Regulation of Hepatic Heme Synthesis by Drugs, Bile Acids and Nutrition

A transcriptional network regulating δ-aminolevulinic acid synthase 1 (ALAS1)

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Abbreviations

AIP	Acute Intermittent Porphyria
ALA	Aminolevulinic acid
ALAS1	δ-Aminolevulinic acid synthase 1
CA	Cholic acid
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
ChIP	Chromatin Immunoprecipitation
CNG	Conserved Non-Genic Sequence
CYP	Cytochrome (s) P450
DHA	4,6-dioxoheptanoic acid
DR	Direct repeat
EMSA	Electromobility shift assay
FOXO1	Forkhead box protein O1A
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HNF4α	Hepatocyte nuclear factor 4 α
IR	Inverted repeat
LCA	Lithocholic acid
NRF1	Nuclear respiratory factor 1
PB	Phenobarbital
PBG	Porphobilinogen
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator activated receptor γ coactivator α
PPAR	Peroxisome proliferator activated receptor
PXR	Pregnane X receptor
RXR	9-*cis* Retinoic acid receptor
SHP	Short heterodimer partner
siRNA	Small interfering RNA
UDCA	Ursodeoxycholic acid

Abstract

ALAS1 is the rate limiting enzyme of heme synthesis. It is highly inducible in liver in cases of increased heme demand, such as drug metabolism or in inducible hepatic porphyrias. The clinical hallmark of these rare genetic disorders of enzymes of heme synthesis are neuropsychiatric attacks precipitated by drugs, hormones and fasting. The xenosensing nuclear hormone receptors CAR and PXR have been previously shown to mediate ALAS1 induction by classical inducer drugs (Podvinec et al. PNAS, 2004). The molecular details of the action of other precipitating factors, however, have not been elucidated so far. Studying the molecular mechanism of the fasting response as well as the involvement of additional nuclear hormone receptors in the transcriptional regulation of ALAS1 was the aim of this study.

We show that ALAS1 is regulated by the peroxisome proliferator-activated receptor γ-coactivator α (PGC-1α) via an insulin sensitive FOXO1 site within the promoter of ALAS1. In vivo studies confirm that ALAS1 is induced at the transcriptional level upon fasting. This effect is lost in liver specific PGC-1α knock-out animals, clearly demonstrating that PGC-1α is the master regulator of the fasting response. Interestingly, induction of ALAS1 by classical inducer phenobarbital is preserved, indicating that PGC-1α is not essential for the drug mediated induction of the gene.

In an attempt to identify additional factors regulating ALAS1, we have analyzed by phylogenetic footprinting the genomic sequence of human ALAS1. At minus 14kb of the transcriptional start site a novel response element for the bile acid receptor FXR, a nuclear receptor involved in lipid and glucose homeostasis as well as detoxification, was identified. Based on the molecular characterization of the FXR response element, and functional data in primary human hepatocytes and in mice as well as on genomic sequence analysis, we demonstrate that the response of ALAS1 to bile acid is unique to primates.

In addition, multiple enhancer modules identified by cross species sequence comparison were shown to be activated by hepatocyte nuclear factor 4α (HNF4α). Chromatin immunoprecipitation confirmed the binding of this factor in a native chromatin context. We therefore hypothesize that the liver enriched transcription factor HNF4α is required for basal expression and liver specific induction of ALAS1.

In conclusion, in the present work we describe and discuss a complex regulatory network consisting of FOXO1, the nuclear receptors CAR, PXR, FXR and HNF4α as well as the coactivator PGC-1α, which mediate the transcriptional regulation of ALAS1 to various stimuli, such as fasting, or to the exposure to endo- and xenobiotics.

1 Introduction

1.1 Heme synthesis

Heme is a pivotal molecule for life, and its synthesis takes place in every cell of the body except the mature erythrocyte. Hemeproteins are involved in a numerous crucial biologic functions such as oxygen binding (hemoglobin, myoglobin), oxygen metabolism (oxidases, peroxidases and catalases) and electron transfer (respiratory cytochromes) (Atamna, 2004). Moreover heme plays an important role by providing carbon monoxide (CO) through its catabolism, a signaling molecule whose wide biological function only started to be disclosed (Kim et al., 2006). Recent findings also revealed that heme may function as a circadian modulator of various cellular processes (Kaasik and Lee, 2004). To avoid heme accumulation and cellular toxicity heme levels need to be tightly controlled; this is achieved by a fine balance between heme biosynthesis and its catabolism by the enzyme heme oxygenase.

