of this process may lead to cholestasis and liver injury.
For decades PB has been used to treat pruritis, a side effect of elevated serum bile acids associated with cholestasis. Recent studies showed that activation of CAR is necessary and sufficient to protect from the hepatotoxicity of lithocholic acid, a secondary bile acid [118, 132], thus explaining the beneficial effect of PB in the treatment of cholestasis. This finding implies that CAR agonist may be useful in the treatment of bile acids disorders.

CAR in Endocrine Homeostasis and Disruption

CAR affects endocrine homeostasis by altering hormone metabolism. Indeed, several CAR target genes are responsible for hormone metabolism, such as CYP2B or UGT1A1, which hydroxylate androgens and estrogens or glucuronidate estrogens to their inactive metabolites, respectively. Thus, CAR activation by xenobiotics or endobiotics increases steroid hormone catabolism affecting the endocrine homeostasis. Moreover, CAR activity was shown to be affected by steroids, such as androstanol and androstenol, which inhibit the receptor [133], or the active estrogen 17β-estradiol (E2), which activates CAR [134, 135]. Interestingly, CAR is not activated by inactive estrogens, suggesting a positive role of CAR-mediated estrogen metabolism against toxicity, because high doses of E2 often cause cholestasis in rodent as well as in humans [136].
The importance of CAR in endocrine homeostasis became even more prominent when CAR was found to be a primary glucocorticoid receptor (GR) target gene. A glucocorticoid response element (GRE) was identified in the human *CAR* gene and dexamethasone was shown to potentiate PB induction of CYP2B2 and Cyp2b10 via GR [137, 138].

CAR, Thyroid Hormone Homeostasis and Obesity

Thyroid hormones act to increase the metabolic rate; high level of thyroxine (T4) and triiodothyronine (T3) in the serum is correlated to an increased metabolic rate. Chronic treatment with PB or PB-like inducers is known to promote thyroid hypertrophy in humans [139] and to decrease the level of total serum T4, thus decreasing the metabolic rate. Indeed, PB and similar drugs are known to increase enzymes involved in thyroid hormone metabolism, such as UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), decreasing their serum levels in both animals and humans [140-143]. Obese individuals trying to lose weight have to face with the homeostatic resistance mechanisms that operate to resist weight loss. In fact, during periods of reduced caloric intake or fasting the level of thyroid hormones and consequently the basal metabolic rate are decreased, which results in low energy expenditure and low caloric loss. This major homeostatic barrier prevents weight loss under reduced caloric intake, making the life of obese individuals trying to diminish their weight very difficult. The importance of understanding the molecular basis of this homeostatic resistance pathway is huge: the opportunity to create pharmacological treatments for obesity.
It is well documented that CAR targets genes, such as CYP2B, but more interestingly UGT and SULT, which can metabolize thyroid hormones, are upregulated during fasting [144]. It was thus reasonable to test if CAR plays a role in these events. Two recent studies reported that CAR is required for the increased expression of sulfo- and glucuronyl-transferases that accelerate the clearance of thyroid

hormones, in turn resulting in decreased serum T4 levels. Indeed, fasted CAR-null mice under calorie-restricted diet for 12 weeks lost more than twice as much weight as the wildtype animals [144, 145]. These studies indicate that CAR contributes to the homeostatic resistance to weight loss, and thus its inverse agonists might be useful in the treatment of obesity.

CAR and Tumorigenesis

Acute treatment with PB causes hepatomegaly apparently due to both hypertrophy and hyperplasia [78]. In the 1970s it was reported that chronic PB treatment promotes hepatocellular carcinoma (HCC) in rats [146]. This drug is the prototype of so-called nongenotoxic carcinogens that cause tumors without mutating DNA. These tumor promoters increase the probability of cancer by accelerating the clonal expansion of cells transformed during tumor initiation. In humans, chronic PB treatment was never connected to liver tumor promotion probably due to the higher resistance of humans to tumorigenesis for a variety of reasons, including shorter telomeres. Indeed, telomerase-deficient mice are resistant to chemically induced hepatocarcinoma [147]. However, the potential of human CAR as tumor promoter cannot be ruled out, because intriguing data in mice suggest that not all strains are susceptible to HCC promotion by PB. A locus on chromosome 1 was associated to the HCC formation susceptibility and the corresponding locus on the human chromosome 1 is amplified in more than half of human HCC samples. In two recent publications, CAR-null mice were used to investigate the role of CAR in the development of HCC. The data present experimental evidence that CAR has an essential role in the liver tumor promotion induced by TCPOBOP, another strong tumor promoter [79, 80].

Thus, a short and transient hepatomegalic response promotes xenobiotic clearance, but chronic CAR activation can create a tumorigenic environment. For full understanding, further studies on the relationship of human CAR to HCC are necessary.

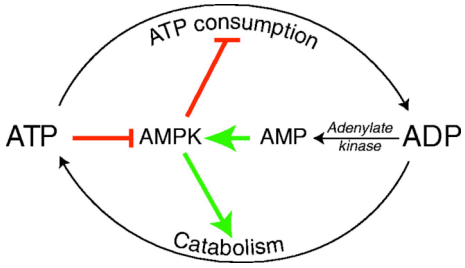
CAR in Glucose Metabolism

Several experimental observations point to a link between drug induction of CYPs and glucose homeostasis. Cyp2b10 expression is upregulated during fasting and in diabetes [144, 148]. Insulin was shown to repress drug induction in rat hepatocytes [144, 149], whereas glucagon was reported to increase CYP expression in chicken embryo hepatocytes [150]. In addition, treatment with PB decreases plasma glucose level in diabetic patients hence enhancing the insulin sensitivity [151] and inhibits gluconeogenic genes, such as phosphoenolpyruvate kinase (PEPCK) or glucose-6-phosphatase (G6P) in mice and rats [152, 153] via a CAR-mediated mechanism [17].

Kodama et al. recently showed that CAR and a forkhead transcription factor, FOXO1, interact coregulating reciprocally CAR and FOXO1 target genes, affecting both drug metabolism and gluconeogenesis [154]. These results shed light on the molecular mechanism of PB-induced repression of gluconeogenic enzymes and indicate a coordinate regulation of two major liver functions, the drug and the glucose metabolism.

AMP-Activated Protein Kinase

The AMP-activated protein kinase (AMPK) is a metabolic-stress-sensing protein kinase that regulates metabolism in response to energy demand and supply by directly phosphorylating rate-limiting enzymes in metabolic pathways as well as controlling gene and protein expression. If the energy charge decreases, AMP/ATP ratio increases followed by activation of AMPK which subsequently turns off anabolic pathways such as fatty acid, triglycerides and cholesterol synthesis as well as protein synthesis and transcription, and switches on catabolic pathways such as glycolysis and fatty acid oxidation (FIGURE 1 and 2). Thus, all stresses that affect AMP/ATP ratio might activate AMPK and given that all physiological processes depend on energy, there are potentially many links with AMPK. In addition, hyperosmotic stress and the antidiabetic drug metformin were shown to increase AMPK activity without detectable changes in the AMP/ATP ratio, raising the possibility that other signals feed into this system, thus increasing the complexity of energy homeostasis regulation (reviewed in [155]).
AMPK is a heterotrimeric enzyme that has been highly conserved throughout evolution as homologues of all three subunits have been identified in plants, yeast, nematodes, flies and mammals [156, 157]. AMPKα is the catalytic subunit, which has to be phosphorylated by upstream kinases to be activated. β and γ are regulatory subunits, which are necessary for catalytic function and stability of the kinase. The β subunit acts as a targeting scaffold, whereas the γ subunit was shown to bind AMP. AMP seems to regulate AMPK activity by two different mechanisms, i.e. by making AMPK a better substrate for the upstream kinase or a worse substrate for protein phosphatases.



From Hardie DG [158]

FIGURE 1

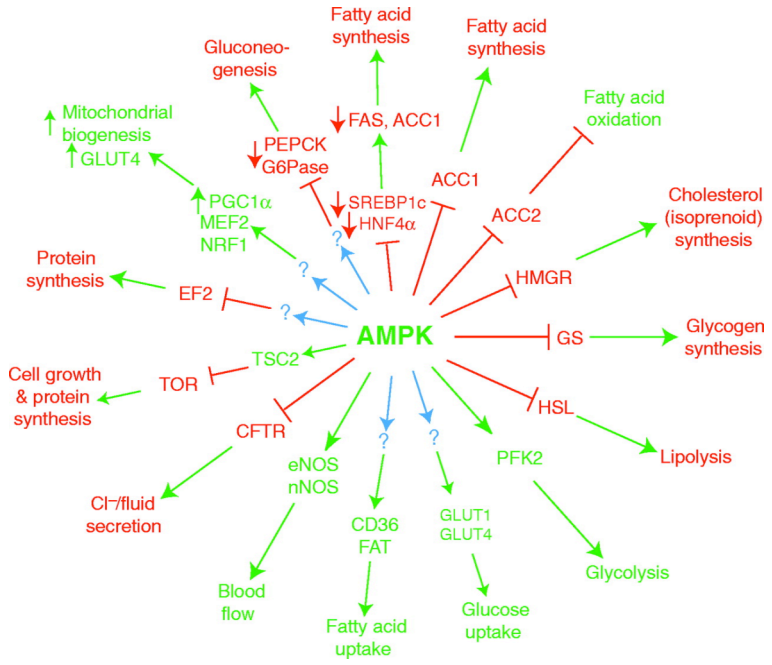
Physiological role of AMPK in the cell. Catabolism charges up the battery by converting ADP to ATP whereas ATP-consuming processes convert ATP to ADP. AMPK acts to restore the energy homeostasis.

Recent data showed that high cellular glycogen represses AMPK activation in muscle in vivo [159, 160], suggesting that this kinase may be a sensor of glycogen content. Supporting this thesis, AMPKβ subunits contain glycogen binding domains that could associate AMPK to glycogen in overexpression studies [161].
Until today three AMPK upstream kinases were identified, LKB1, CaMKKα and CaMKKβ, which by phosphorylation of threonine172 of AMPKα activate the kinase in cells and *in vivo* [155]. LKB1 is ubiquitously expressed and was shown to be required for activation of AMPK in response to AMP/ATP ratio changes, both in cultured cells and in vivo [162, 163]. In contrast, increases in AMP do not stimulate phosphorylation of Thr172 by the CaMKKs, which is triggered instead by a rise in Ca^{2+} [164-

166]. CaMKKs are mainly expressed in neural tissue, therefore their role in AMPK regulation in other tissue is not clear.

In liver cells, activation of AMPK decreases the expression of gluconeogenic enzymes, PEPCK and G6P both in cell culture [167] and in vivo [168]. In type 2 diabetes the blood glucose level is elevated mainly due to increased gluconeogenesis. For this reason, AMPK-activating drugs are successful in the treatment of this condition.

Much of the previous work on AMPK was focused on its effects on energy homeostasis within individual cells. However, recent results suggest that AMPK might affect whole-body energy metabolism by its activation by cytokines such as adiponectin and leptin, [168, 169], muscle contraction [170, 171] and its regulation of insulin secretion and expression in pancreatic β cells [172].



From Hardie DG [158]

FIGURE 2

Targets of AMPK. Target proteins and processes activated by AMPK are shown on green, and those inhibited by AMPK activation are shown in red.

LKB1, the AMPK Upstream Kinase in the Liver

LKB1, which was identified in humans as a tumor suppressor, is the gene mutated in the rare autosomal dominant human genetic disorder, Peutz-Jeghers syndrome (PJS) [173, 174]. PJS is an autosomal dominantly inherited disorder characterized by the occurence of intestinal polyps, which are benign tumors caused by disorganized growth of cells. LKB1 exists in a complex with two accessory proteins, Ste20-related adaptor protein (STRAD) and mouse protein 25 (MO25), which were shown to be important for LKB1 activity. STRAD, which is an inactive pseudokinase, anchors LKB1 in the cytoplasm, whereas MO25 appears to stabilize the LKB1:STRAD complex [175, 176].

LKB1 was shown to be necessary and sufficient for AMPK activation by most stimuli in HeLa cells, which do not express LKB1, and in immortalized fibroblast from $LKB1^{-/-}$ mouse embryos (MEFs) [162, 177]. Recent evidence was presentated that LKB1 is the upstream kinase not only of AMPKα1 and α2 subunits, but also of at least other twelve AMPK-related kinases, whose function is poorly understood. Apparently, LKB1 is a constitutive active kinase, which is not directly activated by stimuli. In fact, AMP binding to AMPK seems to make this kinase a better substrate for LKB1 [177, 178].

Furthermore, specific deletion of LKB1 in the liver resulted in a nearly complete loss of AMPK activity, providing evidence that LKB1 is the major AMPK upstream kinase in liver [179].

Conceptual Considerations

Hepatic cytochrome P450 enzyme activities and gene expression can be profoundly altered in disease states causing documented impairment of drug clearance and clinical drug toxicity. In order to make these effects more predictable, it will be important to gain a better understanding in the major physiological, cellular and molecular factors responsible for these changes. In the last years, the physiological role of CAR has expanded to include responses to metabolic and nutritional stresses. Particularly, two of the major liver functions, drug and glucose metabolism seem to be coordinately regulated by this receptor. The full elucidation of this interplay will help to understand the consequences of hepatic drug induction on the physiology and pathophysiology of the liver and the influence of metabolic and nutritional state on drug metabolism. Basal and drug-induced CYP expression is strongly affected by factors influencing the liver metabolic state, such as diabetes and energy starvation, and by phosphorylation/dephosphorylation events. Since AMP-activated protein kinase is an important energy sensor and regulates genes, which are affected by phenobarbital and CAR, too, its involvement in the molecular mechanism of cytochrome P450 enzyme induction was investigated in this thesis.

Aims of the Thesis

The initial goal of this thesis was to investigate the molecular mechanism of cytochromes P450 drug-mediated induction, particularly by applying differential 2-dimensional electrophoresis to detect changes in the phosphoproteome of control versus phenobarbital samples. Since AMP-activated protein kinase was reported to be activated by phenobarbital in a human hepatoma cell line [180], we then focused on this protein kinase as possible mediator of the phenobarbital induction in liver.
The chicken hepatoma cell line LMH was characterized and validated in previous studies showing that PB responses are preserved and evolutionary conserved in this system. For this reasons, this thesis firstly focused on this system to further unravel the pathway leading to the phenomenon of drug induction, particularly:

1. Characterization of CXR in comparison to mammalian CAR and PXR (Results – Part I)

2. Investigation of the role of AMP-activated protein kinase in drug induction (Results – Part II)

3. Characterization of proteins involved in this drug response (Results – Part VII)

4. Confirmation and extension of the notion of evolutionary conservation of liver drug responses (Results – Part I to VII)

5. Confirmation of data from LMH cells in other systems, for instance human and mouse hepatocytes (Results – Part V and VI)

Results

Part I

Nuclear Receptor

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Research

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The evolution of drug-activated nuclear receptors: one ancestral gene diverged into two xenosensor genes in mammals

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Abstract

Background: Drugs and other xenobiotics alter gene expression of cytochromes P450 (CYP) by activating the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) in mammals. In non-mammalian species, only one xenosensor gene has been found. Using chicken as a model organism, the aim of our study was to elucidate whether non-mammalian species only have one or two xenosensors like mammals.

Results: To explore the evolutionary aspect of this divergence, we tried to identify additional xenobiotic sensing nuclear receptors in chicken using various experimental approaches. However, none of those revealed novel candidates. Ablation of chicken xenobiotic receptor (CXR) function by RNAi or dominant-negative alleles drastically reduced drug-induction in a chicken hepatoma cell line. Subsequently, we functionally and structurally characterized CXR and compared our results to PXR and CAR. Despite the high similarity in their amino acid sequence, PXR and CAR have very distinct modes of activation. Some aspects of CXR function, e.g. direct ligand activation and high promiscuity are very reminiscent of PXR. On the other hand, cellular localization studies revealed common characteristics of CXR and CAR in terms of cytoplasmic-nuclear distribution. Finally, CXR has unique properties regarding its regulation in comparison to PXR and CAR.

Conclusion: Our finding thus strongly suggest that CXR constitutes an ancestral gene which has evolved into PXR and CAR in mammals. Future studies should elucidate the reason for this divergence in mammalian versus non-mammalian species.

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Background

A gene superfamily of heme-proteins, the cytochromes P450 (CYP), encodes the main enzymatic system for metabolism of structurally diverse lipophilic substrates [1]. A subset of these CYPs can be activated or inhibited in the liver by a variety of xenobiotic and endobiotic compounds. Transcriptional activation of these CYPs is part of an adaptive response to exposure to drugs and other xenobiotics and has major clinical and toxicological implications. The enzymatic capacities of the affected CYPs are changed, leading to an altered metabolic profile in the liver [2]. The barbiturate phenobarbital (PB) is prototypical for a class of compounds that induce or repress hepatic CYPs and many other genes [3]. PB-responsive enhancer units (PBRU) have been identified in the 5'-flanking regions of several of these CYPs and transcription factors binding to those units could be isolated (reviewed in [4-7]). In mammals, the pregnane X receptor (PXR, official nomenclature NR1I2) and the constitutive androstane receptor (CAR, NR1I3), both belonging to the gene superfamily of nuclear receptors, have been identified to be involved in hepatic drug-induction [8-12].

Strikingly, in contrast to the two xenobiotic-sensing nuclear receptors in mammals, only one xenosensor has been found in non-mammalian species, e.g. chicken [13], fish (fugu *Fugu rubripes* [14] and zebrafish *Danio rerio* [15]) or the nematode *Caenorhabditis elegans* [16]. The amino acid sequence of the full-length chicken xenobiotic receptor (CXR, NR1I3) is about equally related to those of mammalian PXRs and CARs [17]. Moreover, chicken CXR and mammalian PXR and CAR as well as drug-inducible CYP enhancer elements from these species could be freely interchanged in transactivation and electrophoretic mobility shift assays suggesting evolutionary conservation of the fundamental hepatic drug-induction mechanisms from birds to man [18].

In this report, we studied the evolutionary aspects of these findings. Despite using various methods and techniques, we were unable to isolate further genes that encode chicken xenobiotic-sensing nuclear receptors confirming the hypothesis that non-mammalian genomes only have one xenosensor gene. Since PXR and CAR exhibit different typical features concerning their activation, localization and regulation [6,19], we examined the properties of CXR to see whether on the functional and structural level, the chicken xenosensor shares common aspects with one or both of the mammalian receptors. Our findings give important insights the evolution of hepatic detoxification systems that protect different species from toxic compounds in their particular diet and environment.

Results and Discussion

Orthologs of PXR and CAR have been isolated from man, monkey, pig, dog, rabbit, mouse and rat [15]. In non-mammalian species, only one xenosensor gene is found and sequence-wise, the corresponding receptors from chicken, zebrafish, fugu fish and *C. elegans* are about equally related to the mammalian PXRs and CARs (Fig. 1A). Of the 18 nuclear receptors in the fruitfly *Drosophila melanogaster* genome, DHR96 shares considerable similarity to the xenosensors but the functions of this receptor have not been elucidated yet. Although the African clawed frog *Xenopus laevis* has two nuclear receptors, benzoate X receptor α and β (BXRα/β, NR1I2), that are related to the xenobiotic-sensing nuclear receptors, the BXRs are pharmacologically distinct from PXR and CAR and do not respond to xenobiotics [15,20]. No drug-sensing nuclear receptors have thus been isolated in amphibians so far. Figure 1A shows the phylogeny of the xenobiotic-sensing nuclear receptors from different species. The completion of the rat genome allowed a global analysis of the nuclear receptors from three mammalian species, man, mouse and rat. In the nuclear receptor subfamily NR1I which includes the 1,25-dihydroxyvitamin D_3 receptor (VDR, NR1I1) in addition to PXR and CAR, intron-exon junctions are highly conserved [21]. Human and rodent CARs and PXRs have the same number of introns. Moreover, apart from one intron which is found in the variable region 5' of the DNA-binding domain, all other seven introns are located in the same position on the corresponding genes, even in the ligand-binding domains that in the case of CAR and PXR are unusually divergent for nuclear receptor orthologs [22]. Using a chicken genomic library, we isolated the gene encoding CXR and analyzed its structure. Again, the number of introns in the CXR coding sequence was the same as those in the mammalian xenosensors and the intron-exon junctions occur at the same locations (Figure 1B). The apparent conservation of gene structures between the single chicken xenosensors and the two mammalian orthologs suggest a close relationship between these receptors and supports the hypothesis that CXR constitutes an ancestral gene in chicken from which two receptors diverged in mammals.

To further test this hypothesis, we used different experimental approaches in order to isolate additional chicken xenobiotic-sensing nuclear receptors. Neither high- and low-stringency screening of a chicken liver cDNA library using CXR, CAR and PXR fragments as probes nor PCR-based strategies with degenerate primers designed on CAR and PXR alignments or degenerate primers based on generic nuclear receptor DNA-binding domains [23] resulted in the identification of novel chicken xenobiotic-sensing receptors (data not shown). The sequences of the previously unknown chicken orthologs for estrogen-related receptor γ (ERRγ, NR3B3) and a partial fragment of

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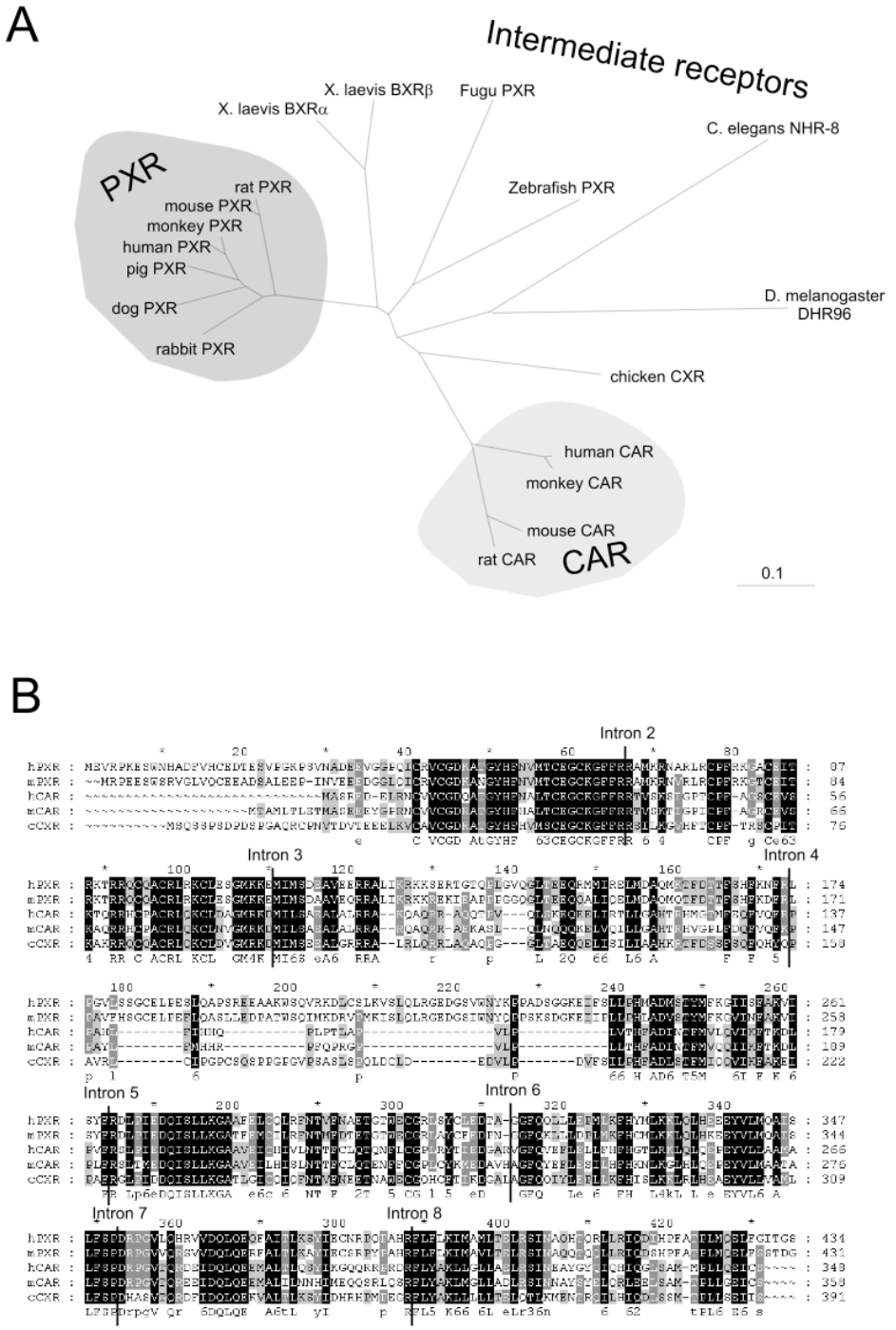

Figure 1

Phylogeny of xenobiotic-sensing nuclear receptors from different species. *A*, A non-rooted phylogenetic tree depicts the relationship between mammalian CARs and PXRs and non-mammalian intermediate receptors. The scale bar represents 0.1 amino acid substitutions per site. *B*, The sites of intron-exon junctions in the coding regions of CXR, PXR and CAR are highly conserved as depicted in an alignment of the amino acid sequences of these receptors.

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ear2 (NR2F6) that were found in these screens have been deposited (Genbank accession numbers AY702438 and AY702439, respectively).

If CXR in fact is the only chicken xenobiotic-sensing nuclear receptor, ablation of CXR expression or function is predicted to drastically reduce drug-induction of CYPs and other target genes. To reduce CXR expression, we designed RNAi oligonucleotides targeting CXR and stably expressed those in the chicken hepatoma cell line leghorn male hepatoma (LMH). LMH cells express endogenous CXR and retain induction of genes by PB-type inducer compounds and other drugs [18]. As shown in Figure 2A, endogenous mRNA levels of CXR were reduced about 60% by the RNAi. LMH cells expressing either control vector or CXR RNAi were subsequently transfected with drug-responsive enhancer elements from CYP2H1 [17], CYP3A37 [24], CYP2C45 [25] and δ-aminolevulinate synthase (ALAS-1) [26] and treated with vehicle or 400 μM PB for 16 hours. ALAS-1 is the first and rate-limiting enzyme in heme biosynthesis and its transcription is regulated by a variety of factors and stimuli, including PB-type inducers and other drugs [26,27]. In the case of ALAS-1, the 2-fold PB-induction was completely abolished by the CXR RNAi (Figure 2E). In contrast, PB-activation of the CYP2H1, CYP3A37 and CYP2C45 PBRUs was only partially reduced by 50 to 60% (Figure 2B,2C,2D). In these cases, reduction of CXR levels by 60% might not be enough. Alternatively, these findings could also be explained by the presence of additional drug-sensing signalling mechanism independent of CXR.

Thus, we used an alternative method that aimed at reducing CXR activity by designing dominant-negative CXR alleles. These CXR mutants were then tested in reporter gene assays on drug-responding enhancer elements. In our case, we generated three different CXR alleles (Figure 3A): first, we deleted the N-terminus since in some nuclear receptors, this part harbours a ligand-independent activation domain AF-1 [28,29]. Second, site-directed mutagenesis of the cysteine residues in the zinc-fingers of the DNA-binding domain results in a CXR mutant that is expected to lack DNA-binding but to retain its ability to bind activators and to heterodimerize with its partner retinoid X receptor (RXR, NR2B1/2/3). Third, helix 12 in the ligand-binding domain was deleted which harbours a ligand-dependent activation domain AF-2. Nuclear receptors that act as dominant-negative alleles due to the absence of a functional AF-2 domain have been observed in some diseases (e.g. see refs. [30,31]). These findings were subsequently used to generate various dominant-negative nuclear receptor mutants for cellular assays [32].

First, the three CXR mutants were tested for their ability to bind to and activate a 264-bp PBRU isolated from the 5'-flanking region of chicken CYP2H1 [17,33]. As shown in electrophoretic mobility shift assays (Figure 3B), CXR can heterodimerize with RXR and bind to the 264-bp PBRU as wild-type, full-length receptor and when the N-terminal region from amino acid 1–29 (called ΔN-term) or the C-terminal region from amino acid 383–391 (referred to as ΔAF-2) are deleted, respectively (Figure 3B, lanes 8, 10 and 12). As expected, site-directed mutagenesis of four cysteine within the DNA-binding domain into alanine residues (denominated DBD) that participate in forming the zinc-finger domains abolishes protein-DNA interaction (lane 11). These results show that removal of the N-terminus or the C-terminus of CXR does not influence its binding to DNA. Subsequently, the CXR mutants were tested in CV-1 transactivation assays for functionality. The CV-1 monkey kidney cells constitute an excellent tool to study nuclear receptor function in a cellular system which does not express endogenous xenosensors, is not drug-inducible and thus has a very low background in these assays. Neither CXR lacking its C-terminal activation domain AF-2 (ΔAF-2) nor CXR with the mutated DNA-binding domain (DBD) are able to transactivate the CYP2H1 264-bp PBRU in CV-1 cell assays (Figure 3C). In contrast, removal of the N-terminus of CXR (ΔN-term) has no effect on its transactivation potential suggesting that no activation function AF-1 is present in these 29 amino acids. Finally, the test whether any of these CXR mutant alleles acts in a dominant-negative fashion is performed in the LMH cells which do express endogenous CXR and which are drug-inducible [18]. When co-transfected with the 264-bp PBRU, the CXR allele lacking a functional AF-2 domain (ΔAF-2) drastically decreases PB-induction of the PBRU (Figure 3D). In contrast, the DNA-binding domain (DBD) and the N-terminal truncated (ΔN-term) mutants have no effect. Similar results were obtained with PBRUs from other drug-responsive genes (data not shown). Together, the RNAi experiments and the findings using the dominant-negative CXR mutants show that functionally, CXR is the major drug-sensing nuclear receptor in chicken.

A significant difference between PXR and CAR in mammals is their mode of activation and their cellular localization [19]. PXR is strongly activated by a huge number of compounds. In contrast, CAR exhibits less promiscuity but high constitutive activity in most cellular assays [34]. However, CAR activity can be modulated by inverse agonists, agonists and different protein phosphorylation events [35]. In terms of activation, CXR is also highly promiscuous and normally has a low basal activity, thus pharmacologically more resembling a PXR-type than a CAR-type receptor [13,15]. Regulation of CAR activity can in part be explained by its unusual cellular localization. Both PXR and CAR undergo cytoplasmic-nuclear shuttling upon activation [36-40]. However, in contrast to

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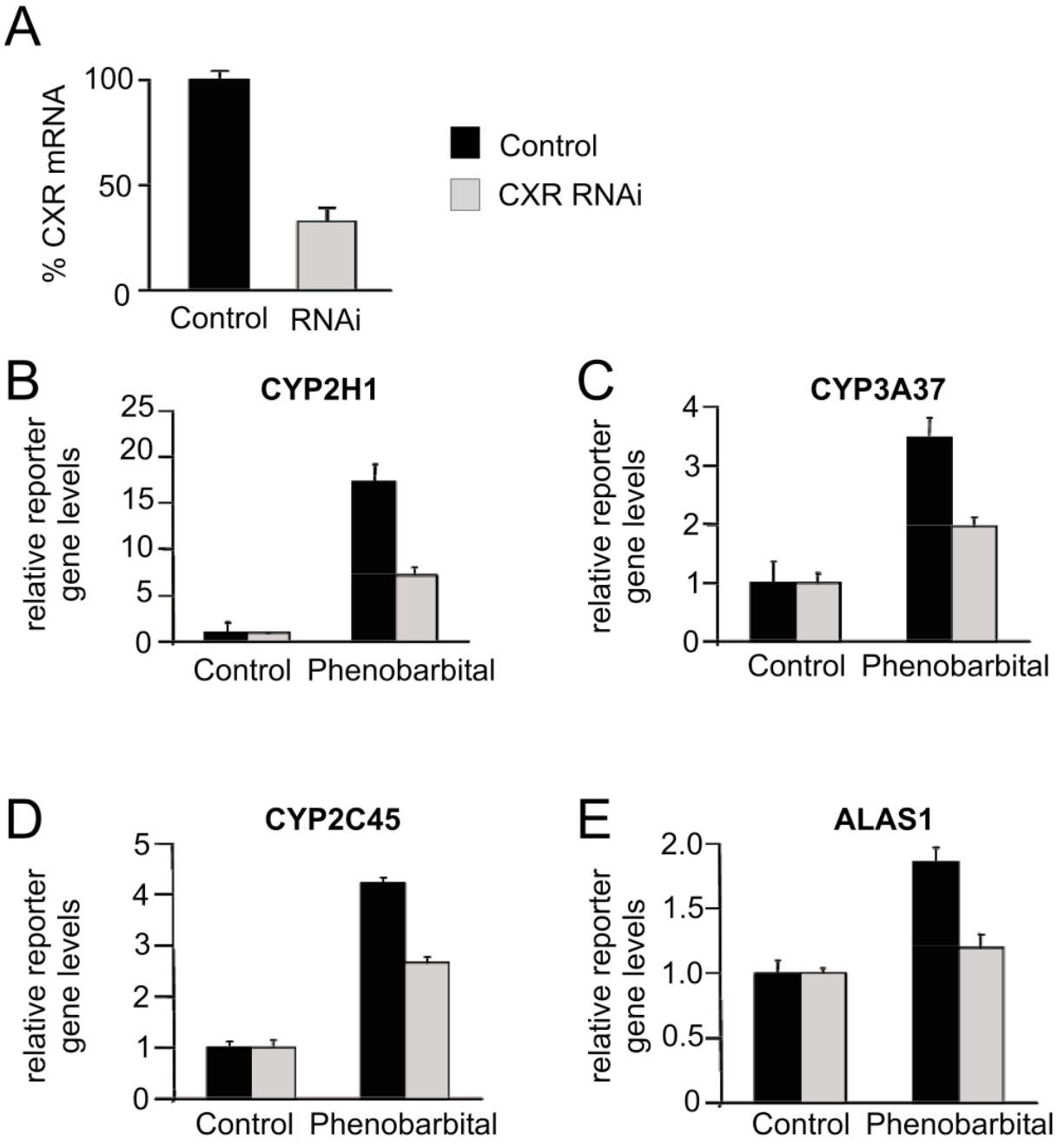

Figure 2

Reduced drug-induction of drug-responsive enhancer elements from CYP2H1, CYP3A37, CYP2C45 and ALAS1 in LMH cells stably expressing CXR RNAi. *A*, mRNA levels of endogenous CXR in LMH cells expressing pSUPER expression vector or CXR RNAi. CXR levels were measured by real-time PCR in LMH cells that stably express control vector or CXR RNAi. *B–E*, Phenobarbital-induction of drug-responsive enhancer elements from CYP2H1 (B), CYP3A37 (C), CYP2C45 (D) and ALAS1 (E) in LMH cells expressing pSUPER or CXR RNAi. LMH cells were transfected with the reporter gene plasmids and subsequently treated with vehicle or 400 μM PB for 16 hours before reporter gene levels were determined.

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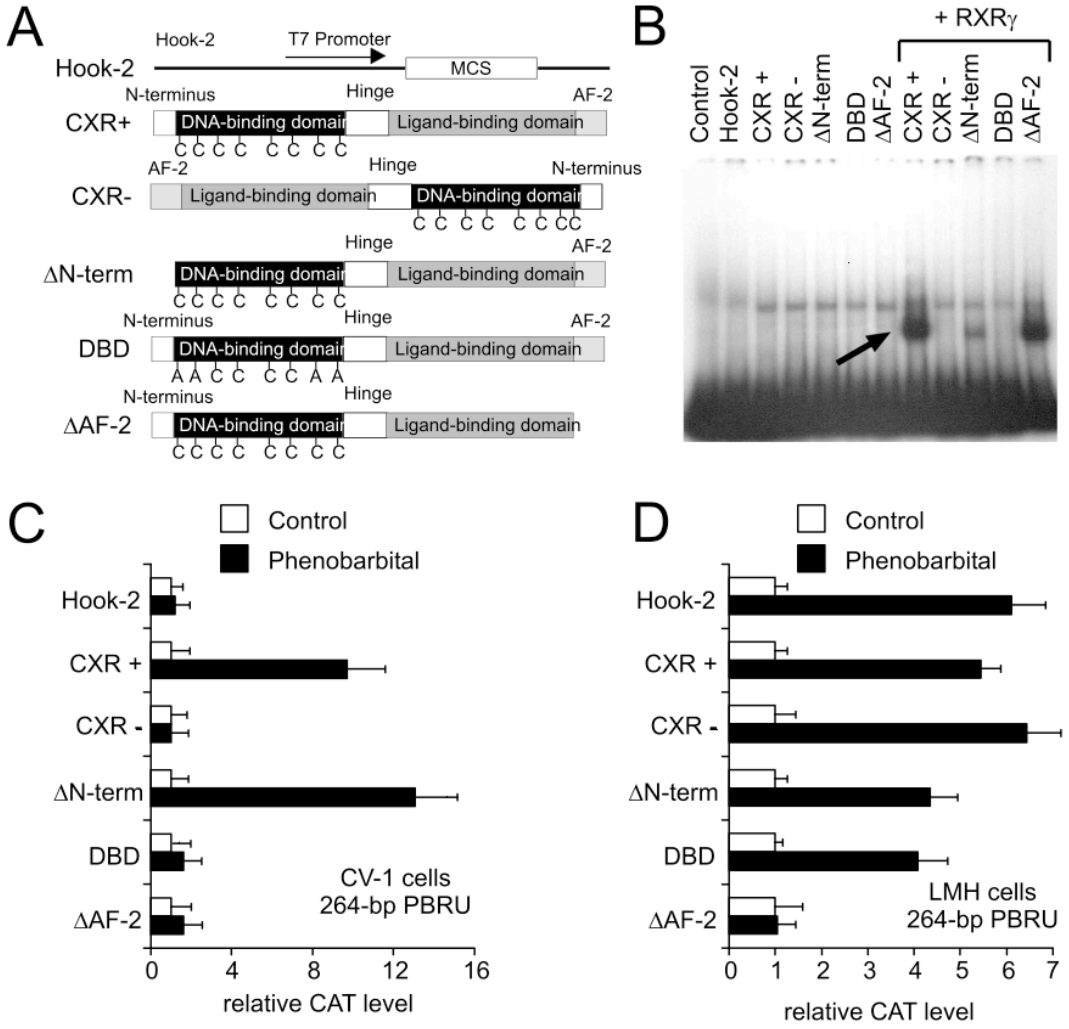

Figure 3

Drug-induction of the 264-bp PBRU is abolished by a dominant-negative CXR allele. *A*, CXR was subcloned into the pHook-2 expression plasmid (Hook-2) either full-length CXR in positive orientation (CXR+), negative orientation (CXR-), lacking its N-terminal amino acids 1–29 (ΔN-term), full-length CXR with four of its cysteine residues (cysteine 31, 34, 83 and 86) in the DNA-binding domain mutated (DBD) or lacking its C-terminal amino acids 383–391 containing the activation function AF-2 (ΔAF2). *B*, Electrophoretic mobility shift assays with mock *in vitro* transcribed/translated reticulocyte lysate (lane 1), expression plasmid pHook-2 (lane 2) and either expression plasmids for the different CXR alleles alone (lanes 3–7) or together with a pSG5-expression plasmid for chicken RXRγ (lanes 8–12). The arrow indicates the specific shift of CXR/RXR complexes with the radiolabeled CYP2H1 264-bp PBRU. *C*, pHook-2 expression plasmids without insert or containing the various CXR alleles were co-transfected with the CYP2H1 264-bp PBRU in the pBLCAT5 reporter vector as well as a lacZ-expression vector for normalization of transfection efficiencies into non-drug responsive CV-1 cells. After transfection, the cells were treated with either vehicle or 400 μM PB for 24 hours before cells were lysed and analysed for reporter gene expression and β-galactosidase expression. Values are the average of the relative CAT expression normalized for β-galactosidase levels of three independent experiments and error bars represent the standard deviation. *D*, pHook-2 expression plasmids without insert or containing the various CXR alleles were co-transfected with the CYP2H1 264-bp PBRU in the pBLCAT5 reporter vector into drug-responsive LMH cells expressing endogenous CXR. After transfection, the cells were treated with either vehicle or 400 μM PB for 24 hours before cells were lysed and analysed for reporter gene expression and β-galactosidase expression. Values are the average of the relative CAT expression normalized for β-galactosidase levels of three independent experiments and error bars represent the standard deviation.

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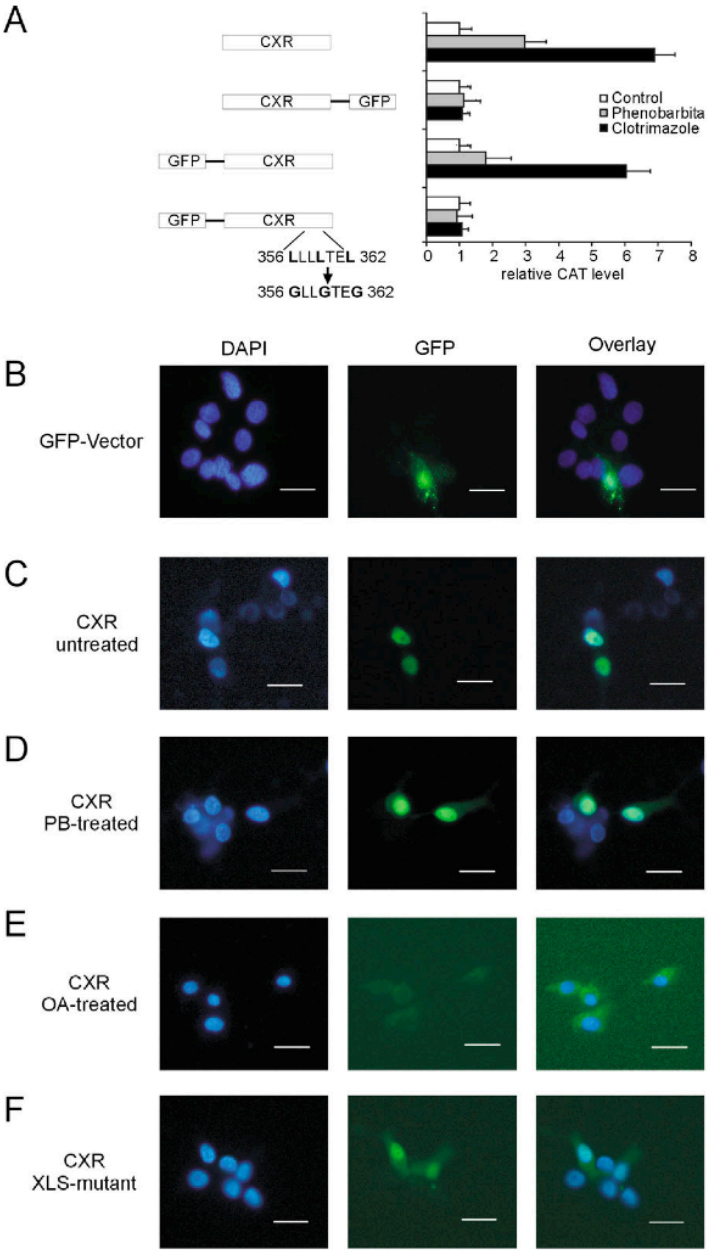

Figure 4

Cellular localization of CXR in transiently transfected LMH cells. *A*, Full-length CXR, CXR with GFP attached at its C-terminus or at its N-terminus or N-terminal GFP-CXR fusion protein mutated in its xenochemical response signal (XRS) at positions 356, 359 and 362 were expressed in CV-1 cells together with a reporter plasmid containing the CYP2H1 264-bp PBRU. After transfection, the cells were treated with either vehicle, 400 μM PB or 10 μM clotrimazole before cells were lysed and analysed for reporter gene expression. Values are the average of three independent experiments and error bars represent standard deviations. *B–F*, LMH cells were transfected with either pEGFP-vector alone (B), expression vector for N-terminal GFP-CXR fusion protein treated with vehicle (C), 400 μM PB (D) or 0.1 μM okadaic acid (E) for 16 hours or GFP-CXR fusion protein with the xenochemical response signal mutation as described in Fig. 4A (F). Cells were stained with 300 nM DAPI in PBS and analysed for DAPI and GFP-specific light emissions at 461 nm and 507 nm using excitation wavelengths of 358 nm and 488 nm, respectively. Size bars stand for 20 μm.

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PXR, CAR translocates after activation by PB, other xenobiotics or bilirubin for which no direct binding to the ligand-binding pocket was found. Although some progress in identifying CAR-interaction partners have been made recently [41,42], the mechanisms controlling the cytosolic-nuclear translocation are not clear. Interestingly, CAR translocation is independent of the C-terminal AF-2 function but instead requires the xenochemical response signal (XRS) LXXLXXL located between leucine 313 and leucine 319 in the human CAR sequence [37]. In contrast, cytoplasmic-nuclear translocation of VDR, the glucocorticoid receptor (GR, NR3C1) and the progesterone receptor (PR, NR3C3) is dependent on AF-2 suggesting a different mechanism for CAR shuttling [37,43]. A putative XRS (LLLLTEL) is also found in the CXR sequence between leucine 356 and leucine 362. Thus, to assess the relatedness of CXR to PXR and CAR in terms of cellular localization, we engineered different CXR-green fluorescent protein (GFP) fusion proteins. These were subsequently tested for functionality in CV-1 cell transactivation assays using the 264-bp PBRU as drug-sensitive enhancer. CXR with N-terminal, but not C-terminal GFP is activated by 400 μM PB and 10 μM clotrimazole after 16 hours of incubation (Figure 4A). Site-directed leucine to glycine mutagenesis in the CXR XRS reduces its ability to confer drug activation in these assays (Figure 4A).

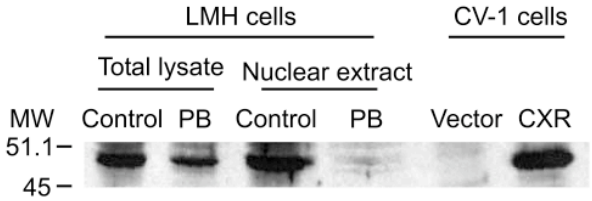

Figure 5
Nuclear CXR protein levels decrease after PB-treatment. LMH cells were treated with vehicle or 400 μM PB for 16 hours before cells were lysed and CXR protein levels in the total lysate (lanes 1 and 2) and in nuclear extracts (lanes 3 and 4) determined by Western blot. As controls, CV-1 cells were transfected with control vector or CXR expression vector (lanes 5 and 6). MW, molecular weight in kDa.

Subsequently, N-terminal GFP-CXR was transiently expressed in LMH cells, the cells were counterstained with DAPI to stain the nuclei and GFP-CXR localization was compared to that of GFP-expression vector without insert. GFP was found to be evenly distributed throughout the cell (Figure 4B). As depicted in Figure 4C, GFP-CXR in vehicle-treated LMH cells is exclusively in the nucleus. Treatment of transiently transfected LMH cells with 400 μM PB for 16 hours leads to an increase of GFP-staining in the cytosol (Figure 4D). Similar observations have been made for a variety of nuclear receptors where activation stimulates their export from the nucleus and subsequent degradation in the cytosol [44,45]. Accordingly, PB-treatment of LMH cells results in decreased CXR protein levels in total cell lysates (Figure 5, lanes 1 and 2) and even more dramatic in nuclear extracts (Figure 5, lanes 3 and 4) suggesting that activated CXR protein is more rapidly exported from the nucleus and degraded of this receptor. Most nuclear receptors that are exported and degraded upon activation share a conserved KXFF$^{K}/_{R}$R motif between the two zinc-fingers in the DNA-binding domain that can serve as binding site for calreticulin which is involved in the nuclear export [45]. PXR, CAR and CXR also contain a KGFFRR-motif but whether calreticulin plays a role in nuclear export of these receptors remains to be investigated. The protein phosphatase inhibitor okadaic acid inhibits PB-induction of mammalian and chicken PBRUs [13,18,46,47]. In transiently transfected LMH cells, 100 nM okadaic acid prevents nuclear localization of CXR after 16 hours (Figure 4E). Moreover, protein levels of the GFP-CXR fusion protein were reduced. Okadaic acid treatment prevents the drug-induced cytosolic-nuclear translocation of CAR [36]. Our findings regarding CXR are therefore very reminiscent of those results. Furthermore, site-directed mutagenesis of the XRS reduces the nuclear localization of CXR (Figure 4F) but not as completely as XRS mutations of CAR in mouse primary hepatocyte cultures [37]. The nuclear-cytoplasmic redistribution of this CXR mutant correlates with the decrease in its ability to activate the 264-bp PBRU in transactivation assays (Figure 4A). Thus, although CXR is normally found in the nucleus like PXR, it shares some features with CAR concerning its localization after treatment with okadaic acid or when its XRS is mutated.

In primary human hepatocytes, glucocorticoids have a dual effect on the expression of the drug-inducible CYP3A4 that is regulated by both PXR and CAR [48]. At low concentrations, these compounds activate GR which subsequently induces transcript levels of PXR and CAR [49,50] whereas higher concentrations of glucocorticoids directly activate PXR [9,51]. We thus wanted to test whether the chicken CXR is regulated in the same way as the mammalian xenobiotic-sensing receptors. Treatment of LMH with 50 μM dexamethasone (Dex) for 16 hours did not alter CXR expression (Figure 6). Moreover, dexamethasone does not activate CXR directly, at least at this concentration [13]. In contrast, dexamethasone increases transcription of the chicken peroxisome-proliferator activated receptor α (PPARα, NR1C1), the chicken liver X receptor (LXR, NR1H3) and the chicken farnesoid X receptor (FXR, NR1H4). These receptors play important roles in maintaining hepatic bile acid, cholesterol and

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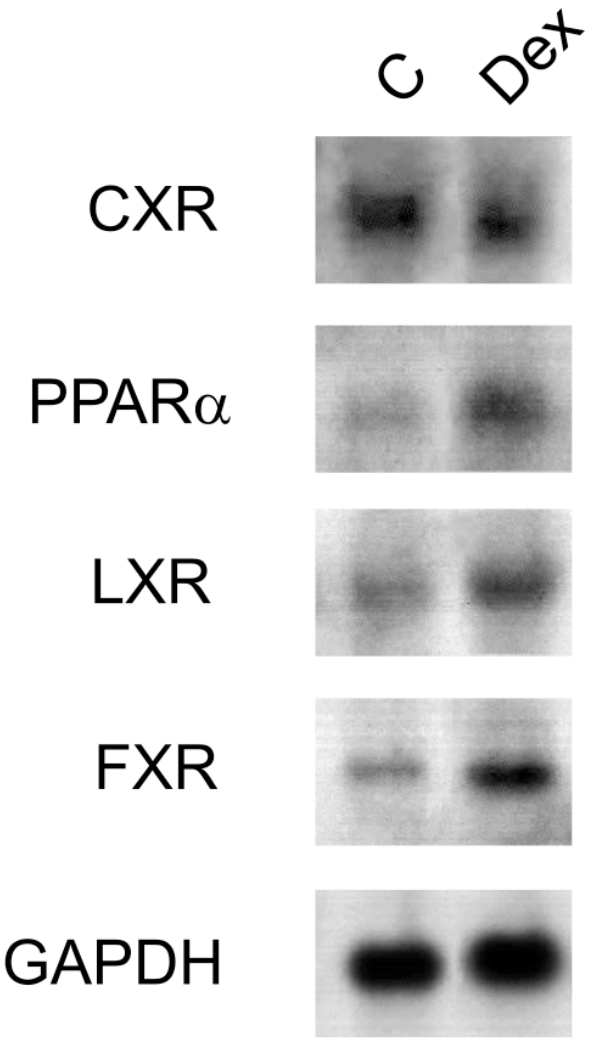

Figure 6
Transcriptional regulation of chicken CXR, FXR, LXR and PPARα in LMH cells by glucocorticoids. LMH cells were treated for 16 hours with vehicle or 50 μM dexamethasone before cells were lysed and RNA was analysed by Northern blotting with probes for CXR, chicken PPARα, chicken LXR, chicken FXR or chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

lipid homeostasis, respectively [52]. PXR, CAR and CXR have been found to be activated by bile acids and thus are involved in the regulation of the intrahepatic levels of lipid soluble compounds by stimulating metabolism and subsequent excretion of these compounds [12,53,54]. Therefore, activation of one of these receptors leads to changes in intrahepatic lipid levels which then potentially affects transcription of the other receptors. However, the regulatory network of these receptors is still under investigation.

Molecular modelling studies confirm the close relationship between chicken and fish xenosenors to mammalian PXR. X-ray structures of human PXR revealed several peculiarities of the PXR ligand-binding domain which are not found in other nuclear receptors [55,56]. First, PXR has an expanded β-sheet with two more strands. Moreover, helix 6 is completely and helix 7 partially unwinded which leaves a solvent-accessible hole in the ligand-binding pocket that is capped by an extension in helix 1–3. Although an extended β-sheet is not obvious in chicken and fish xenosensors, both receptors have long helix 1–3 inserts which could potentially induce partial unwinding of helix 6 and 7. Thus, molecular modelling of aligned amino acid sequences suggest enlarged ligand binding pockets for both fish and chicken xenobiotic-sensing receptors which could explain their high promiscuity [15]. In striking contrast, CARs not only lack an extended β-sheet but also have a much shorter helix 1–3 resulting in a more rigid and less promiscuous ligand binding pocket [15,57,58]. Therefore, the relatively high degree of promiscuity of CAR could at least partially be due to the ability of different compounds to trigger cytoplasmic-nuclear translocation of this receptor independent of direct binding [35]. The loop connecting helix 11 and 12 is much shorter in the CAR sequence than most other nuclear receptors [15,57]. This short loop might reduce the ability of helix 12 and AF-2 to reach an inactive conformation and thus could explain the constitutive activity of CAR [57]. CAR also has a shorter helix 12 than most other nuclear receptors [57]. Interestingly, helix 12 of CXR is very conserved to that of mammalian CARs in terms of amino acid composition and of length whereas the length of the zebrafish xenosensor helix 12 is intermediate between CARs and PXRs.

Conclusions

In summary, our results confirm that in contrast to mammals which have two xenobiotic-sensing receptor PXR and CAR, the genome of other species encodes for only one xenosensor. This hypothesis is supported by analysis in the fugu fish genome (data not shown), unsuccessful attempts to isolate further xenosenors in chicken and functional assays showing that ablation of CXR function drastically reduces drug-inducibility in a chicken hepatoma cell line. Our findings presented here and those of other laboratories imply that PXR and CAR origin from one ancestral gene which diverged into two genes in mammals. This ancestral gene, in chicken coding for CXR, is a promiscuous, PXR-like receptor. Thus, CXR and related receptors from fish are activated by a variety of dif-

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ferent compounds [13,15]. Interestingly, in a comprehensive study of different classes of ligands on xenosensors from man, monkey, pig, dog, mouse, chicken and fish, CXR was one of the most promiscuous receptors in regard to the compounds tested [15]. Therefore, the ancestral xenosensors in non-mammalian species might have a broader substrate spectrum than their mammalian counterparts where the task for detoxification is split between two receptors [59]. On the other hand, CXR also shares some features with CAR that are not found in PXR: its short helix 12, the xenochemical response signal and in part its cellular localization after okadaic acid treatment. Finally, in contrast to both PXR and CAR, CXR is not regulated by glucocorticoid treatment in the chicken LMH cells suggesting that this regulation was acquired only after birds and mammals diverged from a common ancestor.

Evolution of drug-metabolizing CYPs and xenobiotic-sensing nuclear receptors is influenced by diet and exposure to other environmental chemicals. Accordingly, drug-induction is very species specific. This is reflected in the unusually divergent ligand-binding domains of PXRs and CARs orthologs [22]. When comparing PXRs and CARs from human, mouse and rat, nonsynonymous nucleotide substitution rates are considerably higher in comparison to any other nuclear receptor [21] and reflect the different evolutionary adaptations of these species to their specific environment. It is thus extremely puzzling why in non-mammalian species, one xenosensor is sufficient whereas two xenobiotic-sensing nuclear receptors have evolved in mammals. Furthermore, it is unclear why in addition to the ligand-activated PXR, mammalian genomes encode CAR, a nuclear receptor that is unorthodox in many ways. On one hand, CAR and PXR might just share the workload in hepatic detoxification of xenobiotics. On the other hand, evidence accumulated in recent years that both PXR and CAR have functions that go beyond detoxification. As example, PXR and CAR form an intricate network with other nuclear receptors and transcription factors to regulate hepatic cholesterol and bile acid homeostasis [60]. It is thus conceivable that these receptors have so-far unidentified functions in mammals which require two receptors and that are thus absent in non-mammalian species. Therefore, further insights into the evolution of drug-sensing nuclear receptors are extremely important in order to gain novel insights into the role of these factors in the physiology and pathophysiology of the liver.

Methods

LMH and CV-1 cell culture, transfection and reporter gene assays

Culture and transfection of LMH cells with FUGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland) were performed as published [17,33]. Before transfections, LMH cells were kept in serum-free medium for 24 hours. CV-1 cell transactivation assays have been described in detail [17,33]. Sixteen or twenty-four hours after drug-treatment, cells were harvested and assays for CAT expression using a CAT ELISA Kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland). CAT concentrations were normalized against β-galactosidase activities to compensate for different transfection efficiencies.

Isolation of the CXR gene

Chicken BAC filters (UK Human Genome Mapping Project Resource Center, UK) were hybridised with a probe encoding for CXR. Positive clones were purchased, digested with different restriction enzymes and Southern blots obtained using the same probe. Bands hybridising with the CXR probe were isolated, subcloned and CXR genomic information obtained by PCR using primers designed after the CXR mRNA sequence.

Site-directed mutagenesis

Mutagenesis was carried out using overlapping primers as described [17]. Mutated fragments were excised, cloned into new vectors and verified by sequencing.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays have been described in detail [33]. Proteins were expressed using the TNT *in vitro* transcription/translation kit (Promega, Wallisellen, Switzerland) before being subjected to non-denaturing SDS-polyacrylamide gel electrophoresis with [^{32}P]-radiolabeled CYP2H1 264-bp PBRU.

Targeting of CXR in LMH cells by RNAi

Expression of CXR in LMH cells was repressed by RNAi as described [61]. In brief, a 19 bp fragment ranging from position 857 to 875 in the open reading frame of CXR was chosen for targeting. A double-stranded oligonucleotide containing this sequence and compatible ends for cloning into pSUPER was obtained by annealing single stranded oligonucleotides for the sense (GATCCCCGGATGGGGCTCTGGCCGGCTTCAAGAGAGCCGGCCAGAGCCCCATCCTTTTTGGAAA) and the anti-sense strand (AGCTTTTCCAAAAAGGATGGGGCTCTGGCCGGCTCTCTTGAAGCCGGCCAGAGCCCCATCCGGG) and subsequent ligation into pSUPER cut with BglII and HindIII (underlined letters refer to CXR-specific targeting sequence). After verification of the ligation product the pSUPER-CXR-RNAi expression cassette was cut out using BamHI and XhoI and subcloned into BglII/XhoI-digested pcDNA3 (Invitrogen, Carlsbad, USA). The ScaI-linearised construct was transfected into LMH cells using FUGENE 6 (Roche Molecular Biochemicals, Rotkreuz, Switzerland). Stable transfectants were selected by addition of 175 μg/

ml G418 (PAA Laboratories, Pasching, Austria) to the cell culture medium. A control cell line was selected in parallel which was stably transfected with pcDNA3 carrying the empty pSUPER expression cassette. Reporter gene assays in LMH cells using the CXR-RNAi clones were performed using reporter constructs for CYP2H1, CYP3A37, CYP2C45 and ALAS-1 described previously [17,24-26].

Cellular localization studies

LMH cells were cultivated on glass cover slips and subsequently transfected with pEGFP-C1 or pEGFP-N1 expression plasmids (Clontech, Allschwil, Switzerland) before cells were either treated with vehicle, 400 μM PB or 0.1 μM okadaic acid for 16 hours. Cells were washed with PBS, fixed in 3% formaldehyde for 30 minutes, washed again with PBS, stained with 300 nM DAPI and subsequently mounted on glass slides. Digital images were captured using a Leica DC 300F camera (Leica, Nidau, Switzerland) mounted on a Leitz DMRB microscope with the Leica IM50 Image Manager program version 1.20. Figures were assembled with Adobe Photoshop version 5.0.

CXR antibodies, nuclear extracts and Western blots

CXR ligand-binding domain was expressed in bacteria, purified and injected into rabbits for antibody production according to standard procedures. Anti-CXR-ligand-binding domain antibody from rabbit serum was subsequently used in Western blots. LMH cells were grown under standard conditions and treated with vehicle or 400 μM PB overnight. Cells were subsequently washed with PBS and protein extracts prepared using RIPA buffer. As control, CV-1 cells were transfected with empty pSG5 expression vector or vector expressing CXR and subsequently lysed with RIPA buffer. Nuclear extracts were prepared as published [62].

Northern hybridisation

LMH cells were treated with the indicated compounds for 16 hours before total RNA was isolated using the TRIZOL Reagent (Life Technologies, Basel, Switzerland). Twenty μg of total RNA were subjected to electrophoresis and analysed in Northern hybridisations as described [17,33].

List of Abbreviations

CYP, cytochrome P450; PB, phenobarbital; PXR, pregnane X receptor; CAR, constitutive androstane receptor; CXR, chicken xenobiotic receptor; PBRU, phenobarbital-responsive enhancer unit; ALAS-1, δ-aminolevulinate synthase; AF-1/2, activation function-1/2; LXR, liver X receptor; PB, phenobarbital; XRS, xenochemical response signal; GFP, green fluorescent protein; PPAR, peroxisome-proliferator activated receptor; FXR, farnesoid X receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CH carried out the cellular localization assays, cloned the various CXR mutants, performed the reporter gene and the electrophoretic-mobility shift assays as well as the transcriptional regulation studies. SB did the various screens for further chicken xenobiotic-sensing nuclear receptors. AR performed the RNAi experiments. RL and MO isolated the CXR antibody and carried out the protein stabilization and localization assays. MRK helped with the RNAi experiments. MP and CG helped with the CV-1 cell transactivation assays. UAM conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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The mRNA sequences for chicken estrogen-related receptor γ (ERRγ) and a partial fragment of ear2 have been submitted to Genbank under accession numbers AY702438 and AY702439, respectively.

References

1. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW: **P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature.** *Pharmacogenetics* 1996, **6:**1-42.
2. Waxman DJ, Azaroff L: **Phenobarbital induction of cytochrome P-450 gene expression.** *Biochem J* 1992, **281:**577-592.
3. Frueh FW, Zanger UM, Meyer UA: **Extent and Character of Phenobarbital-Mediated Changes in Gene Expression in Liver.** *Mol Pharmacol* 1997, **51:**363-369.
4. Sueyoshi T, Negishi M: **Phenobarbital response elements of cytochrome p450 genes and nuclear receptors.** *Annu Rev Pharmacol Toxicol* 2001, **41:**123-143.
5. Goodwin B, Redinbo MR, Kliewer SA: **Regulation of cyp3a gene transcription by the pregnane x receptor.** *Annu Rev Pharmacol Toxicol* 2002, **42:**1-23.
6. Handschin C, Meyer UA: **Induction of drug metabolism: the role of nuclear receptors.** *Pharmacol Rev* 2003, **55:**649-673.
7. Handschin C, Podvinec M, Meyer UA: **In silico approaches, and in vitro and in vivo experiments to predict induction of drug metabolism.** *Drug News Perspect* 2003, **16:**423-434.
8. Honkakoski P, Zelko I, Sueyoshi T, Negishi M: **The Nuclear Orphan Receptor CAR-Retinoid X Receptor Heterodimer Activates the Phenobarbital-Responsive Enhancer Module of the CYP2B Gene.** *Mol Cell Biol* 1998, **18:**5652-5658.
9. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM: **An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway.** *Cell* 1998, **92:**73-82.
10. Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD: **The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism.** *Nature* 2000, **407:**920-923.
11. Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, Evans RM: **Humanized xenobiotic response in mice expressing nuclear receptor SXR.** *Nature* 2000, **406:**435-439.
12. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA: **The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity.** *Proc Natl Acad Sci U S A* 2001, **98:**3369-3374.
13. Handschin C, Podvinec M, Meyer UA: **CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR).** *Proc Natl Acad Sci U S A* 2000, **97:**10769-10774.

