Crossroads between drug and energy metabolism: Role of constitutive androstane receptor and AMP-activated kinase.

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Abstract

Phenobarbital (PB) is a prototype inducer of genes encoding drug metabolizing enzymes including the cytochromes P450 (CYPs). Additionally, phenobarbital was found to repress genes that encode enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK). Constitutive androstane receptor (CAR) is known to play a fundamental role in the phenobarbital-mediated regulation of cytochromes P450 and in gluconegenesis in the liver. Phenobarbital was recently shown in our laboratory to activate a known inhibitor of hepatic glucose production, the energy sensor AMP-activated kinase (AMPK). In the present thesis, we investigated the role of AMPK in the phenobarbital-mediated inhibition of gluconeogenesis. Our experiments reveal that both CAR and AMPK are necessary to mediate the PB inhibitory effect on PEPCK mRNA expression. Furthermore, our study indicates that AMPK and CAR physically interact in this process. We speculate that once activated by PB, the CAR-AMPK complex may prevent coactivators such as PGC-1α to interact with partners at the PEPCK promoter. However, overexpression of exogenous CAR dose-dependently increased PEPCK mRNA expression and its promoter activity in a human hepatoma cell line. The co-transfection of CAR with PGC-1α, a master regulator of PEPCK, clearly increases PEPCK promoter activity. Moreover, we show that the cotransfection of CAR and protein kinase A (PKA), a well established inducer of gluconeogenic pathways, dose dependently activates PEPCK.

Our results also indicate that similarily to PEPCK, CAR mRNA expression is induced during fasting and in the absence of glucose. CAR also induces genes that encode for glucose transport during fasting. On the other hand, insulin represses CAR mRNA expression suggesting that CAR plays a significant role in the fasting-feeding transition. Finally, we demonstrate that CAR regulates the expression of genes encoding for of acetyl-CoA carboxylase (ACC), an enzyme known to be involved in the control of lipogenesis and beta oxidation of fatty acids. Altogether, these studies indicate that CAR is involved in the regulation of glucose and lipid metabolism and the regulation of its activity may be crucial to understand the molecular mechanisms that link drug metabolism to energy metabolism.

Introduction

Cytochromes P450 in drug metabolism

We are daily exposed to various exogenous potentially harmful compounds. Fortunately, our organism possesses very sophisticated mechanisms to metabolize these toxic substances. In the liver, primary detoxification process occurs, where hydrophobic compounds are converted to more polar molecules to be further excreted into body fluids. This process is controlled by a whole arsenal of drug metabolizing enzymes including the cytochromes P450 (CYPs) which catalyze the so-called phase I in the detoxification process. In phase II and III, many other enzymes further modify these substances, which are then transported for excretion by urine faeces and bile [1].

CYPs are probably the biggest and the most studied drug metabolizing enzymes. In eukaryotic organisms, CYPS are essential and many different endogenous compounds have been identified to be substrate of these enzymes including steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, and retinoids [2, 3]. Interestingly, CYPs are present from bacteria to humans. However, in higher animals the enzymes are membrane-bound and located in the endoplasmic reticulum or in the inner mitochondrial membrane. In most species, and in addition to their role in metabolizing endogenous compounds, some of these CYPs are important in the metabolism of various substrates such as drugs, carcinogens, food additives, pollutants, pesticides, or environmental chemicals [4]. Since CYPs play key roles in metabolizing pathways of a variety of compounds, their expression must be highly regulated.

Drug-mediated induction of cytochromes P450

A common characteristic of CYPs is their relatively low basal expression in the absence of substrate and their highly elevated expression in the presence of their own substrates or other inducer compounds, a phenomenon called drug induction [4-6]. This process is tissue-specific, rapid, dose-dependent and reversible. In higher animals, it occurs mainly in the liver, in the intestine and to a lesser extent in kidney, lung and brain. More than 40 years ago, CYPs increased concentration has been shown upon increases doses of the barbiturate phenobarbital (PB) treatment in rat livers. Those animals progressively adapted to PB due to enhanced drug metabolism clearance. In rat, smooth endoplasmic reticulum of hepatocytes was found to proliferate upon PB treatment [7, 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 hormonal disposal [9-13].

Many drugs and chemicals have the ability to induce their own metabolism by transcriptional activation of CYPs. Moreover, inducers can drastically alter the pattern of gene expression in the liver giving raise to pleiotropic hepatic responses. For instance, in a recent study, PB was shown to affect more than 100 genes in mouse livers [14].

Drug-metabolizing enzymes mainly belong to the CYP1, CYP2, CYP3 and CYP4 families and their prototypical inducers are classified 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 CYPs3As induction, clofibrate acting on the CYP4As which metabolize fatty acids and ethanol that increases the activity of CYP2Es [15, 16].

The molecular mechanism of hydrocarbon induction of CYP1As by the polyaromatic hydrocarbon receptor (AhR) was elucidated already in the 1980s [17], 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 receptor 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.

The first insights in the transcriptional regulation of CYPs came from a negative regulated element in the proximal promoter of P450 from BM-3 Bacillus megaterium [18, 19]. Sequences homologous to this element referred to as Barbie Box were also found in proximal promoters of mammalian CYPs [20, 21]. Several proteins binding to the promoter were identified and shown to regulate the basal levels of expression and the tissue specific expression. However, a growing body of evidence suggested an important role of a distant enhancer element in drug regulation of CYPs. The first discovery of such regulatory sequence was made in 5'-flanking regions of the CYP2H1 gene in chicken embryo hepatocytes [22] and later in rat CYP2B2 [23] and mouse Cyp2b10 [24] gene. 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 [25] [26] as well as a 264-bp in CYP2H1 [27] and a 51-bp in the cyp2b10 [28] were shown to be sufficient to mediate PB induction. Analysis of these sequences revealed the presence of putative hexamer half-sites suggesting for the first time that transcription factors may be involved in drug regulated expression of CYPs (discussed below).

The pleiotropic effects of phenobarbital

For many years, PB has been extensively used to treat epileptic patients, but the real impact of this drug has been underestimated. Several decades ago PB was shown to induce drug metabolism [29]. Since then, many different groups have described numerous other biological processes affected by PB.

Tumour promotion

One of the most disturbing effects of PB exposure is the development of liver tumours in rodents. Mice acutely treated with PB develop hepatomegaly, cellular hypertrophy and hyperplasia [30]. Moreover, in the early 70s PB was shown to promote hepatocellular carcinoma (HCC) [31]. However, in humans chronic PB treatment has never been shown to induce tumor promotion. This human resistance is probably due to many different

reasons, including shorter telomeres [32]. Indeed, telomerase-deficient mice are resistant to chemically induced hepatocarcinogenesis [33].

Steroids and thyroid metabolism

Chronic treatment with PB is known to promote thyroid hypertrophy in humans and in rats [34, 35]. Levels of thyroid hormones (TH) are determined by a tight control of the balance between their synthesis, metabolism and secretion. Thyroid stimulating hormone (TSH) regulates synthesis of inactive tetraiodothyronine (T4). T4 is converted to the bioactive triiodothyronine (T3) by deiodinases in the peripheral target tissues, such as liver and kidney. Recently PB was shown to decrease the level of T4 in mice [36].

Bile acid metabolism

Bile acids are the major products of cholesterol catabolism and, acting as physiological detergents, promotes intestinal absorption of dietary lipids. The levels of bile acids are regulated by the balance of synthesis and elimination. CYP7A1 is the rate-limiting enzyme of bile acid synthesis, while other enzymes are involved in transport and elimination [37] [38]. Accumulation of bile acid in the hepatocyte impairs liver function and causes intrahepatic cholestasis. Indeed, PB has been extensively used to treat pruritis, a side effect of elevated serum bile acids associated with cholestasis [39].

A recent study has suggested that PB represses CYP7A1 gene expression in mouse liver by interfering with the recruitment of hepatic nuclear factor 4α (HNF4α), an important transcription factor in CYP7A1 regulation [40]. Moreover, our group has demonstrated that PB represses chicken HNF4α transcript levels concomitant with a reduction in CYP7A1 expression [41]. However, mammalian HNF4*α* expression is not repressed by drugs. Altogether these results show that, besides the importance of HNF4alpha in CYP7A1 regulation in all species, birds and mammals use different signaling pathways to adjust CYP7A1 levels after exposure to xenobiotics.

Glucose metabolism

Chronic PB treatment reduced plasma glucose levels in diabetic patients [42]. Hepatic gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are repressed in PB-treated rats and mice [43, 44]. Interestingly, in the early 60s insulin was reported to repress drug metabolism [45]. Hepatic levels of CYP2B and CYP3A were increased in diabetic mice and rats and insulin treatment decreased them to normal levels [46] [47]. In support of these observations, Yoshida et al., showed that insulin blocks PB induction of CYP2B in rat primary hepatocytes in culture [48] [49]. These results suggest a strong link between drug metabolism and glucose homeostasis. Unfortunately, the molecular mechanisms by which insulin influences drug metabolism and the mechanisms by which PB affects hepatic glucose production remain unclear. This thesis approaches some of the remaining questions.

Phosphorylation/dephosphorylation events in phenobarbital-mediated induction of cytochromes P450

Several experimental observations point to a role of phosphorylation and dephosphorylation events in the indirect mechanism of PB induction of CYPs.

For many years it was assumed that PB induction requires *de novo* protein synthesis. However, evidence has accumulated which indicates that *de novo* protein synthesis is not required for PB induction. In fact, post-transcriptional modifications have been shown to regulate PB signalling in hepatocytes, i.e. protein kinases/phosphatases. 2-Aminopurine, a general inhibitor of serine/threonine protein kinases, blocked CYP2B1/2 induction in rat hepatocytes [50], and CYP2H1 and ALAS1 induction in chick embryo hepatocytes by PB [51]. Genistein, a tyrosine protein kinase inhibitor, also fully blocked Cyp2b10 induction by PB in mouse hepatocytes [52]. However, other studies reported no effect of genistein on CYP2H or CYP2B induction by PB [51, 53, 54]. 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 [55] and of Cyp2b10 in mouse hepatocytes [56], 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 primary mouse hepatocytes did not find any effect of forskolin and dibutyryl cAMP on Cyp2b10 inducibility by PB [57]. Moreover, no direct effect of PB on cAMP levels or PKA activity could be detected in mouse and rat hepatocytes [57, 58] and in avian LMH cells [27]. However, data obtained by the use of forskolin have to be interpreted with caution, because this PKA modulator is a ligand and activator of human and mouse PXR [59]. 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 [56]. In this regard, Yamamoto et al. blocked the TCPOBOB-mediated induction of Cyp2b10 in mouse hepatocytes by KN-62 [60]. 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 [61, 62]. Moreover, our group has pointed out a new target of PB in the cell. We showed that PB increases the activity of an energy sensor, AMP-activated kinase (AMPK). Indeed, this kinase is necessary to mediate the induction of CYPs by PB in human and in mouse livers [63, 64]. A growing body of evidence suggests that phosphorylation is involved in PB-mediated induction of CYPs. Nevertheless, further functional studies are required to elucidate the role of phosphorylation in this process.

Nuclear receptors

Cell-cell communication within an organism is necessary in all phases from 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 often interact with members of the family of nuclear receptors, which act as transcription factors. This family is subdivided into three classes, the classical hormone receptors, the sensor receptors and the orphan nuclear receptors [65]. 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. The third class of "sensor" receptors bind endogenous compound 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 as a metabolic sensor 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 its origin and was well diversified even before the arthropod/vertebrate split [66]. NRs control the activity of target genes directly by interacting as monomers, homodimers or heterodimers with the distal enhancer elements, located in the promoter of their target gene. 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 monomers, usually bind to an extended half-site, the extension being rich in A and T [67-69].

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 recruitment of coactivators or corepressors (Figure 1A). The role of the cofactors is to facilitate the communication between NRs, the basal transcriptional machinery and the chromatin environment. Because the NRs need to gain access to the DNA, which is wrapped around histones forming nucleosomes, they recruit coactivators which stabilize 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 inhibits transcription [70-74].

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 (Figure 1) [75, 76].

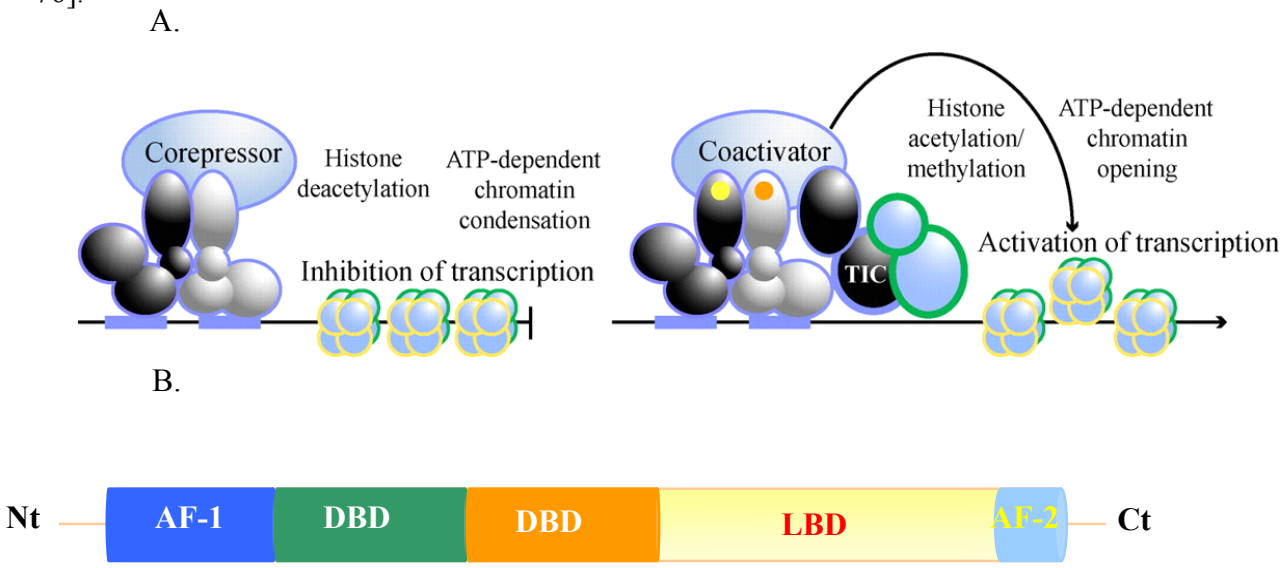


Figure 1: **Nuclear receptors**

Pregnane X receptor

PXR, alternatively called steroid and xenobiotic receptor, was discovered in mice and humans by three independent groups [77-80]. 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 [81]. 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 [82]. Similarly, PXR knockout mice that express the human PXR exhibit a typical human response to different inducers [83]. 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 [82]. 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 [78, 79, 84, 85]. PXR is found normally in the nucleus, where it is activated by ligand binding [86]. However, PXR was recently proposed to accumulate also in the cytoplasm in complex with Hsp90 and CCRP by overexpression of the latter [87]. The first identified targets of PXR were CYP3A1 and CYP3A4 in rat and human, respectively [79, 80, 83]. Later, other genes were shown to be regulated by PXR, such as CYP2Bs, MDR1, MRP2, OATP2 among others (for a recent review, see [88, 89]).
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 [83, 90], 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 by one DR-4 repeat used in affinity purification for isolation of proteins binding to this sequence and thereby mediating drug induction. This approach led to the observation that murine CAR binds to Cyp2b10 [91]. 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 [92, 93], but DR-1, ER-6 and ER-8 can also serve as CAR binding sites, too [40, 94, 95]. 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 1, 4-Bis[2-(3,5-dichloropyridyloxy)]benzene (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 [92, 93, 96] since transiently transfection of CAR in HepG2 cells elicited high basal activity of Cyp2b10 or CYP2B6 PBRU in reporter gene assays [95]. In these cells CAR is already located in the nucleus

and its activity 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 [56]. 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 xenobiotics response signal (XRS), which is located in the C-terminal region of CAR [97]. 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 [98, 99]. 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 [100].

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, CAR and PXR, which regulate the expression of CYP2B and CYP3A genes, respectively [101, 102]. 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 other sensor receptors (LXR, FXR, and PPAR).

CAR mediates PB effects

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 [30, 103, 104].

Thus, CAR is not only responsible for the transcriptional up-regulation of CYP genes caused by PB, but also for other hepatic responses. Despite much progress in understanding CAR-dependent hepatic responses, the molecular mechanisms of CAR-mediated signal transduction remains enigmatic.

CAR and drug metabolism

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 [14, 30, 105, 106]. A recent cDNA microarray analysis showed that, upon PB CAR up-regulates a set of genes that encode CYP2B, CYP2C, CYP3A, NADPH-cytochrome P450 reductase, sulfotransferases, glucuronyltransferases and glutathione S-transfereases [14]. In addition to these phase I and II drug-metabolizing enzymes, CAR also regulates genes encoding transporters such as Mrp2 and Mrp4 [94]. As CAR can be activated by many different drug and xenobiotics, this receptor may be a central gatekeeper against toxicity and carcinogenicity in the liver. [60].

CAR and bilirubinemia

Because all the enzymes and transporters up-regulated by CAR are also involved in the metabolism of endobiotics secretion, the protective role of CAR is also extended to endogenous compounds. Bilirubin is the catabolic byproduct of heme derived from β-globin and cytochromes. It is one of the most toxic endobiotic compounds. Its accumulation causes hyperbilirubinemia which can leads to neuronal diseases. However, in a non pathological state, *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. The group of Masahiko Negishi found that CAR regulates the PB-induced transcription of the UGTA1 gene [107]. In addition CAR also mediates the PB induction of glutathione S-transferase A1/A2 and the transporters OATP and MRP2 to increase bilirubin clearance [108].

CAR and tumor promotion

The potential of human CAR as tumor promoter cannot be ruled out, because intriguing data in rodents suggest that not all mouse strains are susceptible to hepatocellular carcinoma (HCC) promotion by PB. Because CAR is activated by PB it was logical to examine whether CAR is involved in PB promotion of HCC. In two recent publications, CAR-null mice (CARKO) were used to investigate the role of CAR in the development of HCC. The data of the first publication present experimental evidence that CAR has an essential role in the liver tumor promotion induced by TCPOBOP, another strong tumor promoter [103, 104]. In the second study, liver tumors were initiated by a single injection of a typical genetoxic carcinogen diethylnitrosamine (DEN) in CAR wild type and CAR KO mice, subjected to chronic treatment with PB. After 35 weeks of PB treatment, HCC only developed in wild type mice. After 50 weeks, all wild type mice died of liver tumors, whereas CAR KO stayed protected from tumor or death [103].

Moreover, a locus on chromosome 1 associated to the HCC formation susceptibility is amplified in more than half of human HCC samples. 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 and thyroid function

As previously mentioned, the chronic treatment with PB or PB-like inducers is known to promote thyroid hypertrophy in humans [35] and to decrease the level of total serum T4, thus decreasing the metabolic rate. 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. It is well established that CAR target genes, such as UGT and SULT, which can metabolize thyroid hormones, are up-regulated during fasting [109]. It was thus reasonable to test if CAR plays a role in these events. 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 wild type animals [36, 109]. 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 glucose homeostasis

As previously mentioned PB has been shown to lower glyceamia in diabetic patients. Moreover, it was found that CAR regulates the PB-induced repression of PEPCK [14]. Kodama et al. recently showed that CAR and forkhead transcription factor, FOXO1, interact, thereby coregulating reciprocally CAR and FOXO1 target genes and affecting both drug metabolism and gluconeogenesis [110]. However these datas have to be interpreted with caution because no *in vivo* evidence supports these findings. The discrepancy of these results with our study will be discussed below.

Glucose metabolism

Few physiological parameters are more tightly and acutely regulated in humans than blood glucose concentration. Elevated glucose levels are rapidly returned to normal (5–6 mM) even after huge caloric ingestions, and they are maintained at only slightly lower levels during long-term starvation. Such control prevents severe dysfunctions such as loss of consciousness due to hypoglycemia and toxicity to peripheral tissues in response to the chronic hyperglycemia of diabetes.

Pancreas

High glucose levels influence gene expression either directly or through the stimulation of insulin production by the β-cells. Pro-insulin is synthesized in the β-cells of the pancreatic Langerhans islets and is cleaved by proconvertases into insulin and peptide C. Insulin is stored in secretory vesicles, and its secretion is directly linked to a sensing glucose-sensing mechanism availability via an increase in the intracellular ATP/ADP ratio that correlates with the entry and metabolism of glucose in the β-cells [111]. The entry of glucose into the β -cells requires a glucose transporter, Glut2 in rodents but Glut1 rather than Glut2 in humans [112], whose expression and membrane localization are

independent of glucose or insulin signalling. The posttranscriptional control of insulin expression and processing, as well as the control over the secretory mechanism, which are dependent on glucose sensing, are key features of the regulation of insulin signalling.

On the other hand, in response to stressful stimuli, such as hypoglycemia, glucagon is processed from a large precursor, proglucagon, in pancreatic alpha-cells. Glucagon primarily acts on liver to initiate glycogenolysis and gluconeogenesis, resulting in a rapid increase in endogenous production of glucose. With longer stimulation, glucagon action at the liver results in a glucose-sparing activation of free fatty acid oxidation and production of ketones (Figure 2). During hypoglycemia, glucagon secretion is clearly a protective feed-back, defending the organism against damaging effects of low glucose in brain and nerves (neuroglycopenia).

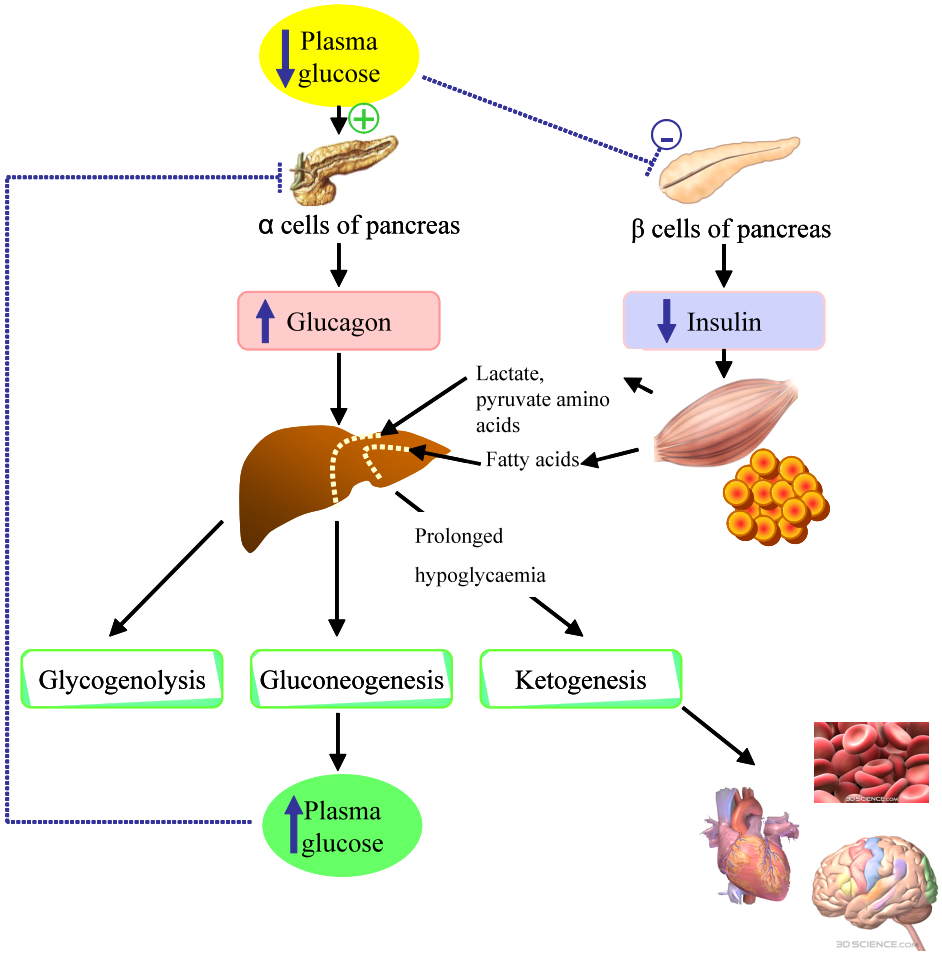


Figure 2: **Regulations of blood glucose**

Glyceamia is permanently regulated by the glucagon/insulin ratio. In the post-absorptive state, insulin promotes glucose uptake and glycogen synthesis in muscles, whereas during fasting glucagon induces glycogenelysis, hepatic glucose production and ketogenesis in sustained fasting.

Muscle

The major cellular mechanism that diminishes blood glucose when carbohydrates are ingested is insulin-stimulated glucose transport into skeletal muscle. Skeletal muscle both stores glucose as glycogen and oxidizes it to produce energy following the transport step. The principal glucose transporter protein that mediates this uptake is GLUT4, which plays a key role in regulating whole body glucose homeostasis [113]. Early studies using indirect calorimetry in combination with femoral vein catheterization and the euglycemic-hyperinsulinemic clamp suggested that nonoxidative glucose metabolism was the major pathway for glucose disposal in healthy subjects [114, 115]. These data suggested that during a hyperglycemic-hyperinsulinemic clamp, skeletal muscle accounts for the vast majority of glucose uptake in normal humans and that over 80% of this glucose is then stored as muscle glycogen [116]. Moreover, during fasting, the muscle provides lactate, pyruvate and amino acids from protein catabolism to the liver. In the hepatocytes, these compounds will then be used as gluconeogenic substrate.

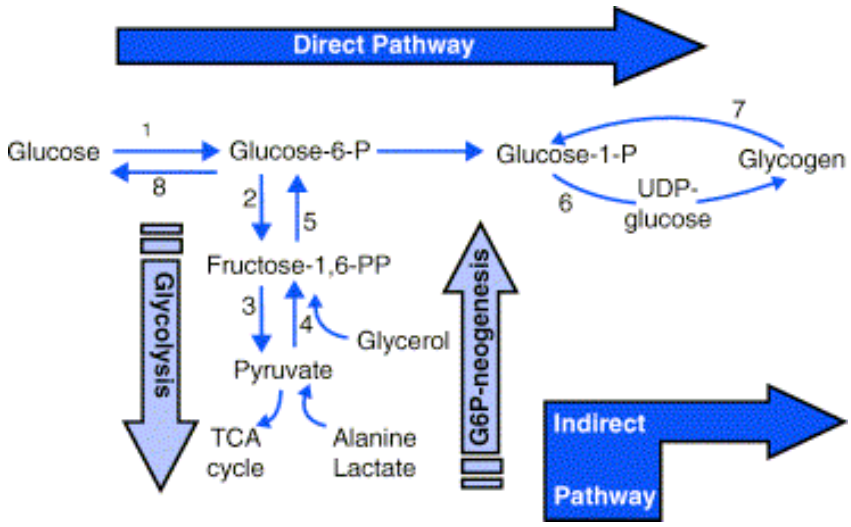
Adipose tissue

Traditionally, the major function of adipose tissue is considered to be energy storage. When fuel is required, (e.g. during periods of fasting, starvation, or exercise) free fatty acids (FFAs) are released from adipose triglyceride stores into the circulation by lipolysis and oxidized, primarily by skeletal muscle, to provide energy. However, a whole arsenal of evidence suggests that adipose tissue also plays an important part in other physiological processes; for example, as an endocrine organ. Adipocytes secrete proteins involved in a variety of functions, including glucose homeostasis. Early studies determined that adipose tissue accounts for only a fraction of glucose disposal after a meal (about 10–15%), with most of the rest taken up by muscle [117]. Nonetheless, it was equally clear that alterations in adiposity have profound implications for glucose homeostasis; too much fat (obesity) and too little fat (lipodystrophy) are both associated with insulin resistance and hyperglycemia. Moreover, Adipocytes secrete proteins with anti-diabetic action including leptin, adiponectin, omentin and visfatin, whereas other factors tend to raise blood glucose, such as resistin and TNF-α [118]

Liver

The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in a normal range, expressing a number of enzymes that are alternatively turned on or off depending on blood glucose levels. In the post-absorptive state, 75 % of the total glucose disposal occurs in insulin-independent tissues, approximately 50 % in the brain and 25 % in the splanchnic area including the liver [119]. Glucose utilization, which averages approximately 2.0 mg/kg/min, is exactly balanced with the production of glucose from the liver. Gluconeogenesis accounts for a substantial fraction (64 %) of total glucose production even during the first 22 hours of a fast in humans [120]. In the post-prandial period, the liver takes up a portion of ingested carbohydrates to restore glycogen stores, however the majority (approximately 80-85 %) of glucose disposal occurs in muscle tissue. After glucose enters a cell it can be either converted to glycogen (mainly in muscles (70 %)), or engaged in the pentose phosphate pathway or enters the glycolytic pathway. In addition, the liver is the principal site of insulin degradation, eliminating up to 70 % of the portal insulin [121].

Upon entry into the cells, glucose is phosphorylated to Glucose 6-Phosphate (G6P) by Glucokinase (GK) in hepatocytes and by hexokinase in all other cells [122]. This step is required for glucose to undergo glycolysis, be used in the glycogen synthesis pathway, or enter the pentose phosphate pathway (Figure 2). This first metabolic transformation of glucose is also required for generating the signal that acts in transcriptional regulation. Some reports suggest that G6P itself might be the signal for glucose uptake in the cell [123]. Alternatively, other metabolites such as xylitol produced by the pentose phosphate pathway or intermediates of the hexosamine biosynthetic pathway might also act in tissue-specific regulations [124].



From Roden et al. [117]

Figure 3: **The fate of glucose in the hepatocyte**. [1]glucokinase, [2]phosphofructo-1-kinase, [3]pyruvate kinase, [4]phosphoenolpyruvate carboxykinase (PEPCK), [5]fructose-1,6-biphosphatase, [6]glycogen synthase, [7]glycogen phosphorylase, [8]glucose-6-phosphatase.

Glucose homeostasis

Metabolic role of gluconeogenesis

Gluconeogenesis provides glucose to the tissues of the body in the fasted state when dietary carbohydrates are not available, by formation of glucose from noncarbohydrate sources. The gluconeogenic process clears metabolic products, such as lactate and amino acids from protein catabolism produced by muscle from circulation and erythrocytes as well as glycerol produced by adipose tissue. The regulation of endogenous glucose production is crucial to the control of blood glucose concentrations, and the liver and kidney are the principal organs responsible for gluconeogenesis.

Many of the enzymes of glycolysis and gluconeogenesis are shared, including those from phosphoenolpyruvate to fructose 1,6-diphosphate (Figure 2). In liver, glucose-6-phosphatase catalyzes the rate-limiting step of gluconeogenesis. However, for gluconeogenesis to occur, the enzymes pyruvate carboxylase (PC), phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) must be present and can limit flux through the gluconeogenic pathway [125].

PEPCK

Metabolic role of PEPCK

Cytosolic form of phosphoenol pyruvate carboxykinase (PEPCK-C) (EC 4.1.1.32) is a key enzyme in the synthesis of glucose in the liver and kidney [126]. In fact, the gene for PEPCK-C has become a marker gene for hepatic glucose output during fasting or in diabetes, or where the mechanism of action of hormones, transcription factors, or drugs of virtually every category is being assessed. However, PEPCK gene expression also occurs in tissues that do not make glucose, such as white and brown adipose tissue, and tissues where its role is unknown, such as: lung, mammary gland during lactation, skeletal muscle and the pancreas [127]. Despite the general lack of attention to the metabolic role of PEPCK in these tissues, there is a growing body of evidence regarding the alternative biological role for PEPCK in tissues other than the liver. In white [128] and brown adipose tissue [129], for example, PEPCK-C has been shown to participate in an abbreviate version of gluconeogenesis, termed glyceroneogenesis, a process that generates 3-phosphoglycerol from pyruvate and its precursors.