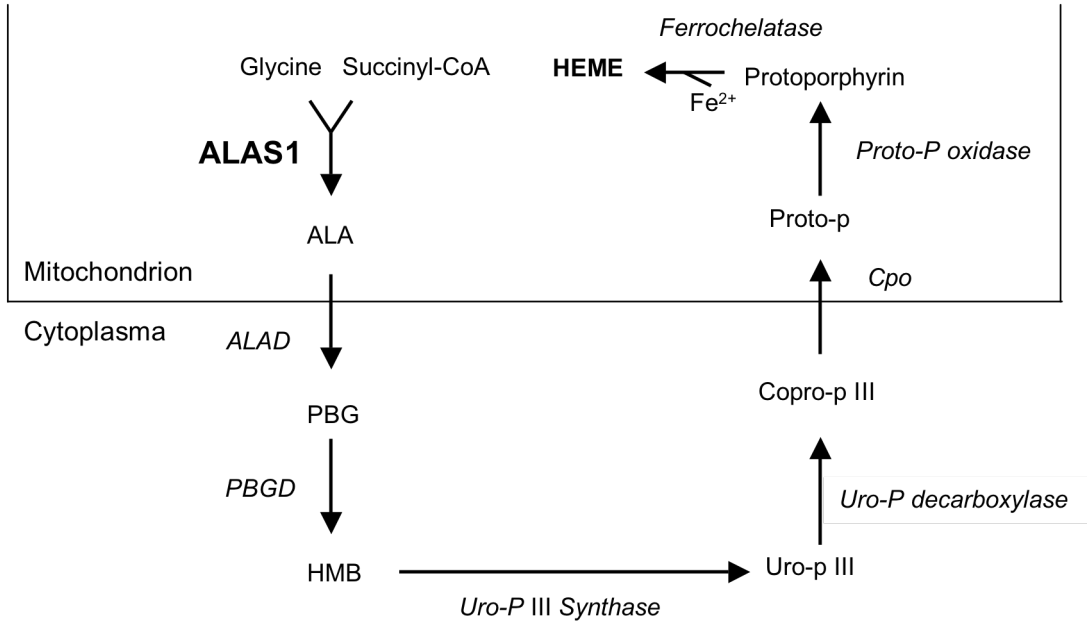

Figure 1 Heme synthesis pathway involves eight enzymatic steps. ALA, 5-aminolevulinic acid; ALAD, ALA dehydratase; PBG, porphobilinogen; PBGD, PBG deaminase; HMB, hydroxymethylbilane; Uro-P, uroporphyrinogen; Copro-P, coproporphyrinogen; Cpo, coproporphyrinogen oxidase; Proto-P, protoporphyrinogen

The heme synthesis pathway is schematically represented in Fig. 1. It consists of 8 enzymatic steps, partly located in the mitochondria, partly in the cytoplasm. The pathway starts at the mitochondrial matrix with the condensation of glycine and succinyl CoA to δ-aminolevulinic acid (ALA), a step, driven by the enzyme δ-aminolevulinic acid synthase (ALAS). ALA is subsequently transported to the cytoplasm, where 2

molecules of ALA are used to form the pyrolle ring of porphobilinogen (PBG). 4 PBGs are finally converted to protoporphyrin IX through decarboxylation and oxidation steps, which begin in the cytosol and end in the mitochondria. Ferrochelatase catalyzes the last step of the synthesis by incorporating iron into protoporphyrin-IX generating Fe-protoporphyrin IX, or alternatively called heme b.