14. Maglich JM, Caravella JA, Lambert MH, Willson TM, Moore JT, Ramamurthy L: **The first completed genome sequence from a teleost fish (Fugu rubripes) adds significant diversity to the nuclear receptor superfamily.** *Nucleic Acids Res* 2003, **31:**4051-4058.
15. Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH, Moore JT: **Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR), and Benzoate X Receptor (BXR) Define Three Pharmacologically Distinct Classes of Nuclear Receptors.** *Mol Endocrinol* 2002, **16:**977-986.
16. Lindblom TH, Pierce GJ, Sluder AE: **A C. elegans orphan nuclear receptor contributes to xenobiotic resistance.** *Curr Biol* 2001, **11:**864-868.
17. Handschin C, Meyer UA: **A Conserved Nuclear Receptor Consensus Sequence (DR-4) Mediates Transcriptional Activation of the Chicken CYP2H1 Gene by Phenobarbital in a Hepatoma Cell Line.** *J Biol Chem* 2000, **275:**13362-13369.
18. Handschin C, Podvinec M, Stöckli J, Hoffmann K, Meyer UA: **Conservation of signaling pathways of xenobiotic-sensing orphan nuclear receptors, chicken xenobiotic receptor, constitutive androstane receptor, and pregnane x receptor, from birds to humans.** *Mol Endocrinol* 2001, **15:**1571-1585.
19. Honkakoski P, Sueyoshi T, Negishi M: **Drug-activated nuclear receptors CAR and PXR.** *Ann Med* 2003, **35:**172-182.
20. Grün F, Venkatesan RN, Tabb MM, Zhou C, Cao J, Hemmati D, Blumberg B: **Benzoate X receptor alpha and beta are pharmacologically distinct and do not function as xenobiotic receptors.** *J Biol Chem* 2002, **277:**43691-43697.
21. Zhang Z, Burch PE, Cooney AJ, Lanz RB, Pereira FA, Wu J, Gibbs RA, Weinstock G, Wheeler DA: **Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome.** *Genome Res* 2004, **14:**580-590.
22. Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT: **The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution.** *Mol Endocrinol* 2000, **14:**27-39.
23. Becker-Andre M, Andre E, DeLamarter JF: **Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences.** *Biochem Biophys Res Commun* 1993, **194:**1371-1379.
24. Podvinec M, Kaufmann MR, Handschin C, Meyer UA: **NUBIScan, an in Silico Approach for Prediction of Nuclear Receptor Response Elements.** *Mol Endocrinol* 2002, **16:**1269-1279.
25. Baader M, Gnerre C, Stegeman JJ, Meyer UA: **Transcriptional activation of cytochrome P450 CYP2C45 by drugs is mediated by the chicken xenobiotic receptor (CXR) interacting with a phenobarbital response enhancer unit.** *J Biol Chem* 2002, **277:**15647-15653.
26. Fraser DJ, Podvinec M, Kaufmann MR, Meyer UA: **Drugs Mediate the Transcriptional Activation of the 5-Aminolevulinic Acid Synthase (ALAS1) Gene via the Chicken Xenobiotic-sensing Nuclear Receptor (CXR).** *J Biol Chem* 2002, **277:**34717-34726.
27. Fraser DJ, Zumsteg A, Meyer UA: **Nuclear receptors constitutive androstane receptor and pregnane X receptor activate a drug-responsive enhancer of the murine 5-aminolevulinic acid synthase gene.** *J Biol Chem* 2003, **278:**39392-39401.
28. Mangelsdorf DJ, Evans RM: **The RXR heterodimers and orphan receptors.** *Cell* 1995, **83:**841-850.
29. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM: **The nuclear receptor superfamily: the second decade.** *Cell* 1995, **83:**835-839.
30. Damm K, Thompson CC, Evans RM: **Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist.** *Nature* 1989, **339:**593-597.
31. Bamberger CM, Schulte HM, Chrousos GP: **Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids.** *Endocr Rev* 1996, **17:**245-261.
32. Kocarek TA, Shenoy SD, Mercer-Haines NA, Runge-Morris M: **Use of dominant negative nuclear receptors to study xenobiotic-inducible gene expression in primary cultured hepatocytes.** *J Pharmacol Toxicol Methods* 2002, **47:**177-187.
33. Handschin C, Podvinec M, Looser R, Amherd R, Meyer UA: **Multiple enhancer units mediate drug induction of cyp2h1 by xenobiotic-sensing orphan nuclear receptor chicken xenobiotic receptor.** *Mol Pharmacol* 2001, **60:**681-689.
34. Moore JT, Moore LB, Maglich JM, Kliewer SA: **Functional and structural comparison of PXR and CAR.** *Biochim Biophys Acta* 2003, **1619:**235-238.
35. Swales K, Negishi M: **CAR, Driving into the Future.** *Mol Endocrinol* 2004, **18:**1589-1598.
36. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M: **Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene.** *Mol Cell Biol* 1999, **19:**6318-6322.
37. Zelko I, Sueyoshi T, Kawamoto T, Moore R, Negishi M: **The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver.** *Mol Cell Biol* 2001, **21:**2838-2846.
38. Huang W, Zhang J, Chua SS, Qatanani M, Han Y, Granata R, Moore DD: **Induction of bilirubin clearance by the constitutive androstane receptor (CAR).** *Proc Natl Acad Sci U S A* 2003, **100:**4156-4161.
39. Squires EJ, Sueyoshi T, Negishi M: **Cytoplasmic localization of PXR and ligand-dependent nuclear translocation in mouse liver.** *J Biol Chem* 2004, **in press:**.
40. Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K, Kawajiri K: **Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR.** *Mol Pharmacol* 2003, **63:**524-531.
41. Kobayashi K, Sueyoshi T, Inoue K, Moore R, Negishi M: **Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells.** *Mol Pharmacol* 2003, **64:**1069-1075.
42. Yoshinari K, Kobayashi K, Moore R, Kawamoto T, Negishi M: **Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital.** *FEBS Lett* 2003, **548:**17-20.
43. Zelko I, Negishi M: **Phenobarbital-Elicited Activation of Nuclear Receptor CAR in Induction of Cytochrome P450 Genes.** *Biochem Biophys Res Commun* 2000, **277:**1-6.
44. Black BE, Holaska JM, Rastinejad F, Paschal BM: **DNA binding domains in diverse nuclear receptors function as nuclear export signals.** *Curr Biol* 2001, **11:**1749-1758.
45. Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, Paschal BM: **Calreticulin Is a Receptor for Nuclear Export.** *J Cell Biol* 2001, **152:**127-140.
46. Sidhu JS, Omiecinski CJ: **An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures.** *J Pharmacol Exp Ther* 1997, **282:**1122-1129.
47. Honkakoski P, Negishi M: **Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes.** *Biochem J* 1998, **330:**889-895.
48. Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ: **Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor.** *Eur J Biochem* 2001, **268:**6346-6358.
49. Pascussi JM, Drocourt L, Fabre JM, Maurel P, Vilarem MJ: **Dexamethasone Induces Pregnane X Receptor and Retinoid X Receptor-alpha Expression in Human Hepatocytes: Synergistic Increase of CYP3A4 Induction by Pregnane X Receptor Activators.** *Mol Pharmacol* 2000, **58:**361-372.
50. Pascussi JM, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ: **Dexamethasone Enhances Constitutive Androstane Receptor Expression in Human Hepatocytes: Consequences on Cytochrome P450 Gene Regulation.** *Mol Pharmacol* 2000, **58:**1441-1450.
51. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA: **The Human Orphan Nuclear Receptor PXR Is Activated by Compounds That Regulate CYP3A4 Gene Expression and Cause Drug Interaction.** *J Clin Invest* 1998, **102:**1016-1023.
52. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ: **Nuclear Receptors and Lipid Physiology: Opening the X-Files.** *Science* 2001, **294:**1866-1870.
53. Xie W, Evans RM: **Orphan nuclear receptors: the exotics of xenobiotics.** *J Biol Chem* 2001, **276:**37739-37742.

54. Handschin C, Podvinec M, Amherd R, Looser R, Ourlin JC, Meyer UA: **Cholesterol and Bile Acids Regulate Xenosensor Signaling in Drug- mediated Induction of Cytochromes P450.** *J Biol Chem* 2002, **277:**29561-29567.
55. Watkins RE, Maglich JM, Moore LB, Wisely GB, Noble SM, Davis-Searles PR, Lambert MH, Kliewer SA, Redinbo MR: **2.1 A crystal structure of human PXR in complex with the St. John's wort compound hyperforin.** *Biochemistry* 2003, **42:**1430-1438.
56. Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR: **The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity.** *Science* 2001, **292:**2329-2333.
57. Dussault I, Lin M, Hollister K, Fan M, Termini J, Sherman MA, Forman BM: **A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity.** *Mol Cell Biol* 2002, **22:**5270-5280.
58. Xiao L, Cui X, Madison V, White RE, Cheng KC: **Insights from a three-dimensional model into ligand binding to constitutive active receptor.** *Drug Metab Dispos* 2002, **30:**951-956.
59. Willson TM, Kliewer SA: **PXR, CAR and drug metabolism.** *Nat Rev Drug Discov* 2002, **1:**259-266.
60. Goodwin B, Kliewer SA: **Nuclear receptors. I. Nuclear receptors and bile acid homeostasis.** *Am J Physiol Gastrointest Liver Physiol* 2002, **282:**G926-31.
61. Brummelkamp TR, Bernards R, Agami R: **A system for stable expression of short interfering RNAs in mammalian cells.** *Science* 2002, **296:**550-553.
62. Dignam JD, Lebovitz RM, Roeder RG: **Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei.** *Nucleic Acids Res* 1983, **11:**1475-1489.

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Supplemental data

Human and mouse PXR and CAR genes contain 9 exons, 8 introns and highly conserved intron-exon junctions. Their amino acid sequences were aligned and compared with CXR highlighting conservation of the amino acids at the intron-exon boundaries (FIGURE 1.1, red amino acids). For identification and characterization of CXR introns, several primer pairs were designed at putative junctions (FIGURE 1.1, blue amino acids). A chicken genomic library was used as template in PCR reactions with different primer pairs. The PCR products were cloned, sequenced and examined on the existence of introns between the putative intron-exon junctions. After PCR condition improvements, all the primer pairs amplified pieces of CXR DNA sequences containing intron, only exception was the first intron because it is too large for amplification. Indeed, the position of the first intron-exon junction is not conserved among species and the intron length is very variable.

FIGURE 1.1

Intron-exon junctions of CARs, PXRs and CXR. Amino acid sequences of human and mouse CAR or PXR, and CXR were aligned. The exon-intron boundaries are shown in red and the primers used for intron amplification in blue.

Part II

Chicken AMP-Activated Protein Kinase Alpha Characterization

To test if AMPK catalytic subunits are expressed in LMH cells, chicken cDNA was used as template in PCR reactions with primers specific for AMPKα1 or AMPKα2. In order to design the primers, human AMPKα1 or AMPKα2 DNA sequences were used in a Blast search in the partial sequenced chicken genome (at that time). Several Contig sequences highly similar to the human sequences used as baits were found. The putative AMPKα1 was found on the chicken chromosome Z, whereas AMPKα2 was on the chromosome 8. Luckily, the 5' and the 3' end containing the putative chicken start and stop codons of the two subunits were found in the Blast search, allowing specific primer design on the basis of the Blast retrieved sequences.

Sequencing of the PCR fragments amplified with these primers revealed the identity of the amplified DNA sequences as chicken AMPKα1 and AMPKα2. The sequences of the newly characterized avian AMPK catalytic subunits were compared to the human and rat sequences on the amino acid level highlighting extremely high identity. The amino acid conservation is very high through the all sequence, but especially the kinase domain resulted almost completely conserved (FIGURE 2.0).

A

```
hAMPKα1          -MATAEKQKHDGRVKIGHYILGDTLGVGTFGKVKVGKHELTGHKVAVKILNRQKIRSLDV
cAMPKα1          MAAADKQKHEHGRVKIGHYILGDTLGVGTFGKVKVGKHELTGHKVAVKILNRQKIRSLDV
                   *: ::::..***************************************************

hAMPKα1          VGKIRREIQNLKLFRHPHIIKLYQVISTPSDIFMVMEYVSGGELFDYICKNGRLDEKESR
cAMPKα1          VGKIRREIQNLKLFRHPHIIKLYQVISTPTDIFMVMEYVSGGELFDYICKNGRLDEKESR
                 ********************************:*******************************

                                                                        172
hAMPKα1          RLFQQILSGVDYCHRHMVVHRDLKPENVLLDAHMNAKIADFGLSNMMSDGEFLRTSCGSP
cAMPKα1          RLFQQILSGVDYCHRHMVVHRDLKPENVLLDAHMNAKIADFGLSNMMSDGEFLRTSCGSP
                 ************************************************************

hAMPKα1          NYAAPEVISGRLYAGPEVDIWSSGVILYALLCGTLPFDDDHVPTLFKKICDGIFYTPQYL
cAMPKα1          NYAAPEVISGRLYAGPEVDIWSSGVILYALLCGTLPFDDDHVPTLFKKICDGIFYTPQYL
                 ************************************************************

hAMPKα1          NPSVISLLKHMLQVDPMKRASIKDIREHEWFKQDLPKYLFPEDPSYSSTMIDDEALKEVC
cAMPKα1          NPSVISLLKHMLQVDPMKRATIRDIREHEWFKQDLPKYLFPEDPSYSSTMIDDEALKEVC
                 **********************:*:*********************************

hAMPKα1          EKFECSEEEVLSCLYNRNHQDPLAVAYHLIIDNRRIMNEAKDFYLATSPPDSFLDDHHLT
cAMPKα1          EKFECTEEEVLSCLYSRNHQDPLAVAYHLIIDNRRIMNEAKDFYLATSPPDSFLDDHHLS
                 *****:********* .*****************************************:

hAMPKα1          RPHPERVPFLVAETPRARHTLDELNPQKSKHQGVRKAKWHLGIRSQSRPNDIMAEVCRAI
cAMPKα1          RPHPERVPFLVAEAPRPRHTLDELNPQKSKHQGVRRAKWHLGIRSQSRPNDIMAEVCRAI
                 *************:** .*****************:************************

hAMPKα1          KQLDYEWKVVNPYYLRVRRKNPVTSTYSKMSLQLYQVDSRTYLLDFRSIDDEITEAKSGT
cAMPKα1          KQLDYEWKVVNPYYLRVRRKNPVTSAYSKMSLQLYQVDSRTYLLDFRSIDDEIIETKSGT
                 *************************:*************************** *:****

hAMPKα1          ATPQRSGSVSNYRSCQRSDSDAEAQGKSSEVSLTSSVTS-LDSSPVDLTPRPGSHTIEFF
cAMPKα1          ATPQRSGSVSNYRSCQK-DSDGDAQGKSADTSLTSSMTSSLDSSTADLTPRPGSHTIEFF
                 ****************: *** .:*****::.*****:** **** ..**************

hAMPKα1          EMCANLIKILAQ
cAMPKα1          EMCANLIKILAQ
                 ************
```

FIGURE 2.0A

Chicken AMPKα subunits are similar to the mammalian orthologs. AMPKα subunits were characterized in LMH cells. AMPKα1 and α2 were amplified from LMH cell cDNA. The amplified products were sequenced and the derived amino acid sequences compared to human and rat sequences. **(A)** Chicken AMPKα1 amino acid sequence is compared to the human AMPKα1 sequence. The kinase domain is evidenced by red amino acids.

B

```
hAMPKα2          -MAEKQKHDGRVKIGHYVLGDTLGVGTFGKVKIGEHQLTGHKVAVKILNRQKIRSLDVVG
cAMPKα2          MADKQKHEHGRVKIGHYILGDTLGVGTFGKVKVGEHQLTGHKVAVKILNRQKIRSLDVVG
                    ::::..********:**************:****************************

hAMPKα2          KIKREIQNLKLFRHPHIIKLYQVISTPTDFFMVMEYVSGGELFDYICKHGRVEEMEARRL
cAMPKα2          KIKREIQNLKLFRHPHIIKLYQVISTPTDFFMVMEYVSGGELFDYICKHGRVEEAEARRL
                 ************************************************** *****

                                                                    172
hAMPKα2          FQQILSAVDYCHRHMVVHRDLKPENVLLDAHMNAKIADFGLSNMMSDGEFLRTSCGSPNY
cAMPKα2          FQQILSAVDYCHRHMVVHRDLKPENVLLDAHMNAKIADFGLSNMMSDGEFLRTSCGSPNY
                 **************************************************T*******

hAMPKα2          TAPEVISGRLYAGPEVDIWSCGVILYALLCGTLPFDDEHVPTLFKKIRGGVFYIPEYLNR
cAMPKα2          AAPEVISGRLYAGPEVDIWSCGVILYALLCGTLPFDDEHVPTLFKKIRGGVFYIPEYLNR
                 :************************************************************

hAMPKα2          SVATLLMHMLQVDPLKRATIKDIREHEWFKQGLPSYLFPEDPSYDANVIDDEAVKEVCEK
cAMPKα2          SVATLLMHMLQVDPLKRATIKDIREHEWFKEELPSYLFPEDPSYDATVIDDDAVREVCEK
                 ******************************: ***************.****:**:*****

hAMPKα2          FECTESEVMNSLYSGDPQDQLAVAYHLIIDNRRIMNQASEFYLASSPPSGSFMDDSAMHI
cAMPKα2          FECTESEVMNSLYSGDPQDQLAVAYHLVIDNRRIMNQASEFYLASSPPTGSFMDD-TMHI
                 ***************************:********************:******  :***

hAMPKα2          PPGLKPHPERMPPLIADSPKARCPLDALNTTKPKSLAVKKAKWRQGIRSQSKPYDIMAEV
cAMPKα2          PPGVKPHPERMPPLIADSPKARCPLDALNTTKPKPLTVKKAKWHLGIRSQSKPYDIMAEV
                 ***:*******************************.*:******: ***************

hAMPKα2          YRAMKQLDFEWKVVNAYHLRVRRKNPVTGNYVKMSLQLYLVDNRSYLLDFKSIDDEVVEQ
cAMPKα2          YRAMKQLDFEWKVVNSYHLRVRRKNPVTGNYVKMSLQLYQVDNRSYLLDFKSIDDEVMEQ
                 ***************:********************** ****************:**

hAMPKα2          RSGSSTPQRSCSAAGLHRPRSSFDSTTAESHSLSGSLTGSLTGSTLSSVSPRLGSHTMDF
cAMPKα2          RSGSSTPQRSCSAAGLHRPRLSIDAAAAAECQSLMGSLSGSFVGSISSVPPRLGSHTMDF
                 ******************** *:*:::* . .   .  ..   .::***.**********

hAMPKα2          FEMCASLITTLAR
cAMPKα2          FEMCASLIMALAR
                 ******** :***
```

	alpha1	alpha2
chicken AMPK		
human AMPK	95%	93%
rat AMPK	94%	93%

FIGURE 2.0B/C

Chicken AMPKα subunits are similar to the mammalian orthologs. (B) Chicken AMPKα2 amino acid sequence is compared to the human AMPKα2 sequence. The kinase domain is evidenced by red amino acids. **(C)** The amino acid identity between chicken, human or rat AMPKα sequences is shown in percentage.

In the Regulation of Cytochrome P450 Genes, Phenobarbital Targets LKB1 for Necessary Activation of AMP-Activated Protein Kinase

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Abbreviations

ACC, acetyl-CoA carboxylase; AICAR, 5'-phosphoribosyl-5-aminoimidazol-4-carboxamide; ALAS1, δ-aminolevulinic acid synthase 1; AMPK, AMP-activated protein kinase; CAR, constitutive androstane receptor; CXR, chicken xenobiotic receptor; CYPs, cytochromes P450; DNP, dinitrophenol; LMH, leghorn male hepatoma; mtDNA, mitochondrial DNA; NAC, N-acetyl L-cysteine; PB, phenobarbital; PXR, pregnane X receptor; ROS, reactive oxygen species; RT-PCR, real-time PCR; UCP-1, uncoupling protein 1.

Abstract

Transcriptional activation of cytochrome P450 (CYP) genes and various drug metabolizing enzymes by the prototypical inducer phenobarbital (PB) and many other drugs and chemicals is an adaptive response of the organism to exposure to xenobiotics. The response to PB is mediated by the nuclear receptor constitutive androstane receptor (CAR), whereas the chicken xenobiotic receptor (CXR) has been characterized as the PB mediator in chicken hepatocytes. Our previous results suggested an involvement of AMP-activated protein kinase (AMPK) in the molecular mechanism of PB induction. Here we show that the mechanism of AMPK activation is related to an effect of PB-type inducers on mitochondrial function with consequent formation of reactive oxygen species (ROS) and phosphorylation of AMPK by the upstream kinase LKB1. Gain- and loss-of-function experiments demonstrate that LKB1-activated AMPK is necessary in the mechanism of drug induction and that this is an evolutionary conserved pathway for detoxification of exogenous and endogenous chemicals. The activation of LKB1 adds a new proximal target to the so far elusive sequence of events by which PB and other drugs induce the transcription of multiple genes.

Introduction

Evolution has provided organisms with an elaborate defense system against foreign compounds (xenobiotics). The liver of vertebrates contains numerous enzymes that can transform potentially toxic xenobiotics (e.g. nutrients or drugs) or endobiotics (e.g. bile acids) to inactive and extretable metabolites. The expression of these enzymes can be adapted to the needs for detoxification by a process called induction. Phenobarbital (PB) is the prototype of a number of drugs that induce their own and the metabolism of other xenobiotics. Induction of drug metabolism is part of a pleiotropic response of the liver to xenobiotic exposure, which includes liver hypertrophy, tumor promotion, and induction of numerous genes in addition to those encoding for drug-metabolizing enzymes and drug transporters [1, 2]. PB also was shown to decrease the transcription of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase 1 (PEPCK1) and glucose-6-phosphatase (G6P) [3], and of several hepatic genes responsible for fatty acid metabolism [4]. Moreover, PB increases the transcription of δ-aminolevulinic acid synthase 1 (ALAS1), the rate-limiting enzyme in the synthesis of heme, the prosthetic group of cytochromes P450 (CYPs) [5-8]. The molecular details of the mechanisms by which PB causes these effects are incompletely understood.

The transcriptional activation by PB of genes encoding drug-metabolizing enzymes, such as *Cyp2b10* in mouse and *CYP2B6* in human, is mediated by the nuclear receptor constitutive androstane receptor (CAR) [9, 10]. Another xenobiotic-sensing receptor, the pregnane X receptor (PXR) also responds to drugs like pregnenolone-16alpha-carbonitrile (PCN), dexamethasone or rifampicin by enhancing the expression of *Cyp3a11* in mouse and *CYP3A4* in human as well as many other genes [11-13]. The role of CAR and PXR was clearly established in transgenic mice, as PB-mediated induction of *Cyp2b10* was completely abolished in $CAR^{-/-}$ mice [4], and PCN-mediated induction of *Cyp3a11* was absent in $PXR^{-/-}$ mice [14]. However, the interaction of PB with CAR is complex. PB apparently does not bind directly to CAR, but rather triggers its translocation from cytoplasm to the nucleus by as yet unknown mechanisms [15-17]. In addition, phosphorylation and dephosphorylation events strongly affect PB induction of CYPs [18-20].

Interestingly, some of the effects of PB on energy metabolism in the liver were found to be CAR-mediated. *Cyp2b10* is upregulated during fasting and in diabetes [21, 22] and insulin has a repressive effect on induction of CYPs [23]. These and other observations point to an interaction between the energy state of liver cells and expression of CYPs and to a physiological role of CAR in the responses to metabolic and nutritional stress.

An important energy sensor is AMP-activated protein kinase (AMPK). AMPK responds to any cellular stress that threatens to lower ATP levels by arresting non-essential ATP-utilizing functions and stimulating ATP-generating pathways [24]. Among the several genes regulated by AMPK is PEPCK1 [25] an effect also exerted by PB. Because the effect of PB on CAR is influenced by phosphorylation and dephosphorylation events and the regulation of some CYPs can be affected by metabolic and nutritional stress, we investigated the role of AMPK in the induction response. AMPK indeed was

shown to be activated during PB-mediated induction of *CYP2B6* in human hepatoma-derived cells [26] and in primary cultures of human and mouse hepatocytes [27]. However, the mechanism by which these drugs increase AMPK activity was unknown.

The phenomenon of PB induction appears conserved in evolution and has been observed in bacteria, insects, fish and birds [2]. We have recently shown that in chicken hepatoma cells, the CAR- and PXR-related chicken X receptor (CXR) confers PB-type induction by functionally identical or exchangeable signaling pathways triggered by the nuclear receptors CAR and PXR in mammals [28, 29]. In contrast to mammalian hepatoma cells, the chicken leghorn male hepatoma (LMH) cell line maintains a large spectrum of CYP gene induction by PB as well as by other drugs providing an accessible model for induction research [2]. Interestingly, PB responsiveness is a uniquely sensitive indicator of hepatocytes differentiation status [30].

In the present study, we have explored the mechanism by which AMPK is involved in the induction of three drug-inducible genes in chicken liver, namely *CYP2H1*, *CYP3A37* and *ALAS1*. Our data confirm dose-dependent increase of AMPK activity after exposure of LMH cells to PB and extend this effect to metyrapone. The role of AMPK is further established through downregulation of AMPK expression by siRNA or decreasing AMPK activity by an inhibitor, which strongly reduce the drug-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1* genes. Most importantly, we observed that PB and metyrapone increase mitochondrial reactive oxygen species (ROS) generation and trigger the interaction of AMPK with one of its upstream kinases, LKB1, a protein kinase encoded by the Peutz-Jegher syndrome tumor suppressor gene. Our experiments confirm and extend the involvement of AMPK signaling in liver drug responses as an evolutionary conserved system from birds to mammals and suggest a mechanism by which inducer drugs activate AMPK.

Results

AMPK activation by phenobarbital and metyrapone in LMH cells

Because AMPK signaling pathways have not been characterized previously in LMH cells, AMPKα1 and AMPKα2 protein expression, localization and activation were analyzed in these cells. The endogenous expression of the two protein subunits in LMH cells was investigated through western blots using antibodies for the human, rat and mouse kinases (SUPPLEMENTAL FIGURE 2.1A). Expression of the avian α1 and α2 subunits was detected albeit at a relatively lower level than in primary human hepatocytes, which were used as control for the antibodies. A thick unspecific band, whose identity is unknown, was observed with the antibody against AMPKα2 in LMH cells, in contrast to human hepatocytes. Apparently, these antibodies recognize the chicken AMPK subunits, meaning that the AMPKα amino acid conservation between chicken and mammals is very high. In fact, the LKB1/AMPK cascade in chicken was recently characterized, showing a high degree of evolutionary conservation with values of 92% and 99% amino acids identity for LKB1 and AMPKα kinase domains, respectively (Results – Part II p.38 and [31]).

The intracellular localization of the two proteins was studied by immunostaining using the same antibodies (SUPPLEMENTAL FIGURE 2.1B). Like in mammalian organisms [32], the protein recognized by α1 subunit antibodies was localized mostly in the cytoplasm, whereas the one recognized by α2 subunit antibodies was also present in the nucleus.

In order to test if a classical mammalian AMPK activator [33] would also increase chicken AMPK activity, LMH cells were exposed to AICAR. In mammalian cells, AICAR can usually double the kinase activity; the magnitude of activation in LMH cells is considerably lower with a 40-50% increase (FIGURE 2.1A). AICAR is a cell-permeable compound that is phosphorylated within cells by adenosine kinase to 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP), an AMP analogue. This molecule mimics the effect of AMP and activates AMPK. Thus, AICAR conversion in LMH cells might not be as efficient or as fast as in other cells, explaining the lower AMPK activation. Another possible explanation is that LMH cells are not as sensitive as other cell lines to AMP increases. In fact, when we measured AMP/ATP ratio in these cells, the AMP level was considerably higher than in several mammalian cell lines. Western blots with antibodies against phosphorylated AMPK-Thr172 and ACC-Ser79, which is an indication of AMPK activation, confirmed the increased AMPK activity upon AICAR treatment (FIGURE 2.1B).

These results clearly show that AMPKα1 and α2 are expressed in LMH cells and that they are comparable to the mammalian counterparts both in subcellular localization and activation by AICAR.

Recent studies have shown that PB activates AMPK in a human hepatoma-derived cell line [26]. To examine if this is also the case in our chicken cell line, two classical inducers of *CYP2H1*, *CYP3A37* and *ALAS1* [34], PB and metyrapone, were tested for their capacity to activate AMPK in LMH cells. Both compounds increased the AMPK activity after 1h treatment in a dose-dependent manner (FIGURE 2.1A), PB showing a higher activation level than metyrapone. Increased activity was confirmed to be

due to higher AMPK-Thr172 phosphorylation by western blots (FIGURE 2.1B). Phosphorylation of the AMPK target ACC also was increased. In this experiment, we thus demonstrate that two different inducers of CYPs trigger dose-dependent AMPK activation.

Activation or overexpression of AMPKα subunits affect *CYP2H1*, *CYP3A37* and *ALAS1* gene expression

In order to test if modulation of AMPK activity alone affects *CYP2H1*, *CYP3A37* and *ALAS1* gene expression, compounds known to activate AMPK by different mechanisms were tested. AICAR is a compound which mimics AMP accumulation [33], metformin was shown to enhance ROS production [35], and NaN_3 inhibits the complex IV of the respiratory chain provoking ATP depletion [36]. DNP uncouples the mitochondrial respiration leading to changes in the mitochondrial membrane potential [36], and rotenone is known to inhibit the complex I producing an initial increase in ROS production followed by ATP depletion [37]. After confirming that these compounds can effectively activate AMPK in LMH cells and lead to phosphorylation of AMPK-Thr172 and its target ACC-Ser79 (DATA NOT SHOWN 2.1), their effects on expression of CYP genes were examined. CYP2H1, CYP3A37 and ALAS1 mRNA were indeed increased by all compounds, but to a lower extent than by PB and metyrapone (FIGURE 2.2A + DATA NOT SHOWN FIGURE 2.6). By treating LMH cells with different AMPK activators, we could mimic the PB induction of *CYP2H1*, *CYP3A37* and *ALAS1*, but since it cannot be excluded that these compounds are direct inducers, we decided to overexpress AMPKα1 and AMPKα2 subunits.

When LMH cells were transiently transfected with rat AMPKα1 and AMPKα2 subunits, a strong increase of *CYP2H1, CYP3A37* and *ALAS1* induction by PB and metyrapone was observed (FIGURE 2.2B). The latter showed a more pronounced effect most likely due to the difference in transfection efficiency of the two constructs. The basal expression level of the three genes was not altered. The increase in CYP induction obtained by AMPKα transfection was moderate probably as a result of the limited availability of AMPKβ and AMPKγ subunits, which were not co-transfected, but are known to be necessary for AMPK activation. These data show that induction of *CYP2H1*, *CYP3A37* and *ALAS1* is enhanced by increasing the expression of AMPKα.

Downregulation of AMPKα activity by siRNA or Compound C decreases phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1*

To establish if AMPK expression is necessary for drug-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1*, AMPKα1 and AMPKα2 subunits were downregulated by gene-specific siRNA duplexes produced *in vitro* by recombinant Dicer enzyme [38-41]. mRNA levels of AMPKα1 and AMPKα2 were reduced to approximately 40% of the corresponding mRNAs of the control cells (SUPPLEMENTAL FIGURE 2.2A). None of the negative control genes (GAPDH and cyclophilin) were affected by AMPK-targeted siRNA duplexes (DATA NOT SHOWN FIGURE 2.2). The efficiency of AMPKα downregulation was tested also at the protein level by both activity assay and western blot. The AMPK activity was decreased to about 50% of the control by α1 and α2 specific d-siRNA both separately as well as by the combination of the

two (SUPPLEMENTAL FIGURE 2.2A). In addition, the protein expression levels were clearly reduced as shown by western blot (FIGURE 2.3A). MAPK protein expression was used as negative control for the d-siRNA specificity. Even if the downregulation of the two AMPKα subunits was not complete, it drastically reduced the effect of both PB and metyrapone on CYP2H1 and CYP3A37 mRNA (FIGURE 2.3B). A complete inhibition of the drug induction was not expected, as there was residual AMPK activity. In the case of ALAS1, the effect of siRNA was weaker, suggesting that ALAS1 is subject to other regulatory mechanisms.

Another way to modulate the AMPK activity is Compound C (cell-permeable pyrrazolopyrimidine compound), which is a specific and well-studied inhibitor of the kinase [42]. Preincubation of LMH cells with Compound C abolished the AMPK activation by DNP completely (SUPPLEMENTAL FIGURE 2.2B), confirming the potency of this inhibitor. The increased phosphorylation of AMPK-Thr172 and ACC-Ser79 achieved by incubating LMH cells with PB or metyrapone could be prevented by preincubation of the cells with Compound C without affecting the AMPKα total protein expression (FIGURE 2.3C). Preincubation of LMH cells with Compound C drastically reduced the increase in mRNA expression of *CYP2H1*, *CYP3A37* and *ALAS1* genes without changing the basal activity (FIGURE 2.3D).

All these studies firmly establish that an activation of AMPK is necessary for the effect of PB and metyrapone on the transcriptional activation of CYPs and also influences the regulation of ALAS1. Knowing that PB and metyrapone activate AMPK and that this kinase is essential to mediate their drug effects on CYP gene expression, we now focused on how these drugs can switch on AMPK activity.

The AMPK upstream kinase LKB1 interacts with AMPKα upon phenobarbital and metyrapone treatment

Several mechanisms of AMPK activation have been described, including ATP depletion, changes in mitochondrial membrane potential and formation of ROS. These mechanisms are not mutually exclusive and all involve the activation of AMPK by the upstream kinase LKB1. Since it is known that LKB1 is the upstream kinase of AMPK in the liver [43], we tested its function on the effects of PB and metyrapone on CYPs and ALAS1 mRNA. LKB1 and a LKB1 dominant negative (DN) form were transiently overexpressed in LMH cells. After treatment with PB or metyrapone, no statistically significant change in the CYP2H1, CYP3A37 and ALAS1 mRNA expression was detected with neither the wildtype nor the dominant negative form of LKB1 (SUPPLEMENTAL FIGURE 2.3A). The overexpression of the protein was confirmed by western blot with an antibody against the HA-tag and the AMPK activity was measured in LMH cells overexpressing LKB1 but no change in AMPK activity was detected by transfection of the LKB1 constructs (SUPPLEMENTAL FIGURE 2.3B). However, an LKB1 involvement cannot be excluded by this experiment, because it is known that this kinase forms an active heterotrimeric complex with two accessory proteins, Ste20-related adaptor protein (STRAD) and the mouse protein 25 (MO25) in the cytosol, but if overexpressed on its own, it is localized mainly in the nucleus [44]. Thus, STRAD and MO25 could be the limiting factors in LMH cells preventing the activation of transfected LKB1.

In addition to overexpression experiments, we also measured LKB1 activity upon PB and metyrapone treatment, but we could not detect a clear and statistically significant increase in activity (DATA NOT SHOWN FIGURE 2.3), probably due to the fact that LKB1 is constitutively active. LKB1, although necessary for the activation of AMPK by several mechanisms, including AICAR, metformin, phenformin, muscle contraction, or H_2O_2, in all these cases does not influence directly the LKB1 activity [45-48].

Therefore, a direct interaction between AMPK and LKB1 was considered. Coimmunoprecipitation experiments were performed to find out if PB and metyrapone trigger an LKB1-AMPKα interaction. Western blots of lysates immunoprecipitated with anti-LKB1 antibodies revealed the appearance of a protein corresponding to AMPKα after 20min of PB and metyrapone treatment, which disappeared at 40min of exposure (FIGURE 2.4A). Since AMPKα showed weak binding affinity to the protein G agarose beads in the preclearing step, we performed the vice versa experiment by immunoprecipitating AMPKα and assaying LKB1 by western blots. As a result a band after 20min appeared, which corresponds to LKB1 (FIGURE 2.4B). These experiments demonstrate that PB and metyrapone cause an interaction between LKB1 and AMPKα and establish LKB1 as a new target of these drugs.

Phenobarbital and metyrapone affect mitochondrial membrane potential, ROS production and phosphorylation of LKB1-Ser428

The involvement of LKB1 in the drug effects on CYPs led us to investigate by which mechanism PB and metyrapone prompt LKB1-AMPKα interaction and hence activate the latter by phosphorylation at Thr172. It was reported that some stimuli like AICAR provoke changes in the AMP/ATP ratio leading to AMPK activation by LKB1 [33]. Another recently described mechanism is the AMPK activation by metformin [35], which triggers mitochondrial ROS formation resulting in AMPK phosphorylation by LKB1. Troglitazone, an anti-diabetic drug, activates AMPK by a mechanism involving mitochondrial membrane depolarization, but in this case, LKB1 involvement was not investigated [49].

In our experiments, we could not detect changes in the AMP/ATP ratio caused by treatment with PB and metyrapone (DATA NOT SHOWN FIGURE 2.4), suggesting an AMP/ATP ratio-independent mechanism in this system. We then tested for effects of PB and metyrapone on the mitochondrial membrane potential applying the JC-1 dye, which can enter the mitochondrial membrane and form aggregates. If the potential drops, the dye is released into the cytoplasm as monomers. Aggregates and monomers emit fluorescence at two different wavelengths that can be monitored.

DNP, an uncoupler that leads to a membrane potential drop, was used as positive control. Exposure of LMH cells to 500μM PB or metyrapone, 1mM AICAR or 0.4mM DNP, caused changes in mitochondrial membrane potential (FIGURE 2.5A), whereas AICAR as expected had no effect.

Because an increase in ROS production can lead to AMPK activation [50], PB and metyrapone were then tested for their capacity to enhance intracellular ROS production. The DCFH-DA dye, a nonfluorescent and cell-permeable compound that is oxidized by intracellular ROS to yield the highly fluorescent product DCF, was employed for the measurements, the effects of rotenone served as

positive control. Both PB and metyrapone increased ROS production to a similar extent as did rotenone (FIGURE 2.5B). As expected, AICAR did not affect ROS levels, because of its different AMPK activating mechanism. ROS were recently shown to activate AMPK by promoting the phosphorylation of LKB1 at Ser428 [51]. PB and metyrapone also caused an increase in LKB1-Ser428 phosphorylation (FIGURE 2.5C). These data clearly show that PB and metyrapone affect mitochondrial functions.

Interference with ROS production affects phenobarbital- and metyrapone-mediated *CYP2H1*, *CYP3A37* and *ALAS1* gene expression and AMPK activation

To assess if PB- and metyrapone-stimulated ROS increase plays an important role in CYP induction, we decided to modulate the cellular ROS production by either overexpression of uncoupling protein 1 (UCP-1) or ROS scavenging by N-acetyl L-cysteine (NAC). UCP-1 is known to block the electron transfer of the respiratory chain and prevent $O_2^{\bar{}\bullet}$ formation [52]. For this reason, its effect on the drug-mediated *CYP2H1*, *CYP3A37* and *ALAS1* gene expression was tested. Transfection of UCP-1 in LMH cells provoked a considerable decrease of the effect of PB and metyrapone on mRNA levels of both CYP2H1 and CYP3A37 (FIGURE 2.6A), whereas the effect on ALAS1 was smaller. As expected, the AICAR effect on CYP induction was not altered. To establish if ROS production is indeed critical for AMPK activation by PB and metyrapone, the phosphorylation of AMPK-Thr172 and ACC-Ser79 was assessed in LMH cells overexpressing UCP-1 (FIGURE 2.6A). The AMPK and ACC phosphorylation normally triggered by PB and metyrapone was diminished by UCP-1, indicating that the increase of ROS produced by these drugs is linked to their ability to activate AMPK. In addition, ROS scavenging by NAC [53] strongly diminished the PB and metyrapone induction of *CYP2H1*, *CYP3A37* and *ALAS1* (FIGURE 2.6B) without altering the AICAR effect on these genes. Cotreatment of PB- or metyrapone-induced cells with NAC decreased AMPK-Thr172 and ACC-Ser79 phosphorylation (FIGURE 2.6B). These experiments show that ROS generation is necessary for activation of AMPK by PB and metyrapone. Since the mitochondrial respiration chain is the major site of ROS production, we investigated the role of mitochondria in drug induction.

Phenobarbital and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1* is mediated by effects on mitochondria

Variation of mitochondrial membrane potential and ROS production could be detected in response to PB and metyrapone. To provide evidence that mitochondria act as mediators of PB and metyrapone in the cascade, which leads to increased ROS production and subsequently to AMPK activation, mitochondrial DNA (mtDNA) was destroyed and consequently the number of functional mitochondria was lowered. Ethidium bromide is an inhibitor of DNA/RNA synthesis, which at low concentration more affects transcription and replication of extrachromosomal genetic components by intercalating into circular DNA without any detectable effect on nuclear DNA division. Treatment of cells with this reagent prevents mitochondrial DNA synthesis, leading to a smaller number of functional mitochondria. These cells are dependent on glycolysis to produce ATP and have to be supplied with

uridine and pyruvate to survive [54, 55]. In order to produce LMH cells with a decreased number of functional mitochondria, these cells were incubated for 12 weeks with ethidium bromide giving rise to the modified cell line LMHρ°. At the beginning of the treatment LMH cells grew at a slower rate, presumably because the cells have to switch first to glycolysis for ATP production as a consequence of their deficient mitochondrial respiration. After a short adaptation period, the cells were perfectly viable and normally dividing. Mitochondrial markers were monitored and NADH dehydrogenase 1 (ND1), cytochrome c oxidase 1 (COX1) and cytochrome b (Cytb) mRNA levels decreased to approximately 40% of the level of wildtype LMH cells (DATA NOT SHOWN 2.5), in contrast to GAPDH, which is expressed by the nuclear genome that was not affected by ethidium bromide treatment. After PB or metyrapone treatment, LMHρ° cells showed a drastically decreased induction of *CYP2H1*, *CYP3A37* and *ALAS1* gene expression (FIGURE 2.7) without a change of the basal level of the three genes in the LMHρ° cells. A complete inhibition of drug induction was not observed presumably due to still functional mitochondria present in the cells.

The effect of the mitochondrial poison rotenone and of AICAR was also tested in these cells. As expected, the AICAR induction of *CYP2H1, CYP3A37* and *ALAS1* was not affected in the LMHρ° cells indicating that the AICAR effect is not mediated by mitochondria. On the other hand, the rotenone effect on induction decreased, which was expected since this compound targets mitochondria (SUPPLEMENTAL FIGURE 2.4). These data imply an important role for mitochondria in the mechanism of drug induction and identify these organelles as targets of inducer drugs.

Discussion

In the present study we demonstrate that PB induces drug-metabolizing enzymes such as CYPs by a cascade of events that involves an initial interaction with mitochondrial function and leads to phosphorylation of LKB1, the upstream kinase of AMPK. LKB1 then phosphorylates the AMPKα subunit at Thr172 and activates AMPK. This activation of AMPK is a necessary step in the induction of these genes by PB, as recently shown in human hepatoma cells, primary mouse and human hepatocytes [27] and here in LMH cells.

The direct interaction of the two kinases LKB1 and AMPK was evidenced by coimmunoprecipitation experiments and was associated with the drug-induced phosphorylation of LKB1 at Ser428. The activation of LKB1 adds a new proximal target to the so-far elusive sequence of events by which PB induces the transcription of multiple genes.

Our findings also raise numerous new questions. For instance, do all PB-type inducers work by affecting LKB1 and thereby AMPK? Is activation of AMPK a necessary step of all inducers of CYPs? Is activation of AMPK sufficient to explain the pleiotropic effect of PB on gene transcription? Which kinase(s) activate LKB1 and how are these mechanisms related to mitochondrial functions?

The fact that metyrapone, another inducer drug, in all our experiments mimicked the dose-dependent response of PB, suggests an identical mechanism for this and possibly other inducers. An interesting drug is metformin, used in the treatment of diabetes, which increases AMPK activity by a similar or identical mechanism [35]. Recently, some studies proposed that this drug increases mitochondrial ROS production leading to AMPK activation. Clearly, if a compound like PB activates AMPK in a similar way than metformin, the question arises if PB can be used to treat diabetes. Indeed, PB has been used beneficially to improve glycemic control in patients with non-insulin-dependent diabetes mellitus by enhancing glucose utilization in the liver and glucose storage in muscle [56]. However, even if these two drugs activate AMPK by ROS increase, they do not share all their effects. Probably, metformin also has antidiabetic effects, which are not AMPK-mediated. In our experiments in cell culture metformin activated expression of CYPs as did PB and metyrapone, supporting the role of AMPK activation in the induction process. However, metformin apparently is not an inducer in animal or human liver *in vivo*. This is most probably due to its rapid renal excretion, which prevents sustained accumulation in the liver required for induction.

LKB1-AMPK activation is necessary for induction of CYP genes

The experiments reported here confirm and extend previous studies, which suggest that induction of CYPs by PB requires increased AMPK activity. This interpretation is derived from the following results: (a) AMPK activity is dose-dependently increased upon PB or metyrapone treatment; (b) AMPK overexpression enhances induction of CYPs by PB and metyrapone; (c) downregulation of AMPK expression by siRNA drastically reduced induction. These experiments in avian cells establish that

AMPK is necessary for the PB induction of CYPs and reveal evolutionary conservation of the mechanism of drug-mediated induction of CYPs.

Phenobarbital interacts with mitochondria

An important observation in explaining the effect of PB and metyrapone on LKB1 was that these drugs lead to increased production of ROS. This is suggested by the observation that overexpression of UCP-1 inhibits drug induction of CYPs and that ROS scavenging by NAC decreases this effect on drug induction. In addition, we prepared LMH cells with a decreased number of functional mitochondria and the PB and metyrapone effect on expression of CYPs was strongly decreased, indicating the role of these organelles in the drug-elicited effect. ROS are commonly thought to be toxic, resulting in oxidation of various cell constituents such as DNA, lipids and proteins and consequently these oxidations may cause damage to the cellular machinery leading to cell death as the ultimate consequence. ROS have been implicated in the etiology of a wide array of human diseases, including cancer. But evidence is now also accumulating that ROS might play a role as signaling molecules if tightly regulated. In particular, ROS affect stress-activated pathways like NF-κB signaling, which interestingly also is upregulated by PB [57]. Our experiments therefore strongly suggest a role for ROS in drug induction but the precise mechanism remains unknown and further studies are needed to unravel the direct downstream targets of these molecules.

Cell lines are tumour-derived cells and for this reason they may be less dependent than primary cells on the respiratory chain to generate ATP (aerobic metabolism). It is well known that cell lines usually have predominant anaerobic metabolism (glucose uptake and glycolysis) and low mitochondrial respiration. Our results show that mitochondrial functions are required by PB and metyrapone to exert their inducing effects on CYPs. Since in most cell lines the phenomenon of drug induction is not maintained, we speculate that this may be due to reduced mitochondrial function. If this is the case, LMH cells, which are highly inducible, should have higher aerobic metabolism in comparison to other cell lines. This hypothesis is currently under investigation. Our speculation could also explain why cell lines need higher drug doses to respond and induce CYPs in comparison to primary cells, which are more dependent on mitochondrial respiration. A correlation between mitochondrial dysfunctions/oxidative stress and diabetes has been repeatedly reported [58] and knowing that transcriptional regulation of CYPs is affected in diabetes suggests that these two effects may be somehow related. Moreover, the mechanism of PB and metyrapone-mediated induction of CYPs via effects of these drugs on mitochondria may also be considered in regard to the recent findings that mitochondrial dysfunction is involved in aging [59] and to the observation that in elderly there is a decline in drug metabolism capacity [60-62]. Mitochondria also are the site where ALAS1 is located and this enzyme is quickly upregulated upon drugs not only at the mRNA level but also at the activity level (Peyer AK, unpublished results). An interaction between drugs and mitochondria could allow a rapid ALAS1 activity regulation. All these observations strongly support a role of mitochondria in the response to inducer drugs.

How is AMPK activated?

AMPK is activated by several stimuli, which are sensed as stress for the cells/organism. Previous studies in a hepatoma cell line [26] and experiments done in our laboratory in primary cultures of human hepatocytes [27] detected AMP/ATP ratio changes in response to PB treatment. In LMH cells we could not detect changes in AMP/ATP ratio in response to PB and metyrapone but when we measured AMP, ADP and ATP levels by HPLC in these cells, we noticed that AMP is much higher in LMH cells as compared to other cell lines. This could mask the presence of an effect on the AMP/ATP ratio or make the cells less sensitive to AMP/ATP changes. We therefore cannot exclude that inducer drugs change the AMP/ATP ratio under specific conditions.

In this study we demonstrate changes in mitochondrial membrane potential and ROS generation caused by PB and metyrapone. These effects as well as AMP/ATP ratio changes are not mutually exclusive, suggesting that if inducer drugs target the mitochondria, several changes may occur at the same time. In fact, an inhibition of the mitochondrial respiration chain could explain all of these effects.

As a novel concept, our results propose mitochondria as a target for inducer drugs. Obviously, further studies are required to understand how exactly drugs affect these organelles.

AMPK targets in the mechanism of drug induction

Our results implicate LKB1/AMPK in the drug induction mechanism and also raise new questions about the target/s of this cascade. Which proteins does AMPK phosphorylate and how does this lead to drug-mediated increased expression of CYPs? Major efforts in our lab are directed to answer this question. Preliminary results show that the xenobiotic-sensing nuclear receptor CAR is not a direct target of AMPK (Matis M. et al, unpublished observation). However, dephosphorylation of CAR by the protein phosphatase 2A (PP2A) was recently shown to be necessary for nuclear translocation triggered by PB [63]. Is this dephosphorylation related to the AMPK activation caused by drugs? If CAR has to be dephosphorylated to translocate, which kinase does phosphorylate CAR?

AMPK activation by inducers could play a role in different ways. An interesting hypothesis is that AMPK affects transcriptional coactivators or corepressors important for nuclear receptor-mediated transcriptional regulation. Does AMPK phosphorylate cofactors interacting with CAR? This may be possible since AMPK was already shown to affect p300 [64] and peroxisome proliferators-activated receptor γ coactivator 1α (PGC-1α) [65], two transcriptional coactivators. Otherwise, AMPK may affect the CAR cytosolic complex and change the localization of the nuclear receptor. Does AMPK phosphorylate a component of this complex thus releasing CAR? Is AMPK activation affecting the nuclear export of CAR? In this regard, it was previously reported that importin alpha1, an adaptor protein involved in nuclear import, is a downstream target of AMPK [66]. Importin alpha1 interacts with the nuclear localization signal (NLS) of proteins and translocates them through the nuclear pore complex. It may be interesting to test if this protein also mediates CAR nuclear translocation, since two NLS were recently identified in the amino acid sequence of this nuclear receptor [67].

If we consider that AMPK is usually activated in stress situations, it is not unreasonable that this kinase is activated by compounds which upregulate CYPs. Drugs are probably sensed by organisms as a stress, because switch to drug metabolism for detoxification purposes is an energy consuming process. For this reason rapid initial AMPK activation by drugs could switch off unnecessary pathways allowing the organism to concentrate on the disposal of these compounds.

Transcriptional regulation of ALAS1

ALAS1 is the rate-limiting enzyme in heme synthesis and more than 80% of the heme synthesized in the liver is incorporated into CYPs. This enzyme is upregulated by drugs when the demand for reconstitution of CYPs increases. ALAS1 is transcriptionally regulated by the same nuclear receptors that drive drug-mediated induction of CYPs [6]. Thus it was reasonable to test if AMPK is also involved in ALAS1 drug upregulation. In our experiments, both by overexpression of AMPKα subunits or by siRNA dowregulation or by ROS scavenging, the effect on ALAS1 was present but not as strong as that on CYPs. This suggests that other mechanisms act on ALAS1 transcriptional regulation. This is likely, since the PB effect on ALAS1 was detected also in CAR-/- mice, indicating a CAR-independent mechanism. Since ALAS1 has a central role in heme production, it is reasonable to assume that this enzyme is tightly regulated by several pathways. In support of these data, we recently observed that the transcription coactivator PGC-1α, which is a target of AMPK, is responsible for the nutritional regulation of ALAS1 [68].

In conclusion, we demonstrate that PB- and metyrapone-mediated transcriptional regulation of three chicken hepatic drug-inducible genes, *CYP2H1*, *CYP3A37* and *ALAS1*, is achieved by a signaling cascade involving mitochondrial functions leading to ROS generation, LKB1 phosphorylation and consequent interaction with AMPK, which is in turn phosphorylated and activated. These findings add new knowledge regarding the mechanism by which PB and metyrapone lead to transcriptional regulation of CYPs via AMPK. To our knowledge this is the first time that inducers of CYPs have been revealed to affect the AMPK upstream kinase LKB1.

Future studies are needed to understand how ROS production leads to activation of AMPK, which is/are the downstream target/s of AMPK and which is the LKB1 upstream kinase in this pathway. The biotransformation and detoxification of xenobiotics and endobiotics metabolism evolved over millions of years. Understanding the molecular mechanism of drug-mediated induction of CYPs is of importance for the molecular links between expression of CYPs and the metabolic state of the liver, for the problems caused by drug-drug interactions and adverse drug reactions, and for the crosstalk between disposal of endogenous and exogenous molecules.

Materials and Methods

For methods used in these studies see the Material and Methods section at page 123.

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References

1. Frueh FW, Zanger UM, Meyer UA: **Extent and character of phenobarbital-mediated changes in gene expression in the liver**. *Mol Pharmacol* 1997, **51**(3):363-369.
2. Handschin C, Meyer UA: **Induction of drug metabolism: the role of nuclear receptors**. *Pharmacol Rev* 2003, **55**(4):649-673.
3. Kodama S, Koike C, Negishi M, Yamamoto Y: **Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes**. *Mol Cell Biol* 2004, **24**(18):7931-7940.
4. Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM, Negishi M: **Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital**. *Mol Pharmacol* 2002, **61**(1):1-6.
5. Fraser DJ, Podvinec M, Kaufmann MR, Meyer UA: **Drugs mediate the transcriptional activation of the 5-aminolevulinic acid synthase (ALAS1) gene via the chicken xenobiotic-sensing nuclear receptor (CXR)**. *J Biol Chem* 2002, **277**(38):34717-34726.
6. Podvinec M, Handschin C, Looser R, Meyer UA: **Identification of the xenosensors regulating human 5-aminolevulinate synthase**. *Proc Natl Acad Sci U S A* 2004, **101**(24):9127-9132.
7. Kakizaki S, Yamamoto Y, Ueda A, Moore R, Sueyoshi T, Negishi M: **Phenobarbital induction of drug/steroid-metabolizing enzymes and nuclear receptor CAR**. *Biochim Biophys Acta* 2003, **1619**(3):239-242.
8. Dogra SC, May BK: **Phenobarbital-induced activation of CYP2H1 and 5-aminolevulinate synthase genes in chick embryo hepatocytes is blocked by an inhibitor of protein phosphorylation**. *Arch Biochem Biophys* 1996, **327**(2):271-278.
9. Honkakoski P, Zelko I, Sueyoshi T, Negishi M: **The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene**. *Mol Cell Biol* 1998, **18**(10):5652-5658.
10. Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M: **The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene**. *J Biol Chem* 1999, **274**(10):6043-6046.
11. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA: **The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions**. *J Clin Invest* 1998, **102**(5):1016-1023.
12. Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES, Evans RM: **SXR, a novel steroid and xenobiotic-sensing nuclear receptor**. *Genes Dev* 1998, **12**(20):3195-3205.

13. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A: **Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction**. *Proc Natl Acad Sci U S A* 1998, **95**(21):12208-12213.
14. Staudinger J, Liu Y, Madan A, Habeebu S, Klaassen CD: **Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor**. *Drug Metab Dispos* 2001, **29**(11):1467-1472.
15. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M: **Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene**. *Mol Cell Biol* 1999, **19**(9):6318-6322.
16. Kobayashi K, Sueyoshi T, Inoue K, Moore R, Negishi M: **Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells**. *Mol Pharmacol* 2003, **64**(5):1069-1075.
17. Swales K, Negishi M: **CAR, driving into the future**. *Mol Endocrinol* 2004, **18**(7):1589-1598.
18. Sidhu JS, Omiecinski CJ: **An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures**. *J Pharmacol Exp Ther* 1997, **282**(2):1122-1129.
19. Honkakoski P, Negishi M: **Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes**. *Biochem J* 1998, **330 (Pt 2)**:889-895.
20. Joannard F, Galisteo M, Corcos L, Guillouzo A, Lagadic-Gossmann D: **Regulation of phenobarbital-induction of CYP2B and CYP3A genes in rat cultured hepatocytes: involvement of several serine/threonine protein kinases and phosphatases**. *Cell Biol Toxicol* 2000, **16**(5):325-337.
21. Sakuma T, Honma R, Maguchi S, Tamaki H, Nemoto N: **Different expression of hepatic and renal cytochrome P450s between the streptozotocin-induced diabetic mouse and rat**. *Xenobiotica* 2001, **31**(4):223-237.
22. Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, Moore JT: **The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction**. *J Biol Chem* 2004, **279**(19):19832-19838.
23. Yoshida Y, Kimura N, Oda H, Kakinuma A: **Insulin suppresses the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital in adult rat cultured hepatocytes**. *Biochem Biophys Res Commun* 1996, **229**(1):182-188.
24. Kahn BB, Alquier T, Carling D, Hardie DG: **AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism**. *Cell Metab* 2005, **1**(1):15-25.
25. Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C: **5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase**. *Diabetes* 2000, **49**(6):896-903.
26. Rencurel F, Stenhouse A, Hawley SA, Friedberg T, Hardie DG, Sutherland C, Wolf CR: **AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line**. *J Biol Chem* 2005, **280**(6):4367-4373.
27. Rencurel F, Foretz M, Kaufmann MR, Stroka D, Looser R, Leclerc I, da Silva Xavier G, Rutter GA, Viollet B, Meyer UA: **Stimulation of AMP-activated protein kinase is essential for the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver**. *Molecular Pharmacology* 2006, **in press**.
28. Handschin C, Podvinec M, Meyer UA: **CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR)**. *Proc Natl Acad Sci U S A* 2000, **97**(20):10769-10774.
29. Handschin C, Podvinec M, Stockli J, Hoffmann K, Meyer UA: **Conservation of signaling pathways of xenobiotic-sensing orphan nuclear receptors, chicken xenobiotic receptor, constitutive androstane receptor, and pregnane X receptor, from birds to humans**. *Mol Endocrinol* 2001, **15**(9):1571-1585.
30. Sidhu JS, Liu F, Omiecinski CJ: **Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and**

extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes. *Exp Cell Res* 2004, **292**(2):252-264.
31. Proszkowiec-Weglarz M, Richards MP, Ramachandran R, McMurtry JP: **Characterization of the AMP-activated protein kinase pathway in chickens**. *Comp Biochem Physiol B Biochem Mol Biol* 2006, **143**(1):92-106.
32. Salt I, Celler JW, Hawley SA, Prescott A, Woods A, Carling D, Hardie DG: **AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform**. *Biochem J* 1998, **334 (Pt 1)**:177-187.
33. Corton JM, Gillespie JG, Hawley SA, Hardie DG: **5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells?** *Eur J Biochem* 1995, **229**(2):558-565.
34. Handschin C, Meyer UA: **A conserved nuclear receptor consensus sequence (DR-4) mediates transcriptional activation of the chicken CYP2H1 gene by phenobarbital in a hepatoma cell line**. *J Biol Chem* 2000, **275**(18):13362-13369.
35. Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WGt, Schlattner U, Neumann D, Brownlee M, Freeman MB, Goldman MH: **Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species**. *J Biol Chem* 2004, **279**(42):43940-43951.
36. Witters LA, Nordlund AC, Marshall L: **Regulation of intracellular acetyl-CoA carboxylase by ATP depletors mimics the action of the 5'-AMP-activated protein kinase**. *Biochem Biophys Res Commun* 1991, **181**(3):1486-1492.
37. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ: **Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism**. *Diabetes* 2000, **49**(4):527-531.
38. Hagerkvist R, Mokhtari D, Myers JW, Tengholm A, Welsh N: **siRNA produced by recombinant dicer mediates efficient gene silencing in islet cells**. *Ann N Y Acad Sci* 2005, **1040**:114-122.
39. Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ: **Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy**. *Nat Biotechnol* 2005, **23**(2):222-226.
40. Myers JW, Ferrell JE: **Silencing gene expression with Dicer-generated siRNA pools**. *Methods Mol Biol* 2005, **309**:93-196.
41. Myers JW, Jones JT, Meyer T, Ferrell JE, Jr.: **Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing**. *Nat Biotechnol* 2003, **21**(3):324-328.
42. Kim EK, Miller I, Aja S, Landree LE, Pinn M, McFadden J, Kuhajda FP, Moran TH, Ronnett GV: **C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase**. *J Biol Chem* 2004, **279**(19):19970-19976.
43. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC: **The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin**. *Science* 2005, **310**(5754):1642-1646.
44. Boudeau J, Scott JW, Resta N, Deak M, Kieloch A, Komander D, Hardie DG, Prescott AR, van Aalten DM, Alessi DR: **Analysis of the LKB1-STRAD-MO25 complex**. *J Cell Sci* 2004, **117**(Pt 26):6365-6375.
45. Sakamoto K, Goransson O, Hardie DG, Alessi DR: **Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR**. *Am J Physiol Endocrinol Metab* 2004, **287**(2):E310-317.
46. Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, Alessi DR: **Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction**. *Embo J* 2005, **24**(10):1810-1820.
47. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC: **The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress**. *Proc Natl Acad Sci U S A* 2004, **101**(10):3329-3335.
48. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D: **LKB1 is the upstream kinase in the AMP-activated protein kinase cascade**. *Curr Biol* 2003, **13**(22):2004-2008.

49. Konrad D, Rudich A, Bilan PJ, Patel N, Richardson C, Witters LA, Klip A: **Troglitazone causes acute mitochondrial membrane depolarisation and an AMPK-mediated increase in glucose phosphorylation in muscle cells**. *Diabetologia* 2005, **48**(5):954-966.
50. Hwang JT, Ha J, Park OJ: **Combination of 5-fluorouracil and genistein induces apoptosis synergistically in chemo-resistant cancer cells through the modulation of AMPK and COX-2 signaling pathways**. *Biochem Biophys Res Commun* 2005, **332**(2):433-440.
51. Xie Z, Dong Y, Zhang M, Cui MZ, Cohen RA, Riek U, Neumann D, Schlattner U, Zou MH: **Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells**. *J Biol Chem* 2006, **281**(10):6366-6375.
52. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP *et al*: **Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage**. *Nature* 2000, **404**(6779):787-790.
53. Oeda T, Henkel R, Ohmori H, Schill WB: **Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility?** *Andrologia* 1997, **29**(3):125-131.
54. King MP, Attardi G: **Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation**. *Science* 1989, **246**(4929):500-503.
55. Zylber E, Vesco C, Penman S: **Selective inhibition of the synthesis of mitochondria-associated RNA by ethidium bromide**. *J Mol Biol* 1969, **44**(1):195-204.
56. Lahtela JT, Arranto AJ, Sotaniemi EA: **Enzyme inducers improve insulin sensitivity in non-insulin-dependent diabetic subjects**. *Diabetes* 1985, **34**(9):911-916.
57. Li Y, Leung LK, Spear BT, Glauert HP: **Activation of hepatic NF-kappaB by phenobarbital in rats**. *Biochem Biophys Res Commun* 1996, **229**(3):982-989.
58. Brownlee M: **Biochemistry and molecular cell biology of diabetic complications**. *Nature* 2001, **414**(6865):813-820.
59. Chomyn A, Attardi G: **MtDNA mutations in aging and apoptosis**. *Biochem Biophys Res Commun* 2003, **304**(3):519-529.
60. Loi CM, Vestal RE: **Drug metabolism in the elderly**. *Pharmacol Ther* 1988, **36**(1):131-149.
61. Sotaniemi EA, Arranto AJ, Pelkonen O, Pasanen M: **Age and cytochrome P450-linked drug metabolism in humans: an analysis of 226 subjects with equal histopathologic conditions**. *Clin Pharmacol Ther* 1997, **61**(3):331-339.
62. Woodhouse K, Wynne HA: **Age-related changes in hepatic function. Implications for drug therapy**. *Drugs Aging* 1992, **2**(3):243-255.
63. Hosseinpour F, Moore R, Negishi M, Sueyoshi T: **Serine-202 regulates the nuclear translocation of constitutive active/androstane receptor CAR**. *Mol Pharmacol* 2005.
64. Leff T: **AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins**. *Biochem Soc Trans* 2003, **31**(Pt 1):224-227.
65. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI: **AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation**. *Proc Natl Acad Sci U S A* 2002, **99**(25):15983-15987.
66. Wang W, Yang X, Kawai T, Lopez de Silanes I, Mazan-Mamczarz K, Chen P, Chook YM, Quensel C, Kohler M, Gorospe M: **AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1: involvement in the nuclear import of RNA-binding protein HuR**. *J Biol Chem* 2004, **279**(46):48376-48388.
67. Kanno Y, Suzuki M, Nakahama T, Inouye Y: **Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear receptor CAR**. *Biochim Biophys Acta* 2005, **1745**(2):215-222.
68. Handschin C, Lin J, Rhee J, Peyer AK, Chin S, Wu PH, Meyer UA, Spiegelman BM: **Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1alpha**. *Cell* 2005, **122**(4):505-515.

Figures

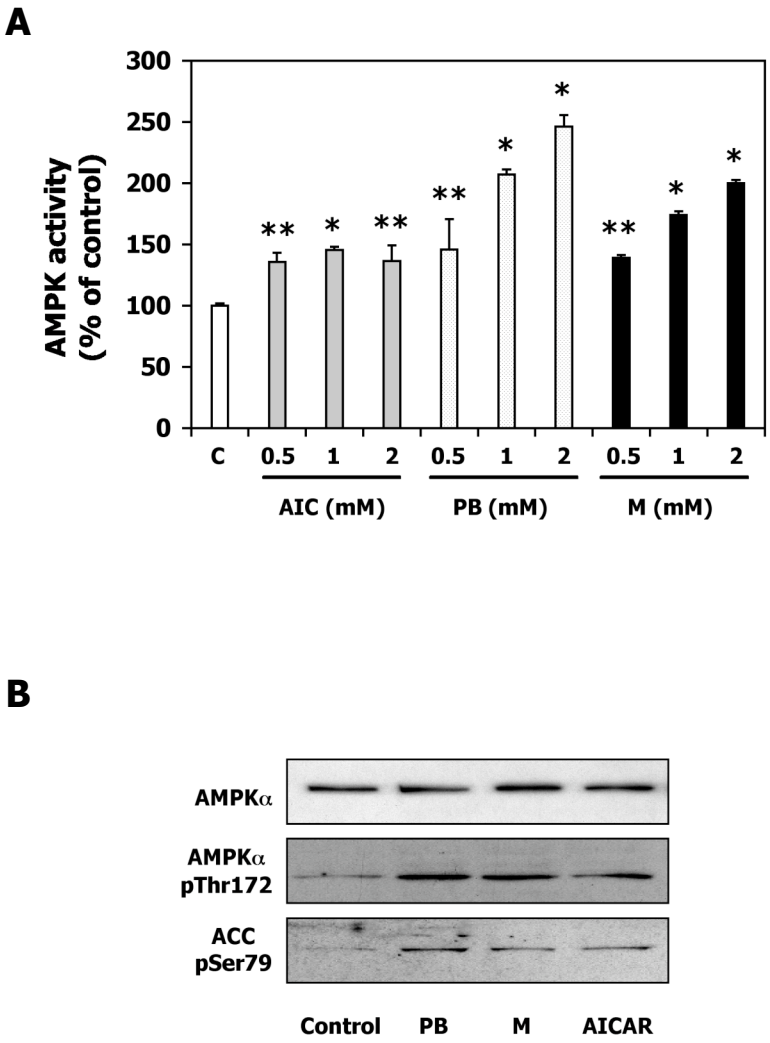


FIGURE 2.1

Chicken AMPKα subunits are activated by the classical activator AICAR and by phenobarbital-type inducers. (A) LMH cells were treated with increasing doses of AICAR, PB or metyrapone for 1h. AMPK activity was measured by SAMS peptide assay and shown as percentage of the control. *p<0.01, **p<0.05 (control versus treatment). **(B)** Phosphorylation of AMPK-Thr172 and ACC-Ser79 is shown by western blot using 500μM for all three compounds and the same conditions as for the activity measurements. M, metyrapone.