In both liver and kidney various factors increase PEPCK transcription, including glucagon, glucocorticoids and thyroid hormone, whereas insulin inhibits this process.

Regulation of PEPCK by the insulin/glucagon ratio

In mammals glycaemia is controlled by tight regulated ration between glucagon and insulin. Indeed, in healthy animals, a decrease in plasma glucose results in the release of glucagon by pancreatic α-cells and glucocortioids by the adrenal gland. These hormones both increase the rate of PEPCK gene transcription. The released glucagon, reaches the liver by the portal vein and bind to a protein G coupled receptor to induce the cAMP pathway. The glucagon-induced production of intracellular cAMP activates protein kinase A (PKA). Roesler and colleagues have developed a model for the cAMP control of PEPCK-C gene transcription that involves a cAMP regulatory unit (CRU), which encompasses five CREs that have been described in the PEPCK-C gene promoter [130]. Since a number of transcription factors can bind to these sites in the CRU (see above for details), and all are present in the liver, their specific interaction on the promoter is considered as a critical element in determining the response of the PEPCK-C gene to

changes in the concentration of hepatic cAMP. However, the most important factor that mediates the cAMP response on endogenous glucose production (EGP) is the cAMP Response Element Binding protein (CREB). This transcription factor is phosphorylated by PKA, thereby leading to its binding to the PEPCK promoter.

Glucocorticoids play a fundamental role in the maintenance of energy homeostasis in mammals. The removal of the adrenals severely compromises the ability of animals to withstand fasting and greatly diminishes the symptoms that are characteristic of diabetes, such as an elevated concentration of blood glucose. A key aspect of this response is the control of PEPCK-C gene transcription by glucocorticoids in several target tissues. Glucocorticoids stimulate transcription of genes in the liver that leads to the elevated level of hepatic glucose output noted during fasting. The concept of a large and complex Glucocorticoid Response Unit (GRU) was introduced by Granner and colleagues [131, 132]. They first described the existence of a GRU in the PEPCK-C gene promoter, using rat hepatoma cells, and then characterized this unit using a relatively short segment of the PEPCK-C gene promoter (-600 to +68) [133]. The GRU co-ordinates the hormonal response of the PEPCK-C gene to glucocorticoids and interacts with the CRU to mediate the cAMP control of gene transcription. Altogether, these datas suggest a cooperative action of glucagon and glucocorticoids on PEPCK gene transcription.

High plasma glucose stimulates insulin secretion and insulin will inhibit the action of glucagon and glucocorticoids in a dominant fashion [133-136]. The mechanisms responsible for the regulation of PEPCK-C gene transcription by insulin have been a major area of interest almost since the discovery of the enzyme in 1953 [137] and the realization that it was a critical step in hepatic glucose output in mammals. In 1963, Shrago and colleagues [138] published the first systematic analysis of the regulation of PEPCK-C gene expression by hormones, including insulin. They demonstrated that the injection of insulin into diabetic rats caused a decrease in the activity of hepatic PEPCK-C to below basal levels. Latter on, The isolation and characterization of the gene for PEPCK-C provided the opportunity to directly study the mechanisms involved in the effect of insulin on PEPCK-C gene transcription. Soon after the isolation of the gene, it was demonstrated, using "run off" transcription assays, that insulin decreased the rate of PEPCK-C gene transcription in hepatoma cells [139] and in the livers of diabetic rats [140]. Magnuson and coworkers reported that insulin blocked the induction of

transcription from the PEPCK-C gene promoter (+69 to -2100) by dexamethasone and cAMP in H4IIE hepatoma cells [141]. An insulin response element (IRE) was later mapped at -416 to -407 in the PEPCK-C gene promoter. When this putative IRE was mutated, transcription from the PEPCK-C gene promoter in hepatoma cells was reduced by 50% [142]. This IRE lies within the glucocorticoid regulatory unit (GRU), overlapping an Accessory Factor binding region (AF2) that is required for glucocorticoid stimulation of PEPCK-C gene transcription (Figure 3).

Partial anatomy of the PEPCK gene promoter

The sequence of the PEPCK gene promoter from the rat [143], human [144] and mouse [145] have been described. Interestingly, the sequence of these three mammalian species share more than 95% of similarity from the start site of transcription to -1500. The regulatory elements responsible for the dietary and hormonal control of PEPCK-C gene transcription in the liver that have been studied to date bind within a region between -1500 and +73 of the PEPCK promoter. This part of the promoter can be arbitrarily divided into three regions based on the clustering of regulatory elements (Figure 4) and the functional role of the binding sites in these regions.

The *Region 1* contains the CRE and an adjacent NF-1 site (P1) (Figure 3). Mutation of the CRE markedly reduces the cAMP responsiveness of the PEPCK gene promoter in the livers of transgenic mice [136], whereas a mutation of the NF-1 site causes a marked increase in the level of basal gene transcription [146, 147]. The CRE from the PEPCK-C gene promoter binds transcription factors, including CREB [148], CREM [149], C/EBPα [130], C/EBPβ [150] and others.

In addition the several sites which have been shown to be critical for the transcription of the PEPCK gene in the kidney, *region 2* contains a C/EBP binding domain, termed P3(I) [130, 151]. The C/EBP binding site at P3(I) is required for the full transcriptional responsiveness of the promoter to cAMP [136] and for the liver-specific expression of the PEPCK-C gene [127] (figure 4).

Region 3 is probably the most studied segment of the PEPCK gene promoter. It contains the Glucocorticoids Regulatory Unit (GRU) which conveys the glucorticoids-mediated induction of PEPCK. The GRU was originally mapped between -321 to -455 of the PEPCK gene promoter [133, 152] but recent studies have demonstrated that it extends to

-1500 [153]. The AF1 site binds the hepatic-enriched orphan receptors HNF-4α [154], COUP-TFII [155], PPARγ [156], the retinoic acid receptorg α (RARα) [157] and retinoid X receptor α (RXR α) [158]. The AF2 site binds members of the Forkhead family including HNF-3β (Foxo2a) and the phosphorylated form of Foxo1 [159]. The deletion of this site results in the inhibition of the diabetes-induced increase of PEPCK gene transcription in the livers of transgenic mice and renders the PEPCK gene promoter refractory to induction by glucocorticoids [160]. The AF3 site binds the thyroid hormone receptor [161, 162] and overlaps a SREBP-1c binding domain on the PEPCK-C gene promoter [163]. There are two additional upstream binding sites in the extended GRU that we have termed distal AF1 (dAF1) and distal AF2 (dAF2) that are required for optimal response of the hepatic gene promoter to. The dAF1 (a PPARγ 2 binding site) binds PPAR/RXR and HNF4α and is required for the expression of the gene for PEPCK-C in adipose tissue. Finally, there are also two SREBP-1c binding domains in this region of the PEPCK-C gene promoter, at -322 and -590, that are both *potentially* involved in the insulin inhibition of PEPCK-C gene transcription. The regulation of PEPCK by insulin will be discussed latter.

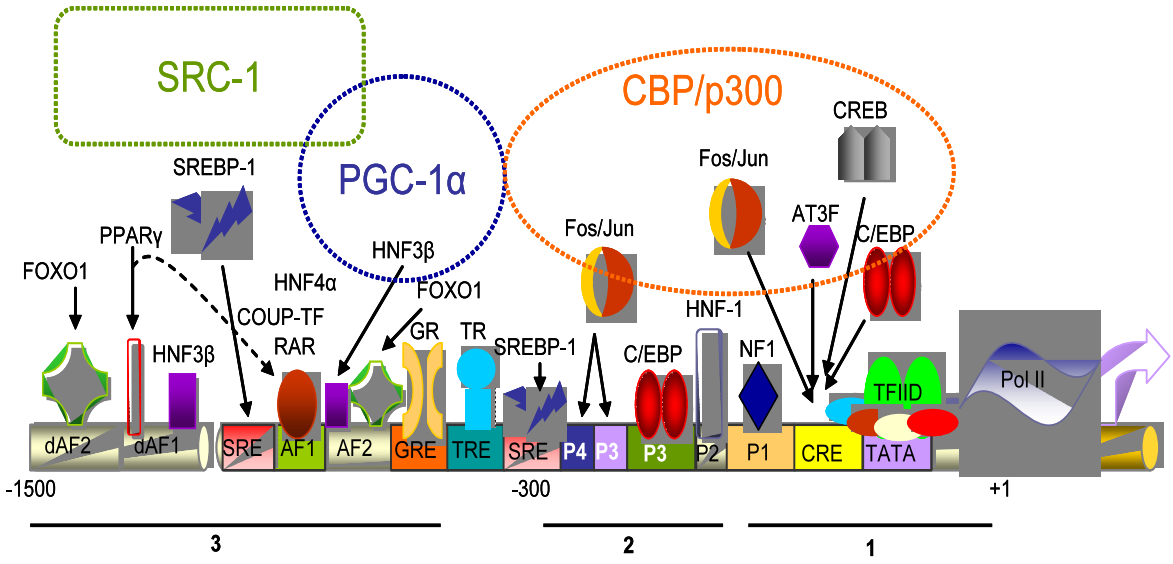


Figure 4: **A diagram of the PEPCK-C promoter showing the transcription factors and co-activators that regulate the transcription of this gene.**

CREB = cAMP Regulatory Element Binding Protein; ATF3 = Activating Transcription Factor 3; COUP-TF = Chicken Ovalbumin Upstream Promoter-Transcription Factor; PPARγ 2 = Peroxisome Proliferator-Activated Receptorγ 2; SREBP-1 = Sterol Regulatory Element Binding Protein; HNF = Hepatic Nuclear Factor; GRU = Glucocorticoid Regulatory Unit; T_3R, Thyroid Hormone Receptor; C/EBP = CAAT/Enhance Binding Protein; NF1 = Nuclear Factor 1; CREM = cAMP Regulatory Element Modifier; CBP/p300 = CREB Binding Protein/p300; PGC-1α = Peroxisome Proliferator-Activated Receptor Coactivator 1α; SRC-1 = Sterol Receptor Coactivator-1; RAR = Retinoic Acid Receptor; AF1 = Accessory Factor 1; AF2 = Accessory Factor 2; dAF1 = distal Accessory Factor 1; dAF2 = distal Accessory Factor 2; TRE = Thyroid Hormone Regulatory Element; Pol II = RNA polymerase II; GRE = Glucocorticoid Regulatory Element; CRE = cAMP Regulatory Element; P1, P2, P3(I). P3(II) and P4 are protein binding sites

Regulation of PEPCK by co-factors

Several transcriptional co-activators control PEPCK-C gene transcription; these include CREB Binding Protein (CBP) [147], sterol receptor co-activator 1 (SRC-1) [164] and PPARγ co-activator-1a (PGC-1α) [159]. CBP interacts with NF-1 [147], C/EBPβ and with SREBP-1c [165] to inhibit PEPCK-C gene transcription. Some reports suggested that SRC-1 interacts with HNF-4α, COUP-TFII and with HNF-3β, all of which are required for the maximal induction of PEPCK-C gene transcription by glucocorticoids and that the recruitment of SRC-1 is part of a transcriptional complex with CBP. PGC-1α coordinates the control of transcription of genes in the gluconeogenic pathway (PEPCK-C and G-6-Pase) [166]. Glucagon induces the levels of PGC-1α mRNA, and over-expression of PGC-1α stimulates transcription of the gene for PEPCK-C [166]. It is likely that PGC-1α is involved in coordinating the transcriptional response of hepatic PEPCK-C

to inductive stimuli, such as occurs during fasting. PGC-1α has also been proposed to be involved, together with Foxo-1 and HNF-4α, in the insulin-regulated inhibition of PEPCK-C gene transcription [159].

AMP-Activated Protein Kinase

The AMP-activated protein kinase (AMPK) is a ubiquitously expressed 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 stock decreases, AMP/ATP ratio increases followed by activation of AMPK which subsequently turns off ATP-consuming 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 5). AMPK activity is activated by a wide array of metabolic stresses, including hypoxia, ischemia, and oxidative and hyperosmotic stresses. Furthermore, exercise and glucose deprivation also activate AMPK, which suggests a role in exercise adaptations and pancreatic β-cell function (reviewed in [167]).

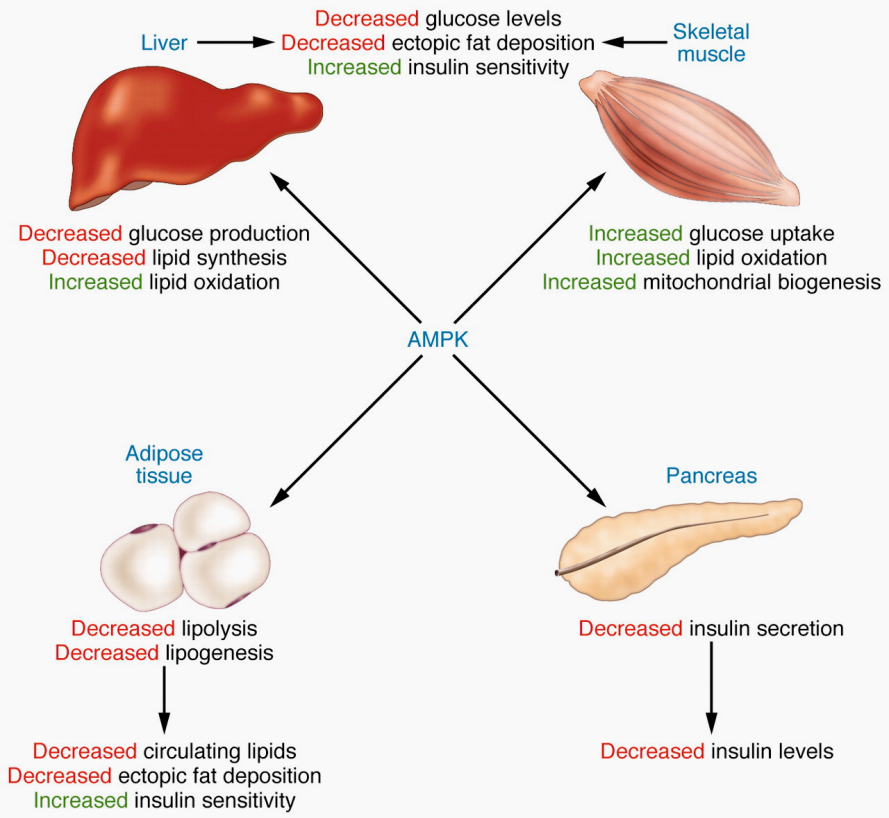
AMPK is a heterotrimeric enzyme that has been highly conserved throughout evolution as homologues of all three subunits that have been identified in plants, yeast, nematodes, flies and mammals [168, 169]. AMPKα is the catalytic subunit, which has to be phosphorylated by upstream kinases to be activated. β and γ are regulatory subunits, 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.

Recent data showed that high cellular glycogen represses AMPK activation in muscle in vivo [170, 171], suggesting that AMPK may regulates cellular glycogen content. Supporting this thesis, AMPK β subunits contain glycogen binding domains that could associate AMPK to glycogen metabolism in overexpression studies [172].

To date three AMPK upstream kinases were identified, LKB1, CaMKK α and CaMKK β, which by phosphorylation of threonine172 of AMPK α subunit activate the kinase in cells and *in vivo* [167]. 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 [173, 174]. In contrast, increases in AMP do not stimulate phosphorylation of Thr172 by the CaMKKs, which is triggered instead by a rise in Ca^{2+} [175-177]. CaMKKs

are mainly expressed in neural tissue; therefore their role in AMPK regulation in other tissue is not clear.

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, [178, 179], muscle contraction [180, 181] and by its regulation of insulin secretion in pancreatic β-cells [182].



from [183]

Figure 5: **Role of AMPK in the regulation of whole-body glucose homeostasis**.

Activation of AMPK turns on ATP-generating processes, while switching off ATP-consuming processes. In skeletal muscle, acute activation of AMPK increases glucose uptake and lipid oxidation, while chronic activation of AMPK is associated with mitochondrial biogenesis. Activation of AMPK inhibits glucose and lipid synthesis in the liver but increases lipid oxidation. Lipolysis and lipogenesis in adipose tissue are also reduced by AMPK activation

Aims

The aims of the present thesis are to clarify the molecular mechanisms by which phenobarbital regulates gluconeogenesis and to explore the role of CAR and AMPK in this process. Particularly, we investigated the role of CAR during fasting and its diverse effect on PEPCK promoter activation. Finally, we studied the complex interplay between CAR, AMPK and PGC-1α. The specific aims of this thesis can be divided into four parts:

Part I

Study of CAR mRNA expression and its target genes during fasting and in the absence of glucose in a mouse hepatoma cell line.

Part II

In vitro investigations of the role of CAR in the regulation of the PEPCK gene.

Part III

Study of the impact of fasting and high carbohydrate diet in transgenic mice in which CAR has been deleted.

Part IV

Investigations of the role of CAR in the regulation of AMPK expression in primary mouse hepatocytes.

Results

CAR as a glucose sensor ?

Animal studies reveal that fasting and caloric restriction produce increased activity of specific metabolic pathways involved in resistance to weight loss in liver. Evidence suggests that this phenomenon may in part occur through the action of the constitutive androstane receptor. However, currently, the precise molecular mechanisms that activate CAR during fasting are unknown and the role of CAR in the regulation of weight remains controversial. A recent study show that fasting coordinately induces expression of genes encoding PGC-1α, Cyp2b10, Ugt1a1, sulfotransferase Sult2a1, and organic anion-transporting polypeptide 2 (Oatp2), all CAR target genes mouse liver [184]. In the next section, we described experiments showing that, besides the induced CAR mRNA levels during fasting; CAR may also be involved in the regulation of glucose homeostasis.

CAR mRNA is induced by fasting and glucose depletion.

The expression of CAR (Figure 1.1A) and PEPCK (Figure 1.1B) increased following a 16 hours fasting period. Interestingly, the induction of both CAR and PEPCK is significantly reduced in the PB-treated animals. This result confirms that, in liver PEPCK is repressed by phenobarbital. However the repression of CAR is more intriguing especially because CAR has been previously shown to mediate PB repression on PEPCK [185]. In order to point out the molecular mechanism by which CAR expression is induced during fasting, we used a mouse hepatoma cell line (mAT3F). This cell line is very interesting because it has been previously described as a glucose-responsive system [186]. In fact, we were able to induce PEPCK (Figure 1.1D) and CAR (Figure 1.1C) in mAT3F cells cultured in glucose free medium for 48H. To allow gluconeogenesis to occur, we supplemented the medium with lactate and pyruvate (Cf material and methods). Surprisingly, phenobarbital induced PEPCK and CAR mRNA in the presence of 5mM glucose, whereas PB reduced their mRNA levels in glucose free condition. Here, we demonstrated that fasting and glucose depletion both can induce CAR and PEPCK, and this induction is attenuated upon PB treatment in mouse liver and in a hepatoma cell line.

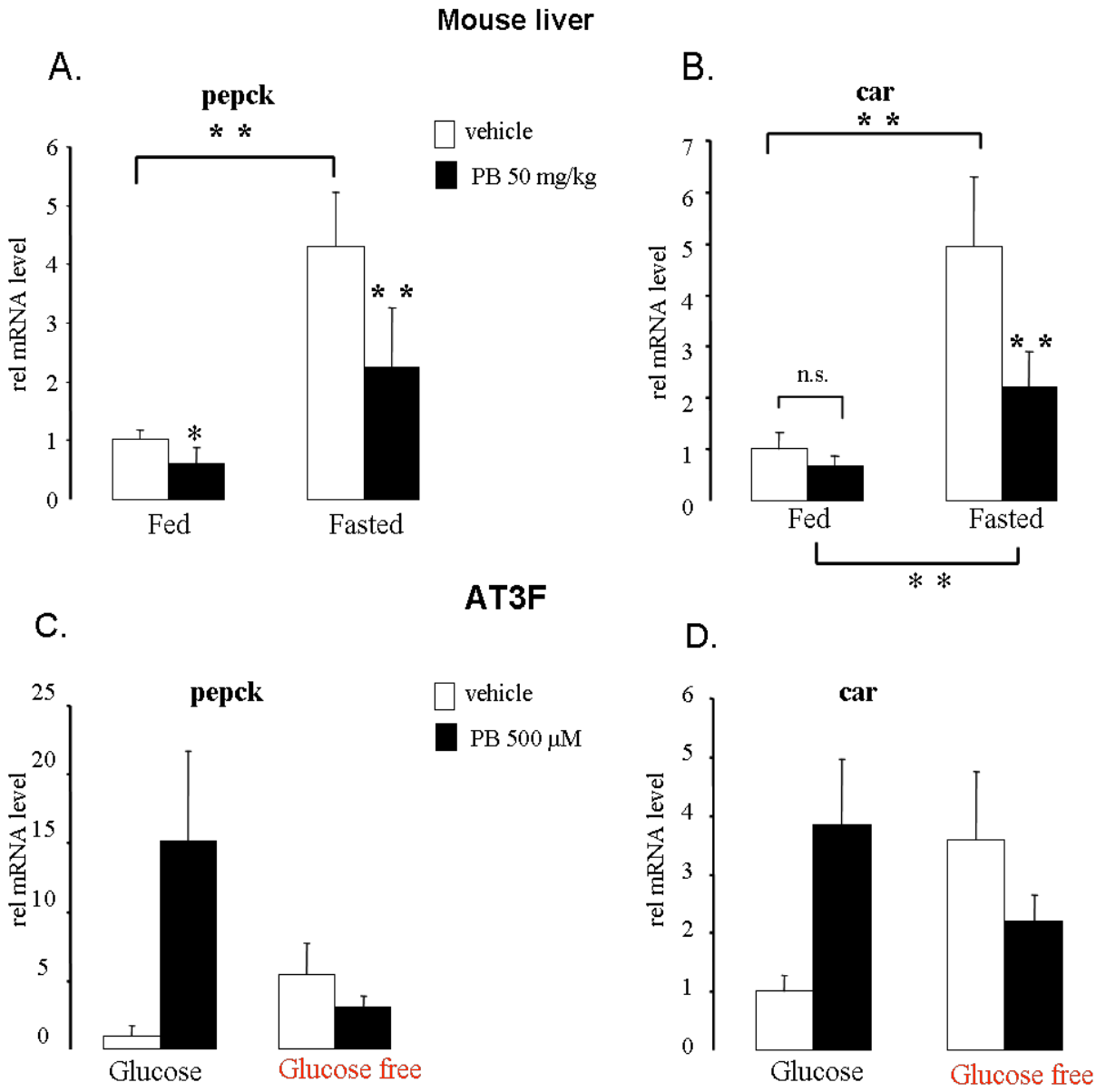


Figure 1.1 **CAR and PEPCK induced gene expression by fasting and glucose depletion.**
Total RNA was isolated from the livers of 16 hour fasted mice and injected with vehicle or PB 50 mg/kg. pepck (A) and car (B) mRNA were quantified by RT-PCR and standardized to GAPDH. Results represent means of five different mice ± S.D. with each determination done in triplicates (*, $p<0.05$). AT3F mouse hepatoma cells were cultured with or without glucose for 24h and treated with PB 500 µM for 16h. pepck (C) and car (D) mRNA were quantified by RT-PCR and standardized to GAPDH.

Interestingly, glucose depletion from the medium induces AMPK activation in both cytosol and nucleus of mAT3F cells (Figure 1.2). This activation is correlated with the phosphorylation of

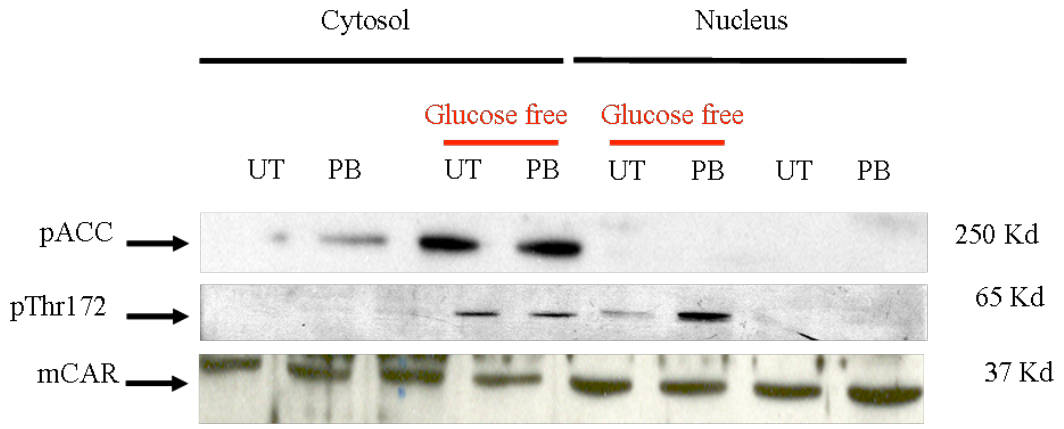


Figure 1.2 **AMPK activation by PB in the absence of glucose.**

AT3F mouse hepatoma cells were cultured with or without glucose for 24h and treated with PB 500 µM for 4h. Nuclear and cytosolic fractions were prepared according to the protocol described in material and methods. Expression of activated AMPKαt172, pACC and mouse CAR were shown by western blot.

acetyl-coA carboxylase (ACC), an important enzyme in the regulation of fatty acid oxidation and lipogenesis and a target of AMPK in the liver. Furthermore, AMPK activation only occurs in the absence of glucose. More interestingly, in the absence of glucose, phenobarbital activates AMPK more efficiently in the nuclei than in the cytosol of AT3F cells. Altogether, these results show that AMPK is activated by glucose depletion and that PB may differently activates AMPK according to its location in the cell. Suprisingly, in this experiments, neither glucose depletion nor PB treatment changed CAR location in the cell, even though the western blot showed that, in the presence of glucose and upon PB treatment, CAR has the tendency to accumulate in the nucleus (Figure 1.2 *lower pannel*).

CAR dependent effect induced by glucose depletion

It is well established that the induction of cyp2b10 during fasting is CAR dependent [109]. Here in, for the first time, we demonstrate that like CAR and PEPCK mRNA, cyp2b10 is induced by the absence of glucose in the culture medium in AT3F cells (Figure 1.3A). Unfortunately, no induction of cyp2b10 has been seen upon PB. Moreover, our experiments show that, upon glucose depletion, the cyp2b10 induction is

blunted by treatment with phenobarbital. It is likely, that glucose interferes with the PB induction in this mouse hepatoma cell line.

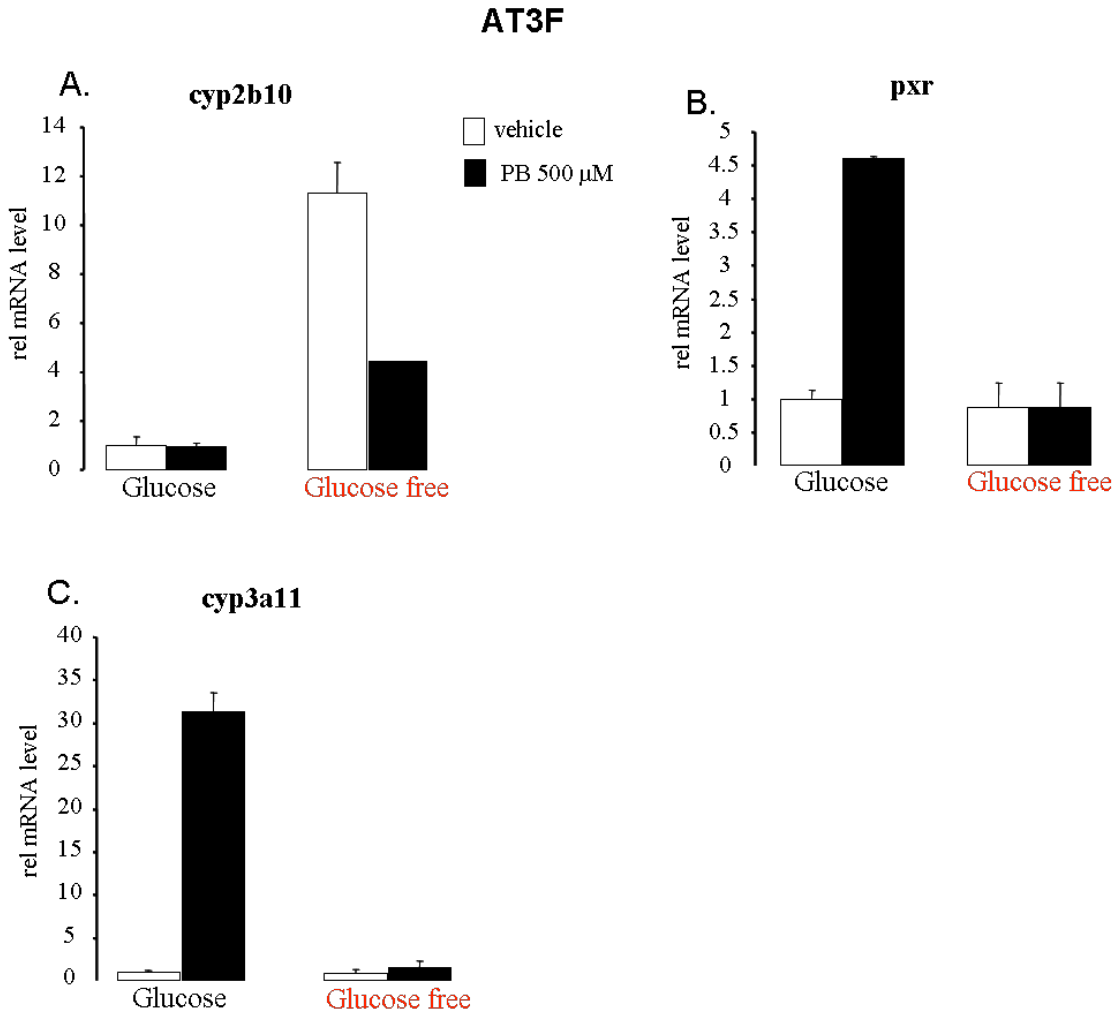


Figure 1.3 **cyp2b10 induction in the absence of glucose.**

AT3F mouse hepatoma cells were cultured with or without glucose for 24h and treated with PB 500 µM for 16h. cyp2b10 (A), pxr (B) and cyp3a11 (C) mRNA were quantified by RT-PCR and standardized to GAPDH.

The nuclear receptor pxr (Figure 1.3B) and its target gene cyp3a11 (Figure 1.3C) are both induced upon PB treatment, whereas no induction has been seen upon glucose depletion. Taken together, these results suggest that CAR is the only xenosensor whose expression as well as its target gene expression is regulated by glucose levels in the cell.

Effect of insulin on CAR mRNA expression

Since fasting and glucose depletion induced CAR expression, the effect of insulin was also tested. As previously mentioned (*introduction*), dexamethasone and cAMP mimics the fasting effects in the cell. Dexamethasone mimics corticoids effects and cAMP mimics the glucagon induced effect. The combinatorial effect of dexamethasone and cAMP induced PEPCK gene transcription (Figure 1.4A), PGC-1α (Figure 1.4B) and CAR (Figure 1.4C) expression in primary human hepatocytes in culture. It is well established that, in the post-prandial state insulin inhibits endogenous glucose production by repressing the expression of numerous gluconeogenic genes including PEPCK and PGC-1α. Here, we show that the effect of insulin is extended to CAR mRNA levels. Insulin represses CAR mRNA which suggests that CAR is an insulin responsive gene and it may be involved in the molecular mechanism that regulates the fasting-feeding transition. The possible role of CAR in glucose homeostasis will be discussed below (*discussion*).