There are 3 functional forms of heme known in eukaryotes: heme a, b and c, which slightly differ in substituents of the porphyrin ring system and the way they are incorporated into apoproteins. Heme b is the most abundant form and, like heme a, is bound through coordination to the iron ion to amino acid side chains of the proteins. Heme b is found in cytochromes P450, hemoglobin, myoglobin and respiratory complexes II and III. In contrast heme c is covalently bound through the two vinly side chains to the protein itself, such as in cytochrome c and nitric oxid synthetase. Formation of heme a requires two major modification: farnesylation and addition of a formyl group to position 8 of the Fe-protoporphyrin-IX. Mitochondrial complex IV is the only protein in the cell that contains heme-a. Ageing and neurodegenerative diseases such as Alzheimer disease have been connected to deficiency of heme-a and subsequent reduction in complex IV (Atamna, 2004) highlighting the pivotal role of heme in the maintenance of crucial cellular processes.

Thunnell compared the heme synthesis pathway with a pipeline having components of variable diameter according to the enzymes capacities (Thunell, 2000). The bottleneck and therefore rate limiting step of heme synthesis is its first step driven by ALAS. There are two isoforms of this enzyme encoded by different genes on separate chromosomes, erythrocyte specific ALAS2, expressed in erythroid cells only, and the ubiquitously expressed ALAS1. The two isoformes are completely differently regulated according to the need of the different tissues. In erythroid progenitor cells, where the bulk of body's heme is synthesized, heme is constantly demanded for the incorporation into globin chains to form hemoglobin. The acquisition of iron is the rate limiting step stimulating ALAS2 transcription via an iron response element in the promoter .

The liver is the organ where the second highest amount of heme is synthesized. The majority of it is incorporated into cytochromes P450, microsomal monooxygenases responsible for steroid hormone and bile acid synthesis and for detoxification of endogenous and exogenous substances.

In contrast to the bone marrow, where cell differentiation and cell division regulate the expression of heme synthesis enzymes, heme synthesis in liver must be flexible to adapt to the ever changing metabolic demand of the hepatocyte. Therefore ALAS1 is highly inducible by a variety of stimuli and simultaneously under the control of a negative feedback exerted by the end-product heme. Together with a free heme induced catabolism these mechanism ensure a fine tuning of cellular hepatic heme levels according to the current metabolic need.

1.2 Hereditary Defects of Heme Synthesis: The Porphyrias

The porphyrias are clinically very heterogeneous diseases resulting from defects in one of the enzymes of the heme synthesis pathway. Partial defects for all the enzymes of the heme synthesis pathway except ALAS1 are known. Depending on the location of the enzymatic defect within the pathway, different patterns of accumulation of intermediates and accordingly different clinical symptoms are observed. The location of the different enzymatic defects and corresponding porphyrias are given in Fig. 2.