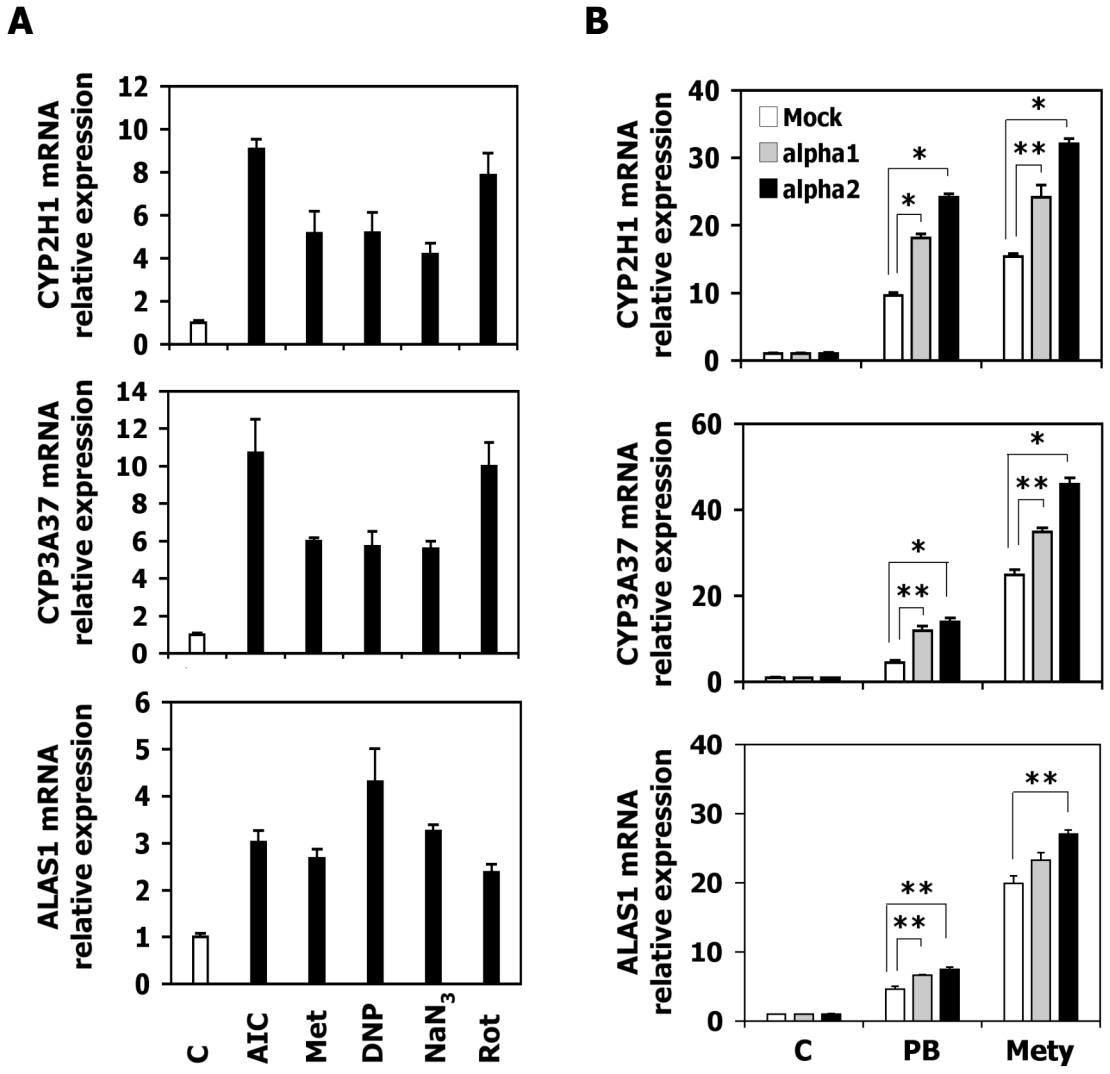


FIGURE 2.2

Activation or overexpression of AMPKα subunits affect *CYP2H1, CYP3A37* and *ALAS1* gene expression. (A) LMH cells were treated with 1mM AICAR (AIC) or metformin (Met), 0.2mM DNP, 1mM NaN_3, or 1μM rotenone (Rot) for 16h. Gene expression of *CYP2H1, CYP3A37* and *ALAS1* was analyzed by RT-PCR. $p<0.01$. **(B)** LMH cells transiently transfected with AMPKα subunits were treated with 500μM PB or metyrapone (Mety) for 16h. Gene expression of *CYP2H1*, *CYP3A37* and *ALAS1* was analyzed by RT-PCR. *$p<0.01$, **$p<0.05$.

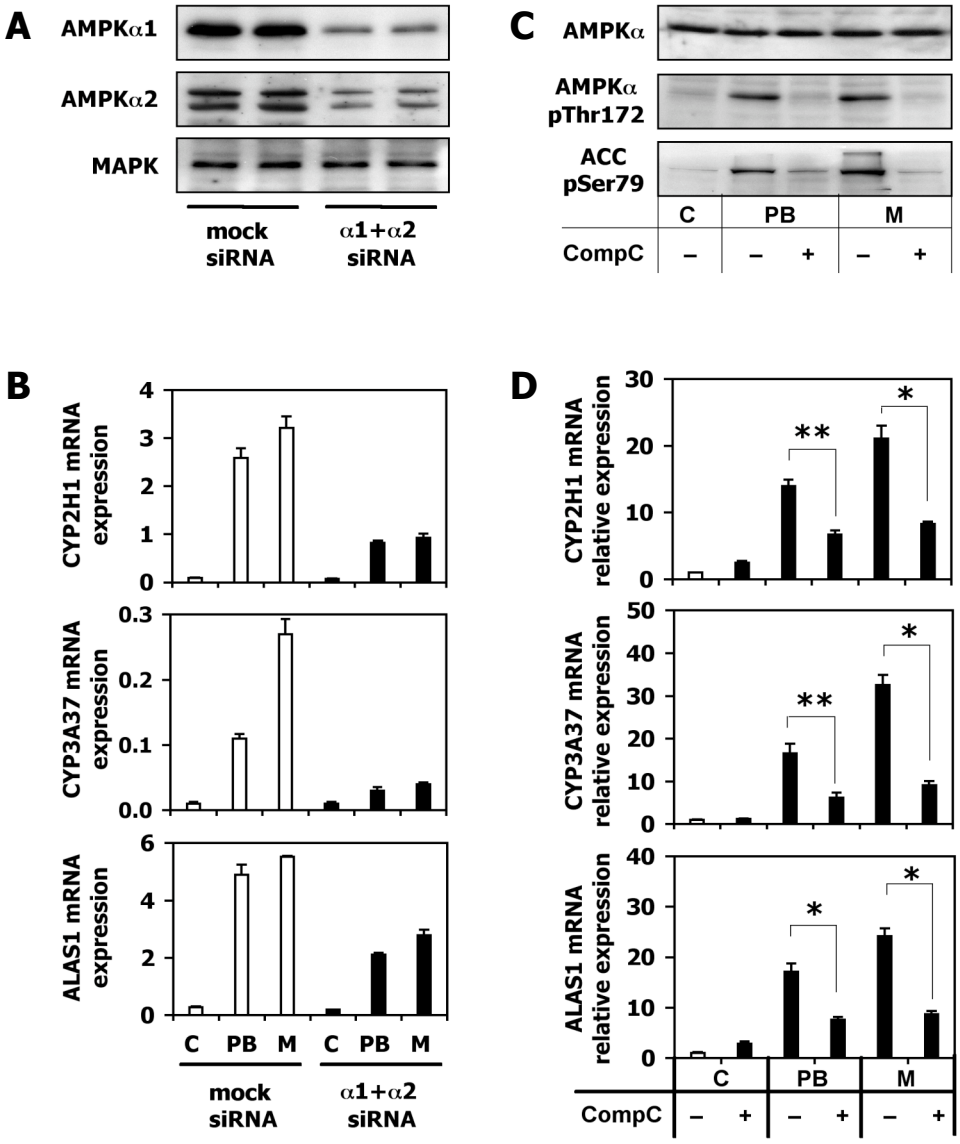


FIGURE 2.3

Downregulation of AMPKα activity by d-siRNA or Compound C decreases phenobarbital- and metyrapone-mediated induction of *CYP2H1, CYP3A37* and *ALAS1*. (A) LMH cells were transiently transfected with AMPKα1 and AMPKα2 specific d-siRNA. AMPKα protein expression was detected by western blots with antibodies against mammalian AMPKα1 or AMPKα2. MAPK protein expression was detected as negative control for d-siRNA unspecific downregulation. **(B)** LMH cells transiently transfected with d-siRNA were treated with 500μM PB or metyrapone for 16h. Expression of *CYP2H1*, *CYP3A37* and *ALAS1* was measured by RT-PCR. $p<0.01$ (mock d-siRNA versus α1+α2 d-siRNA). **(C)** Activation of AMPK by 500μM PB or metyrapone with or without 30min pretreatment with 20μM Compound C is shown by western blot evidencing the phosphorylation of AMPK-Thr172 and ACC-Ser79. **(D)** LMH cells were incubated 16h with 500μM PB or metyrapone with or without pretreatment for 30min by 20μM Compound C. mRNA level of CYP2H1, CYP3A37 and ALAS1 was measured by RT-PCR. *$p<0.01$, **$p<0.05$. M, metyrapone.

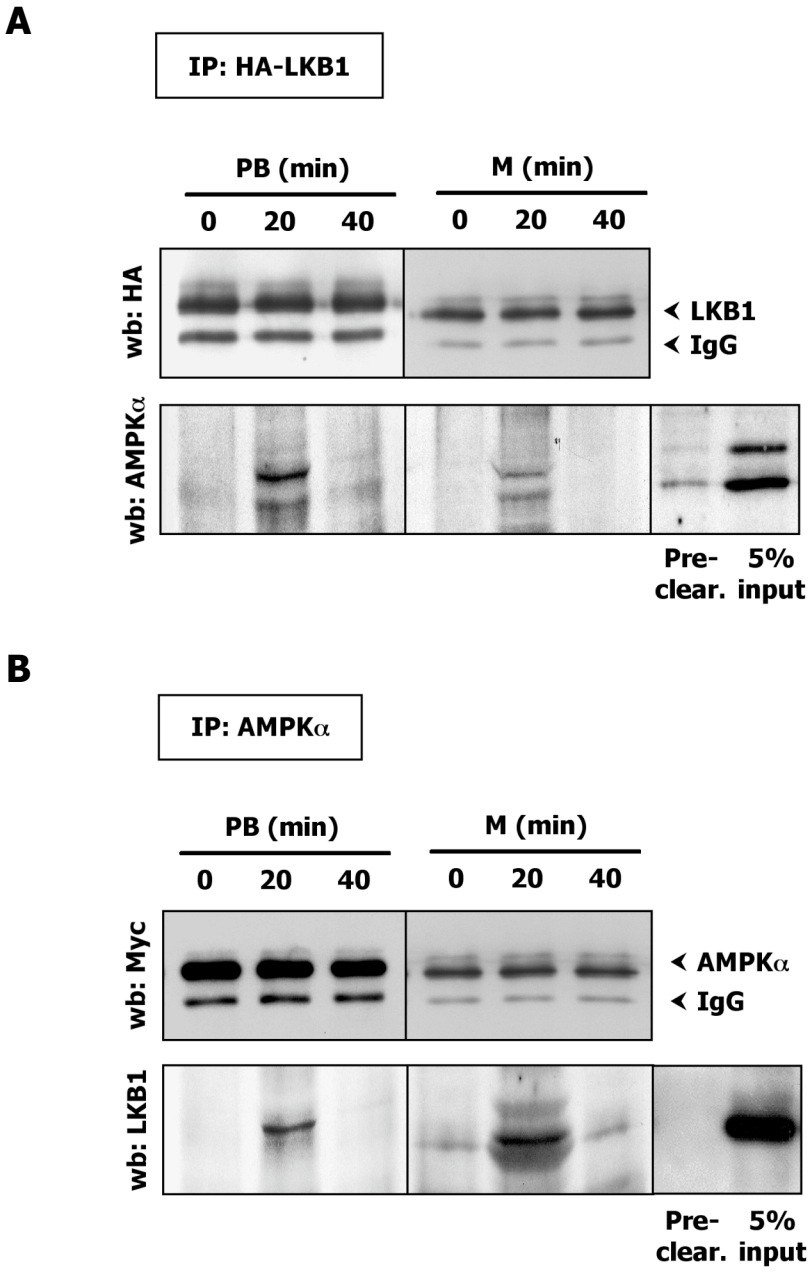


FIGURE 2.4

Phenobarbital and metyrapone trigger the interaction of AMPKα with its upstream kinase LKB1. (A) Immunoprecipitation of overexpressed HA-LKB1 by anti-HA antibody upon 500μM PB or metyrapone treatment at three different time points. Immunoprecipitation of HA-LKB1 was checked by western blot with anti-HA antibody. The western blot with anti-AMPKα antibody shows co-immunoprecipitation of AMPKα subunits with LKB1. **(B)** Immunoprecipitation of overexpressed Myc-AMPKα upon 500μM PB or metyrapone treatment. Immunoprecipitation of Myc-AMPKα was checked by western blot with anti-Myc antibody. The western blot with anti-LKB1 antibody shows co-immunoprecipitation of LKB1 with AMPKα. M, metyrapone; wb, western blot.

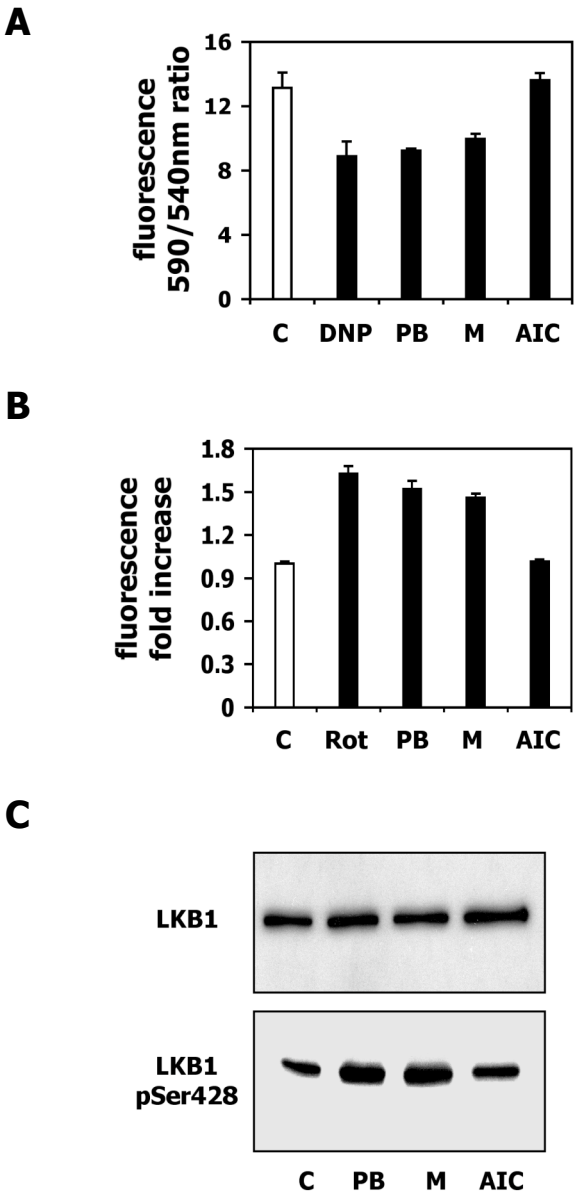


FIGURE 2.5

Phenobarbital and metyrapone affect mitochondrial membrane potential, ROS production and LKB1 phosphorylation. (A) LMH cells were incubated with the JC-1 fluorescent dye. Fluorescence was detected after 1h treatment with 500μM PB or metyrapone, 0.4mM DNP, or 1mM AICAR. The mitochondrial membrane potential is expressed as the 590nm/540nm fluorescence ratio in comparison to the control sample. $p<0.05$. **(B)** Intracellular ROS was detected by the DCFDA fluorescence dye. LMH cells were treated with 500μM PB or metyrapone, 5μM rotenone and 1mM AICAR for 1h and fluorescence was detected. The result is shown as fold increase in comparison to the control sample. $p<0.01$. **(C)** Phosphorylation of LKB1-Ser428 upon PB and metyrapone is proven by western blot with antibodies specific for the LKB1 phosphorylated form. M, metyrapone; Rot, rotenone; AIC, AICAR.

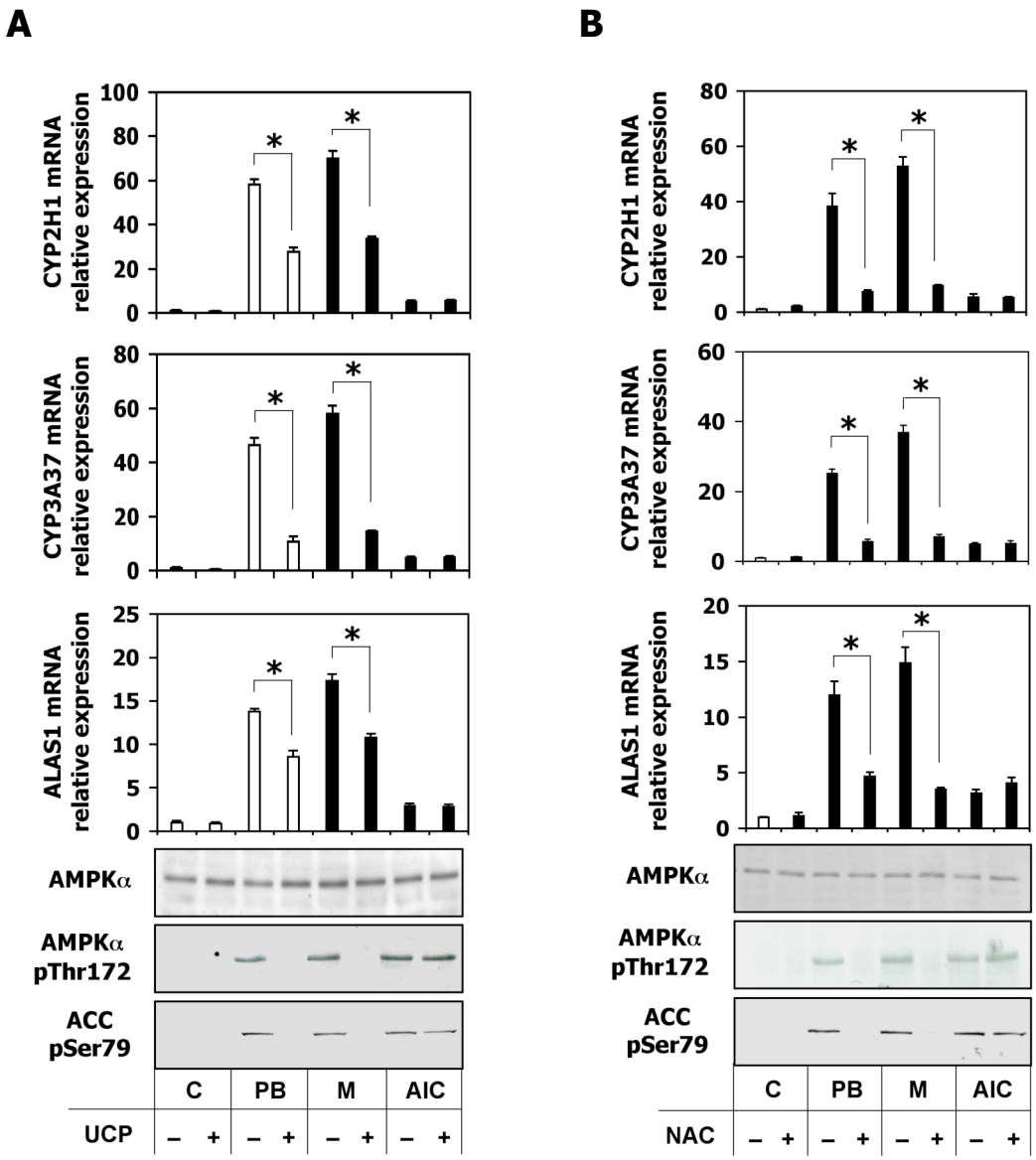


FIGURE 2.6

Decrease in intracellular ROS production by UCP-1 overexpression or by NAC-mediated scavenging attenuates drug-mediated increase of *CYP2H1*, *CYP3A37* and *ALAS1* gene expression and AMPK activity. (A) LMH cells were transiently transfected with UCP-1 and then incubated with 500μM PB, metyrapone or 1mM AICAR for 16h. *CYP2H1*, *CYP3A37* and *ALAS1* gene expression was detected by RT-PCR. *p<0.01. Activation of AMPK after 1h treatment with 500μM PB or metyrapone and 1mM AICAR is shown by western blot evidencing the phosphorylation of AMPK-Thr172 and ACC-Ser79. **(B)** LMH cells were incubated with 500μM PB or metyrapone, or 1mM AICAR, with or without 10mM NAC for 16h. CYP2H1, CYP3A37 and ALAS1 mRNA level was measured by RT-PCR. *p<0.01. Activation of AMPK after 1h treatment with 500μM PB or metyrapone, or 1mM AICAR with or without NAC is shown by phosphorylation of AMPK-Thr172 and ACC-Ser79. M, metyrapone; AIC, AICAR.

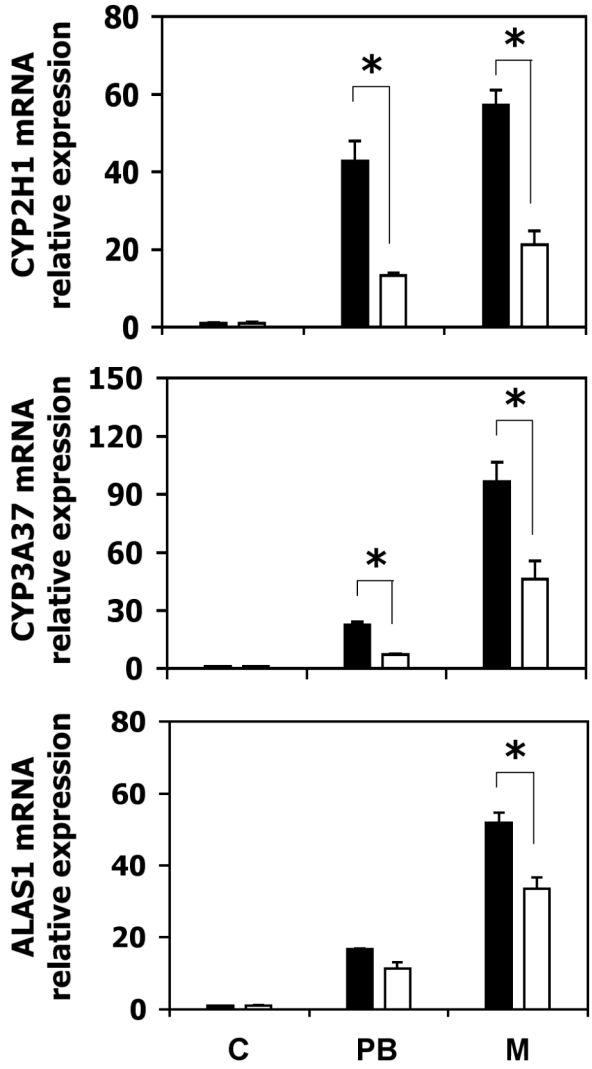
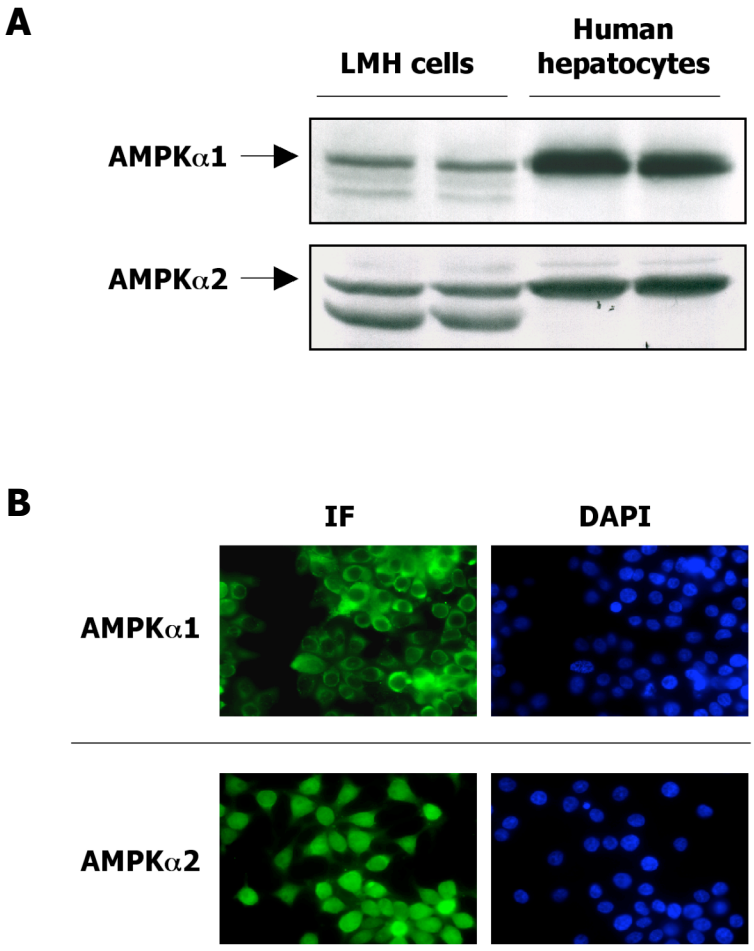


FIGURE 2.7

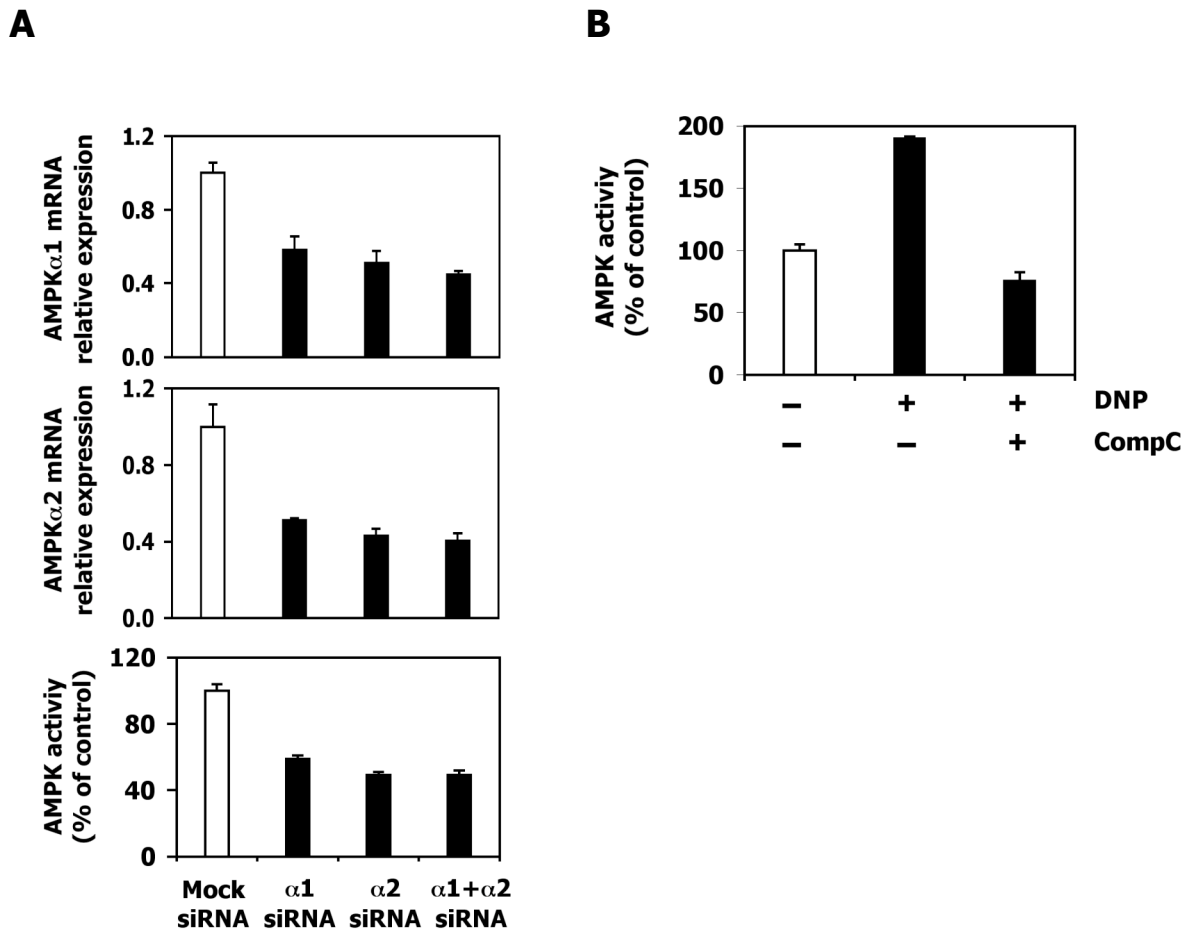
Mitochondrial functions are necessary for phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1* gene expression. LMH wildtype (■) or LMHρ^0 cells (□) were incubated with 500μM PB or metyrapone for 16h. CYP2H1, CYP3A37 and ALAS1 mRNA level was detected by RT-PCR. *p<0.01. M, metyrapone.

Supplemental Figures



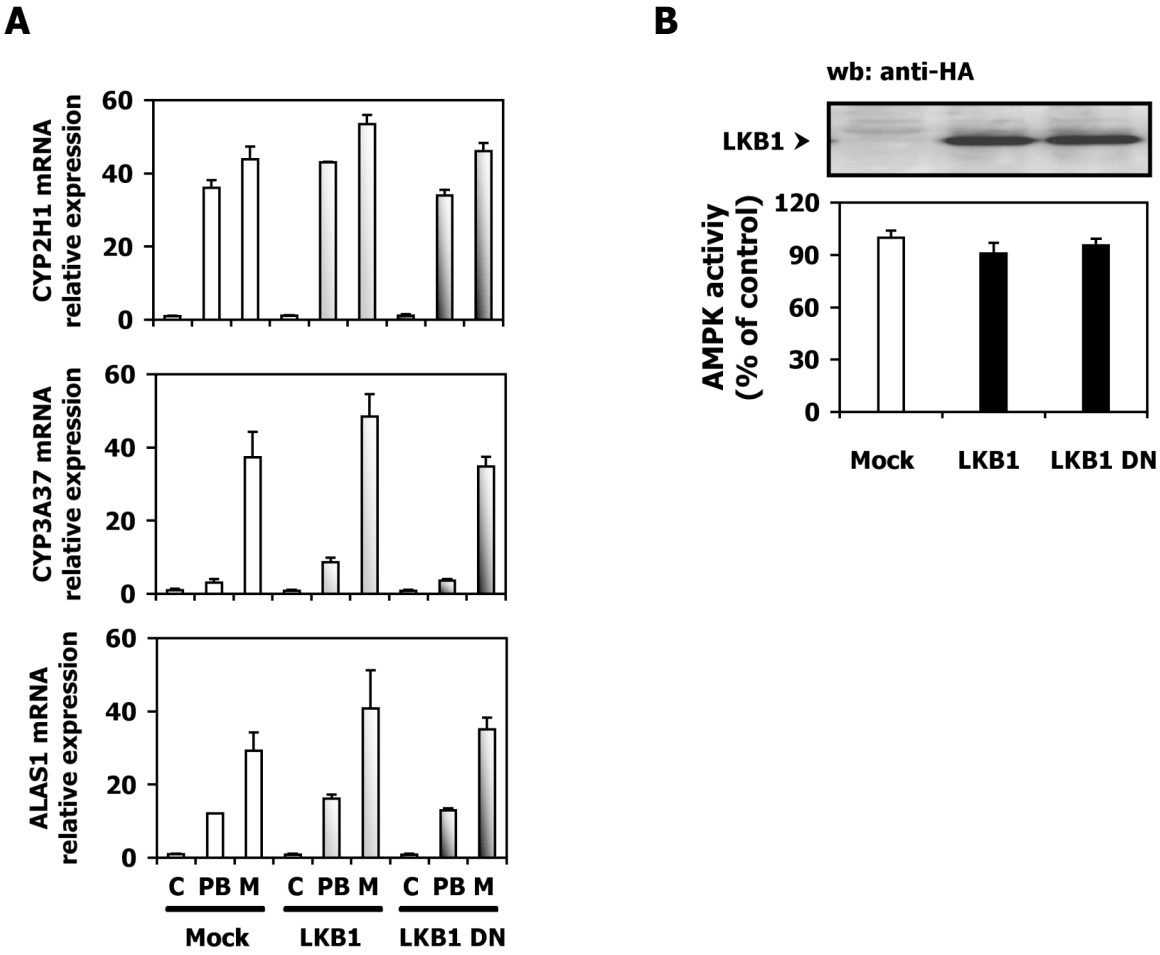
SUPPLEMENTAL FIGURE 2.1

Chicken AMPKα subunits are similar to the mammalian orthologs. AMPKα subunits were characterized in LMH cells. **(A)** Endogenous protein expression of the two chicken subunits was assessed by western blot with antibodies against human AMPKα1 and AMPKα2. Human hepatocytes were used as positive control for the antibodies. **(B)** The subcellular localization of the chicken AMPKα subunits was determined by immunofluorescence (IF) staining. The left panels show the immunostaining of AMPKα1 and AMPKα2 using the same antibodies as for the western blots. DAPI staining of the nuclei is shown in the right panels.



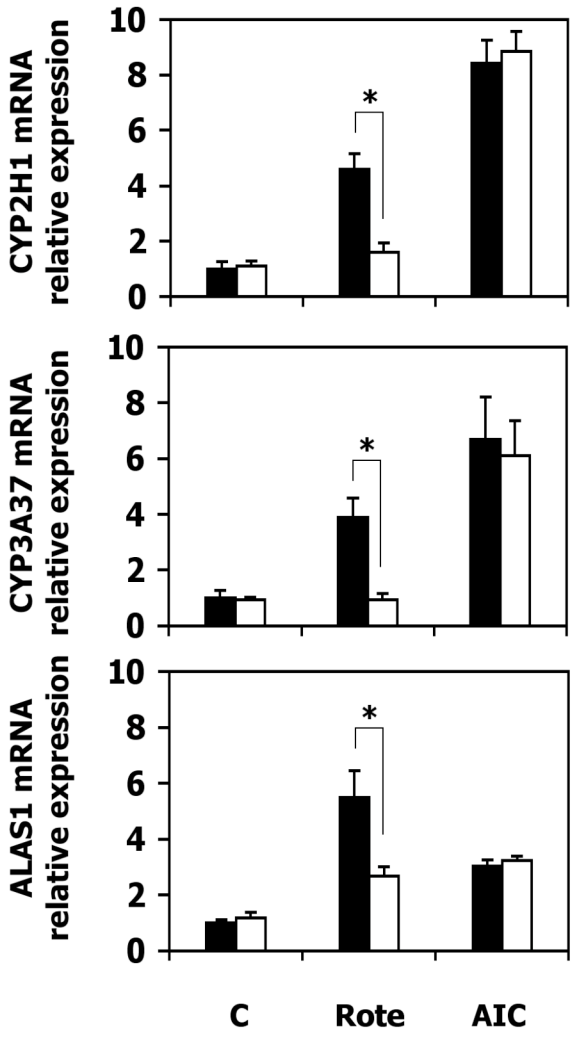
SUPPLEMENTAL FIGURE 2.2

Downregulation of AMPKα subunits expression or activity by d-siRNA and Compound C. (A) Gene expression of AMPKα subunits was measured by RT-PCR. The results are shown as fold expression relative to the control sample (upper panels). In the lower panel AMPK activity of d-siRNA transfected cells was measured and is shown as percentage of the control activity. $p<0.01$. **(B)** LMH cells were pretreated with 20μM Compound C for 30min. The potency of Compound C was tested by measuring the AMPK activity upon 0.4mM DNP with or without Compound C preincubation and is shown as percentage of the control.



SUPPLEMENTAL FIGURE 2.3

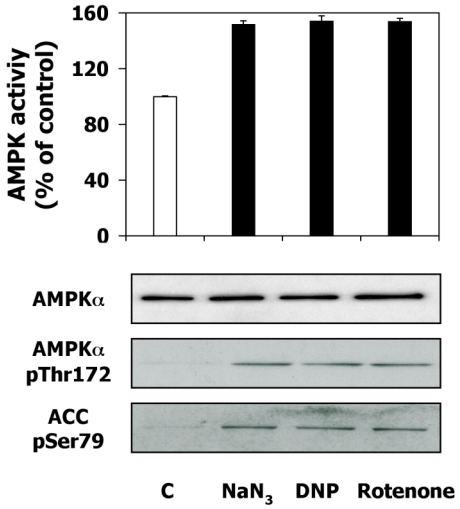
LKB1 overexpression does not affect phenobarbital- and metyrapone-mediated CYP induction and AMPK activity. (A) LMH cells transiently transfected with LKB1 wildtype or LKB1 dominant-negative (DN) form were treated with 500μM PB or metyrapone (M) for 16h. CYP2H1, CYP3A37 and ALAS1 mRNA level was analyzed by RT-PCR. **(B)** Expression of LKB1 protein was checked by western blot with antibodies against the HA-tag of the constructs (upper panel). AMPK activity was measured in LKB1-overexpressing cells (lower panel).



SUPPLEMENTAL FIGURE 2.4

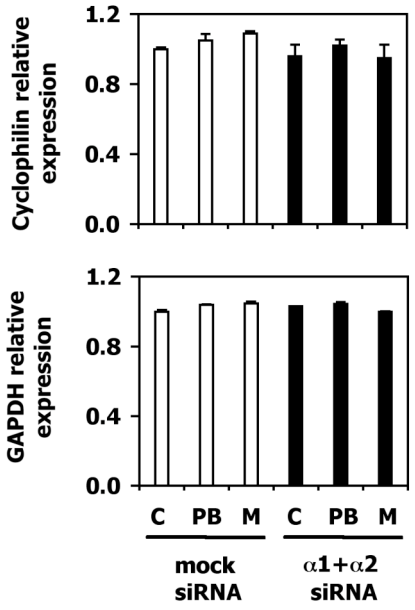
Rotenone and AICAR induction in LMHρ^0 cells. LMH wildtype (■) or LMHρ^0 cells (□) were incubated with 1mM AICAR or 2μM rotenone for 16h. CYP2H1, CYP3A37 and ALAS1 mRNA level was detected by RT- PCR. *p<0.05. Rote, rotenone; AIC, AICAR.

Data not shown



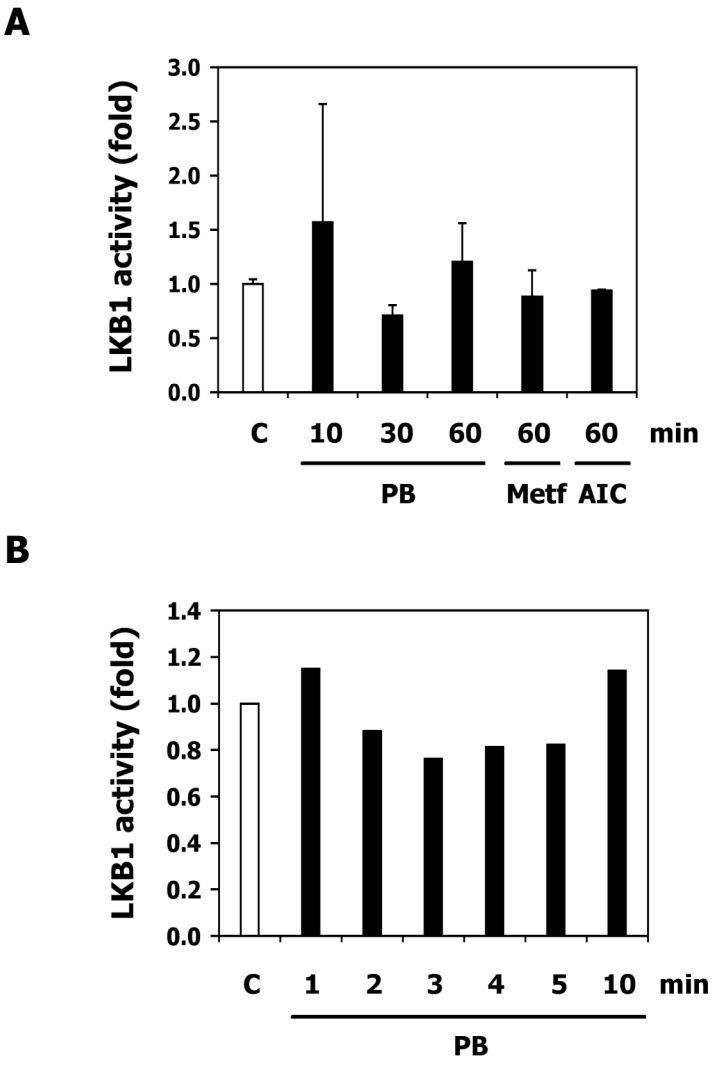
DATA NOT SHOWN FIGURE 2.1

AMPK activation by different compounds. LMH cells were treated with 1mM NaN_3, 0.2mM DNP or 1μM rotenone for 1h. AMPK activity was measured by SAMS peptide assay and shown as percentage of the control. $p<0.01$ (control versus treatment). Phosphorylation of AMPK-Thr172 and ACC-Ser79 is shown by western blot using the same compound concentration and conditions as for the activity measurement.



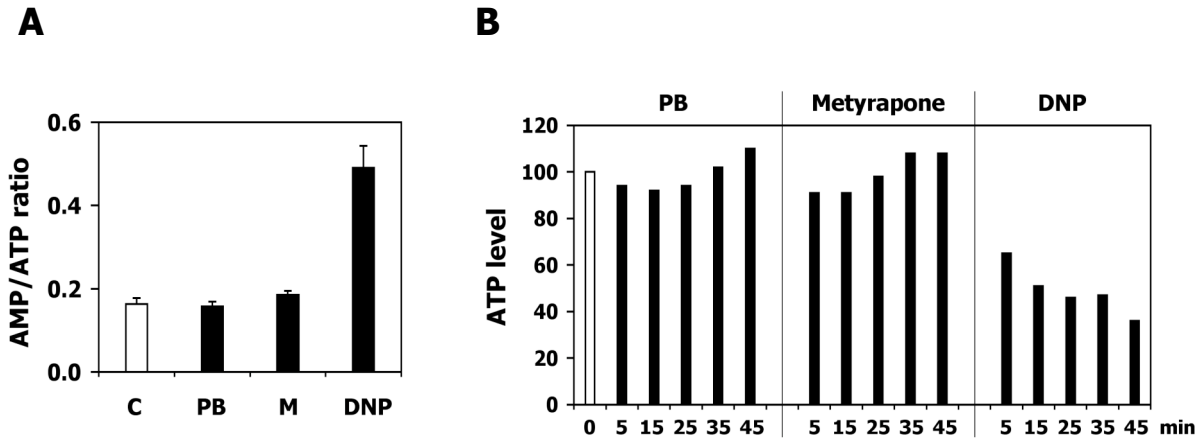
DATA NOT SHOWN FIGURE 2.2

Downregulation of AMPKα subunits expression by d-siRNA is specific. Transfection of siRNA specific against AMPKα subunits does not affect the expression of other genes. Gene expression of *GAPDH* and *cyclophilin* was measured by RT-PCR. The results are shown as fold expression relative to the control sample.



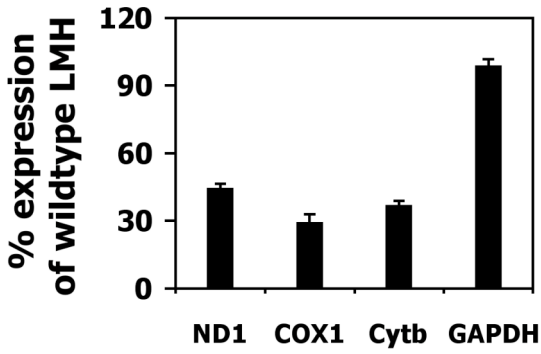
DATA NOT SHOWN FIGURE 2.3

LKB1 is not activated by phenobarbital, metformin or AICAR. (A) LMH cells were treated with 1mM PB, 1mM metformin (Metf) or 1mM AICAR (AIC) for different times. LKB1 activity was measured by LKB1 peptide assay and shown as fold in comparison to the control. **(B)** LMH cells were treated with 1mM PB in a time-course. LKB1 activity was measured by LKB1 peptide assay and shown as fold in comparison to the control.



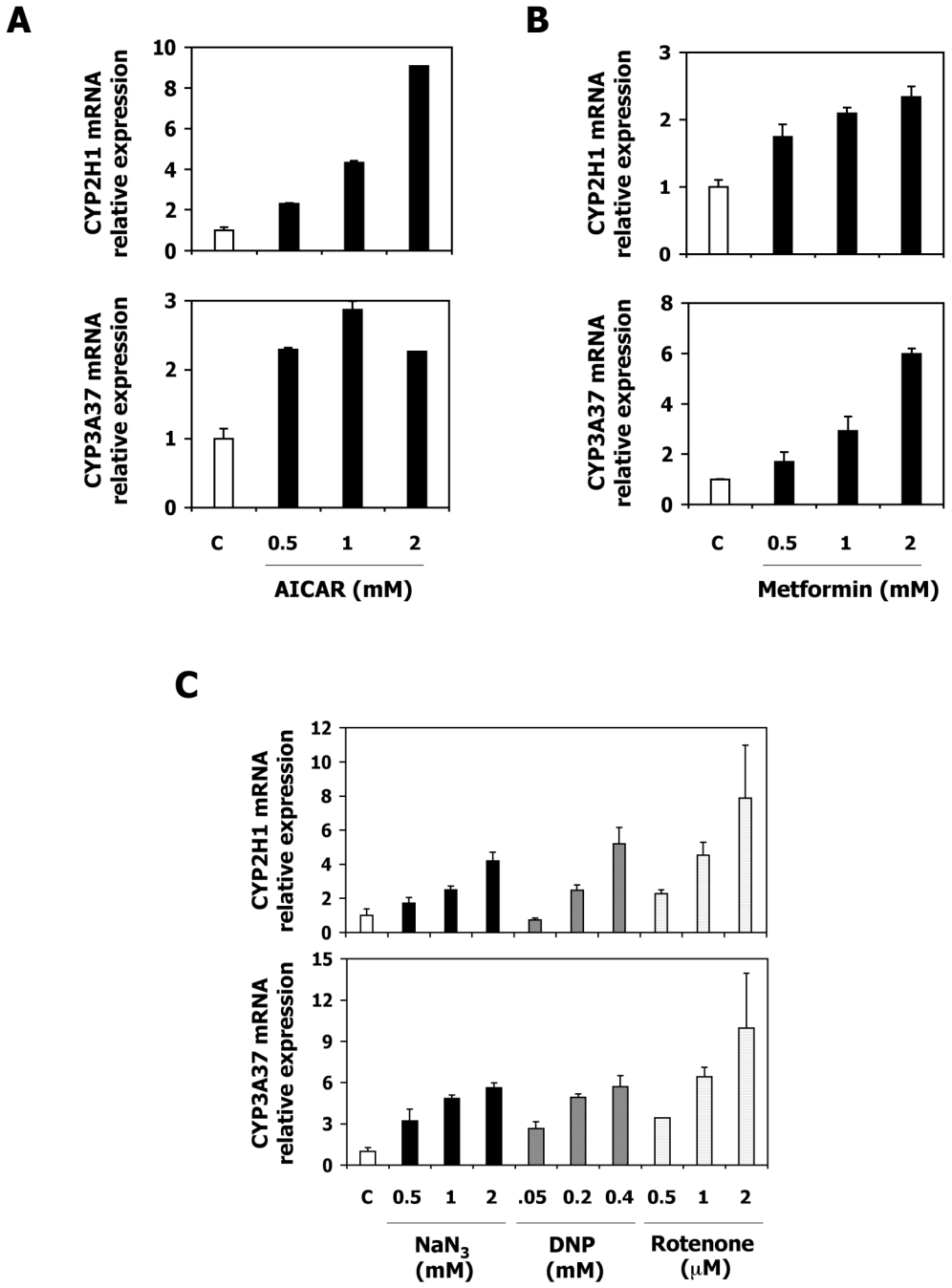
DATA NOT SHOWN FIGURE 2.4

Phenobarbital and metyrapone do not affect AMP/ATP ratio. (A) LMH cells were treated with 500μM PB, 500μM metyrapone (M) or 0.2mM DNP for 1h. AMP and ATP level were measured by HPLC as described in material and methods. AMP/ATP ratio is shown. **(B)** LMH cells were treated with 500μM PB, 500μM metyrapone or 0.2mM DNP for different times. ATP level was measured by ATP Bioluminescence Assay Kit as described in material and methods.



DATA NOT SHOWN FIGURE 2.5

Expression level of mitochondrial markers in LMHρ^0 cells. Mitochondrial DNA markers (ND1, COX1, Cytb) and one nuclear DNA marker (GAPDH) were measured in LMH wildtype and LMHρ^0 cells. The expression level in LMHρ^0 cells is shown as percentage in comparison to wildtype cells.



DATA NOT SHOWN 2.6

AMPK activators dose-dependently enhance *CYP2H1* and *CYP3A37* gene expression. LMH cells were treated with increasing doses of AICAR **(A)**, metformin **(B)**, NaN_3, DNP and rotenone **(C)** for 16h. Gene expression of *CYP2H1* and *CYP3A37* was measured by RT-PCR. The results are shown as fold expression relative to the control sample.

Part III

Additional Mechanistic Studies of the Phenobarbital-Mediated Induction of Cytochromes P450

We demonstrated that AMPK activation is necessary for the drug-mediated increase of CYP expression (Results – Part II and [181]). In the next section, experiments to elucidate the mechanism by which AMPK affects the transcriptional regulation of CYPs are described.

AMPK positive effect on CYP transcriptional regulation is mediated by distal PB-responsive units in LMH reporter gene assay

By reporter gene assay it is possible to link effects on transcription to a definite regulatory region in the 5' flanking region of the gene of interest. The CYP2H1 264-bp and the CYP3A37 159-bp PB-responsive units (PBRUs) were transfected along with increasing quantity of AMPKα1 (FIGURE 3.1A) or α2 (FIGURE 3.1B) followed by measurement of the luciferase expression after 16h of treatment with 500μM PB or 500μM metyrapone. Drug-mediated induction of both reporter genes was increased by overexpression of AMPKα subunits without affecting the basal level.

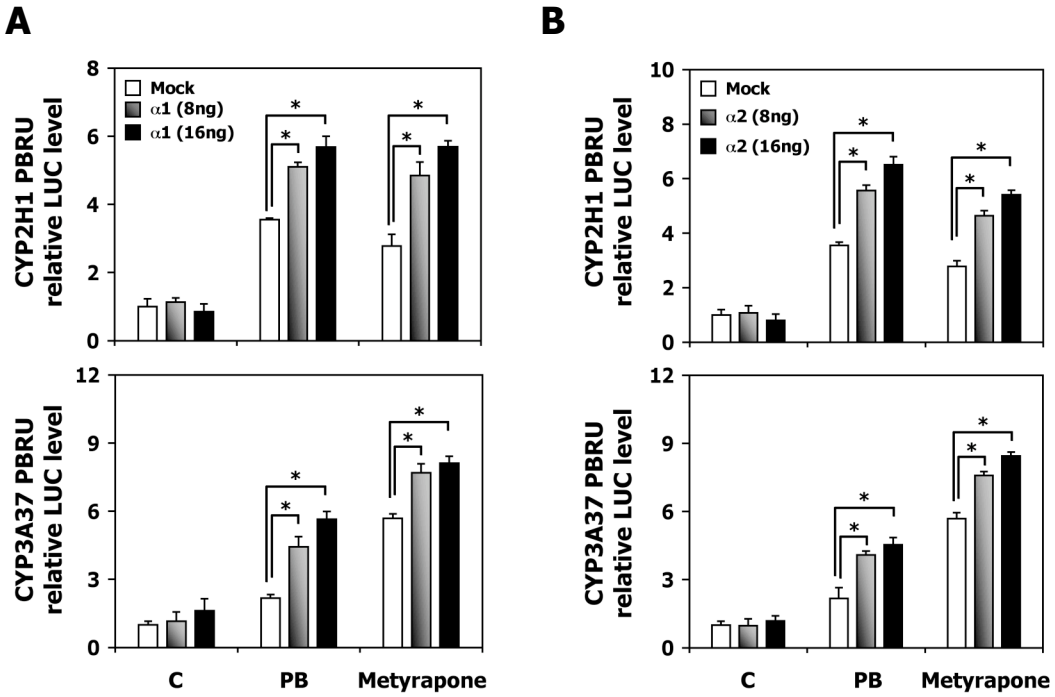


FIGURE 3.1

Phenobarbital- and metyrapone-mediated activation of CYP2H1 264-bp and CYP3A37 159-bp PBRU reporter gene constructs is increased by AMPKα overexpression. LMH reporter gene assays. LMH cells co-transfected with either tk-luc CYP2H1 264-bp or tk-luc CYP3A37 159-bp PBRU along with or without increasing concentrations of AMPKα1 **(A)** or AMPKα2 **(B)** were treated with 500μM PB or metyrapone (M) for 16h. Luciferase expression level is shown as relative expression to the control *p<0.01.

These experiments are evidence for an AMPK transcriptional regulation of CYPs via the short distal PBRUs, meaning that CYP2H1 264-bp and CYP3A37 159-bp PBRU are sufficient to mediate a direct or indirect effect of AMPK on gene expression. These findings suggest that AMPK may affect proteins directly involved in the transcriptional machinery binding to PBRUs, such as transcription factors, corepressors or coactivators.

As already observed on mRNA level, the overexpression of LKB1 wildtype or the dominant negative form did not affect the reporter gene expression under any condition (FIGURE 3.2).

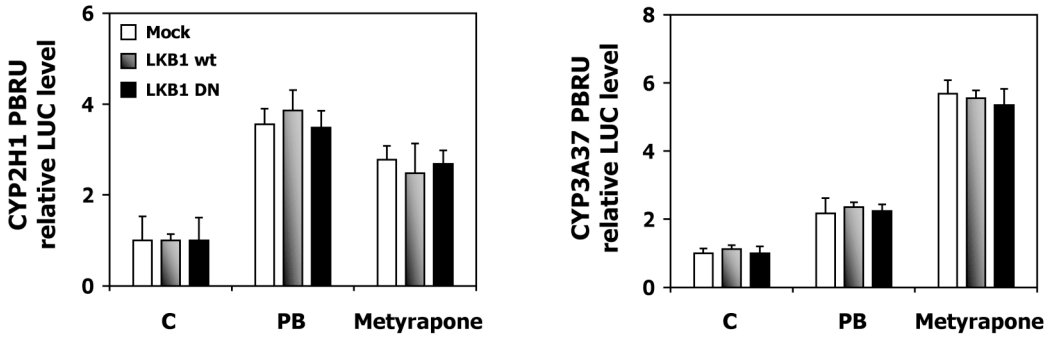


FIGURE 3.2

Phenobarbital- and metyrapone-mediated activation of CYP2H1 264-bp and CYP3A37 159-bp PBRU reporter gene constructs is not affected by LKB1 overexpression. LMH reporter gene assays. LMH cells co-transfected with either tk-luc CYP2H1 264-bp or tk-luc CYP3A37 159-bp PBRU along with or without LKB1 wildtype or LKB1 dominant-negative (DN) form were treated with 500μM PB or metyrapone for 16h. Luciferase expression level is shown as relative expression to the control. *p<0.01.

AMPK positive effect on CYP transcriptional regulation is mediated by distal PB-responsive elements in mammalian cells

In order to assess if distal drug responsive elements are sufficient to mediate the AMPK effect on CYP gene regulation also in mammalian systems, Cyp2b10 51-bp and CYP2B6 51-bp PBREM reporter gene constructs were transfected into CV-1 cells with mouse or human CAR and increasing quantity of AMPKα1 or α2. After 16h of treatment cells were lysed and luciferase measurement was carried out. Both mouse (FIGURE 3.3A) and human (FIGURE 3.3B) PB-induced reporter gene expression were increased by AMPKα overexpression, which did however not affect the basal levels. Furthermore, an increase was also observed with the Cyp2b10 51-bp PB responsive enhancer module (PBREM) upon TCPOBOP (FIGURE 3.3A). In contrast, CITCO induction of CYP2B6 PBRU was not affected by increased AMPKα expression (FIGURE 3.3B). As already discussed in the manuscript (Results – Part II), the availability of AMPKβ and γ subunits may be the explanation for the limited effect of AMPKα overexpression on the transcription of CYPs. The distal drug responsive elements of mammalian CYP genes are thus the mediator of the AMPK effect on CYP gene upregulation by PB, like in avian cells, but not all inducers (for instance CITCO) seem to signal through this kinase.

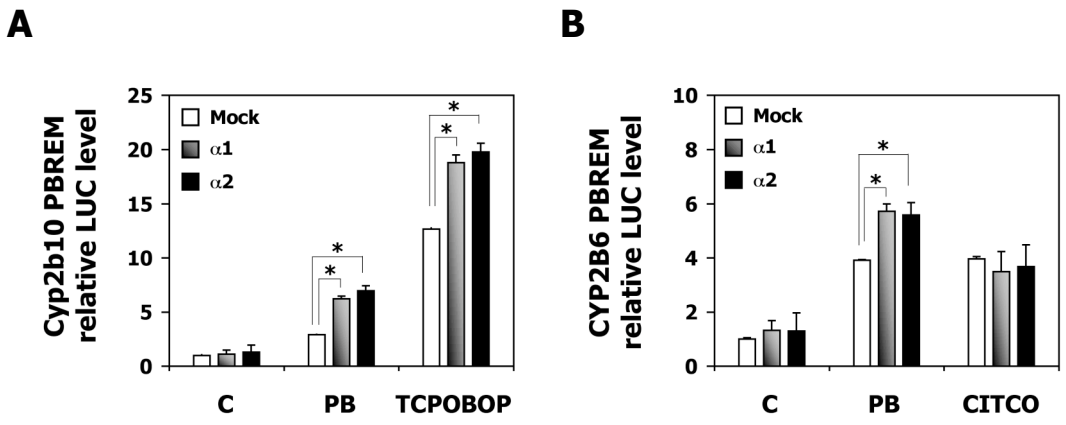


FIGURE 3.3

Phenobarbital-mediated transactivation of mouse Cyp2b10 51-bp and human CYP2B6 51-bp PBREM by CAR is increased by AMPKα overexpression. CV-1 cells transactivation assays. **(A)** CV-1 cells co-transfected with mouse tk-luc Cyp2b10 51-bp PBREM along with mouse CAR were treated with 500μM PB or 10μM TCPOBOP for 16h in the presence of increasing concentration of AMPKα1 or α2. Luciferase expression level is shown as relative expression to the control. **(B)** CV-1 cells co-transfected with human tk-luc CYP2B6 51-bp PBREM along with human CAR were treated with 500μM PB or 50nM CITCO for 16h in the presence of increasing concentration of AMPKα1 or α2. Luciferase expression level is shown as relative expression to the control. *p<0.01.

Since AICAR and metformin increased expression of *CYP2H1*, *CYP3A37* and *ALAS1* in LMH cells, their effects in transactivation assays on Cyp2b10 51-bp PBREM were tested. Both compounds enhanced the reporter gene expression considerably as did the mouse inducer TCPOBOP (FIGURE 3.4). Interestingly, in contrast to TCPOBOP, AICAR and metformin could not reverse the inverse agonistic effect of androstanol suggesting that their effect on CYP expression is not via binding to CAR but via an indirect mechanism that cannot compete with the repression of CAR by androstanol.

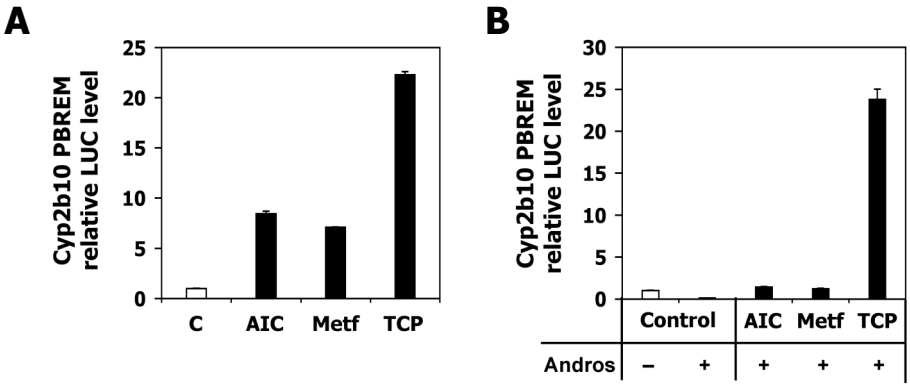


FIGURE 3.4

Transactivation of mouse Cyp2b10 51-bp PBREM by CAR is increased by AICAR and metformin. CV-1 cells transactivation assays. **(A)** CV-1 cells co-transfected with the reporter gene vector tk-luc Cyp2b10 51-bp PBREM along with mouse CAR were treated with 500μM AICAR, 500μM metformin or 10μM TCPOBOP (as positive control) for 16h. Luciferase expression level is shown as relative expression to the control. p<0.01. **(B)** CV-1 cells co-transfected with tk-luc Cyp2b10 51-bp PBREM along with mouse CAR were treated with 500μM AICAR (AIC), 500μM metformin or 10μM TCPOBOP for 16h in the presence of the inverse agonist androstanol. Luciferase expression level is shown as relative expression to the control. AICAR, AIC; metformin, Metf; TCPOBOP, TCP. p<0.01.

Phenobarbital and metformin effects on *CYP2H1*, *CYP3A37* and *ALAS1* expression are not additive

Our data demonstrate that PB shares a part of the mechanism of AMPK activation with metformin, for instance ROS formation and LKB1 recruitment to AMPKα. Cotreatment with both compounds should thus result in no additive effect if they use the same mechanism, in contrast an additive effect may appear if the compounds use two different mechanisms.

Cotreatment of LMH cells with PB and metformin did not cause an increase in the PB induction of *CYP2H1*, *CYP3A37* and *ALAS1*, whereas addition of NaN_3, which is an inhibitor of mitochondrial complex IV and thus of ATP synthesis, strongly increased their expression upon PB (FIGURE 3.5). These results suggest that PB and metformin share the signaling pathway leading to AMPK activation and CYP induction, whereas NaN_3 activates AMPK by a different mechanism resulting in a additive effect. Knowing that metformin targets the mitochondrial complex I, we can speculate that PB may have the same mitochondrial target.

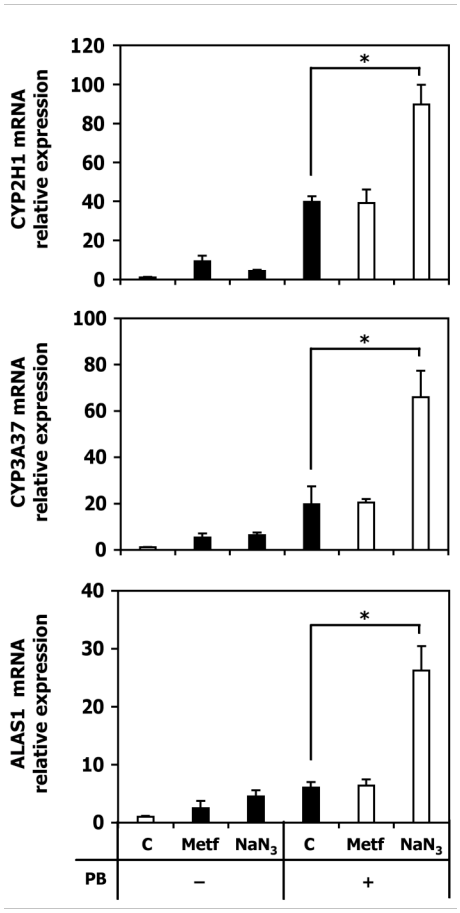


FIGURE 3.5

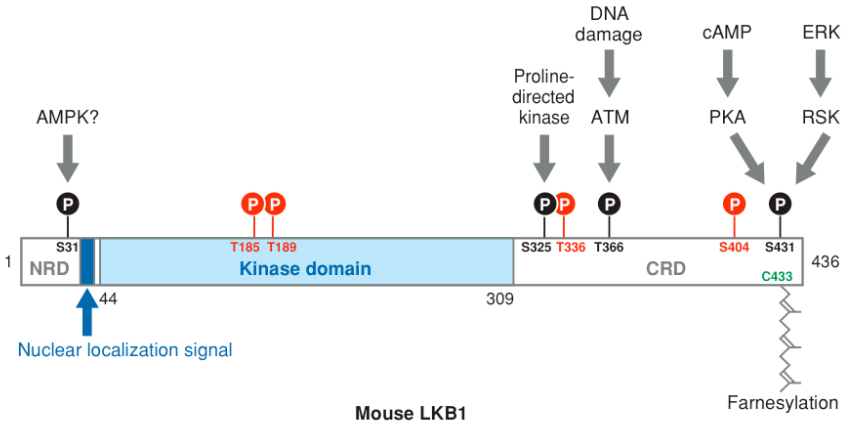
Phenobarbital and metformin effect on CYP expression are not additive. LMH cells were treated with 500μM metformin (Metf), 1mM NaN_3 with or without 500μM PB for 16h. Gene expression of *CYP2H1*, *CYP3A37* and *ALAS1* was measured by RT-PCR. *p<0.01.