CAR is involved in the regulation of PEPCK

Regulation of endogenous PEPCK

In order to test whether CAR can regulates PEPCK gene transcription, we transfected increasing amount of hemaglutinin-tagged human CAR into HepG2 cells (Figure 1.5A). This hepatoma cell line has been chosen because its glucose insensitivy. The activation of gluconeogenic genes and especially PEPCK has never been reported in this cell line. Thus, we then assessed PEPCK, by measuring mRNA levels via reversed-transcription experiments. PEPCK mRNA dose-dependently increased upon CAR transfection (Figure 1.5B). Furthermore, the PEPCK induction seems to be potentiated by dexamethasone and cAMP upon transfection of 5 μg of CAR cDNA. This result is another demonstration of CAR potency in dex/cAMP induction of PEPCK gene transcription. Interestingly, in HepG2 cells increased transfected amount of CAR repressed PGC-1α expression even in the presence of dexamethasone and cAMP (Figure 1.5C). Since PEPCK gene transcription is induced upon human CAR transfection without any correlation with PGC-1α expression, we can argue that PGC-1α mRNA induction is not necessary for the CAR-mediated induction of PEPCK promoter activity.

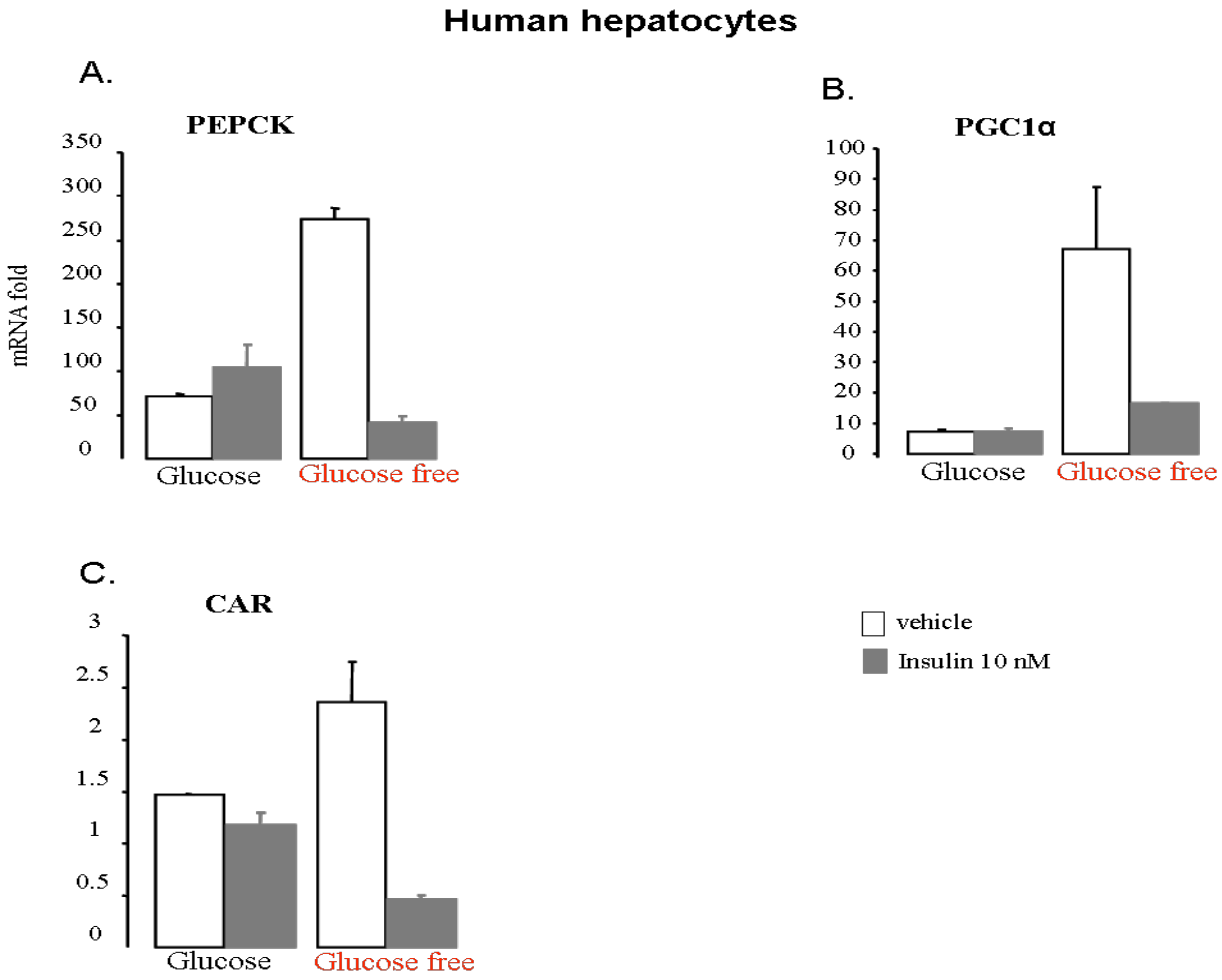


Figure 1.4 **CAR, PEPCK and PGC-1α expression in primary human hepatocytes in culture.**

Primary human hepatocytes were cultured with 5 mM or without glucose for 24h and treated with PB 500 µM for 16h. PEPCK (A), PGC-1α (B) and CAR (C) mRNA were quantified by RT-PCR and standardized to 18S. Results represent means of three different donors ± S.D. with each determination done in triplicates (*, $p<0.05$).

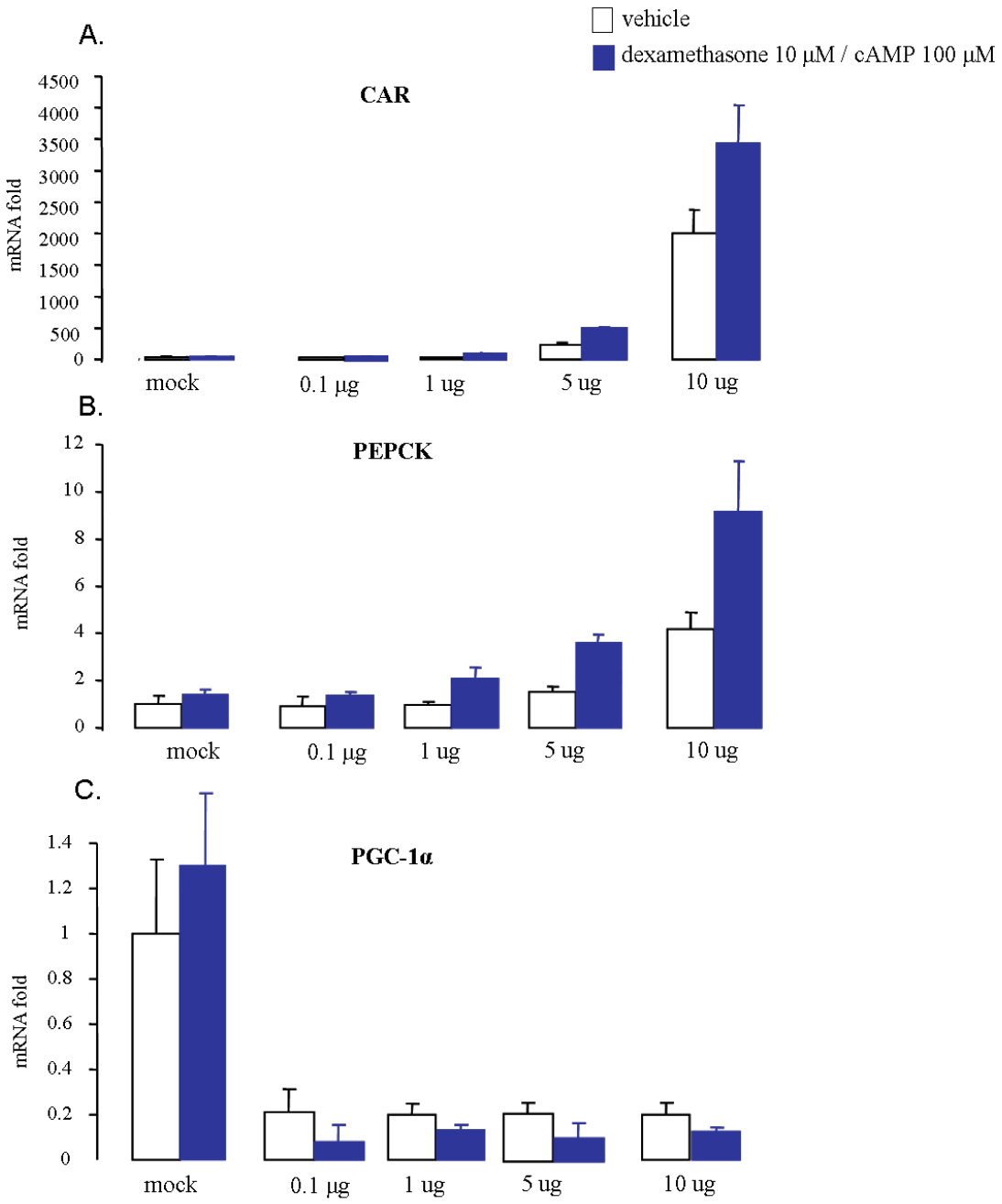


Figure 1.5 **CAR, PEPCK and PGC-1α expression in HepG2 cells.**

Human hepatoma cell line (HepG2) were cultured in DMEM 5 mM glucose and transfected with psG5 empty vector or 0.1, 1, 5 and 10 µg of psG5-hCAR. The cells were then treated with dexamethasone 10 µM and dibutyryl-cAMP 100 µM for 24h. CAR (A), PEPCK (B) and PGC-1α (C) mRNA were quantified by RT-PCR and standardized to 18S. Each determination was done in triplicates (*, $p<0.05$).

Positive effect of CAR on PEPCK transcriptional regulation is mediated by the -467/+67 enhanceosome

Since we showed that CAR induces endogenous PEPCK in HepG2 cells, it was logical to locate the targeted region of the PEPCK promoter. We then co-transfected human CAR and the -467/+67 fragment of the rat PEPCK promoter (PEPCK-LUC) in CV-1 cells. This fragment of the PEPCK promoter was shown to mediate most of the hormonal response that trigger gluconeogenesis during fasting. CAR dose-dependently increased PEPCK activity in HepG2 cells (Figure 1.6). This induction was even more potent upon dexamethasone/cAMP treatment. More importantly, the repressive effect of phenobarbital on induced-PEPCK promoter activity reached its maximum when the highest amount of CAR is transfected (Figure 1.6A). This finding confirms the role of CAR as mediator of the PB effect on PEPCK promoter activity. Interestingly, the combination of CAR transfection, phenobarbital and dex/cAMP treatment leads to the strongest repression of PEPCK activity.

In order to show that the activation of CAR by a ligand may also affect PEPCK promoter, we repeated the previous experiment in cells treated with a ligand of human CAR, 50 nM of 6-(4-chlorophenyl) imidazo [2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO). Suprisingly, CITCO did not repress PEPCK promoter activity, even in the presence of CAR (Figure 1.6B). Moreover, CITCO potentiates the inductive role of CAR on induced PEPCK promoter activity.

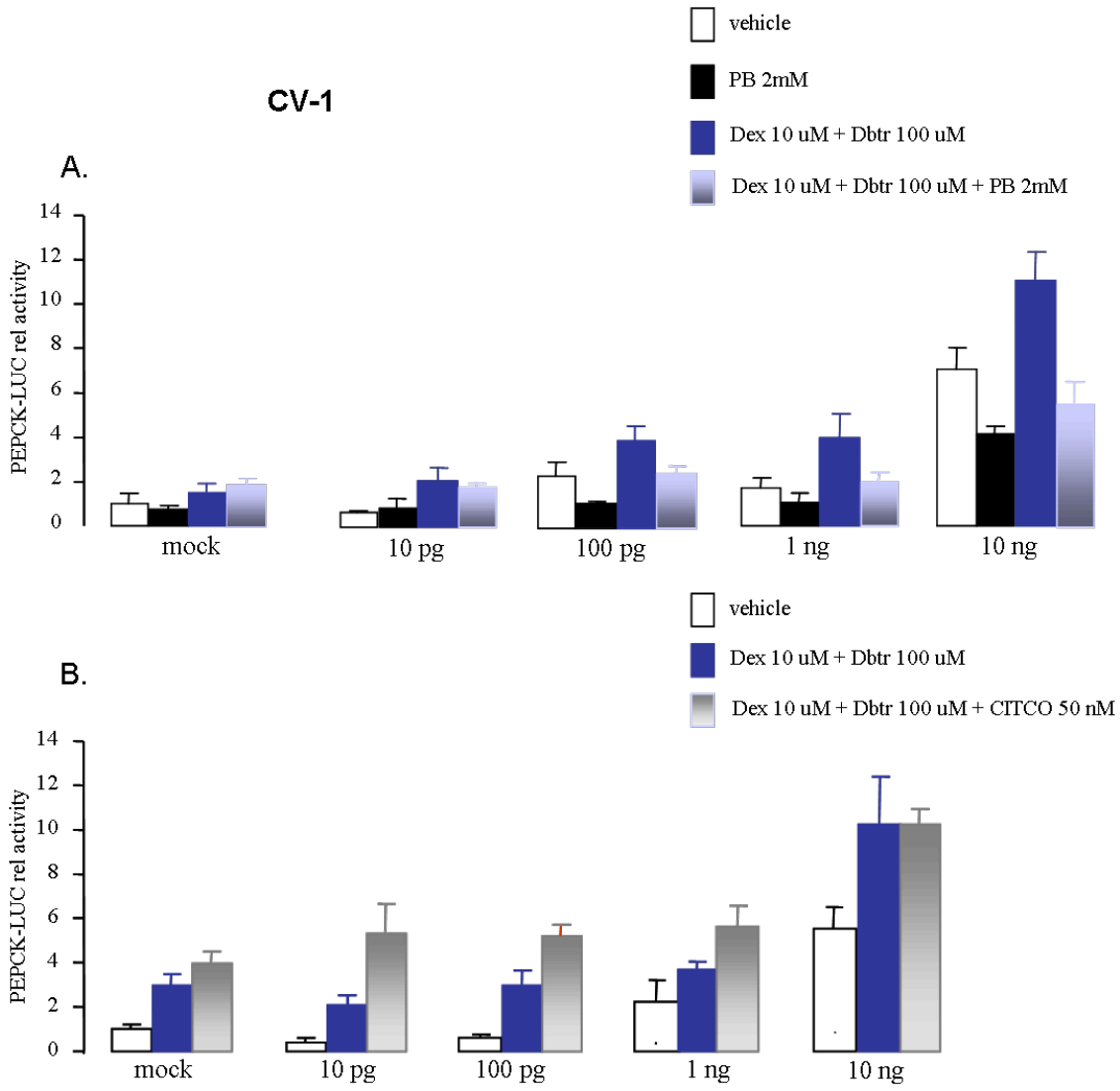


Figure 1.6 **CAR transactivates PEPCK promoter.**

CV-1 cells were co- transfected with psG5hCAR and -465+67 PEPCK-LUC promter. The cells were then treated with dexamathasone 10 µM (Dex), dibutyrylcAMP 100 µM (Dbtr) and PB 500 µM (A) or CITCO 50 nM (B) for 24h. The cells were lysed and luciferase activity was determined as described under material and methods. Each determination was done in fourplicates (*, $p<0.05$).

Protein Kinase A (PKA), a potential upstream regulator of CAR

Protein Kinase A (PKA) is the key enzyme that links the cytosolic increased cAMP concentration enhanced by glucagon, and the transcriptional activity mediated by CREB (cf introduction). To test whether this enzyme could be the upstream regulator of CAR-mediated induction of PEPCK, we co-transfected CAR and the catalytic subunit of PKA with PEPCK-LUC in CV-1 cells (Figure 1.7A). Here we show an additive effect of CAR and PKA on PEPCK promoter activity. However, no significant differences are noted between vehicle, CITCO or dexamethasone/cAMP treatment.

The treatment of the cells by meclizine, a described antagonist of human CAR, completely reversed the effect of cAMP and dexamethasone on PEPCK (Figure 1.7B). Interestingly, this effect was shown only when both CAR and PKA are co-transfected. Altogether these findings strengthen the role of CAR as an important mediator in fasting response, especially in the regulation of PEPCK.

This cooperation between CAR and PKA was intriguing; we thereby decided to test the effect of this co-transfection on CAR target DNA elements. We co-transfected CAR and the catalytic subunit of PKA with NR1-LUC, a 51 base pairs element found in the promoter of CAR target genes. Here we show that the cotransfection of CAR and PKA dose dependently activates NR1-LUC, suggesting that both CAR and PKA cooperates in the regulation of CAR target genes (Figure 1.7C). These findings reveal a potential cross-talk between drug metabolism and endogenous glucose production regulation.

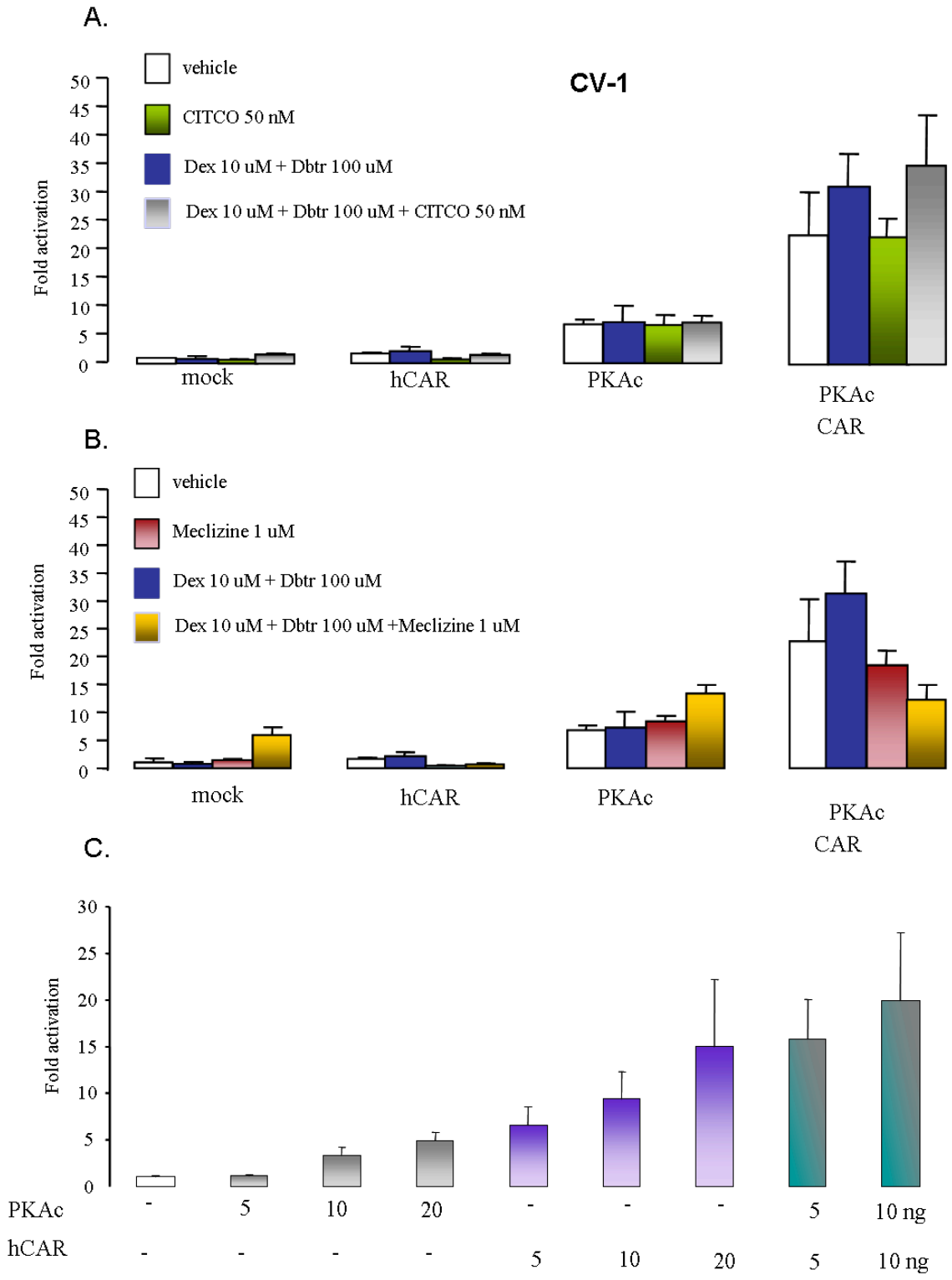


Figure 1.7 **PKA and CAR additive effects.**

CV-1 cells were co- transfected with psG5hCAR, active catalytic subunit of PKA (PKAc) and -465+67 PEPCK-LUC promter. The cells were then treated with dexamathasone 10 µM (Dex), dibutyrylcAMP 100 µM (Dbtr) and CITCO 50 nM (A) or meclizine 1 µM for 24h. The cells were lysed and luciferase activity was determined as described under material and methods.

Role of fasting and diet.

Metabolic parameters: Glyceamia and body weight

Most of the previous data presented in this thesis were achieved in human or mouse hepatoma cell line. However, the physiological relevance of CAR function in glucose homeostasis remains unclear. In order to understand, how CAR regulates PEPCK in vivo, wild type (wt) and CAR KO mice were fed, fasted and refed on a high carbohydrate diet (HC) according to the protocol showed in Figure 2.1 and described under material and methods.

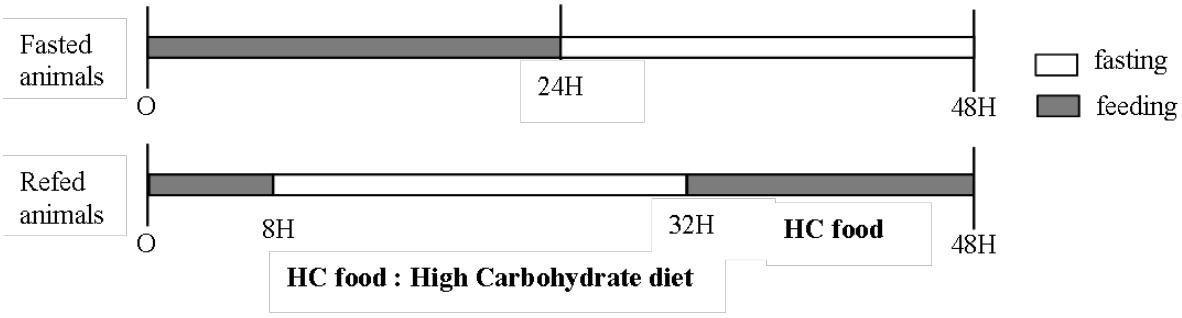


Figure 2.1 **High carbohydrate diet protocol.**

Mice were refed upon HC diet following fasting to boost endogenous glucose production. In our experiment, all the CARKO mice were slightly lighter than the wild type mice (Figure 2.2A). Interestingly, the refed animals are slightly heavier than the fasted ones, even though 16 hours of HC diet may not be enough to note a significant increase in total body weight. Nonetheless, the liver weight of refed animals is significantly higher than the fasted animals (Figure 2.2B), which suggest that 16h upon this diet compensates the fasting liver weight loss. These animals may have stored the excess of carbohydrates into glycogen and latter on into fat. Unfortunately, no accumulation of lipid droplet has been seen in those livers.

We also measured glycaemia in these animals. Surprisingly, in fed state, CAR KO had a lower glycaemia than wild type animals (Figure 2.2C). However, during fasting, glycaemia in both wt and CARKO decreased to 4 mM and, when refed on HC, glycaemia reaches back the same levels as in the fed state. These findings suggest that CAR is involved in the regulation of glycaemia during the fasting-feeding transition.

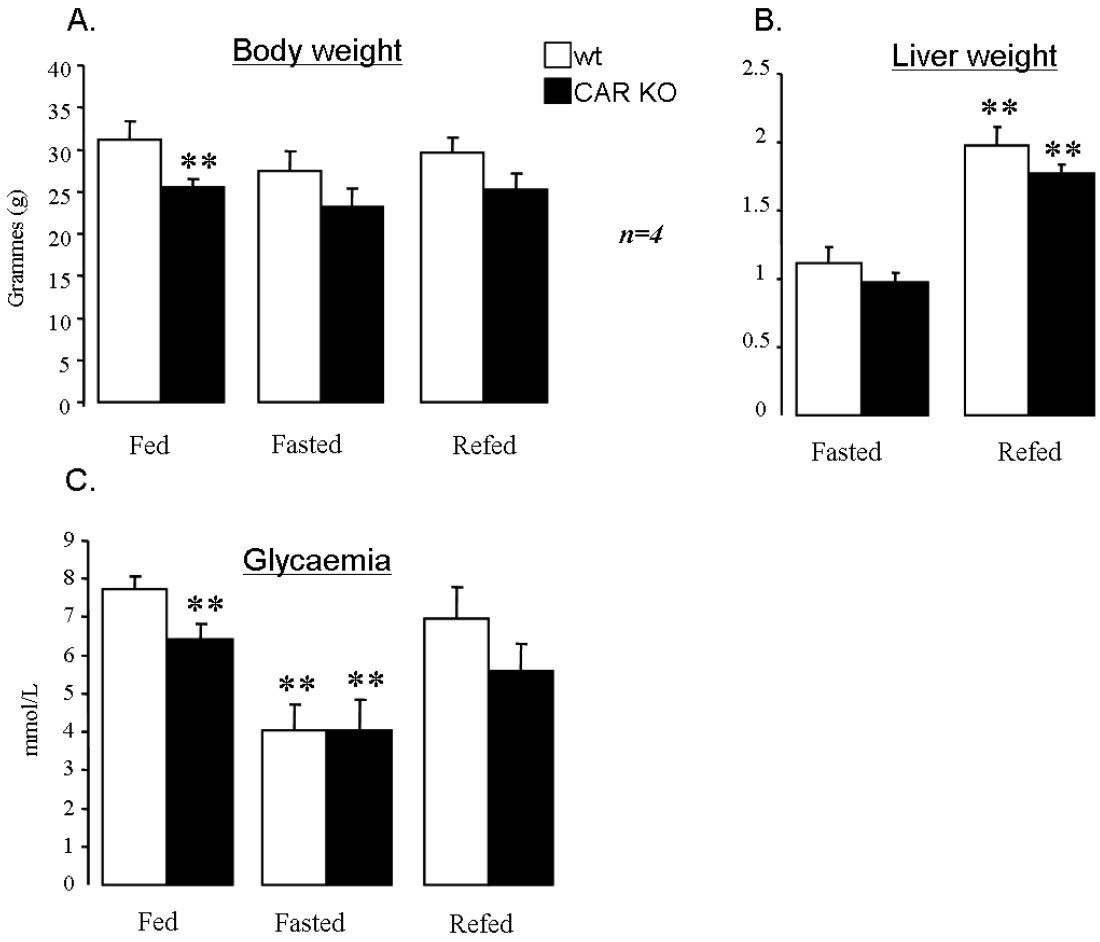


Figure 2.1 **CAR regulates glycaemia during fasting/feeding transition.**

Wild-type mice (□, open bars) and CAR KO mice (■, black bars) were fasted and refed according to the protocol previously described. Total body weight (A) and liver weight (B) were assessed the day of the sacrifice. Blood glucose (C) was measured at each nutritional transition. Results represent means of four different mice ± S.D. All values obtained are compared to the wild type fed animals. (**, $p<0.01$)

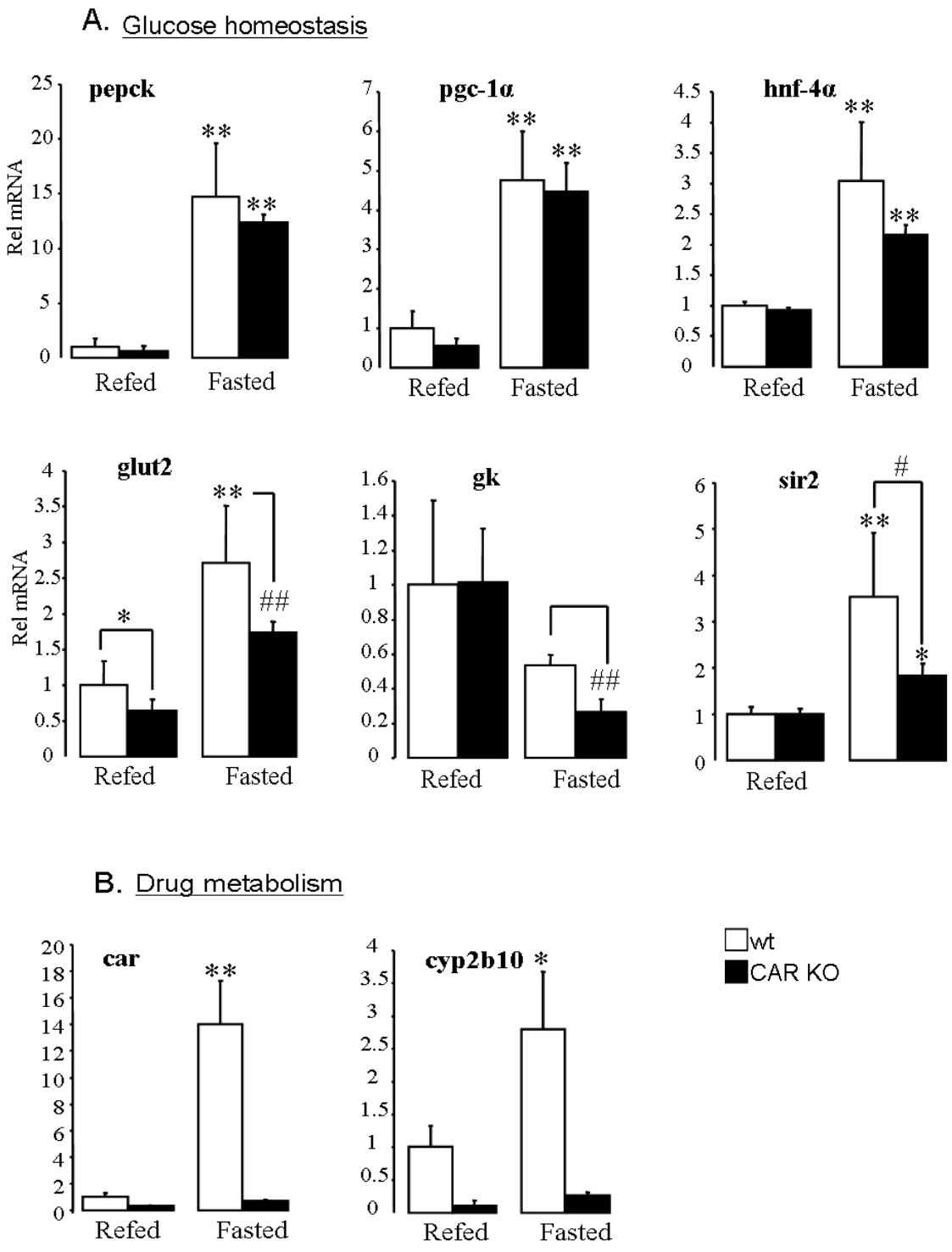
Glucose homeostasis and lipogenesis regulation by CAR.