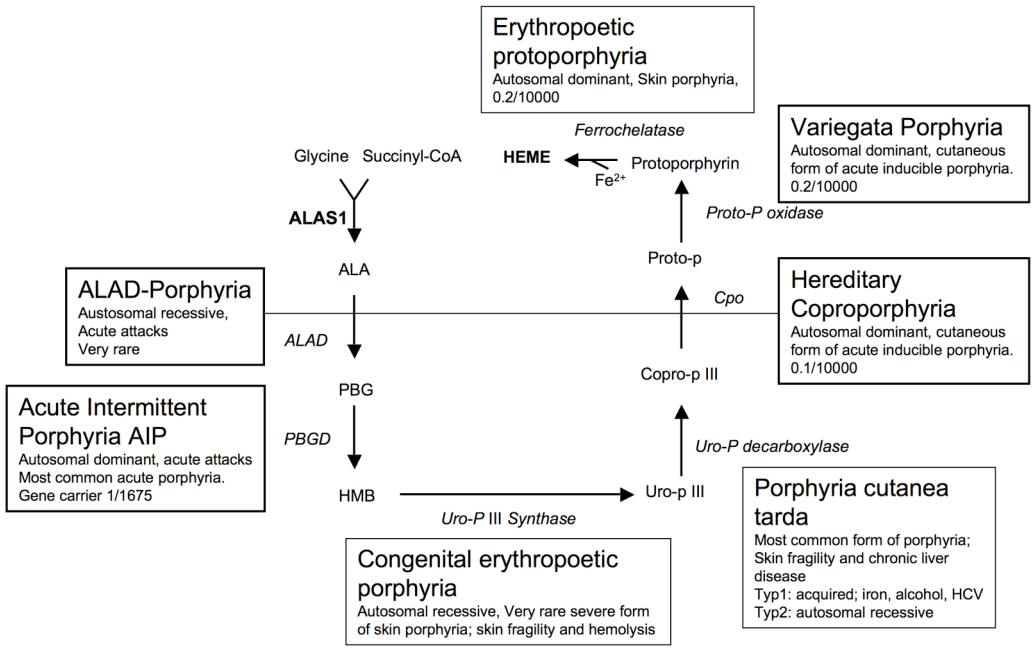

Figure 2 Hereditary enzyme deficiencies of heme synthesis pathway leading to 'Porphyrias' Partial defect of all of the enzmyes (except ALAS1) are known and the diseases are referred to as porphyrias. Clinically they are classified in acute, inducible porphyrias (bold) and/or skin porphyrias (light). Adapted from Thunell et al. 2000.

Photosensitivity due to accumulation of porphyrins in skin is the main clinical manifestation of the so called acute or chronic skin porphyrias. In contrast inducible, acute hepatic porphyrias are characterized by intermittent attacks of neuropsychiatric dysfunction always accompanied by increased porphyrin precursors ALA and PBG (Kauppinen, 2005, Anderson et al., 2005).

There are four different forms of acute porphyrias. Acute intermittent porphyria (AIP), variegata porphyria (VP) , hereditary coproporphyrinuria (HC) and the extremely rare ALAD deficiency (ALAD-P). The attacks of these hepatic porphyrias are indistinguishable from each other but all show increased urinary excretion of porpyhrin precursors. The acute attack is a potentially fatal condition characterized by severe abdominal pain, autonomic dysfunction and a motor neuropathy that may progress to

paralysis. Clinical manifestation are present in only a subset of gene carriers and arise after puberty and are more frequent in women (Kauppinen, 2005). The acute attacks may be precipitated by a number of known triggering factors, such as drugs, which induce cytochromes P450, fasting, hormones and any form of stress but most often remain unexplained. Acute attacks are treated by withdrawal of the causative agent, high carbohydrate load and most efficiently by intravenous administration of hemin. If dehydration and imbalance of electrolytes ensue, attacks can be life threatening and require intensive care. It is still under debate if increased ALA concentration in the nervous system or a neuronal heme deficiency account for symptoms in acute attacks (Sengupta et al., 2005) (Meyer et al., 1998). Liver transplantation has been reported to be successful in long term treatment of severe recurrent episodes. In a recent report, porphyrin precursors in the patients urine returned to normal already 24h after transplantation, and remained so for a follow-up of one and a half year (Soonawalla et al., 2004). These data clearly demonstrate, that the liver is the major source of excess porphyrin precursor production.

In order to understand the molecular basis of this liver specific and excessive overproduction of porpyhrin precursors, the regulation of ALAS1 has to be studied.

1.3 ALAS1 and its regulation

An important aspect of ALAS1 regulation in liver is the strong negative feedback, which is exerted by the end product heme. Heme represses its own synthesis via three different mechanism. It inhibits import of cytosolic ALAS1 precursor protein into the mitochondrial matrix, directly inhibits ALAS1 transcription (Kolluri et al., 2005) and reduces its mRNA stability (Thunell, 2000, May et al., 1995). After exposure of cells to heme, ALAS1 mRNA half-life decreases from 130 minutes to 40 minutes, illustrating the highly efficient repression (May et al., 1995). However, the exact mechanisms are still not understood and subject of ongoing research (Roberts et al., 2005). In the past it has been assumed that depletion of this so-called regulatory heme pool indirectly increases ALAS1 activity upon heme consuming stimuli. However, studies of our laboratory and others shed light into several aspects of transcriptional regulation of ALAS1, which demonstrate a direct stimulatory effects on ALAS1. What is known so far about the transcriptional regulation of ALAS1 will be briefly summarized below.