Part IV

Role of Protein Kinase C in the Phenobarbital-Mediated Induction of CYPs

The findings presented in the manuscript (Results - Part II) demonstrate that LKB1 is involved in AMPK activation by PB-type drugs. In order to find a player further upstream in this cascade, the identity of the LKB1 upstream kinase was investigated.

So far, eight LKB1 phosphorylation sites (four of which are autophosphorylated) were identified. The kinases that phosphorylate LKB1-Ser31 and Ser325 (numbers referred to the human sequence) are not known, whereas LKB1-Thr366 was shown to be phosphorylated by checkpoint ataxia telangiectasia mutated (ATM) kinases and Ser428 by protein kinase A (PKA) or by the 90kDa ribosomal S6 kinases (p90RSK) [182-184]. ATM kinases are key players in the cell cycle regulation, whereas PKA and p90RSK are activated by extracellular signal-regulated protein kinases in response to many stimuli [185]. Since these last two kinases phosphorylating LKB1-Ser428 are rather responding to extracellular stress stimuli (such as drugs), the next experiments focused on Ser428. In accordance with these considerations, a ROS-dependent phosphorylation of LKB1-Ser428 was recently shown to lead to an interaction between LKB1 and AMPKα, which is in turn activated [186]. Because that study identified protein kinase C zeta (PKCζ) as the LKB1 upstream kinase in endothelial cells, we focused our experiments on this kinase.



From Alessi DR et al. [187]

FIGURE 4.0

Posttranslational modification sites of the mouse LKB1 protein. Autophosphorylation sites are depicted in red, and the sites phosphorylated by other kinases are in black. The Cys433 farnesylation site is depicted in green. Residues Thr366, Ser404, Ser431, and Cys433 in the mouse sequence correspond to human LKB1 residues Thr363, Thr402, Ser428, and Cys430, respectively. The noncatalytic domains are in white, and the kinase domain is light blue.

Downregulation of PKCζ activity by its pseudosubstrate decreases phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1*

To assess if PKCζ may be a player in the signaling cascade leading to increased expression of CYPs by drugs, LMH cells were treated with 500μM PB or 500μM metyrapone in combination with increasing doses of PKCζ pseudosubstrate, a peptide whose effect is to decrease the kinase activity. The inhibitor alone did not affect the basal expression of *CYP2H1*, *CYP3A37* and *ALAS1*, but it dose-dependently reduced their PB- and metyrapone-mediated increase in mRNA expression (FIGURE 4.1A).

The decrease in CYP induction by PB and metyrapone caused by PKCζ pseudosubstrate was associated with less phosphorylated AMPK-Thr172 as shown by western blot suggesting an involvement of PKCζ in AMPK activation upon drugs (FIGURE 4.1B).

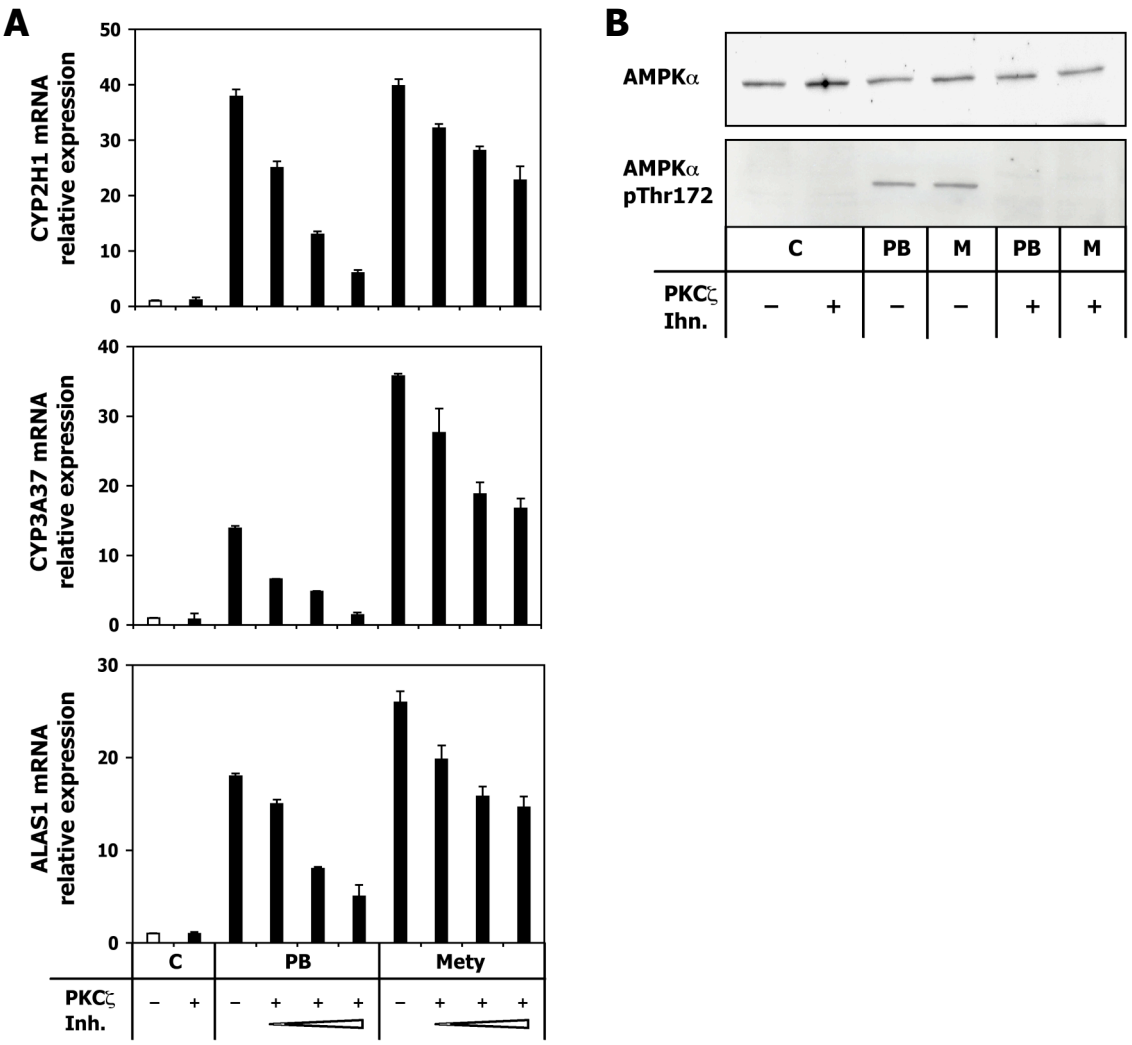


FIGURE 4.1

PKCζ activity is necessary for phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1* gene expression. (A) LMH cells were treated with 500μM PB or metyrapone (Mety) for 16h in the presence of increasing concentration of PKCζ inhibitor (PKCζ Inh.). Gene expression of *CYP2H1*, *CYP3A37* and *ALAS1* was analyzed by RT-PCR. **(B)** Phosphorylation of AMPK-Thr172 by treatment with PB or metyrapone (M) with or without PKCζ inhibitor is shown by western blot.

Overexpression of PKCζ increases phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1*

When LMH cells were transiently transfected with PKCζ, a strong increase in *CYP2H1*, *CYP3A37* and *ALAS1* induction by PB and metyrapone was observed (FIGURE 4.2). The basal expression level of the three genes was not altered. These data show that induction of *CYP2H1*, *CYP3A37* and *ALAS1* is enhanced by increasing the expression of PKCζ.

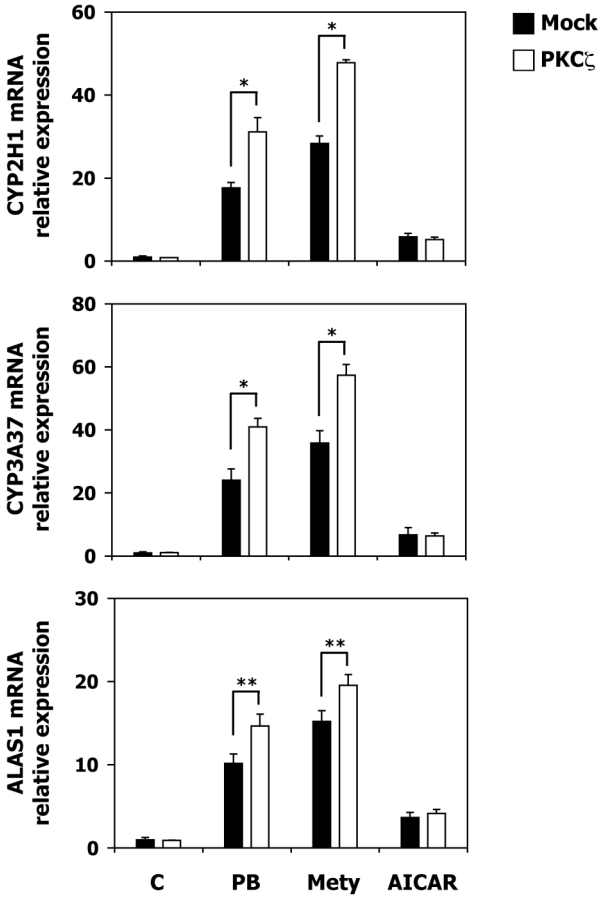


FIGURE 4.2

PKCζ overexpression increases phenobarbital- and metyrapone-mediated induction of *CYP2H1*, *CYP3A37* and *ALAS1*. LMH cells transiently transfected with mock or PKCζ DNA were treated with 500μM PB, 500μM metyrapone (Mety) or 1mM AICAR for 16h. Gene expression of *CYP2H1*, *CYP3A37* and *ALAS1* was analyzed by RT-PCR. *p<0.01, **p<0.05.

Phosphorylation of PKCζ occurs prior to LKB1 and AMPK phosphorylation

To test if PKCζ is upstream of LKB1 and AMPK, LMH cells were treated with 500μM PB and activation of PKCζ, LKB1 and AMPK was assessed by detection of their increased phosphorylation state at different time points. In the PB time-course, phosphorylation and thus activation of PKCζ occurred at early time points, already after 15min of PB treatment, whereas LKB1 and AMPKα phosphorylation were increased later at 30min and 45min, respectively (FIGURE 4.3), indicating that PKCζ is upstream of LKB1/AMPK.

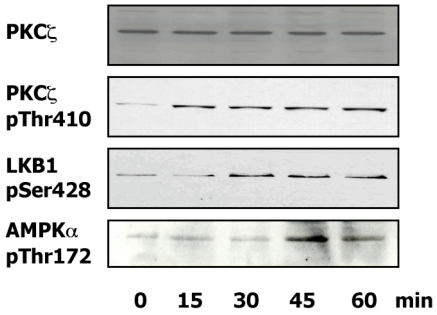


FIGURE 4.3

PKCζ phosphorylation caused by phenobarbital occurs before LKB1 and AMPKα phosphorylation. LMH cells were treated with 500μM PB for different times. Phosphorylation of PKCζ, LKB1-Ser428 and AMPK-Thr172 upon treatment with PB is shown by western blot.

Phenobarbital treatment triggers PKCζ translocation to the plasma membrane

PKCζ is known to translocate to the plasma membrane upon activation by several stimuli [188]. To test if PB is able to trigger PKCζ translocation, western blot of cytoplasmic and membrane proteins were performed, which showed increased PKCζ-Thr410 membrane localization upon exposure of LMH cells to 500μM PB for 10min (FIGURE 4.4A). Immunofluorescence studies also detected increased amount of PKCζ localized at the plasma membrane (FIGURE 4.4B).

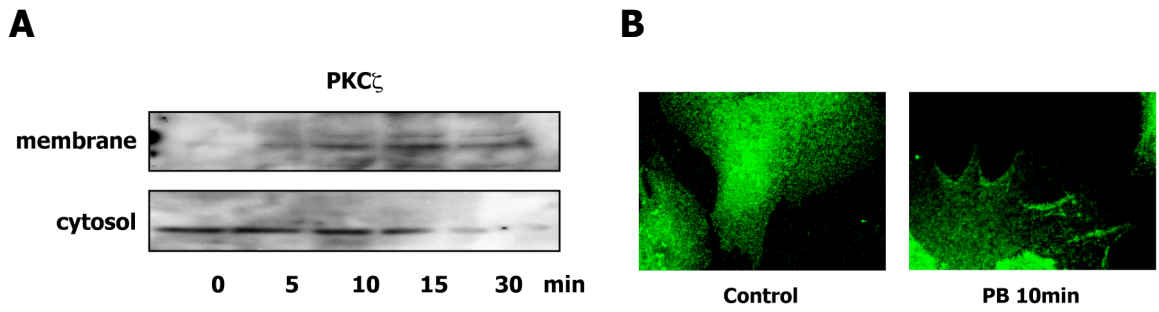


FIGURE 4.4

Phenobarbital causes PKCζ translocation to the plasma membrane. (A) Western blot of LMH cytosolic and membrane fractions after exposure to 500μM PB for different times. **(B)** Immunofluorescence staining of PKCζ in LMH cells treated with 500μM PB for 10min.

Part V

Human Experiments

Phenobarbital dose-dependently activates AMPK in primary cultures of human hepatocytes

In the attempt to test if the molecular mechanism of PB-mediated CYP induction via PKCζ-LKB1-AMPK derived from the studies in chicken hepatoma cells is conserved through evolution, primary culture of human hepatocytes were exposed to AICAR or metformin. Thereafter, AMPK activity was increased by PB treatment in a dose-dependent manner, as well as *CYP2B6* and *CYP3A4* expression [181]. Since these data demonstrate that the AMPK signaling pathway is triggered by PB in human hepatocytes, too, the involvement of mitochondrial functions in the mechanism of PB induction of CYPs was tested in human hepatocytes and hepatoma cells.

Phenobarbital affects mitochondrial membrane potential and ROS formation in primary cultures of human hepatocytes and in human hepatoma cells

The results in LMH cells clearly showed that mitochondrial membrane potential and ROS formation are affected by treatment with drugs. Since this is a fundamental finding for the proposed mechanism of drug-mediated CYP induction, the corresponding experiments were also carried out in human primary cultures of hepatocytes and in human G2F hepatoma cells (G2F cells were described in [180]). The effect of PB on the mitochondrial membrane potential was measured by applying the JC-1 dye. Exposure of human hepatocytes to 500μM PB and 0.4mM DNP, or of G2F cells to 2mM PB and 0.4mM DNP caused changes in the mitochondrial membrane potential (FIGURE 5.1).

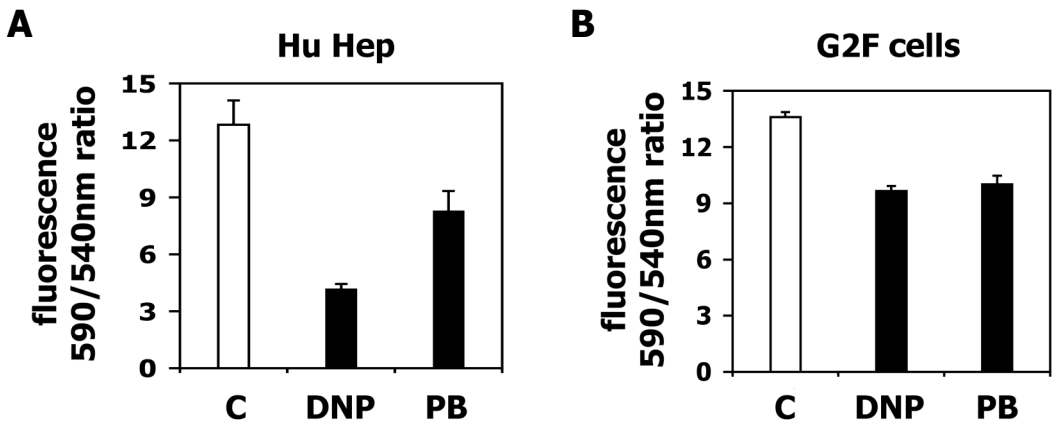


FIGURE 5.1

Phenobarbital affects mitochondrial membrane potential in primary cultures of human hepatocytes and in G2F hepatoma cells. Cells were incubated with the JC-1 fluorescent dye. Fluorescence was detected after 1h treatment **(A)** with 500μM PB or 0.4mM DNP in human hepatocytes and **(B)** with 2mM PB and 0.4mM DNP in G2F cells. The mitochondrial membrane potential is expressed as the 590nm/540nm fluorescence ratio in comparison to the control sample. $p<0.01$.

PB was then tested for its capacity to enhance intracellular ROS production by the DCFH-DA dye. PB at 500μM increased ROS production to a similar extent as the positive control rotenone in human hepatocytes (FIGURE 5.2), whereas ROS could not be measured in G2F cells. These experiments clearly show that mitochondrial functions are affected by PB in human hepatocytes and at least in part in G2F cells, too.

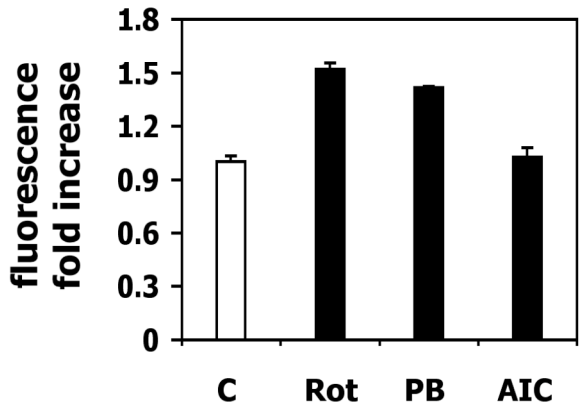


FIGURE 5.2

Phenobarbital affects ROS formation in primary cultures of human hepatocytes. Human hepatocytes were incubated with the DCFH-DA dye. Fluorescence was detected after 1h treatment with 500μM PB, 500μM AICAR (AIC) or 2μM rotenone (Rot). The mitochondrial membrane potential is expressed as the 590nm/540nm fluorescence ratio in comparison to the control sample. $p<0.01$.

Activation of AMPK increases *CYP2B6*, *CYP3A4* and *ALAS1* gene expression in primary cultures of human hepatocytes and in human hepatoma cells

In order to investigate if modulation of AMPK activity alone affects CYP gene expression in human cells, like in LMH cells, compounds known to activate AMPK were tested for their effects on CYP2B6, CYP3A4 and ALAS1 mRNA levels. The expression of all three genes was increased by DNP, NaN_3 and rotenone in primary cultures of primary hepatocytes (FIGURE 5.3A), in addition to AICAR and metformin [181]. Furthermore, the same effect was observed in G2F cells (FIGURE 5.3B)

Inhibition of AMPK decreases *CYP2B6*, *CYP3A4* and *ALAS1* gene expression in primary cultures of human hepatocytes

Inhibition of AMPK activity by Compound C completely blocked PB-mediated induction of CYP2B6, CYP3A4 and ALAS1 in human hepatocytes (FIGURE 5.4). In addition, transfection of a dominant negative form of AMPKα decreased the PB induction of CYP2B6 and CYP3A4 [181]. These data showed that AMPK activity is necessary for PB-mediated induction of CYP2B6, CYP3A4 and ALAS1 in human hepatocytes.

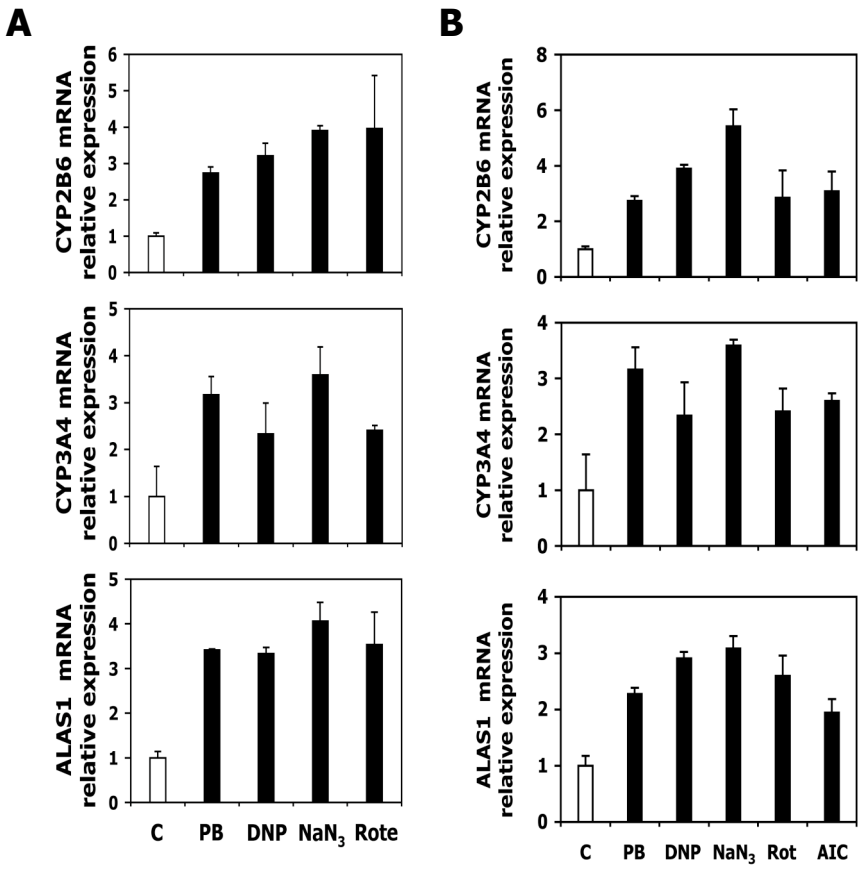


FIGURE 5.3

Activation of AMPK increases *CYP2B6*, *CYP3A4* and *ALAS1* gene expression in primary cultures of human hepatocytes and in human hepatoma cells. (A) Human hepatocytes were treated with 500μM PB, 1mM NaN_3, 0.2mM DNP or 2μM rotenone (Rote) for 16h. CYP2B6, CYP3A4 and ALAS1 mRNA expression was analyzed by RT-PCR. $p<0.01$. **(B)** G2F cells were treated with 2mM PB, 2mM NaN_3, 0.4mM DNP, 4μM rotenone (Rot) or 2mM AICAR (AIC) for 16h. CYP2B6, CYP3A4 and ALAS1 mRNA expression was analyzed by RT-PCR. $p<0.01$.

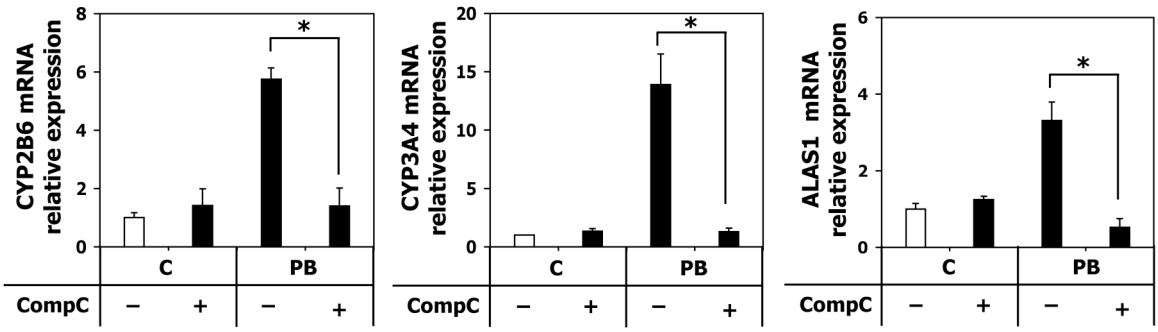


FIGURE 5.4

Inhibition of AMPK decreases phenobarbital-mediated induction of *CYP2B6*, *CYP3A4* and *ALAS1* gene expression in primary cultures of human hepatocytes. Human hepatocytes were incubated with 500μM PB with or without pretreatment for 30min by 20μM Compound C. After 16h treatment, mRNA level of CYP2B6, CYP3A4 and ALAS1 was measured by RT-PCR. *$p<0.01$.

Downregulation of PKCζ activity by its pseudosubstrate decreased phenobarbital-mediated induction of *CYP2B6*, *CYP3A4* and *ALAS1* in primary cultures of human hepatocytes

Human hepatocytes were treated with 500μM PB or AICAR in combination with increasing doses of the PKCζ pseudosubstrate for 16h. The basal expression of *CYP2B6*, *CYP3A4* and *ALAS1* was not affected by the inhibitor, which could however dose-dependently reduce the PB-mediated induction but not the AICAR effect on mRNA expression of *CYP2B6*, *CYP3A4* and *ALAS1* (FIGURE 5.5A). This experiment demonstrates the involvement of PKCζ in the PB induction of CYPs in human hepatocytes and the xenobiotic-specificity of this effect, since AICAR-induced upregulation of *CYP2B6*, *CYP3A4* and *ALAS1* was not affected.

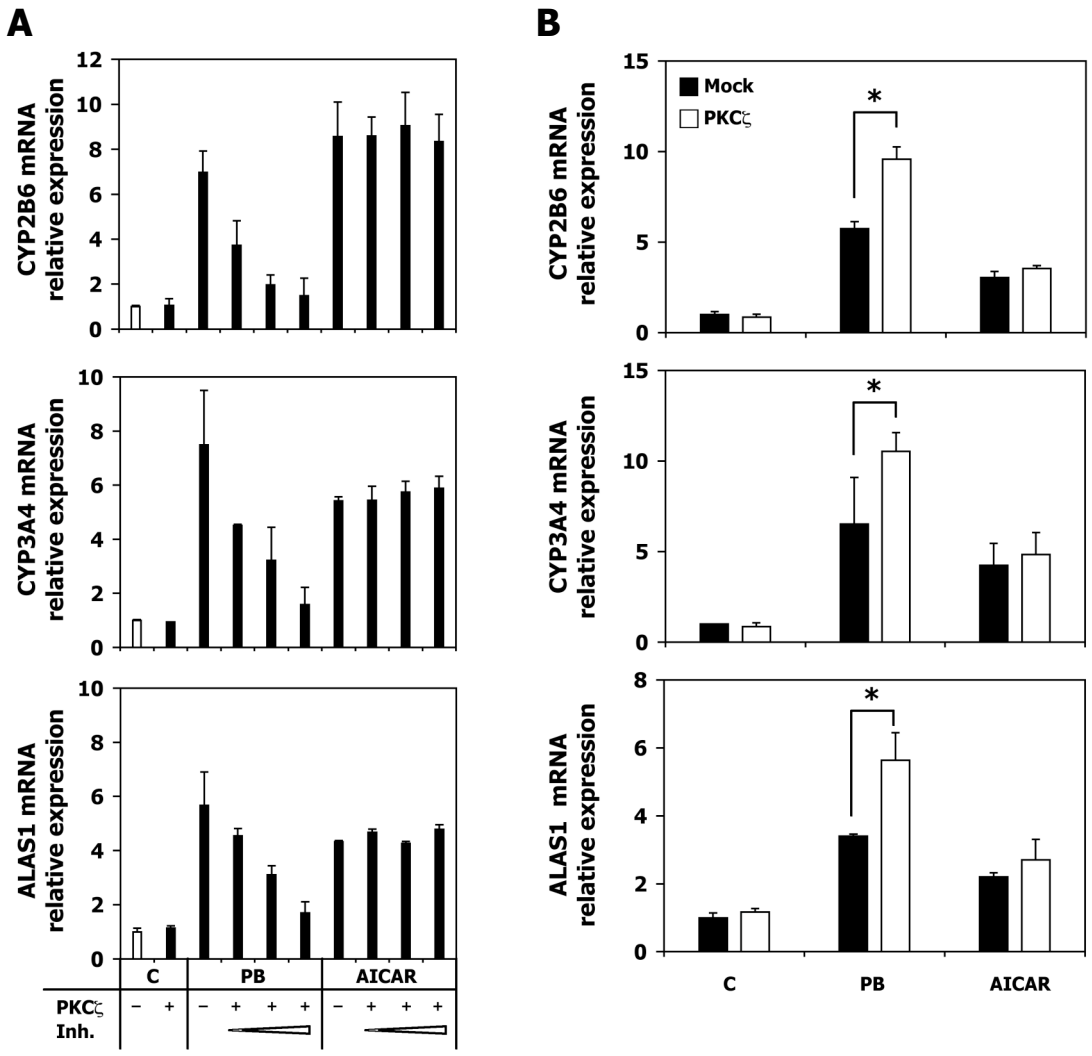


FIGURE 5.5

PKCζ activity modulation affects phenobarbital-mediated induction of *CYP2B6*, *CYP3A4* and *ALAS1* gene expression. (A) Human hepatocytes were treated with 500μM PB or AICAR for 16h in the presence of increasing concentration of PKCζ inhibitor (PKCζ Inh.). Gene expression of *CYP2B6*, *CYP3A4* and *ALAS1* was analyzed by RT-PCR. **(B)** PKCζ transiently transfected G2F cells were treated with 2mM PB or AICAR for 16h. Gene expression of *CYP2B6*, *CYP3A4* and *ALAS1* was analyzed by RT-PCR. *p<0.01.

PKCζ overexpression increases phenobarbital-mediated induction of *CYP2B6*, *CYP3A4* and *ALAS1* in human hepatoma cells

G2F cells were transfected with PKCζ and then treated with 2mM PB or 2mM AICAR for 16h. The basal expression of *CYP2B6*, *CYP3A4* and *ALAS1* was not affected by PKCζ overexpression, whereas the PB-mediated induction of *CYP2B6*, *CYP3A4* and *ALAS1* was increased. As expected, the AICAR effect on these genes was not affected (FIGURE 5.5B). This experiment shows that PB induction of CYPs and ALAS1 in G2F cells is potentiated by increasing the availability PKCζ.

Phenobarbital and metformin effects on *CYP2B6*, *CYP3A4* and *ALAS1* expression are not additive in primary cultures of human hepatocytes

Our data demonstrate that PB shares a part of the mechanism of AMPK activation with metformin and since the effect of metformin and PB were not additive in LMH cells, human hepatocytes were treated with 500μM PB, 500μM metformin or the combination of the two. Cotreatment did not cause an increase in the PB induction of *CYP2B6*, *CYP3A4* and *ALAS1*, whereas addition of NaN_3 strongly increased their expression upon PB treatment (FIGURE 5.6). These results suggest that PB and metformin share the signaling pathway leading to AMPK activation and CYP induction, whereas NaN_3 shows a strong additive effect in combination with PB in primary cultures of human hepatocytes, like in LMH cells.

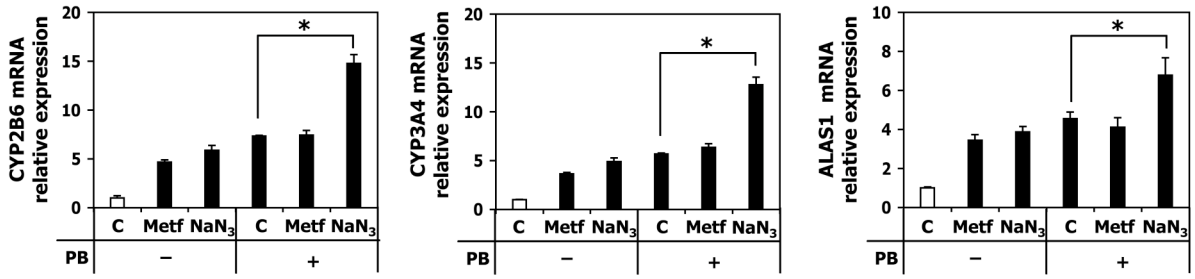


FIGURE 5.6

Phenobarbital and metformin effect on CYP expression are not additive. Human hepatocytes were treated with 500μM metformin (Metf), 1mM NaN_3 with or without 500μM PB for 16h. Gene expression of *CYP2B6*, *CYP3A4* and *ALAS1* was measured by RT-PCR. *p<0.01.

Part VI

Mouse Experiments

AICAR increases Cyp2b10, Cyp3a11, ALAS1 and CAR mRNA level in primary cultures of mouse hepatocytes

The data presented in this thesis were achieved in LMH cells and confirmed in primary cultures of human hepatocytes. To further emphasize that the proposed molecular mechanism of PB induction is conserved among species, some experiments were also carried out in mouse hepatocytes or mouse liver. Exposure of primary cultures of mouse hepatocytes to AICAR increased the gene expression of *Cyp2b10*, *Cyp3a11* and *ALAS1* (FIGURE 6.1A and [181] in APPENDIX 3). In addition, *CAR* expression was also strongly enhanced, in contrast to the expression of two other nuclear receptors, *PXR* and *HNF4α*, which was not affected by AICAR (FIGURE 6.1B).

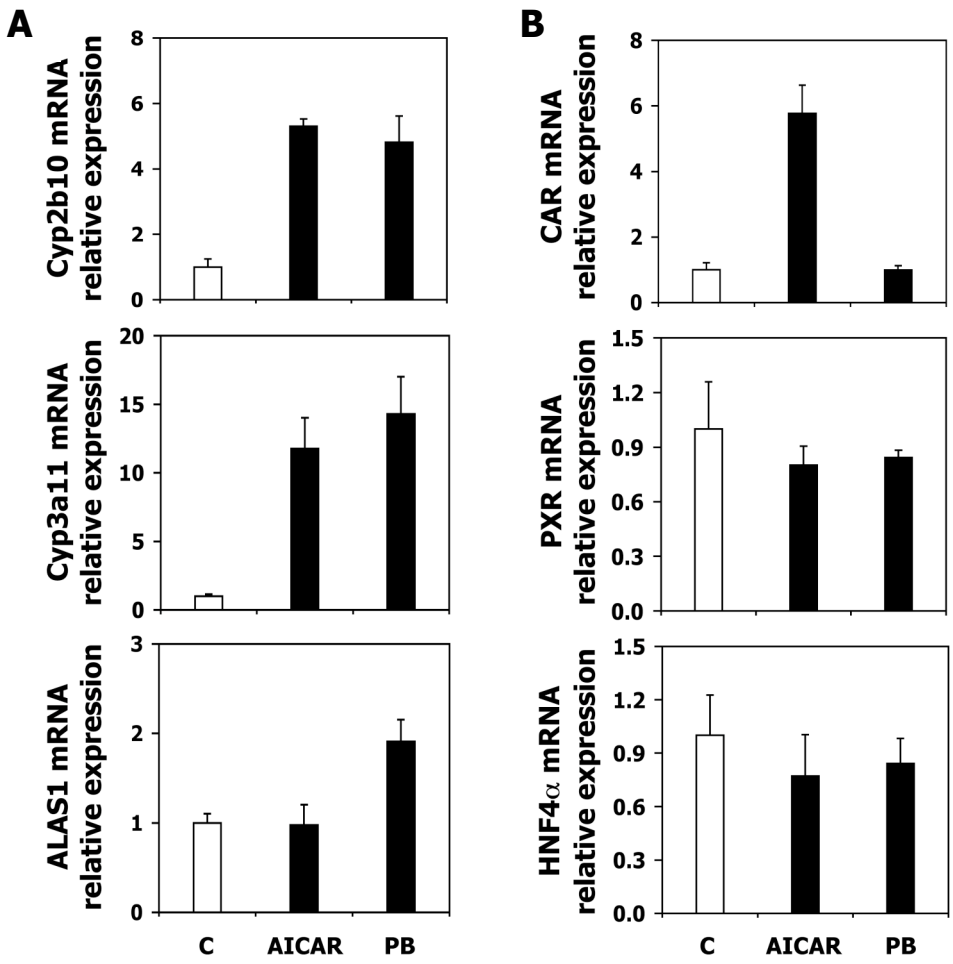


FIGURE 6.1

AICAR increases *Cyp2b10*, *Cyp3a11*, *ALAS1* and *CAR* expression in primary cultures of mouse hepatocytes. Mouse hepatocytes were treated with 500μM AICAR or PB for 16h. Gene expression of **(A)** *Cyp2b10*, *Cyp3a11* and *ALAS1* and **(B)** *CAR*, *PXR* and *HNF4α* was measured by RT-PCR. $p<0.01$.

Phenobarbital induction of *Cyp2b10* is abolished in AMPK knockout mice

To prove that AMPK is indeed necessary for the *in vivo* PB induction of CYPs, AMPK knockout mice were treated with 100mg/kg for 16h and Cyp2b10 mRNA expression was measured. PB induction of *Cyp2b10* was completely abolished in AMPK knockout mice (FIGURE 6.2), but the basal level of this gene was dramatically increased (FIGURE 6.2, insert graph). To reduce the effect of potential circulating factors on CYP basal expression in AMPK knockout, liver-specific AMPKα1 or AMPKα2 knockout mice were used for further experiments, which are described in [181] (APPENDIX 3) and will be reviewed in the Discussion.

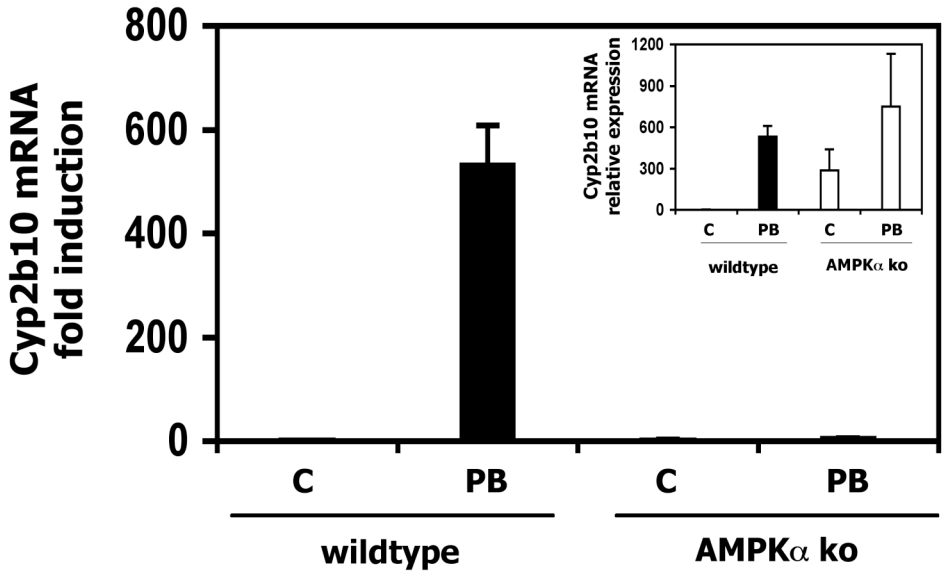


FIGURE 6.2

Phenobarbital induction of *Cyp2b10* is abolished in AMPKα knockout mice. Mice were treated with 100mg/kg PB for 16h. Gene expression of *Cyp2b10* was measured by RT-PCR. The results are expressed as fold induction (big graph) or as relative expression (small graph). $p<0.01$.

Part VII

Chicken X Receptor Immunoprecipitation and Phosphorylation

Several proteins interact with CXR: studies by immunoprecipitation and mass spectrometry

It was shown that CAR forms a complex with heat-shock protein 90 (Hsp90), protein phosphatase 2A (PP2A) and a cytosolic CAR retention protein (CCRP) in the cytoplasm of uninduced cells [73, 74]. Though size of this complex could be approximately 500kDa, many proteins in this complex remain unknown. Since the signaling pathway triggered by PB is conserved from birds to mammals, in order to identify new potential CAR interacting proteins, we tried to isolate the chicken X receptor (CXR) complex from LMH cells and to characterize it by mass spectrometry. To this purpose, HA-CXR was transfected for 24h into LMH cells, which were subsequently treated with 500μM PB for 3h. CXR was immunoprecipitated by anti-HA antibodies. Under these conditions several proteins were coimmunoprecipitated together with CXR (FIGURE 7.1), among which Hsp90 (band 4) and PP2A (band 6), were clearly identified in complex with CXR (band 8), too (TABLE 7.1). In addition, Hsp70 (band 5) and Hsp40 (band 8) were shown to coimmunoprecipitate with CXR, which was never shown for CAR. These heat shock proteins were previously identified in the estrogen receptor and glucocorticoid receptor complexes as essential chaperone. Interestingly, several proteins involved in miRNA-mediated gene expression regulation were identified, such as trinucleotide repeat-containing 6B protein (TNRC6B, band 1), translation initiation factor 2C1 and 2C2 (band 3), which are necessary for rapid decay of mRNAs by miRNA. Furthermore, tubulin (band 6), several DEAD box proteins (band 4 and 5), pyruvate carboxylase (band 2), aldehyde dehydrogenase (band 7) and alpha enolase (band 8) were shown to precipitate with CXR. No difference between control and PB treatment could be established because of the difference in total protein level between control and PB.

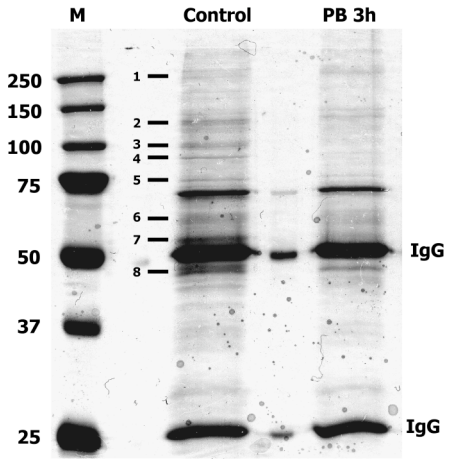


FIGURE 7.1

CXR immunoprecipitation from LMH cells. LMH cells transiently transfected with HA-CXR were treated with 500μM PB for 3h. Immunoprecipitated proteins were loaded on a 10% SDS gel, which was stained by cosmassie. Bands 1-8 were cut and analyzed by mass spectrometry.

TABLE 7.1 Mass spectrometry identification of SDS gel bands

1	**PREDICTED: similar to KIAA1093 protein [Gallus gallus]**	**R.MVSALQQQQQR.Q** **K.MENAGVNFVSGR.E**	**200 kDa**
2	**pyruvate carboxylase [Gallus gallus]**	**K.ALALGDLNAAGQR.E** **R.GPQELEESFSR.A** **R.GTPLDTGIALER.V** **K.GQVGAPMPGEVVEVR.V**	**120 kDa**
3	**eukaryotic translation initiation factor 2C, 1**	**K.YAQGADSVEPMFR.H** **K.YAQGADSVEPMFR.H** **K.DYQPGITYIVVQK.R**	**100 kDa**
	RABIT Eukaryotic translation initiation factor 2C 2	**K.AIATPVQGVWDMR.N** **K.NLYTAMPLPIGR.E** **R.SASFNTDPYVR.E** **K.NLYTAMPLPIGR.E**	
4	**heat shock protein 90-beta [validated] - human**	**R.TLTLVDTGIGMTK.A** **R.NPDDITQEEYGEFYK.S**	**90 kDa**
	DEAD box protein DDX1 [Gallus gallus]	**K.SQHTGNAQVVQTQNLPNAPK.A**	
	DEAD box polypeptide 1; DEAD box-1, human	**R.ELLIIGGVAAR.D**	
	heterogeneous nuclear ribonucleoprotein U-like	**R.NYILDQTNVYGSAQR.R** **K.YNILGTNAIMDK.M** **K.YNILGTNAIMDK.M**	
	heterogeneous nuclear ribonucleoprotein U	**R.NFILDQTNVSAAAQR.R** **K.QMADTGKLNTLLQR.A** **K.YNILGTNTIMDK.M**	
5	**heat shock 70kDa protein**	**K.SQIFSTASDNQPTVTIK.V** **K.NQLTSNPENTVFDAK.R** **R.TWNDPSVQQDIK.F** **K.NQLTSNPENTVFDAKR.L**	**70 kDa**
	mouse hsp70	**KSQVFSTAADGQTQVEIKV** **RETGVDLTKDNMALQRV** **KDAGQISGLNVLRV** **KVQQTVQDLFGRA** **RTTPSVVAFTADGERL** **RQATKDAGQISGLNVLRV**	
	heat shock protein 70 [Gallus gallus]	**KDAGTITGLNVMRI** **KNSLESYTYNMKQ** **KNQVAMNPTNTIFDAKR** **RKYDDPTVQSDMKH**	
	DEAD box polypeptide 5; DEAD box-5 [Homo sapiens]	**K.APILIATDVASR.G**	
	poly A binding protein	**R.ALDTMNFDVIK.G** **R.KEFSPFGTITSAK.V** **R.ALDTMNFDVIK.G** **R.YQGVNLYVK.N** **K.FGPALSVK.V** **K.FSPAGPILSIR.V** **K.VDEAVAVLQAHQAK.E** **R.SLGYAYVNFQQPADAER** **K.SGVGNIFIK**	

6	**Alpha isoform of regulatory subunit A, protein phosphatase 2, Mus musculus**	**R.LAGGDWFTSR.T** **R.YMVADKFTELQK.A**	**55 kDa**
	tubulin beta	**RIMNTFSVVPSPKV** **RMSMKEVDEQMLNVQNKN** **RALTVPELTQQVFDAKN**	
	alpha-tubulin [Homo sapiens]	**K.VGINYQPPTVVPGGDLAK.V** **RNLDIERPTYTNLNRL**	
	Tubulin beta Mus musculus	**RALTVPELTQQMFDSKN** **RIMNTFSVMPSPKV**	
7	**CHICK Aldehyde dehydrogenase 1A1**	**RLTLATMEAIDGGKL** **RANNTTYGLAAAVFTKD** **RELGEYGLQEYTEVKT** **KKFEVFNPANEEKI** **KQGSPSNPAPVLPALPEPLKD** **KAFELGSPWRT**	**50 kDa**
8	**nuclear receptor subfamily 1, group I, member 3 (CXR) [Gallus gallus]**	**K.DMIMSEEALGR.R** **R.GLPIDDQISLLK.G** **RCPNVTDVTEEELKV** **KDMIMSEEALGRR** **RGLPIDDQISLLKG** **RKDMIMSEEALGRR**	**45 kDa**
	similar to protein 40kD [Homo sapiens]	**R.AIVAIENPADVSVISSR.N**	
	CHICK Alpha enolase	**K.LMLEMDGTENK.S**	
	similar to Human Alpha enolase, (2-phospho-D-glycerate hydro-lyase)	**K.DATNVGDEGGFAPNILENK.G** **RGNPTVEVDLYTNKG** **RYISPDQLADLYKS**	

Phenobarbital treatment affects the phosphorylation of several proteins immunoprecipitating with CXR

Preliminary studies performed in our group suggested that AMPK does not phosphorylate CAR peptides *in vitro* (Matis M, unpublished observation). Nevertheless, dephosphorylation of CAR upon PB treatment was shown to be necessary for CYP induction. To test if CXR/CAR are phosphorylated or dephosphorylated upon PB treatment, immunoprecipitated CXR was checked for phosphorylation by anti-phosphoThr/Ser/Tyr antibodies. LMH cells transiently transfected with HA-CXR were exposed to PB for 1h, 2h, 3h, 6h and 10h. After immunoprecipitation, CXR was immunoblotted and phosphorylation was visualized. In the control western blot immunoprecipitated lysates were assayed by anti-HA antibodies to ascertain that the same amount of CXR was transferred to the nitrocellulose membrane (FIGURE 7.2). Both threonine and serine phosphorylation of CXR were detected, without observing a significant change upon PB treatment (FIGURES 7.3 and 7.4). In contrast, only a weak tyrosine phosphorylation was observed (FIGURE 7.5). Other proteins coimmunoprecipitated with CXR were also found to be phosphorylated at threonine and serine residues (FIGURES 7.3 and 7.4, entire blot), whereas no significant band was detected with the anti-pTyr antibody (FIGURE 7.5, entire blot).

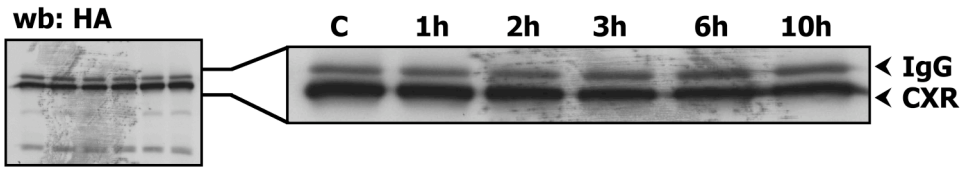


FIGURE 7.2

CXR immunoprecipitation from LMH cells. LMH cells transiently transfected with HA-CXR were treated with 500μM PB for different times. Immunoprecipitated proteins were loaded on a 10% SDS gel and blotted into nitrocellulose membrane. The blot was incubated with anti-HA antibodies.

Western blot: pThr

1. In the first magnification no significant change in the band intensity was detected.
2. Interestingly, in the second magnification an increase in threonine phosphorylation of an approximately 100kDa protein is detected after PB treatment for 6h.
3. CXR phosphorylation at threonine residue is detected.
4. Threonine phosphorylation of an approximately 35kDa protein was also increased after 6h of PB (third band), whereas the other bands showed a slightly time-dependent increase beginning at 2h until 6h PB, which disappeared after 10h PB.

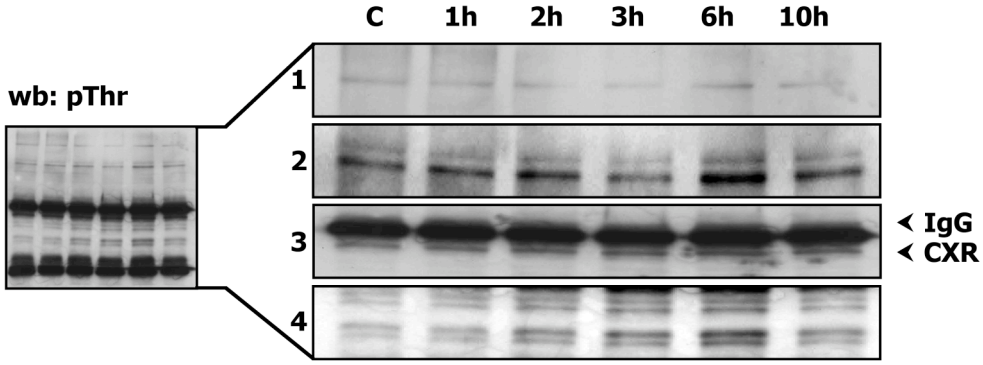


FIGURE 7.3

CXR immunoprecipitation from LMH cells. LMH cells transiently transfected with HA-CXR were treated with 500μM PB for different times. Immunoprecipitated proteins were loaded on a 10% SDS gel and blotted into nitrocellulose membrane. The blot was incubated with anti-pThr antibodies.

Western blot: pSer

1. Serine phosphorylation of an approximately 200kDa protein was slightly enhanced at 6h and 10h PB.
2. Serine phosphorylation of an approximately 100kDa protein was slightly increased beginning at 2h PB.
3. CXR serine phosphorylation
4. The first two and the fourth bands did not change significantly, whereas the serine phosphorylation of the third band was increased after 2h until 10h PB. Note: these four bands may correspond to the bands in the fourth magnification of the pThr western blot.

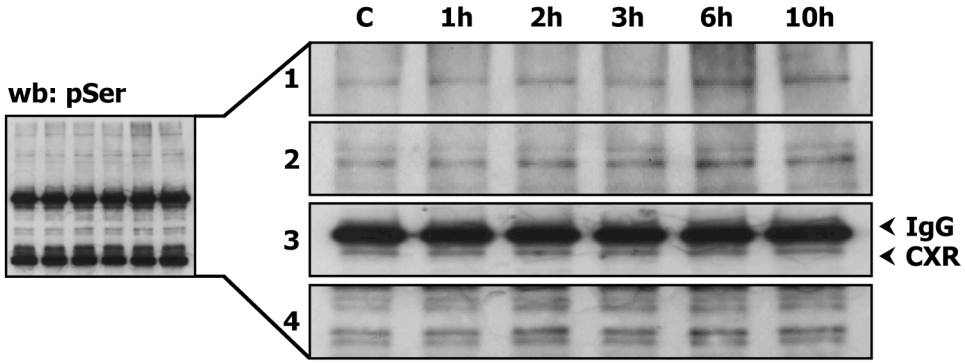


FIGURE 7.4

CXR immunoprecipitation from LMH cells. LMH cells transiently transfected with HA-CXR were treated with 500μM PB for different times. Immunoprecipitated proteins were loaded on a 10% SDS gel and blotted into nitrocellulose membrane. The blot was incubated with anti-pSer antibodies.

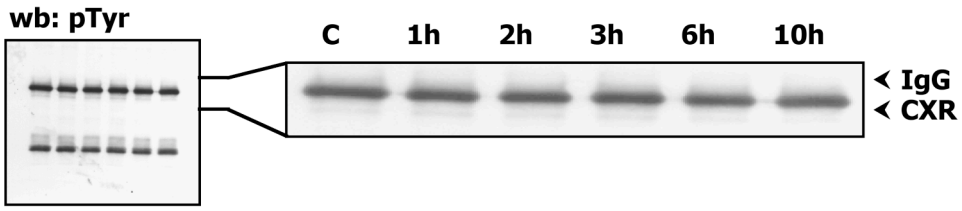


FIGURE 7.5

CXR immunoprecipitation from LMH cells. LMH cells transiently transfected with HA-CXR were treated with 500μM PB for different times. Immunoprecipitated proteins were loaded on a 10% SDS gel and blotted into nitrocellulose membrane. The blot was incubated with anti-pTyr antibodies.

Discussion

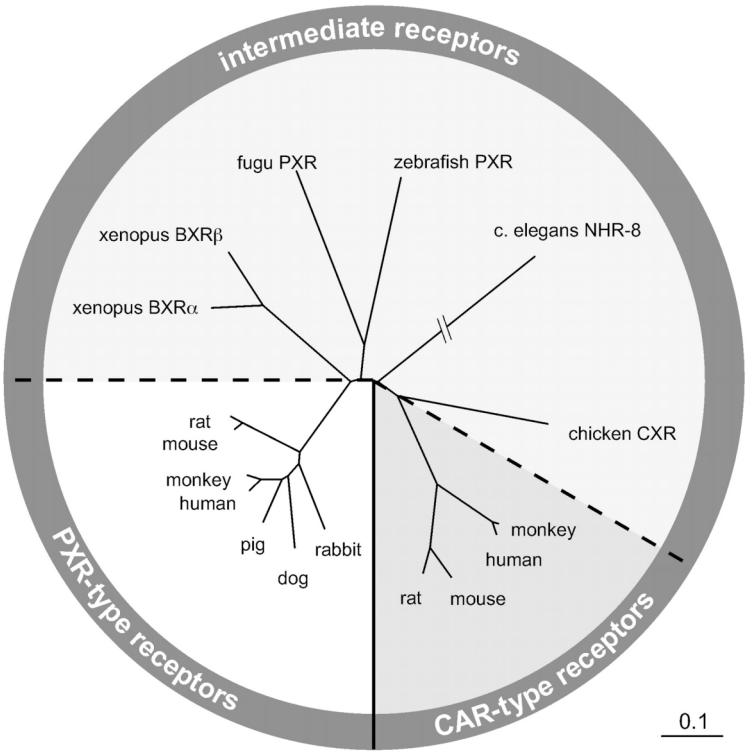
CXR is the Ancestral Gene of CAR and PXR

CXR represents the ancestral gene that diverged into CAR and PXR in the course of evolution as an adaptive response to different environmental and nutritional pressures (Results – Part I). Consistent with this view, CXR is one of the most promiscuous receptors with regard to the spectrum of drugs and endobiotics that activate or inhibit it compared with the mammalian xenosensors [189-190]. In contrast to the two xenobiotic-sensing receptors in mammals, only one xenosensor has been found in non-mammalian species, e.g. chicken, fish (fugu and zebrafish) or nematodes (FIGURE 1). The majority of these non-mammalian receptors resemble PXR more closely than CAR, suggesting that CAR may be a mammalian receptor that diverged from PXR later in the evolution.

A recent phylogenetic study by Krasowski et al. aimed to find evidence of a positive selection (molecular adaptation) in the CAR and PXR genes among vertebrates [191]. Nuclear receptor (NR) genes generally show strong sequence conservation and little positive selection, especially in the DNA binding domain (DBD), characterized by $\omega<1$ (usually less than 0.01), the ratio between non-synonymous (changes in amino acid sequence encoded by the codon) versus synonymous nucleotide variation (no change in amino acid sequence). $\omega<1$ reflects a negative or purifying selection to maintain a conserved amino acid sequence (applying for most gene comparisons). $\omega=1$ means neutral selection (usually for pseudogenes), whereas $\omega>1$ suggests positive selection. Among the NR superfamily, CAR and PXR are an exception and show relatively high ω values, probably due to the adaptation to cross-species differences in toxic compound exposure of the ligand binding domain (LBD) for functional advantage. For these two genes the positive selection may be directed at fine-tuning the ligand specificity towards the most important toxic compounds for a given species. One example of positive selection is the PXR activation by bile acids, which has expanded from narrow specificity for C27 bile alcohol sulfates (early fish) to a broader specificity for recent bile acids (birds, mammals). PXR specificity for bile acids has thus paralleled the increasing complexity of the bile acids synthetic pathway during vertebrate evolution, an unusual example of ligand-receptor coevolution in the nuclear hormone receptor superfamily [192].

The ω ratio for CAR DBD is also on the higher end for the NR superfamily. According to the authors, this may reflect the recent divergence of the CAR gene following duplication of a pre-mammalian PXR gene, which supports our findings that CXR is the ancestral gene of PXR and CAR (Results – Part I). In fact, functional diversification with higher rates of non-synonymous to synonymous substitutions is common in a recently duplicated gene [193, 194]. Once two copies of a gene exist, one of the two genes can diversify its function to evolutionary advantage although the two genes still may share overlapping functions (like in the case of CAR and PXR). The high ω ratio of the CAR DBD suggests that it is diversifying to recognize other binding elements, in accordance with the recently new proposed roles of CAR, such as in glucose and energy homeostasis, which are not exerted by PXR.

Our previous findings and the results presented in this thesis show that LMH cells are an excellent system to study the molecular mechanism of PB induction.



Handschin and Meyer, Pharmacological Reviews (2003) [62]

FIGURE 1

Phylogeny of the nuclear receptor subfamilies NRI2 and NRI3. Full-length amino acid sequences of the NR1I2 (CAR) and NR1I3 (PXR) subfamily members were compared, and an unrooted phylogenetic tree was derived. The scale bar represents 0.1 substitutions per site.

The Molecular Mechanism of Drug Induction

In the present thesis, we demonstrated that PB transcriptionally induces CYP enzymes by a cascade of events that involves an initial impact on mitochondrial functions and triggers drug-induced phosphorylation of PKCζ-Thr410 and LKB1-Ser428. LKB1 subsequently interacts with AMPKα and phosphorylates AMPK-Thr172 increasing its activity and thus CYP gene expression (FIGURE 2). The results are based on experiments carried out in chicken and human hepatoma cells (LMH and G2F), in human and mouse primary cultures of hepatocytes and in mouse liver (this thesis and [181] in APPENDIX 3).

The assumption of mitochondria involvement in the PB induction is based on the following results: (a) mitochondrial functions were affected by PB treatment leading to changes in the membrane potential, ROS generation and ATP level (in human hepatocytes); (b) genetic and pharmacological ROS scavenging reduced AMPK activation and CYP induction by PB; (c) PB induction was strongly inhibited

in cells with decreased number of functional mitochondria. These data strongly suggest a role of ROS and mitochondria in drug induction, however further studies are required to unravel the direct downstream targets of ROS and if mitochondria are the primary target of drugs.

PKCζ activity also seems to affect PB induction based on the following observations: (a) PKCζ was phosphorylated and translocated to the plasma membrane upon PB treatment; (b) pharmacological inhibition of PKCζ decreased PB activation of AMPK and CYP induction; (c) overexpression of PKCζ enhanced CYP induction by PB treatment. Though all these results point to a role of PKCζ activity in PB induction, genetic ablation is necessary to clearly show whether or not it is essential.

The role of LKB1 in the mechanism of drug induction was difficult to establish due to the constitutive activity of this kinase. Nevertheless, immunoprecipitation studies demonstrated that LKB1 is phosphorylated and interactes with AMPKα in a PB-induced manner.

Additionally, we prove that AMPKα activity is necessary in the induction of CYP genes by PB in LMH cells, in mouse liver and primary cultures of hepatocytes, and in primary cultures of human hepatocytes (this thesis and [181] in APPENDIX 3). This interpretation is derived from the following results: (a) AMPK activity is dose-dependently increased upon PB treatment; (b) AMPK overexpression enhances induction of CYPs by PB; (c) downregulation of AMPK expression by siRNA or by transfection of an AMPKα dominant negative form drastically reduced induction in LMH cells and human hepatocytes; (d) pharmacological inhibition of AMPK activity blocks PB induction; (e) PB induction is abolished in AMPKα knockout mice. All these data establish that AMPK is essential for the PB induction of CYPs and reveal evolutionary conservation of this mechanism from birds to mice and humans.

From mechanistic studies, some suggestions about the molecular mechanism by which AMPK affects transcriptional regulation of CYPs could be derived. Our data suggest that AMPK positively regulates PB induction of *CYP2H1*, *CYP3A37*, *CYP2B6* and *Cyp2b10* expression in chicken, human and mouse, respectively, through an effect on the PB-responsive enhancer element (PBREM) in the promoter showing that these short elements are sufficient to mediate AMPK effect on CYP expression. This interpretation is derived from the results of reporter gene and transactivation assays. Furthermore, the mitochondrial complex I may be the PB target on these organelles, since the effects of metformin, which was shown to affect complex I activity, and PB on CYP gene expression are not additive, whereas NaN_3, which inhibits complex IV, and PB show additive effects. Further studies need to be done to confirm this assumption.

Immunoprecipitation of the CXR complex from LMH cells with subsequently identification of the interacting proteins led to the identification of two proteins Hsp90 and PP2A known to bind to CAR, for instance, and of several other proteins, which are potentially CAR interacting partners, too. Among them, many are involved in mRNA posttranscriptional regulation, which may be an additional way for nuclear receptors to affect gene expression. Additional experiments aimed to detect CXR phosphorylation changes upon PB, showed that many proteins immunoprecipitating with CXR are phosphorylated in a PB-induced manner. The identification of these proteins will be the focus of future experiments.

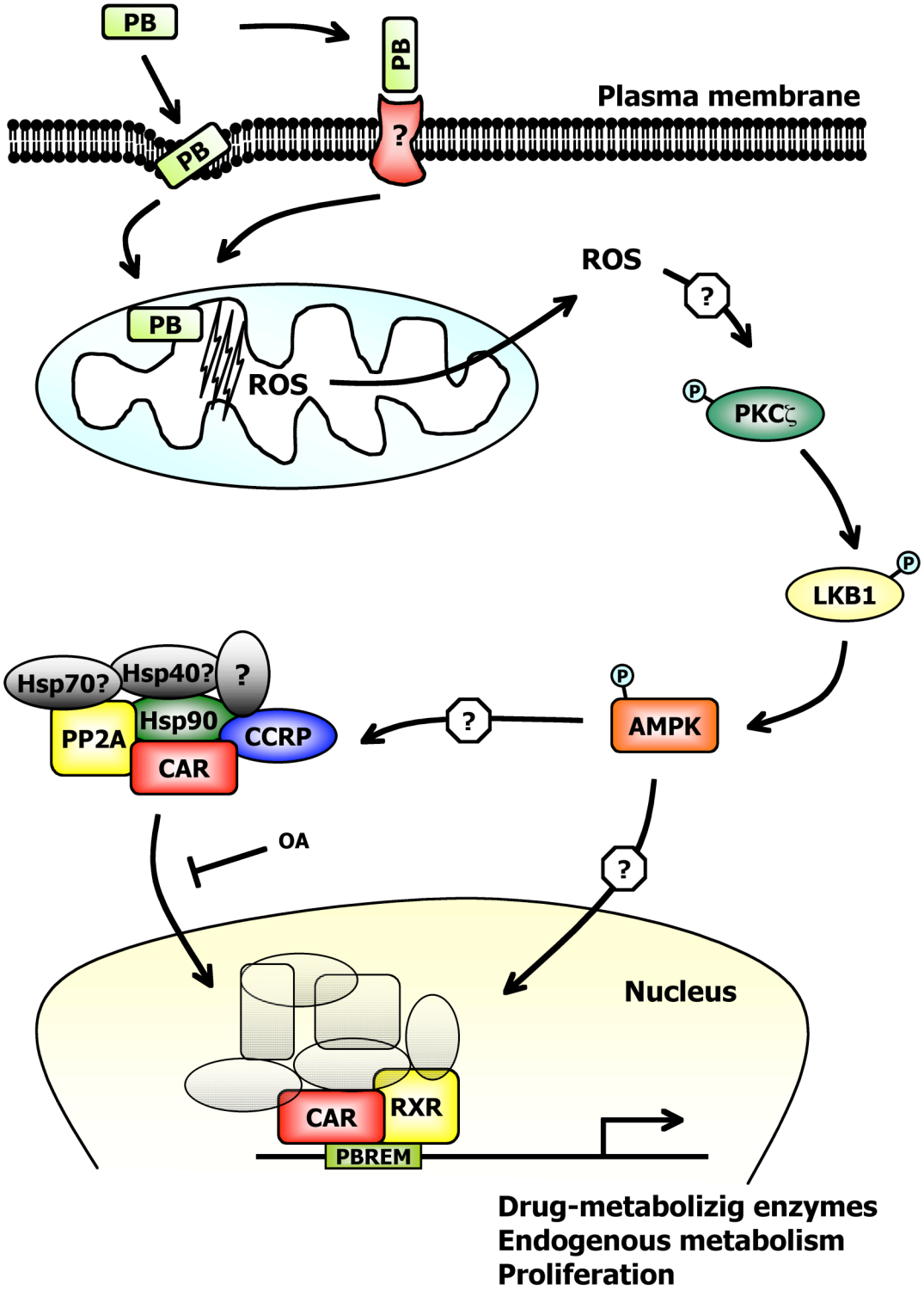


FIGURE 2

The molecular mechanism of drug induction. Model for PB-type induction of CYP genes.