To understand the role of CAR in the regulation of glycaemia, we performed quantitative PCR (qPCR) to assess the expression of several genes encoding for proteins involved in glucose homeostasis from the liver of wt and CARKO animals. Our data show that in both genotypes, pepck, pgc-1α and hnf-4α were nicely inducible upon fasting (Figure 2.2A *upper pannel*). No significant differences were noted between wt and CARKO mice, which suggest that the enhancement of gluconeogenesis is not affected in CARKO animals. However, in the fasted state, the expression of genes encoding for proteins involved in glucose transport (glut2) and phosphorylation (gk) in the liver is significantly

lower in CARKO livers (Figure 2.2A, *lower pannel*). Taken together, these data suggest a role of CAR in the regulation of glucose transport and phosphorylation in the hepatocyte. It is well established that the deacetylase sir2 modulates ageing in several species and controls the gluconeogenic/glycolytic pathways in liver in response to fasting. Here in, we found that sir2 was also downregulated in CARKO samples (Figure 2.2A, *lower pannel*).

As previously mentioned (results part I), fasting induces the expression of CAR and its target gene cyp2b10 in the liver (Figure 2.2B). Moreover, in CARKO livers, no induction of cyp2b10 was observed neither in refed nor in fasted state. This finding strongly supports the CAR dependency in cyp2b10 induction.

It is well established, that chronic feeding upon high carbohydrate diet can induce lipogenesis in the liver, because the excess of carbohydrate will ultimately be stored as fat. We then measured the genes encoding for proteins involved in lipogenesis in wt and CARKO livers. Surprisingly, in the fed state, the expression of srebp-1c is higher in CARKO livers (Figure 2.2C *upper panel*). However, in the fed state, the expression of all the other genes involved in lipogenesis (s14, acc1, acc2 and fas) is significantly lower in CARKO livers (Figure 2.2C *lower panel*). More importantly, during fasting, the expression of acc1, acc2, fas and s14 is higher in CARKO livers as compared to the wt livers. All these findings point out a new role of CAR in the regulation of nutritional transition. This role of CAR will be discussed below.


A. Glucose homeostasis
pepck
pgc-1α
hnf-4α
glut2
gk
sir2
Rel mRNA
Refed
Fasted
B. Drug metabolism
car
cyp2b10
wt
CAR KO

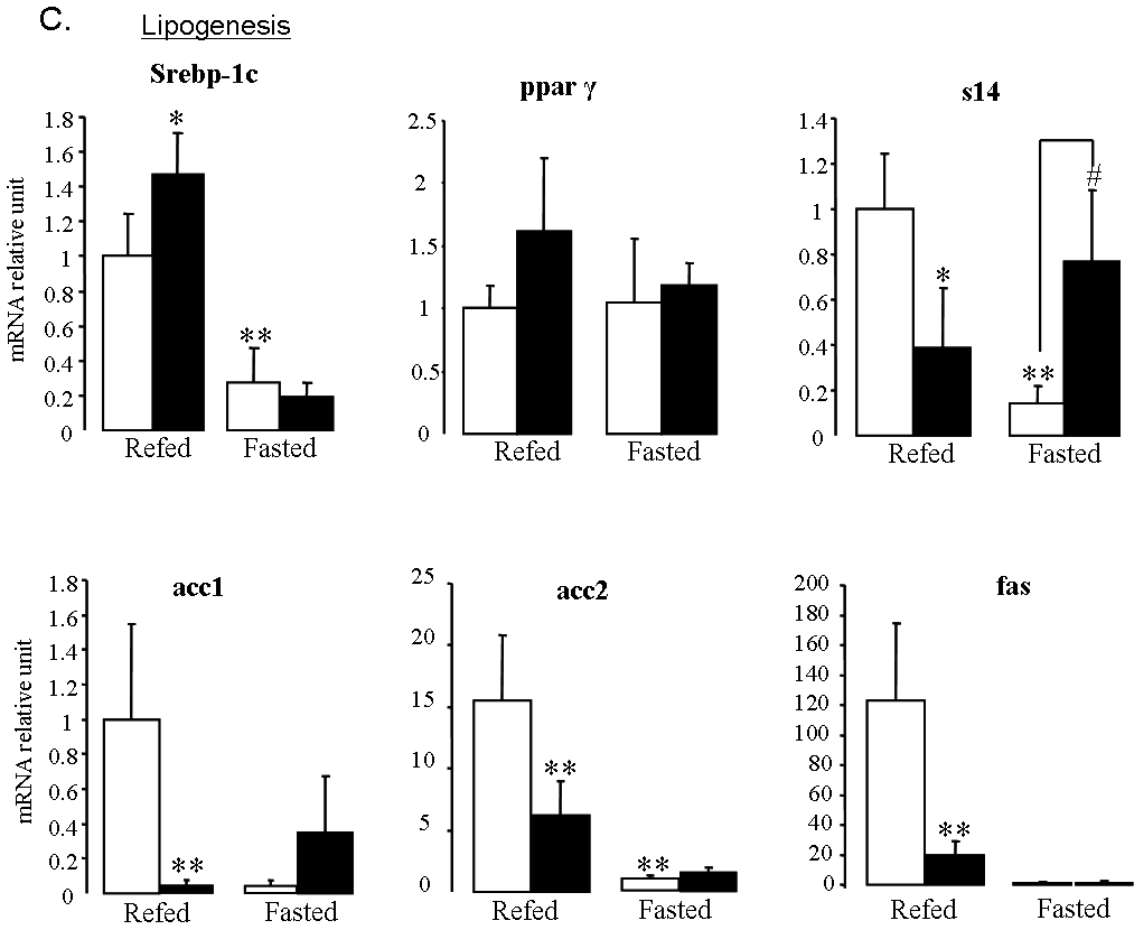


Figure 2.2 **CAR regulations of glucose homeostasis and lipogenesis.**

Wild-type mice (□, open bars) and CAR KO mice (■, black bars) were fasted and refed according to the protocol previously described. Total RNA was isolated from the livers and mRNA product from genes encoding for proteins involved in glucose homesotasis (A), drug metabolism (B) and lipogenesis (C) were quantified by RT-PCR and standardized to GAPDH. Results represent means of four different mice ± S.D. with each determination done in triplicates (*, $p<0.05$:**,$p<0.01$).

High carbohydrate diet

We previously showed that CAR regulates the expression of genes involved in glucose homeostasis and in lipogenesis. To understand how CAR regulates glucose homeostasis, we fed wild type and CARKO mice with a high carbohydrate diet / Low fat (HC diet) for 12 weeks (cf material and methods).

We measured glycaemia over 80 days, and surprisingly, HC diet did not significantly increase glycaemia in both groups. Blood glucose has been taken every morning at 8:00 am, unfortunately, in both groups, a lot of variations occurred from one mouse to another

(Figure 2.3A). More importantly, After 80 days under HC diet, CARKO mice exhibit a significant higher glycaemia than the wild type mice, which suggest than CAR may controls blood glucose concentration under chronic HC diet. Another surprising observation is the relative constancy of CARKO mice glycaemia over time as compared to the normal animals (Figure 2.3B)

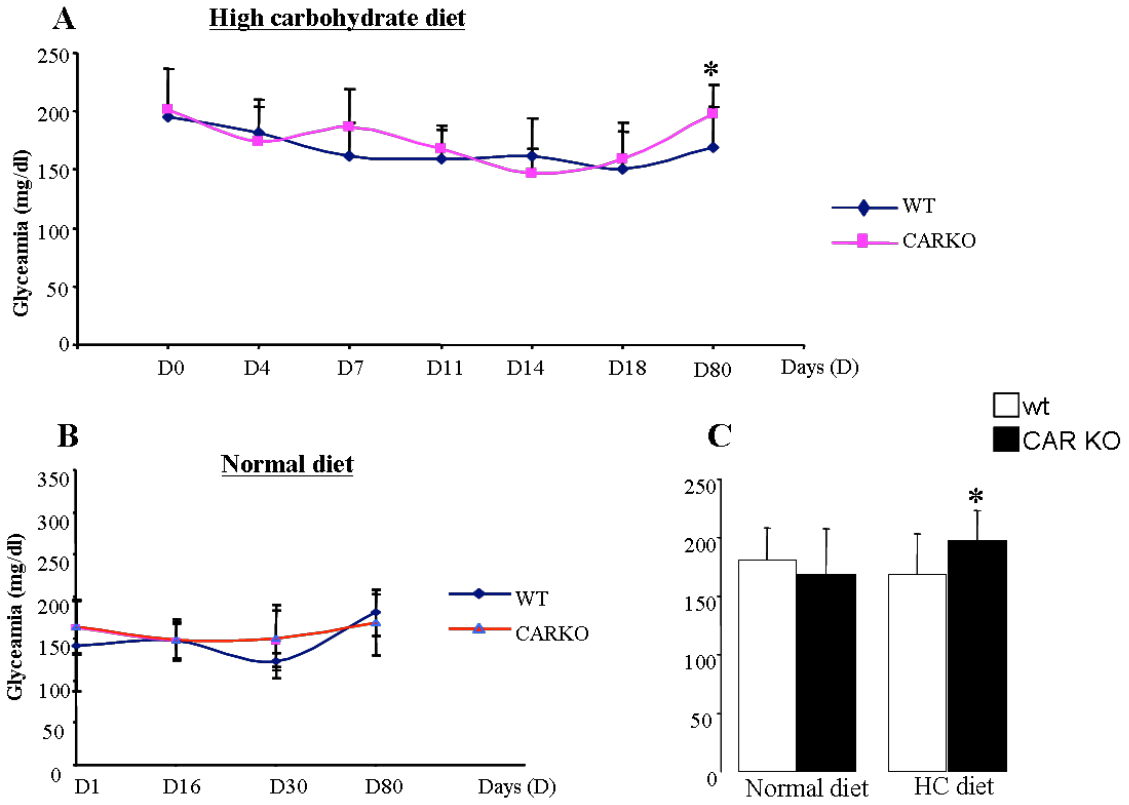
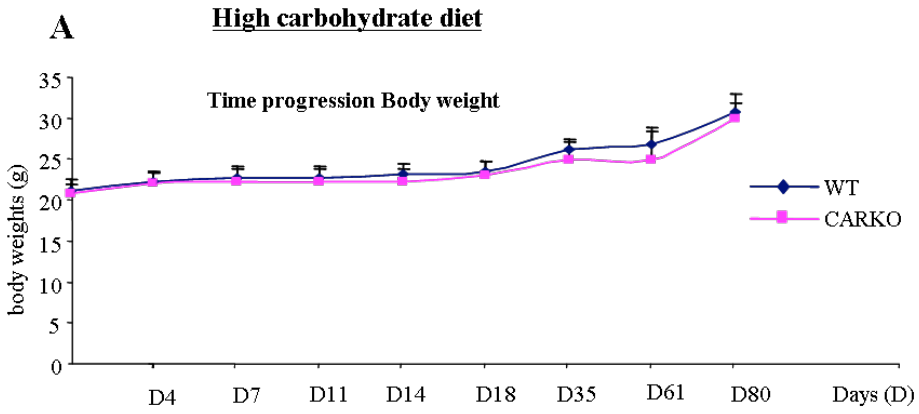


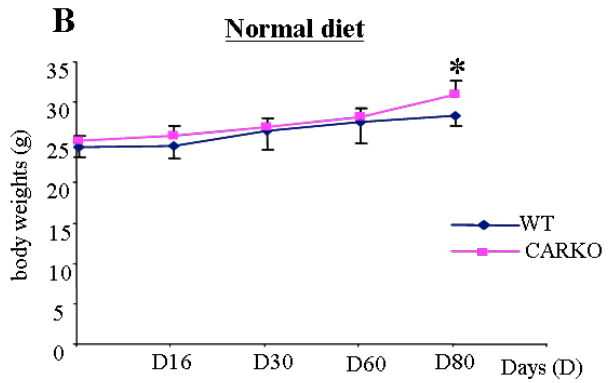
Figure 2.3 **Glycaemia.**

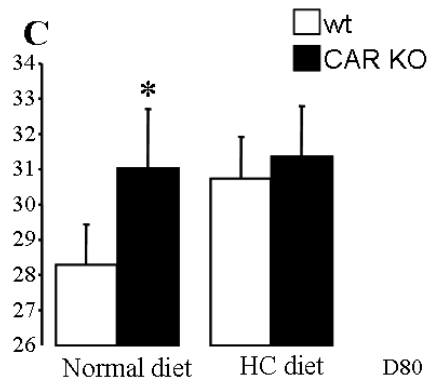
Wild-type mice (□, open bars) and CAR KO mice (■, black bars) were fed with a normal lab chow or with high carbohydrate diet (HC) according to the protocol described under mat et methods. Glycaemia was assessed over 80 days in both groups and under both diets (A) and (B). Blood glucose concentration measured the day of the sacrifice (D80) (C). Results represent means of five different mice ± S.D. All values obtained are compared to the wild type fed animals. (*, $p<0.05$).

As expected, during the study, total body weight increased over time in normal and CARKO mice (Figure 2.4 A,B). Moreover, normal animals fed under HC gained more weight than those fed under normal diet. (Figure 2.4D). Interestingly, only when fed under a normal diet, after 80 days, CARKO mice became heavier than the normal mice (Figure 2.4C). However, during the whole study, and independently of the diet, CARKO

animals gained more weight than the wild type ones, even though, this difference is bigger in the normal diet fed group (Figure 2.4D).







D

Normal diet

				weight gain (g)
WT	Day 1	mean	**24.38**	
		SD	1.21	
				3.89
	Day 80	mean	28.28	
		SD	1.15	

				weight gain (g)
CARKO	Day 1	mean	**25.17**	
		SD	0.64	
				5.85
	Day 80	mean	31.02	
		SD	1.70	

HC diet

WT	Day 1	mean	21.2	
		SD	1.31301	
				9.52
	Day 80	mean	30.7167	
		SD	1.19736	

CARKO	Day 1	mean	20.85	
		SD	1.06615	
				10.52
	Day 80	mean	31.3667	
		SD	1.41892	

Figure 2.4 **Total body weight.**

Wild-type mice (□, open bars) and CAR KO mice (■, black bars) were fed with a normal lab chow or with high carbohydrate diet (HC) according to the protocol described under mat et methods. Total body weight was assessed over 80 days in both groups and under both diets (A) and (B). Total body weight measured the day of the sacrifice (D80) (C). Results represent means of five different mice ± S.D. All values obtained are compared to the wild type fed animals. (*, $p<0.05$).

We then performed qPCR to assess, in the liver, the expression of genes encoding for proteins involved in glucose and lipid metabolism. After, 12 weeks under a normal chow diet, glut2 mRNA level is higher in CARKO as compared to wild type animals (figure 2.5A). Surprisingly, glut2 and carbohydrate response element binding protein (chrebp) (figure 2.5C) expression were not induced by HC diet, whereas glucokinase (gk) (figure 2.5B) expression tended to increase. These observations are may be due to a negative regulatory mechanism enhanced by a chronic ingestion of carbohydrate and can also explain why 12 weeks of HC diet did not increase glycaemia in these animals.

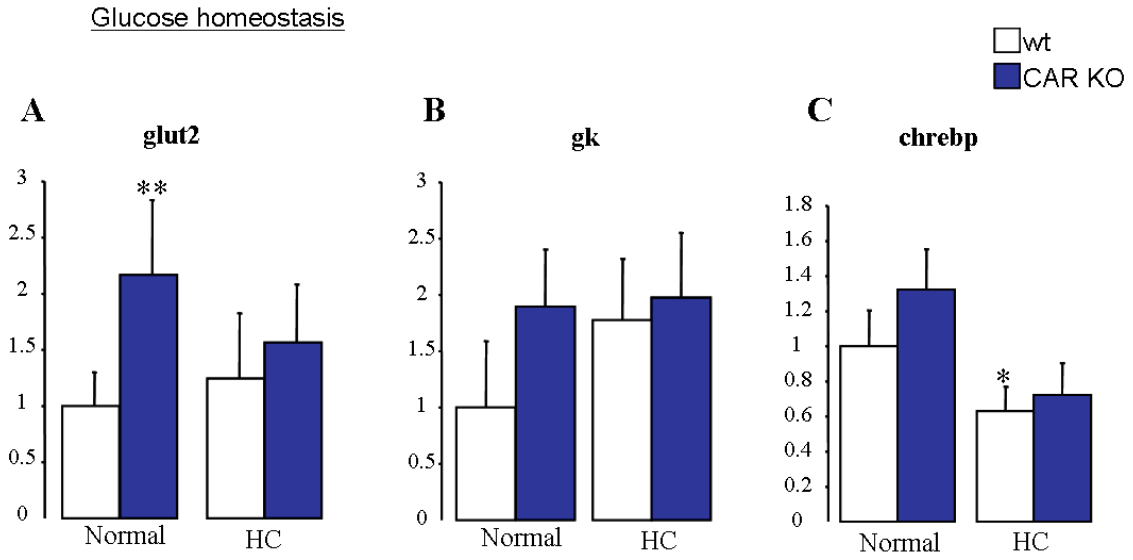


Figure 2.5 **High carbohydrate diet and glucose homeostasis.**

Wild-type mice (□, open bars) and CAR KO mice (■, blue bars) were fed with a normal lab chow or with high carbohydrate diet (HC) according to the protocol described under mat et methods. Total RNA was isolated from the livers and mRNA product from genes encoding glut2 (A), glucokinase (gk) (B) and carbohydrate response element binding protein (chrebp) (C) were quantified by RT-PCR and standardized to GAPDH. Results represent means of 6 different mice ± S.D. with each determination done in triplicates (*, $p<0.05$:**,$p<0.01$).

Interestingly, the expression of genes encoding for proteins involved in lipid metabolism such as fatty acid synthase (fas) (Figure 2.6A), s14 (Figure 2.6B) and acetyl-coA carboxylase-1 (acc1) (Figure 2.6D) are all induced upon HC diet in CARKO livers. Except for s14 expression, under normal diet, no differences in the expression of fas, cpt-1, acc1, acc2 and srebp1-c have been noted between wild type and CARKO livers. The mRNA levels of carnitine palmitoyl transferase (cpt-1) (Figure 2.6C) and acc2 (Figure 2.6F), two proteins involved in fatty acid beta oxidation, are induced under HC diet in

livers of normal mice. However, the mRNA level of cpt-1 is lower in CARKO livers, fed with HC diet. Finally, srebp-1c, an essential gene for lipogenesis was shown to be induced by insulin and by high fat diet. Unexpectedly, in our study, and as for most of the genes that we have measured, srebp-1c mRNA expression was not induced by HC diet (Figure 2.6E).

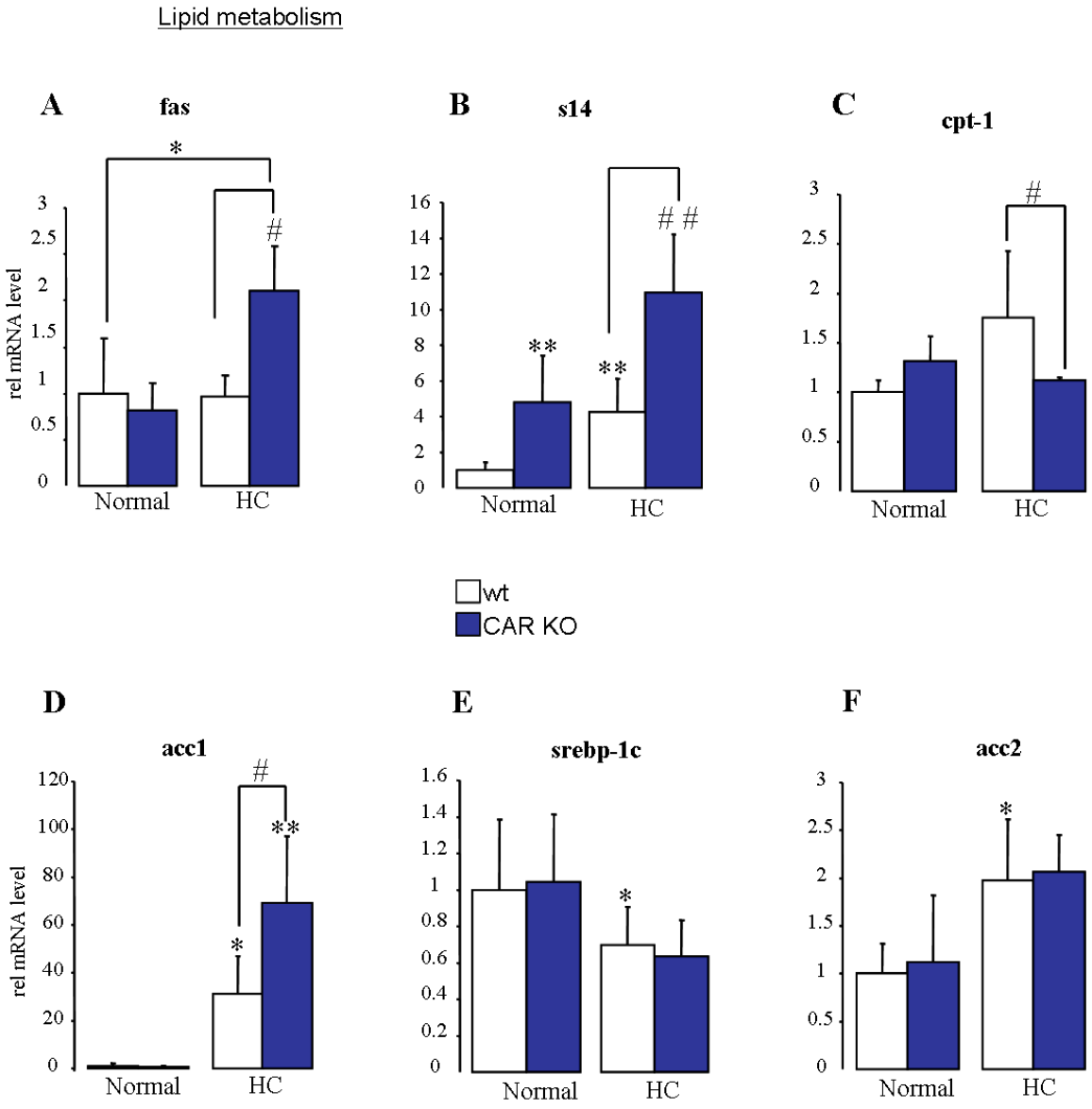


Figure 2.6 **High carbohydrate diet and lipid homeostasis.**

Wild-type mice (□, open bars) and CAR KO mice (■, blue bars) were fed with a normal lab chow or with high carbohydrate diet (HC) according to the protocol described under mat et methods. Total RNA was isolated from the livers and mRNA product from genes encoding fatty acid synthase (A), s14 (B), carnitine-palmitoyle-transferase (cpt-1) (C), acetycoA carboxylase (acc1) (D), srebp-1c (E) and acc2 (F) were quantified by RT-PCR and standardized to GAPDH. Results represent means of 6 different mice ± S.D. with each determination done in triplicates (*, $p<0.05$:**,$p<0.01$).

Interplay between CAR and AMPK

CAR was well established as a xenosensor in mediating the effect of PB in drug metabolism. However, as we previously mentioned, CAR was also described as a regulator of glucose homeostasis in mouse. Interestingly, we showed that PB increases the activity of AMP-activated kinase (AMPK). Indeed, this kinase is necessary to mediate the induction of CYPs by PB in human and in mouse livers [63, 64]. As AMPK was also found to regulate gluconeogenesis, and in order to point out a relationship between AMPK and CAR, we tested the regulation of AMPK activation in primary hepatocytes in culture from CARKO animals. The hepatocytes were cultured in glucose free or in 25 mM glucose containing medium for 24H and were infected with an adenovirus encoding for β-galactosidase (βgal) or for hematoglutinin-tagged human CAR (HAhCAR). We treated the cells with 500 μM phenobarbital or 1 mM metformin (Met) (Figure 3.1) and assessed AMPK activation by western blot.

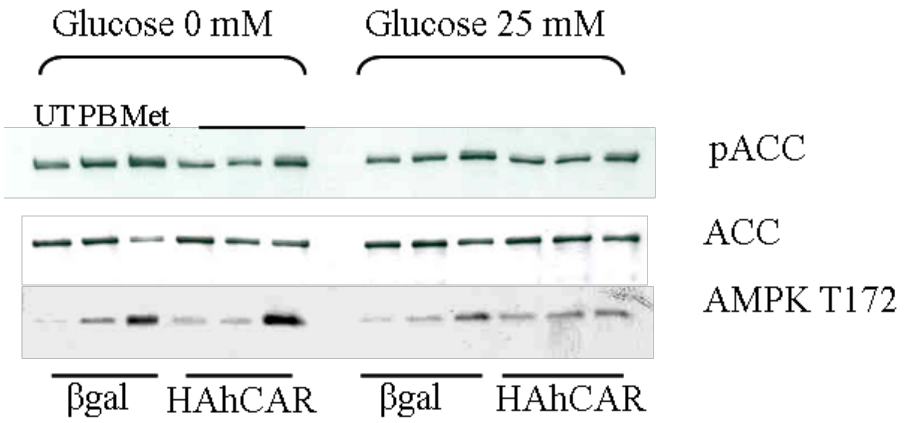


Figure 3.1 **AMPK activation in CARKO hepatocytes.**

Primary hepatocytes in culture from CARKO animals were cultured in glucose free or in a 25 mM glucose containing medium for 24H. The hepatocytes were infected with adenovirus encoding for HAhCAR or for an adenovirus encoding the β-galactosidase protein (βgal). The cells were treated for 4H with 500 uM of phenobarbital (PB) or metformin 1 mM (Met). The expression of phosphorylated AMPK Thr172 (ampkT172), acetyl-coA carboxylase (ACC) and the phosphorylated form of acc (pACC) were assessed by western blot.

In the absence of glucose, metformin is a better activator of AMPK than phenobarbital. However, upon 25 mM glucose, both drugs lose their efficacy in inducing AMPK. This effect seems to be even more pronounced in the presence of exogenous human CAR (Figure 3.1 *right pannel*). In this study, the activation of AMPK by phenobarbital and metformin leads to the phosphorylation of one of the AMPK target, the acetyl-coA carboxylase (pacc). Interestingly, the PB activation of AMPK is blunted in the presence of human CAR. This effect of PB on AMPK activation can be correlated with the phoshporylation of acc. Altogether, this data suggest that the PB-mediated activation of AMPK is glucose and CAR dependent.

To avoid the utilization of viruses and to strictly compare the activation of AMPK in CARKO and normal animals, we repeated the previous experiment without metformin treatment in hepatocytes from CARKO animals. Surprisingly, in this experiment PB did not have any effect nor on AMPK neither on pACC (Figure 3.2). However, it was intriguing to see that the expression of the AMPK α1 subunit was clearly higher in CARKO hepatocytes, whereas the expression of ACC and pACC were lower. In CARKO hepatocytes, the higher expression of AMPK α1 subunit did not correlate with a stronger phosphorylation of ACC.

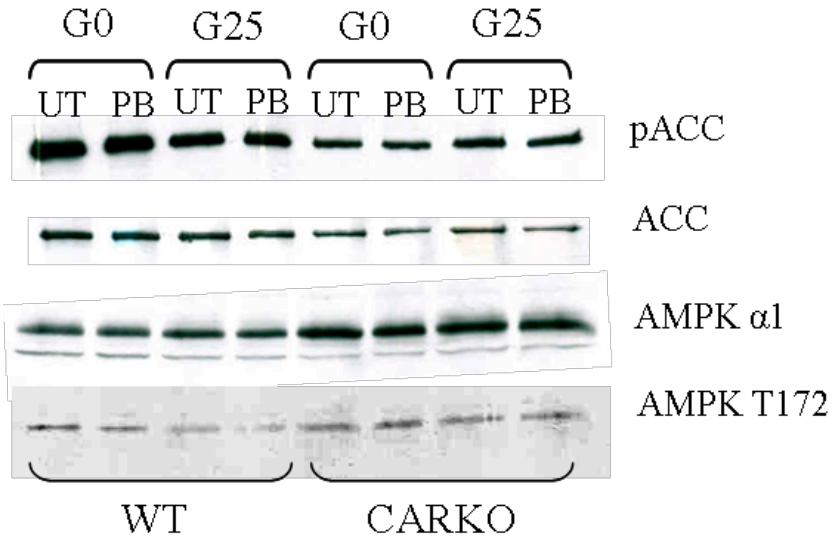


Figure 3.2 **AMPK expressions in CARKO hepatocytes.**

Primary hepatocytes in culture from CARKO animals were cultured in glucose free or in a 25 mM glucose containing medium for 24H. The hepatocytes were treated for 4H with 500 uM of phenobarbital (PB). The expression of phosphorylated AMPK Thr172 (ampkT172), AMPK α1 subunit (AMPK α1), acetyl-coA carboxylase (ACC) and the phosphorylated form of acc (pACC) were assessed by western blot.

This result suggests that CAR may regulate the expression of AMPK α1 subunit, thereby regulating its activity. Moreover, this experiment show for the first time a CAR dependent and AMPK independent regulation of the phosphorylation of acetyl-coA carboxylase (pACC).

The Repression by Phenobarbital of PEPCK Transcription Requires AMP-Activated Kinase and Constitutive Androstane Receptor

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Running title: Repression by Phenobarbital of PEPCK

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Abbreviations: AICAR, 5'-phosphoribosyl-5-aminoimidazol-4-carboxamide; AMPK, AMP-activated protein kinase; CAR, constitutive androstane receptor; CYPs, cytochromes P450; CV-1, PB, phenobarbital; PEPCK, Phosphoenolpyruvate Carboxykinase. Peroxisome proliferator-activated receptor γ coactivator 1 α PGC-1α.

Abstract:

Phenobarbital (PB) lowers glyceamia in patients with diabetes by repressing genes that encode enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK). Recently, phenobarbital was shown to activate the energy sensor AMP-activated kinase (AMPK) protein. In this study, we first show that PB dose-dependently decreases PEPCK in primary human hepatocytes. Activation of AMPK by phenobarbital and 5-aminoimidazole-4-carboxamide-1-D-ribofuranoside (AICAR), inhibited PEPCK expression whereas an inhibitor of AMPK, compound C, reversed this inhibition in mouse and human hepatocytes in primary culture. Phenobarbital has no effect on PEPCK expression in the liver of mice deleted of AMPK alpha1 and alpha2 catalytic subunits. In mouse liver, activation of AMPK is necessary but not sufficient to trigger the effect of phenobarbital on PEPCK mRNA. Constitutive androstane receptor (CAR), a nuclear receptor activated by PB and was shown to down-regulate PEPCK expression. In coactivator assays, phenobarbital and AICAR repress the interaction between CAR and the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α), a known regulator of PEPCK.

On the basis of these findings, we propose a new mechanism by which phenobarbital represses PEPCK. The activation of AMPK by phenobarbital simultaneously potentiates nuclear accumulation of CAR and increases AMPK-CAR interaction. Once activated, the AMPK-CAR complex prevents PGC-1α from its recruitment to the PEPCK promoter.

Introduction

Phenobarbital (PB) is an anticonvulsant and hypnotic drug and the prototype of chemicals which induce pleiotropic effects in the liver, including liver hypertrophy, tumor promotion and the regulation of genes coding for enzymes such as cytochrome P450s (CYPs) or transporters that regulate drug disposition. Drug metabolism is mainly controlled by transcriptional activation of hepatic genes encoding drug metabolizing enzymes and transporters.