The ALAS1 promoter contains a consensus TATA box and two nuclear respiratory factor 1 (NRF1) sites, important for basal expression as demonstrated by site directed mutagenesis (Braidotti et al., 1993). 5'RACE of mRNA isolated from different tissues in rat revealed that up to six different transcription start sites exist, some of them independent of the TATA box (Roberts and Elder, 2001). The authors conclude that depending on the tissue, different start sites are used, which may account for the tissue specific expression and regulation of the enzyme. In liver, transcription is initiated almost exclusively via the TATA box.

The phenomenon of a repressive effect of glucose and insulin on ALAS1 has been known for long (Giger and Meyer, 1981). Canepa and coworkers described two different cyclic AMP response elements binding CREB in the ALAS1 rat promoter responding to protein kinase A (PKA) activation (Giono et al., 2001) in cell based transactivation assays. In addition they demonstrated that hepatocyte nuclear factor 3 beta (HNF3beta), a factor negatively regulated by insulin, was binding to two adjacent sites within the promoter (Scassa et al., 2004). However, mutation of the two HNF3beta sites did not completely abolish the repressive effect of insulin on ALAS1, indicating, that additional factors are involved in the transcriptional repression by insulin.

More than 80% of the heme synthesized in liver is used for incorporation into cytochromes P450 necessary for the metabolism of endogenous and exogenous substances. Hence the highest demand for heme is clearly exerted by the administration of drugs. The mechanisms how drugs directly upregulate heme synthesis has been deciphered only recently by different members of our group. Drug response elements binding the xenosensing nuclear receptors chicken X receptor (CXR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in the far upstream region of ALAS1 have been characterized in three different species: chicken, mouse and human (Fraser et al., 2002, Fraser et al., 2003, Podvinec et al., 2004). Together these data demonstrate that the principle action of nuclear receptors with respect to the drug response of ALAS1 is conserved over evolution.

1.4 The Nuclear Hormone Receptor Family of Transcription Factors

Nuclear hormone receptors are ligand activated transcription factors that work in concert with coactivators and corepressors to regulate gene expression. Nuclear receptor proteins have a characteristic modular structure, consisting of (from the N-terminus to C-terminus) a modulatory A/B domain, a highly conserved DNA binding domain containing two zinc fingers (DBD), a flexible hinge region and a ligand binding domain (LBD) responsible for dimerization and ligand dependent transactivation (Mangelsdorf et al., 1995). Nuclear receptors bind either as homodimers or as heterodimers to characteristic DNA response elements, consisting of a hexameric consensus halfsite AGGTCA arranged as direct (DR), inverted (IR) or everted repeats (ER). Full activation of nuclear receptors is achieved upon binding of ligands, such as hormones, fatty acids, bile acids, oxysterols or a variety of xenobiotics. Nuclear receptors thus exert diverse crucial roles in metabolic homeostasis, development, inflammation and detoxification (Francis et al., 2003).

Nuclear hormone receptors constitute one of the largest groups of transcription factors in animals. In mammals the superfamily of nuclear receptors is composed of approximately 50 different functional genes, with 48 in humans, 47 in rats and 49 in

mice (Zhang et al., 2004c). The current official nomenclature divides the superfamily into 6 subfamilies (NR0-6) based on their phylogenetic tree (NUCLEAR RECEPTORS NOMENCLATURE COMMITTEE, 1999). Most prominent members of the nuclear receptors, to mention only a few, are the steroid hormone receptors, such as estrogen receptor (ER), androgen receptor (AR) and the mineralocorticoid receptor (MR) and glucocortiocid receptor (GR). The nuclear receptors controlling fat, glucose, cholesterol, and bile acid metabolism include the peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs) and farnesoid X receptor (FXR).

Several nuclear receptors are involved in detoxification processes. They include members of the NR1 subfamily, such as CAR, PXR, vitamin D receptor (VDR) and farnesoid X receptor (FXR) as well as one member of the NR2 family, hepatocyte nuclear factor 4α HNF4α. The ones addressed in this study will be briefly introduced below.