AMPK Targets in the Mechanism of Drug Induction

The data presented in this thesis demonstrate that AMPK is necessary for the PB-mediated induction of CYPs, but the targets of AMPK in this mechanism are unknown (see also manuscript discussion in Results – Part II). Recent studies demonstrated okadaic acid-sensitive PP2A recruitment to the CAR complex upon PB treatment in mice [74] and involvement of dephosphorylation of CAR at Ser202 in its nuclear translocation. However, these observations are not sufficient to explain the translocation, which needs probably other factors, like CCRP or the xenobiotic responsive signal (XRS) [195]. Preliminary experiments in our group showed that CAR peptides containing putative AMPK target sequences incubated with AMPK *in vitro* were not phosphorylated, however the experimental conditions are far from the *in vivo* situation. Though we cannot yet exclude definitely that CAR is not phosphorylated by AMPK, other potential AMPK targets have to be considered, starting from proteins known to be phosphorylated by AMPK. One potential candidate is p300, a transcriptional coactivator with acetyltransferase activity, which acts not only as an adaptor between basal transcription machinery and transcription factors, but also plays a role in acetylation of histones to increase DNA accessibility or other proteins for their activation, nuclear retention or modulation of half-life [196]. AMPK was shown to phosphorylate p300 [197] inhibiting thus its interaction with several nuclear receptors, such as PPARγ, RAR, RXR and TR, but not with proteins, like p53 or GATA4. However, in this study it was not demonstrated that abrogation of this interaction has an effect on the transcriptional activation by these nuclear receptors, thus leaving unknown whether or not phosphorylation of p300 by AMPK may affect transcriptional regulation. But, even if this assumption would be true, it is impossible to predict if this effect would also hit CAR-p300 interaction, because apparently this effect seems to be selective. Nevertheless, it is worth noticing that p300 activation is responsible for histone hyperacetylation, which generally increases transcription. In this regard, CBP/p300 activity is related to the extent of PB-induced CYP2H1 gene expression in chick embryo primary hepatocytes [198].

Recent experiments performed in cell culture demonstrated that AMPK triggers the nuclear import of a member of the Hu family, which plays a very important role in mRNA stability, via increased p300-mediated acetylation and phosphorylation of importinα [199]. The importinα-importinβ pathway transports cargo proteins containing nuclear localization signals (NLS) through the nuclear pore complex. The authors proposed that under stress conditions activation of AMPK occurs leading to phosphorylation/acetylation of importinα and thus to an increase in the efficiency of the nuclear transport of cargo proteins. Until now this has been demonstrated only for one member of the Hu family, but because the nuclear translocation of CAR is necessary in the mechanism of drug induction, in which AMPK is clearly involved, the possibility that CAR may be a cargo protein for importinα is very fascinating. Consistent with this hypothesis, two nuclear localization signals were identified in the mouse CAR [200]. Activation of importinα-mediated nuclear import by AMPK/p300 upon xenobiotics or other stresses would be a means for AMPK to alter gene expression patterns.

Another candidate protein is the PPARγ coactivators 1α (PGC1α), which plays a role in mitochondria biogenesis and oxidative metabolism, keeping thus an energy homeostasis, for example by promoting responses to fasting. PGC1α mRNA expression was shown to increase upon AMPK activation in mice skeletal muscle and to be necessary for the AMPK-stimulated fatty acid oxidation [201]. In addition, AMPK-mediated enhancement of PGC1α mRNA and protein is important in mitochondria biogenesis [202-205]. Hence, drug treatment may increase the coactivator PGC1α protein expression via AMPK activation leading to potentially higher transcriptional power. However, there is no evidence that PGC1α is important for CAR target gene expression besides the observation that PGC1α coactivates a chimerical GAL4-CAR fusion protein on the artificial luciferase reporter gene UAS-LUC under basal conditions in a ligand-independent manner [206]. Nevertheless, a role for PGC1α as transcriptional regulator of *CAR* in fasting was shown. The group of Jeff Staudinger in collaboration with Frank Gonzalez identified an interaction between PGC1α and hepatic nuclear factor 4α (HNF4α) that directly regulates *CAR* gene expression. Expression of PGC1α in cells increases *CAR* expression and ligand-independent activity, whereas fasting-induced *CAR* and CAR target gene expression is abolished and basal expression of CAR was severely diminished in HNF4α knockout mice [207].

In the light of these results, it is plausible that PB-mediated activation of AMPK enhances PGC1α expression, increasing thus *CAR* gene expression and consequently the expression of CYPs. However, in experiments done in collaboration with the Bruce Spiegelman group we could show that PGC1α knockout mice retain *Cyp2b10*, *Cyp3a11* and *ALAS1* PB-mediated induction [208] suggesting against an essential role for this coactivator in the drug induction mechanism. If the role of PGC1α in the coactivation of HNF4α on the CAR promoter can be replaced by another cofactor is not known yet, but it is clear that cofactors are very redundant in their function.

Despite a double role for CAR in metabolic and in xenobiotic stress, it is still not clear if this two responses are mediated by the same factors. It is possible that CAR xenobiotic response is different from its metabolic response and is initiated through interaction with other cofactors. Nevertheless, CAR is activated in response to metabolic or nutritional stress in a ligand-independent manner acting as an energy sensor, which is exactly the role of AMPK.

While PGC1α is apparently not necessary for drug-mediated CYP induction, HNF4α was demonstrated to be important. In HNF4α knockout mice CAR expression is drastically reduced and Cyp2b10 induction by CAR activators is abolished [209, 210]. Additionally, HNF4α response elements present in several CYP genes are essential for maximal CAR-mediated drug induction [211-213]. So, HNF4α would be a perfect AMPK target since both are essential for induction. However, Leclerc *et al.* showed in 2001 that treatment of rat hepatocytes by AICAR decreases HNF4α protein expression (but not its mRNA level) and some HNF4α target genes, which is not compatible with the possibility of AMPK regulating drug induction by increasing the HNF4α expression or activity. Despite of this, it is worth noticing that AICAR is not a specific AMPK activator and also that those experiments showed transiently HNF4α protein decrease without long-term transcriptional regulation upon AICAR.

Many other potential targets for drug-activated AMPK could be considered, from proteins binding to CAR in the cytosol, such as the chaperone Hsp90 or the cochaperone CCRP, to unidentified proteins in the complex, which is thought to be in the 500kDa range implying the existence of other proteins in this complex (see Discussion on the CXR complex).

AMPK-LKB1 Signaling

Our data are consistent with a model, in which PB-type drugs affect mitochondrial functions leading to LKB1-AMPK activation necessary for the transcriptional regulation of CYPs by drugs. These findings prompt to consider why PB-type drugs activate AMPK and if this activation favors drug induction and can explain the pleiotropic effects of PB in the liver.

AMPK-LKB1-mediated inhibition of mTOR-regulated protein synthesis

Protein synthesis accounts for approximately 20% of energy turnover in growing cells and is particularly sensitive to decreases in ATP level, wherefore AMPK could link translation and energy metabolism. Recently, it was demonstrated that AMPK inhibits the mammalian target of rapamycin (mTOR) pathway by phosphorylating the tuberous sclerosis complex 2 (TSC2 or tuberin), which regulates mTOR activity [214]. Furthermore, LKB1 was shown to be necessary for AMPK-mediated inhibition of mTOR [215], which can also be directly phosphorylated by AMPK [216]. All these data indicate that in case of low energy AMPK inhibits protein synthesis by decreasing mTOR activity (FIGURE 3).

The distinction between short- and long-term PB effects could explain the apparent incongruence that PB activation of AMPK may inhibit translation, knowing that PB treatment in rodents causes liver size increase, wherefore protein synthesis is needed. In fact, our studies indicate that PB activates AMPK within 1h, but even if AMPK activation can still be detected after 3h (data not shown), long-term effects of PB on AMPK were not studied. Thus, a short-term AMPK activation by PB would not preclude a long-term liver size increase.

Another consideration is that in response to many types of stimuli or stress, global changes in protein synthesis are relatively modest (up to twofold), but the translation rates of a small population of mRNAs is dramatically modulated. In fact, the general translation apparatus can discriminate between different mRNA, giving raise to the so-called "translational discrimination". mRNA species can be divided into two categories, the 5′ TOP mRNA and the 5′ cap mRNA. On one hand the 5′ TOP (terminal oligopyrimidine tracts) mRNAs code for ribosomal proteins, translation factors involved in mRNA translation, such as elongation factors eEF1A and eEF2, and poly A binding proteins amongst others. The abundance of 5′ TOP mRNAs varies, but can account for up to 15–20% of total cell mRNA. On the other hand, the nuclear encoded mRNAs are capped with a 7-methyl guanosine residue at the 5′ end, which necessarily binds to initiation factors in order to start translation (cap-dependent translation). Within 5′ cap mRNAs huge differences exist in the cap-proximal 5' UTR secondary structure. Because 40S ribosomal subunit binding appears to require a region of single-stranded

mRNA, mRNAs with structured and complicated secondary structures in the cap-proximal 5' UTR need high helicase activity to unwind RNA. Therefore, their translational efficiency is reduced by rendering the mRNA less accessible to a ribosome, and by impeding the scanning process. In fact, while eIF4F helicase activity is low, translation of mRNAs with low 5' UTR secondary structure is affected to a lesser degree than mRNAs possessing extensive 5' UTR secondary structure, and eIF4E overexpression preferentially enhances the translation of mRNAs with structured 5' UTRs [217, 218].

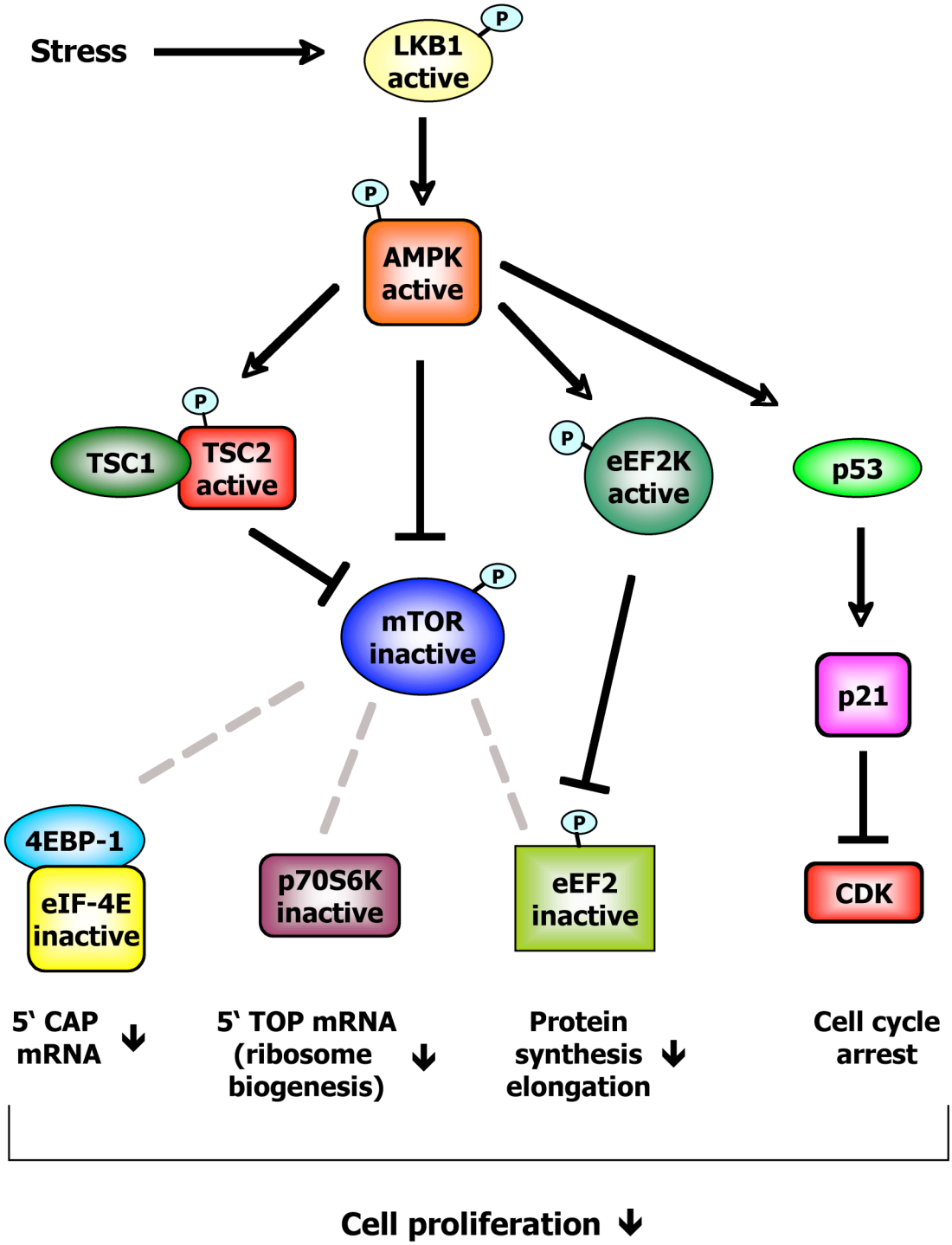


FIGURE 3

AMPK inhibition of protein synthesis. mTOR pathways controlling protein synthesis are shown.

Recent data demonstrate that the synthesis of all proteins is not repressed equally. Fibronectin, for example, was shown to stimulate cap-dependent translation but not 5′ TOP mRNA translation [219]. In conditions of nutrient deprivation, lack of growth factors or after initiation of a differentiation program, the translation of 5' TOP mRNAs is potently repressed, whereas other proteins are not affected, suggesting that the 5' TOP tract confers very stringent translation regulation of 5′ TOP mRNAs [220, 221]. This is because the production of ribosomes is an energetically very costly process that requires the action of all three RNA polymerases and involves more than hundred gene products [222].

In the view of this "translational discrimination" occurring in cells, it may be possible that short-term PB inhibits (via AMPK-mTOR pathway) translation of 5′ TOP mRNAs in order to enhance the translation efficiency of 5′ cap mRNAs coding for proteins involved in drug metabolism. Only later, the 5′ TOP mRNAs are translated to increase ribosome content and protein synthesis capacity, explaining the long-term PB effect on the liver size. Consistent with this, exposure of LMH and G2F cells to PB in combination with rapamycin, which is a mTOR inhibitor and consequently also an inhibitor of protein synthesis, strongly increases the PB effect on CYP gene expression (FIGURE 4). Interestingly, rapamycin was shown to possess a particular inhibitory effect on 5′ TOP mRNA translation, whereas its effect on non-5′ TOP mRNA was weaker [223]. By addition of the protein synthesis inhibitor cycloheximide (data not shown) PB induction of *CYP2H1*, *CYP3A37* and *ALAS1* expression in LMH cells is potentiated in chick embryo hepatocytes and liver [224, 225]. Discriminatory inhibition of translation by AMPK activation may then be compatible with the need of protein synthesis for drug-metabolism and immediate-early gene induction does not require protein synthesis since it is activated by transcription factors that preexist in a latent form.

AMPK-LKB1-mediated inhibition of mTOR-regulated cell growth and proliferation

Several results show that mTOR plays a role in protein synthesis and cell growth and responds to several different types of stresses to ensure that growth occurs only when overall conditions are favorable. Stress conditions other than nutrient limitation may be heat shock, H_2O_2, osmotic stress, oxidative stress, pH changes and DNA damage.

The LKB1-AMPK-TSC2 pathway negatively regulates mTOR during cellular stress leading to protection against apoptosis and decreased protein synthesis and cell growth. These recent findings open intriguing opportunities for the AMPK signaling to act as an energy checkpoint that determines if cellular energy status is sufficient before the cell starts growth triggered by the mTOR pathway.

Studies by Jones et al. [226] demonstrated that AMPK couples glucose availability to the cell-cycle machinery by promoting cell cycle arrest in the G1 phase under low glucose. AMPK activation in HepG2 cells causes a cell cycle arrest in the G1 phase through stabilization of p53 [227] and treatment of cells with AICAR protects cells from cell death in response to different stimuli. Furthermore, reduction of AMPK levels was recently shown to decrease cellular viability after glucose deprivation in a number of human tumor cell lines [214], whereas LKB1-deficient cells are hypersensitive to apoptosis induced by energy stress [177, 228, 229]. In a proposed mechanism,

stress induces the LKB1-AMPK system, which leads to direct or indirect p53 phosphorylation and to overexpression of $p21^{CIP}$ and $p27^{KIP}$, two cyclin-dependent kinase inhibitors (CDKIs), followed by cell cycle arrest and inhibition of apoptosis. This pathway allows the cells to gain time to attempt restoration of cellular homeostasis caused by metabolic stress.

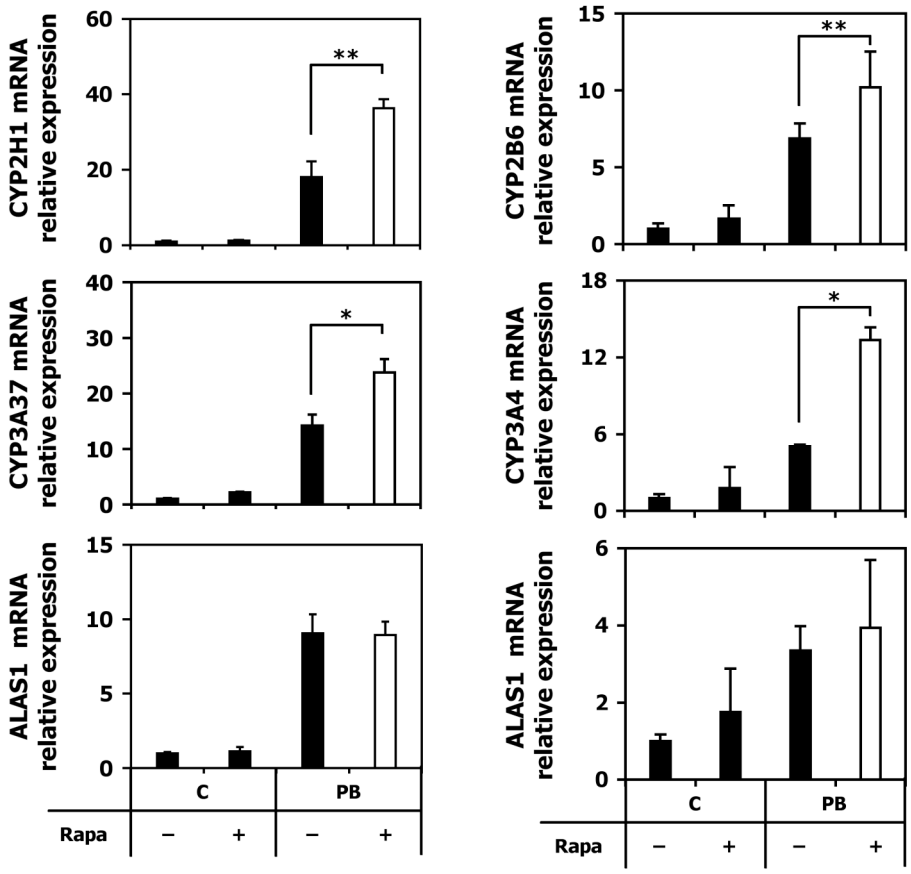


FIGURE 4

The mTOR inhibitor rapamycin potentiates the phenobarbital induction of *CYP* genes in chicken and human hepatoma cells. LMH and G2F cells were treated for 16h with 500μM and 2mM PB, respectively, with or without 100nM rapamycin. Gene expression of *CYP2H1*, *CYP3A37*, *CYP2B6*, *CYP3A4* or *ALAS1* was measured by RT-PCR. *p<0.01, **p<0.05.

Taken that AMPK exerts anti-proliferative and anti-apoptotic effects, the question raises if this is compatible with the pleiotropic responses triggered by PB. Effects caused by PB and TCPOBOP (two non-genotoxic carcinogens) include decreased apoptosis, increased DNA replication and tumor promotion, which are all CAR-mediated effects, too. The anti-apoptotic effect of these drugs correlates nicely with the cell survival promotion by AMPK activation, giving a speculative explanation for this effect.

In regard to the effect of PB on DNA replication, no reports exist that link AMPK activation to DNA replication, but in low energy conditions AMPK may repress this energy-demanding task. The majority of hepatocytes are tetraploid (4N) under normal conditions with small numbers of diploid (2N) and octoploid (8N) cells. PB and TCPOBOP were shown to increase the 8N/4N ratio (thus DNA replication)

in wildtype mice but not in CAR knockout mice [79]. Furthermore, in recent studies exposure of mice to PB effectively promoted liver growth, which was however mainly due to cell enlargement (hypertrophy) accompanied by hepatocytes polyploidization, and not to cell proliferation (hyperplasia) [230]. In contrast, PB-mediated liver growth in long-term experiments was due to cell proliferation and led to tumor promotion. This fast response to PB stress leading to hypertrophy is not incongruent with its AMPK activation. Indeed, when AMPK was activated by putting cells in low-glucose medium for 24h, surprisingly the cells exhibited a larger size, although AMPK-dependent cell-cycle arrest was detected [226]. Cell cycle arrest was also caused by low quantity of the AMPK activation AICAR, while rapamycin-sensitive cell growth was maintained, whereas high AICAR concentration was associated with both cell cycle and cell growth arrest. The data presented in this study demonstrate that low glucose can induce an AMPK-dependent cell cycle arrest under conditions where mTOR remains active and stimulates cell growth, indicating that cell cycle and cell growth can be differentially regulated by nutrients availability. The tempting speculative proposal that this could also happen upon PB treatment may explain some PB effects. In conclusion, short-term PB stress leads to transient increased liver size due to cell enlargement (and not to cell division), which should promote drug clearance, particularly because an increase in hepatocyte ploidy has been associated with higher metabolic activity [231]. Importantly, these effects of PB are not incompatible with its AMPK activation, on the contrary they can be explained by increased AMPK activity.

In contrast with this conclusion, it is apparently paradoxal that PB, which activates AMPK, promotes tumorigenesis (as mentioned in the Introduction) since AMPK is correlated with tumor suppressor genes, such as LKB1 and TSC2 that tightly control mTOR signaling to protein synthesis and cell growth.

Recent results show that PB or TCPOBOB treatment enhances murine double minute-2 (Mdm2) expression by CAR binding to a DR4 element, implicating that *Mdm2* is a primary target gene [79]. Mdm2 is an E3 ubiquitin ligase, which keeps p53 level low by targeting it for proteasome degradation, thus inhibiting the p53-induced apoptosis under normal conditions. Upon stress, p53 is phosphorylated and released from Mdm2, leading to apoptosis. If xenobiotics induce overexpression of Mdm2, p53 level is constantly kept low hampering its response in case of necessary apoptosis. Therefore, putative transiently activation of AMPK by PB with following p53-mediated antiapoptotic effect helps cells going through a threat (drug exposure) and allows them to recover, whereas drug-mediated transcriptional upregulation of Mdm2 by CAR creates a tumorigenic environment by inhibition of p53-dependent cell cycle surveillance and apoptosis in the long-term. As discussed in the Introduction, tumor promoter effects of PB in humans were never found, but interestingly, CAR knockout mice expressing hCAR exposed to PB showed all the acute responses observed with mCAR, for instance, liver size and hepatocytes octoploidy increase, *Mdm2* upregulation and antiapoptotic effects. In addition, preliminary results from the David Moore's group indicate that chronic xenobiotic stress promotes tumorigenesis in the hCAR mice suggesting that the human receptor is able to create a tumorigenic environment in a mouse background. Furthermore, not all mice strains are susceptible

to non-genotoxic carcinogens at the same extent, pointing again to the importance of the background. For all these reasons, the role of hCAR in tumorigenesis must not be underestimated.

In addition, the phenomenon of hormesis should also be considered. Hormesis is a biphasic dose-response relationship, in which a chemical exerts opposite dose-dependent effects [232]. Even if PB is a well-known non-genotoxic carcinogen and tumor promoter in rodents, in many studies, beneficial effects were shown from low-level exposure of rats to PB, whereas high doses turned out to promote tumor, a phenomenon being known as hormesis (FIGURE 5) [233, 234]. This hormetic effect of PB in the liver indicates the existence of a threshold for its carcinogenicity and possibly also for other effects. Interestingly, a dose of 10μM PB was recently shown to inhibit cell growth in human breast cancer cells [235], which fits perfectly in a hormetic effect of PB.

In summary, chronic PB treatment leads to liver size increase both by hyperplasia and hypertrophy, inhibition of apoptosis, DNA replication and tumorigenesis. Even if some of these effects are apparently incompatible with the AMPK-mediated inhibition of protein synthesis, speculative but strongly supported explanations were presented in the last pages.

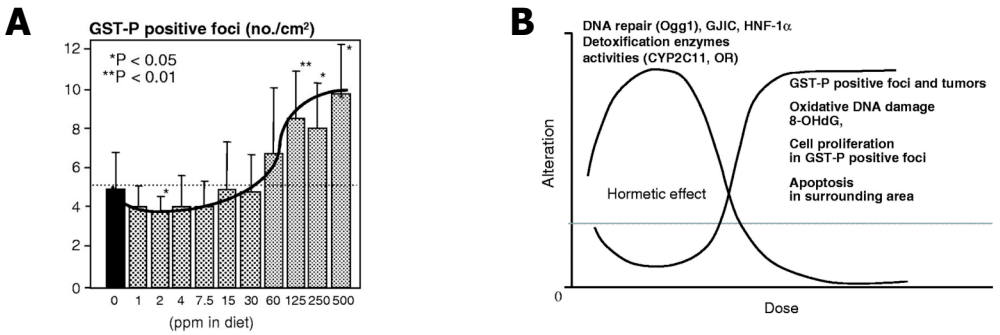


FIGURE 5

Hormesis and dose-response-mediated mechanisms in carcinogenesis. (A) Induction of GST-P positive foci in the liver of rats treated with phenobarbital. **(B)** Potential mechanisms mediating hormesis in carcinogenesis. Figure adapted from Fukushima, S. et al. Carcinogenesis 2005 26:1835-1845 [233].

Phenobarbital and Mitochondria

Many years ago, barbiturates were shown to depolarize neuronal mitochondria [236] and depress mitochondrial respiration [237, 238]. In isolated mitochondria, compounds of the oxybarbiturate class (including secobarbital, amobarbital, and amytal) inhibit respiration by interfering with complex 1 of the electron transport chain, whereas thiobarbiturates (including thiamylal and thiopental) may additionally have an uncoupling effect on the oxidative phosphorylation.

In consideration of our studies, we propose that PB and metyrapone affect mitochondrial functions leading to changes in the mitochondrial membrane potential, ROS generation (in LMH cells and human hepatocytes) and in the AMP/ATP ratio that increased in G2F cells and human hepatocytes. However, it is still unknown if drugs influence the mitochondria by direct binding to these organelles

or by an indirect effect via unknown signaling pathways. Considering that mitochondria isolation is a challenging step and that the drug doses used in induction experiments are quite high for a possible detection of specific binding, this questions could remain unanswered for a long time. Interestingly, metformin was shown to inhibit mitochondrial complex I, but its binding to mitochondria was found to be very weak [239].

It is also worth noticing that androstanol, which is an inverse agonist of CAR, inhibits *iNOS* expression via binding of CAR to a DR4 response element in its flanking region [240], thus keeping ROS formation low. However, PB does not seem to affect iNOS mRNA expression, but an effect on the protein level cannot be excluded. In this regard, estradiol was recently shown to induce a Hsp90-eNOS interaction, thus activating eNOS-mediated generation of NO in an AMPK-dependent and estrogen receptor-independent manner [241].

Since PB increases ROS formation, the inhibition of apoptosis caused by PB could allow the cell to evade from the p53-mediated apoptotic pathway triggered by transient ROS overproduction. ROS in low quantity was also shown to promote proliferation via the mitogen-activated protein kinase (MAPK) pathways [242, 243], which may be related with the PB-mediated increase in proliferation since recent evidence showed that PB activates p38 MAPK in rat hepatocytes [244].

The Phenobarbital "Receptor"

Despite many efforts to characterize the phenobarbital receptor, the primary target of this drug still remains unidentified. The search for a cytosolic receptor has failed, at least in part because of the apparent low potency of PB-type inducers. Interestingly, after exposure of cells to radioactively labeled PB, only a minor amount of radioactivity was detected inside the cells (earlier unpublished experiments). This finding would suggest a membrane receptor for PB, which triggers a signaling cascade ending in the nucleus. Years ago, our group started an effort to test if PB binds to the peripheral benzodiazepine receptor (PBR), which was shown to be involved among others in porphyrin and cholesterol transport and heme biosynthesis. PBR is a multiprotein complex located at the contact site between inner and outer mitochondrial membranes but it is also found at the plasma membrane. Unfortunately, PBR increased expression upon PB treatment could never be detected and stereobenzodiazepines, which do not bind to the receptor, induced CYP expression (unpublished observations). The plasma membrane $GABA_A$ receptor complex contains benzodiazepine binding sites, which bind among others barbiturates. Recently, different $GABA_A$ and PBR ligands were tested for CYP induction, but no correlation between binding affinity to the receptor and potency to induce CYPs was observed [245].

Metformin is an antihyperglycemic agent widely used in the treatment of type 2 diabetes. This drug stimulates glucose transport and utilization by peripheral tissue sensitive to insulin and inhibits hepatic gluconeogenesis. In studies by the Leverve's group, metformin was shown to inhibit mitochondrial complex I in intact cells but not in isolated mitochondria of rat hepatocytes, oocytes and in a human carcinoma-derived cell line (KG cells) [239, 246, 247]. Metformin treatment of permeabilized cells or

direct injection inside the cells had no effect on complex I activity and in addition, the direct binding to mitochondria is very weak. Leverve also demonstrated that a prolonged incubation was necessary to detect the radioactive-labeled metformin in the cytosol. Though less than 0.1% of radioactive metformin was associated to whole cells after 60 minutes, metformin potentiation of insulin effects was observed even before [248]. All these findings indicate that metformin has probably a mitochondrial target, which can be reached only in living cells via plasma membrane related events, suggesting the cell membrane itself as primary site for metformin action. This phenomenon was suppressed at low temperatures (21°C) and completely insensitive to any inhibitors of signaling pathways supporting the role of the plasma membrane in the metformin transport [246]. Interestingly, estrogen uptake by clathrin-coated pits was detected in rat liver and HepG2 cells [249, 250], indicating membrane-mediated endocytosis. It is in fact established that estrogen has also estrogen receptor-independent non-genomic effects.

Some observations indicate that the plasma membrane is involved in PB signaling, too. In older studies, PB was shown to interact with the outer layer of the plasma membrane changing thereby its fluidity [251, 252]. In addition, in overexpression experiments performed by the AMAXA device, which is based on electroporation, the PB induction was strongly decreased possibly due to damages in the plasma membrane destroying some microdomains important for the PB effect. In contrast, when using Lipofectamine, which is a cationic lipid transfection reagent, no difference in the induction range was detected (FIGURE 6). Furthermore, barbiturates suppress glucose transport across the blood-brain barrier (BBB) in rats [253-255] and in cultured mammalian cells and human erythrocytes by interacting directly with purified GLUT-1 [256], suggesting that they interact with proteins at the plasma membrane.

These three experimental observations support the speculative role of the plasma membrane in PB signaling, although, in the light of the PB and metyrapone effects on mitochondria presented in this thesis, mitochondrial targets must also be considered.

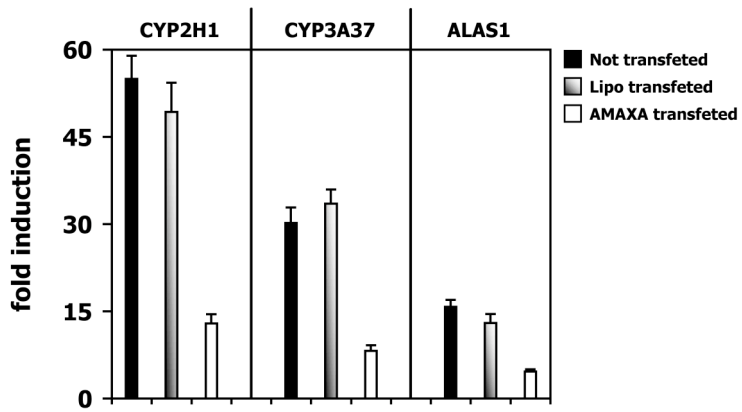


FIGURE 6

Effect of plasma membrane disturbance on phenobarbital-mediated CYP induction. The effect of transfection by lipofectamine2000 or electroporation (AMAXA) on PB induction of CYP2H1, CYP3A37 and ALAS1 is shown as fold induction. Transfection by electroporation drastically decreases PB induction.

Cancer Cells

Tumor-derived cell lines usually loose the ability to induce CYP gene expression upon drugs, which is a huge problem for mechanistical studies on the molecular level of this transcriptional regulation. It is thought that the rapid cell division and dedifferentiation of hepatoma cells are the basis of the loss of PB responses in cell lines. Cell lines differ from hepatocytes in various points. Firstly, cell lines usually express lower levels of CAR and PXR, but since G2F cells, which express more CAR than HepG2 cells (that have no PB responses), are not particularly inducible, this can not completely explain the loss of drug-mediated induction in cell lines.

Secondly, as already mentioned in the discussion of the manuscript (Results – Part II), cell lines are less dependent on mitochondrial respiration for ATP production since the glycolysis is strongly increased. Reduced mitochondrial functions may attenuate the effect of PB on these organelles and thus leading to lower or abolished drug induction.

Thirdly, the most common and profound phenotype of cancer cells is their propensity to utilize and catabolize glucose at high rates. Cancer cells have higher glucose uptake, glycolytic rate and lactic acid production because of higher hexokinase and glucose transporter 1 (GLUT1) expression, a glucose transporter that is quickly saturated at low plasma glucose concentrations. Hepatocytes, in contrast, express only GLUT2, which transports glucose much better that GLUT1 because it is a low-affinity transporter that is not easily saturated at even high plasma glucose levels. Hepatoma derived cell lines loose GLUT2 expression and overexpress GLUT1, which was also observed in G2F cells in comparison to human hepatocytes (Rencurel F, unpublished observation). Knowing that PB can bind glucose transporter and block the glucose uptake, and that CAR was recently shown to be involved in glucose homeostasis, interplay between PB and glucose may occur. If PB blocks GLUT2 in human hepatocytes, which express only this transporter, the level of glucose uptake is drastically decreased, thus the cells sense glucose starvation. In cell lines, which express also GLUT1, the effect is probably not as strong. It is therefore possible that PB leads to AMPK activation by causing glucose deprivation, which is sensed by the kinase as low energy state.

Interestingly, exposure of cells to cytochalasin B, which is a GLUT inhibitor, increases the expression of *CYP2B6* and *CYP3A4* (Rencurel F, unpublished observation).

LMH cells, which are hepatoma cells, should be somehow different from other cell lines because they retain drug responses very well. In preliminary experiments, GLUT1 and GLUT2 turned out to be similarly expressed on mRNA level in LMH cells (Blättler S, unpublished observation) in contrast to G2F, which express low level of GLUT2 and are maybe therefore not strongly inducible. The reason for this higher GLUT2 expression could be the fact that LMH cells are cultured in medium with higher glucose concentration, which keeps the GLUT2 expression higher.

To verify the hypothesis that PB causes ATP depletion leading to AMPK induction, several ATP-depleting agents were tested for their capacity of inducing CYP gene expression (Results – Part II, NaN_3 effect). Among them, glucosamine and 2-deoxyglucose, which are rapidly phosphorylated by hexokinase without being further metabolized by glycolysis to produce ATP, were used to deplete ATP. Indeed, both compounds increased the *CYP2H1*, *CYP3A37* and *ALAS1* expression, but an

attenuation of PB induction of these genes was observed by glucosamine or 2-deoxyglucose addition, the last having a stronger effect possibly due to the higher affinity binding to GLUT (FIGURE 7) [257]. Considering that both of them are transported by GLUT, we can speculate that competition with PB for binding to the transporter occurs dampening thus the PB effect. This would support a role for glucose transporters in PB signaling, but obviously further experiments are needed to prove this possibility. However, the experimental observation that glucose attenuates CYP gene inducibility in hepatocytes, the so-called glucose effect, is in good correlation with a competition between PB and glucose for binding to GLUT and with a decreased AMPK activity in high glucose state.

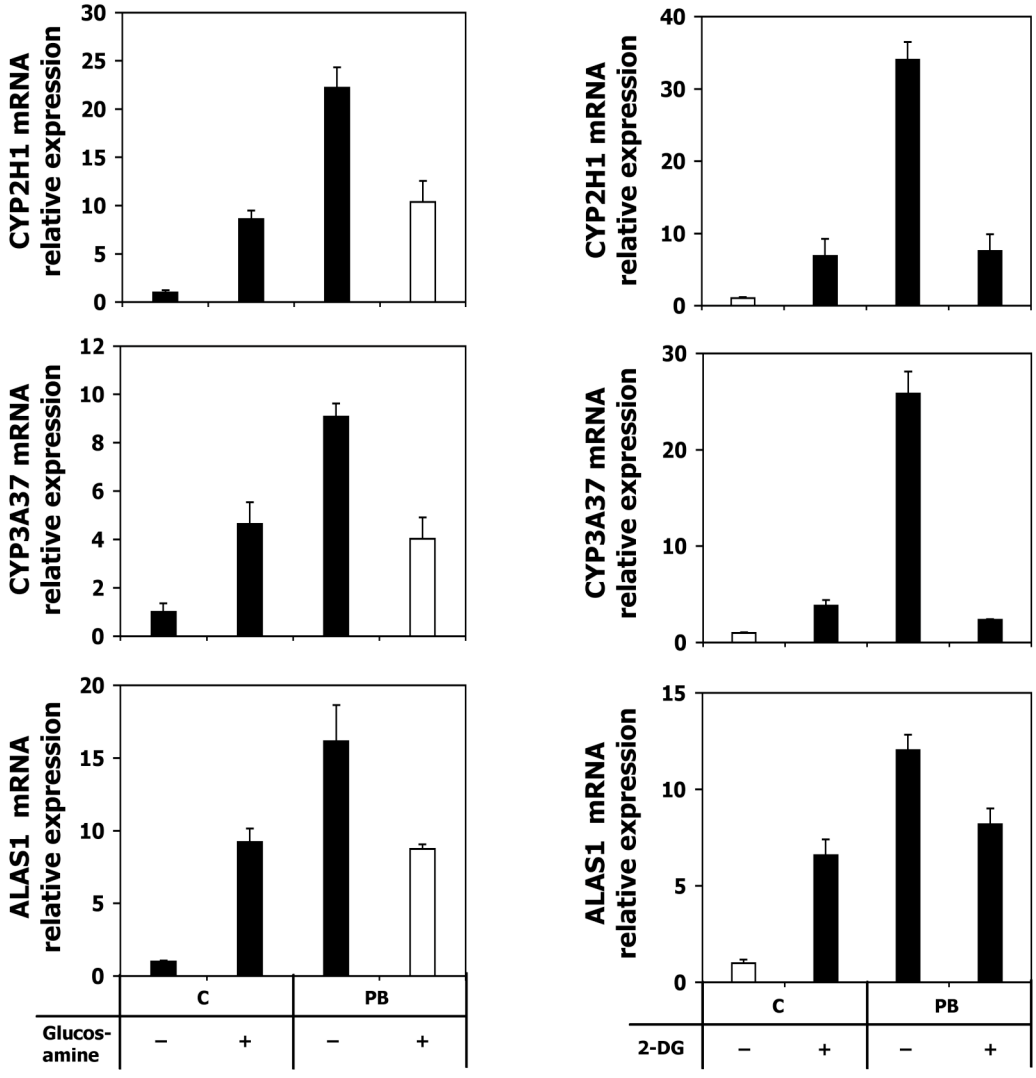


FIGURE 7

Glucosamine and 2-deoxyglucose decrease PB induction in LMH cells. Exposure of LMH cells to 500μM PB in combination with glucosamine or 2-deoxyglucose (2-DG) decreases PB induction of *CYP2H1*, *CYP3A37* and *ALAS1*.

Role of Mitochondria and/or AMPK in Drug Metabolism in the Elderly

It has been reported that aging diminishes hepatic capacity, particularly the clearance of drugs. Liver drug metabolism decreases with age in animals [258-261]. In human, the rate of drug elimination of several substances was shown to decline with age *in vivo* [262, 263], whereas *in vitro* data showed unaltered CYP content. For long time there was a discrepancy on the effect of age on CYP activity probably due to interindividual variation in restricted numbers of liver samples and the difficulty of getting representative liver samples from healthy subjects. In 1997, Sotaniemi could show a reduction of *in vitro* and *in vivo* drug metabolism with age in humans in a study with 226 patient samples. The reduction of drug metabolism (-30%) after 70 years of age indicates that the prescription of drugs for elderly people is very delicate [264]. In addition, aging causes a significant reduction in liver volume and blood flow [265-267], where drug clearance appears to be reduced parallel with diminished liver volume in healthy aging man [268, 269]. Moreover, some data suggest a loss of inducibility [270, 271] and a loss of hepatic smooth endoplasmic reticulum associated with aging in animals [272-274]. Generally, lower drug doses are required to achieve the same effect with advancing age.

Studies in male Fischer 344 rats with PB showed a significant decline in the non-induced and induced activities of liver drug-metabolizing enzymes and in the amount of CYPs between maturity (16 months) and senescence (27 months), whereas essentially no differences exist between very young (1 month) and mature animals. The livers of young and mature animals treated with PB exhibit greater hepatomegaly, faster rates of induction and post-induction recovery of normal enzyme activities and higher maximally induced levels of CYPs in comparison to senescent rats [275].

In the last years, researchers have speculated that mitochondria might contribute to aging, either by releasing tissue-damaging reactive oxygen molecules or by deteriorating and depriving the cell of its needed energy. Attardi et al. reported some of the first hard evidence that mitochondria deteriorate during aging. His team found that mutations in the 16,500-base mitochondrial genome accumulate with time in a particularly important 1000-base segment that controls the genome's replication [276]. Acquired mitochondrial dysfunction and age-associated accumulation of mutations in mitochondrial DNA may play a role in aging, as well as in age-related metabolic and degenerative diseases (such as diabetes, Parkinson’s disease, Alzheimer disease, cancer) [276-278]. For example, mice with a defective mitochondrial DNA polymerase have been shown to age prematurely [279] and a mitochondrial DNA (mtDNA) polymorphism, eventually related to a low production of ROS, is often found among centenarians [280].

Our data show an important role of mitochondria in the mechanism of drug induction, especially changes in the mitochondrial potential and in ROS formation are triggered by PB and metyrapone in LMH cells and by PB in human primary cultures of hepatocytes and in human hepatoma cells. In the light of recent observations, mitochondria dysfunction may be related to aging and to the decrease of drug metabolism in elderly. The observation that in aged cells mitochondrial potential is reduced by decreased respiration and that endogenous ROS production rises with aging [281, 282] support this

speculation, meaning that the sensitivity to PB-mediated changes in potential or ROS level may be attenuated in aged cells leading to reduced drug induction and metabolism.

In this regard, new findings showed that changes in expression or regulation of AMPK are related to aging. Hyperphosphorylated AMPK was found only in old but not in young muscles and the reduced muscle hypertrophy typically found in aged-muscles was correlated to high phosphorylation extent of AMPK [283, 284]. Also increases in aortic superoxide anion with aging were significantly correlated with changes in AMPK expression and regulation [285]. Another study in old mice demonstrated that the diminished stress tolerance characteristic for aging is associated to age-related changes in hepatic AMPKα1 basal activity, which was increased, and to reduced capacity of hypoxia to activate the kinase [286]. All these data suggest that old mice were generally less tolerant to stress with a clear correlation to AMPK expression and activity. So, an age-related elevation in AMPK phosphorylation may partly contribute to the attenuated drug responsiveness in elderly.

The CXR and CAR Complexes

By immunoprecipitation several proteins were found in the complex with CXR. Among them, two were also coimmunoprecipitated with mouse CAR, Hsp90 and PP2A, supporting evolutionary conservation also in this aspect. However, two new cochaperones, Hsp70 and Hsp40, were identified in the CXR complex. The Hsp90/Hsp70 chaperone machinery is a multiprotein complex, which assists the assembly of receptors or of other signal transduction proteins into heterocomplexes. The minimal system to assemble stable receptor heterocomplex is composed of five proteins, for instance Hsp90, Hsp70, Hsp40, Hop and p23 [287]. Three out of these five proteins were identified in complex with CXR. It was shown that J domain-containing proteins play important regulatory roles as cochaperones, recruiting Hsp70 and Hsp40 [288]. CCRP, which was shown to interact with CAR, contains a J domain [73] supporting our findings. Hsp90 was shown to regulate the nuclear retention of the glucocorticoid receptor (GR) because Hps90 inhibitors, such as geldanamycin and radicicol that bind to the ATP/ADP binding site of the chaperone interrupting its function, accelerate GR nuclear export [289]. Furthermore, geldanamycin inhibits TCPOBOP-mediated Cyp2b10 induction by decreased CAR nuclear accumulation [74]. In addition to their role as chaperones, heat shock proteins (Hsp) are activated by cellular stress, and likely help to maintain viability under stress conditions. Barrett et al. showed that treatment of a human cell line with micromolar concentration of a mitochondrial poison resulted in inhibition of cytochrome oxidase with subsequent ROS formation. Increase in ROS caused Hsp70 nuclear translocation and activation [290]. The authors concluded that mitochondrial electron transport chain dysfunction activates an Hsp response, which is mediated by oxidative stress. The purpose of this nuclear translocation is unknown but most likely the presence of Hsp in the nucleus affects gene expression, maybe by transporting transcription factors, such as CXR/CAR into the nucleus. It was also shown that Hsp70 binds histone deacetylase complex (HDAC), one way how mitochondria may affect gene expression via ROS.

Another identified CXR interacting protein is tubulin, which is quite interesting in consideration of the observation that colchicine, which destroys the tubulin network, decreases basal and PB-induced *CYP2B6* and *CYP3A4* expression in human hepatocytes [291]. In addition, CAR was suggested to colocalize with tubulin in mouse liver [73], wherefore CXR/CAR nuclear translocation mediated by the tubulin network is plausible

Two bands (1 and 3) were related to three proteins involved in microRNA (miRNA)-mediated gene downregulation, eukaryotic translation initiation factor 2C1 (or Ago1), eukaryotic translation initiation factor 2C2 (or Ago2) and trinucleotide repeat-containing 6B protein (TNRC6B). miRNAs are small noncoding RNAs that repress gene expression by recruiting effector complexes (miRNPs) to miRNA complementary sites on mRNAs and thus blocking translation initiation. In animals, nearly all miRNAs investigated to date appear to regulate gene expression by imperfect base-pairing at the 3' UTR of target mRNAs. miRNAs assemble into the RNA-induced silencing complex (RISC), which is the effector of RNAi. The key component of the RISC complex is an argonaute protein. Repressed mRNAs, Ago proteins, and miRNAs accumulate in so-called cytoplasmic processing bodies (p-bodies), which are stress granules that bear sites for mRNA degradation or mRNA storage of translationally repressed mRNA. Interestingly, it was recently shown that this miRNA-mediated repression can be relieved suggesting that this is a rather dynamic regulation [292]. It is estimated that 20-30% of all genes are miRNA targets (for reviews on miRNA [293, 294]). In support to an interaction between CXR and these three proteins, the identified TNRC6B was shown to bind to Ago1 and Ago2. These data suggest that CXR interacts with proteins involved in miRNA repression of translation extending the CXR potential regulation to post-transcriptional events. Consistently, in band 4 and 5 several proteins associated to RNA were identified, for instance, DEAD box proteins (RNA helicases), heterogeneous nuclear ribonucleoproteins and the poly A binding protein, which was also linked to mRNA translation inhibition. If this data will be confirmed by coimmunoprecipitation studies followed by western blot, new players in the complicated xenosensor-mediated gene expression regulation will be added.

Band 2 was identified as pyruvate carboxylase, an enzyme of the gluconeogenesis pathway. Since it is known that CAR regulates another enzyme of this pathway, PEPCK, it is possible that several enzymes involved in gluconeogenesis are regulated by CAR. In band 7, aldehyde dehydrogenase, an enzyme involved in phase II drug metabolism, was identified, whereas band 8 was recognize as alpha enolase, an enzyme of the glycolytic pathway. The significance of this last interaction is completely unknown.

Although to date no differences in proteins interacting with CXR between control and PB samples could be observed due to the lower protein content of the PB-treated sample, our findings encourage future investigation of the CXR/CAR interacting proteins.

Protein Kinase C Zeta: Physiological Role

In the present study, we established an involvement of atypical protein kinase C zeta (PKCζ), a protein kinase of the AGC family, in the LKB1-dependent activation of AMPK upon PB treatment. Inhibition of PKCζ with a pharmacological inhibitor reduced AMPK activation caused by PB. This AMPK activation was LKB1-dependent, involved LKB1 phosphorylation at Ser428, and led to an association of LKB1 with AMPK. In addition, overexpression of PKCζ in LMH or G2F cells increased PB induction. At the present time, the physiological relevance of the role of PKCζ in drug induction is unknown.

There are at least 12 PKC isoforms classified into three subfamilies according to the structure of the N-terminal regulatory domain, the classical, the novel and the atypical isoforms, which are surprisingly non-redundant despite the high degree of homology [295]. PKCζ belongs to the last category, which controls cell proliferation, apoptosis and insulin-stimulated glucose transport [296-300]. Recently, overexpression of PKCζ in carcinomas of liver and urinary bladder [301, 302] and its involvement in the phosphorylation of the mTOR downstream target p70SK6 were reported [303]. Interestingly, PKCζ nuclear translocation in mouse was associated with hyperproliferation [304] and the AMPK activator metformin was reported to activate PKCζ in muscle of diabetic mice [305]. PKCζ overexpression increases PPARα activity in reporter gene assays with suggested increase in PPARα phosphorylation [306]. In addition, PKCζ was shown to promote p300 interaction with the hypoxia-inducible factor 1α increasing its activity in transcriptional regulation [307]. If this applies also for CAR will be investigated in future studies aimed to unravel the link between PKCζ and drug induction mechanism.

Outlook

Although our findings clearly demonstrate that AMPK is essential for hepatic PB induction of CYPs, they also raise many new questions, which must be addressed in the future.

We showed that PB induction is abolished in AMPKα liver-specific knockout mice and their primary hepatocytes. Knowing that PB has pleiotropic effects, it would be interesting to investigate if AMPK mediates these effects, particularly the liver size increase, the tumor promoter and the antiapoptotic effect. In response to PB exposure, many CAR-independent mechanisms occur, which may be mediated by AMPK. Investigation of the mechanism, by which liver cells can alter gene expression in response to PB in a CAR-independent way, is important to understand the adaptation of liver cells to drugs. From microarray data in wildtype and CAR knockout mice it emerged that a large spectrum of genes are regulated by CAR upon PB treatment but many others resulted to be CAR-independent raising the question if and which of these genes are also AMPK-dependent. Studies aimed to investigate the role of AMPK on genes, such as transporters or phase II drug-metabolizing enzymes, would help to answer these questions. In addition, a PB time-course experiment *in vivo* should be performed to investigate the AMPK activity at several time-points acquiring insights about differences between short-term and long-term PB treatment.

Furthermore, the role of the mTOR pathway on PB-mediated proliferation and tumorigenesis should be tested, since our preliminary experiment with rapamycin suggests mTOR involvement in CYP gene expression regulation in the short-term induction, but analysis of the long-term effect of PB on mTOR could elucidate if the tumor promoter effect of PB is linked to a mTOR dysregulation.

Another important experiment is the investigation of PB induction of CYPs in LKB1 knockout mice. Since this kinase was shown to be the major AMPK upstream kinase in the liver [179], its genetic deletion should strongly impact PB-mediated CYP gene expression assuming that its role is essential.

Obviously, a very interesting topic are the downstream AMPK targets. AMPK phosphorylates unknown proteins leading to increased CYP expression upon PB treatment. To definitively exclude the possibility of CAR as an AMPK target, experiments using the entire CAR protein, and not only peptides, should be performed. In fact, conformational features or the presence of other proteins may be necessary to allow or to prime phosphorylation. For these kinds of experiments, Shokat and collaborators developed a combined chemical/genetic method that consents to identify protein kinase substrates. This approach is based on a particular functionally silent point mutation in the ATP binding site of a kinase that allows the mutant kinase to use an ATP analogue, N^6-(benzyl)-ATP, which is a poor substrate for all other protein kinases in the cell. By adding the radioactive labeled ATP analogue to a cell extract containing the mutated kinase, radiolabeling is detected exclusively in the mutant kinase direct targets. This elegant experiment allows to work in cell extract where all the proteins are present. However, since the radiolabeled ATP analogue cannot cross the cell membrane, it is not possible to perform experiments in intact cells [308]. Currently, modifications of ATP analogues that can cross the cell membrane are tested.

As already mentioned in the discussion, proteins like p300 and HNF4α, which were already identified as AMPK substrate, should also be considered as potential AMPK targets upon PB treatment potentiating CAR transcriptional regulation or CAR expression.

In immunoprecipitation experiments, PP2A was shown to interact with mouse CAR [74] and with its chicken ortholog CXR (Results – Part VII), but the significance of this interaction is unknown. Low doses of okadaic acid, a potent PP2A inhibitor, completely block PB induction of CYP2Bs in mouse and rat hepatocytes by preventing CAR nuclear translocation, wherefore a role of this phosphatase in the nuclear CAR accumulation is suggested. Interestingly, Keen et al. showed that PP2A regulates mRNA stability of estrogen receptor (ER) via its 3' UTR region. In fact, okadaic acid reduced ER mRNA to 25% and activation of PP2A stabilized ER mRNA [309]. In preliminary experiments, hCAR mRNA level was strongly decreased by okadaic acid treatment down to 35% in primary cultures of human hepatocytes (FIGURE 1) suggesting that PP2A stabilizes CAR mRNA, possibly enhancing its translation and consequently its nuclear translocation.

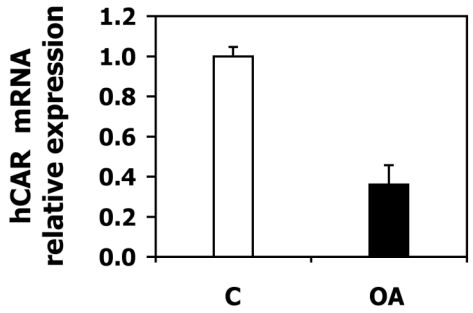


FIGURE 1

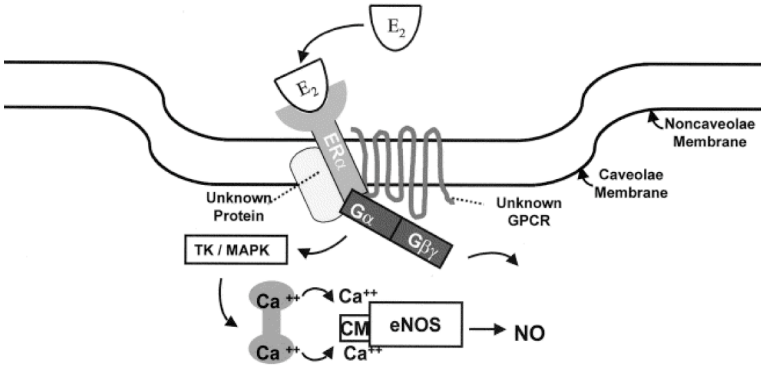
Okadaic acid decreases CAR mRNA level in primary cultures of human hepatocytes. Human hepatocytes were treated with 100nM okadaic acid (OA) for 16h. CAR mRNA was measured by RT-PCR. $p<0.01$.

CAR was colocalized with PGC1α in nuclear speckles [206], which are highly dynamic sites for storage and assembly of splicing factors and other proteins involved in regulation of transcription, which are then released upon gene activation targeting mRNAs to transcriptionally active sites. It is intriguing that okadaic acid was shown to affect the composition of these subnuclear regions [310] potentially leading to splicing and mRNA export regulation, which may be the reason for the decreased CAR mRNA level upon okadaic acid treatment. Interestingly, according to our results in AMPK liver-specific knockout mice, CAR seems to be localized in these transcriptionally silent nuclear speckles upon PB treatment, whereas more diffuse localization was observed in wildtype mice ([181] in APPENDIX 3) explaining possibly the abolished PB induction in AMPK knockout mice. However, no difference was observed in CAR nuclear transfer in wildtype versus knockout mice. These results imply that AMPK is not involved in the PB-induced cytoplasmic-nuclear transfer of CAR, but possibly in its subnuclear localization.

As mentioned in the discussion, the efficiency of importinα-mediated nuclear transport is increased by AMPK suggesting that nuclear translocation of proteins involved in gene expression regulation may be

enhanced affecting transcriptional and post-transcriptional events. Therefore, gene expression pattern are potentially affected by AMPK and proteins involved in drug-mediated CYP gene regulation could be importinα cargoes. Future studies should reveal whether or not CAR is a cargo for importins.

CAR was found to localize at the plasma membrane in an approximately 160kDa complex and this association was increased by PB treatment in mouse liver suggesting that CAR may exert non-genomic effects on signaling cascades that potentially indirectly affect gene expression [311]. Interestingly, CAR translocation to the nucleus and to the plasma membrane occurred simultaneously supporting the notion that CAR membrane localization is not a prerequisite for the nuclear transfer. The elucidation of potential CAR non-genomic effects in the liver may lead to important new insights into the regulation of drug metabolism by xenobiotics. Furthermore, AMPK is possibly involved in this pathway, thus the search for its targets may be directed in this direction. Such non-genomic effects were shown for estrogen receptor α, thyroid hormone receptor and vitamin D receptor, which were shown to affect G-protein coupled receptor, ion channels and Src, respectively [312, 313]. Interesting is the case of the non-genomic effects of estrogen on eNOS mediated by a subpopulation of estrogen receptor localized at caveolae in the plasma membrane (FIGURE 2): estrogen receptor and eNOS are coupled in a functional signaling module in caveolae. By binding of estrogen to the estrogen receptor on the caveolae, G-protein coupled receptors are activated, which mediates downstream events including activation of MAPK and Akt signaling, stimulation of Hsp90 binding to eNOS, and perturbation of the local calcium environment, leading to eNOS phosphorylation and calmodulin-mediated eNOS stimulation. The potential generation of ROS is consequently increased. Other studies indicate that estrogen is transported into mitochondria by both passive diffusion and by receptor-mediated endocytosis and that it enhances mitochondrial transcription. Estrogen inhibition of the electron transport chain were also observed possibly caused by a estrogen-mediated change in membrane fluidity, which as consequence enhances the rate of electron transfer (due to increased collision between respiratory chain complex and electron carriers) and ROS formation [314, 315]. Some of these events are reminiscent with some PB effects, wherefore it would be interesting to study the hypothesis of a membrane-mediated PB-CAR signaling.



Form Chambliss et al. [315]

FIGURE 2

Non-genomic effect of estrogen. Activation of eNOS by estrogen at the plasma membrane via G-coupled receptors.

From the CXR immunoprecipitation studies, several proteins were identified with functions that could be related to CYP gene regulation. For example, the heterogeneous nuclear ribonucleoprotein U (hnRNP U) is known to associate with the histone acetylase (HAT) and transcription activator CBP/p300 affecting transcription regulation. Additionally, hnRNP U was shown to be associated with the phosphorylated C-terminal domain (CTD) of polymerase II (Pol II) promoting transcription [316]. These observations prompt studies to investigate whether this interaction between CXR and hnRNP U has functional relevance impacting CYP expression. The interaction between CXR and the proteins involved in miRNA gene regulation (Ago1/2 and TNRC6B) is also interesting in terms of function, leading possibly to a new strategy of nuclear receptors to fine tuning the transcription of drug-metabolizing enzymes and other proteins involved in detoxification pathways. The interaction of CXR with these new partners should therefore be further investigated to unravel its physiological relevance.

In addition, identification of the differential phosphorylated proteins detected by western blot of CXR immunoprecipitated lysates may reveal potential AMPK targets in the PB induction mechanism.

Summary

During the past several years, important advances have been made towards the understanding of the mechanisms regulating the expression of genes that determine drug clearance, including phase I and phase II drug-metabolizing enzymes and drug transporters. Orphan nuclear receptors have been recognized as key mediators of drug-induced changes in both metabolism and efflux mechanisms.
Characteristically, xenobiotic-metabolizing members of the cytochrome P450 (CYP) superfamily are transcriptionally induced upon exposure to xenobiotics. This phenomenon increases the ability of the organism to adaptively defend itself against toxic foreign compounds. However, the induction of drug-metabolizing CYPs has important clinical consequences, such as impacting drug treatment, causing drug-drug interactions and influencing endogenous regulatory pathways. Therefore, understanding how drugs lead to induction of drug-metabolizing enzymes is of huge importance for the human pharmacotherapy with a future aim to develop predictive tests for personalized medicine.
In this thesis, we demonstrated that phenobarbital-type drug-mediated transcriptional regulation of CYP genes is achieved by a signaling cascade involving mitochondrial functions leading to reactive oxygen species generation, PKCζ and LKB1 phosphorylation and subsequent interaction between LKB1 and AMPK, which is in turn phosphorylated and activated. The activation of PKCζ, LKB1 and AMPK adds new proximal targets to the so-far elusive sequence of events, by which phenobarbital-type drugs induce the transcription of multiple genes. In addition, our results propose mitochondria as a target for inducer drugs as a novel concept.
Obviously, further studies are required to understand the mechanism more in detail. Especially the activation of AMPK by ROS production, the downstream targets of AMPK and the effect of phenobarbital on mitochondrial functions need further investigation.

The molecular mechanism of hepatic drug induction is closely linked to endogenous regulatory pathways, raising the speculation that xenobiotic-sensing receptors evolved from nuclear receptors that handled toxic lipophilic metabolites, such as cholesterol metabolites. Since xenobiotic compounds have similar hydrophobic properties, these nuclear receptors may have extended their substrate specificity to include xenobiotics. Thus, organisms could sense like toxic endogenous metabolites, for example bile acids.
The evolutionary origin of AMPK may also be similar to the one of the drug metabolism. In single-celled eukaryotes, AMPK orchestrates the response to glucose starvation, which may have been its original "raison d'être". However, during evolution of multicellular organisms AMPK became involved in the regulation of energy intake and expenditure at the whole body level, while hormones and cytokines have acquired the ability to regulate AMPK. Furthermore, AMPK may have turned from an energy-sensing kinase to a stress-sensing protein. Therefore, it is reasonable that this kinase is activated by compounds which upregulate CYPs. Drugs are probably sensed by AMPK as stress,

because drug metabolism is an energy consuming process. For this reason rapid initial AMPK activation by drugs could downregulate unnecessary pathways allowing the organism to concentrate on the disposal of these compounds.

To survive environmental stress like phenobarbital exposure, cells activate a variety of adaptive mechanisms, including inhibition of the energy-costly mRNA translation and promotion of cell survival in the short-term, later liver size increase and cell proliferation in the long-term. These mechanisms produce a transient hepatomegalic response that promotes drug clearance, but clearly also creates a tumorigenic environment. The proliferative and antiapoptotic environment induced by CAR activators, such as phenobarbital and TCPOBOP, may be an important contributor to early stages of hepatocarcinogenesis, for example by promoting accumulation of cells carrying tumorigenic genetic changes.

As a result of drug-mediated CAR activation, drug-metabolizing enzymes are induced, whether gluconeogenesis, energy metabolism and fatty acid oxidation are inhibited allowing liver cells to increase their drug metabolism potential and lower their energy metabolism, wherefore the cells have ultimately more capacity to metabolize drugs and xenobiotics.

The question of how our body has adapted itself to deal with foreign compounds that it has never encountered before (reminiscent of the immnune system) is a fascinating challenge for the future.

Materials and Methods

Reagents

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), N-acetyl L-cysteine (NAC), metformin and protein G agarose beads were obtained from Sigma (Buchs, Switzerland) and AICAR (5'-phosphoribosyl-5-aminoimidazol-4-carboxamide) from Toronto Research (North York, Canada). Phenobarbital sodium salt (5-ethyl-5-phenyl-barbituric acid sodium salt), dinitrophenol (DNP), rotenone, and sodium azide (NaN_3) were purchased from Merck (Dietikon, Switzerland) and Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine) from Calbiochem (Laufelfingen, Switzerland). Antibodies against AMPKα1 and AMPKα2 were purchased from Upstate Biotechnology (Luzern, Switzerland) and antibodies against ACC-pSer79, AMPK-pThr172, HA-tag, MAPK, AMPKα, LKB1, LKB1-pSer428, PKCζ and PKCζ-pThr410 from Cell Signaling Technology (Allschwil, Switzerland). All other reagents and supplies were obtained from standard sources.

Cell Culture Methods

Culture and transient transfection of LMH cells

LMH cells were obtained from ATCC (catalogue no. 2117-CRL, batch no. F12213). They were cultivated in William's E medium supplemented with 10% fetal calf serum, 1% glutamine (2mM), and 1% penicillin/streptomycin (50IU/ml) on gelatin-coated dishes. Cells were seeded for 3 days and when they reached approximately 80% surface density, they were transfected for 48h using the NucleofectorTM Kit T (AMAXA Biosystems, Köln, Germany) with Solution T according to the AMAXA protocol. Alternatively, cells were transfected for 48h by Lipofectamine2000 (Invitrogen, Nivelles, Belgium) as described in the manufacturer's protocol.

Preparation of LMH ρ^0 cells

LMH cells were grown for 12 weeks in medium containing 50ng/ml ethidium bromide, 50mg/ml uridine, and 1mM pyruvate. The ρ^0 status of the cells was confirmed by the decrease of mitochondrial marker genes via RT-PCR and by the incapacity of surviving without uridine and pyruvate. RT-PCR primer sequences are summarized in Table 1 in the supplemental data.