The induction of human cytochrome P450 CYP2B6 and its mouse ortholog Cyp2b10 by PB is mediated by the nuclear receptor constitutive androstane receptor (CAR, NR1I3) which binds to its cognate sequence named phenobarbital response enhancer module (PBREM) [187]. In untreated primary mouse hepatocytes, CAR is retained in the cytoplasm within a protein complex of chaperones and co-chaperones such as heat shock protein 90 (HSP90) [188], and a protein called cytoplasmic CAR retaining protein (CCRP)[189]. 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 its consensus DNA sequences of target genes [190]. However, this mechanism is incompletely understood and cannot explain all PB effects on gene transcription. Earlier studies have shown that PB lowers glycaemia of patient with diabetes as well as in animal models of diabetes such as streptozotocin injected rats [191, 192]. Furthermore, it has been reported that gluconeogenesis was inhibited in liver of mice fed on a normal chow diet and treated with PB [110]. Active gluconeogenesis and increased hepatic glucose production is the main cause of post-prandial hyperglycemia observed in Non-Insulin Dependant Diabetic (NIDDM) patients.

The gluconeogenic pathway converts non carbohydrate precursors into glucose and helps sustain a normal blood glucose concentration under fasting conditions. PEPCK catalyses the first irreversible step in this process, the conversion of oxaloacetate to phosphoenolpyruvate. The PEPCK gene is tightly regulated by glucagon, glucocorticoids, retinoic acid, thyroid hormone, and insulin [134, 193], as well as glucose [194]. Each of the hormones exerts its effect through a hormone response unit located within the proximal 500 bp of the PEPCK promoter and consisting of DNA elements and associated transcription factors [195]. Each of these DNA elements was also shown to bind multiple

transcription factors, such as retinoic acid receptor (RAR)[196], fork-head box (FOX) family members [197], CCAATT/enhancer binding protein (C/EBP α), cAMP response element binding protein (CREB) [198] and hepatic nuclear transcription factor 4α (HNF4α) [199]. These DNA-binding transcription factors function by recruiting coregulators to the promoter, which in turn results in the activation or repression of transcription. However, it is not clear whether any or all of these proteins serve obligatory roles, which combinations are sufficient for regulation, or if some act to amplify or suppress an existing lower level response.

Recently, it has been shown that CAR is another player in the regulation of PEPCK transcription. In mouse liver, CAR binds to the PEPCK promoter upon PB treatment [200]. Moreover, CAR may compete with HNF-4α for its DNA binding site and for its interaction with coactivators. Many coactivators were described to be recruited to the promoter of PEPCK by ligand-activated transcription factors to form a functional complex with other components of the transcription machinery. For example, the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) is a critical regulator of gluconeogenesis in response to fasting [201]. PGC-1α induces basal and hormone-induced PEPCK gene transcription by promoting a complex formation between HNF4 α and glucocorticoid receptor (GR). More importantly, CAR was shown to interact with PGC-1α, but no functionnal assay has been previously described regarding this interaction.

Gluconeogenesis is a energy-demanding process, consuming 4 ATP molecules per molecule of glucose produced. When gluconeogenesis is sustained it results in ATP depletion in the cell. It is now well established that AMP-activated protein kinase (AMPK) is a master switch of energy demand by inhibiting anabolic pathways and stimulating catabolic pathways to control ATP levels [167]. AMPK is a heterotrimeric complex ubiquitously expressed consisting of a catalytic subunit α, and two regulatory subunits, β and γ [202]. All three subunits have been characterized, and each subunit is encoded by two (α1α2, β1β2) or three genes (γ1, γ2, γ3). Formation of the trimeric complex is necessary for optimal kinase activity [203]. Changes in the cellular energy state activate AMPK through mechanisms involving an AMP allosteric regulation and phosphorylation by an upstream kinase of threonine residue 172 within the activation loop of the alpha subunit [204]. Suppression of expression of gluconeogenic genes such

as PEPCK and glucose-6-phosphatase (G-6-Pase) was observed in rat hepatoma cells (H4IIE) in response to the non specific AMPK activator, 5-aminoimidazole-4-carboxamide riboside (AICAR) [205] and in mice after adiponectin administration [179] or in obese diabetic db/db mice after adenovirus-mediated delivery of a constitutively active AMPK [206, 207].

The inhibitory effect of PB on gluconeogenesis is not observed in transgenic mice in which the CAR gene is deleted [14]. Furthermore, we reported that PB activates AMPK and this activation is necessary for the transcription of cytochrome P450s in human and mouse liver [64]. Based on these observations we have designed experiments to test whether inhibition of PEPCK expression by PB is mediated in an AMPK/CAR dependent manner. We have established that this effect occurs in human hepatocytes in primary culture and that AMPK and CAR are necessary to trigger the effect of PB on PEPCK expression in mouse liver. Moreover, we demonstrate a functional interaction and cooperation between CAR and PGC-1α to transactivate the PEPCK promoter. Finally, our results suggest that in primary human hepatocytes, AMPK binds to CAR to prevent PGC-1α from its recruitment to PEPCK promoter, thereby inhibiting PEPCK transactivation.

Results

PB and AICAR repress PEPCK in mouse and human hepatocytes in primary culture

In primary culture of human hepatocytes we observed a dose dependent inhibition of PEPCK expression by phenobarbital (Fig. 1A). In order to be able to detect PEPCK mRNA in cell culture, cells were kept in serum free and glucose free medium. The induction of CYP2B6 by PB further validated our culture system of human hepatocytes for this study (Fig.1B). Intraperitoneal injection of 50 mg/Kg of PB in mice fasted for 16 hours also led to an inhibition of PEPCK expression in the liver at 10 hours of exposure to the drug (datas not shown). This confirms both *in vivo* and *in vitro* the inhibitory effect of PB on the PEPCK gene.

AICAR is a cell permeable compound phosphorylated to the monophosphate form Z-Mono- Phosphate (ZMP) which can accumulate in the cell, mimicking the effect of AMP on the AMPK phosphorylation and activation [208]. In cells exposed to dexamethasone (1μM) and cAMP (10μM) in order to induce PEPCK, the pharmacological activation of AMPK with AICAR dose-dependently inhibited PEPCK expression in primary culture of human and mouse hepatocytes (Fig 1C, 1D) This inhibitory effect was antagonized by pre-incubation of cells with Compound C, an inhibitor of AMPK. Inhibition of PEPCK expression by AICAR and the antagonistic effect of compound C suggest an AMPK dependent repression of the PEPCK gene by PB *in vitro*.

AMPK is necessary for the inhibitory effect of phenobarbital on PEPCK mRNA in mice

To test the role of AMPK in the inhibition of PEPCK by PB, we injected PB (100mg/Kg) in wild type (wt) and mice where the α1 and α2 AMPK subunits were specifically deleted in the liver (AMPK $\alpha1/\alpha2^{LS}$ KO mice) (Fig. 2A). PB represses PEPCK in the liver of wild type mice, whereas no inhibition was observed in $\alpha1/\alpha2^{LS}$ KO mice confirming the important role of AMPK in the repression of PEPCK by PB (Fig.2A). To rule out possible effects of metabolic changes as a consequence of the

AMPKα1 and α2 deletion in the liver, we performed primary culture of hepatocytes from wt and $\alpha1/\alpha2^{LS}$ KO mice (Fig. 2B). The inhibition of PEPCK was only observed in hepatocytes from wt animals after 12 hours exposure to 500μM PB (Fig. 2B) tending to a strong support for the role of AMPK in the regulation of the basal transcription of the PEPCK gene. It is important to note that PEPCK expression was much higher in hepatocytes from $\alpha1/\alpha2^{LS}$ KO mice as compared to hepatocytes from wt mice. Moreover, PB was unable to repress PEPCK gene expression in hepatocytes from $\alpha1/\alpha2^{LS}$ KO mice. The AMPK inhibitor Compound C (CompC) was able to reverse the inhibitory effect of PB on PEPCK expression only in hepatocytes from wt mice but not in hepatocytes from $\alpha1/\alpha2^{LS}$ mice. Taken together these results confirm the necessary role of AMPK in the effect of PB on PEPCK in human and mouse hepatocytes in primary culture.

The role of AMPK in PEPCK regulation was further tested in human hepatocytes in primary culture infected with an adenovirus expressing a constitutively active, truncated form of AMPK α2 subunit (ad-AMPK-CA-α2, Fig. 2C). The ad-AMPK-CA-α2 was able to partially repress PEPCK expression even in the presence of dexamethasone (1μM) and cAMP (10μM) (Fig.2C).

Interestingly, the inhibition of PEPCK by PB and AICAR were much stronger in cells infected with ad-AMPK-CAα2 than in cells infected with an adenovirus expressing β-galactosidase (AdβGal).

These results again support the necessary role of AMPK in the inhibition of PEPCK expression by PB in mouse and human hepatocytes in primary culture.

CAR mediates the PB effect on gluconeogenesis in mouse primary hepatocytes.

Previous studies described a CAR dependency of the PB effect on gluconeogenesis [110]. To test this hypothesis, we cultured primary mouse hepatocytes from wild type (wt) and CAR -/- animals under conditions where PEPCK and G6Pase are induced before exposure to PB (500 uM) (Fig. 3A). Unexpectedly, in absence of dexamethasone and cAMP, PB was able to significantly induce PEPCK expression in hepatocytes from CAR -/- mice (Fig. 3A). The same tendency is observed for G6Pase mRNA (Fig. 3B). The inhibition of PEPCK and G6Pase expression by PB was observed only after 8 hours of

culture in hepatocytes from wt mice. The efficiency of dexamethasone and cAMP to induce PEPCK and G6Pase expression and insulin to repress PEPCK is similar in hepatocytes from wt and CAR -/- mice. Addition of Compound C (CompC) reversed the inhibitory effect of PB on both enzymes only in hepatocytes from CAR -/- mice confirming the involvement of AMPK in the repression of gluconeogenesis by PB. Suprisingly, in hepatocytes from wild type animals compC failed to reverse the inhibitory effect of PB on G6Pase expression (Fig. 4B). The lack of an effect of PB on gluconeogenic genes in CAR -/- hepatocytes cannot be attributed to a difference in AMPK expression since both AMPK alpha 1 and alpha 2 subunits were equally expressed in wild type and in CAR -/- mice livers (Fig.3C).

To test whether this regulation of PEPCK by PB reflects inhibition of glucose production, we measured glucose produced in culture medium in hepatocytes from wild type and CAR -/- mice (Fig. 3D). Glucose production is significantly decreased upon PB treatment in wt hepatocytes, whereas no significant changes have been observed in CAR -/- cells. Moreover, AICAR and metformin, both activators of AMPK, decreased by almost 60% glucose production in wt hepatocytes but their effect is attenuated in CAR -/-cells. This result shows that CAR is necessary to mediate the PB repressive effect on glucose production in hepatocytes. Furthermore, the attenuated effect of metformin and AICAR on glucose production in CAR -/- suggest that CAR may also play a role in mediating the effect of AMPK on gluconeogenesis.

Interaction of PGC-1α, CAR and AMPK

A large number of hormones and transcription factors have been shown to regulate the expression of PEPCK. Among them, FOXO1 has been proposed to be the mediator of the effect of PB on PEPCK (Kodama et al., 2003). We co-expressed hCAR and hFOXO1 in CV-1 cells and tested the transactivation of the -467/+69 fragment of the PEPCK promoter. We observed that hFOXO1 significantly increased PEPCK promoter transactivation, whereas hCAR had no effect. Surprisingly, we were unable to reproduce the co repressive effect of CAR and FOXO1 for the transactivation of the -467/+69 fragment of PEPCK (Fig. 4A). The possible reasons for this discrepancy will be discussed below. Because the interaction between CAR and PGC1a has previously been

described (Shiraki et al., 2003) and because PGC-1α is a master regulator involved in the amplification of PEPCK induction by fasting (Lin et al., 2005), we decided to investigate the role played by PGC-1α on PEPCK promoter regulation by CAR. No functional assay has previously been performed on the CAR- PGC-1α interaction. The direct binding of PGC-1α to CAR was first tested by a co-factor assay as previously described (Shiraki et al., 2003). Briefly, the human CAR ligand binding domain (CAR LBD) fused to the Gal4 DNA binding domain was used as bait and cotransfected with hPGC-1α and GAL4 response element (Gal4RE) fused to luciferase. Luciferase activity reflects binding of CAR LBD to Gal4RE. Without any exogenous coactivator hCAR LBD shows a constitutive activity (Fig. 4B *gray bar*). hPGC-1α co-transfection clearly increases the binding of CAR LBD Gal4 to Gal4RE demonstrating an increase in hCAR LBD activity. As expected, addition of 500μM AICAR strongly disrupts this effect (Fig. 4B). Thus, we designed experiments to test the effects of PGC-1α in combination with hCAR on the activity of the -467/+69 fragment of the PEPCK promoter in CV-1 cells (Fig 4B). The co-activator hPGC-1α dose-dependently transactivated the PEPCK promoter in a hCAR dependent manner. More importantly, the potentializing effect of hPGC1a on PEPCK transactivation was blunted by addition of 500μM AICAR. Meanwhile the AICAR effect suggests a possible role of AMPK on hCAR and/or PGC1a activity.

Altogether, these results indicate a functional binding of hPGC-1α to hCAR and suggest a role of this interaction on PEPCK transactivation. Furthermore, the effect of AICAR on this interaction suggests that AMPK may interact both with CAR and PGC-1α in the regulation of PEPCK.

We now considered a possible direct interaction between AMPKα and CAR. Primary human hepatocytes were infected with Adenoviruses expressing hemagglutinin tagged hCAR (ad-HAhCAR) and myc tagged AMPKα2. Western blots of HAhCAR-immunoprecipitated lysates show an increased interaction between CAR and AMPKα2 protein upon PB treatment. In a second step, we performed the vice versa experiment by immunoprecipitating AMPK and assaying HA-hCAR by Western blot. This experiment shows the binding of AMPK to CAR enhanced by PB in human hepatocytes (Fig. 5A). In addition to coimmunoprecipitation experiment we analysed the CAR-AMPK interaction by a mammalian two-hybrid assay (Fig. 5B). The reporter activity was increased by 3 fold when AMPKα2 is co-transfected with the human CAR ligand binding domain (CAR

LBD) fused to Gal4 DNA binding domain. This experiment shows that AMPK strongly enhances CAR activity. Furthermore, we used a recombinant HIS-AMPKα1 as a bait to bind hCAR on a Nickel affinity gel. Incubation of HIS-AMPKα1 with human hepatocytes protein lysates expressing Ad-HAhCAR revealed a binding between AMPKα1 and HAhCAR as shown by the co-elution profile detected by western blot (Fig. 5C). HAhCAR protein is exclusively eluted in the presence of HIS-AMPKα1. Without AMPK, hCAR is washed out in the first fraction. Finally, in human hepatocytes expressing hCAR–EGFP, AMPKα2 subunit was detected in proximity of CAR suggesting a colocalisation of these two proteins in the nucleus upon PB treatment (Fig. 5D).

As we have previously mentioned, the cotransfection of CAR and PGC-1α increased PEPCK promoter activity in CV-1 cells. The cotransfection of AMPK leads to the repression of PEPCK (Fig. 5E). Interestingly, AMPK 1α dead kinase subunit inhibits the PEPCK transactivation, which suggests that the kinase acitiviy is not necessary to mediate the repressive effect of AMPK on PEPCK promoter activity. Altogether, these results strongly suggest that hCAR directly binds to human AMPKα subunit and that PB does enhance this interaction thereby leading to prevention of PGC-1α recruitment on PEPCK promoter.

Discussion

The present study identifies a new molecular mechanism by which phenobarbital (PB) inhibits gluconeogenesis. We describe, for the first time a PB-enhanced interaction between human CAR and AMPK α2 subunit. This interaction leads to a synergistic action of CAR, PGC-1α and AMPK in the control of the regulation of gluconeogenesis. Furthermore, here in, we show that CAR and AMPK are both necessary to mediate the effect of PB on PEPCK expression.

PEPCK is the rate limiting enzyme of gluconeogenesis process and is regulated by hormones involved in the fasting feeding transition. These hormones exert their effects through a hormone response unit located within the promoter (Chakravarty, et al. 2005). The deregulation of the transcriptional activity of PEPCK gene is associated with hyperglycaemia and insulin resistance observed in non insulin dependent diabetic patients (NIDDM). Moreover, earlier studies have shown that chronic PB treatment lowers glycaemia of patients with diabetes [191, 192]. Furthermore, others reported that hepatic gluconeogenic enzymes, such as PEPCK and G6Pase are repressed in PB-treated rats and mice [43, 44]. In this context, our study shows this inhibitory effect of PB in human hepatocytes in primary culture.

However, the mechanism by which PB regulates gluconeogenesis remains unclear. It was found that CAR regulates the PB-induced repression of PEPCK [14]. CAR directly binds and represses FOXO1, a known positive regulator of PEPCK. Unfortunately our datas do not support this finding. This discrepancy of results may be attributed to the difference in the systems that have been used. It is not excluded that the molecular mechanisms that regulate PEPCK in mouse and in human are different which can also explain the discrepancy between the studies. Furthermore, Kodama et al., worked on an Insulin response sequence (IRS) which consist of 40 base pairs, whereas we used a 500 bp fragment of the PEPCK promoter.

Here in, for the first time, we demonstrate a functional interaction and cooperation between CAR and PGC-1α to regulate the PEPCK promoter activity. Interestingly, the potentializing effect of PGC-1α on PEPCK transactivation was blunted by addition of AICAR, confirming the role of AMPK in such regulation. Meanwhile, there is no

evidence of phosphorylation of PGC-1α by AMPK or interaction between these two proteins despite a suggested link in muscle beta oxidation [209].

We previously reported that PB activates AMPK in mouse and human livers and this activation is necessary for the PB-induced transcription of cytochrome P450s [63, 64]. Besides mediating the PB effect in CYPs regulation, AMPK also conveys the PB message on PEPCK since PB does not repress PEPCK in mice where α1 and α2 AMPK subunits were specifically deleted in the liver (AMPK α 1/ α 2LS KO mouse). Moreover, the AMPK inhibitor Compound C was able to reverse the inhibitory effect of PB on PEPCK expression only in hepatocytes from wt mice but not in hepatocytes from α 1/ α 2LS mouse which confirm the role of AMPK in mediating the PB effect on PEPCK. As PB-independent pharmacological activation of AMPK leads to inhibition of gluconeogenesis, AMPK may be the key enzyme in the complex linked regulation of energy and drug metabolism. Nevertheless, the AMPK downstream targets in the regulation of CYPs and PEPCK have still to be identified.

Hence we point out a new mechanism by which AMPK interact with CAR to repress PEPCK gene transcription. It was previously reported that AMPK, independently of its kinase activity, binds and co-activates the nuclear receptor peroxisome proliferator-activated receptor α (PPAR α) in rat hepatocytes [210]. Herein, we demonstrated that AMPKα2 is co-localized with human CAR in primary human hepatocytes. Moreover, we showed that AMPKα1 directly binds to CAR and co-regulates its activity. This interaction may interfere with the complex interplay of proteins that regulate PEPCK, thereby leading to its inhibition.

A recent study suggested that mouse CAR binds to PEPCK promoter upon PB treatment in mouse liver [200], thereby leading to competition between CAR and HNF4α for a DR1 binding site and for co-activators recruitment. The authors showed that mouse CAR and HNF4α bind with the same affinity to PEPCK promoter. Therefore, in our system, low levels of endogenous HNF4α would be easily displaced by exogenous hCAR. However, PEPCK promoter activation requires important co-regulators such as PGC-1α. PB activates AMPK thereby leading to an interaction with CAR, which reduces the amount and/or the specificity of PGC-1α recruitment to induce PEPCK. One could assume that activation of AMPK by PB simultaneously potentiates nuclear accumulation of CAR and

increases AMPK-CAR interaction. Hence activated, the AMPK-CAR complex prevents PGC-1α from its recruitment to the PEPCK promoter.

Our study indicate that AMPK and CAR are both necessary to mediate the PB inhibitory effect on PEPCK. This study describes for the first time an interaction between CAR and AMPK. Our experiments provide a new mechanism by which PB controls the energy sensor activation, thereby controlling the regulation of synthesis of *de novo* glucose molecules. It is clear that CAR is more than a xenobiotic sensor, but can also control the expression of gluconeogenic genes by interacting with AMPK. It would be of interest to test if CAR interferes with AMPK on the regulation of other target genes such as CYPs. We still do not know whether AMPK interaction with CAR involves phosphorylation or not. An increase binding of CAR to AMPKα1 upon PB treatment suggest the activation of AMPK as a step in this process. Meanwhile, we are currently investigating the CAR phosphorylation by AMPK. Nowadays, AMPK is considered as potential target for the development of an anti-diabetic drug [211]. Our laboratory showed recruitment of LKB1 to AMPK upon PB treatment [212]. LKB1 was described to be responsible of the anti diabetic action of metformin. It is worth of interest to test if some of the pleiotropic effect of PB can be explained by activation of the LKB1-AMPK-CAR cascade.

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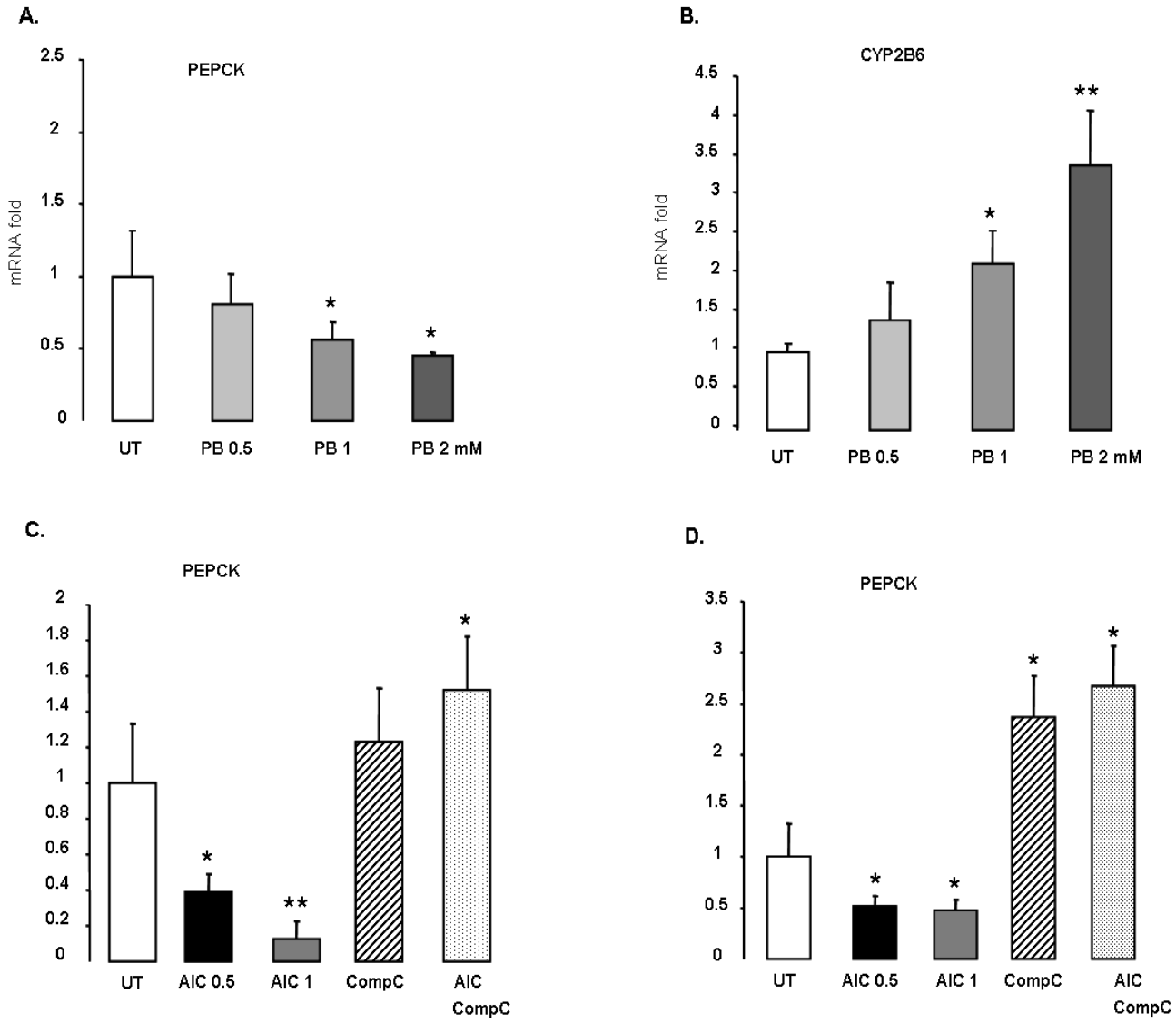

Figure 1 **PB represses PEPCK in primary human and mouse hepatocytes**

hepatocytes in primary culture were cultured in glucose free medium and exposed for 16h to 0.5, 1 and 2 mM of phenobarbital (PB), 0.5 and 1 mM AICAR or 5 uM of Compound C (Comp C) or a combination of AICAR and comp C. PEPCK (A, C) and CYP2B6 (B) mRNA were quantified by RT-PCR and standardized to 18S.

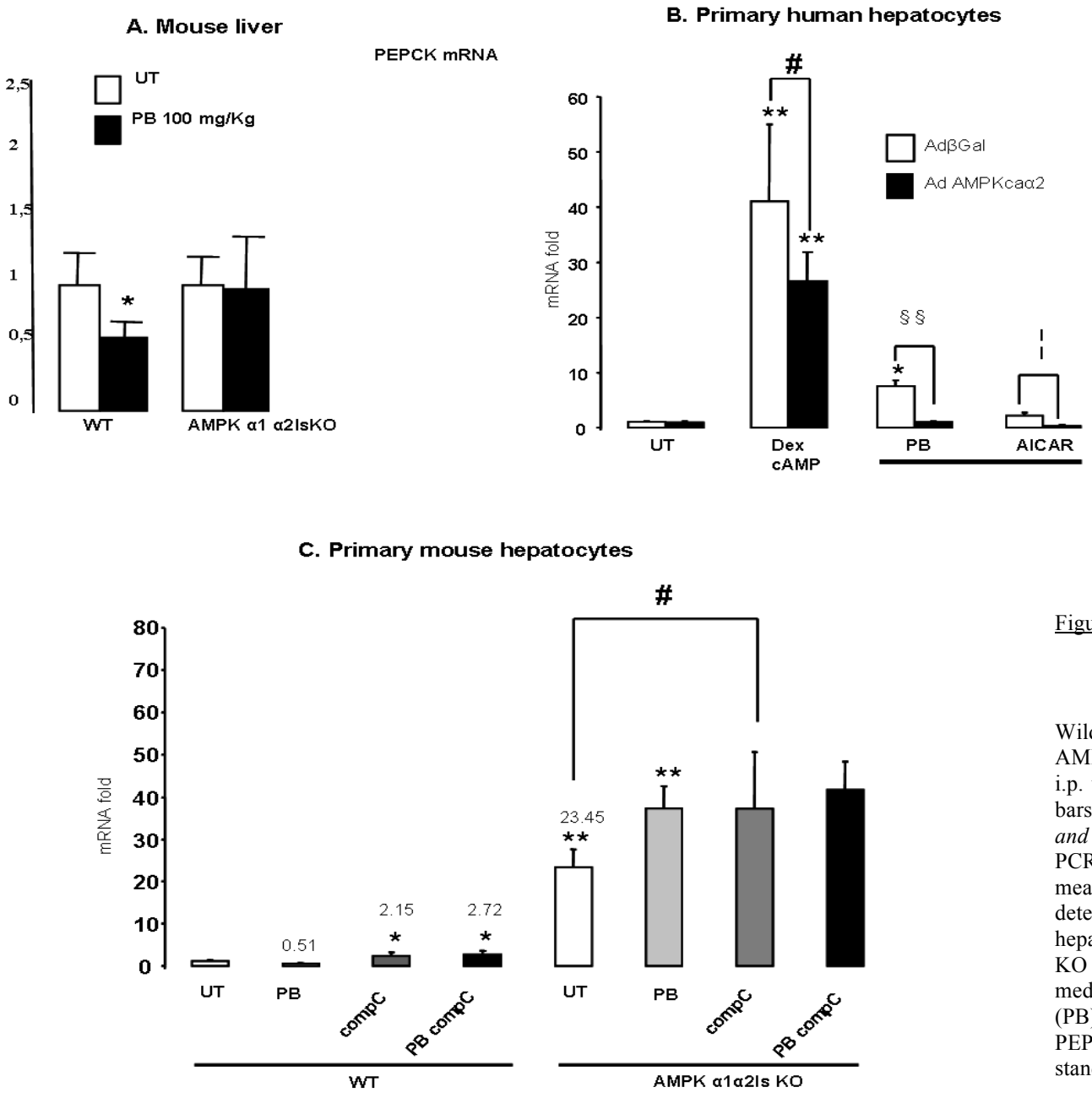

Figure 2 **Liver specific deletion of AMPKα1 and α2 subunits in mice blunts the repression of PEPCK by PB**

Wild type mice and mice with liver specific deletion of AMPKα1 and α2 subunits (AMPKα1lsα2) were injected i.p. with saline (□, open bars) or PB 100 mg/kg (■, black bars) and sacrificed 16h later as described under *Materials and Methods.* PEPCK mRNA (A) was quantified by RT-PCR and standardized to GAPDH. Results represent means of five different mice ± S.D. with each determination done in triplicates. (*,$p<0.05$). Mouse hepatocytes prepared from wild type and AMPKα1lsα2 KO mouse liver were cultured in Williams E glucose free medium and exposed for 12h to 500 uM phenobarbital (PB) or 5 uM of Compound C (comp C). As indicated PEPCK mRNA (B) quantified by RT-PCR and standardized to GAPDH

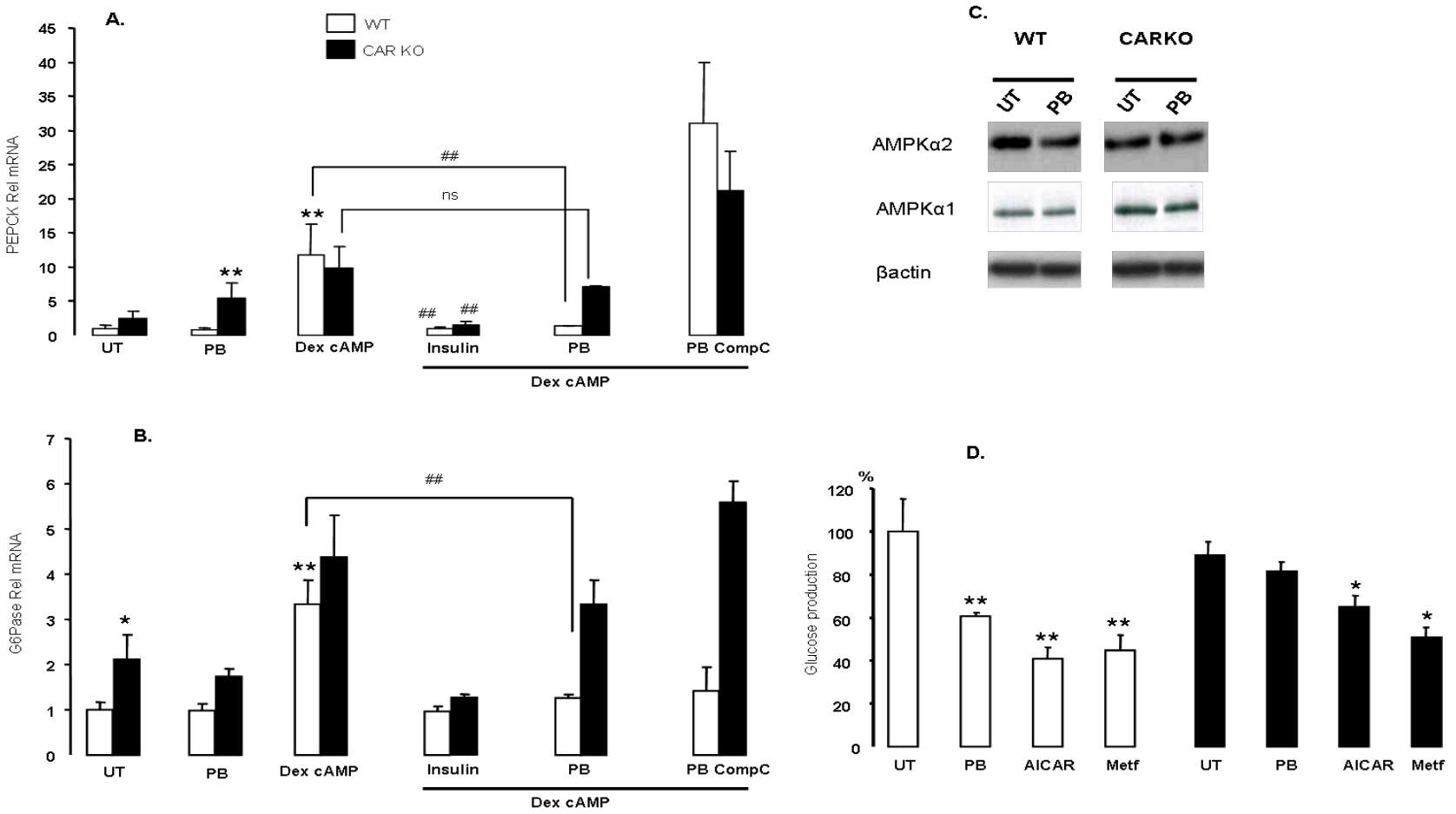

Figure 3 **CAR mediates the inhibition of PEPCK mRNA by phenobarbital**

Primary mouse hepatocytes from wild type (□, open bars) or from mice with a total deletion of constitutive androstane receptor (CARKO) (■, black bars) were exposed for 8h to 500 uM phenobarbital (PB), 1 uM deamethasone (dex), 10 uM dibutyryl-cAMP (cAMP), 10 nM insulin or 5 uM Compound C (CompC). PEPCK mRNA (A) and G6Pase (B) were quantified by RT-PCR and standardized to GAPDH. Expression of mouse AMPKα1, AMPKα2 and β actin were shown by western blot (C) after 4 h treatment with 500 µM PB. Glucose concentration was measured in the medium of wild type and CARKO hepatocytes cultured in glucose free medium and treated for 16h with PB 500 uM or AICAR 500 uM or metformin 1 mM (Metf).