1.4.1 The xenosensing nuclear receptors PXR and CAR

The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) belong to the NR1H subfamily and are the classical xenosensing nuclear hormone receptors activated by array of structurally diverse ligands (Handschin and Meyer, 2003). PXR has a broader substrate specificity than CAR and PXR ligands include commonly used drugs, such as rifampicin, natural and synthetic steroids as well as plant products. Only a few direct CAR ligands are known, e.g. Citco and TCPOBOP for human and mouse CAR respectively. Other CAR activators, such as phenobarbital (PB) or bilirubin, do not directly bind to the receptor, but induce its translocation to the nucleus by a mechanism which involves phosphorylation and/or dephosphorylation events (Kawamoto et al., 1999) (Hosseinpour et al., 2006). However the detailed mechanism is not fully understood so far. Together with their obligate heterodimerization partner retinoic X receptor RXR, CAR as well as PXR bind to DR3, DR4 or ER6 type of response elements. Members of the cytochrome P450 family are their prototypical target genes, such as human CYP3A4 for PXR and CYP2B6 for CAR. Other target genes include sulfotransferases, glucuronosyltransferases and transport proteins such as multidrug resistant protein 3 (MRP3) all involved in detoxification processes (reviewed in (Tirona and Kim, 2005)).

1.4.2 The bile acid activated receptor Farneosid X receptor - FXR

Soon after the nuclear hormone receptor FXR (NR1H4) was cloned, it was found to be activated by farnesols, intermediates of the cholesterol synthesis pathway, hence its name (Forman et al., 1995). Later primary and secondary bile acids as well as their gluco- and tauroconjugates were shown to effectively activate FXR, among them configures the primary bile acid chenodeoxycholic acid as the most potent natural ligand (Wang et al., 1999). FXR also heterodimerizes with RXR and binds mostly to inverted repeats spaced by 1 nucleotide (IR1) in the flanking region of its target genes.

In response to ligand binding, FXR regulates a variety of genes involved in bile acid, cholesterol, triglyceride and lipoprotein metabolism (for a recent review and a comprehensive list of target genes see (Kalaany and Mangelsdorf, 2006).

Targeted disruption of the FXR gene in mice confirmed its critical role in bile acid and lipoprotein metabolism. FXR knock-out mice exhibit elevated plasma cholesterol and triglycerides levels and excessive accumulation of fat in the liver (Sinal et al., 2000). More recently several reports highlight an effect of FXR activation on glucose homeostasis. Its final role however, remains controversial (Claudel et al., 2005). Interestingly, FXR, CAR and PXR share a common set of target genes, namely cytochrome P450 CYP3A4 (Gnerre et al., 2004), UDP-glucuronosyltransferases (Barbier et al., 2003), sulfotransferases (Song et al., 2001), or the transport proteins, such as MRP2 (Kast et al., 2002), all involved in the elimination and excretion of potentially toxic bile acids as well as xenobiotics.

Of note, researchers recently have identified four isoforms of the murine and human FXR (FXRα1, α2, α3, and α4) due to alternative promoter usage and alternative splicing (Huber et al., 2002, Zhang et al., 2003). The four isoforms differ by their N terminus and by the existence of a four-amino acid insertion in the hinge region. Interestingly, FXR isoforms lacking the four amino acid insertion (α2 and α4) bind FXR response elements (FXREs) with higher affinity and display stronger transactivation of the promoters of some FXR target genes, i.e. ileal bile acid binding protein (IBABP), but not others, i.e. short heterodimer partner (SHP). However the precise differences of the four isoforms with respect to various FXR signaling pathways is not yet fully understood.

1.4.3 Hepatocyte Nuclear Factor 4 – HNF4α

HNF4α is a nuclear hormone receptor (NR2A1) highly conserved from men to insects. HNF4α belongs to the liver enriched transcription factors and is considered to be the major regulator of the hepatocyte phenotype (Watt et al., 2003). In mammals it is expressed at highest level in liver, kidney, intestine and pancreas (Sladek et al., 1990). HNF4α null mice die during early embryogenesis due to a failure in endoderm development (Chen et al., 1994). Liver specific knock-out animals have been generated, which show a severely disturbed lipid homeostasis at the onset of 5 weeks of age and an increased mortality of >70% by 8 weeks of age (Hayhurst et al., 2001). These reports demonstrate the pivotal role of HNF4α in early development as well as for the maintenance of proper adult liver function.