Preparation of primary culture of mice hepatocytes

Mouse hepatocytes were prepared from animals anaesthetized with Ketamin/Xylazine (Sigma, Buchs, Switzerland). The hepatic portal vein was canulated and perfused with HEPES-EGTA pH 7.4 at a flow rate of 8ml/min for 5min. The liver was then perfused for 6min with a collagenase solution (type 2 Worthington, Lakewood NJ, USA) continuously gassed with carbogen. The livers were extracted and put in Leibowitz L-15 medium (Sigma, Buchs, Switzerland) supplemented with 10% newborn calf serum (Invitrogen, Basel, Switzerland). Hepatocytes were then filtered through a nylon mesh and centrifuged three times for 5min at 50xg and 4°C. After determination of viability, cells were allowed to attach at a density of 200'000 cell per well of 12-well dishes in Williams'E medium supplemented

with 10% fetal calf serum, 4μg/ml insulin, 200μM glutamine and 1% penicillin/streptomycin (50U/ml) on collagen-coated dishes. After overnight incubation, the medium was replaced with serum-free Williams'E medium for 24h and the cell were subsequently exposed to compounds.

Preparation of primary cultures of human hepatocytes

Liver tissue (50-200mg) was obtained from consented patients undergoing surgical resections in the clinic of visceral surgery in Bern (Switzerland). Tissue was perfused with two cannulea with buffers heated to 37°C at a rate of 100ml/min. Firstly, PBS containing 10mM HEPES for 5min followed by 500ml of PBS/HEPES containing 2mM EGTA were used. EGTA was then removed by 500ml of the first buffer. The enzyme solution (0.05% collagenase, 0.02% dispase, 0.017% hyaluronidase and 0.02%DNase in HBSS containing 5mM $CaCl_2$) was recirculated through the liver piece for a period of 7-12min until it was sufficiently softened. The tissue was mechanically disrupted in DMEM containing 10% fetal calf serum, filtered through 50micron sterile gauze and washed twice in DMEM by keeping the suspension on ice. Hepatocytes were subsequently seeded on rat-tail collagen coated plastic dishes at a density of 100'000 cells per well of a 12-well dish in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1mM dexamethasone and 50IU/ml penicillin/streptomycin. After overnight attachment, the medium was replaced by serum-free Williams'E medium supplemented with 100nM hydrocortisone, 0.5x ITS solution (Sigma) and 50IU/ml penicillin/streptomycin. Cells were incubated under these conditions for 24h and subsequently exposed to the compounds of interest.

AMPKα1 and AMPKα2 downregulation by diced siRNA (d-siRNA) duplexes

RNAi analysis was performed by BLOCK-iT Dicer RNAi Kit (Invitrogen, Nivelles, Belgium). Primers used for the sense and antisense DNA templates amplification are summarized in Table 1 in the supplemental informations. RNA transcription was done by BLOCK-iT T7 Enzyme Mix and the double-stranded RNA transcripts were cleaved by BLOCK-iT Dicer Enzyme into d-siRNA duplexes, which were transiently transfected into LMH cells by Lipofectamine2000 according to standard protocols. After 48h of transfection, LMH cells were treated with 500μM PB or metyrapone for 16h in order to perform RT-PCR measurements, or 1h for AMPK activity measurements and western blots.

Assays

Western blots

LMH lysates were prepared as described above, but lysis was performed in 200μl buffer. The lysates were centrifuged at 14000rpm and 4°C for 10min, the supernatant transferred to a fresh tube, snap-frozen in liquid nitrogen, and stored at -80°C. Protein concentration was determined by BCA assay and 30μg of total proteins were loaded. Proteins were separated on 10% SDS-PAGE and blotted for 1h onto a nitrocellulose membrane using a Biorad Minigel system with transfer buffer containing 39mM glycine, 48mM Tris, 0.0375% SDS, and 20% methanol. The blots were incubated overnight at

4°C with a 1/1000 dilution of the primary antibody in Roche Blocking Reagent. Proteins were visualized according to the enhanced chemiluminescence protocol (Amersham Pharmacia Biotech, Zürich, Switzerland).

Immunoprecipitation

LMH cells were cultivated on 10cm dishes, transfected with HA-LKB1 or Myc_AMPKα1/2 by Lipofectamine2000 for 48h, and treated with PB or metyrapone for 20 or 40min. The cells were scraped in 150mM NaCl, 50mM Tris pH 8, 1% Triton X-100 and protease inhibitors (Complete Mini EDTA-free), left 15min on ice, sonicated two times for 5 seconds, and centrifuged 10min at 4°C and 14000rpm. BCA assay was used to determine the protein content of the supernatant, which was then incubated for 30min at 4°C under rotation with protein G agarose beads for the preclearing. After removal of the beads, antibody was added to the lysates, which were incubated under rotation at 4°C for 2h. Finally, protein G agarose beads were rotated with lysates under the same conditions. The beads were washed three times with lysis buffer, two times with PBS before being resuspended in protein loading buffer.

AMPK activity measurement

AMPK activity was measured using the SAMS peptide (HMRSAMSGLHLVKRR) phosphorylation assay kit from Upstate Biotechnology (Luzern, Switzerland) according to the manufacturer's protocol. Briefly, cells were grown in 6cm dishes until they reached about 80% confluence before being put in serum-free medium for 24h. Treatments were added straight to the culture dishes and cells incubated for 1h at 37°C. Culture medium was quickly removed, the cells were washed once with ice-cold PBS and harvested in 400μl lysis buffer containing 50mM Tris pH 7.5, 50mM NaF, 1mM EDTA, 1mM EGTA, 1mM sodium pyrophosphate, 250mM mannitol, 1% Triton X-100, protease inhibitors (Complete Mini EDTA-free, Roche Molecular Biochemicals, Rotkreuz, Switzerland), 5μg/ml soybean trypsin inhibitors, 0.2mM sodium orthovanadate, and 1mM DTT. After PEG precipitation and BCA protein assay (Biorad, Reinach, Switzerland), 15μg of total proteins were used in a 40μl reaction in the presence of 75mM $MgCl_2$, 0.5mM ATP, 0.3mM AMP, 0.2mM SAMS, 0.4mM DTT and 1mCi/100μl [γ-^{32}P]ATP for 10min at 30°C. At the end of the incubation, 35μl of supernatant from the reaction mixture were spotted on Whatman filter papers (P81) which were then washed three times with 0.75% phosphoric acid, once with acetone and then allowed to dry before scintillation counting in 5ml scintillation cocktail.

LKB1 activity measurement

LKB1 activity was measured using the LKBtide peptide and LKB1 enzyme mix from Upstate Biotechnology (Luzern, Switzerland). Cells were grown in 6cm dishes until they reached about 80% confluence before being put in serum-free medium for 24h. Treatments were added straight to the culture dishes and cells incubated for 1h at 37°C. Culture medium was quickly removed, the cells were washed once with ice-cold PBS and harvested in 400μl lysis buffer containing 50mM Tris pH 7.5, 50mM NaF, 1mM EDTA, 1mM EGTA, 1mM sodium pyrophosphate, 250mM mannitol, 1% Triton X-100,

protease inhibitors (Complete Mini EDTA-free, Roche Molecular Biochemicals, Rotkreuz, Switzerland), 5μg/ml soybean trypsin inhibitors, 0.2mM sodium orthovanadate, and 1mM DTT. After PEG precipitation and BCA protein assay (Biorad, Reinach, Switzerland), 15μg of total proteins were used in a 40μl reaction in the presence of 75mM $MgCl_2$, 0.5mM ATP, 0.3mM AMP, 0.2mM SAMS, 0.4mM DTT and 1mCi/100μl [γ-^{32}P]ATP for 10min at 30°C. The reaction was stopped on ice water and 20μl of supernatant from the reaction mixture were spotted on Whatman filter papers (P81) which were then washed three times with 0.75% phosphoric acid, once with acetone and then allowed to dry before scintillation counting in 5ml scintillation cocktail.

RNA isolation and real-time PCR analysis

RNA from LMH cells was isolated by Trizol Reagent. 1μg of total RNA was reverse-transcribed with the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase assay (Roche Molecular Biochemicals, Rotkreuz, Switzerland). PCR was performed using the real-time PCR core reagent kit (PE Applied Biosystems, Rotkreuz, Switzerland), and the mRNA levels were quantified with an ABI PRISM 7700 sequence detection system according to the manufacturer's protocol. Different genes were measured in separate tubes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization of the results (APPENDIX TABLE 2 for primer sequences). The data are shown as relative expression to control sample.

LMH reporter gene assays

LMH cells were cultured onto 96-well dishes coated with gelatin at a density of 40'000 cell per well. Transfections were performed using 0.2μl/well FUGENE6 transfection reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland) in serum-free Williams' E medium for 6h and then the medium was changed to serum-supplemented Williams' E. The transfection mix contained 100ng reporter gene construct, 25ng β-gal vector and 16/32ng of AMPKα1, AMPKα2 or LKB1 vector.

Transactivation assay

Transactivation assay were carried out in monkey kidney CV-1 cells. Cells were maintained in DMEM-F12 supplemented with 10% fetal bovine serum. Before experiments, cells were splitted 1:10 in DMEM-F12 without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum. After 3 days of growth, cells were plated onto 96-well dishes at a density of 25'000 cells per well and grown overnight. CV-1 cells were transiently transfected in OptiMEM medium with 1μl of Lipofectamine reagent per well. Transfection mixes containing 8ng receptor expression vector, 20ng reporter vector, 60ng pRSV-βgal and 8-16ng of AMPKα1 or AMPKα2 vector were added to the cells. 24h after transfection, the medium was replaced by DMEM-F12 without phenol red, supplemented with 10% delipidated, charcoal-stripped fetal calf serum containing the inducer compounds of interest. Cells were then incubated for additional 24h, after which cell extracts were prepared with 200μl of passive lysis buffer (Promega). Luciferase expression was assayed by the Luciferase assay kit (Promega) with 10μl of cell lysates. At the same time, β-galactosidase activities were determined by adding 180μl of

chlorophenol red-1β-D-galactopyra (CPRG) substrate solution (Roche Molecular Biochemicals) to 20µl of the cell lysates. After 10-30min of incubation at 37°C, absorption at 550nm wavelength was measured using a Labsystems Multiscan RC microplate reader (Labsystems, Frankfurt). Luciferase expression was then normalized against measured b-gal activities to compensate for variations in transfection efficiency.

Immunofluorescence

LMH cells were cultivated on LabTek dishes coated with gelatin. Cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) for 30min at RT, and permeabilized in 4% PFA/0.5% Triton X-100 for 5min at RT. Cells were rinsed for 5min in 10mM Tris pH 7.5/10mM NaCl, subsequently twice in PBS and blocked in 2% BSA/10% serum/0.1% Tween20 for 30min at RT. Staining with primary and secondary antibody were done in blocking solution for 1h each at RT. After extensive washing with PBS, cells were stained with 0.5µg/ml DAPI and subsequently analyzed by fluorescence microscopy.

Mitochondrial membrane potential measurement

Cells were cultivated in serum-free medium for 24h were detached by trypsin and resuspended in serum-free Williams'E medium. Approximately $4x10^5$ cells were transferred to an Eppendorf tube and incubated at 37°C for 20min with 1µl of a 5mg/ml JC-1 (Invitrogen, Nivelles, Belgium) stock in DMSO, and either with 500µM PB or 0.2mM DNP. After two washing steps with 1ml PBS and 5min centrifugation at 400g the cells were resuspended in 300µl PBS and the fluorescence was measured in triplicates with 100µl of cell suspension. The JC-1 dye can enter the mitochondrial membrane and form aggregates. If the potential drops, the dye is released into the cytoplasm as monomers. Aggregates and monomers emit fluorescence at two different wavelengths that can be monitored, 590nm and 540nm, respectively (FIGURE 1A). The ratio of the fluorescence at 590nm and at 540nm was calculated and depicted on a graph. The results were also confirmed by FACS analysis, which clearly showed two different shifted populations (FIGURE 1B).

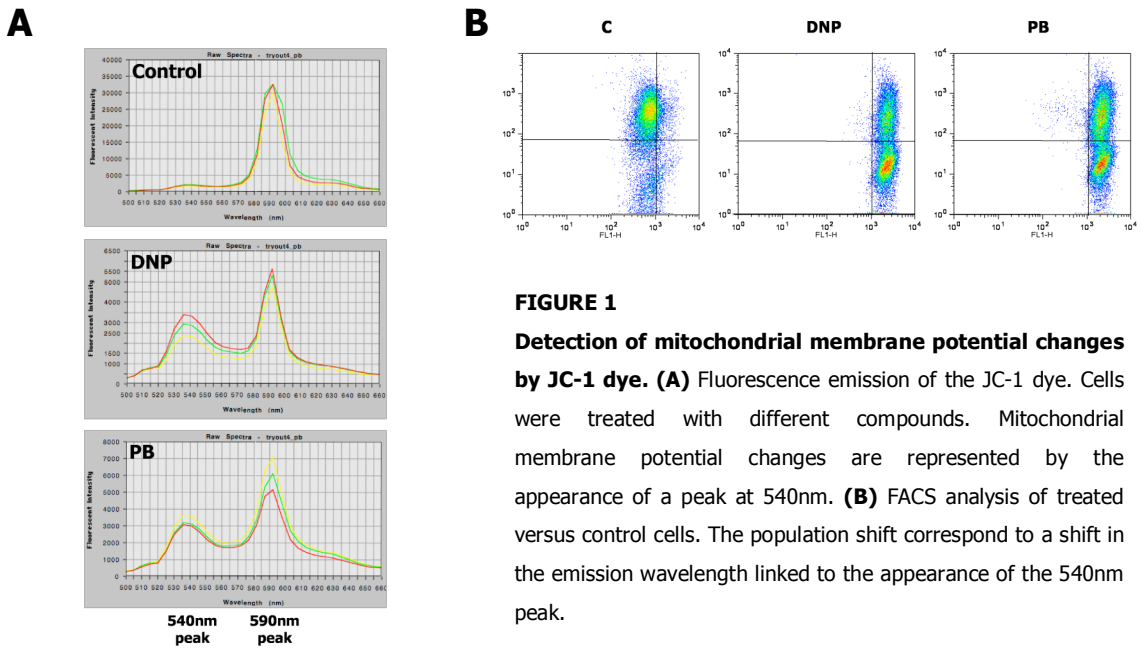


FIGURE 1

Detection of mitochondrial membrane potential changes by JC-1 dye. (A) Fluorescence emission of the JC-1 dye. Cells were treated with different compounds. Mitochondrial membrane potential changes are represented by the appearance of a peak at 540nm. **(B)** FACS analysis of treated versus control cells. The population shift correspond to a shift in the emission wavelength linked to the appearance of the 540nm peak.

Reactive oxygen species formation measurement

Human hepatocytes were cultivated for 24h in serum-free medium, detached with trypsin, washed with PBS, and incubated at 37°C with carboxydichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes, Nivelles, Belgium) for 30min. After treatment for 1h with 500μM PB or 5μM rotenone, cells were washed once with PBS and the fluorescence was measured at 535nm wavelenght.

ATP level measurement

ATP was measured by ATP Bioluminescence Assay Kit HS II (Roche Diagnostic, Penzberg, Germany) as described in the manufacturer's protocol. Cells were cultivated on 96-well dishes, treated with the compounds of interest for different times and lysed with the kit cell lysis reagent. 50μl of luciferase reagent were added to the multiwell plate and luciferase activity was measured.

Animals

C57/Bl6 wild-type animals maintained on standard laboratory chow with food and water ad libitum were used for the experiments. Ten to twelve-week-old female mice (n=5) were injected i.p. with vehicle alone (sterile NaCl) or AICAR 50mg/kg. After different treatment time, animals were killed and the liver was extracted. Approximately 100mg of the liver were solubilized in 1ml Trizol Reagent (Invitrogen) and total RNA was extracted according to the manufacturer protocol.

Statistics

Significant differences between means were determined by the two-tailed Student's *t*-test for paired samples. Error bars represent standard deviation of at least three experiments.

References

1. Xu C, Li CY, Kong AN: **Induction of phase I, II and III drug metabolism/transport by xenobiotics**. *Arch Pharm Res* 2005, **28**(3):249-268.
2. Nelson DR: **Cytochrome P450 and the individuality of species**. *Arch Biochem Biophys* 1999, **369**(1):1-10.
3. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW *et al*: **P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature**. *Pharmacogenetics* 1996, **6**(1):1-42.
4. Omura T: **Forty years of cytochrome P450**. *Biochem Biophys Res Commun* 1999, **266**(3):690-698.
5. Nelson DR: **Cytochrome P450 nomenclature, 2004**. *Methods Mol Biol* 2006, **320**:1-10.
6. Waxman DJ, Azaroff L: **Phenobarbital induction of cytochrome P-450 gene expression**. *Biochem J* 1992, **281**(Pt 3):577-592.
7. Nebert DW, Dieter MZ: **The evolution of drug metabolism**. *Pharmacology* 2000, **61**(3):124-135.
8. Okey AB: **Enzyme induction in the cytochrome P-450 system**. *Pharmacol Ther* 1990, **45**(2):241-298.
9. Bolton AE, Peng B, Hubert M, Krebs-Brown A, Capdeville R, Keller U, Seiberling M: **Effect of rifampicin on the pharmacokinetics of imatinib mesylate (Gleevec, STI571) in healthy subjects**. *Cancer Chemother Pharmacol* 2004, **53**(2):102-106.
10. Chan WK, Nguyen LT, Miller VP, Harris RZ: **Mechanism-based inactivation of human cytochrome P450 3A4 by grapefruit juice and red wine**. *Life Sci* 1998, **62**(10):PL135-142.
11. Fugh-Berman A: **Herb-drug interactions**. *Lancet* 2000, **355**(9198):134-138.
12. Meyer UA, Gut J: **Genomics and the prediction of xenobiotic toxicity**. *Toxicology* 2002, **181-182**:463-466.
13. Pirmohamed M, Park BK: **Cytochrome P450 enzyme polymorphisms and adverse drug reactions**. *Toxicology* 2003, **192**(1):23-32.
14. Remmer H, Merker HJ: **Drug-Induced Changes in the Liver Endoplasmic Reticulum: Association with Drug-Metabolizing Enzymes**. *Science* 1963, **142**:1657-1658.
15. Remmer H, Merker HJ: **[Enzyme induction and increase of endoplasmic reticulum in liver cells during phenobarbital (Luminal) therapy.]**. *Klin Wochenschr* 1963, **41**:276-282.
16. Frueh FW, Zanger UM, Meyer UA: **Extent and character of phenobarbital-mediated changes in gene expression in the liver**. *Mol Pharmacol* 1997, **51**(3):363-369.
17. Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM, Negishi M: **Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital**. *Mol Pharmacol* 2002, **61**(1):1-6.
18. Dogra SC, Whitelaw ML, May BK: **Transcriptional activation of cytochrome P450 genes by different classes of chemical inducers**. *Clin Exp Pharmacol Physiol* 1998, **25**(1):1-9.
19. Waxman DJ: **P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR**. *Arch Biochem Biophys* 1999, **369**(1):11-23.
20. Whitlock JP, Jr., Okino ST, Dong L, Ko HP, Clarke-Katzenberg R, Ma Q, Li H: **Cytochromes P450 5: induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription**. *Faseb J* 1996, **10**(8):809-818.
21. Meyer UA, Hoffmann K: **Phenobarbital-mediated changes in gene expression in the liver**. *Drug Metab Rev* 1999, **31**(2):365-373.
22. Handschin C, Meyer UA: **A conserved nuclear receptor consensus sequence (DR-4) mediates transcriptional activation of the chicken CYP2H1 gene by phenobarbital in a hepatoma cell line**. *J Biol Chem* 2000, **275**(18):13362-13369.
23. Shaw GC, Fulco AJ: **Barbiturate-mediated regulation of expression of the cytochrome P450BM-3 gene of Bacillus megaterium by Bm3R1 protein**. *J Biol Chem* 1992, **267**(8):5515-5526.
24. Shaw GC, Fulco AJ: **Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site on the barbiturate-inducible cytochrome P450BM-3 gene of Bacillus megaterium**. *J Biol Chem* 1993, **268**(4):2997-3004.

25. He JS, Fulco AJ: **A barbiturate-regulated protein binding to a common sequence in the cytochrome P450 genes of rodents and bacteria**. *J Biol Chem* 1991, **266**(12):7864-7869.
26. Liang Q, He JS, Fulco AJ: **The role of Barbie box sequences as cis-acting elements involved in the barbiturate-mediated induction of cytochromes P450BM-1 and P450BM-3 in Bacillus megaterium**. *J Biol Chem* 1995, **270**(9):4438-4450.
27. Hahn CN, Hansen AJ, May BK: **Transcriptional regulation of the chicken CYP2H1 gene. Localization of a phenobarbital-responsive enhancer domain**. *J Biol Chem* 1991, **266**(26):17031-17039.
28. Ramsden R, Sommer KM, Omiecinski CJ: **Phenobarbital induction and tissue-specific expression of the rat CYP2B2 gene in transgenic mice**. *J Biol Chem* 1993, **268**(29):21722-21726.
29. Honkakoski P, Moore R, Gynther J, Negishi M: **Characterization of phenobarbital-inducible mouse Cyp2b10 gene transcription in primary hepatocytes**. *J Biol Chem* 1996, **271**(16):9746-9753.
30. Park Y, Li H, Kemper B: **Phenobarbital induction mediated by a distal CYP2B2 sequence in rat liver transiently transfected in situ**. *J Biol Chem* 1996, **271**(39):23725-23728.
31. Trottier E, Belzil A, Stoltz C, Anderson A: **Localization of a phenobarbital-responsive element (PBRE) in the 5'-flanking region of the rat CYP2B2 gene**. *Gene* 1995, **158**(2):263-268.
32. Honkakoski P, Negishi M: **Characterization of a phenobarbital-responsive enhancer module in mouse P450 Cyp2b10 gene**. *J Biol Chem* 1997, **272**(23):14943-14949.
33. May BK, Dogra SC, Sadlon TJ, Bhasker CR, Cox TC, Bottomley SS: **Molecular regulation of heme biosynthesis in higher vertebrates**. *Prog Nucleic Acid Res Mol Biol* 1995, **51**:1-51.
34. Fraser DJ, Podvinec M, Kaufmann MR, Meyer UA: **Drugs mediate the transcriptional activation of the 5-aminolevulinic acid synthase (ALAS1) gene via the chicken xenobiotic-sensing nuclear receptor (CXR)**. *J Biol Chem* 2002, **277**(38):34717-34726.
35. Fraser DJ, Zumsteg A, Meyer UA: **Nuclear receptors constitutive androstane receptor and pregnane X receptor activate a drug-responsive enhancer of the murine 5-aminolevulinic acid synthase gene**. *J Biol Chem* 2003, **278**(41):39392-39401.
36. Podvinec M, Handschin C, Looser R, Meyer UA: **Identification of the xenosensors regulating human 5-aminolevulinate synthase**. *Proc Natl Acad Sci U S A* 2004, **101**(24):9127-9132.
37. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al*: **The nuclear receptor superfamily: the second decade**. *Cell* 1995, **83**(6):835-839.
38. Laudet V: **Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor**. *J Mol Endocrinol* 1997, **19**(3):207-226.
39. Khorasanizadeh S, Rastinejad F: **Nuclear-receptor interactions on DNA-response elements**. *Trends Biochem Sci* 2001, **26**(6):384-390.
40. Mangelsdorf DJ, Evans RM: **The RXR heterodimers and orphan receptors**. *Cell* 1995, **83**(6):841-850.
41. Rastinejad F: **Retinoid X receptor and its partners in the nuclear receptor family**. *Curr Opin Struct Biol* 2001, **11**(1):33-38.
42. Collingwood TN, Urnov FD, Wolffe AP: **Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription**. *J Mol Endocrinol* 1999, **23**(3):255-275.
43. Lee JW, Lee YC, Na SY, Jung DJ, Lee SK: **Transcriptional coregulators of the nuclear receptor superfamily: coactivators and corepressors**. *Cell Mol Life Sci* 2001, **58**(2):289-297.
44. Lee KC, Lee Kraus W: **Nuclear receptors, coactivators and chromatin: new approaches, new insights**. *Trends Endocrinol Metab* 2001, **12**(5):191-197.
45. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L: **Nuclear receptor coactivators and corepressors**. *Mol Endocrinol* 1996, **10**(10):1167-1177.
46. Glass CK, Rose DW, Rosenfeld MG: **Nuclear receptor coactivators**. *Curr Opin Cell Biol* 1997, **9**(2):222-232.

47. Whitfield GK, Jurutka PW, Haussler CA, Haussler MR: **Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function**. *J Cell Biochem* 1999, **Suppl**(32-33):110-122.
48. Kumar R, Thompson EB: **The structure of the nuclear hormone receptors**. *Steroids* 1999, **64**(5):310-319.
49. Honkakoski P, Sueyoshi T, Negishi M: **Drug-activated nuclear receptors CAR and PXR**. *Ann Med* 2003, **35**(3):172-182.
50. Moore JT, Moore LB, Maglich JM, Kliewer SA: **Functional and structural comparison of PXR and CAR**. *Biochim Biophys Acta* 2003, **1619**(3):235-238.
51. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A: **Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction**. *Proc Natl Acad Sci U S A* 1998, **95**(21):12208-12213.
52. Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES, Evans RM: **SXR, a novel steroid and xenobiotic-sensing nuclear receptor**. *Genes Dev* 1998, **12**(20):3195-3205.
53. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH *et al*: **An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway**. *Cell* 1998, **92**(1):73-82.
54. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA: **The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions**. *J Clin Invest* 1998, **102**(5):1016-1023.
55. Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM *et al*: **The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution**. *Mol Endocrinol* 2000, **14**(1):27-39.
56. Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR: **The Human Nuclear Xenobiotic Receptor PXR: Structural Determinants of Directed Promiscuity**. *Science* 2001, **14**:14.
57. Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, Evans RM: **Humanized xenobiotic response in mice expressing nuclear receptor SXR**. *Nature* 2000, **406**(6794):435-439.
58. Goodwin B, Redinbo MR, Kliewer SA: **Regulation of cyp3a gene transcription by the pregnane x receptor**. *Annu Rev Pharmacol Toxicol* 2002, **42**:1-23.
59. Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, Evans RM: **Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR [In Process Citation]**. *Genes Dev* 2000, **14**(23):3014-3023.
60. Sueyoshi T, Negishi M: **Phenobarbital response elements of cytochrome p450 genes and nuclear receptors**. *Annu Rev Pharmacol Toxicol* 2001, **41**:123-143.
61. Squires EJ, Sueyoshi T, Negishi M: **Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver**. *J Biol Chem* 2004, **279**(47):49307-49314.
62. Handschin C, Meyer UA: **Induction of drug metabolism: the role of nuclear receptors**. *Pharmacol Rev* 2003, **55**(4):649-673.
63. Orans J, Teotico DG, Redinbo MR: **The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges**. *Mol Endocrinol* 2005, **19**(12):2891-2900.
64. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J *et al*: **The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity**. *Proc Natl Acad Sci U S A* 2001, **98**(6):3369-3374.
65. Honkakoski P, Negishi M: **Regulatory DNA elements of phenobarbital-responsive cytochrome P450 CYP2B genes**. *J Biochem Mol Toxicol* 1998, **12**(1):3-9.
66. Baes M, Gulick T, Choi HS, Martinoli MG, Simha D, Moore DD: **A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements**. *Mol Cell Biol* 1994, **14**(3):1544-1551.
67. Choi HS, Chung M, Tzameli I, Simha D, Lee YK, Seol W, Moore DD: **Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR**. *J Biol Chem* 1997, **272**(38):23565-23571.
68. Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA: **Regulation of multidrug resistance-associated protein 2**

(ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002, **277**(4):2908-2915.
69. Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M: **The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene**. *J Biol Chem* 1999, **274**(10):6043-6046.
70. Yoshinari K, Sueyoshi T, Moore R, Negishi M: **Nuclear receptor CAR as a regulatory factor for the sexually dimorphic induction of CYB2B1 gene by phenobarbital in rat livers**. *Mol Pharmacol* 2001, **59**(2):278-284.
71. Marc N, Galisteo M, Lagadic-Gossmann D, Fautrel A, Joannard F, Guillouzo A, Corcos L: **Regulation of phenobarbital induction of the cytochrome P450 2b9/10 genes in primary mouse hepatocyte culture. Involvement of calcium- and cAMP-dependent pathways**. *Eur J Biochem* 2000, **267**(4):963-970.
72. Zelko I, Sueyoshi T, Kawamoto T, Moore R, Negishi M: **The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver**. *Mol Cell Biol* 2001, **21**(8):2838-2846.
73. Kobayashi K, Sueyoshi T, Inoue K, Moore R, Negishi M: **Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells**. *Mol Pharmacol* 2003, **64**(5):1069-1075.
74. Yoshinari K, Kobayashi K, Moore R, Kawamoto T, Negishi M: **Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital**. *FEBS Lett* 2003, **548**(1-3):17-20.
75. Zelko I, Negishi M: **Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes [In Process Citation]**. *Biochem Biophys Res Commun* 2000, **277**(1):1-6.
76. Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA: **Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification**. *Mol Pharmacol* 2002, **62**(3):638-646.
77. Wei P, Zhang J, Dowhan DH, Han Y, Moore DD: **Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response**. *Pharmacogenomics J* 2002, **2**(2):117-126.
78. Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD: **The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism**. *Nature* 2000, **407**(6806):920-923.
79. Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G, Moore DD: **Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor**. *Mol Endocrinol* 2005, **19**(6):1646-1653.
80. Yamamoto Y, Moore R, Goldsworthy TL, Negishi M, Maronpot RR: **The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice**. *Cancer Res* 2004, **64**(20):7197-7200.
81. Handschin C, Blattler S, Roth A, Looser R, Oscarson M, Kaufmann MR, Podvinec M, Gnerre C, Meyer UA: **The evolution of drug-activated nuclear receptors: one ancestral gene diverged into two xenosensor genes in mammals**. *Nucl Recept* 2004, **2**(1):7.
82. Handschin C, Podvinec M, Meyer UA: **CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR)**. *Proc Natl Acad Sci U S A* 2000, **97**(20):10769-10774.
83. Handschin C, Podvinec M, Stockli J, Hoffmann K, Meyer UA: **Conservation of signaling pathways of xenobiotic-sensing orphan nuclear receptors, chicken xenobiotic receptor, constitutive androstane receptor, and pregnane X receptor, from birds to humans**. *Mol Endocrinol* 2001, **15**(9):1571-1585.
84. Burt DW: **The chicken genome and the developmental biologist**. *Mech Dev* 2004, **121**(9):1129-1135.
85. Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, Bork P, Burt DW, Groenen MA, Delany ME *et al*: **Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution**. *Nature* 2004, **432**(7018):695-716.
86. Nirodi CS, Sultana S, Ram N, Prabhu L, Padmanaban G: **Involvement of synthesis and phosphorylation of nuclear protein factors that bind to the positive cis-acting**

element in the transcriptional activation of the CYP2B1/B2 gene by phenobarbitone in vivo. *Arch Biochem Biophys* 1996, **331**(1):79-86.
87. Dogra SC, May BK: **Phenobarbital-induced activation of CYP2H1 and 5-aminolevulinate synthase genes in chick embryo hepatocytes is blocked by an inhibitor of protein phosphorylation**. *Arch Biochem Biophys* 1996, **327**(2):271-278.
88. Posti K, Leinonen S, Tetri S, Kottari S, Viitala P, Pelkonen O, Raunio H: **Modulation of murine phenobarbital-inducible CYP2A5, CYP2B10 and CYP1A enzymes by inhibitors of protein kinases and phosphatases**. *Eur J Biochem* 1999, **264**(1):19-26.
89. Joannard F, Galisteo M, Corcos L, Guillouzo A, Lagadic-Gossmann D: **Regulation of phenobarbital-induction of CYP2B and CYP3A genes in rat cultured hepatocytes: involvement of several serine/threonine protein kinases and phosphatases**. *Cell Biol Toxicol* 2000, **16**(5):325-337.
90. Kawamura A, Yoshida Y, Kimura N, Oda H, Kakinuma A: **Phosphorylation/Dephosphorylation steps are crucial for the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital**. *Biochem Biophys Res Commun* 1999, **264**(2):530-536.
91. Sidhu JS, Omiecinski CJ: **cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures**. *J Biol Chem* 1995, **270**(21):12762-12773.
92. Honkakoski P, Negishi M: **Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced Cyp2b10 gene transcription in mouse primary hepatocytes**. *Biochem J* 1998, **330 (Pt 2)**:889-895.
93. Beck NB, Omiecinski CJ: **Lack of modulation by phenobarbital of cyclic AMP levels or protein kinase A activity in rat primary hepatocytes**. *Biochem Pharmacol* 1999, **58**(7):1109-1114.
94. Ding X, Staudinger JL: **Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway**. *J Pharmacol Exp Ther* 2005, **312**(2):849-856.
95. Yamamoto Y, Kawamoto T, Negishi M: **The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity**. *Arch Biochem Biophys* 2003, **409**(1):207-211.
96. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M: **Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene**. *Mol Cell Biol* 1999, **19**(9):6318-6322.
97. Sidhu JS, Omiecinski CJ: **An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures**. *J Pharmacol Exp Ther* 1997, **282**(2):1122-1129.
98. Makinen J, Frank C, Jyrkkarinne J, Gynther J, Carlberg C, Honkakoski P: **Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors**. *Mol Pharmacol* 2002, **62**(2):366-378.
99. Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P, Vilarem MJ: **The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors**. *Biochim Biophys Acta* 2003, **1619**(3):243-253.
100. Kocarek TA, Kraniak JM, Reddy AB: **Regulation of rat hepatic cytochrome P450 expression by sterol biosynthesis inhibition: inhibitors of squalene synthase are potent inducers of CYP2B expression in primary cultured rat hepatocytes and rat liver**. *Mol Pharmacol* 1998, **54**(3):474-484.
101. Kocarek TA, Reddy AB: **Negative regulation by dexamethasone of fluvastatin-inducible CYP2B expression in primary cultures of rat hepatocytes: role of CYP3A**. *Biochem Pharmacol* 1998, **55**(9):1435-1443.
102. Kocarek TA, Schuetz EG, Guzelian PS: **Regulation of phenobarbital-inducible cytochrome P450 2B1/2 mRNA by lovastatin and oxysterols in primary cultures of adult rat hepatocytes**. *Toxicol Appl Pharmacol* 1993, **120**(2):298-307.
103. Ourlin JC, Handschin C, Kaufmann M, Meyer UA: **A Link between cholesterol levels and phenobarbital induction of cytochromes P450**. *Biochem Biophys Res Commun* 2002, **291**(2):378-384.
104. Plewka A, Kaminski M: **Influence of cholesterol and protein diet on liver cytochrome P-450-dependent monooxygenase system in rats**. *Exp Toxicol Pathol* 1996, **48**(4):249-253.

105. Blouin RA, Bandyopadhyay AM, Chaudhary I, Robertson LW, Gemzik B, Parkinson A: **Cytochrome P450 2B enzyme (CYP2B) induction defect following phenobarbital treatment in the fa/fa Zucker rat: molecular characterization**. *Arch Biochem Biophys* 1993, **303**(2):313-320.
106. Thomas KD: **Plasma cholesterol levels in adult male and female rats after chronic treatment with phenobarbitone**. *Isr J Med Sci* 1984, **20**(3):240-241.
107. Heller FR, Desager JP, Harvengt C: **Changes in plasma activities of lipolytic enzymes and lipids of normolipidemic subjects given phenobarbital, a strong microsomal inducer, alone or in combination with fenofibrate**. *Int J Clin Pharmacol Ther Toxicol* 1988, **26**(3):138-142.
108. Aynaci FM, Orhan F, Orem A, Yildirmis S, Gedik Y: **Effect of Antiepileptic drugs on plasma lipoprotein (a) and other lipid levels in childhood**. *J Child Neurol* 2001, **16**(5):367-369.
109. Eiris JM, Lojo S, Del Rio MC, Novo I, Bravo M, Pavon P, Castro-Gago M: **Effects of long-term treatment with antiepileptic drugs on serum lipid levels in children with epilepsy**. *Neurology* 1995, **45**(6):1155-1157.
110. Schwaninger M, Ringleb P, Annecke A, Winter R, Kohl B, Werle E, Fiehn W, Rieser PA, Walter-Sack I: **Elevated plasma concentrations of lipoprotein(a) in medicated epileptic patients**. *J Neurol* 2000, **247**(9):687-690.
111. Back P: **Therapeutic use of phenobarbital in intrahepatic cholestasis. Inductions in bile acid metabolism**. *Pharmacol Ther* 1987, **33**(1):153-155.
112. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME *et al*: **A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis**. *Mol Cell* 2000, **6**(3):517-526.
113. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ: **Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors**. *Mol Cell* 2000, **6**(3):507-515.
114. Staudinger J, Liu Y, Madan A, Habeebu S, Klaassen CD: **Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor**. *Drug Metab Dispos* 2001, **29**(11):1467-1472.
115. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J *et al*: **The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity**. *Proc Natl Acad Sci U S A* 2001, **98**(6):3369-3374.
116. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ: **Vitamin D receptor as an intestinal bile acid sensor**. *Science* 2002, **296**(5571):1313-1316.
117. Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB, Jr., Kliewer SA, Gonzalez FJ, Sinal CJ: **Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity**. *J Biol Chem* 2003, **278**(46):45062-45071.
118. Saini SP, Sonoda J, Xu L, Toma D, Uppal H, Mu Y, Ren S, Moore DD, Evans RM, Xie W: **A novel constitutive androstane receptor-mediated and CYP3A-independent pathway of bile acid detoxification**. *Mol Pharmacol* 2004, **65**(2):292-300.
119. Handschin C, Podvinec M, Amherd R, Looser R, Ourlin JC, Meyer UA: **Cholesterol and bile acids regulate xenosensor signaling in drug-mediated induction of cytochromes P450**. *J Biol Chem* 2002, **277**(33):29561-29567.
120. Flockhart DA, Rae JM: **Cytochrome P450 3A pharmacogenetics: the road that needs traveled**. *Pharmacogenomics J* 2003, **3**(1):3-5.
121. Keser G, Capar I, Aksu K, Inal V, Danaoglu Z, Savas R, Oksel F, Tunc E, Kabasakal Y, Kitapcioglu G *et al*: **Pulmonary hypertension in rheumatoid arthritis**. *Scand J Rheumatol* 2004, **33**(4):244-245.
122. Nebert DW, Russell DW: **Clinical importance of the cytochromes P450**. *Lancet* 2002, **360**(9340):1155-1162.
123. Bremmelgaard A, Alme B: **Analysis of plasma bile acid profiles in patients with liver diseases associated with cholestasis**. *Scand J Gastroenterol* 1980, **15**(5):593-600.
124. Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM: **An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids**. *Proc Natl Acad Sci U S A* 2001, **98**(6):3375-3380.

125. Gnerre C, Blattler S, Kaufmann MR, Looser R, Meyer UA: **Regulation of CYP3A4 by the bile acid receptor FXR: evidence for functional binding sites in the CYP3A4 gene**. *Pharmacogenetics* 2004, **14**(10):635-645.
126. Drocourt L, Ourlin JC, Pascussi JM, Maurel P, Vilarem MJ: **Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes**. *J Biol Chem* 2002, **277**(28):25125-25132.
127. Gnerre C, Schuster GU, Roth A, Handschin C, Johansson L, Looser R, Parini P, Podvinec M, Robertsson K, Gustafsson JA *et al*: **LXR deficiency and cholesterol feeding affect the expression and phenobarbital-mediated induction of cytochromes P450 in mouse liver**. *J Lipid Res* 2005, **46**(8):1633-1642.
128. Zhang J, Huang W, Chua SS, Wei P, Moore DD: **Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR**. *Science* 2002, **298**(5592):422-424.
129. Yaffe SJ, Levy G, Matsuzawa T, Baliah T: **Enhancement of glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital**. *N Engl J Med* 1966, **275**(26):1461-1466.
130. Huang W, Zhang J, Chua SS, Qatanani M, Han Y, Granata R, Moore DD: **Induction of bilirubin clearance by the constitutive androstane receptor (CAR)**. *Proc Natl Acad Sci U S A* 2003, **100**(7):4156-4161.
131. Sugatani J, Yamakawa K, Yoshinari K, Machida T, Takagi H, Mori M, Kakizaki S, Sueyoshi T, Negishi M, Miwa M: **Identification of a defect in the UGT1A1 gene promoter and its association with hyperbilirubinemia**. *Biochem Biophys Res Commun* 2002, **292**(2):492-497.
132. Zhang J, Huang W, Qatanani M, Evans RM, Moore DD: **The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity**. *J Biol Chem* 2004, **279**(47):49517-49522.
133. Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM, Moore DD: **Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta [see comments]**. *Nature* 1998, **395**(6702):612-615.
134. Kawamoto T, Kakizaki S, Yoshinari K, Negishi M: **Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse Cyp2b10 gene [In Process Citation]**. *Mol Endocrinol* 2000, **14**(11):1897-1905.
135. Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM *et al*: **Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands**. *J Biol Chem* 2000, **275**(20):15122-15127.
136. Schreiber AJ, Simon FR: **Estrogen-induced cholestasis: clues to pathogenesis and treatment**. *Hepatology* 1983, **3**(4):607-613.
137. Pascussi JM, Busson-Le Coniat M, Maurel P, Vilarem MJ: **Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element**. *Mol Endocrinol* 2003, **17**(1):42-55.
138. Pascussi JM, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ: **Dexamethasone Enhances Constitutive Androstane Receptor Expression in Human Hepatocytes: Consequences on Cytochrome P450 Gene Regulation**. *Mol Pharmacol* 2000, **58**(6):1441-1450.
139. Curran PG, DeGroot LJ: **The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland**. *Endocr Rev* 1991, **12**(2):135-150.
140. Hood A, Allen ML, Liu Y, Liu J, Klaassen CD: **Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers**. *Toxicol Appl Pharmacol* 2003, **188**(1):6-13.
141. Hood A, Hashmi R, Klaassen CD: **Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy**. *Toxicol Appl Pharmacol* 1999, **160**(2):163-170.
142. Hood A, Klaassen CD: **Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation**. *Toxicol Sci* 2000, **55**(1):78-84.
143. Klaassen CD, Hood AM: **Effects of microsomal enzyme inducers on thyroid follicular cell proliferation and thyroid hormone metabolism**. *Toxicol Pathol* 2001, **29**(1):34-40.

144. Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, Moore JT: **The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction**. *J Biol Chem* 2004, **279**(19):19832-19838.
145. Qatanani M, Zhang J, Moore DD: **Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism**. *Endocrinology* 2005, **146**(3):995-1002.
146. Peraino C, Fry RJ, Staffeldt E: **Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene**. *Cancer Res* 1971, **31**(10):1506-1512.
147. Farazi PA, Glickman J, Jiang S, Yu A, Rudolph KL, DePinho RA: **Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma**. *Cancer Res* 2003, **63**(16):5021-5027.
148. Sakuma T, Honma R, Maguchi S, Tamaki H, Nemoto N: **Different expression of hepatic and renal cytochrome P450s between the streptozotocin-induced diabetic mouse and rat**. *Xenobiotica* 2001, **31**(4):223-237.
149. Yoshida Y, Kimura N, Oda H, Kakinuma A: **Insulin suppresses the induction of CYP2B1 and CYP2B2 gene expression by phenobarbital in adult rat cultured hepatocytes**. *Biochem Biophys Res Commun* 1996, **229**(1):182-188.
150. Giger U, Meyer UA: **Induction of delta-aminolevulinate synthase and cytochrome P-450 hemoproteins in hepatocyte culture. Effect of glucose and hormones**. *J Biol Chem* 1981, **256**(21):11182-11190.
151. Lahtela JT, Arranto AJ, Sotaniemi EA: **Enzyme inducers improve insulin sensitivity in non-insulin-dependent diabetic subjects**. *Diabetes* 1985, **34**(9):911-916.
152. Argaud D, Halimi S, Catelloni F, Leverve XM: **Inhibition of gluconeogenesis in isolated rat hepatocytes after chronic treatment with phenobarbital**. *Biochem J* 1991, **280 (Pt 3)**:663-669.
153. Manenti G, Dragani TA, Della Porta G: **Effects of phenobarbital and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene on differentiated functions in mouse liver**. *Chem Biol Interact* 1987, **64**(1-2):83-92.
154. Kodama S, Koike C, Negishi M, Yamamoto Y: **Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes**. *Mol Cell Biol* 2004, **24**(18):7931-7940.
155. Hardie DG, Hawley SA, Scott JW: **AMP-activated protein kinase--development of the energy sensor concept**. *J Physiol* 2006, **574**(Pt 1):7-15.
156. Davies SP, Hawley SA, Woods A, Carling D, Haystead TA, Hardie DG: **Purification of the AMP-activated protein kinase on ATP-gamma-sepharose and analysis of its subunit structure**. *Eur J Biochem* 1994, **223**(2):351-357.
157. Mitchelhill KI, Stapleton D, Gao G, House C, Michell B, Katsis F, Witters LA, Kemp BE: **Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase**. *J Biol Chem* 1994, **269**(4):2361-2364.
158. Hardie DG: **The AMP-activated protein kinase pathway--new players upstream and downstream**. *J Cell Sci* 2004, **117**(Pt 23):5479-5487.
159. Wojtaszewski JF, Jorgensen SB, Hellsten Y, Hardie DG, Richter EA: **Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle**. *Diabetes* 2002, **51**(2):284-292.
160. Wojtaszewski JF, MacDonald C, Nielsen JN, Hellsten Y, Hardie DG, Kemp BE, Kiens B, Richter EA: **Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle**. *Am J Physiol Endocrinol Metab* 2003, **284**(4):E813-822.
161. Hudson ER, Pan DA, James J, Lucocq JM, Hawley SA, Green KA, Baba O, Terashima T, Hardie DG: **A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias**. *Curr Biol* 2003, **13**(10):861-866.
162. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG: **Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade**. *J Biol* 2003, **2**(4):28.

163. Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, Alessi DR: **Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction**. *Embo J* 2005, **24**(10):1810-1820.
164. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG: **Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase**. *Cell Metab* 2005, **2**(1):9-19.
165. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA: **The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases**. *J Biol Chem* 2005, **280**(32):29060-29066.
166. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: **Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells**. *Cell Metab* 2005, **2**(1):21-33.
167. Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C: **5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase**. *Diabetes* 2000, **49**(6):896-903.
168. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K *et al*: **Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase**. *Nat Med* 2002, **8**(11):1288-1295.
169. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB: **Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase**. *Nature* 2002, **415**(6869):339-343.
170. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: **Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport**. *Diabetes* 1998, **47**(8):1369-1373.
171. Mu J, Brozinick JT, Jr., Valladares O, Bucan M, Birnbaum MJ: **A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle**. *Mol Cell* 2001, **7**(5):1085-1094.
172. Rutter GA, Da Silva Xavier G, Leclerc I: **Roles of 5'-AMP-activated protein kinase (AMPK) in mammalian glucose homoeostasis**. *Biochem J* 2003, **375**(Pt 1):1-16.
173. Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P *et al*: **A serine/threonine kinase gene defective in Peutz-Jeghers syndrome**. *Nature* 1998, **391**(6663):184-187.
174. Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, Muller O, Back W, Zimmer M: **Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase**. *Nat Genet* 1998, **18**(1):38-43.
175. Boudeau J, Baas AF, Deak M, Morrice NA, Kieloch A, Schutkowski M, Prescott AR, Clevers HC, Alessi DR: **MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm**. *Embo J* 2003, **22**(19):5102-5114.
176. Boudeau J, Scott JW, Resta N, Deak M, Kieloch A, Komander D, Hardie DG, Prescott AR, van Aalten DM, Alessi DR: **Analysis of the LKB1-STRAD-MO25 complex**. *J Cell Sci* 2004, **117**(Pt 26):6365-6375.
177. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC: **The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress**. *Proc Natl Acad Sci U S A* 2004, **101**(10):3329-3335.
178. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D: **LKB1 is the upstream kinase in the AMP-activated protein kinase cascade**. *Curr Biol* 2003, **13**(22):2004-2008.
179. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC: **The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin**. *Science* 2005, **310**(5754):1642-1646.
180. Rencurel F, Stenhouse A, Hawley SA, Friedberg T, Hardie DG, Sutherland C, Wolf CR: **AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line**. *J Biol Chem* 2005, **280**(6):4367-4373.
181. Rencurel F, Foretz M, Kaufmann MR, Stroka D, Looser R, Leclerc I, da Silva Xavier G, Rutter GA, Viollet B, Meyer UA: **Stimulation of AMP-activated protein kinase is essential for**

the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver. *Mol Pharmacol* 2006.

182. Collins SP, Reoma JL, Gamm DM, Uhler MD: **LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo**. *Biochem J* 2000, **345 Pt 3**:673-680.
183. Sapkota GP, Boudeau J, Deak M, Kieloch A, Morrice N, Alessi DR: **Identification and characterization of four novel phosphorylation sites (Ser31, Ser325, Thr336 and Thr366) on LKB1/STK11, the protein kinase mutated in Peutz-Jeghers cancer syndrome**. *Biochem J* 2002, **362**(Pt 2):481-490.
184. Sapkota GP, Kieloch A, Lizcano JM, Lain S, Arthur JS, Williams MR, Morrice N, Deak M, Alessi DR: **Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at Ser431 by p90(RSK) and cAMP-dependent protein kinase, but not its farnesylation at Cys(433), is essential for LKB1 to suppress cell vrowth**. *J Biol Chem* 2001, **276**(22):19469-19482.
185. Frodin M, Gammeltoft S: **Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction**. *Mol Cell Endocrinol* 1999, **151**(1-2):65-77.
186. Xie Z, Dong Y, Zhang M, Cui MZ, Cohen RA, Riek U, Neumann D, Schlattner U, Zou MH: **Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells**. *J Biol Chem* 2006, **281**(10):6366-6375.
187. Alessi DR, Sakamoto K, Bayascas JR: **LKB1-Dependent Signaling Pathways**. *Annu Rev Biochem* 2006.
188. Farese RV, Sajan MP, Standaert ML: **Atypical protein kinase C in insulin action and insulin resistance**. *Biochem Soc Trans* 2005, **33**(Pt 2):350-353.
189. Krasowski MD, Yasuda K, Hagey LR, Schuetz EG: **Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts**. *Mol Endocrinol* 2005, **19**(7):1720-1739.
190. Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH, Moore JT: **Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors**. *Mol Endocrinol* 2002, **16**(5):977-986.
191. Krasowski MD, Yasuda K, Hagey LR, Schuetz EG: **Evolutionary selection across the nuclear hormone receptor superfamily with a focus on the NR1I subfamily (vitamin D, pregnane X, and constitutive androstane receptors)**. *Nucl Recept* 2005, **3**:2.
192. Krasowski MD, Yasuda K, Hagey LR, Schuetz EG: **Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts**. *Mol Endocrinol* 2005, **19**(7):1720-1739.
193. Lynch M, Conery JS: **The evolutionary fate and consequences of duplicate genes**. *Science* 2000, **290**(5494):1151-1155.
194. Wagner A: **Birth and death of duplicated genes in completely sequenced eukaryotes**. *Trends Genet* 2001, **17**(5):237-239.
195. Hosseinpour F, Moore R, Negishi M, Sueyoshi T: **Serine-202 regulates the nuclear translocation of constitutive active/androstane receptor CAR**. *Mol Pharmacol* 2005.
196. Kalkhoven E: **CBP and p300: HATs for different occasions**. *Biochem Pharmacol* 2004, **68**(6):1145-1155.
197. Yang W, Hong YH, Shen XQ, Frankowski C, Camp HS, Leff T: **Regulation of transcription by AMP-activated protein kinase: phosphorylation of p300 blocks its interaction with nuclear receptors**. *J Biol Chem* 2001, **276**(42):38341-38344.
198. Dogra SC, Tremethick D, May BK: **Evidence that the coactivator CBP/p300 is important for phenobarbital-induced but not basal expression of the CYP2H1 gene**. *Mol Pharmacol* 2003, **63**(1):73-80.
199. Wang W, Yang X, Kawai T, Lopez de Silanes I, Mazan-Mamczarz K, Chen P, Chook YM, Quensel C, Kohler M, Gorospe M: **AMP-activated protein kinase-regulated phosphorylation and acetylation of importin alpha1: involvement in the nuclear import of RNA-binding protein HuR**. *J Biol Chem* 2004, **279**(46):48376-48388.

200. Kanno Y, Suzuki M, Nakahama T, Inouye Y: **Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear receptor CAR**. *Biochim Biophys Acta* 2005, **1745**(2):215-222.
201. Lee WJ, Kim M, Park HS, Kim HS, Jeon MJ, Oh KS, Koh EH, Won JC, Kim MS, Oh GT *et al*: **AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1**. *Biochem Biophys Res Commun* 2006, **340**(1):291-295.
202. Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, Wackerhage H: **Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation**. *Faseb J* 2005, **19**(7):786-788.
203. Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI: **Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis**. *Am J Physiol Endocrinol Metab* 2001, **281**(6):E1340-1346.
204. Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I: **Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle**. *Biochem Biophys Res Commun* 2002, **296**(2):350-354.
205. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI: **AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation**. *Proc Natl Acad Sci U S A* 2002, **99**(25):15983-15987.
206. Shiraki T, Sakai N, Kanaya E, Jingami H: **Activation of orphan nuclear constitutive androstane receptor requires subnuclear targeting by peroxisome proliferator-activated receptor gamma coactivator-1 alpha. A possible link between xenobiotic response and nutritional state**. *J Biol Chem* 2003, **278**(13):11344-11350.
207. Ding X, Lichti K, Kim I, Gonzalez FJ, Staudinger JL: **Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha**. *J Biol Chem* 2006, **281**(36):26540-26551.
208. Handschin C, Lin J, Rhee J, Peyer AK, Chin S, Wu PH, Meyer UA, Spiegelman BM: **Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1alpha**. *Cell* 2005, **122**(4):505-515.
209. Briancon N, Weiss MC: **In vivo role of the HNF4alpha AF-1 activation domain revealed by exon swapping**. *Embo J* 2006, **25**(6):1253-1262.
210. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ: **Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis**. *Mol Cell Biol* 2001, **21**(4):1393-1403.
211. Chen Y, Kissling G, Negishi M, Goldstein JA: **The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promoter**. *J Pharmacol Exp Ther* 2005, **314**(3):1125-1133.
212. Ferguson SS, Chen Y, LeCluyse EL, Negishi M, Goldstein JA: **Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha**. *Mol Pharmacol* 2005, **68**(3):747-757.
213. Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ *et al*: **The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4**. *Nat Med* 2003, **9**(2):220-224.
214. Inoki K, Zhu T, Guan KL: **TSC2 mediates cellular energy response to control cell growth and survival**. *Cell* 2003, **115**(5):577-590.
215. Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC: **The LKB1 tumor suppressor negatively regulates mTOR signaling**. *Cancer Cell* 2004, **6**(1):91-99.
216. Cheng SW, Fryer LG, Carling D, Shepherd PR: **Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status**. *J Biol Chem* 2004, **279**(16):15719-15722.
217. Koromilas AE, Lazaris-Karatzas A, Sonenberg N: **mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E**. *Embo J* 1992, **11**(11):4153-4158.

218. Svitkin YV, Pause A, Haghighat A, Pyronnet S, Witherell G, Belsham GJ, Sonenberg N: **The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure**. *Rna* 2001, **7**(3):382-394.
219. Gorrini C, Loreni F, Gandin V, Sala LA, Sonenberg N, Marchisio PC, Biffo S: **Fibronectin controls cap-dependent translation through beta1 integrin and eukaryotic initiation factors 4 and 2 coordinated pathways**. *Proc Natl Acad Sci U S A* 2005, **102**(26):9200-9205.
220. Hornstein E, Tang H, Meyuhas O: **Mitogenic and nutritional signals are transduced into translational efficiency of TOP mRNAs**. *Cold Spring Harb Symp Quant Biol* 2001, **66**:477-484.
221. Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G: **Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k**. *Embo J* 1997, **16**(12):3693-3704.
222. Warner JR: **The economics of ribosome biosynthesis in yeast**. *Trends Biochem Sci* 1999, **24**(11):437-440.
223. Kawasome H, Papst P, Webb S, Keller GM, Johnson GL, Gelfand EW, Terada N: **Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity**. *Proc Natl Acad Sci U S A* 1998, **95**(9):5033-5038.
224. Dogra SC, Hahn CN, May BK: **Superinduction by cycloheximide of cytochrome P4502H1 and 5-aminolevulinate synthase gene transcription in chick embryo liver**. *Arch Biochem Biophys* 1993, **300**(1):531-534.
225. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE: **Inhibition of protein synthesis increases the transcription of the phenobarbital-inducible CYP2H1 and CYP2H2 genes in chick embryo hepatocytes**. *Arch Biochem Biophys* 1992, **298**(1):96-104.
226. Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB: **AMP-activated protein kinase induces a p53-dependent metabolic checkpoint**. *Mol Cell* 2005, **18**(3):283-293.
227. Imamura K, Ogura T, Kishimoto A, Kaminishi M, Esumi H: **Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line**. *Biochem Biophys Res Commun* 2001, **287**(2):562-567.
228. Karuman P, Gozani O, Odze RD, Zhou XC, Zhu H, Shaw R, Brien TP, Bozzuto CD, Ooi D, Cantley LC *et al*: **The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death**. *Mol Cell* 2001, **7**(6):1307-1319.
229. Kato K, Ogura T, Kishimoto A, Minegishi Y, Nakajima N, Miyazaki M, Esumi H: **Critical roles of AMP-activated protein kinase in constitutive tolerance of cancer cells to nutrient deprivation and tumor formation**. *Oncogene* 2002, **21**(39):6082-6090.
230. Bursch W, Grasl-Kraupp B, Wastl U, Hufnagl K, Chabicovsky M, Taper H, Schulte-Hermann R: **Role of apoptosis for mouse liver growth regulation and tumor promotion: comparative analysis of mice with high (C3H/He) and low (C57Bl/6J) cancer susceptibility**. *Toxicol Lett* 2004, **149**(1-3):25-35.
231. Rajvanshi P, Liu D, Ott M, Gagandeep S, Schilsky ML, Gupta S: **Fractionation of rat hepatocyte subpopulations with varying metabolic potential, proliferative capacity, and retroviral gene transfer efficiency**. *Exp Cell Res* 1998, **244**(2):405-419.
232. Lave LB: **Hormesis: implications for public policy regarding toxicants**. *Annu Rev Public Health* 2001, **22**:63-67.
233. Fukushima S, Kinoshita A, Puatanachokchai R, Kushida M, Wanibuchi H, Morimura K: **Hormesis and dose-response-mediated mechanisms in carcinogenesis: evidence for a threshold in carcinogenicity of non-genotoxic carcinogens**. *Carcinogenesis* 2005, **26**(11):1835-1845.
234. Kinoshita A, Wanibuchi H, Morimura K, Wei M, Shen J, Imaoka S, Funae Y, Fukushima S: **Phenobarbital at low dose exerts hormesis in rat hepatocarcinogenesis by reducing oxidative DNA damage, altering cell proliferation, apoptosis and gene expression**. *Carcinogenesis* 2003, **24**(8):1389-1399.
235. Olsen CM, Meussen-Elholm ET, Roste LS, Tauboll E: **Antiepileptic drugs inhibit cell growth in the human breast cancer cell line MCF7**. *Mol Cell Endocrinol* 2004, **213**(2):173-179.

236. Anderson CM, Norquist BA, Vesce S, Nicholls DG, Soine WH, Duan S, Swanson RA: **Barbiturates induce mitochondrial depolarization and potentiate excitotoxic neuronal death**. *J Neurosci* 2002, **22**(21):9203-9209.
237. Aldridge WN, Parker VH: **Barbiturates and oxidative phosphorylation**. *Biochem J* 1960, **76**:47-56.
238. Chance B, Hollunger G: **Energy-linked reduction of mitochondrial pyridine nucleotide**. *Nature* 1960, **185**:666-672.
239. Detaille D, Guigas B, Leverve X, Wiernsperger N, Devos P: **Obligatory role of membrane events in the regulatory effect of metformin on the respiratory chain function**. *Biochem Pharmacol* 2002, **63**(7):1259-1272.
240. Toell A, Kroncke KD, Kleinert H, Carlberg C: **Orphan nuclear receptor binding site in the human inducible nitric oxide synthase promoter mediates responsiveness to steroid and xenobiotic ligands**. *J Cell Biochem* 2002, **85**(1):72-82.
241. Schulz E, Anter E, Zou MH, Keaney JF, Jr.: **Estradiol-mediated endothelial nitric oxide synthase association with heat shock protein 90 requires adenosine monophosphate-dependent protein kinase**. *Circulation* 2005, **111**(25):3473-3480.
242. Torres M: **Mitogen-activated protein kinase pathways in redox signaling**. *Front Biosci* 2003, **8**:d369-391.
243. Torres M, Forman HJ: **Redox signaling and the MAP kinase pathways**. *Biofactors* 2003, **17**(1-4):287-296.
244. Joannard F, Rissel M, Gilot D, Anderson A, Orfila-Lefeuvre L, Guillouzo A, Atfi A, Lagadic-Gossmann D: **Role for mitogen-activated protein kinases in phenobarbital-induced expression of cytochrome P450 2B in primary cultures of rat hepatocytes**. *Toxicol Lett* 2006, **161**(1):61-72.
245. Roberge C, Beaudet MJ, Anderson A: **GABA(A)/central benzodiazepine receptor and peripheral benzodiazepine receptor ligands as inducers of phenobarbital-inducible CYP2B and CYP3A**. *Biochem Pharmacol* 2004, **68**(7):1383-1389.
246. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X: **Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I**. *J Biol Chem* 2000, **275**(1):223-228.
247. Guigas B, Detaille D, Chauvin C, Batandier C, De Oliveira F, Fontaine E, Leverve X: **Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study**. *Biochem J* 2004, **382**(Pt 3):877-884.
248. Detaille D, Wiernsperger N, Devos P: **Cellular and molecular mechanisms involved in insulin's potentiation of glycogen synthase activity by metformin**. *Biochem Pharmacol* 1999, **58**(9):1475-1486.
249. Moats RK, 2nd, Ramirez VD: **Rapid uptake and binding of estradiol-17beta-6-(O-carboxymethyl)oxime:125I-labeled BSA by female rat liver**. *Biol Reprod* 1998, **58**(2):531-538.
250. Moats RK, 2nd, Ramirez VD: **Electron microscopic visualization of membrane-mediated uptake and translocation of estrogen-BSA:colloidal gold by hep G2 cells**. *J Endocrinol* 2000, **166**(3):631-647.
251. Dipple I, Gordon LM, Houslay MD: **The activity of 5'-nucleotidase in liver plasma membranes is affected by the increase in bilayer fluidity achieved by anionic drugs but not by cationic drugs**. *J Biol Chem* 1982, **257**(4):1811-1815.
252. Houslay MD, Dipple I, Gordon LM: **Phenobarbital selectively modulates the glucagon-stimulated activity of adenylate cyclase by depressing the lipid phase separation occurring in the outer half of the bilayer of liver plasma membranes**. *Biochem J* 1981, **197**(3):675-681.
253. Gjedde A, Rasmussen M: **Pentobarbital anesthesia reduces blood-brain glucose transfer in the rat**. *J Neurochem* 1980, **35**(6):1382-1387.
254. Otsuka T, Wei L, Acuff VR, Shimizu A, Pettigrew KD, Patlak CS, Fenstermacher JD: **Variation in local cerebral blood flow response to high-dose pentobarbital sodium in the rat**. *Am J Physiol* 1991, **261**(1 Pt 2):H110-120.
255. Otsuka T, Wei L, Bereczki D, Acuff V, Patlak C, Fenstermacher J: **Pentobarbital produces dissimilar changes in glucose influx and utilization in brain**. *Am J Physiol* 1991, **261**(2 Pt 2):R265-275.
256. Honkanen RA, McBath H, Kushmerick C, Callender GE, Scarlata SF, Fenstermacher JD, Haspel HC: **Barbiturates inhibit hexose transport in cultured mammalian cells and human**

erythrocytes and interact directly with purified GLUT-1. *Biochemistry* 1995, **34**(2):535-544.
257. Hresko RC, Heimberg H, Chi MM, Mueckler M: **Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP**. *J Biol Chem* 1998, **273**(32):20658-20668.
258. Durnas C, Loi CM, Cusack BJ: **Hepatic drug metabolism and aging**. *Clin Pharmacokinet* 1990, **19**(5):359-389.
259. Kamataki T, Maeda K, Shimada M, Kitani K, Nagai T, Kato R: **Age-related alteration in the activities of drug-metabolizing enzymes and contents of sex-specific forms of cytochrome P-450 in liver microsomes from male and female rats**. *J Pharmacol Exp Ther* 1985, **233**(1):222-228.
260. Kato R, Vassanelli P, Frontino G, Chiesara E: **Variation in the Activity of Liver Microsomal Drug-Metabolizing Enzymes in Rats in Relation to the Age**. *Biochem Pharmacol* 1964, **13**:1037-1051.
261. Loi CM, Vestal RE: **Drug metabolism in the elderly**. *Pharmacol Ther* 1988, **36**(1):131-149.
262. Abernethy DR, Greenblatt DJ: **Impairment of lidocaine clearance in elderly male subjects**. *J Cardiovasc Pharmacol* 1983, **5**(6):1093-1096.
263. O'Malley K, Crooks J, Duke E, Stevenson IH: **Effect of age and sex on human drug metabolism**. *Br Med J* 1971, **3**(5775):607-609.
264. Sotaniemi EA, Arranto AJ, Pelkonen O, Pasanen M: **Age and cytochrome P450-linked drug metabolism in humans: an analysis of 226 subjects with equal histopathologic conditions**. *Clin Pharmacol Ther* 1997, **61**(3):331-339.
265. Woodhouse K, Wynne HA: **Age-related changes in hepatic function. Implications for drug therapy**. *Drugs Aging* 1992, **2**(3):243-255.
266. Woodhouse KW, Wynne HA: **Age-related changes in liver size and hepatic blood flow. The influence on drug metabolism in the elderly**. *Clin Pharmacokinet* 1988, **15**(5):287-294.
267. Wynne HA, Cope LH, Mutch E, Rawlins MD, Woodhouse KW, James OF: **The effect of age upon liver volume and apparent liver blood flow in healthy man**. *Hepatology* 1989, **9**(2):297-301.
268. Bach B, Hansen JM, Kampmann JP, Rasmussen SN, Skovsted L: **Disposition of antipyrine and phenytoin correlated with age and liver volume in man**. *Clin Pharmacokinet* 1981, **6**(5):389-396.
269. Swift CG, Homeida M, Halliwell M, Roberts CJ: **Antipyrine disposition and liver size in the elderly**. *Eur J Clin Pharmacol* 1978, **14**(2):149-152.
270. Twum-Barima Y, Finnigan T, Habash AI, Cape RD, Carruthers SG: **Impaired enzyme induction by rifampicin in the elderly**. *Br J Clin Pharmacol* 1984, **17**(5):595-597.
271. Vestal RE, Wood AJ, Branch RA, Shand DG, Wilkinson GR: **Effects of age and cigarette smoking on propranolol disposition**. *Clin Pharmacol Ther* 1979, **26**(1):8-15.
272. Schmucker DL, Wang RK: **Age-related changes in liver drug metabolism: structure vs function**. *Proc Soc Exp Biol Med* 1980, **165**(2):178-187.
273. Schmucker DL, Mooney JS, Jones AL: **Stereological analysis of hepatic fine structure in the Fischer 344 rat. Influence of sublobular location and animal age**. *J Cell Biol* 1978, **78**(2):319-337.
274. Schumucker DL, Mooney JS, Jones AL: **Age-related changes in the hepatic endoplasmic reticulum: a quantitative analysis**. *Science* 1977, **197**(4307):1005-1008.
275. Schmucker DL, Wang RK: **Effects of animal age and phenobarbital on rat liver glucose-6-phosphatase activity**. *Exp Gerontol* 1980, **15**(1):7-13.
276. Michikawa Y, Mazzucchelli F, Bresolin N, Scarlato G, Attardi G: **Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication**. *Science* 1999, **286**(5440):774-779.
277. Wallace DC: **A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine**. *Annu Rev Genet* 2005, **39**:359-407.
278. Modica-Napolitano JS, Singh KK: **Mitochondrial dysfunction in cancer**. *Mitochondrion* 2004, **4**(5-6):755-762.
279. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly YM, Gidlof S, Oldfors A, Wibom R *et al*: **Premature ageing in mice expressing defective mitochondrial DNA polymerase**. *Nature* 2004, **429**(6990):417-423.