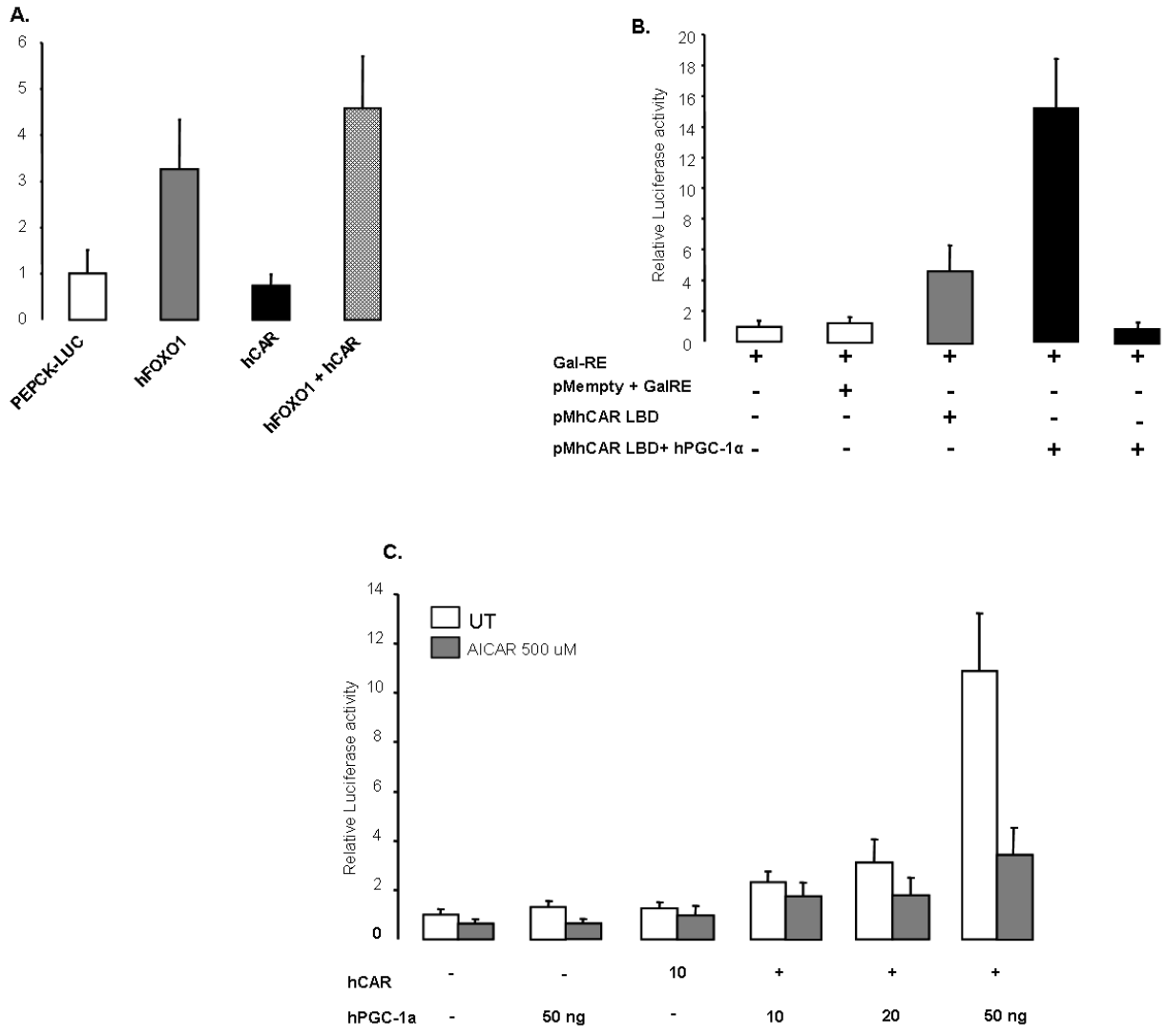

Figure 4: **PGC-1α and CAR co transfection enhance PEPCK transactivation**

The -467+69 PEPCK-LUC reporter and pcDNA3hCAR were cotransfected with pEBG2T-hFOXO1 in CV-1 cells (A). Cell extracts were analyzed for luciferase activity normalized against β-galactosidase activity. Fold induction indicates relative luciferase activity standardized against control cells infected with 467+69 PEPCK-LUC reporter alone. The data represent means of triplicate measurements from a representative experiment, and error bars indicate SD. CV-1 cells were co-transfected with Gal4 DNA binding domain fused to hCAR LBD (pMhCAR LBD) and human PGC-1α, the reporter construct Gal4 response element (Gal-RE), and pRSVβ-galactosidase (B). Cell extracts were analyzed for luciferase activity normalized against β-galactosidase activity

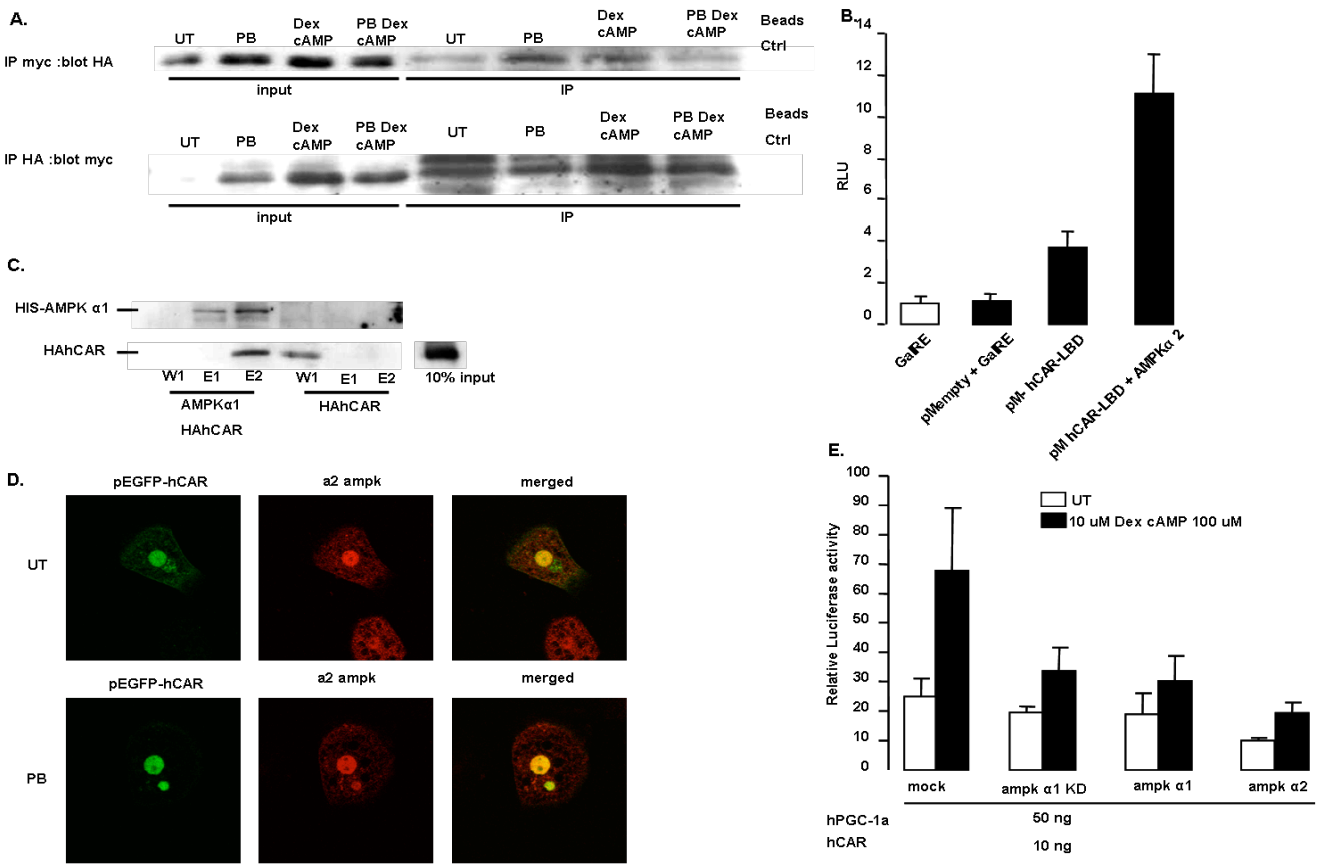

Figure 5 **Immunoprecipitaiton of human CAR and AMPKα2**

Immunoprecipitation of HA-hCAR by anti-HA antibody and of myc-AMPKα2 by anti-myc antibody (A). Cofactor assay: AMPK α2 subunit binds to hCAR (B). HAhCAR pulled down with AMPK α1 subunit (C). human CAR and AMPK α2 colocalized in primary human hepatocytes (D). AMPK represses PEPCK transactivation by hCAR and PGC-1α

Figure legends

Figure 1: PB represses PEPCK in primary human and mouse hepatocytes

Human hepatocytes in primary culture were cultured in glucose free medium and exposed for 16h to 0.5, 1 and 2 mM of phenobarbital (PB), 0.5 and 1 mM AICAR or 5 uM of Compound C (Comp C) or a combination of AICAR and comp C. PEPCK (A, C) and CYP2B6 (B) mRNA were quantified by RT-PCR and standardized to 18S as described under *Materials and Methods*. Results are means of three different donors ± S.D. with each determination done in triplicates Mouse hepatocytes in primary culture were cultured in DMEM containing 5 mM glucose and exposed for 12h to 0.5 and 1 mM AICAR or 5 uM of Compound C (Comp C) (D) PEPCK mRNA was quantified by RT-PCR. Results are means of three different mice ± S.D. with each determination done in triplicates. (*,$p<0.05$; **,$p<0.01$). Values obtained with PB, AICAR and CompC are compared with values obtained from untreated cells.

Figure 2: Liver specific deletion of AMPKα1 and α2 subunits in mice blunts the repression of PEPCK by PB.

Wild type mice and mice with liver specific deletion of AMPKα1 and α2 subunits (AMPKα1lsα2) were injected i.p. with saline (□, open bars) or PB 100 mg/kg (■, black bars) and sacrificed 16h later as described under *Materials and Methods*. PEPCK mRNA (A) was quantified by RT-PCR and standardized to GAPDH. Results represent means of five different mice ± S.D. with each determination done in triplicates. (*,$p<0.05$). Mouse hepatocytes prepared from wild type and AMPKα1lsα2 KO mouse liver were cultured in Williams E glucose free medium and exposed for 12h to 500 uM phenobarbital (PB) or 5 uM of Compound C (comp C). As indicated PEPCK mRNA (B) quantified by RT-PCR and standardized to GAPDH. Results are means of three different mice from each genotype ± S.D. with each determination done in triplicates. (*,$p<0.05$; **,$p<0.01$). Values obtained with PB or compC or their combinations are compared with values obtained from untreated cells. Values obtained with compC in the AMPKα1lsα2 KO mice are compared to the untreated cells from the same group (#,$p<0.05$). Human

hepatocytes in primary culture were cultured in medium containing 5 mM glucose and infected with adenovirus containing β-galactosidase (Ad βGal) (□, open bars) or a constitutively active form of AMPKα2 subunit (Ad AMPKcaα2) (■, black bars) as described under *Materials and Methods*. Twenty four hours after infection cells were exposed for 16h to 1 uM dexamethsaone and 10 uM dibutyryl-cAMP, 500 uM AICAR or 500 uM phenobarbital (PB). PEPCK (C) mRNA was quantified by RT-PCR and standardized to 18S. Results are compared with values from cells not exposed to drugs and infected with Ad βGal (*,$p<0.05$; **,$p<0.01$). Values obtained with cells infected with Ad βGal are compared to cells infected with Ad AMPKα2 for each drug treatment (#,$p<0.05$; §§,$p<0.01$; ¦,$p<0.05$).

Figure 3: CAR mediates the inhibition of PEPCK mRNA by phenobarbital.

Primary mouse hepatocytes from wild type (□, open bars) or from mice with a total deletion of constitutive androstane receptor (CARKO) (■, black bars) were exposed for 8h to 500 uM phenobarbital (PB), 1 uM deamethasone (dex), 10 uM dibutyryl-cAMP (cAMP), 10 nM insulin or 5 uM Compound C (CompC). PEPCK mRNA (A) and G6Pase (B) were quantified by RT-PCR and standardized to GAPDH. All values obtained with drugs exposure are compared to values from cells from wild type animals and not exposed to drugs (**,$p<0.01$). All values obtained with the combination of Dex and cAMP are compared to the values obtained from Dex cAMP wild type treated cells (##,$p<0.01$). Primary mouse hepatocytes from wild type and CARKO animals were cultured in serum free medium for 24h. Results are means of three different mice ± S.D. with each determination done in triplicates. (*, $p<0.05$). Expression of mouse AMPKα1, AMPKα2 and β actin were shown by western blot (C) after 4 h treatment with 500 μM PB. Glucose concentration was measured in the medium of wild type and CARKO hepatocytes cultured in glucose free medium and treated for 16h with PB 500 uM or AICAR 500 uM or metformin 1 mM (Metf).

Figure 4: PGC-1α and CAR co transfection enhance PEPCK transactivation

The -467+69 PEPCK-LUC reporter and pcDNA3hCAR were cotransfected with pEBG2T-hFOXO1 in CV-1 cells (A). Cell extracts were analyzed for luciferase activity normalized against β-galactosidase activity. Fold induction indicates relative luciferase activity standardized against control cells infected with 467+69 PEPCK-LUC reporter alone. The data represent means of triplicate measurements from a representative experiment, and error bars indicate SD. CV-1 cells were co-transfected with Gal4 DNA binding domain fused to hCAR LBD (pMhCAR LBD) and human PGC-1α, the reporter construct Gal4 response element (Gal-RE), and pRSVβ-galactosidase (B). Cell extracts were analyzed for luciferase activity normalized against β-galactosidase activity. The data represent means of triplicate measurements from a representative experiment, and error bars indicate SD. The -467+69 PEPCK-LUC reporter and pcDNA3hCAR were cotransfected with human PGC-1α in CV-1 cells, treated with 500 uM AICAR for 24h (C)

Figure5: Immunoprecipitaiton of human CAR and AMPKα2

Immunoprecipitation of HA-hCAR by anti-HA antibody and of myc-AMPKα2 by anti-myc antibody (A). CV-1 cells were co-transfected with Gal4 DNA binding domain fused to hCAR LBD (pMhCAR LBD) and human AMPKα2, the reporter construct containing Gal4 response element (Gal-RE), and pRSVβ-galactosidase (B). Incubation of recombinant histidine tagged AMPKα1 subunit (HIS-AMPKα1) with protein lysates expressing an adenovirus encoding for HA tagged hCAR (Ad-HAhCAR). Binding between AMPKα1 and HAhCAR is shown by the co-elution profile detected by western blot (C). HIS-AMPKα1 is detected with an antibody against AMPKα1 and Ad-HAhCAR is detected with an antibody against HA. Primary human hepatocytes infected with adenovirus expressing EGFP human CAR (D) (pEGFP-hCAR). Exogenous EGFP-hCAR was detected with an anti-GFP antibody and endogenous AMPKα2 was detected with an anti AMPKα2 antibody. The -467+69 PEPCK-LUC reporter, pcDNA3hCAR, hPGC-1α were cotransfected with AMPKα1, AMPK dead kinase α1 subunit (AMPKα1 KD) or AMPKα2 in CV-1 cells (E). Cell extracts were analyzed for luciferase activity

normalized against β-galactosidase activity. The data represent means of triplicate measurements from a representative experiment, and error bars indicate SD.

Supplemental data

CAR and AMPK interaction

We previously showed that AMPKα subunit binds to human CAR. However, the domains by which CAR and AMPK interacts remain unclear. Furthermore, the nature of this interaction is also complex because it may involve a simple protein-protein connection or a “hit and run” phosphorylation process. We then decided to preform competition experiments, where a known substrate of AMPK, SAMS peptide is challenged with a peptide contained in the ligang binding domain of human CAR.The CAR peptide is depicted in figure 1. We assessed AMPK activity by measuring SAMS peptide phosphorylation by a recombinant AMPK as described in material methods.

As shown in figure 2A, AMPK reaches the maximum of its acitvity in the presence of 100 uM of SAMS. Interestingly, 50 uM of the CAR T248 peptide blunt this activation, suggesting that the T248 peptide may compete with SAMS peptide for phosphorylation by AMPK. It worth noting, that the T248 peptide alone does not increase the AMPK activity. Increassing amount of T248 peptide dose dependently inhibits SAMS peptide phosphorylation by AMPK (Figure 2B). Indeed, 10 uM of T248 peptide completely abolished AMPK activity on.

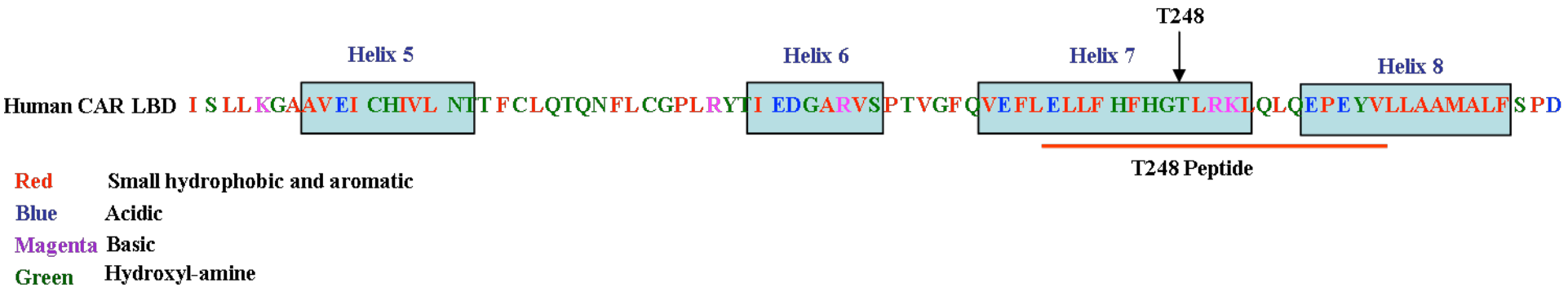

Figure 1 **Human CAR Ligand Binding Domain**

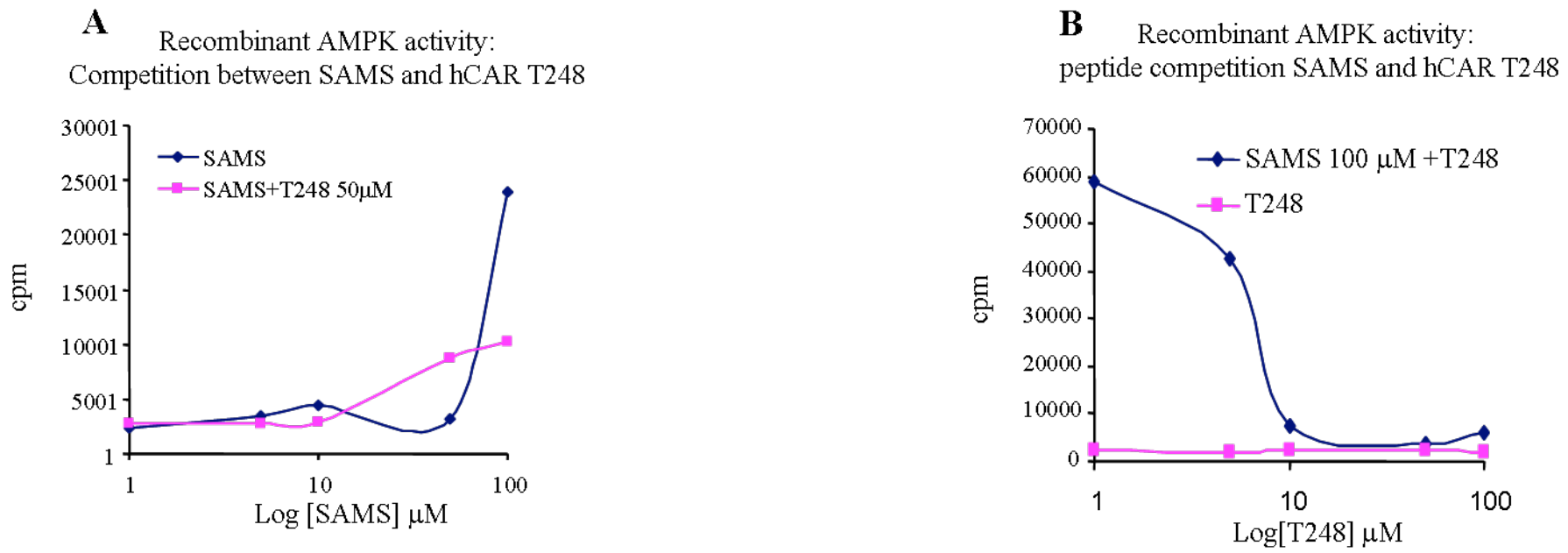

Figure 2 **Competition between SAMS and hCAR T248 peptides**

In order, to test the specificity of the competition between the two peptides, we incubated SAMS with a control peptide from a non AMPK protein target. The activity of AMPK was not disturbed by the control peptide, whereas T248 blunted AMPK activity. This result suggests that CAR T248 peptide may specifically compete with SAMS peptide for the AMPK phosphorylation.

Finally, we used the full ligand bind domain of CAR (LBD) as competitor for SAMS phosphorylation. In picomolar range, human CAR LBD can compete with SAMS (Figure 3A). When CAR LBD and SAMS are incubated together, the AMPK activity is decerased by ~ 30 % (Figure 3B). Taken together, these results demonstrate that the human CAR ligand binding domain may be a potential target for the AMPK.

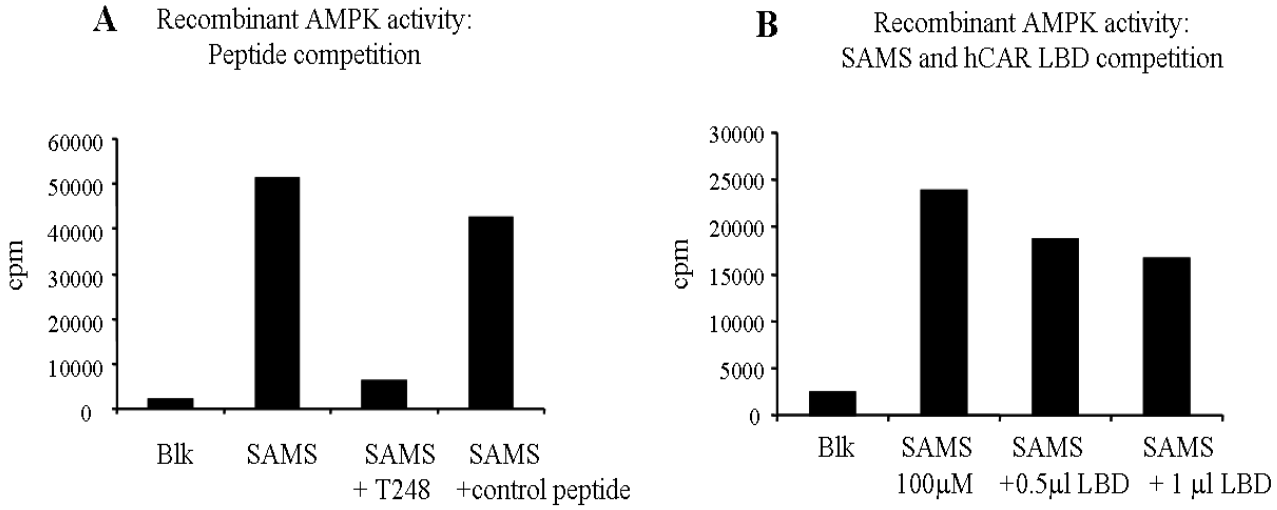

Figure 3: **hCAR LBD and SAMS competition**

Discussion

CAR as a glucose sensor

We have previously shown (cf Results part I) that CAR expression is induced during fasting and more specifically in the absence of glucose in human hepatocytes in primary culture (cf results I). A previous study by Bauer et al. also showed an increase of CAR mRNA expression in the liver of mice fasted for 24 hours [213]. By contrast, in this study, 24 hours of high carbohydrate diet did not affect CAR mRNA expression. Additionally, in the absence of glucose insulin represses CAR mRNA in human hepatocytes. This may indicate that CAR is regulated somehow by the fasting-feeding transition, when dramatic changes in insulin concentration occur. The G-6-Phosphatase and PEPCK promoters are known to be insulin targets and both contain so-called insulin-responsive sequences [214]. These sequences [T(G/A)TTT] have been functionally characterized, and several transcription factors that bind to these regions have been identified. It is thus possible that the CAR promoter contains such sequences and consequently is directly regulated by transcription factors induced by insulin. However, to discriminate between the glucose and the insulin effect, CAR expression and activity should be assessed *in vivo* for instance using hyperglycaemic-hyperinsulinemic clamps [215-217].

Interestingly, a recent study reported that CAR mRNA and protein levels were increased in diabetic db/db mouse livers [218]. However, the same group also demonstrated that CAR expression was markedly impaired in the livers of genetically obese Zucker rats which seemed to result in the reduced expression of CYP2Bs in these rats [219]. This discrepancy is very intriguing because both animals have mutation in the leptin receptor gene and show similar characteristics of hyperphagia, hyperglyceamia, hyperlipidemia and obesity [220]. As db/db mice and Zucker rats have mutations at a different position of the leptin receptor gene [221-223], this may explain the discrepancy in CAR expression.

During fasting, the expression of genes encoding for proteins involved in glucose transport and phosphorylation in the liver is significantly lower in the liver of mice

lacking CAR, which indicates that CAR may promote glucose uptake and subsequently enhance the glycolytic pathway. However, this result is very curious, because why would CAR upregulate already low levels of expression of these genes during fasting ? It is possible that CAR function consists of providing a minimum of energy required for drug metabolism to occur. Furthermore, since GLUT2 [224] and CAR expression are both regulated by the fasting/feeding transition in Shepatocytes, one could assume that CAR may be the mediator of insulin action on the regulation of GLUT2 expression during nutritional stress.

It is well established that a decrease of glyceamia during fasting leads to glucagon-mediated increase of cAMP levels in the liver [225]. Consequently, Ding et al., hypothesized that the activation of CAR during fasting is a cAMP-dependent phenomenon. They provided evidence that both CAR target genes, including Cyp2b10, Oatp2, Sult2a1, and Ugt1a1, as well as the CAR gene itself, are induced in mouse liver during fasting. Treatment of primary mouse hepatocytes with the β-adrenergic agonist epinephrine induces both CAR and its target genes. This effect is mimicked by the cAMP analogue 8-Br-cAMP [184]. The authors also demonstrated by gene reporter assays that the CAR promoter is regulated by the hepatocyte nuclear factor 4α (HNF4α) (figure 1). Additional fasting experiments performed using liver-specific HNF4α -knock-out (HNF4α-KO) mice demonstrated that HNF4α is required *in vivo* for both basal and fasting-inducible CAR gene expression. Another interesting study by Pascussi et al. investigated the effect of HNF4α isoforms (HNF4α1 and HNF4α7) on the human CAR promoter [226]. HNF4α7 is highly expressed in the pancreas and HNF4α1 is essentially expressed in the liver and both HNF4α isoforms bind to CAR promoter with similar affinity. However, HNF4α1 strongly enhanced hCAR promoter activity whereas HNF4α7 repressed the HNF4α1-mediated transactivation of the hCAR promoter. Briancon and al. also confirmed *in vivo* the specificity of HNF4α1 in the regulation of the CAR promoter [227]. Furthermore, the fasting- and cAMP-inducible coactivator PPARγ coactivator 1α (PGC-1α) coactivates HNF4α on the CAR promoter. The extent to which chronic overexpression of PGC-1α in liver such as it occurs during caloric restriction may contribute to CAR induction remains to be investigated. The data presented by the authors cannot rule out the possibility that other PGC-1α-independent mechanisms also are involved in the activation of CAR during fasting. For example, a recent study shows

that the coactivator PPAR-binding protein (PBP) is critical for the induction of CAR target genes by xenobiotics [228], but these data do not address a role for PBP protein in the activation of CAR during fasting. Another study describes the coactivator PBP to be essential for nuclear translocation of CAR [229], but the role of this interaction in fasting has not yet been studied. Adenoviral expression of both PBP and EGFP-CAR restored phenobarbital-mediated nuclear translocation of exogenously expressed CAR in PBP-null livers and in PBP-null primary hepatocytes. Unfortunately, functionnal interaction of PBP and CAR has not been assessed. It is possible that the xenobiotic response of CAR is different from its metabolic response and is produced through interaction with distinct cofactors, such as PGC-1α, during fasting. The respective CAR-cofactor complexes might then function to regulate the expression of different groups of genes in response to metabolic *versus* xenobiotic stress. The positive regulation of CAR expression by HNF4α and PGC-1α during fasting highlights the complexity of the role played by CAR since its activation by phenobarbital leads to a repression of gluconeogenic genes. This finding contributes to the concept of different mechanisms involved in fasting and drug induction.

Pascussi et al. also reported that glucocorticoid agonists, such as dexamethasone, prednisolone, or hydrocortisone, specifically increase human CAR mRNA expression. Additionally, dexamethasone increases both basal and phenobarbital-mediated nuclear translocation of CAR protein in human hepatocytes. The up-regulation of CAR mRNA and protein in response to dexamethasone explains the synergistic effect of this glucocorticoid on phenobarbital-mediated induction of CYP2B genes [230]. Moreover, another study describes the binding of the glucocorticoid receptor to the distal region of the human CAR promoter in cultured hepatocytes [231]. On the basis of cotransfection experiments, mutagenesis, and gel shift assays, the authors identified a glucocorticoid response element at -4447/-4432, which was recognized and transactivated by the human glucocorticoid receptor. Thus, we speculate that CAR expression *in vivo* increases in situations where glucocorticoid hormone levels are high, *e.g.* in stress conditions, during the peak of the corticosterone circadian rhythm or during fasting [232, 233]. Unfortunately, there exist no *in vivo* data in support of the role of endogenous glucocorticoids such as cortisol in the induction of CAR mRNA during fasting.

Nonetheless, similary to HNF4α and PGC-1α, GR is a crucial factor in the sustained induction of PEPCK during prolonged fasting and is a positive regulator of CAR.

Inflammation and/or infection are frequently associated with a reduced capacity of liver to metabolize xenobiotics/drugs [234]. Cytokines and bacterial endotoxins are likely to be responsible for this effect [235], and were shown to reduce CAR gene expression in primary human hepatocytes [236, 237]. Cytokines, oxidative stress, and chemicals are known to trigger NFκB activation [238] and this pro-inflammatory factor has been shown to bind and inactivate GR [239]. Thus, activation of NFkB in human hepatocytes leads to inhibition of GR transcriptional activity, and may cause the downregulation of CAR. The mechanism by which NFκB inhibits CAR transcription is unknown. A model for the induction of CAR mRNA during fasting is depicted in Figure 1.

In experiments where CARKO mice were fasted and refed upon a high carbohydrate diet, a lower expression of lipogenic genes was observed. This corroborates with lower levels of triglycerides in the liver of CARKO mice fed with a high carbohydrate diet for 12 weeks (unpublished data). Suprisingly, no difference in the amount of white adipose tissue has been observed between CARKO and normal mice. Interestingly, CARKO animals did not loose significantly more weight than normal animals during the experiment. This result is very suprising since Maglich et al., demonstrated that CARKO animals lost more weight than wild type animals under caloric restriction. More importantly, the data of Maglich and co-workers indicate that the weight loss observed in the CARKO animals was normal loss of stored fat from adipose tissue and not from lean mass [109]. The authors explained this result, in part, by the role of CAR in the regulation of thyroid hormone metabolism (cf introduction). However, thyroid hormone action alone is not sufficient to explain the role of CAR in the control of weight. Although their data demonstrate that CAR is required for the downregulation of thyroid metabolism upon caloric restriction, it was notable that a partial response was still observed in the CAR knockout mice, indicating that other mechanisms contribute to the process. The discrepancy between the studies may be attributed to the differences in the experimental conditions. We fed mice with a high carbohydrate /low fat diet at 15 MJ/kg which can be converted to approximatively 11.5 kcal/day/mouse, whereas the authors used a diet of 8 kcal/day/mouse. Consequently, the high carbohydrate diet used in our study might not provide enough energy to be assimilated as an energy-rich diet.

Nevertheless, further investigations are needed to find out how carbohydrates regulate weight in a CAR dependent manner and what the molecular mechanisms as well as factors underlying CAR regulation during nutritionnal stress may be.

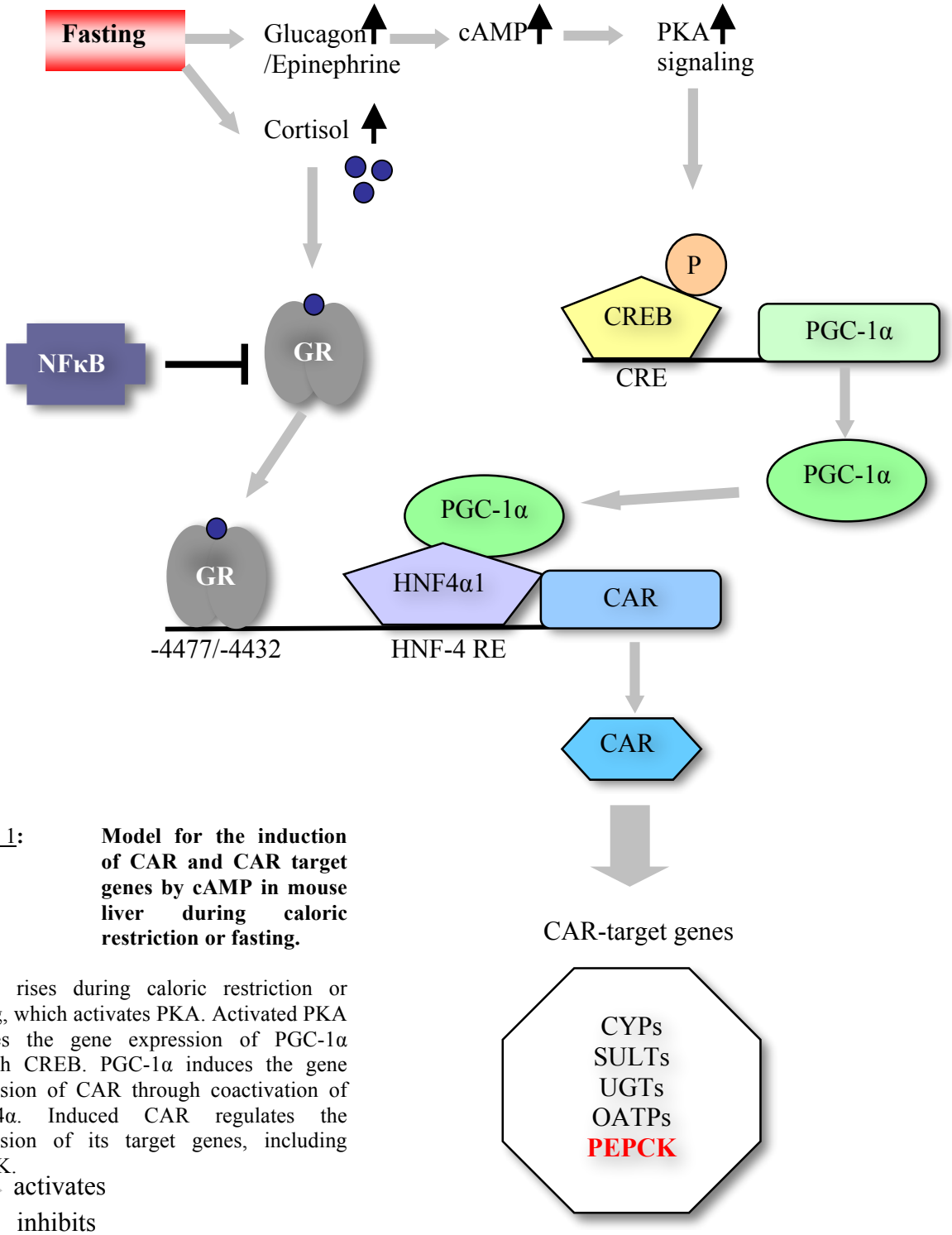

Figure 1: **Model for the induction of CAR and CAR target genes by cAMP in mouse liver during caloric restriction or fasting.**

cAMP rises during caloric restriction or fasting, which activates PKA. Activated PKA induces the gene expression of PGC-1α through CREB. PGC-1α induces the gene expression of CAR through coactivation of HNF-4α. Induced CAR regulates the expression of its target genes, including PEPCK.

Role of CAR in the regluation of PEPCK

As we previously described, PEPCK is a key protein in the regulation of hepatic glucose production during fasting (cf introduction). Suprisingly, our data indicate that basal expression of PEPCK mRNA is unaffected in livers of mice lacking CAR which suggests that endogenous CAR does not seem to be a constitutive repressor of PEPCK. Furthermore, the transfection of increasing amount of human CAR alone or with PGC-1α induces PEPCK promoter activity and mRNA in hepatoma cells. It has also been confirmed recently that the transfection of human CAR did not repress PEPCK mRNA in human hepatocytes [240]. The authors discovered a new variant of human CAR expressed from internal protein translation start sites. The resulting CAR protein lacks the N-terminal DNA-binding domain (DBD) of the receptor, yielding a ΔCAR variant with unique biological function. Although ΔCAR maintains full retinoid X receptor alpha (RXRα) heterodimerization capacity it is inactive on classical CAR-inducible direct repeat (DR)-4 elements, but efficiently transactivates a DR-1 element derived from the endogenous PPAR-inducible acyl-CoA oxidase gene promoter. The authors propose a new model of CAR DBD-independent transactivation, where only the RXRα portion of the CAR-RXRα heterodimer binds directly to DNA. It might explain the regulation of PEPCK expression by CAR since it has been shown that CAR competes with HNF4α for binding to a DR-1 site in the PEPCK promoter [40]. Taken together, these studies highlight a new role of CAR where it can serve as a cofactor for other transcription factors.

It is well established that protein kinase A (PKA) mediates the hormonal control of gluconeogenesis during fasting [241-243]. Our data indicate an additive effect of PKA and CAR on PEPCK promoter activity. This effect explains at least partially the role of CAR mRNA induction during fasting. However, it remains to be determined in which physiological context and how CAR and PKA cooperate in the regulation of PEPCK. A recent study shows that phosphorylation/dephosphorylation of serine 202 regulates subcellular localization of CAR [244]. Therefore, CAR may be the target of PKA and it may modulate CAR-cofactor interactions through alterations in phosphorylation status of co-factor proteins, CAR, or both. But, these data should be interpreted with caution

because the serine 202 does not match with a PKA phosphorylation site and the experimental conditions were far from the *in vivo* situation.

The CAR-mediated transactivation of PEPCK is blunted by phenobarbital but not by the CAR ligand CITCO. It is now clear that pharmacological activation of CAR by PB may not necessarily reflects the constitutive endogenous activation of CAR or its direct activation by a ligand. On one hand, CAR dose-dependently induces PEPCK mRNA and on the other hand, PB represses PEPCK in a CAR dependent manner. Since no endogenous ligand has yet been identified, it is very difficult to distinguish between the endogenous role of CAR and its pharmacological activation by PB. The difference between fasting and PB-mediated CAR activation can be attributed to interaction with specific cofactors, such as PGC-1α, during fasting. This hypothesis is supported by a co-factor assay showing that PB blunts the interaction between CAR and PGC-1α (cf Results Part). Although CAR and PGC-1α clearly interact, the physiological relevance of such interaction remains unknown. In collaboration with Christoph Handschin and Bruce Spiegelman we could show that PGC-1α knock out mice retain cyp2b10 PB-mediated induction [245] speeking againt an essential role of this coactivator in drug metabolism. PGC-1α is not essential in the regulation of PEPCK since transcription of the PEPCK gene can occur in the absence of PGC-1α (but at a lower level) suggesting that it serves as a "transcriptional amplifier" for this gene [246]. PGC-1α promotes transcription by the assembly of a transcription factor complex. This interaction has been proposed to cause a conformational change in PGC-1α, which permits the binding of other cofactors such as SRC-1 and CBP/p300 [247]. If the role of PGC-1α in the coactivation of CAR on the PEPCK promoter can be replaced by another cofactor is not known yet, but it is clear that cofactors are very redundant in their function.

Stafford and coworkers have suggested that SRC-1 interacts with HNF-4α and with COUP-TFII, both of which are required for the maximal induction of PEPCK gene transcription by glucocorticoids [164]. Transfection, analysis demonstrated that mouse CAR interacts with SRC-1 [248], while another study reported that SRC-1 enhances both constitutive and xenobiotic-induced CAR-mediated transactivation via the CYP2B1 PB-response element in rat transfected primary hepatocytes [249]. Furthermore, transient transfection assays with increasing amounts of SRC-1 reveal that amino acid at position 176 could regulate the interaction of mCAR with SRC-1 in the presence and absence a

ligand [250]. These data strongly suggest that SRC-1 contributes to the translocation and activation of CAR during CYPs induction. However, no evidence supports a role of a SRC-1-CAR interaction in the regulation of gluconeogenesis. Nevertheless, the recruitment of specific co-factors by CAR may then explain the dual role of CAR in the regulation of PEPCK. The nutritional context may influence the affinities between CAR and other transcription factors involved in the regulation of PEPCK.

A variety of other agents, in addition to insulin and glucose, repress PEPCK gene transcription [134]. In fact, a number of changes in glucose metabolism occur during infection, neoplastic growth, or tissue damage. Hypoglycemia is a major complication in the septic shock syndrome and is believed to result from the repression of PEPCK transcription by the concerted actions of TNF-α, IL-1, and IL-6 in the liver [251, 252]. Waltner-Law et al. reported that the p65 subunit of NF-κB represses the increase of PEPCK gene transcription mediated by glucocorticoids and cAMP in a concentration-dependent manner in H4IIE cells. Further analysis suggested that p65 represses PEPCK gene transcription through a protein-protein interaction with the coactivator, CBP [253]. Thus another possibility that may link NF-κB to CAR is competition for CBP/P300 or SRC-1 in the regulation of PEPCK. However, a recent study does not support this finding. The authors showed that moderate inhibition of NF-κB improved glucose tolerance through decreased gluconeogenesis associated with reduced PGC-1α gene expression in db/db mice, and suggest that inhibition of NF-κB activity in liver is a potentially suitable strategy for the normalisation of blood glucose concentration in type 2 diabetes [254]. Further studies are needed to confirm the role of NF-κB in the regulation of gluconeogenesis and the possible link between CAR and NF-κB signaling cascade.

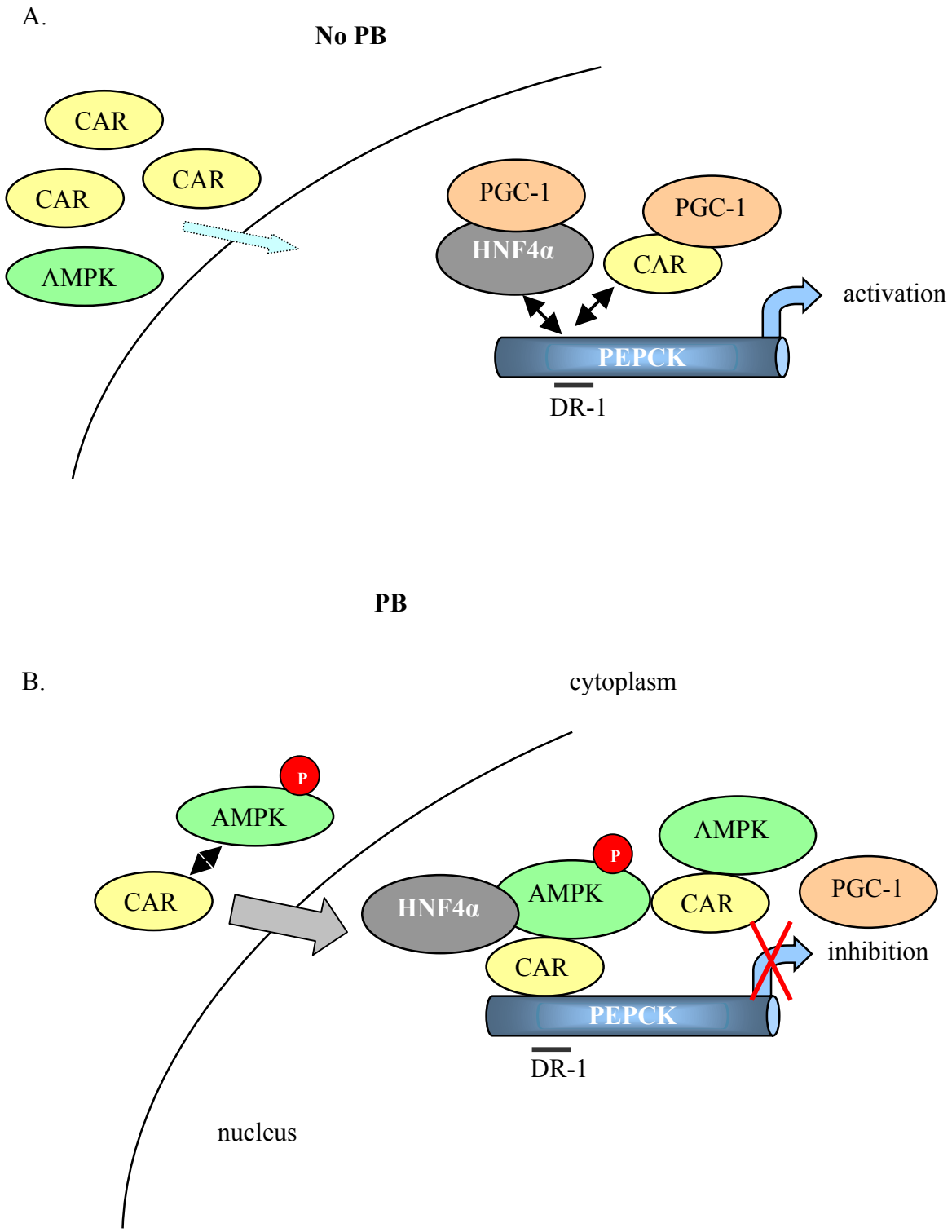

Figure 2: **Hypothetic model for the regulation of PEPCK promoter by phenobarbital**

A. In the absence of PB, PGC-1α coactivates CAR on PEPCK promoter and weak competition between CAR and HNF-4α occurs. B. Once activated by PB, CAR accumulates in the nucleus and binds to AMPK thereby preventing PGC-1α from its recruitment to the PEPCK promoter.

The CAR-AMPK connection

The data presented in this thesis demonstrate that AMPK and CAR are both necessary for the PB-mediated repression of PEPCK. Furthermore, we provide evidence for direct interaction between CAR and the AMPKα catalytic subunit by coimmunoprecipitation and pull-down experiments. Our group also reported that AMPK plays an essential role in the induction of CYP2B and CYP3A forms by phenobarbital, and that among several CAR and PXR agonists (including notably rifampicin, CITCO, and TCPOBOP), only phenobarbital increased the AMP/ATP ratio and activated AMPK [255, 256]. AMPK was not necessary for controlling the nuclear translocation of CAR, although CAR was concentrated in nuclear speckles in the hepatocytes of AMPKα1/α2 (−/−) mice, whereas its accumulation was homogeneous in cell nuclei from wild-type animals. Since AMPK and CAR co-localize in the nucleus of human hepatocytes in primary culture, CAR may be a target of AMPK in the PB-mediated regulation of CYPs and PEPCK. Preliminary experiments in our group indicated that a CAR peptide containing a consensus AMPK target sequence competed for phosphorylation when incubated with SAMS peptide, a known synthetic substrate of AMPK. However, this finding must be confirmed in an *in vivo* situation and we cannot yet exclude definitively that CAR is not phosphorylated by AMPK. Hosseinpour et al. reported that the dephosphorylation of Ser-202 is required in the regulation of the xenobiotic-dependent nuclear translocation of mCAR [244], but the phosphorylation as well as the putative up-stream kinase(s) have not yet been evidenced. Furthermore, the serine-202 does not match with an AMPK consensus site of phosphorylation.

We also show in this thesis that exogenous expression of CAR blunts the PB-mediated activation of AMPK in mouse hepatocytes in primary cultures. AMPK is activated by PB and more importantly was shown to be essential in the PB-mediated regulation of CYPs. Furthermore, our group showed that the phosphorylation of AMPK is required in the regulation of CYPs [255] whereas kinase activity seems to be facultative in the repression of PEPCK. Therefore, it is possible that a protein-protein interaction between CAR and AMPK may be the master signal in the regulation of PEPCK. Since AMPK expression is lower in hepatocytes lacking CAR, another possibility might be that exogenous CAR may interfere with the complex interplay of proteins, thereby regulating the expression of

AMPK and subsequently its activation. Finally, PXR is known to regulate several CAR target genes, thus it is not excluded that in the absence of CAR, the role of PXR may be extended to the regulation of AMPK activity.

Interestingly, the expression and consequently the phosphorylation of acetyl-coA carboxylase, a known AMPK target is lower in the hepatocytes from CARKO animals suggesting a lower AMPK activity in the liver of these mice. This result corroborates the lower expression of acc-1, acc-2 and fas during the fasting refeeding experiment and with a lower accumulation of triglycerides, whereas it does not correlate with the expresssion of acc-1, acc-2 and fas during the high carbohydrate experiment. However, Savage et al. suggested that both acc-1 and acc-2 are involved in regulating fat oxidation in the liver, whereas only acc-1 synthesizes malonyl-CoA for fat synthesis [257-259]. Altogether, these findings are consistent with a role of CAR in the regulation of lipid metabolism via acetyl-coA carboxylase isoforms 1 and 2.

Malonyl CoA is a regulator of carnitine palmitoyl transferase 1 (CPT-1), the enzyme that controls the transfer of long chain fatty acyl-CoA into mitochondria where it is oxidized. The formation of malonyl-CoA is regulated acutely (in minutes) by changes in the activity of acetyl CoA carboxylase, the enzyme that catalyzes malonyl CoA synthesis. An increase in the concentration of malonyl-CoA leads to the inhibition of CPT-1 activity and subsequently to the inhibition of beta oxidation of fatty acids. A decrease in ACC-1 and/or 2 phosphorylation leads to an increase in malonyl-CoA synthesis and ultimately to the inhibtion of CPT-1. Whether the CAR-mediated regulation of ACCs is reflected in malony-CoA synthesis and activation of CPT-1 is not known. Furthermore, our study did not discriminate between ACC-1 and ACC-2 isoforms. Thus, the relative importance of each isoforms in the regulation of lipogenesis and fatty acid oxidation may complicate the comprehension of the role of CAR. Previous reports described that the repeated treatment of rats with phenobarbital downregulates a number of genes involved in energy metabolism, including CPT-1. Additionally, Ueda et al. showed by cDNA microarray analysis that PB-mediated downregulation of CPT-1 was CAR dependent in mouse liver [14]. However, a recent study indicated that the serum concentration of total ketone bodies was increased after seven days of PB treatment [260]. It is worth noting that all these data have to be interpreted with caution, since no experiments demonstrate CAR as a regulator of beta oxidation of fatty acids or ketogenesis in the liver.

Although ACC is a known target of AMPK, in CARKO hepatocytes, the higher expression of AMPK α1 subunit does not correlate with a stronger phosphorylation of ACC. This finding indicates that CAR may regulate ACC expression and activity independently of AMPK α1 subunit. Interestingly, a recent study reported that caloric restriction decreased threonine-172-phosphorylated AMPKα levels in the liver, and the levels of ACC phosphorylation did not alter in parallel with the AMPK phosphorylation level, particularly in the liver [261]. Another study showed that the expression of ACC and its phosphorylation increased independently of AMPK activation upon high carbohydrate diet [262]. Altogether, these results suggest that other AMPK independent mechanisms are involved in the regulation of ACCs. Moreover, the activities of both AMPK α1 and α2 subunits and their relative contribution to the phosphorylation of ACC in the liver have not yet been studied.

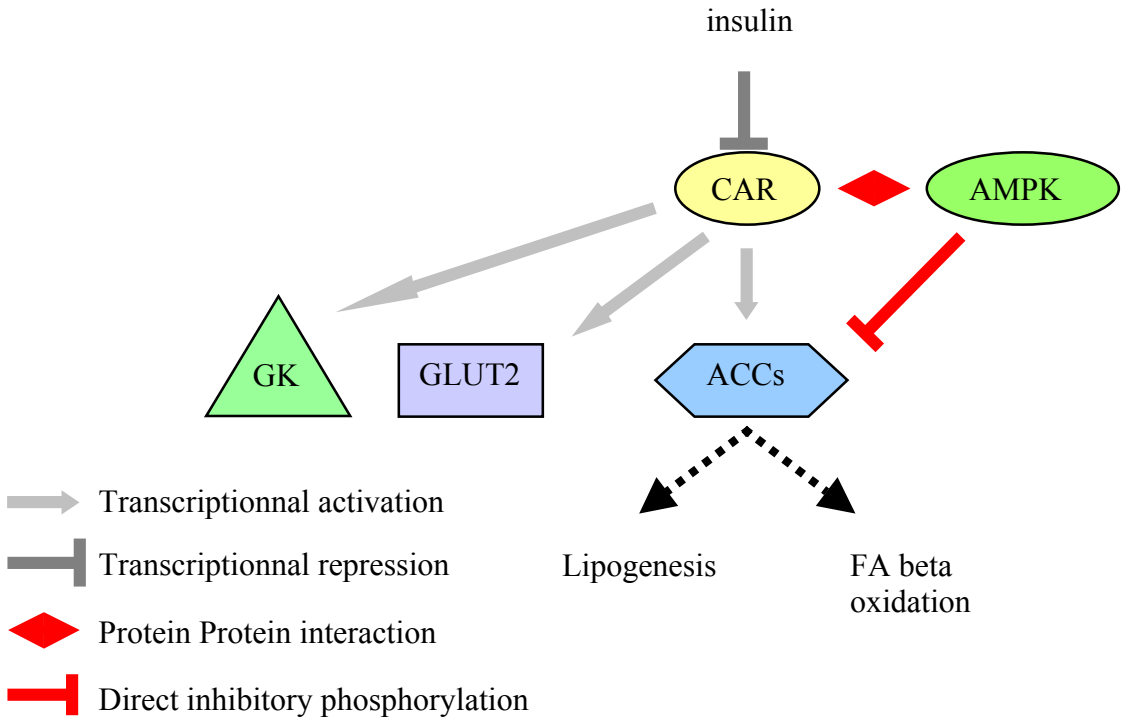

Figure 3: **Role of CAR in hepatic metabolism**

In the fed state insulin represses CAR mRNA expression which may indirectly impair the regulation of glucose homeostasis and lipid metabolism.

Nuclear receptor cross-talk and glucose homeostasis

A major breakthrough in bile acids (BAs) metabolism came with the demonstration that BAs bind to and activate the farnesoid X receptor (FXR), an adopted member of the nuclear receptor family [263-265]. The physiological role of FXR is to protect liver cells from the deleterious effects of BA overload by decreasing their endogenous production and accelerating their biotransformation and excretion, thereby acting as an intracellular bile acids sensor [266]. Despite some unresolved discrepancies, several sets of data are also in favor of a role for FXR in the control of gluconeogenic gene expression. Some results indicate that FXR might decrease PEPCK expression through the induction of SHP, which inhibits the activation of the PEPCK promoter by HNF-4α and Foxo1 in response to BAs treatment [267], whereas other *in vitro* data showed that BAs decrease the activity of HNF-4α, which is a positive regulator of PEPCK gene expression, in an FXR-independent manner [268]. Other studies demonstrated that FXR activation by an agonist induces PEPCK expression, leading to an increased glucose output in rodent primary hepatocytes *in vitro* [269]. Interestingly, SHP has been shown to bind to and inhibit the transcriptional activity of CAR [270]. Although, the interaction between CAR and FXR has never been evidenced, previous reports indicated that several sequences known to be responsive elements in the promoter of CAR and PXR target genes are shown to be targets of FXR [94, 271]; we might then speculate that CAR and FXR may cross-talk via SHP to regulate PEPCK during nutritional stress.

AMPK in hepatic glucose metabolism

The activation of AMPK suppresses the transcription of PEPCK and G6Pase in hepatoma cells [205], providing clues for the role of AMPK in gluconeogenesis. Furthermore, AMPK α2 liver-specific knockout mice are glucose intolerant and display fasting hyperglycemia, presumably because of elevated gluconeogenesis associated with increased PEPCK and G6Pase activity [272]. Recent data have provided evidence that inhibition of gluconeogenesis by AMPK involves a transcriptional coactivator, the transducer of regulated CREB activity 2 (TORC2) [273]. Glucagon and fasting induce hepatic TORC2 nuclear translocation, which enhances CREB-dependent transcription of gluconeogenic genes such as PGC-1α. Conversely, AMPK activation causes TORC2

phosphorylation and sequesters the coactivator in the cytoplasm, thus blunting gluconeogenesis. Deletion of hepatic LKB1, an upstream kinase of AMPK, abolishes AMPK activation and leads to nuclear accumulation of TORC2, which in turn drives gluconeogenesis [274] (Figure 4). Consistently, liver LKB1 knockout mice on a high-fat diet exhibit fasting hyperglycemia and glucose intolerance and are unresponsive to metformin treatment [274]. Interestingly, our group demonstrated that phenobarbital promotes the formation of an LKB1-AMPK complex, resulting in the phosphorylation of LKB1 which in turn leads to the phosphorylation of AMPK [212]. Whether this mechanism can be generalized to mammalian species and extended to the regulation of gluconeogenesis is presently unknown. Nevertheless, this finding is consistent with the previously reported observation that PB lowers blood glucose in patients with noninsulin-dependent diabetes, as mentionned above (introduction).

Inoue et al. cloned and characterizated a novel zinc finger transcription factor referred to as AICAR response element binding protein (AREBP) [275]. AREBP is phosphorylated at Serine 470 by AMPK. Transient transfection experiments indicate that wild-type AREBP represses PEPCK gene expression. However, the authors did not provide any evidence for the role of AREBP in the *in vivo* regulation of PEPCK. Furthermore, whether phenobarbital induces the AMPK-mediated phosphorylation of AREBP is presently unknown.

Many of the studies validating AMPK stimulation as a potential target for the treatment of type-2 diabetes, obesity and the metabolic syndrome have utilized AICAR as a tool to induce AMPK activation. Since AICAR is metabolized to ZMP, an AMP mimetic, it clearly has additional effects that are independent of AMPK activation. In both rat hepatocytes [276] and mice *in vivo* [277], AICAR was shown to dose-dependently inhibit fructose-1,6-bisphosphatase activity hence potently inhibiting gluconeogenesis. Consequently, to circumvent this lack of specificity, Cool et al., identified a thienopyridone family of AMPK activators [278]. The authors show that A-769662 directly stimulated partially purified rat hepatic AMPK (EC50 = 0.8 μM) and inhibited fatty acid synthesis in primary rat hepatocytes (IC50 = 3.2 μM). Furthermore, treatment of ob/ob mice with 30 mg/kg A-769662 decreased hepatic expression of PEPCK, G6Pase, and FAS, lowered plasma glucose by 40%, reduced body weight gain and significantly decreased both plasma and hepatic triglyceride levels. However, the

molecular mechanisms underlying AMPK activation by A-769662 are presently unknown, but this compound remains a putative AMPK activator that may be used *in vivo*.