280. Tanaka M, Gong JS, Zhang J, Yoneda M, Yagi K: **Mitochondrial genotype associated with longevity**. *Lancet* 1998, **351**(9097):185-186.
281. Linnane AW, Marzuki S, Ozawa T, Tanaka M: **Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases**. *Lancet* 1989, **1**(8639):642-645.
282. Wei YH, Lu CY, Lee HC, Pang CY, Ma YS: **Oxidative damage and mutation to mitochondrial DNA and age-dependent decline of mitochondrial respiratory function**. *Ann N Y Acad Sci* 1998, **854**:155-170.
283. Thomson DM, Gordon SE: **Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation**. *J Appl Physiol* 2005, **98**(2):557-564.
284. Thomson DM, Gordon SE: **Impaired overload-induced muscle growth is associated with diminished translational signalling in aged rat fast-twitch skeletal muscle**. *J Physiol* 2006, **574**(Pt 1):291-305.
285. Rice KM, Preston DL, Walker EM, Blough ER: **Aging influences multiple incidices of oxidative stress in the aortic media of the Fischer 344/NNiaxBrown Norway/BiNia rat**. *Free Radic Res* 2006, **40**(2):185-197.
286. Mulligan JD, Gonzalez AA, Kumar R, Davis AJ, Saupe KW: **Aging elevates basal adenosine monophosphate-activated protein kinase (AMPK) activity and eliminates hypoxic activation of AMPK in mouse liver**. *J Gerontol A Biol Sci Med Sci* 2005, **60**(1):21-27.
287. Pratt WB, Toft DO: **Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery**. *Exp Biol Med (Maywood)* 2003, **228**(2):111-133.
288. Kelley WL: **The J-domain family and the recruitment of chaperone power**. *Trends Biochem Sci* 1998, **23**(6):222-227.
289. Tago K, Tsukahara F, Naruse M, Yoshioka T, Takano K: **Regulation of nuclear retention of glucocorticoid receptor by nuclear Hsp90**. *Mol Cell Endocrinol* 2004, **213**(2):131-138.
290. Barrett MJ, Alones V, Wang KX, Phan L, Swerdlow RH: **Mitochondria-derived oxidative stress induces a heat shock protein response**. *J Neurosci Res* 2004, **78**(3):420-429.
291. Dvorak Z, Modriansky M, Pichard-Garcia L, Balaguer P, Vilarem MJ, Ulrichova J, Maurel P, Pascussi JM: **Colchicine down-regulates cytochrome P450 2B6, 2C8, 2C9, and 3A4 in human hepatocytes by affecting their glucocorticoid receptor-mediated regulation**. *Mol Pharmacol* 2003, **64**(1):160-169.
292. Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W: **Relief of microRNA-mediated translational repression in human cells subjected to stress**. *Cell* 2006, **125**(6):1111-1124.
293. Carthew RW: **Gene regulation by microRNAs**. *Curr Opin Genet Dev* 2006, **16**(2):203-208.
294. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R: **Control of translation and mRNA degradation by miRNAs and siRNAs**. *Genes Dev* 2006, **20**(5):515-524.
295. Poole AW, Pula G, Hers I, Crosby D, Jones ML: **PKC-interacting proteins: from function to pharmacology**. *Trends Pharmacol Sci* 2004, **25**(10):528-535.
296. Grunicke HH, Spitaler M, Mwanjewe J, Schwaiger W, Jenny M, Ueberall F: **Regulation of cell survival by atypical protein kinase C isozymes**. *Adv Enzyme Regul* 2003, **43**:213-228.
297. Jenny M, Wrulich OA, Schwaiger W, Ueberall F: **Relevance of atypical protein kinase C isotypes to the drug discovery process**. *Chembiochem* 2005, **6**(3):491-499.
298. Mosthaf L, Kellerer M, Muhlhofer A, Mushack J, Seffer E, Haring HU: **Insulin leads to a parallel translocation of PI-3-kinase and protein kinase C zeta**. *Exp Clin Endocrinol Diabetes* 1996, **104**(1):19-24.
299. Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J *et al*: **Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes**. *J Biol Chem* 1999, **274**(36):25308-25316.
300. Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese RV: **Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport**. *J Biol Chem* 1997, **272**(48):30075-30082.

301. Cornford P, Evans J, Dodson A, Parsons K, Woolfenden A, Neoptolemos J, Foster CS: **Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer**. *Am J Pathol* 1999, **154**(1):137-144.
302. Tsai JH, Hsieh YS, Kuo SJ, Chen ST, Yu SY, Huang CY, Chang AC, Wang YW, Tsai MT, Liu JY: **Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma**. *Cancer Lett* 2000, **161**(2):171-175.
303. Romanelli A, Martin KA, Toker A, Blenis J: **p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex**. *Mol Cell Biol* 1999, **19**(4):2921-2928.
304. Umar S, Sellin JH, Morris AP: **Increased nuclear translocation of catalytically active PKC-zeta during mouse colonocyte hyperproliferation**. *Am J Physiol Gastrointest Liver Physiol* 2000, **279**(1):G223-237.
305. Luna V, Casauban L, Sajan MP, Gomez-Daspet J, Powe JL, Miura A, Rivas J, Standaert ML, Farese RV: **Metformin improves atypical protein kinase C activation by insulin and phosphatidylinositol-3,4,5-(PO4)3 in muscle of diabetic subjects**. *Diabetologia* 2006, **49**(2):375-382.
306. Gray JP, Burns KA, Leas TL, Perdew GH, Vanden Heuvel JP: **Regulation of peroxisome proliferator-activated receptor alpha by protein kinase C**. *Biochemistry* 2005, **44**(30):10313-10321.
307. Datta K, Li J, Bhattacharya R, Gasparian L, Wang E, Mukhopadhyay D: **Protein kinase C zeta transactivates hypoxia-inducible factor alpha by promoting its association with p300 in renal cancer**. *Cancer Res* 2004, **64**(2):456-462.
308. Ulrich SM, Kenski DM, Shokat KM: **Engineering a "methionine clamp" into Src family kinases enhances specificity toward unnatural ATP analogues**. *Biochemistry* 2003, **42**(26):7915-7921.
309. Keen JC, Zhou Q, Park BH, Pettit C, Mack KM, Blair B, Brenner K, Davidson NE: **Protein phosphatase 2A regulates estrogen receptor alpha (ER) expression through modulation of ER mRNA stability**. *J Biol Chem* 2005, **280**(33):29519-29524.
310. Sleeman JE, Lamond AI: **Nuclear organization of pre-mRNA splicing factors**. *Curr Opin Cell Biol* 1999, **11**(3):372-377.
311. Koike C, Moore R, Negishi M: **Localization of the nuclear receptor CAR at the cell membrane of mouse liver**. *FEBS Lett* 2005, **579**(30):6733-6736.
312. Farach-Carson MC, Davis PJ: **Steroid hormone interactions with target cells: cross talk between membrane and nuclear pathways**. *J Pharmacol Exp Ther* 2003, **307**(3):839-845.
313. Farach-Carson MC, Nemere I: **Membrane receptors for vitamin D steroid hormones: potential new drug targets**. *Curr Drug Targets* 2003, **4**(1):67-76.
314. Felty Q, Roy D: **Estrogen, mitochondria, and growth of cancer and non-cancer cells**. *J Carcinog* 2005, **4**(1):1.
315. Chambliss KL, Shaul PW: **Estrogen modulation of endothelial nitric oxide synthase**. *Endocr Rev* 2002, **23**(5):665-686.
316. Kukalev A, Nord Y, Palmberg C, Bergman T, Percipalle P: **Actin and hnRNP U cooperate for productive transcription by RNA polymerase II**. *Nat Struct Mol Biol* 2005, **12**(3):238-244.

Appendices

TABLE 1: REAL-TIME PCR PRIMER SEQUENCES

chGAPDH	765F	GGTCACGCTCCTGGAAGATAGT
	838R	GGGCACTGTCAAGGCTGAGA
	788T	ATGGCGTGCCCATTGATCACAAGTTTC
CYP2H1	1076F	AGGGTGGTGAGGGCAAATC
	1140R	ACAGGCATTGTGACCAGCAA
	1096T	TCGCAGTTGCCTCCAGGTCTCCC
CYP3A37	1446F	GTCCCAAAGAAAGGCAATGGT
	1509R	GGCCATTTGGGTTGTTCAAG
	1468T	TTGGCCCAGGAATGCCCAGC
chALAS1	1031F	GCAGGGTGCCAAAACACAT
	1108R	TCGATGGATCAGACTTCTTCAACA
	1051T	TTCCGCCATAACGACGTCAACCATCTT
CXR	432F	GCAGAGCAGCAGGAGCTCAT
	521R	AGGCTGGTAGTGCTGGAACTG
	469T	CGCACAAACGCACCTTCGACTCC
h18s	F	AGTCCCTGCCCTTTGTACACA
	R	CGATCCGAGGGCCTCACTA
	T	CGCCCGTCGCTACTACCGATTGG
CYP2B6	1009F	ACATCGCCCTCCAGAGCTT
	1090R	GTCGGAAAATCTCTGAATCTCATAGA
	1032T	ACCGAGCCAAAATGCCATACACAGAGG
CYP3A4	F	CATTCCTCATCCCAATTCTTGAAGT
	R	CCACTCGGTGCTTTTGTGTATCT
	T	CGAGGCGACTTTCTTTCATCCTTTTTACAGATTTTC
hALAS1	7F	ATGATGCCAGGCTGTGAGATTT
	7R	GCTGTTTCGAATCCCTTGGA
	7T	TCTGATTCTGGGAACCATGCCTCCA
hCAR	F	CACATGGGCACCATGTTTGA
	R	AAGGGCTGGTGATGGATGAA
	T	TTTGTGCAGTTTAGGCCTCCAGCTCATCT
mGAPDH	649F	CCAGAACATCATCCCTGCATC
	878R	GGTCCTCAGTGTAGCCCAAGAT
	781T	CCGCCTGGAGAAACCTGCCAAGTATG
Cyp2b10	460F	CAATGTTTAGTGGAGGAACTGCG
	527R	CACTGGAAGAGGAACGTGGG
	488T	CCCAGGGAGCCCCCCTGGA
Cyp3a11	1459F	AGAACTTCTCCTTCCAGCCTTGTA
	1598R	GAGGGAGACTCATGCTCCAGTTA
	1548T	CTAAAGGTTGTGCCACGGGATGCAGT
mALAS1	1002F	GGCCTCCCGGTCATCC
	1068R	TGTTCTTAGCAGCATCGGCA
	1019T	CTGTCCGAGTCACATCATCCCTGTGC
mCAR	440F	CATTGCGGCGAGCCA
	502R	GCTGATTCAGTTGCAAAGATGC
	456T	ACAGGCACAGCGGCGGGC
mPXR	519F	GAGGAAGAAGAGGGAAAAGATTGAG
	584R	TGCTGTTCTTCCGTCAGCC
	545T	CTCCACCGCCTGGAGGGCAG
mHNF4α	1159F	AGATGCTTCTCGGAGGGTCTG
	1246R	TTGGTGCCCATGTGTTCTTG
	1181T	CAGTGATGCACCCCACACCCACC

Abbreviations

2-DG	2-deoxyglucose
AhR	aromatic hydrocarbon receptor
ACC	acetyl-CoA carboxylase
AICAR	5'-phosphoribosyl-5-aminoimidazol-4-carboxamide
ALAS1	δ-aminolevulinic acid synthase 1
AMPK	AMP-activated protein kinase
bp	basepair(s)
CAR	constitutive androstane receptor
CaMK	calcium/calmodulin-dependent protein kinase
CCRP	cytoplasmic CAR retention protein
CXR	chicken xenobiotic receptor
CYP	cytochrome P450
DBD	DNA-binding domain
DN	dominant negative
DNP	dinitrophenol
DR	direct repeat
eEF	eukariotic elongatio factor
eIF	eukariotic initiation factor
ER	everted reapeat
FOXO1	forkhead box protein O1A
FXR	farnesoid X receptor
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GLUT	glucose transporter
GR	glucocorticoid receptor
HNF4α	hepatocyte nuclear factor 4α
HPLC	high pressure liquid chromatography
Hsp90	heat shock protein 90
IF	immunofluorescence
IP	immunoprecipitation
IR	inverted repeat
kDa	kilodalton(s)
LBD	ligand-binding domain
LMH	leghorn male hepatoma
LXR	liver X receptor
MDR1	multidrug resistance gene 1
miRNA	micro RNA

MRP2	multidrug resistance-associated protein 2
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NAC	N-acetyl L-cysteine
NOS	nitric oxide synthase
NR	nuclear receptor
OA	okadaic acid
OATP2	organic anion transport protein 2
PAGE	polyacrylamide gel electrophoresis
PB	phenobarbital
PBREM	phenobarbital-responsive enhancer module
PBRU	phenobarbital-responsive enhancer unit
PCN	5-pregnen-3β-ol-20-one-16α-carbonitrile
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor γ coactivator α
PKCζ	protein kinase C ζ
PKA	cAMP-dependent protein kinase A
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
ROS	reactive oxygen species
RT-PCR	real-time PCR
RXR	9-*cis*-retinoic acid receptor
siRNA	small interfering RNA
SULT	sulfotransferase
TCPOBOP	1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene
TSC2	tuberous sclerosis complex 2
UCP-1	uncoupling protein 1
UGT	UDP-glucuronosyltransferase
5′ UTR	5′ untranslated region
VDR	vitamin D receptor
XLS	xenobiotic localization signal

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Stimulation of AMP-Activated Protein Kinase Is Essential for the Induction of Drug Metabolizing Enzymes by Phenobarbital in Human and Mouse Liver

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ABSTRACT

Our previous studies have suggested a role for AMP-activated protein kinase (AMPK) in the induction of CYP2B6 by phenobarbital (PB) in hepatoma-derived cells (Rencurel et al., 2005). In this study, we showed in primary human hepatocytes that: 1) 5′-phosphoribosyl-5-aminoimidazol-4-carboxamide 1-β-D-ribofuranoside and the biguanide metformin, known activators of AMPK, dose-dependently increase the expression of CYP2B6 and CYP3A4 to an extent similar to that of PB. 2) PB, but not the human nuclear receptor constitutive active/androstane receptor (CAR) ligand 6-(4-chlorophenyl)imidazol[2,1–6][1,3]thiazole-5-carbaldehyde, dose-dependently increase AMPK activity. 3) Pharmacological inhibition of AMPK activity with compound C or dominant-negative forms of AMPK blunt the inductive response to phenobarbital. Furthermore, in transgenic mice with a liver-specific deletion of both the α1 and α2 AMPK catalytic subunits, basal levels of Cyp2b10 and Cyp3a11 mRNA were increased but not in primary culture of mouse hepatocytes. However, phenobarbital or 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene, a mouse CAR ligand, failed to induce the expression of these genes in the liver or cultured hepatocytes from mice lacking hepatic expression of the α1 and α2 subunits of AMPK. The distribution of CAR between the nucleus and cytosol was not altered in hepatocytes from mice lacking both AMPK catalytic subunits. These data highlight the essential role of AMPK in the CAR-mediated signal transduction pathway.

Induction of drug-metabolizing enzymes and drug transporters by drugs and other chemicals is an adaptive response of mammals and other organisms to increase the removal of potentially toxic endobiotics and xenobiotics. Phenobarbital (PB) represents a class of inducers in which the effect on detoxification is part of a pleiotropic response that includes liver hypertrophy, tumor promotion, and induction or repression of multiple genes, in addition to genes coding for enzymes or transporters that regulate drug disposition. The molecular mechanism of the induction response remains incompletely understood. The induction of human cytochrome P450 CYP2B6 and its rat and mouse orthologs CYP2B1 and Cyp2b10 by PB is mediated by the nuclear receptor constitutive active/androstane receptor (CAR, NR1I3) (Honkakoski et al., 1998). In untreated primary mouse hepatocytes, CAR is retained in the cytoplasm within a protein complex of

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ABBREVIATIONS: PB, phenobarbital; CAR, constitutive active/androstane receptor; CCRP, cytoplasmic CAR retaining protein; AMPK, AMP-activated protein kinase; AICAR, 5′-phosphoribosyl-5-aminoimidazol-4-carboxamide 1-β-D-ribofuranoside; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; CITCO, 6-(4-chlorophenyl)imidazol[2,1–6][1,3]thiazole-5-carbaldehyde; Rif, rifampicin; RT, reverse transcription; PCR, polymerase chain reaction; TCPOBOP,1,4 bis[2-(3,5-dichloropyridyloxy)]benzene; PGC1α, peroxisome proliferator-activated receptor γ coactivator-1α.

chaperones and cochaperones, such as the 90-kDa heat shock protein and a protein called cytoplasmic CAR retaining protein (CCRP) (Kobayashi et al., 2003). Exposure to xenobiotics such as PB causes CAR to dissociate from this complex and to transfer into the nucleus, where it forms a heterodimer with retinoid X receptor and binds to cognate DNA sequences of target genes (Kawamoto et al., 1999). This process is influenced by phosphorylation and dephosphorylation of unknown proteins (Hosseinpour et al., 2005). In human hepatoma cells, such as HepG2, CAR is found exclusively in the nucleus and is constitutively active, resulting in CYP2B6 gene expression in cells not exposed to any inducers (Sueyoshi et al., 1999). Coexpression of exogenous CCRP with exogenous CAR in HepG2 cells confirms the cytoplasmic retention of CAR by CCRP, but no nuclear transfer is observed upon drug treatment (Kobayashi et al., 2003). This suggests that additional proteins or reactions are required for drug induced CAR cytoplasmic-nuclear translocations, which are missing or nonfunctional in HepG2 cells. Because hepatoma cells do not reciprocate the physiological activation of CAR or its binding to DNA, we decided to focus on CAR in primary human and mouse hepatocytes.

In the liver of fasted mice, CYP2b10 and mCAR expression are induced (Maglich et al., 2004). AMP-activated protein kinase (AMPK) functions as an energy sensor and is activated when cells experience energy depleting stresses such as nutrient starvation (Carling, 2004). AMPK is also activated by pharmacological manipulations. 5′-Phosphoribosyl-5-aminoimidazol-4-carboxamide 1-β-D-ribofuranoside (AICAR) is a cell-permeant adenosine analog commonly used to activate AMPK. Although activation of AMPK because of an increase in the AMP/ATP ratio is the best-described mechanism, some studies suggest alternative mechanisms of AMPK activation.

AMPK is a heterotrimeric complex ubiquitously expressed and consists of a catalytic subunit, α, and two regulatory subunits, β and γ (Woods et al., 1996). All three subunits have been identified, and each subunit is encoded by two (α1,α2, β1,β2) or three genes (γ1, γ2, γ3) Formation of the trimeric complex is necessary for optimal kinase activity. Changes in the cellular energy state activate AMPK through mechanisms involving an AMP allosteric regulation and phosphorylation by an upstream kinase on threonine residue 172 within the α subunit (Stein et al., 2000). This upstream kinase has recently been identified in liver as LKB1 (Woods et al., 2003).

In a previous study, we showed that PB can activate AMPK in a HepG2-derived hepatoma cell line, WGA, and that activation of AMPK by AICAR induced CYP2B6 (Rencurel et al., 2005). However, the activation of AMPK was observed only at high concentrations of PB (above 1 mM). Questions thus remain as to whether 1) AMPK activation by PB occurs only in transformed cells or 2) only at high concentrations of the inducer. Thus, it has remained unclear whether activation of AMPK is a necessary component of the PB signaling pathway in fully differentiated liver cells either in vitro and or in vivo. We therefore investigated the relationship between AMPK activation and induction of cytochromes P450 in primary human hepatocytes. Moreover, the recent development of transgenic mice with a deletion of AMPK catalytic subunits α1 and α2 in the liver provides a unique experimental model to address the role of AMPK in PB induction of *CYP2b10* and *CYP3a11* gene expression in vivo and in vitro. In primary human hepatocyte culture, we now show a concentration-dependent activation of AMPK by PB. Through ablation of AMPK activity by 1) pharmacological approaches, 2) overexpressing a dominant-negative form of AMPK, or 3) genetic ablation of AMPK α1 and α2 genes in mice. We here provide unequivocal evidence that AMPK activation is a necessary step in the induction of CYP2B6 and Cyp2b10 by PB in both human and mouse hepatocytes.

Materials and Methods

Reagents. Phenobarbital (sodium salt) was purchased from Fluka (Buchs, Switzerland). All other chemicals were from Sigma (Buchs, Switzerland). Cell culture media, fetal bovine serum, other tissue culture reagents, and TRIzol reagent were from Invitrogen (Carlsbad, CA). Antibodies raised against AMPK α1 and α2 subunits and phospho-acetyl-CoA-carboxylase were purchased from Upstate Biotechnology (Lucerne, Switzerland).

Generation of AMPKα1/α2$^{LS-/-}$ Knockout Mice. The liver-specific knock out of both α subunits of AMPK has been described previously (Guigas et al., 2006). In brief, to generate deletion of both catalytic subunits in the liver (α1/α2$^{LS-/-}$), liver-specific AMPKα2-null mice were first generated by crossing floxed AMPKα2 mice (Viollet et al., 2003) and Alfp Cre transgenic mice expressing the Cre recombinase under the control of albumin and α-fetoprotein regulatory elements. A liver-specific AMPKα2 deletion was then produced on an AMPKα1$^{-/-}$ general knockout background by crossing liver-specific α2$^{-/-}$ mice with AMPKα1$^{-/-}$ general knockout mice (Jorgensen et al., 2004).

Recombinant Adenoviruses. Adenovirus encoding constitutively active α1 AMPK subunit (ad-CA-α1^{312}) or dominant-negative mutant α1 AMPK (ad-DNα1) were prepared as described previously (Diraison et al., 2004). Adenoviruses encoding constitutively active α2 AMPK subunit (ad-CA-α2^{312}) or β-galactosidase were also amplified as described previously (Foretz et al., 2005). Adenovirus encoding human CAR in fusion with enhanced green fluorescence protein (ad-hCAR-GFP) was a kind gift of Dr. Ramiro Jover (Hospital La Fe, Valencia, Spain). AMPK adenoviruses also express, under control of a distinct cytomegalovirus promoter. Viral particles were purified by cesium chloride density centrifugation, and human and mouse hepatocytes were infected 12 h after seeding with a multiplicity of infection of 30 to 100.

Culture of Primary Human Hepatocytes. Primary human hepatocytes were isolated from the resected liver tissue of consented patients undergoing liver surgery. Human hepatocytes were enzymatically dissociated from human liver samples using a two-step enzymatic microperfusion technique with collagenase and kept on ice in suspension (Strain, 1994). Hepatocytes were subsequently seeded on plastic dishes coated with rat-tail collagen (25 μg/cm^2) at a density of 130,000 viable cells/cm^2 and cultured in Dulbeco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 μM dexamethasone.

After overnight culture, the medium was replaced by serum-free Williams' E medium supplemented with 100 nM hydrocortisone, 50 U/ml penicillin, 50 μg/ml streptomycin, and solution ITS + 1, containing insulin (5 μg/ml), transferrin (2.75 μg/ml), and selenium (25 ng/ml) (Sigma, Buchs, Switzerland). The medium also was supplemented with 250 μg/ml bovine serum albumin and 2.35 μg/ml linoleic acid. Twenty-four hours after serum deprivation, the human hepatocytes were kept in serum-free medium and exposed to various chemicals for $\leq$16 h as indicated in the figure legends.

Preparation and Culture of Primary Mouse Hepatocytes. Liver cells were prepared by the two-step collagenase method (Berry and Friend, 1969) from postabsorptive male mice (25–30 g) after anesthesia with ketamine/xylazine (1 mg/100 g of body weight).

Hepatocytes were seeded on dishes coated with rat-tail collagen type 1 and cultured overnight in M199 supplemented with 1% Ultroser G (Biosepra SA, Cergy-Saint-Christophe, France) and (50 U/ml penicillin/50 μg/ml streptomycin. After overnight culture, the medium was replaced by a serum free Williams' E medium. Twenty-four hours after serum deprivation, cells were exposed to chemicals for 16 h, or as indicated in the figure legends, and were maintained in serum-free medium.

Real-Time PCR Assays. One microgram of total RNA was reverse-transcribed and used in real-time PCR assays for quantification of different target genes on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Expression levels of these genes were normalized against 18s rRNA for human samples and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for mouse samples. The primers sequences are described in Table 1.

Western Blot Analysis. Cultured hepatocytes were washed in ice-cold PBS and harvested in a 300-μl/6-cm dish of extraction buffer (100 mM KCl, 25 mM HEPES, 7.5 mM $MgCl_2$, and 20% glycerol, pH 7.4) supplemented with protease inhibitor cocktail tablets (Roche, Rotkreuz, Switzerland) containing dithiothreitol (4 mM), aprotinin (2 mg/ml), and β-mercaptoethanol (1 mM). The cell suspension was sonicated for 5 s, and cellular debris was removed by centrifugation (1000*g* for 10 min at 4°C).

Thirty micrograms of total cellular protein were separated by Tris-Tricine glycerol/SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The following primary antibodies were employed: anti-AMPK α1 subunit and anti-AMPK α2 subunit, anti-phospho-ACC (Cell Signaling, Allschwill, Switzerland) and anti-Myc (clone 9E10; Sigma, Buchs, Switzerland). Secondary antibodies anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase were employed for chemiluminescence immunodetection. Blots were developed using ECL reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposure to X-ray films (X-OMAT Kodak; Sigma).

AMP-Activated Kinase Activity Assay. The AMPK assay was performed using the SAMS peptide phosphorylation assay kit from Upstate Biotechnology according to the manufacturer's protocol. In brief, cells were cultured in serum-free medium for 16 h before drug exposure. Chemicals were added straight to cell culture dishes, and cells were incubated for 1 h at 37°C. Culture medium was quickly removed, and cells were washed once with ice-cold PBS and harvested in Tris-HCL (50 mM, pH 7.5), EGTA (1 mM), EDTA (1 mM), dithiothreitol (1 mM), sodium fluoride (50 mM), sodium pyrophosphate (5 mM), benzamidine (1 mM), soybean trypsin inhibitor (4 μg/ml), phenylmethylsulfonyl fluoride (1 mM), mannitol (250 mM), and protease inhibitor tablets (Roche). Cellular debris was removed by centrifugation at 10,000*g* at 4°C for 20 min and the supernatant snap-frozen in liquid nitrogen. Samples were stored at −70°C before AMPK activity assays.

Proteins in the supernatant were concentrated by polyethylene glycol PEG 8000 precipitation, and the AMPK reaction was performed for 10 min at 30°C with 20 μM SAMS peptide, 10 μCi of [γ-^{32}P]ATP and a 10-μg protein sample. The reaction mixture was then spotted on P81 phosphocellulose paper (Upstate Biotechnology), which was washed with 0.75% phosphoric acid and acetone, and the radioactivity of phosphor SAMS peptide was quantified by scintillation counting.

Measurement of ATP Concentration in Primary Hepatocytes. Primary human hepatocytes were seeded in a 96-well plate as described above. Cells were exposed for 1 h with different inducers as stated in the figure legend. ATP concentration was estimated by the luciferase activity using the ATP bioluminescence assay kit (Roche Applied Bioscience, Rothkreuz, Switzerland). Results are expressed as percentage of control cells not exposed to any drugs.

Immunocytochemistry. Hepatocytes were cultured on glass coverslips coated with rat-tail collagen (25 μg/cm^2). Mouse hepatocytes were infected 12 h after seeding in a serum-free medium with Ad-hCAR-GFP at an MOI of 30 to 100. Twelve hours after infection, cells were exposed to chemicals for 6 h then washed twice at room temperature with PBS and fixed for 20 min in 4% (w/v) paraformaldehyde. Cells were visualized in Mowiol mounting medium with a 40× objective (1.40 numerical apertures) by using a Leica TCS NT confocal laser scanning microscope (Leica, Wetzlar, Germany).

Ethical Issues. This work was carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Primary human hepatocytes used in this study were from patients undergoing liver surgery who gave

TABLE 1
Primers and probes sequences used in quantitative real-time PCR (TaqMan)

Human Genes	Sequences
CYP2B6	
Forward	5′-ACATCGCCCTCCAGAGCTT-3′
Reverse	5′-GTCGGAAAATCTCTGAATCTCATAGA-3′
Probe	6FAM-5′-ACCGAGCCAAAATGCCATACACAGAGG-3′TAMRA
CYP3A4	
Forward	5′-CATTCCTCATCCCAATTCTTGAAGT-3′
Reverse	5′-CCACTCGGTGCTTTTGTGTATCT-3′
Probe	6FAM-5′-CGAGGCGACTTTCTTTTCATCCTTTTTACAGATTTTC-3′TAMRA
18s	
Forward	5′-AGTCCCTGCCCTTTGTACACA-3′
Reverse	5′-CGATCCGAGGGCCTCACTA-3′
Probe	6FAM-5′-CGCCCGTCGCTACTACCGATTGG-3′TAMRA
Mouse genes	
Cyp2b10	
Forward	5′-CAATGTTTAGTGGAGGAACTGCG-3′
Reverse	5′-CACTGGAAGAGGAACGTGGG-3′
Probe	6FAM-5′-CCCAGGGAGCCCCCCTGGA-3′TAMRA
Cyp3a11	
Forward	5′-AGAACTTCTCCTTCCAGCCTTGTA-3′
Reverse	5′-GAGGGAGACTCATGCTCCAGTTA-3′
Probe	6FAM-5′-CTAAAGGTTGTGCCACGGGATGCAGT-3′TAMRA
GAPDH	
Forward	5′-CCAGAACATCATCCCTGCATC-3′
Reverse	5′-GGTCCTCAGTGTAGCCCAAGAT-3′
Probe	6FAM-5′-CCGCCTGGAGAAACCTGCCAAGTATG-3′TAMRA

TAMRA, 5-carboxytetramethylrhodamine-labeled probe; FAM, 5-carboxyfluorescein-labeled probe.

consent in accordance with standard procedures approved by an institutional review board, (approval 1.05.01.30.-17).

Results

Induction of CYP2B6 and CYP3A4 by Phenobarbital, Rifampicin, and CITCO in Cultured Primary Human Hepatocytes. Human hepatocytes in primary culture are considered the "gold standard" in vitro model to study drug induction of cytochrome P450 gene expression. Our system of primary human hepatocyte culture was therefore first tested for inducibility of CYP2B6 and CYP3A4 mRNA expression by three typical inducers [i.e., phenobarbital (PB), rifampicin (Rif) and CITCO (6-(4-chlorophenyl)imidazol[2,1–6][1,3]thiazole-5-carbaldehyde)]. Exposure of cells for 16 h to PB (500 μM) and Rif (10 μM) led to an increase of CYP2B6 mRNA levels by 9.97 $\pm$ 2.44-fold (PB) and 13.26 $\pm$ 1.35-fold (Rif), respectively (Fig. 1A), and of CYP3A4 mRNA levels by 12.18 $\pm$ 2.48-fold (PB) and 25.30 $\pm$ 3.37-fold (Rif) (Fig. 1B). CITCO at 50 and 100 nM induced CYP2B6 expression by 2.9 $\pm$ 0.5-fold and 3.3 + 0.43-fold and CYP3A4 mRNA expression by 3.5 $\pm$ 0.38-fold and 3.6 $\pm$ 0.41-fold, respectively. These results validate our model of primary human hepatocytes culture to study the regulation of CYP2B6 and CYP3A4. Our previous study in a hepatoma-derived cell line showed induction of CYP2B6 expression in response to AICAR (Rencurel et al., 2005). In the present study, using primary human hepatocytes, the two AMPK activators AICAR and metformin (Sabina et al., 1985; Hawley et al., 2002) both induced CYP2B6 and CYP3A4 expression in a dose-dependent manner (Fig. 2, A–C). This indicates that classic AMPK activators increase expression of drug metabolizing enzymes such as CYP2B6 and CYP3A4. In addition, induction of CYP2B6 and CYP3A4 by PB and AICAR is additive suggesting involvement of different mechanism of induction (Fig. 2B-D).

Phenobarbital Activates AMPK in Human Hepatocytes. We then tested whether drug inducers such as PB, CITCO, and rifampicin were able to change the AMPK activity in primary human hepatocytes. Activity was assessed in cells exposed for 1 h to PB, AICAR, CITCO, Rif, and the mouse CAR ligand TCPOBOP (Fig. 3A). As expected, a strong increase of AMPK activity was observed with AICAR, and, most importantly, PB strongly activated AMPK (Fig. 3A) in a concentration dependent manner, the highest activity being reached at 1 mM PB (Fig. 3B). PB was as potent as AICAR. It is noteworthy that no changes in AMPK activity were observed with CITCO (50 and 100 nM), Rif (10 μM), and TCPOBOP (250 nM) (Fig. 3A). Western blot analysis of crude hepatocyte lysates revealed no changes in expression of both AMPK α1 and α2 catalytic subunits after treatment with PB or AICAR (Fig. 3D). To confirm AMPK activation by PB, phosphorylation of acetyl-CoA carboxylase at serine 79 was visualized by Western blot using a phospho-Ser79–specific antibody. An increase in the ACC phosphorylation because of AMPK activation is clearly shown (Fig. 3D). Finally, we investigated whether PB activation of AMPK is associated with a decrease in ATP concentration. A significant decrease by 40% of ATP levels was determined in cells after 1-h exposure to 0.5 mM PB (Fig. 3C). We conclude that activation of AMPK by PB may be mediated by a decrease in ATP concentration. We are presently exploring the mechanism by which PB and PB-like inducers may cause this decrease in ATP.

Pharmacological Inhibition of AMPK Activity by Compound C Lowers PB Induction. To further investigate the functional effects of AMPK activation, we examined the effect of Compound C, a selective AMPK inhibitor, in primary human hepatocytes (Zhou et al., 2001). Compound C (10 and 40 μM) was added to the culture medium 30 min before addition of PB. Both concentrations of compound C tested were able to blunt PB induction of CYP2B6 expression, whereas compound C alone at a concentration of 10 μM induced CYP2B6 (Fig. 4A). This inhibitory effect of Compound C was also observed on PB induction of CYP3A4 expression (Fig. 4B). We thus conclude that inhibition of AMPK activity markedly reduces PB induction.

Dominant-Negative Forms of AMP-Kinase Blunt the PB Response. It was previously shown that a truncation of AMPK at residue 312 yields a peptide that no longer associates with the β and γ subunits but retains significant kinase activity (Crute et al., 1998). Moreover, replacement of threonine 172 within the α subunit by an aspartic acid mimics the effect of phosphorylation at this site (Stein et al., 2000). The AMPK mutants used in the present study provide a constitutively active form of AMPK. By contrast, mutation of aspartate 157, an essential residue for MgATP binding within the α1 subunit to alanine, yields an inactive kinase but does not have any effect on the binding to the β and γ subunits (Stein et al., 2000). Inactive α1 subunit (DNα1) acts as a dominant-negative inhibitor by competing with the native α subunits for binding with β and γ, which is essential for AMPK activity. In primary rat hepatocytes, expression of the inactive α1 subunit was able to inhibit both α1- and α2-containing complexes to a similar extent (Woods et al., 2000). Adenoviral infection of primary human hepatocytes caused more than 50% to express the AMPK mutant constructs as visualized by enhanced green fluorescence protein (Fig. 5D).

Western (immuno)blot analysis confirmed the expression of ad-CA-α1, ad-CA-α2, and ad-DNα1 with anti-c-Myc anti-

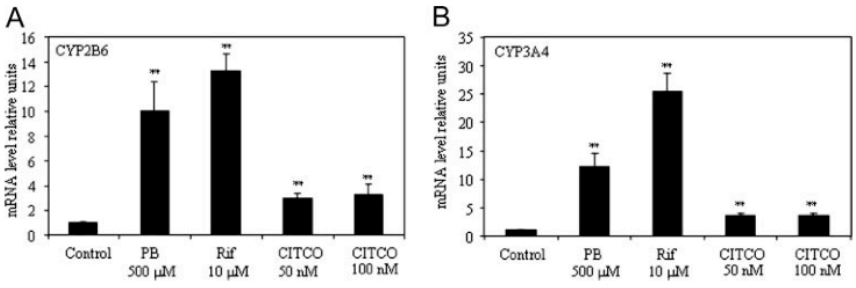

Fig. 1. Regulation of *CYP2B6* and *CYP3A4* in primary human hepatocyte cultures. Human hepatocytes were exposed for 16 h to PB, Rif, or CITCO with the concentrations indicated, and CYP2B6 (A) and CYP3A4 (B) mRNAs were quantified by real-time PCR as described in *Materials and Methods.* Data are expressed as relative units compared with control cells not exposed to any drugs and corrected to 18s rRNA. Results are means of three different donors $\pm$ S.D., with each determination performed in triplicate. (**, $p < 0.01$).

body, which recognizes the Myc epitope tag contained in the three mutants (Fig. 5C). An adenovirus expressing β-galactosidase (ad-βgal) was used as control. Twelve hours after infection, cells were induced with 0.5 mM PB for 16 h and mRNA coding for CYP2B6 quantified by real time RT-PCR. Figure 5, A and B, shows PB induction of CYP2B6 and CYP3A4 expression in ad-βgal infected hepatocytes confirming that adenoviruses do not alter the PB response.

The dominant-negative mutant of α1 AMPK (DN) was able not only to lower the basal expression of CYP2B6 and CYP3A4 genes but also to completely block the PB induction of these two genes. It is noteworthy that the constitutively active mutant, CA-α2, did not significantly change basal levels of the corresponding mRNAs but tended to potentiate the effects of PB effect on CYP2B6 in both donors. More results that are heterogeneous appear with the CA-α1 where no variations in PB induction of CYP2B6 were observed, and even a lower PB induction of CYP2B6 and CYP3A4 was

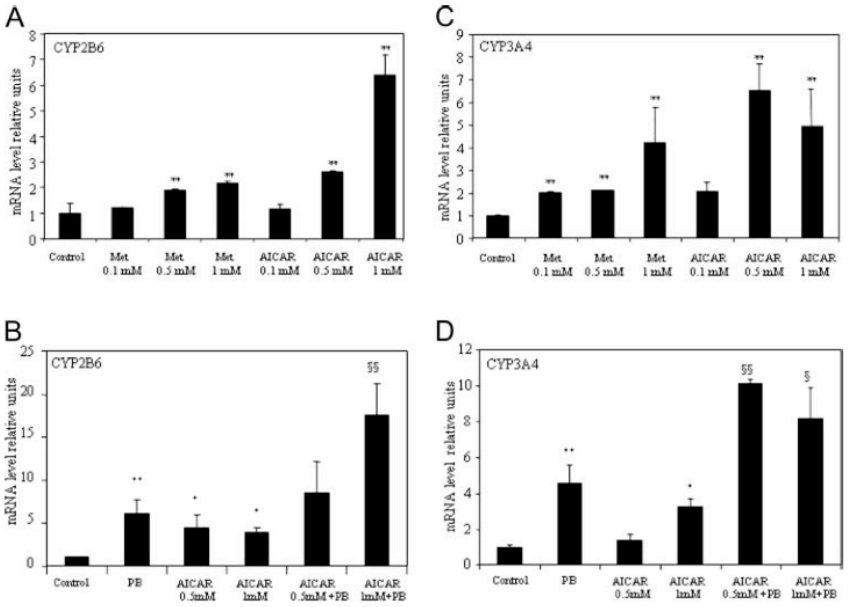

Fig. 2. AICAR and metformin induce *CYP2B6* and *CYP3A4* in primary human hepatocyte cultures. Primary human hepatocytes were exposed for 16 h to different concentrations of metformin (Met) and AICAR as indicated, and *CYP2B6* (A) and *CYP3A4* (C) mRNA were quantified by RT-PCR as described under *Materials and Methods*. Hepatocytes were exposed for 16 h to 500 μM PB alone or in combination with different concentration of AICAR. *CYP2B6* (B) and *CYP3A4* (D) mRNA were quantified by real-time PCR. Results are means of three different donors ± S.D. with each determination done in triplicates. (**, $p < 0.01$). Values obtained with combinations of AICAR and PB treatment are compared with values obtained from cells treated with PB alone (§, $p < 0.05$; §§, $p < 0.01$)

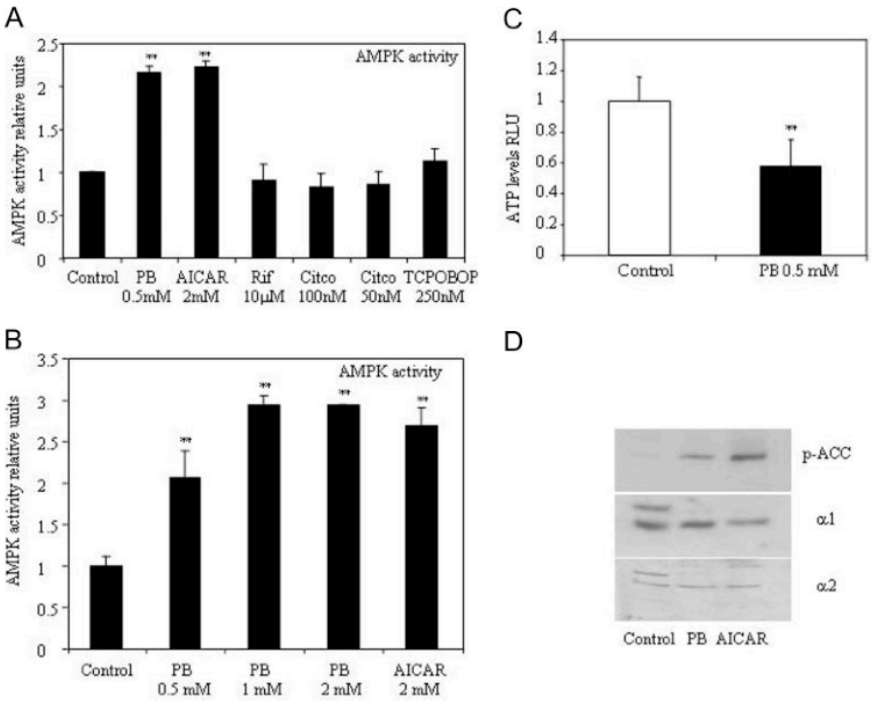

Fig. 3. Phenobarbital activates AMPK in primary human hepatocyte cultures and lowers ATP. Primary human hepatocytes in culture were exposed for 1 h to different concentrations of PB (A and B), AICAR (A and B), Rif (B), CITCO (B), and TCPOBOP (B), as indicated. AMP kinase activity was measured as described under *Materials and Methods*, and results are expressed as -fold stimulation compared with values measured in control cells (Control) not exposed to any drugs. Results are means ± S.D. of three to four different human hepatocyte cultures, and each measurement was performed in triplicate. C, ATP concentration was measured in primary human hepatocytes by luciferase activity as described under *Materials and Methods* after 1-h exposure to PB. Results are expressed as -fold stimulation compared with values obtained in nontreated cells (Control). **, $p < 0.01$. D, human hepatocytes were exposed for 1 h to PB (0.5 mM) or AICAR (2 mM), and expression of AMPK α1, AMPK α2, and phosphorylated acetyl-CoA-carboxylase (p-ACC) was estimated by Western blot using specific polyclonal antibodies.

present in donor 1. These results strongly suggest that AMPK activation is required for PB induction of CYP2B6 and CYP3A4. The specific role played by each AMPK catalytic subunit in such an effect is under further investigation in our laboratory.

Induction of Cyp2b10 and Cyp3a11 Expression by Phenobarbital Depends on AMPK Expression in Mouse Liver and Mouse Hepatocytes. To test further the importance of in vivo AMPK in drug induction, we tested induction of Cyp2b10 and Cyp3a11 by PB and TCPOBOP in AMPK $\alpha1/\alpha2$ liver-specific knockout mice ($\alpha1/\alpha2^{LS-/-}$). As expected, i.p. injection of PB (100 mg/kg body weight) and TCPOBOP (3 mg/kg body weight) in wild-type C57BL/6 mice massively induced Cyp2b10 and Cyp3a11 expression in the liver (Fig. 6B). To our surprise, a strong increase of Cyp2b10 (100-fold) and Cyp3a11 (6-fold) expression occurred in untreated $\alpha1/\alpha2^{LS-/-}$ mice. The PB and TCPOBOP induction of Cyp2b10 and Cyp3a11 was decreased from 155- to 1.54-fold for Cyp2b10 and from 13.6- to 2.42-fold for Cyp3a11 in $\alpha1/\alpha2^{LS-/-}$ knockout mice. To address the question of whether the strong up-regulation of Cyp2b10 and Cyp3A11 observed in vivo in untreated animals is a direct or indirect consequence of the ablation of AMPK catalytic subunits, we chose to perform primary culture of hepatocytes from wild-type and $\alpha1/\alpha2^{LS-/-}$ knockout mice. Primary mouse hepatocytes from wt animals exhibit an induction of Cyp2b10 after exposure to PB (500 μM), TCPOBOP (250 nM), metformin (1 mM), or AICAR (500 μM) (Fig. 6A). Lack of expression of AMPK catalytic subunits $\alpha1$ and $\alpha2$ in primary mouse hepatocytes, resulted in the abolition of Cyp2b10 induction by either PB, TCPOBOP (TCP), metformin (Metf), or AICAR (Fig. 6A). It is noteworthy that contrary to the in vivo situation, the basal expression of Cyp2b10 and Cyp3a11 was not higher in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice compared with hepatocytes from wt mice. This implies a role of circulating factors responsible of high basal *Cyp2b10* and *Cyp3a11* gene expression observed in vivo. Moreover, these results provide further evidence for the important role of AMPK in Cyp2b10 and Cyp3a11 induction by PB AICAR and TCPOBOP.

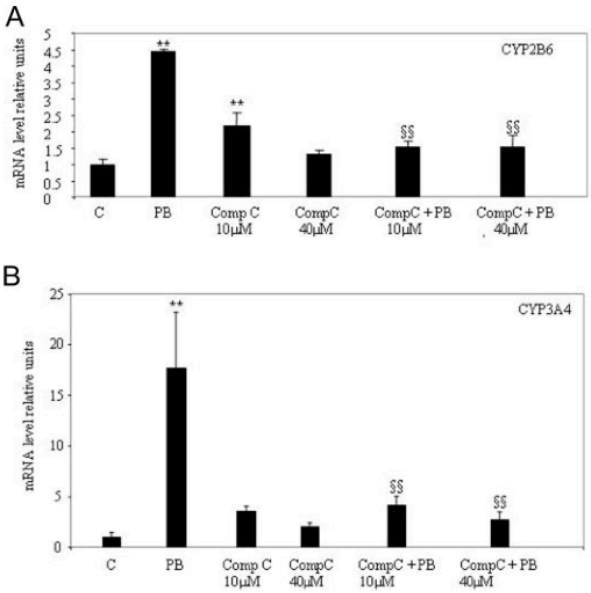

Fig. 4. Pharmacological inhibition of AMPK activity with compound C lowers phenobarbital induction of *CYP2B6* and *CYP3A4* in human hepatocytes. Hepatocytes were exposed for 16 h to 500 μM PB or compound C (10–40 μM). Compound C was added to cells 30 min before addition of PB when both chemicals were used in combination (Comp C+PB). CYP2B6 (A) and CYP3A4 (B) mRNA were quantified by real-time PCR and are expressed as relative units corrected to 18s rRNA. Results represent cultures from two different donors (mean ± S.D.) with each determination done in triplicate. Comparison with values in control cells (C) not exposed to drugs (**, $p < 0.01$). Comparison with values in cells exposed to PB (§§, $p < 0.01$)

The Absence of AMPK Catalytic Subunits Has No Effect on CAR Cytoplasmic-Nuclear Transfer Induced by PB. The capacity of PB to trigger CAR cytoplasmic/nuclear transfer was tested in primary mouse hepatocytes expressing the human CAR in fusion with enhanced GFP. After 6-h exposure to PB, CAR was clearly located predominantly in the nuclei of hepatocytes from wild-type mice. An apparent staining close to the plasma membrane and/or cytoskeleton network was also observed in untreated (Control) and PB-treated hepatocytes from both wild-type and $\alpha1/\alpha2^{LS-/-}$ mice (Fig. 7). Unexpectedly, the cytoplasmic/nuclear shuttling of CAR upon PB treatment was not altered by the absence of AMPK $\alpha1$ and $\alpha2$ catalytic subunits. However, we noted that CAR-GFP fluorescence was located in condensed region of the nucleus in $\alpha1/\alpha2^{LS-/-}$ mice hepatocytes after PB treatment, a phenomenon not observed in hepatocytes from wild-type mice. It is therefore unlikely that the deficiency in the induction of Cyp2b10 and Cyp3a11 by PB observed in $\alpha1/\alpha2^{LS-/-}$ mouse hepatocytes is caused by an alteration in CAR translocation into the nucleus.

Discussion

Despite numerous attempts, the molecular mechanism by which PB exerts its effects on gene expression are still incompletely understood, and no intracellular protein target of PB has been identified. We propose here a new mechanism of drug induction, activation of AMPK, which may ultimately explain some of the diverse effects of PB in human and mouse hepatocytes such as its effect on the transcription of cytochrome P450 genes. We have reported previously that pharmacological activation of AMPK leads to induction of CYP2B6 in a hepatoma-derived cell line (WGA) (Rencurel et al., 2005). However, concentrations of PB above 1 mM were required for CYP2B6 induction and AMPK activation in WGA cells; these concentrations are associated with toxic effects in normal hepatocytes. Thus, the importance of this mechanism in normal hepatocytes was questioned. We therefore chose two experimental systems, primary culture of human hepatocytes and cultures of hepatocytes from AMPK $\alpha1/\alpha2$ liver-specific KO mice ($\alpha1/\alpha2^{LS-/-}$) to investigate the role of AMPK in drug induction. We validated our human hepatocyte culture system by testing classic inducers on *CYP2B6* and *CYP3A4* gene expression and observed robust and reproducible, dose-dependent induction of CYP2B6 and CYP3A4 when cells were exposed to PB and rifampicin for 16 h. It is noteworthy that, compared with PB, CITCO, a human CAR agonist (Maglich et al., 2003), was not a potent CYP2B6 inducer . This could be due to the shorter time of exposure than those described by Maglich et al. (2003) and to its partially different mode of action (i.e., direct activation of CAR) (Maglich et al., 2003).

The two AMPK activators, AICAR and metformin, induced CYP2B6 and CYP3A4 expression in a dose-dependent manner in primary human hepatocytes, a hallmark of drug induction. An additive effect of PB and AICAR on CYP2B6 expression was observed, suggesting the involvement of distinct mechanism of action of these two drugs. AICAR is the most commonly used method of activating AMPK; recently, however, Guigas et al. (2006) highlighted an AICAR effect independent of AMPK activation on glucose phosphorylation. To clearly distinguish AMPK-dependent and AMPK-independent effects on cytochrome P450 induction, we turned to primary hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice. In these hepatocytes, no induction of Cyp2b10 was observed with PB, TCPOBOP, metformin, or AICAR, demonstrating the essential role of AMPK in the induction of CYP2b10. However, minor induction of Cyp3a11 by AICAR was still present in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice, perhaps reflecting the existence of an AMPK-independent mechanism by which AICAR regulates this gene, possibly acting as a ligand of pregnane X receptor.

The activation of AMPK by phenobarbital is unique in the sense that other drug inducers tested so far, such as the CAR ligand/activator CITCO, the mouse CAR ligand/activator TCPOBOP, and the pregnane X receptor ligand/activator rifampicin did not change AMPK activity in human hepatocytes. This suggests that PB and PB-like inducers affect transcription of cytochrome P450 genes by a unique mechanism.

The classic view of AMPK activation is a decrease in cellular energy charge as reflected by the increase in the AMP/ATP ratio. PB is able to significantly lower ATP concentration in hepatocytes within an hour, a finding in agreement

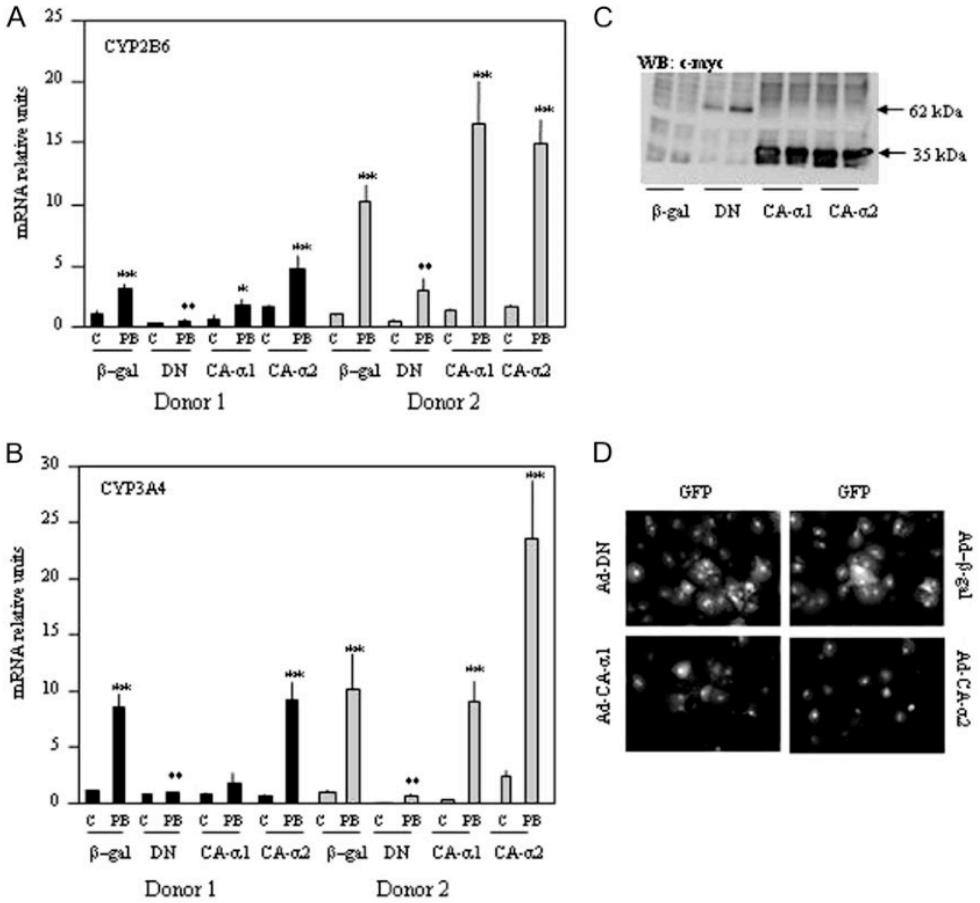

Fig. 5. Exogenous expression of dominant-negative and constitutively active mutants of AMPK in primary human hepatocytes affects phenobarbital induction. Primary human hepatocytes were infected with adenovirus containing β-galactosidase (β-gal), AMPK $\alpha1$ dominant-negative mutant (DN), AMPK constitutively active $\alpha1$ or $\alpha2$ subunits (CA-$\alpha1$; CA-$\alpha2$). Twenty-four hours after infection, cells were exposed for 16 h to 0.5 mM PB. Expression of *CYP2B6* (A) and *CYP3A4* (B) was quantified by real-time PCR, and results were expressed as -fold stimulation compared with cells infected with ad-βgal (β-gal) and not exposed to drug (C = controls). Results are expressed as relative units corrected to 18s rRNA comparison with values in control cells (C) not exposed to drugs and infected with ad-βgal (β-gal) (*, $p < 0.05$; **, $p < 0.01$). C, gene expression in primary human hepatocytes was tested by Western blot. Twenty-four hours after infection, cells were lysed, and 30 μg of total cell lysate was separated in 10% acrylamide gel. The Myc tag of AMPK mutants was detected using a specific monoclonal antibody. The 62-kDa band corresponds to the full-length $\alpha1$ dominant-negative mutant (DN) and the 35-kDa band to the truncated constitutive active $\alpha1$ and $\alpha2$ mutants (CA-$\alpha1$ and CA-$\alpha2$) as described under *Results*. D, the efficiency of infection was estimated by immunocytochemistry in primary human hepatocytes 24 h after infection. Each adenoviral construct expressed GFP under the control of a cytomegalovirus promoter as described under *Materials and Methods*.

with our previous study in hepatoma cells (Rencurel et al., 2005). The lowering effect of PB on ATP in hepatoma cells was observed only at high concentrations (above 1 mM), a level also required for CYP2B6 induction in these cells (Rencurel et al., 2005). The capacity of PB to induce CYP2B6 expression may thus be related to its efficiency to lower ATP and consequently to activate AMPK. Another interesting observation comes from the CYP2B6 induction by metformin in primary human hepatocytes. The biguanide metformin (N^1,N^1-dimethylbiguanide) is prescribed to lower fasting blood glucose in patients with non–insulin-dependent diabetes mellitus, yet its primary site of action is still uncertain. Shaw et al. (2005) described the inhibitory effect of metformin on hepatic glucose production to be dependent on the expression of LKB1 kinase. Experiments in LKB1$^{-/-}$ mice have indeed established that LKB1 is the principal AMPK kinase in the liver (Shaw et al., 2005). Although the molecular mechanisms through which metformin affects cellular energy homeostasis remain disputed, it seems increasingly likely that metformin may act, at least in large part, by inhibiting respiratory chain complex 1 (Owen et al., 2000), hence suppressing mitochondrial ATP synthesis. It is not yet known whether PB activates AMPK via a similar mechanism in the liver, but if so, this could explain the known blood glucose-lowering effect of PB in patients with non–insulin-dependent diabetes mellitus (Sotaniemi and Karvonen, 1989).

The most compelling evidence for the role of AMPK in PB-type induction is reflected in our experiments, which demonstrate that liver-specific deletion of the AMPKα subunit genes in the mouse abolishes the drug induction of CYP2b10 and CYP3a11. It is surprising that markedly higher basal levels of the mRNA of these genes were observed. By contrast, no increase of Cyp2b10 and Cyp3a11 basal expression was observed in primary hepatocytes cultured from the livers of $\alpha1/\alpha2^{LS-/-}$ mice. Such a discrepancy between in vivo and in vitro findings suggests that circulating factors in response to the metabolic changes in the liver might have caused the increased basal expression of Cyp2b10. The streptozotocin-induced diabetic mouse model exhibits a high Cyp2b10 basal expression level that was corrected by insulin treatment to lower hyperglycemia (Sakuma et al., 2001). Guigas et al. (2006) observed a low glucokinase expression in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mouse, which is associated with low glucose phosphorylation and low glucose uptake. A low rate of glucose phosphorylation was described earlier in primary rat hepatocytes from alloxan-induced diabetic rats (Bontemps et al., 1978) and in fasted animals, where Cyp2b10 and CAR expression is induced (Maglich et al., 2004). We therefore speculate that a circulating factor related to the low carbohydrate turnover in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ could be responsible for the high basal expression of Cyp2b10, and we are currently testing this hypothesis.

The blunted induction of Cyp2b10 and CYP3a11 by PB in hepatocyte from $\alpha1/\alpha2^{LS-/-}$ mice was not related to an alteration of CAR nuclear translocation upon drug treatment. It is well documented that CAR translocates into the nucleus upon drug treatment to form an active transcriptional complex with the retinoid X receptor (Zelko et al., 2001). More-

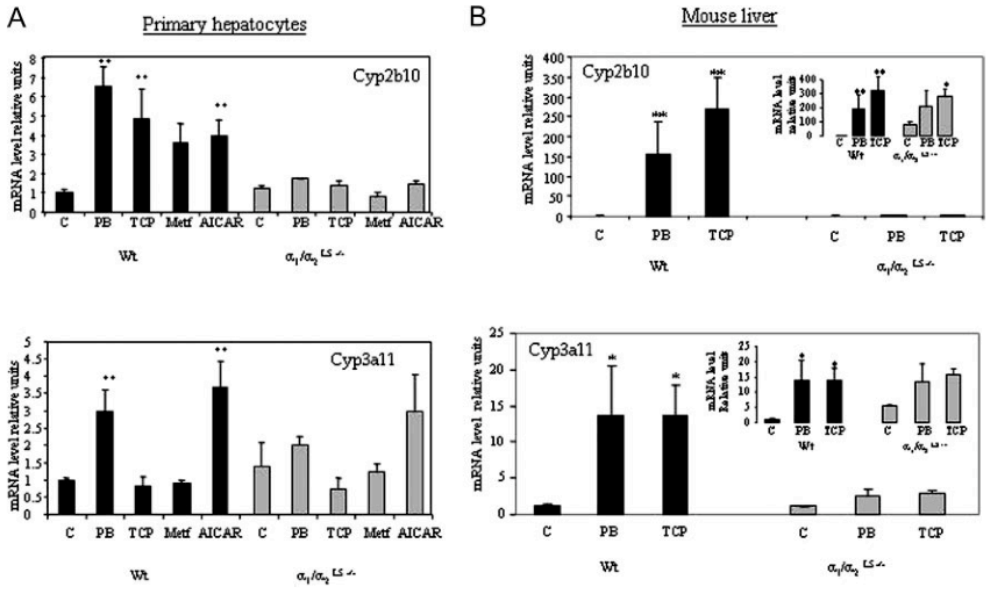

Fig. 6. Liver-specific deletion of AMPK $\alpha1$ and $\alpha2$ subunits in mouse impairs induction of *Cyp2b10* and *Cyp3a11* in vivo and in vitro. Primary hepatocytes from wild type (wt, black bars) mice or from mice with liver-specific deficiencies of AMPK catalytic subunit $\alpha1$ and $\alpha2$ ($\alpha_1/\alpha_2^{LS-/-}$, gray bars) were exposed for 16 h to 500 μM PB, 500 μM TCPOBOP (TCP), 1 mM metformin (Metf) or 500 μM AICAR. Expression of *Cyp2b10* (A, top) and Cyp3a11 (A, bottom) was quantified by real-time PCR, and results were expressed as -fold stimulation compared with non–drug-exposed cells (C = controls) from wild-type mice (wt). Results represent two different cell preparations. (**, $p < 0.01$ unpaired Student's t test). Mice in which AMPK $\alpha1$ and $\alpha2$ were deleted in liver ($\alpha_1/\alpha_2^{LS-/-}$, gray bars) and wild-type mice (wt, black bars) were injected i.p. with saline solution (C = controls), PB or TCPOBOP (TCP) and sacrificed 16 h later as described under *Materials and Methods Cyp2b10* (B, top) and *Cyp3a11* (B, bottom) expression was quantified by RT-PCR and standardized to GAPDH. Results are expressed as -fold stimulation compared with values obtained in saline-injected animals (C = controls) of each group of Wt and $\alpha_1/\alpha_2^{LS-/-}$ mice. The inset results are expressed as -fold induction compared with values obtained in saline-injected (C) wild-type animals (wt). Results are expressed as means ± S.D., *, $p < 0.05$; **, $p < 0.01$ determined by unpaired Student's t test.

over, the translocation and activation of CAR is influenced by phosphorylation/dephosphorylation events (Kawamoto et al., 1999; Yoshinari et al., 2003). For instance, the protein phosphatase 2A inhibitor okadaic acid blocks CAR translocation in mouse hepatocytes exposed to either PB or TCPOBOP (Kawamoto et al., 1999). In addition, okadaic acid induces expression of CYP2B6 in HepG2 cells that were engineered to express the mouse CAR isoform. Again in HepG2 cells, residue serine-202 of mouse CAR was shown to be phosphorylated, and this modification was shown to be important for the retention of CAR in the cytoplasm (Hosseinpour et al., 2005). On the other hand, despite this preliminary evidence that the serine-202 dephosphorylation of mCAR by protein phosphatase 2A affects the cytoplasmic-nuclear transfer of CAR, the identity of the kinase responsible of serine-202 phosphorylation is unknown (Hosseinpour et al., 2005). Our own experiments suggest that AMPK does not phosphorylate serine-202 of mouse CAR or the corresponding serine-192 of human CAR (M. Matis and F. Rencurel, unpublished observation). In hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice, hCAR-GFP was located in condensed regions of the nucleus, whereas a more homogenous staining was observed in nucleus of wild-type mice hepatocytes after PB treatment. These condensed regions are comparable with nuclear speckles described previously in COS-7 cells in which CAR and the cofactor peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) were coexpressed {Shiraki, 2003 #117}. Nuclear speckles contain proteins involved in pre-mRNA splicing but also several kinases (e.g., CLK/STI, PRP4) and phosphatases (e.g., protein phosphatase 1) which can phosphorylate/dephosphorylate components of the splicing machinery (Handwerger and Gall, 2006). Although little or no transcription takes place in nuclear speckles, a set of proteins involved in transcriptional regulation is associated with speckles from where they are shuttled to transcription sites. PB induction of Cyp2b10 was not altered in PGC1α liver-specific knockout mice (Handschin et al., 2005). If and how PGC1α interacts with CAR thus remains an open question. A sequestration of CAR in or near nuclear speckles may control its activity by keeping the nuclear receptor removed from or near the transcription sites. Our data therefore suggest the existence of another control step of CAR signaling independent of CAR intracellular location.