Leclerc and coworkers showed that treatment of rat hepatocytes with AICAR decreases HNF4α protein stability and consequently decreases the expression of some of its target genes. The authors claimed that AMPK phosphorylates HNF4α, thereby reducing its homodimerization and DNA-binding activities and stimulating its degradation, resulting in down-regulation of HNF4α target genes [279]. This is not consistent with the possibility of AMPK regulating drug induction by increasing the HNF4α expression or activity, but it corroborates the role of AMPK in the regulation of PEPCK.

Another potential target of AMPK in the regulation of gluconeogenesis is p300, a transcriptionnal coactivator with strong histone acetyltransferase activity, which acts not only as an adaptor between basal transcription machinery and transcription factors, but also plays a role in the acetylation of histones in order to increase DNA accessibility, or, other proteins for their activation, nuclear retention or modulation of half-life. AMPK phosphorylates p300, reducing its ability to bind to nuclear receptors and thus repressing their target gene expression [280]. As previously mentioned, p300 is simultaneously recruited together with CREB-binding protein (CBP) to the PEPCK promoter during fasting to activate gluconeogenesis [281]. Although, the AMPK-mediated phosphorylation of p300 has never been demonstrated under fasting conditions, p300 remains a potential downstream target of AMPK in the repression of PEPCK.

Another group showed that a dual specificity phosphatase, DUSP-4, is induced by AMPK in H4IIE and Fao hepatoma cells at both mRNA and protein levels [282]. The authors also demonstrated that AMPK induces the immediate early transcription factor EGR-1 (early growth response 1), a known transcriptional activator of DUSP-4, and it directly binds the DUSP-4 promoter at its known binding site. Consequently, the activation of dusp-4 leads to the inhibtion of PEPCK and G6Pase but the mechanistic part of this study remains uncomplete. Furthermore, the *in vivo* importance of EGR1 and DUSP-4 activation in gluconeogenesis remains to be addressed.

The forkhead transcription factor FKHR (now referred to as FOXO1) [283], a known positive regulator of PEPCK has also recently been shown to be down-regulated upon AICAR incubation in H4IIE hepatoma cells [284]. Lower expression of this factor may in

turn repress G6Pase and PEPCK genes [205]. However, as previously mentioned, the data obtained with AICAR should be interpreted with caution as its specificity in the activation of AMPK is limited. Furthermore, the studies never demonstrated the phosphorylation of FOXO1 by AMPK neither *in vitro* nor in *vivo.*

In addition to gluconeogenesis, AMPK has been implicated in the regulation of hepatic lipogenesis, lipid oxidation, and cholesterol synthesis [285]. AMPK suppresses glucose-induced expression of lipogenesis-associated genes such as fatty acid synthase [286], ACC, and pyruvate kinase [287]. In rat primary hepatocytes, activation of AMPK by AICAR or metformin increases fatty acid oxidation by inhibiting ACC [286]. Consistently, in rats treated with metformin, triglyceride levels are reduced, concomitantly with an increase in β-hydroxybutyrate, suggesting elevated hepatic lipid oxidation [286]. These enhanced metabolic profiles are accompanied by downregulation of lipogenic genes such as SREBP-1, fatty acid synthase, and S14 [286]. Moreover, the lipid-lowering effects of metformin in cultured hepatocytes requires AMPK activity [288]. Taken together, these studies provide evidence that AMPK suppresses hepatic gluconeogenesis and lipid production, while decreasing hepatic lipid deposition by increased lipid oxidation, thus improving the glucose and lipid metabolism in type-2 diabetes.

A perplexing aspect of the role of AMPK in the normal regulation of hepatic gene expression is that changes in the activity of the enzyme have been difficult to measure in primary isolated hepatocytes, under conditions in which alterations in lipogenic and/or gluconeogenic gene expression are clearly evident [287, 289]. Moreover, while expression of a constitutively active form of AMPK α1 completely abrogated the effects of glucose on the levels of mRNAs encoding lipogenic enzymes, inhibition of AMPK activity with dominant-negative AMPK α1 failed to alter the suppression of these genes by low glucose concentrations [287]. Thus active AMPK would appear to serve in the liver as an efficient silencer of lipogenic gene expression, and this role may be important in mediating the effects of fatty acids during starvation [290]. On the other hand, it seems likely that additional mechanisms are involved in the normal stimulatory action of high glucose (and insulin) on hepatic expression of lipogenic genes.

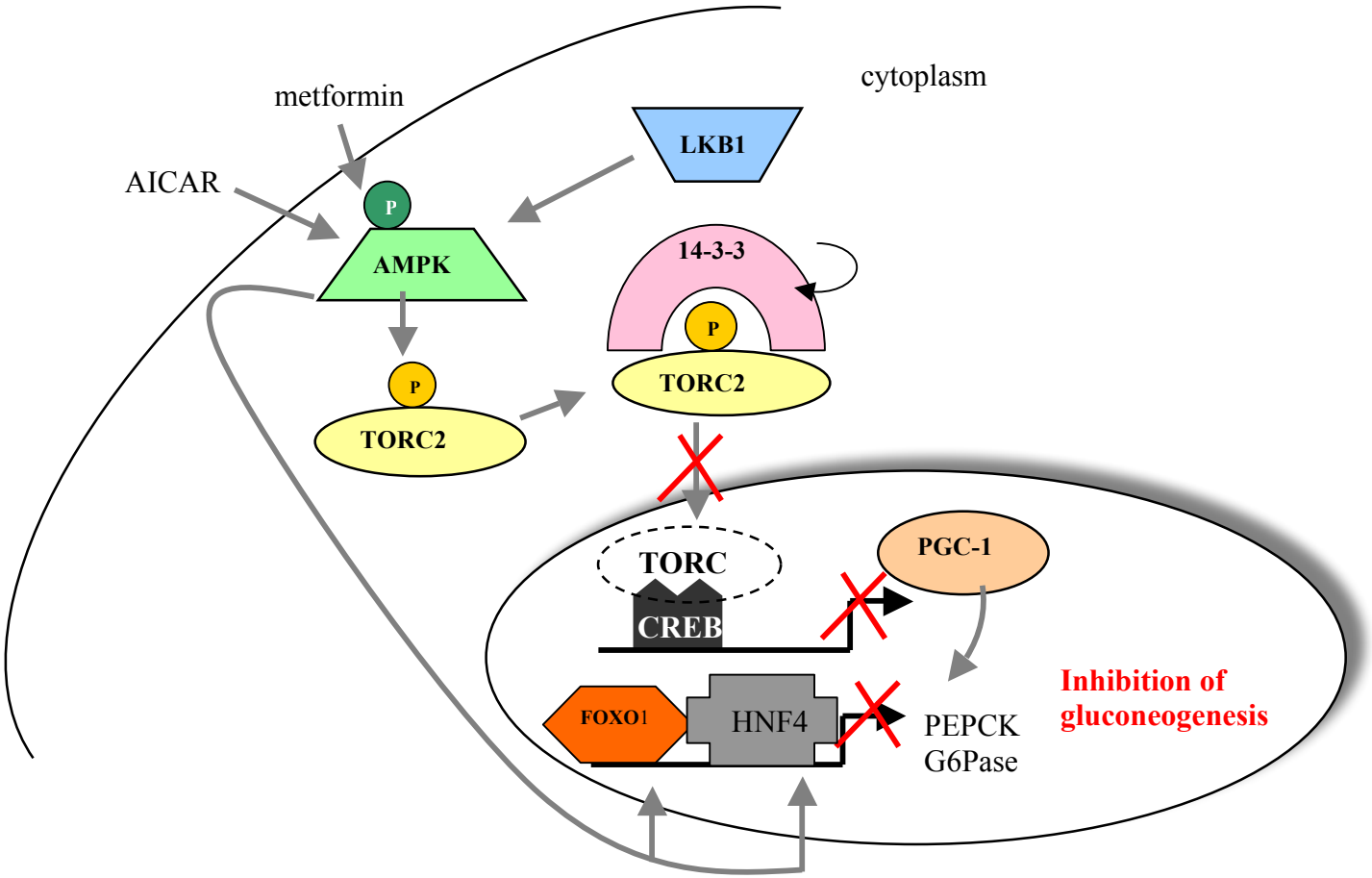

Figure 4: **Transcriptional control of gluconeogenesis by TORC2 and AMPK**

The activity of TORC2 is controlled by AMPK phosphorylation, which determines whether TORC2 becomes localized in the nucleus. Phosphorylated TORC2 is sequestered in the cytoplasm via a phosphorylation-dependent interaction with 14-3-3 proteins. Moreover, AMPK can also control gluconeogenic gene transcription by regulating stability or degradation of HNF4α and FoxO1 transcription factors.

Regulation of gluconeogenesis in diabetes

Type-2 diabetes is characterized by peripheral insulin resistance associated with chronic hyperglyceamia notably due to a gradual and progressive increase in endogenous glucose production during fasting. In the fasted state, gluconeogenesis is increased in diabetic compared with normal individuals and is incompletely suppressed by insulin in the postprandial state [291]. Thus, increased hepatic glucose production contributes to hyperglycemia in both the fasting and postprandial states. High-level expression of the PEPCK-C gene in the liver is a major characteristic of diabetes. Additionally, transgenic mice containing multiple copies of the rat PEPCK-C gene display hyperglyceamia that is resistant to insulin [292], strongly suggesting that overproduction of PEPCK itself can disrupt glucose homeostasis and subsequently lead to diabetes. Furthermore, mice carrying tissue-specific deletion of PEPCK or overexpressed PEPCK displayed type-2 diabetes phenotype, as well as several unexpected phenotypes such as obesity, lipodystrophy, fatty liver, and death. These phenotypes arise from perturbations not only in gluconeogenesis, but in two additional metabolic functions of PEPCK-C: first, cataplerosis [293], which maintains metabolic flux through the Krebs cycle by removing excess oxaloacetate, and second glyceroneogenesis, which produces glycerol-3-phosphate as a precursor for fatty acid esterification into triglycerides [294]. PEPCK-C catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. It is in part the tissue-specificity of this simple reaction that results in the variety of phenotypes listed above. Mice with a total knockout of PEPCK die within 2-3 days from birth, not from hypoglycemia, but probably because the Krebs cycle slows to approximately 10% of normal level in the absence of cataplerosis [295].

However, Burgess et al. recently demonstrated that 90% reduction of PEPCK content in the liver causes 40% reduction in gluconeogenic flux, indicating a lower capacity for PEPCK protein to control gluconeogenesis than expected [296]. This result suggests that other factors such as peripheral substrate supply or insulin-mediated effects on other gluconeogenic enzymes and/or hepatic energy metabolism must coordinate with PEPCK expression to attenuate gluconeogenesis. Although, liver-specific PEPCK knockout mice have markedly impaired hepatic gluconeogenesis, they are able to maintain a near-normal blood glucose concentration during fasting by increasing extrahepatic gluconeogenesis

and diminishing whole-body glucose utilization [297]. Furthermore, variety of studies in mice demonstrate important modulations of hepatic glucose metabolism even in the absence of altered PEPCK expression [298, 299] or disregulated PEPCK expression without significant effect on gluconeogenesis [300]. This incongruence might occur because other factors, such as hepatic energy metabolism [301], can also project substantial control over the rate of gluconeogenesis. Moreover, sustained induction of PEPCK may also occur as an indirect consequence of the decrease in glucose tolerance in diabetes. This is also favors an efficient cooperation between different factors, all involved in intricated pathways that control the endogenous glucose production.

Concluding remarks

Phenobarbital was found to repress genes that encode enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK). Here, we show that both CAR and AMPK are necessary to mediate the PB inhibitory effect on PEPCK mRNA expression. Furthermore, our study indicates that AMPK and CAR interacts in the regulation of PEPCK promoter activity. Once activated by PB, the CAR-AMPK complex may prevents coactivators such as PGC-1α from their recruitment to the PEPCK promoter. Whether the physiological objective of PEPCK repression consists in the prevention of NADPH loss remains to be evaluated. NADPH is essential for cytochrome P450 dependent monoxygenase activities and glutathion recycling. In the liver, the pentose phosphate pathway converts glucose 6-phosphate to ribose 5-phosphate by glucose 6-phosphate dehydrogenase (G6PDH) and generates NADPH. In the gluconeogenesis pathway, glucose 6-phosphate is the last intermediate on the way to glucose, a reaction catalyzed by G6Pase. Thus, repression of gluconeogenesis by xenobiotic-activated CAR might be required to maintain sufficient NADPH levels for xenobiotic metabolism. Alternatively, the decrease in CAR/PXR activity by insulin is consistent with decreased NADPH production through repression of gluconeogenesis.

On the other hand, we demonstrate that endogenous CAR positively regulates PEPCK and the expression of genes encoding for of acetyl-CoA carboxylase (ACC), an enzyme known to be involved in the conrtol of lipogenesis and beta oxidation of fatty acids. Although, pharmacological activation of CAR by PB may not necessarily reflects the constitutive endogenous activation of CAR, the activation of AMPK by PB and the subsequent interaction between CAR and AMPK provide a strong link between energy and drug metabolism. This is also consistent with a hypothesized protection of NADPH stocks. Therefore, the CAR-AMPK connection may serves as an energy sensing platform. In response to nutritional stress or to xenobiotics, the connection may recruits specific cofactors to permanently control the energy status of the cell, suggesting that CAR might be an energy sensor too.

Outlook

Phenobarbital is an excellent pharmacological tool in the comprehension of the molecular mechanisms underlying CAR function in the liver. Since cDNA microarray experiments revealed that more than 140 genes are regulated by PB and CAR regulates only half of them, it is clear that CAR is not the limiting factor mediating the entire phenobarbital effect in the liver. On the other hand, independently of its activation by PB endogenous CAR plays a role in glucose homeostasis even though CAR's precise function in glucose turnover remains unknown. It would be very interesting to measure glucose disposal, production and blood basal parameters such as glyceamia, insulinemia, triglycerideamia and total cholesteroleamia in CARKO mice injected with streptozotocin, a drug that destroys pancreatic cells thereby leading to diabetes. Moreover, it would be very attractive to see whether the suppression of CAR mRNA expression or the inhibition of its activity in db/db and ob/ob diabetic mice would improve the hyperphagia, hyperglyceamia, hyperlipidemia and obesity in these animals. Furthermore, as we previously suggested, CAR may also be involved in the regulation of lipogenesis and/or the beta oxidation of fatty acids in the liver, and since these animals are resistant to hepatic steatosis we might speculate that CAR may indirectly regulate energy expenditure. Consequently it would be useful to measure the ability of CARKO animals to run on a wheel and to measure their respiratory quotient.

To evaluate the physiological relevance of the AMPK-CAR connection it would also be of interest to generate animals with liver-specific deletion of both AMPKα1/2 subunit and CAR. Additionally, AMPK activity should be assessed in livers of CARKO mice, under different nutritional conditions. Unlike the classic model of nuclear receptor action in which a receptor only directly binds to its enhancer element, CAR also exerts its effect by acting as a co-regulator of gene transcription. The specificity of CAR activity may be dependent on the specificity of the interaction with a cofactor including PGC-1α and SRC-1. Thus, the identification of CAR new partners in a particular physiological context will certainly help in the comprehension of the molecular mechanisms underlying CAR function in drug metabolism and in energy metabolism.

Materials and Methods.

Reagents

Phenobarbital (sodium salt) was purchased from Fluka (Buchs, Switzerland). All other chemicals were from Sigma (Buchs, Switzerland). Cell culture media, foetal bovine serum, other tissue culture reagents, and Trizol reagent were from Gibco-BRL Life-Sciences Basel, Switzerland. Antibodies rose against AMPK α1 and α2 subunits and phospho-acetyl-CoAcarboxylase were purchased from Upstate Biotechnology (Lucern, Switzerland).

Plasmid constructs

The eukaryotic expression plasmid for human CAR has previously been described [187]. The proximal promoter of the rat PEPCK-CAT (–467 to +69, with respect to the transcription start site), was described and kindly provided by Dr Granner. PEPCK-CAT was digested with AflIII and BglII and subcloned into the digested luciferase reporter gene vector pGL3-Basic (Promega).

Transient Transfections and Reporter Gene Assays

Transactivation assays with CV-1 cells were performed as previously described.(Handschin et al., 2000) CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Sigma, Buchs, Switzerland). Cells were split 1:10 in DMEM/F12 without phenol red (Invitrogen), supplemented with 10% charcoal-stripped fetal bovine serum. After 3 days' incubation, cells were plated in 96-well plates at a density of 30,000 cells per well and expanded overnight. The medium was then replaced with the transfection mixes in Optimem I without phenol red (Invitrogen). Transfection mixes contained 10 ng of expression construct encoding either human CAR cDNA (kindly provided by Dr M. Negishi, National Institute of Environmental Health Sciences, Research Triangle Park, NC) or 50 ng of human PGC-1α, (kindly provided by Dr A. Kralli, Departments of Cell Biology, The Scripps Research Institute, La Jolla, California), 20 ng of reporter vector PEPCK-LUC containing the -467+69 fragment of the rat PEPCK promoter; 60 ng of

pRSV- β -galactosidase construct; and carrier plasmid to a total of 150 ng deoxyribonucleic acid per well. Cells were transiently transfected with 1 μL/well of Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. After overnight incubation, medium was replaced with DMEM/F12 without phenol red, supplemented with 10% delipidated, charcoal-stripped fetal calf serum (Sigma, Buchs, Switzerland) containing the inducer compounds of interest dissolved in dimethylsulfoxide vehicle at a final concentration of 0.1%. After 24 hours' incubation, cells were lysed in Passive Lysis Buffer (Promega, Madison, Wis). Luciferase and β-galactosidase activities were determined as previously described [27].

Generation of AMPKα1/α2 LS-/- knock out mice.

The liver-specific knock out of both alpha subunits of AMPK has previously been described [302]. Briefly, to generate deletion of both catalytic subunits in the liver (α1α2 LS-/-), liver specific AMPKα2-null mouse 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 knock out background by crossing liver-specific α2-/- mice with AMPKα1-/- general knock out mice [303].

Generation of CAR knock out mice

The targeted deletion of the nuclear constitutive androstane receptor (CAR) in mice has previously been described [30]. Breeding pairs of these CAR knock out mice were kindly provided by David D. Moore (Department of Biochemistry and Molecular Biology, University of Texas Medical School of Houston, Texas USA).

Recombinant adenoviruses

Adenovirus expressing Hemagglutinin tagged human CAR (Ad-HAhCAR) was constructed by using Gateway cloning and the ViraPower Adenoviral Expression System (both from Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. First, the 2560-bp cDNA fragment encoding the human CAR cDNA and a CMV

promoter was sub-cloned by PCR from a pCIneo-hCAR contruct into the pDONOR Gateway vector by the following homologous recombination oligonucleotides:
5'-
GGGGACAGTTTGTACAAAAAAGCAGGCTGCTCACCATGGCTCGACAGATCT-3' and 5'-GGGGACCACTTTGTAACAAGAAAGCTGGGTTTACGCCAGCCCGATCCTT-3'.
The construct was then transferred into the pAdDEST adenoviral Gateway vector by LR Clonase (Invitrogen) reaction to give pAdHAhCAR. Integrity and proper insertion of the cloned hCAR cDNA were confirmed by sequencing. Adenoviral expression plasmids, pADEST and pDONOR, and packaging plasmid mix were purchased from Invitrogen (Invitrogen, Carlsbad, CA). Production of adenoviral particles and subsequent infection of target cells were performed essentially according to the manufacturer's protocols, except that Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of HEK-293T packaging cells. Pseudoviral supernatant harvested from the packaging cells was used directly for cell infection.
Adenovirus encoding constitutively active α1 AMPK subunit (ad-CA-α1 312) or dominant negative mutant α1 AMPK (ad-DNα1) were prepared as described (Diraison et al.,2004). Adenoviruses encoding constitutively active α2 AMPK subunit (ad-CA-α2 312) or β-galactosidase were also amplified as previously described (Foretz et al.,2005). Adenovirus encoding human CAR in fusion with eGFP (ad-hCAR-GFP) was a kind gift of Dr Ramiro
Jover (Hospital La Fe, Valencia, Spain). AMPK adenoviruses also express enhanced green fluorescence protein (eGFP), under control of a distinct CMV promoter.

Culture of primary human hepatocytes.

Cultures of primary human hepatocytes were obtained from patients undergoing liver surgery. All patients gave written consent. Culture from 3 donors were prepared and plated as described [64]. The cultures were maintained 24h in serum free induction medium (William'sE (invitrogen, Basel Switzerland) supplemented with 0.5x ITS (Insulin transferrin Selenium) and 100 nM hydrocortisone (both Sigma, Buchs, Switzerland), when medium was changed and cultures were exposed to chemicals 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 post-absorptive male mice (25-30g) after anaesthesia with ketamin/xylazin (1 mg/100 g body weight). Cell viability (>80%) was checked by trypan blue exclusion. Hepatocytes were seeded on rat tail collagen type1 coated dishes and cultured overnight in DMEM supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin. After overnight culture, the medium was replaced by a serum free DMEM medium. Twelve hours after serum deprivation cells were exposed to chemicals for 8 hours or as indicated in the figure legends, and were maintained in serum free medium.

Real-time PCR assays

One µg 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. 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 primer sequences are described in table 1.

Western blot analysis

Cultured hepatocytes were washed in ice cold PBS and harvested in 300 µl/6-cm dish of extraction buffer (KCl 100 mM, Hepes 25 mM, MgCl2 7.5 mM, glycerol 20%, pH 7.4, supplemented with protease inhibitors cocktail tablets (Roche, Rotkreuz, Switzerland) containing dithiothreitol (4 mM), aprotinin (2 mg/ml) and ß-mercaptoethanol (1 mM). The cell suspension was sonicated for 5 sec and cellular debris removed by centrifugation (1000xg for 10 min at 4°C). Thirty micrograms of total cellular protein were separated by Tris-Tricine glycerol-SDS PAGE and blotted onto nitrocellulose membranes. The following primary antibodies were employed: anti-AMPK α1 subunit and anti-AMPK α2 subunit, anti-phospho-ACC (Cell Signalling, 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

(Amersham Biosciences, Otelfingen, Switzerland) and exposure to Kodak X-ray films (Sigma, Buchs, Switzerland).

Immunoprecipitation.

mAT3F cells were cultured in DMEM F12 medium (Gibco, Chagrin falls, ohio) supplemented with penicillin streptomycin, 0.1 uM insulin, 1uM dexamethasone and 5% fetal bovine serum (Lefrancois-Martinez AM. Et al., 1994). The cells were co-infected with HA-hCAR and Myc-AMPKα1/2 adenoviruses for 48 h and were treated with PB or dexamethasone/cAMP for 1 hour. The cells were scraped in Tris 500 mM pH7.5, NaF 500 mM, EDTA 10 mM, EGTA 10 mM, Mannitol 2.5 M, 5 % Triton X-100, phosphatase inhibitor cocktail I and II (Sigma, Buchs, Switzerland) and protease inhibitors (Roche, Switzerland) left 15 min on ice, vortexed for 5 s, and centrifuged for 10 min at 4°C and 20,800 x *g*. A BCA assay was used to determine the protein content of the supernatant. The HA antibody (Covance, USA) and the myc antibody (Sigma, Buchs, Switzerland) were added to 500 ug of proteins and rotated overnight at 4°C. Finally, protein G agarose beads were rotated with lysates and antibodies for 2h at 4°C. The beads were washed three times with PBS before being resuspended in protein loading buffer.

Immunocytochemistry.

Hepatocytes were cultured on glass coverslips coated with rat-tail collagen (25µg/cm^2). Human hepatocytes were infected 12 h after seeding in a serum-free medium with Ad-hCAR-GFP at an MOI of 30 to 100. Endogenous AMPK α2 was detected with polyclonal antibody (abcam) and a secondary antibody Alexafluor 594 (Molecular probes).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 40x objective (1.40 numerical apertures) by using a Leica TCS NT confocal laser scanning microscope (Leica, Wetzlar, Germany).

Mammalian two hybrid assay

An expression plasmid for GAL4 DNA binding domain (Gal4RE) fused to human CAR ligand binding domain (hCAR LBD) was constructed [304]. This plasmid was cotransfected in CV-1 cells with psG5-hPGC-1α or pCDNA3-AMPK α2 and the reporter plasmid pGal4 response element-Luc. Luciferase was determined as described above.

Glucose production

Primary mouse hepatocytes were serum-starved overnight and stimulated with insulin (100 nm) for 24 h. Cells were washed and incubated in DMEM glucose free medium containing 20 mM lactate and 2 mM pyruvate for 24 h. Culture medium was collected and centrifuged, and glucose levels were determined by glucose-oxidase kit, according to the manufacturer instruction (Sigma-Aldrich) and normalized to protein content of the cells.

Immobilized metal affinity chromatography (IMAC)

One microgram of recombinant his-AMPKα1 subunit (cell signaling) was incubated for 1h at 4°C, in HEPES 50mM pH 7.5, NP140 0.1%, glycerol 10%, KCl 1mM, $MgCl_2$ 2mM with 200 µg of protein lysate from human hepatocytes previously infected with HAhCAR adenovirus. The histidine tagged complex was retained on a Nickel affinity gel (Sigma, Buchs, Switzerland). The complex was washed and eluted with imidazole according to the manufacturer instructions. The different fraction were analysed on 10% SDS PAGE

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 instructions. Briefly, a soluble fraction containing active AMPK is used in a 40 ul reaction in the presence of 75 mM $MgCl_2$, 0.5 mM ATP, 0.3 mM AMP, 0.1 mM SAMS, 0.4 mM DTT and 1 mCi/100 ul [γ-^{32}P] ATP for 10 min at 30°C. At the end of the incubation, 35 ul of supernatant from the reaction mixture were spotted on Whatmann filter papers (P81) which were then washed three times with 0.75% phosphoric acid, once with acetone and then allowed to by dried befor scintillation counting in 5 ml scintillation cocktail.

Animals

C57/B6 wild-type mice and mice with a targeted deletion of the nuclear constitutive androstane receptor were maintained on a standard laboratory chow with food and water ad libitum. Twelve week-old male mice from each genotype were grouped in 4 to 6 animals and were previously injected by phenobarbital 50 mg/kg for 10h. The animals were then fed and fasted as described in the legends of the figures.

Some mice have been refed upon a **high carbohydrate-low fat diet**. The composition of theses pellets is:

Saccharose	75 %
Pork fat	3 %
Protein	13.5 %
Calories	15.2 MJ/kg

Abbreviations

ACC	acetyl-CoA carboxylase
AF2	accessory factor 2
AICAR	5'-phosphoribosyl-5-aminoimidazol-4-carboxamide
ALAS1	d-aminolevulinic acid synthase 1
ATP	adenosuine monophosphate
AMPK	AMP-activated protein kinase
AREBP	AICAR response element binding protein
ATP	adenosine triphosphate
BAs	bile acids
bp	basepair(s)
CAR	constitutive androstane receptor
CaMK	calcium/calmodulin-dependent protein kinase
CCRP	cytoplasmic CAR retention protein
C/EBP α	CCAAT/enhancer-binding protein
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
CRU	cAMP response unit
CPT	carnitine palmitoyl transferase I
CXR	chicken xenobiotic receptor
CYP	cytochrome P450
DBD	DNA-binding domain
DN	dominant negative
DR	direct repeat
DUSP-4	dual specific phosphatase-4
EGFP	Enhanced green fluorescent protein
EGP	Endogenous glucose production
EGR1	Epidermal growth factor receptor 1

ER	everted reapeat
FOXO1	forkhead box protein O1A
FXR	farnesoid X receptor
G-6-Pase	glucose 6-phosphatase
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GK	glucokinase
GLUT	glucose transporter
GR	glucocorticoid receptor
GRU	glucocorticoid response unit
HNF4α	hepatocyte nuclear factor 4α
Hsp90	heat shock protein 90
IF	immunofluorescence
IL-6	interleukine 6
IP	immunoprecipitation
IR	inverted repeat
IRE	insulin response element
IRS	insulin response sequence
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
NFkB	nuclear factor kappaB
NADPH	nicotinamide adenine dinucleotide phosphate
NIDDM	Non-insulin dependent diabetes
NOS	nitric oxide synthase
NR	nuclear receptor
OA	okadaic acid
OATP2	organic anion transport protein 2
PAGE	polyacrylamide gel electrophoresis

PB	phenobarbital
PBP	PPAR-binding protein
PBREM	phenobarbital-responsive enhancer module
PBRU	phenobarbital-responsive enhancer unit
PCN	5-pregnen-3b-ol-20-one-16a-carbonitrile
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator α
PKA	cAMP-dependent protein kinase A
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RT-PCR	real-time PCR
RAR	retinoic
RXR	9-*cis*-retinoic acid receptor
siRNA	small interfering RNA
SREBP-1c	sterol response element binding protein-1c
SULT	sulfotransferase
TCPOBOP	1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene
TNFα	tumor necrosis factor α
TORC	Transducer of regulatory CREB
TR	thyroid hormone receptor
TSH	thyroid stimulating hormone
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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Curriculum vitae

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PERSONAL DETAILS

Date of Birth: 07.06.1977

Place of Birth: Selestat (France)

Citizenship: French-Moroccan

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EDUCATION

2004 – 2007:	Ph.D. at the University of Basel Division of Pharmacology –Neurobiology Programm in Genome Scale Biology and Bioinformatics Biozentrum University of Basel Subject: the regulation of glucose homeostasis by phenobarbital
2003-2004:	Bsc Hon Degree in molecular pharmacology at University Louis Pasteur of Strasbourg (France) - Subject: “The role of an LXRb agonist on the mouse stress response”
2002-2003:	Master in physiology at the University Louis Pasteur – Strasbourg (France)
2001-2002:	Master in molecular biology at the University Louis Pasteur – Strasbourg (France)
1999-2001:	Licence in biology

PROFESSIONAL EXPERIENCE

September 2005:	Education and training of Persons Conducting Animal Experiments.

	LTK Module 1E: Introductory Course in Laboratory Animal Science in Zurich (Switzerland).
Summer 2002:	Lab Training in the laboratory of Pr. Johan Auwerx at Institut de genetique de biologie moleculaire et cellulaire (IGBMC) at Ilkirch (France) Project: Cell culture and PCR
2002-2003:	6 months of Lab training in the laboratory of Pr. Johan Auwerx at Institut de genetique de biologie moleculaire et cellulaire (IGBMC) at Ilkirch (France) Project: Gene trap technology, FACS
June 2002:	Laboratoire de physiologie animale- Strasbourg (France) Study of rat genes expression in suprachiasmatique nuclei (SCN) upon light stimulation.

TECHNICAL EXPERIENCE

Molecular biology:	Genomic DNA isolation; subcloning ; Gateway system for Adenoviral construct (invitrogen); sequencing ; Gene trap technology; Quantitative real-time PCR; Gel shift assay.
Biochemistry:	Western Blot, Northern Blot, Immunofluoresence
Cell culture:	FACS analysis; Preparation of primary mouse and rat hepatocytes Primary human hepatocytes and cell line cultures; Gene reporter assay and mammalian two hybrid assay.
In vivo:	Personal in vivo experiment licence mouse and rat; Genotyping;

AWARDS

Second Outsanding poster award in 16th International Symposium on Microsomes and Drug oxidations at Budapest (Hungary)

SPECIAL SKILLS

Languages: **French** and **Arabic**: Native speaker; **English**: Excellent oral and written skills.

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