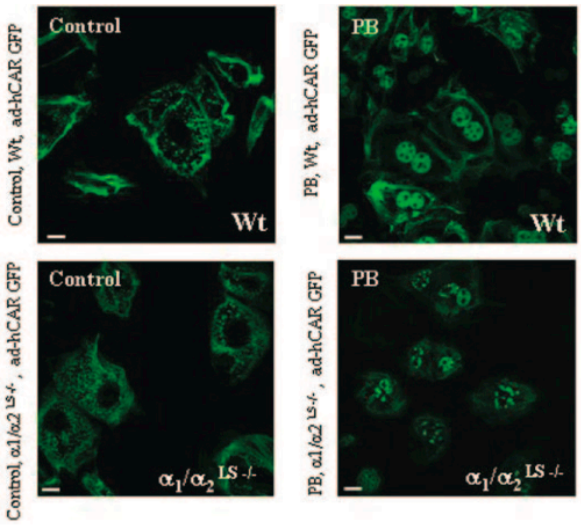

Fig. 7. Human CAR cytoplasmic/nuclear transfer induced by phenobarbital is not altered by the deletion of AMPK $\alpha1$ and $\alpha2$ catalytic subunits in primary mouse hepatocytes. Primary hepatocytes from wild-type (Wt) mice or from mice in which the AMPK catalytic subunits $\alpha1$ and $\alpha2$ were deleted in the liver ($\alpha_1/\alpha_2^{LS-/-}$) were infected with adenovirus coding for human CAR in fusion with enhanced green fluorescence protein (ad-hCAR-GFP) as described under *Materials and Methods* Twelve hours after infection, cells were exposed to PB for 6 h; control mice were not exposed to any drugs. Human CAR-GFP was visualized in cells in Mowiol mounting medium with a 40× objective (1.32 numerical aperture) by using a Leica TCS NT confocal laser scanning microscope. White scale bar, 10 μm.

In conclusion, we demonstrate an essential role of AMPK in induction of CYP2B6 and CYP3A4 by PB in human hepatocytes and of CYP2b10 and CYP3a11 in mouse hepatocytes. This highlights yet another interesting effect of this anticonvulsant and hypnotic drug that also has antidiabetic properties. The role of AMPK in drug induction obviously needs further investigation, and its role in the control of CAR activity is of particular interest. Because AMP-activated kinase is a target for the development of drugs for type II diabetes and obesity, its role in the induction of drug metabolism deserves close attention.

Acknowledgments

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References

Berry MN and Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* **43:**506–520.

Bontemps F, Hue L, and Hers H (1978) Phosphorylation of glucose in isolated rat hepatocytes. Sigmoidal kinetics explained by the activity of glucokinase alone. *Biochem J* **174:**603–611.

Carling D (2004) The AMP-activated protein kinase cascade—a unifying system for energy control. *Trends Biochem Sci* **29:**18–24.

Crute BE, Seefeld K, Gamble J, Kemp BE, and Witters LA (1998) Functional domains of the $\alpha1$ catalytic subunit of the AMP-activated protein kinase. *J Biol Chem* **273:**35347–35354.

Diraison F, Parton L, Ferre P, Foufelle F, Briscoe C, Leclerc I, and Rutter G (2004) Overexpression of sterol-regulatory-element-binding protein-1c (SREBP1c) in rat pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminoimidazole-4-carboxamide ribonucleoside AICAR. *Biochem J* **378:**769–778.

Foretz M, Ancellin N, Andreelli F, Saintillan Y, Grondin P, Kahn A, Thorens B, Vaulont S, and Viollet B (2005) Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* **54:**1331–1339.

Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D, Andreelli F, Viollet B, and Hue L (2006) 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. *Diabetes* **55:**865–874.

Handschin C, Lin J, Rhee J, Peyer A-K, Chin S, Wu P-H, Meyer UA, and Spiegelman BM (2005) Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1α. *Cell* **122:**505–515.

Handwerger KE and Gall JG (2006) Subnuclear organelles: new insights into form and function. *Trends Cell Biol* **16:**19–26.

Hawley SA, Gadalla AE, Olsen GS, and Hardie DG (2002) The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51:**2420–2425.

Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor car-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18:**5652–5658.

Hosseinpour F, Moore R, Negishi M, and Sueyoshi T (2005) Serine-202 regulates the nuclear translocation of constitutive active/androstane receptor CAR. *Mol Pharmacol* **69:**1095–1102.

Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, and Wojtaszewski JFP (2004) Knockout of the $\alpha2$ but not $\alpha1$ 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* **279:**1070–1079.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor car in induction of the CYP2B gene. *Mol Cell Biol* **19:**6318–6322.

Kobayashi K, Sueyoshi T, Inoue K, Moore R, and Negishi M (2003) Cytoplasmic

accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. *Mol Pharmacol* **64:**1069–1075.
Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM, et al. (2003) Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278:**17277–17283.
Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, and Moore JT (2004) The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. *J Biol Chem* **279:**19832–19838.
Owen MR, Doran E, and Halestrap AP (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* **348:**607–614.
Rencurel F, Stenhouse A, Hawley SA, Friedberg T, Hardie DG, Sutherland C, and Wolf CR (2005) AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line. *J Biol Chem* **280:**4367–4373.
Sabina R, Patterson D, and Holmes E (1985) 5-Amino-4-imidazolecarboxamide riboside (Z-riboside) metabolism in eukaryotic cells. *J Biol Chem* **260:**6107–6114.
Sakuma T, Honma R, Maguchi S, Tamaki H, and NN (2001) Different expression of hepatic and renal cytochrome P450s between the streptozotocin-induced diabetic mouse and rat. *Xenobiotica* **4:**223–237.
Shaw RJ, Lamia KA, Vasquez D, Koo S-H, Bardeesy N, DePinho RA, Montminy M, and Cantley LC (2005) The Kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science (Wash DC)* **310:**1642–1646.
Shiraki T, Sakai N, Kanaya E, and Jingami H (2003) Activation of orphan nuclear constitutive androstane receptor requires subnuclear targeting by peroxisome proliferator-activated receptor γ coactivator 1α. A possible link between xenobiotic response and nutritional state. *J Biol Chem* **278:**11344–11350.
Sotaniemi EA and Karvonen I (1989) Glucose tolerance and insulin response to glucose load before and after enzyme inducing therapy in subjects with glucose intolerance and patients with NIDDM having hyperinsulinemia or relative insulin deficiency. *Diabetes Res* **3:**131–139.
Stein SC, Woods A, Jones NA, Davison MD, and Carling D (2000) The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J* **345:**437–443.
Strain A (1994) Isolated hepatocytes: use in experimental and clinical hepatology. *Gut* **4:**433–436.
Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274:**6043–6046.
Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloen A, Flamez D, Mu J, Lenzner C, Baud O, Bennoun M, et al. (2003) The AMP-activated protein kinase α2 catalytic subunit controls whole-body insulin sensitivity. *J Clin Investig* **111:**91–98.
Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferre P, Foufelle F, and Carling D (2000) Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol* **20:**6704–6711.
Woods A, Cheung PCF, Smith FC, Davison MD, Scott J, Beri RK, and Carling D (1996) Characterization of AMP-activated protein kinase β and γ subunits. *J Biol Chem* **271:**10282–10290.
Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LGD, Neumann D, Schlattner U, Wallimann T, Carlson M, and Carling D (2003) LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13:**2004–2008.
Yoshinari K, Kobayashi K, Moore R, Kawamoto T, and Negishi M (2003) Identification of the nuclear receptor CAR: HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Lett* **548:**17–20.
Zelko I, Sueyoshi T, Kawamoto T, Moore R, and Negishi M (2001) The peptide near the C terminus regulates receptor car nuclear translocation induced by xenochemicals in mouse liver. *Mol Cell Biol* **21:**2838–2846.
Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Investig* **108:**1167–1174.

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Regulation of CYP3A4 by the bile acid receptor FXR: evidence for functional binding sites in the *CYP3A4* gene

Carmela Gnerre, Sharon Blättler, Michel R. Kaufmann, Renate Looser and Urs A. Meyer

CYP3A4, the most abundant cytochrome P450 in human liver, is responsible for the metabolism of numerous xenobiotics and endobiotics. CYP3A4 expression is highly variable and is induced by numerous compounds of exogenous and endogenous origin, including elevated concentrations of secondary bile acids via the pregnane X receptor (PXR). We show that physiological concentrations of the primary bile acid chenodeoxycholic acid regulate the expression of CYP3A4 via the bile acid receptor FXR. Experiments performed *in vitro* in different cell culture systems, gel-mobility shift assays and experiments performed *in vivo* in transgenic mice lacking FXR or PXR and treated with the synthetic FXR agonist GW4064 were undertaken to study the implication of FXR in the regulation of CYP3A. Our data provide evidence for the presence of two functional FXR recognition sites located in a 345-bp element within the 5′-flanking region of CYP3A4. Mutational analysis of these sites and experiments in transgenic mice lacking FXR or PXR support the relevance of FXR activation for CYP3A regulation. Thus, whereas elevated concentrations of precursors of bile acids and secondary bile acids induce CYP3A via PXR, primary bile acids can modulate the expression of CYP3A via FXR. These findings may explain elevated CYP3A expression in cholestasis and part of the variability of drug responsiveness and toxicity between individuals.
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Introduction

Cytochromes P450 (P450s) play important roles in the synthesis and degradation of endogenous substances, including steroids hormones, cholesterol or bile acids [1,2] and are essential in eliminating drugs and xenobiotics. Of particular importance for drug metabolism are P450s of the 3A family. It is estimated that more than 50% of clinically used drugs are metabolized, at least in part, by CYP3A4, the predominant human 3A isoform [3]. CYP3A is expressed in several tissues, but is most abundant in the liver and intestine [4].

The expression of CYP3A4 is induced by a variety of drug substrates and other compounds [5], and this has important clinical consequences, affecting the efficacy of drug treatment and causing drug–drug interactions [6]. Moreover, the expression of CYP3A4 in individuals without exposure to inducer drugs also is highly variable. The interindividual variability in the ability to clear a drug by CYP3A4 has been estimated to be between five- and 20-fold [7].

As for many other P450 subfamilies, the transcriptional regulation of *CYP3A4* involves the nuclear hormone receptor family [8]. The pregnane X receptor (PXR) [9,10] and the constitutive androstane receptor (CAR) [11] were shown to mediate activation of CYP3A4 in response to inducer drugs. More recently, the vitamin D receptor (VDR) was also implicated in the regulation of *CYP3A4* gene expression [12]. In addition, HNF4α was identified as an important regulator of the CAR- and PXR-mediated response of CYP3A4 to xenobiotics [13].

Recent studies have demonstrated that bile acids, apart from their role in lipid-soluble and dietary fat absorption, also serve as signalling molecules that activate nuclear receptors and play an important role in the regulation of cholesterol homeostasis [14]. Moreover, bile acids have been identified as endogenous ligands for the bile acid receptor FXR. The hydrophobic bile acid chenodeoxycholic acid (CDCA) activates FXR with the highest potency, followed by the secondary bile acid lithocholic acid (LCA) and deoxycholic acid, while cholic acid is a less potent activator [15]. FXR has been reported to activate gene transcription by dimerizing with the retinoid X receptor (RXR) and binding to response elements found in the 5′-flanking

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regions of target genes [16,17]. Primary bile acids have also been identified as ligands for PXR, but unlike FXR, PXR is not as efficiently activated by CDCA or cholic acid [18].

Indirect evidence suggests that CYP3A induction occurs in response to cholestasis: increased excretion of hyodeoxycholic acid, α-hyocholic acid, the 6α-hydroxylated metabolites of CDCA, occurs in women with cholestasis of pregnancy and in cholestatic patients [19,20]. CYP3A can metabolize bile acids, suggesting that bile acids may positively regulate CYP3A expression to initiate their catabolism when they are in excess. In accordance with this concept, two groups recently demonstrated that the secondary toxic bile acid LCA activates murine *Cyp3a11* transcription via the nuclear receptor PXR [21,22]. Interestingly, Xie *et al.* [22] found *Cyp3a11* induction by LCA also in PXR deficient mice. Moreover, LCA also induces CYP3A expression via activation of VDR [23]. Experiments in a transgenic mouse model of cholestasis incorporating a regulatory human *CYP3A4* transgene provide evidence that adaptive up-regulation of both endogenous and introduced human *CYP3A* genes occurs in response to bile acid accumulation, and that this response is not dependant on increases in circulating LCA [24]. Moreover, GW4064, a selective FXR agonist [25] induced *CYP3A23* mRNA in rat hepatocytes [26], and wild-type mice fed with a diet containing 1% cholic acid had increased Cyp3a11 mRNA in the absence of PXR activation [27,28]. From these experiments, the existence of PXR-independent alternative regulatory mechanisms of CYP3A expression was deduced. We therefore designed experiments performed *in vitro* to analyse the role of FXR in the regulation of CYP3A4. In addition, we report experiments performed *in vivo* in transgenic mice lacking FXR or PXR and treated with the synthetic FXR agonist GW4064.

Methods

Reagents

CDCA was purchased from Sigma (distributed by Fluka AG, Switzerland). GW4064 was kindly provided by Dr T. M. Willson (GlaxoSmithKline, Research Triangle Park, North Carolina, USA).

Plasmids

Reverse-transcribed mRNA from human Huh-7 hepatoma cells cDNA was used in polymerase chain reactions (PCR) reactions to amplifiy the human FXRα isoform (hFXR), which was further cloned into the pSG5 expression plasmid (Stratagene, La Jolla, California, USA). The expression plasmids for human RXRα, human PXR, β-galactosidase, and the monoclonal anti-mouse-RXR rabbit antibody have been described previously [29]. A VP16/hFXR construct was generated by amplification of FXR from pSG5-hFXR and insertion into a pcDNA3/VP16 vector provided by Dr D. Kessler Biozentrum (University of Basel, Switzerland). A pGL3-basic plasmid containing 13 kb of human CYP3A4 5′-flanking region in the context of the natural CYP3A4 promoter was a gift of Dr C. Liddle (University of Sidney at Westmead Hospital, Westmead, Australia). The 13 kb of CYP3A4 5′-flanking region were subfragmented using PCR and standard subcloning procedures. The fragments obtained were cloned into the pGL3-basic plasmid containing the CYP3A4 promoter region from bases −362 to +53. All PCR products were verified by sequencing.

Site-directed mutagenesis

The 345-bp sequence (bases −7836 to −7491) was examined for putative nuclear receptor binding sites using the NUBIScan algorithm [30]. Mutations in the putative binding sites were introduced by PCR using standard overlap techniques [29]. At least five nucleotides per half site were modified. Mutated fragments were generated in the XREM construct (bases −7836 to −7206) containing the CYP3A4 promoter.

Culture of HepG2 cells and RNA isolation

HepG2 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Three days before induction, cells were maintained in DMEM-F12 without phenol red supplemented with 10% charcoal-treated FBS and were plated in six-well dishes. Cells were then exposed to vehicle (DMSO 0.1%), GW4064 (0.1, 1 and 10 μM) or CDCA (10 and 100 μM) for 24 h in DMEM-F12 without phenol red containing 10% of delipidated charcoal stripped FCS (Sigma). Total RNA was isolated from HepG2 cells with the Trizol TM reagent (Invitrogen Co., Carlsbad, California, USA).

Transcriptional activation assays

Transactivation assays were carried out in CV-1 (monkey kidney) cells as previously described [29]. Briefly, cells were expanded for 3 days in DMEM-F12 without phenol red and supplemented with 10% charcoal-treated FBS. Subsequently, cells were plated onto 12-well dishes at a density of 250 000 cells per well and grown overnight. For transfection, cells were maintained in OptiMemI (Invitrogen). Transfection mixes contained 60 ng receptor expression vector, 300 ng reporter vector, 100 ng pRSV-βGal and carrier plasmid to a total of 0.7 μg DNA per well. Cells were transiently transfected using the LipofectAMINE reagent (Invitrogen) according to the manufacturer's protocol. After 24 h of incubation, cells were exposed to drugs or vehicle. After 24 h, cell extracts were prepared using 300 μl of passive lysis buffer (Promega, Corp., Catalys AG, Wallisellen, Switzerland) and the supernatants were assayed for luciferase activities using the luciferase assay kit (Promega) and a Wallac 1420 Multilabel

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Counter. β-galactosidase activities were measured as previously described [31]. Luciferase levels were then normalized against β-galactosidase values to compensate for variation in transfection efficiency. The relative luciferase activity was standardized against untreated control cells. Transcriptional activation assays in HepG2 cells were performed as follows: Cells were kept in DMEM-F12 medium without phenol red, supplemented with 10% charcoal-treated FBS. Cells were plated at a density of 300 000 cells per well in 12-well cell culture dishes. Cells in each well were transfected with 80 ng of pcDNA3Vp16 or VP16/hFXR expression vector, 80 ng of RXRα expression vector, 300 ng of reporter vector, 300 ng of pRSV-βGal, and 240 ng of carrier with FuGene 6 reagent. Cells were harvested after 48 h and reporter gene activities were measured and analysed as described above.

Maintenance and treatment of wild-type, PXR$^{-/-}$ and FXR$^{-/-}$ animals

Wild-type female C57BL6 mice were obtained from Iffa Credo Laboratories (Strasbourg, France). *PXR*$^{+/-}$ mice [21] were obtained from a colony maintained at the Biozentrum, which was started with animals kindly provided by Dr S. A. Kliewer and GlaxoSmithKline, Research Triangle Park (North Carolina, USA). *FXR*$^{-/-}$ mice [32] were kindly provided by Dr F. J. Gonzalez, Laboratory of Metabolism, National Institute of Health (Bethesda, Maryland, USA). All institutional guidelines for animal care and use were applied in this study. Female *PXR*$^{-/-}$, *FXR*$^{-/-}$ and wild-type animals were maintained on standard laboratory chow and were allowed food and water *ad libitum*. Ten to 16-week-old female mice ($n = 5–8$) were injected i.p. with vehicle alone (corn oil with 5% DMSO), GW4064 20 mg/kg or 40 mg/kg. After 16 h, animals were killed, liver tissue samples solubilized in 1 ml TRIzol TM reagent (Invitrogen) and total RNA was extracted.

Taqman analysis

Five μg of HepG2 total RNA or 1 μg of mouse liver total RNA were reverse transcribed with the Moloney murine leukaemia virus reverse transcriptase (Promega) using either random hexamers p(dN)6 (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) or oligo-(dT)15 N primers, respectively. PCR was performed with the qPCR TM Mastermix Plus (Eurogentec GmbH, Köln, Germany). Primers and probes were optimized as follows: human CYP3A4: probe, 5′-FAMCGA GGC GAC TTT CTT TCA TCC TTT TTA CAG ATT TTC-TAMRA-3′ (200 nM); forward primer, 5′-CAT TCC TCA TCC CAA TTC TTG AAG T-3′ (900 nM); reverse primer 5′-CCA CTC GGT GCT TTT GTG TAT CT-3′ (300 nM); 18S: probe, 5′-FAMCGC CCG TCG CTA CTA CCG ATT GG-TAMRA-3′ (300 nM); forward primer, 5′-AGT CCC TGC CCT TTG TAC ACA-3′ (50 nM); reverse primer 5′-CGA TCC GAG GGC CTC ACT A-3′ (300 nM); mouse Cyp3a11: probe, 5′-FAM-CTA AAG GTT GTG CCA CGG GAT GCA GT-TAMRA-3′ (300 nM); forward primer, 5′-AGA ACT TCT CCT TCC AGC CTT GTA-3′ (900 nM); reverse primer 5′-GAG GGA GAC TCA TGC TCC AGT TA-3′ (900 nM); mouse Cyp7a1: probe, 5′-FAM-CAG TCC CGG GCA GGC TTG GGTAMRA-3′ (300 nM); forward primer, 5′-ACA CCA AGT GTC CCC CTC TAG A-3′ (900 nM); reverse primer 5′-CTC AAT ATC ATT TAG TGG TGG CAA A-3′ (900 nM); mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH): probe, 5′-FAM-CCG CCT GGA GAA ACC TGC CAA GTA TG-TAMRA-3′ (300 nM); forward primer, 5′-CCA GAA CAT CAT CCC TGC ATC-3′ (900 nM); reverse primer 5′-GGT CCT CAG TGT AGC CCA AGA T-3′ (900 nM). Transcript levels were quantified with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Boston, Massachusetts, USA) according to the manufacturer's protocol. Briefly, relative transcripts levels in induced livers or cells and untreated controls were determined using the relative quantification method measuring ΔΔCt. The levels of *GAPDH* and *18S* were used for the normalization of the mouse and human genes, respectively.

Electromobility shift assays

The 345-bp fragment labelling and the human RXRα, FXR and PXR proteins were prepared as described previously [29]. The oligonucleotide sequences used for the binding with the individual sites were optimized as follows: IR-1, forward primer 5′-GAT CTC AGC TGA ATG AAC TTG CCT CGA GTC TGC TG-3′, reverse primer 5′-GAT CCA GCA GAC TCG AGG CAA GTT CAT TCA GCT GA-3′, DR-3, forward primer 5′-GAT CTC AAG CTT ATG AAC TTG CTG ACC CTC TGC TG-3′, reverse primer 5′-GAT CCA GCA GAG GGT CAG CAA GTT CAT AAG CTT GA-3′, ER-8, forward primer 5′-GAT CTT CCT GTG TTG ACC CCA GGT GAA TCA CAA GCG-3′, reverse primer 5′-GAT CCG CTT GTG ATT CAC CTG GGG TCA ACA CAG GAA-3′. The reaction mixes containing the nuclear proteins simultaneously introduced, the anti-RXR antibody and/or the radiolabelled probe were incubated at 4°C. The reaction mix was subsequently electrophoresed on a 6% polyacrylamide gel at 4°C followed by autoradiography.

Results

FXR agonists induce CYP3A4 in HepG2 cells

HepG2 were incubated with CDCA for 24 h. Because bile acids, in addition to their potential to activate FXR, may also trigger alternative regulatory pathways, we also used GW4064, a synthetic selective ligand for FXR (Fig. 1a). Messenger RNA levels of endogenous CYP3A4 were quantified and found to be elevated after 24 h exposure to 100 μM CDCA. A significantly larger

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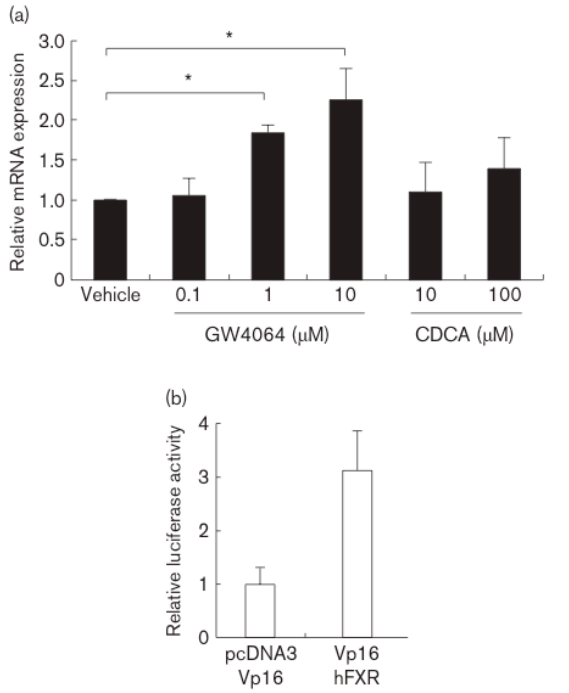
Fig. 1



Regulation of CYP3A4 by FXR in HepG2 cells. (a) HepG2 were treated with increasing concentrations of chenodeoxycholic acid (CDCA) and GW4064. *CYP3A4* levels were determined by real time polymerase chain reaction. (b) HepG2 cells were cotransfected with a reporter plasmid containing 13 kb of CYP3A4 5′-flanking region and either plasmids expressing a Vp16-FXR fusion protein or its empty vector control pcDNA3/Vp16 and exposed to vehicle. Results represent the mean ± SD of three independent experiments. $^*P < 0.05$ (Students' *t*-test).

increase in mRNA was obtained when cells were treated with GW4064 with concentrations as low as 1 μM (1.8-fold induction). In addition, when HepG2 cells were cotransfected with a reporter vector containing 13 kb of CYP3A4 5′-flanking region and an expression plasmid for a chimeric FXR fusion that is constitutively active because it contains a VP16 activation domain (Vp16/hFXR), a three-fold activation of the reporter gene activity was observed. These experiments strongly suggest a role for FXR in CYP3A4 regulation.

Identification of FXR response elements within the CYP3A4 5′-flanking region

The 5′-flanking region of *CYP3A4* was screened for sites mediating response to activated FXR. We subfragmented 13 kb of CYP3A4 5′-flanking region and inserted the resulting fragments into a pGL3basic reporter vector under the control of the *CYP3A4* promoter sequence (bases −362/+53). These constructs were tested for CDCA activation in CV-1 cells cotransfected with human FXR (Fig. 2a). Of all fragments tested, the 630-bp fragment, also known as XREM [33], which was previously shown to mediate drug-induced expression of *CYP3A4* via PXR, elicited the highest activation (5.8-fold) of reporter gene expression. The difference in the activation compared to the parent fragment of 1796 bp in length suggests that there may be inhibitory sequences within this element. Because it was shown to contain a site with a high score for similarity with FXR response elements, as revealed by NUBIScan, the 1796-bp fragment was chosen for further analysis. However, we cannot exclude the presence of additional binding sites within the 13-kb region that may respond to bile acids. This fragment was digested and the resulting 345- and 286-bp subfragments tested in CV-1 cells. This experiment revealed that the FXR responsive sequences are located in the 345-bp fragment, which displays a much higher activation (four-fold) than the 286-bp fragment (1.2-fold).

Functional interaction between FXR and the XREM module

To confirm that activated FXR acts as a positive regulator of *CYP3A4* transcription, we performed transactivation experiments in CV-1 cells that were cotransfected with the XREM reporter construct and an expression plasmid for Vp16/hFXR. The results show a robust activation (eight-fold) of the XREM reporter gene in the presence of the constitutively active FXR construct (Fig. 2b). Additional experiments were carried out in CV-1 cells transfected with the XREM reporter vector and an expression plasmid for FXR or its empty control pSG5. No activation of the reporter gene activity could be measured when CV-1 cells were cotransfected with the pSG5 control vector and treated with CDCA or GW4064 (data not shown). The data (Fig. 2c) demonstrate that the FXR agonist GW4064 induces XREM reporter gene activity in a dose-dependent manner and that activation is already observed with extremely low concentrations of GW4064 (0.05 μM). FXR, activated by a specific ligand or by a fusion with an activation domain, thus activates transcription mediated by a known regulatory module of the *CYP3A4* gene. To determine which concentrations of CDCA can activate either FXR or PXR, we performed a dose–response experiment. Low concentrations of CDCA can activate FXR (Fig. 2d), but activation of PXR only starts to appear at higher concentrations. This demonstrates that FXR is a much higher affinity receptor for CDCA than PXR in our *in vitro* system.

FXR and PXR both bind to a 345-bp sequence

As described above, a 345-bp CDCA responsive unit has been identified in the 5′-flanking region of the human CYP3A4 sequence. Binding sites for the nuclear receptor PXR have already been identified within this same 345-bp sequence [33]. We then decided to

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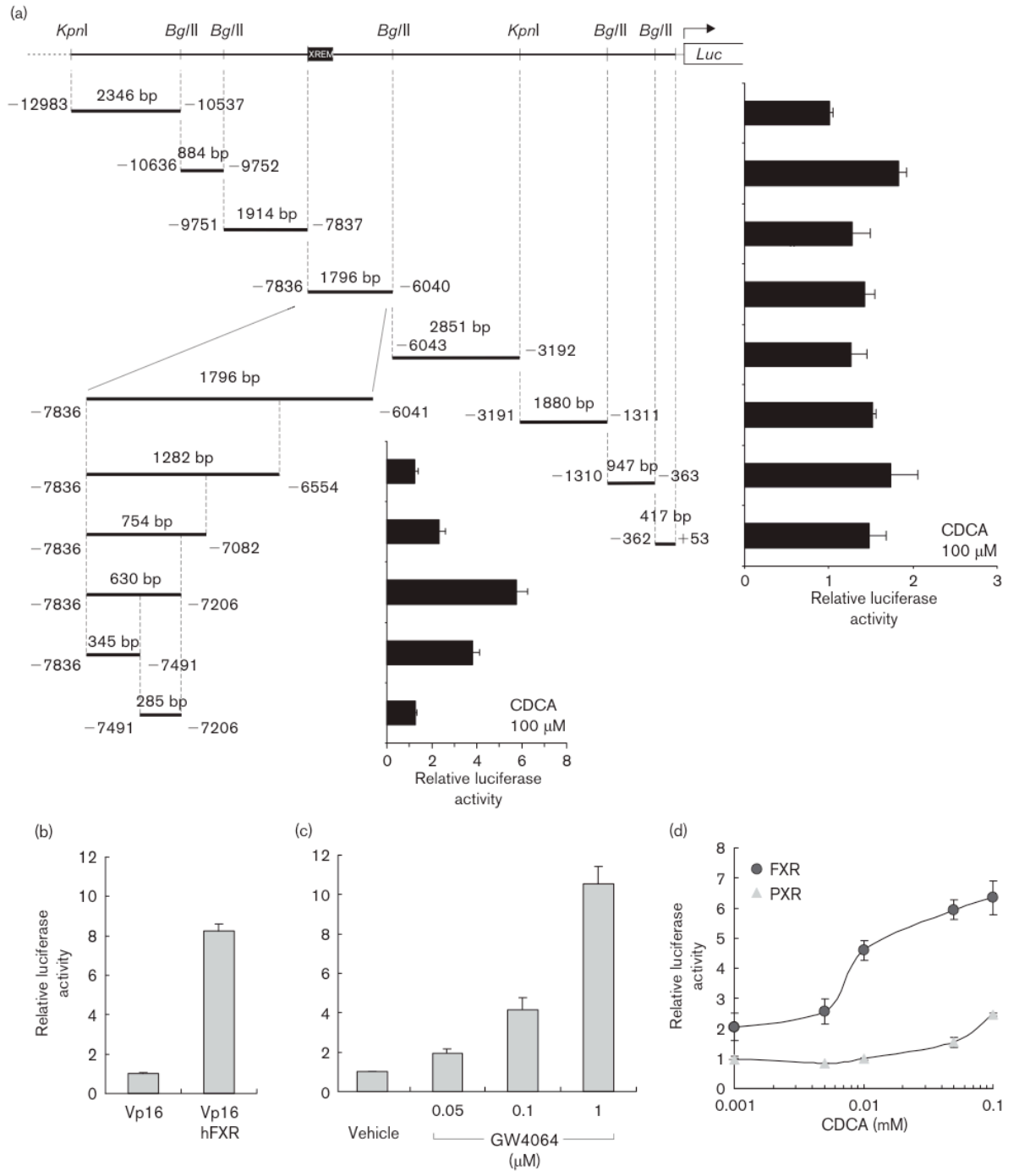
Fig. 2



Analysis of the 5′-flanking region of *CYP3A4*. (a) DNA fragments were tested for induction response in transactivation assays in CV-1, where an expression plasmid for human FXR was cotransfected. Cells were treated with either vehicle or chenodeoxycholic acid (CDCA). (b) CV-1 cells, transfected with the XREM construct and either plasmids expressing a Vp16-FXR fusion protein or its empty vector control pcDNA3/Vp16, were exposed to vehicle. (c) CV-1 cells, transfected with the XREM construct and an expression plasmid for FXR, were treated with increasing concentrations of GW4064. (d) CV-1 cells, transfected with the XREM construct and either plasmids expressing FXR or PXR, were treated as indicated. In all cases, values represent the mean ± SD of at least three independent experiments.

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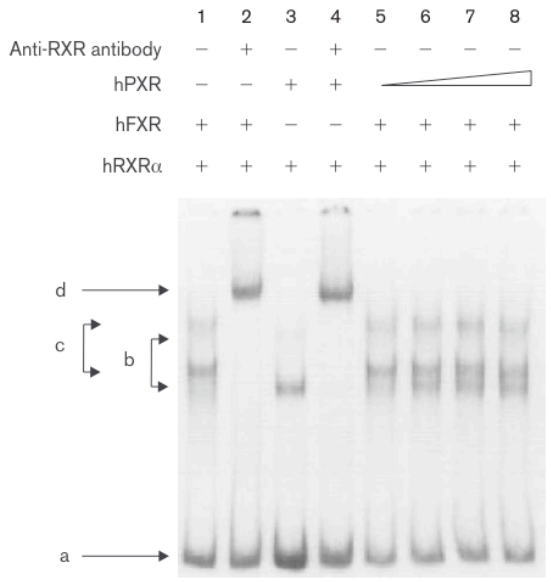
perform gel-mobility shift assays with FXR and PXR (Fig. 3), which both bound as heterodimers with RXRα to the radiolabelled wild-type 345-bp fragment (Fig. 3a, lanes b and c). FXR/RXRα complexes displayed lower mobility due to the larger mass of FXR (55-kDa) compared to PXR (50-kDa). The complexes formed by FXR/RXRα and PXR/RXRα could be supershifted by an antibody against RXR (Fig. 3a, lanes 2 and 4, respectively). In addition, constant concentrations of FXR were titrated against increasing concentrations of PXR (Fig. 3, lanes 5–8). Increasing concentrations of PXR led to the gradual appearance of a band of the size of the PXR/RXRα complex, while no decrease of the FXR/RXRα band could be observed even under excess PXR (Fig. 3, lane 8), suggesting that both nuclear receptor can bind to this element.

ER8-, IR1- and DR3- type response elements are essential for FXR-mediated regulation of CYP3A4

Because CDCA and GW4064 increased CYP3A4 transcription presumably via interactions of FXR with response elements within the 345-bp element, we submitted this sequence to the NUBIScan algorithm [30] searching for ER8 and IR1 type nuclear receptor binding sites, shown to interact with FXR in a number of target genes. We identified a potential IR1 sequence overlapping the DR3 element previously characterized as a PXR binding site and a potential ER8 type element (Fig. 4a). Using site-directed mutagenesis, we investigated the functional role of the ER8, the IR1 and the DR3 motifs within the XREM element in response to FXR ligands.

Fig. 3



FXR and PXR bind to the 345-bp element. Radiolabelled wild-type 345-bp sequence was incubated with *in vitro* transcribed/translated FXR. Increasing PXR amounts were applied as indicated: 0.6-, 1-, 1.4- and two-fold the amount of FXR protein. Arrows depict (a) the unbound probe, (b) shifts of the PXR/RXRα complex with the labelled 345 bp, (c) shifts of the FXR/RXRα complex with the labelled 345 bp, and (d) supershifts of these complexes after addition of the anti-RXR antibody.

In the reporter construct carrying the entire XREM sequence and the endogenous CYP3A4 promoter, we mutated five half-sites (Fig. 4b). To test the effect of the mutations on transcriptional activation, we performed transactivations assays in CV-1 cells. Mutations m4 or m5 in the ER8 binding site (Fig. 4b) reduced the activation of the wild-type by CDCA (6.4-fold) and GW4064 (11.7-fold) to 50% and 60% of their original value. The effect of the double mutant m4/5 on FXR activation was not different from that observed for the single mutant m4. Therefore, we studied the influence of the potential IR1 and the DR3 binding sites in a wild-type or m4/5 background. The three half-sites IR1/hs1 (m1), IR1/hs2 (m2) and DR3/hs2 (m3) were first mutated alone to assess the individual effect of each mutation in respect to activation of the wild-type. Each mutant was able to reduce both CDCA and GW4064 activation, with m2 displaying a stronger effect than m1 or m3. We next combined the double mutation m4/5 with either m1/2 or m2/3 double mutations. The CDCA activation of both mutant constructs (1.4-fold for m1/2,4/5 and 1.3-fold for m2/3,4/5) was identical to the value observed for the promoter construct (1.5-fold in Fig. 2a), whereas GW4064 activation was reduced to 2.1-fold for m1/2,4/5 and 1.8-fold for m2/3,4/5. These data clearly show that FXR interacts at several distinct sites within the 345-bp element.

Binding of human FXR to the 345-bp element is abolished by mutations of the ER8, and either IR1 or DR3 type response elements

To determine which nuclear receptor response elements were important for binding, we performed electromobility shift assays with the different binding sites IR-1, DR-3, ER-8, the 345-bp wild-type and the corresponding mutant fragments m4/5, m2,4/5, m1/2,4/5, m2/3,4/5 in the presence of FXR and/or RXRα, with or without addition of anti-RXR antibody (Fig. 5). FXR/RXRα heterodimers formed two complexes of different size with the wild-type 345-bp element (Fig. 5a, two bands in lane 1, arrow b), denoting the presence of multiple binding sites within this element. These complexes were supershifted by addition of anti-RXR antibody (Fig. 5a, lane 2, arrow c). This multiple binding was further confirmed by the results shown in Fig. 5(b), showing that FXR/RXRα heterodimers were able to bind to the three single response elements IR-1, DR-3 and ER-8. FXR/RXRα binding was reduced but not abolished with the m4/5 fragment (Fig. 5a,

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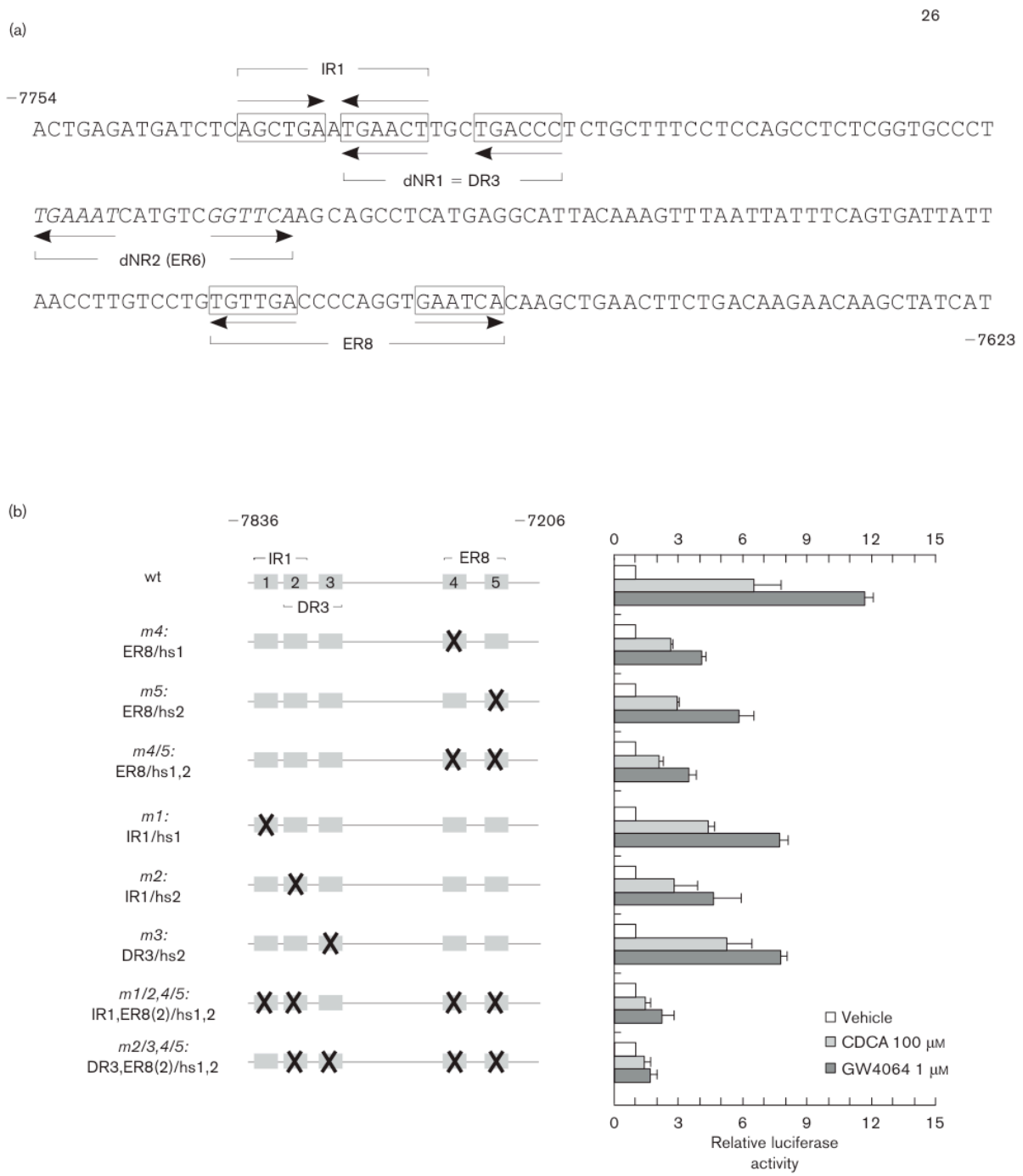
Fig. 4



FXR mediated induction of the XREM occurs via nuclear receptor response elements. (a) Sequence of the 5′-part (131 bp) of the XREM fragment containing FXR-type binding sites predicted by NUBIScan (ER8, IR1). The known PXR binding sites dNR1 (DR3) and dNR2 (ER6) are also indicated. The half sites subjected to site-directed mutagenesis as described in (b) are shaded in grey. (b) Transactivation assays in CV-1 cells with wild-type and mutant XREM fragments. Constructs are labelled in the left column and mutations are depicted by crosses. CV-1 cells, transfected with FXR and the indicated reporter gene vector, were treated as described. Data shown represent the mean ± SD of at least three independent experiments.

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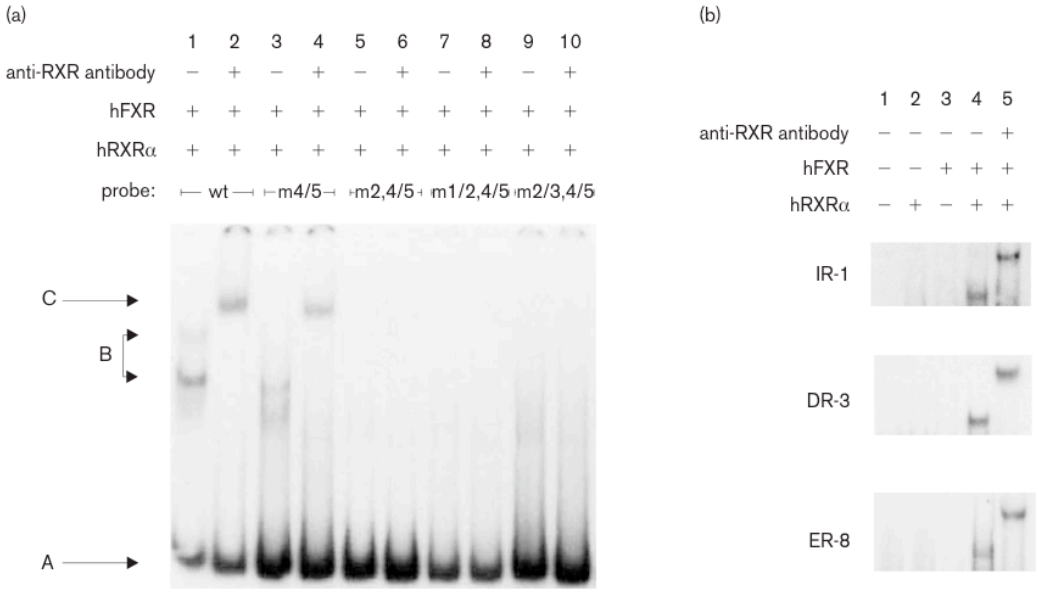
Fig. 5



FXR binds to ER-8, IR1 and DR3 type response elements in the 345-bp element. (a) Mutations of the IR1 or DR3 together with the ER8 response element abolish binding of FXR to the 345-bp element. Radiolabelled wild-type 345-bp element (lanes 1–2), or the corresponding mutants m4/5 (lanes 3–4), m2,4/5 (lanes 5–6), m1/2,4/5 (lanes 7–8), m2/3,4/5 (lanes 9–10) were incubated with *in vitro* transcribed/translated FXR and RXRα (1–8) and anti-RXR antibody (2, 4, 6, 8, 10). Arrows depict A the unbound probe, B shifts of the FXR/RXRα complex with the labelled 345 bp or mutant probes and C supershifts of these complexes after addition of the anti-RXR antibody. (b) FXR/RXRα heterodimers bind to IR-1, DR-3 and ER-8 response elements in the 345 bp fragment. Radiolabelled oligonucleotides for IR-1, DR-3 and ER-8 response elements, as described in Methods, were incubated with *in vitro* transcribed/translated FXR and RXRα. Shifts and supershifts complexes in lane 4 and 5, respectively, are presented for each binding site studied.

lanes 3–4), where the heterodimer formed only one complex with the mutated 345 bp, which could be supershifted by addition of anti-RXR antibody (Fig. 5a, lane 4, arrow c). When constructs harbouring combinations of mutations of the three half-sites composing the IR1 and the DR3 (m1-3) together with m4/5 were examined (Fig. 5a), deletion of either half-site 2 in the IR1, both half-sites 1 and 2 in the IR1, or half-site 1 and 2 in the DR3, in combination with deletions of the ER8 half sites in constructs m2,4/5, m1/2,4/5 and m2/3,4/5, were all able to completely abolish binding of FXR/RXRα heterodimers (Fig. 5a, lanes 5–10). These experiments suggest the presence of two binding sites, ER8 and IR1/DR3, for FXR/RXRα heterodimers within the 345-bp sequence.

GW4064 induces Cyp3a11 expression *in vivo* in mice

We treated wild-type, $FXR^{-/-}$ and $PXR^{-/-}$ mice with either vehicle or GW4064. *PXR* knockout animals were included in this study to eliminate PXR-mediated effects of GW4064 on CYP3A. A significant induction of *Cyp3a11* mRNA was observed in wild-type animals treated with 40 mg/kg GW4064 (Fig. 6a). A similar induction was observed in $PXR^{-/-}$ mice at a dose of 20 mg/kg GW4064. There was no significant difference of *Cyp3a11* basal levels between knockout and wild-type animals in accordance with observations by Xie *et al.* [22], but in contrast to the observations of Staudinger *et al.* [21]. The mRNA levels of mouse *Cyp7a1*, coding for cholesterol 7α-hydroxylase and known to be negatively regulated by FXR [34,35], were measured as a control. Mouse *Cyp7a1* was significantly repressed in wild-type and $PXR^{-/-}$ mice by the FXR agonist (Fig. 6b). The experiments in wild-type and $FXR^{-/-}$ mice (Fig. 6c) reveal a significant increase in *Cyp3a11* basal expression in $FXR^{-/-}$, as previously observed [36]. No further increase in *Cyp3a11* expression was observed in $FXR^{-/-}$ mice after treatment with GW4064. These data suggest that activation of FXR induces *Cyp3a11 in vivo* and that this effect of GW4064 is independent of PXR.

Discussion

In this report, we describe two distal regulatory sequences in the human *CYP3A4* gene that mediate an FXR response. The functionality of these elements was deduced as follows: the exposure of HepG2 cells to FXR ligands such as CDCA or GW4064 resulted in an increase in CYP3A4 mRNA levels. In addition, cotrans-

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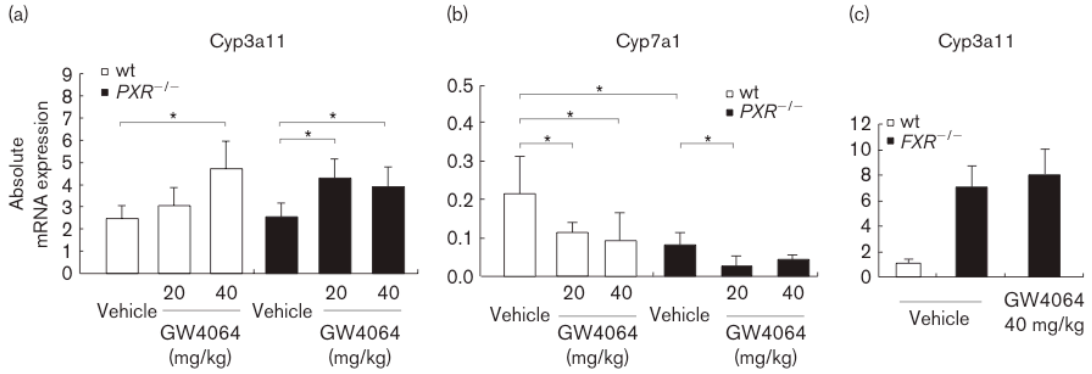
Fig. 6



The FXR agonist GW4064 induces *Cyp3a11* expression in wild-type and *PXR*$^{-/-}$, but not in *FXR*$^{-/-}$ mice. Wild-type, *FXR*$^{-/-}$ and *PXR*$^{-/-}$ mice were injected i.p. as indicated. Real-time polymerase chain reaction analysis was performed with probes specific for (a,c) *Cyp3a11* and (b) *Cyp7a1*. Gene levels are expressed relative to the wild-type vehicle treated control. Error bars indicate SD. *$P < 0.05$ (Students' *t*-test).

fection experiments in HepG2 revealed that 13 kb of CYP3A4 5′-flanking region show activation in the presence of activated FXR. The induction was confirmed in CV-1 cells that showed an increase of the activity of a reporter gene containing a 630-bp element in response to activated FXR. Moreover, FXR heterodimerizes with RXRα to bind to an ER8 and an IR1/DR3 sequence within a 345-bp fragment. The regulatory elements identified are located within a sequence previously shown to mediate drug-response via PXR and CAR [33] and containing a binding site for HNF4α [13]. Cross-talk between members of the nuclear receptor family frequently occurs on common regulatory sequences within the 5′-flanking regions of the target gene. This phenomenon has previously been observed for *CYP2B*, *CYP2H*, and *MRP2* genes [26,37,38] and reflects a common observation regarding gene regulation: transcription factor binding sites tend to cluster into cis-regulatory modules of few hundreds of base pairs in length [39,40]. Thus, the DR-3 element shown here to bind FXR was previously shown to bind PXR [33] and, in the *MRP2* gene, binding of PXR to an ER-8 type response element was reported [26].

To investigate if FXR can activate CYP3A *in vivo*, we administrated GW4064, a specific FXR agonist, to wild-type and *PXR*$^{-/-}$ mice. Our results, showing that GW4064 elicits an induction of *Cyp3a11* mRNA in both wild-type and *PXR* deficient mice, indicate that this effect is PXR-independent. Moreover, the repression of *Cyp7a1* mRNA levels in wild-type and *PXR*$^{-/-}$ mice treated with GW4064 further confirms that FXR was activated by GW4064. The data obtained in *FXR*$^{-/-}$ mice revealed that GW4064 cannot increase *Cyp3a11* mRNA in the absence of FXR. The increase in *Cyp3a11* basal levels in *FXR*$^{-/-}$ compared to wild-type mice could be an indirect consequence of the lack of FXR: it has been shown that *FXR*$^{-/-}$ mice have a higher rate of cholate synthesis compared to wild-type animals [41]. This suggests that increased cholate in the liver could, via PXR, lead to an increase in Cyp3a11 basal expression. Feeding of cholic acid to *FXR*$^{-/-}$ mice [36] may even increase this PXR activation and explain the elevation of Cyp3a11 under these conditions.

FXR is an important regulator of bile acid and lipid homeostasis. When activated by bile acids, FXR initiates a negative feedback-loop on *Cyp7a1* expression, via the small heterodimerization partner (SHP). In addition, FXR can also up-regulate the expression of several genes implicated in the transport of bile acids into the bile (*BSEP*, *MRP2*). Although FXR and PXR share common target genes, they have complementary roles in the protection of the liver against toxic agents. Our study suggests that excess primary bile acids induce their own metabolism via FXR activation of CYP3A, while secondary bile acids, as already shown [22], act via PXR. The *MRP2* gene can be up-regulated by both PXR and FXR to increase the excretion of unwanted substances into the bile. *Cyp7a1* was shown to be repressed by activated FXR [42] and PXR [21], even though the mechanism of PXR repression is still unknown.

CDCA can activate human FXR at 1 μM, while at least 50 μM are required to activate PXR (Fig. 2d). Moreover, the potency of bile acid binding to FXR in cell-free ligand-sensing assays [15] indicated an EC50

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ranging from 4.5 μM for CDCA to 10 μM for its tauro- and glyco-conjugated forms. These concentrations correspond to the estimated physiological range of these bile acids in liver tissue [43,44]. In addition, it has been shown that VDR is not activated by CDCA [23]. Therefore, the present activation and binding data, together with the fact that FXR can modulate CYP3A expression both *in vitro* and *in vivo* in mice, strongly suggest that slightly elevated or even physiological concentrations of primary hepatic bile acids influence the basal level of expression of CYP3A in human liver, and that elevated CYP3A as observed in cholestasis [19,20,24] can now partly be explained by the action of FXR.

In conclusion, our data provide evidence for a role of FXR in CYP3A regulation. They define enhancer sequences responsible for the direct response of human CYP3A4 to primary bile acids. These findings suggest that, although elevated concentrations of precursors of bile acids [45] and secondary bile acids [22] elicit CYP3A activation via PXR, primary bile acids, at only a slightly increased or even at physiological concentration, may modulate the expression of CYP3A via FXR. This phenomenon makes physiological sense because CDCA is the best ligand for FXR, even though it also activates PXR at higher concentrations [22]. The variable expression of CYP3A4 is known to present important interindividual differences, and is associated with dosing problems for physicians. These findings provide evidence for a link between bile acid homeostasis and drug metabolism, and further explain the variability of drug responsiveness between individuals.

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References

1 Waxman DJ, Lapenson DP, Aoyama T, Gelboin HV, Gonzalez FJ, Korzekwa K. Steroid hormone hydroxylase specificities of 11 cDNA-expressed human cytochrome P450s. *Arch Biochem Biophys* 1991; **290**:160–166.
2 Araya Z, Wikvall K. 6alpha-hydroxylation of taurochenodeoxycholic acid and lithocholic acid by CYP3A4 in human liver microsomes. *Biochim Biophys Acta* 1999; **1438**:47–54.
3 Nebert DW, Russell DW. Clinical importance of the cytochromes P450. *Lancet* 2002; **360**:1155–1162.
4 Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* 1997; **29**:413–580.
5 Savas U, Griffin KJ, Johnson EF. Molecular mechanisms of cytochrome P-450 induction by xenobiotics: an expanded role for nuclear hormone receptors. *Mol Pharmacol* 1999; **56**:851–857.
6 Pichard L, Fabre JM, Domergue J, Fabre G, Saint-Aubert B, Mourad G, *et al*. Molecular mechanism of cyclosporine A drug interactions: inducers and inhibitors of cytochrome P450 screening in primary cultures of human hepatocytes. *Transplant Proc* 1991; **23**:978–979.
7 Flockhart DA, Rae JM. Cytochrome P450 3A pharmacogenetics: the road that needs traveled. *Pharmacogenom J* 2003; **3**:3–5.
8 Honkakoski P, Negishi M. Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J* 2000; **347**:321–337.
9 Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, *et al*. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA* 1998; **95**:12208–12213.
10 Goodwin B, Redinbo MR, Kliewer SA. Regulation of cyp3a gene transcription by the pregnane X receptor. *Annu Rev Pharmacol Toxicol* 2002; **42**:1–23.
11 Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, Liddle C. Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* 2002; **62**:359–365.
12 Thummel KE, Brimer C, Yasuda K, Thottassery J, Senn T, Lin Y, *et al*. Transcriptional control of intestinal cytochrome P-4503A by 1alpha, 25-dihydroxy vitamin D3. *Mol Pharmacol* 2001; **60**:1399–1406.
13 Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, *et al*. The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nature Med* 2003; **9**:220–224.
14 Lambert G, Amar MJ, Guo G, Brewer HB Jr, Gonzalez FJ, Sinal CJ. The farnesoid X-receptor is an essential regulator of cholesterol homeostasis. *J Biol Chem* 2003; **278**:2563–2570.
15 Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, *et al*. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284**:1365–1368.
16 Urizar NL, Dowhan DH, Moore DD. The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. *J Biol Chem* 2000; **275**:39313–39317.
17 Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* 2001; **276**:28857–28865.
18 Kliewer SA, Willson TM. Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res* 2002; **43**:359–364.
19 Summerfield JA, Billing BH, Shackleton CH. Identification of bile acids in the serum and urine in cholestasis. Evidence for 6alpha-hydroxylation of bile acids in man. *Biochem J* 1976; **154**:507–516.
20 Bremmelgaard A, Alme B. Analysis of plasma bile acid profiles in patients with liver diseases associated with cholestasis. *Scand J Gastroenterol* 1980; **15**:593–600.
21 Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, *et al*. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* 2001; **98**:3369–3374.
22 Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, *et al*. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA* 2001; **98**:3375–3380.
23 Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, *et al*. Vitamin D receptor as an intestinal bile acid sensor. *Science* 2002; **296**:1313–1316.
24 Stedman C, Robertson G, Coulter S, Liddle C. Feed-forward regulation of bile acid detoxification by CYP3A4: studies in humanized transgenic mice. *J Biol Chem* 2004; **279**:11336–11343.
25 Willson TM, Jones SA, Moore JT, Kliewer SA. Chemical genomics: functional analysis of orphan nuclear receptors in the regulation of bile acid metabolism. *Med Res Rev* 2001; **21**:513–522.
26 Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, *et al*. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002; **277**:2908–2915.
27 Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB Jr, Kliewer SA, *et al*. Complementary roles of Farnesoid X receptor, pregnane X receptor, and constitutive Androstane receptor in protection against bile acid toxicity. *J Biol Chem* 2003; **15**:15.
28 Ourlin JC, Lasserre F, Pineau T, Fabre JM, Sa-Cunha A, Maurel P, *et al*. The small heterodimer partner interacts with the pregnane X receptor and represses its transcriptional activity. *Mol Endocrinol* 2003; **12**:12.
29 Handschin C, Podvinec M, Looser R, Amherd R, Meyer UA. Multiple enhancer units mediate drug-induction of CYP2H1 by the xenobiotic-

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sensing orphan nuclear receptor CXR. *Mol Pharmacol* 2001; **60**: 681–689.
30 Podvinec M, Kaufmann MR, Handschin C, Meyer UA. NUBIScan, an in-silico approach for prediction of nuclear receptor response elements. *Mol Endocrinol* 2002; **16**:1269–1279.
31 Handschin C, Podvinec M, Meyer UA. CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR). *Proc Natl Acad Sci USA* 2000; **97**:10769–10774.
32 Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 2000; **102**:731–744.
33 Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 1999; **56**:1329–1339.
34 Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, *et al*. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell* 2000; **6**:507–515.
35 Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, *et al*. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell* 2000; **6**:517–526.
36 Schuetz EG, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, *et al*. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J Biol Chem* 2001; **276**:39411–39418.
37 Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M. The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* 1999; **274**:6043–6046.
38 Handschin C, Podvinec M, Amherd R, Looser R, Ourlin JC, Meyer UA. Cholesterol and bile acids regulate xenosensor signaling in drug-mediated induction of cytochromes P450. *J Biol Chem* 2002; **277**:29561–29567.
39 Davidson EH, Rast JP, Oliveri P, Ransick A, Calestani C, Yuh CH, *et al*. A genomic regulatory network for development. *Science* 2002; **295**:1669–1678.
40 Berman BP, Nibu Y, Pfeiffer BD, Tomancak P, Celniker SE, Levine M, *et al*. Exploiting transcription factor binding site clustering to identify cis-regulatory modules involved in pattern formation in the Drosophila genome. *Proc Natl Acad Sci USA* 2002; **99**:757–762.
41 Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, *et al*. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. *J Biol Chem* 2003; **278**:41930–41937.
42 Chiang JY, Kimmel R, Weinberger C, Stroup D. Farnesoid X receptor responds to bile acids and represses cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription. *J Biol Chem* 2000; **275**:10918–10924.
43 Setchell KD, Rodrigues CM, Clerici C, Solinas A, Morelli A, Gartung C, *et al*. Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei. *Gastroenterology* 1997; **112**:226–235.
44 Noe J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* 2002; **123**:1659–1666.
45 Goodwin B, Gauthier KC, Umetani M, Watson MA, Lochansky MI, Collins JL, *et al*. Identification of bile acid precursors as endogenous ligands for the nuclear xenobiotic pregnane X receptor. *Proc Natl Acad Sci USA* 2003; **100**:223–228.

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Handschin C, Blättler S, Roth A, Looser R, Oscarson M, Kaufmann MR, Podvinec M, Gnerre C, Meyer UA. "The evolution of drug-activated nuclear receptors: one ancestral gene diverged into two xenosensor genes in mammals". Nucl Recept. 2004 Oct 12;2(1):7.

Meyer UA, Blättler S, Gnerre C, Oscarson M, Peyer AK, Rencurel F, Rifki O, Roth A. "*In vitro*, *in vivo*, and *in silico* approaches to predict induction of drug metabolism". Pharmacokinetic profiling in drug research, 2005, Wiley-VCH.

Blättler SM, Rencurel F, Kaufmann MR, Meyer UA. "In the regulation of cytochrome P450 genes, phenobarbital targets LKB1 for necessary activation of AMP-activated protein kinase". PNAS, 2007 Jan 16;104(3):1045-50.

Courses/symposia

May 2002 Kranyska Gora, Slovenia
International FEBS Advanced Course: "Cytochrome P450 Systems: from Structure to Application"
Poster: Blättler S, Handschin C, Meyer UA, "Search for New Xenobiotic-Sensing Nuclear Receptors".

October 2002 Zürich, Switzerland
Laboratory Animal Course, LTK Modul 1, Institut für Labortierkunde, University of Zürich

September 2003 Munich, Germany
Proteomic Forum 2003, International Meeting on Proteome Analysis, Technical University of Munich

October 2003 Basel, Switzerland
Introduction to Bioinformatics, EMBnet course, Swiss Institute of Bioinformatics (SIB), Biozentrum, University of Basel

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December 2003 Basel, Switzerland

Swiss Proteomics Society 2003 Congress, Integration of proteomic technologies to fully understand complex animal, vegetal and microbial biological systems, Novartis AG Basel

July 2004 Mainz, Germany

15th International Symposium on Microsomes and Drug Oxidations (MDO 2004)

Poster: Gnerre C, Blättler S, Kaufmann MR, Looser R, Meyer UA: "CYP3A4 and Cyp3a11 are regulated by the nuclear receptor FXR and primary bile acids in cell cultures and in mice".

November 2004 Basel, Switzerland

Practical and Legal Aspects of Animal Experiments, Advanced Training, University of Basel

June 2005 Dundee, Scotland

Implications of the LKB1 and AMPK Systems, Cell Signaling Symposium

Poster: Blättler S, Kaufmann MR, Meyer UA, Rencurel F: "Role of AMPK in drug-mediated induction of avian cytochromes P450".

October 2005 Basel, Switzerland

Biozentrum Symposium

Poster: Blättler S, Kaufmann MR, Rencurel F, Meyer UA: "Role of AMPK in the Induction of Cytochromes P450 by Phenobarbital: Studies in Chicken Hepatoma Cells".

September 2006 Budapest, Hungary

16th International Symposium on Microsomes and Drug Oxidations (MDO 2006)

Poster: Blättler S, Kaufmann MR, Rencurel F, Meyer UA: "AMP-activated protein kinase is necessary for induction of cytochromes P450 by phenobarbital and metyrapone: studies in chicken hepatoma cells".

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October 2006 Basel, Switzerland

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Poster: Blättler S, Kaufmann MR, Rencurel F, Meyer UA: "Role of Mitochondria, LKB1 and AMPK in the Phenobarbital-Type Induction of Cytochromes P450".

November 2006 Basel, Switzerland

Animal Models of Disease, Advanced training for animal experiments, University of Basel

December 2006 Basel, Switzerland

Targeting the Kinome

Poster: Blättler S, Kaufmann MR, Meyer UA: "Activation of the PKCζ-LKB1-AMPK signaling cascade by drugs is necessary for transcriptional regulation of cytochromes P450".

Education

1997 - 2002 University of Basel, Switzerland
Master of Science in molecular biology and biochemistry

1993 - 1997 Liceo di Lugano, Switzerland
Maturity (Swiss maturity type C, with focus on science)

1989 - 1993 Scuola media di Lugano, Switzerland

1984 - 1989 Scuola elementare di Montagnola, Switzerland

Personal Interests

Active member of University Basel Volleyball
Trekking, Biking, Ballroom-Dancing
Literature, Music
Foreign Countries and Cultures

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