A large number of HNF4α target genes have been identified, which are involved in key metabolic pathway such as cholesterol, fatty acid and glucose homeostasis as well as xenobiotic metabolism (for an overview: www.sladeklab.ucr.edu/info.html). HNF4α binds DNA exclusively as a homodimer, mostly to direct repeats spaced by one nucleotide (DR1) elements in the promoters of its target genes. The essential fatty acid linoleic acid has been found by affinity purification and subsequent gas chromatography and mass spectrometry analysis to be bound to the HNF4α ligand binding pocket in

mammalian cells (Sladek; EMBO 2005). Functional data, demonstrating increased transcriptional activity upon linoleic acid treatment or increased coactivator recruitment are lacking to date. It is debated, if linoleic acid indeed serves as a classical nuclear hormone receptor ligand. Therefore, HNF4α is still regarded as orphan receptor. In contrast, a number of coactivators have been shown to potently increase HNF4α transcriptional activity in a ligand independent manner, including GRIP1, SRC1 and PGC-1α (Rhee et al., 2003, Wang et al., 1998).

Mutation in and near the human HNF4α gene are associated with an autosomal dominant form of diabetes, MODY1 (Yamagata et al., 1996), and with the common form of type 2 diabetes respectively (Silander et al., 2004), further underlining the role of HNF4α in glucose homeostasis.

The HNF4α gene gives rise to several isoforms, via internal splicing and transcription from two alternative promoters. HNF4α1 and HNF4α7 are the prototypic isoforms derived from the two promoters and differ with respect to their tissue distribution as well as coactivator recruitment. In liver HNF4α1 is by far the most predominant isoform, while HNF4α7 dominates in pancreas (Briancon et al., 2004). An interesting recent paper highlights the central role of HNF4α1 isoform in the regulation of CAR, underlining the link between these nuclear hormone receptors (Briancon and Weiss, 2006).

1.4.4 The versatile coactivator PGC-1α

In 1998, PGC-1α was identified as a PPARγ interacting protein in brown adipose tissue (Puigserver et al., 1998). Since then a variety of transcription factors from inside and outside the family of nuclear hormone receptors were shown to interact with PGC-1α. These experiments document that PGC-1α is a versatile coactivator and affects various aspects of cellular energy status and oxidative metabolism. Its expression is high in all tissues rich in mitochondria, such as brown adipose tissue, heart and skeletal muscle. Through the interaction mainly with nuclear respiratory factor 1 and 2 (NRF1 and NRF2) PGC-1α plays a central role in mitochondrial biogenesis (Scarpulla, 2002).

In normal ‘fed’ liver, expression of PGC-1α is low, however in the fasted state, PGC-1α is robustly increased. PGC-1α is able to activate near all aspects of the hepatic fasting response, including gluconeogenesis, fatty acid β oxidation and ketogenesis (Herzig et al., 2001, Rhee et al., 2003, Yoon et al., 2001). It does so by activating key hepatic transcription factors, such as HNF4α, PPARα, GR, and the forkhead transcription factor FOXO1 (Puigserver et al., 2003). The above described nuclear hormone receptors FXR, CAR and PXR have been added to the list of transcription factors co-activated by PGC-1α (Bhalla et al., 2004, Shiraki et al., 2003, Zhang et al., 2004b).

Together with PGC1β and PGC related coactivator (PRC) PGC-1α builds up the family of PGC1 coactivators. The three members show high homology in their structure: a N-

terminal activation domain, a central regulatory domain and a C-terminal RNA binding domain. Three LXXLL domains are responsible for the interaction with different nuclear hormone receptors. Not much is known about the function of PRC so far. PGC-1β shows a similar tissue distribution to PGC-1α and a number of transcription factors known to interact with PGC1α were also shown to be co-activated by PGC-1β. In liver PGC-1β also is highly induced in fasted state, but while having only a minimal effect of gluconeogenic genes, this factor potently enhances the expression of genes involved in fatty acid oxidation and ketogenesis (Lin et al., 2003). In addition PGC-1β, and not alpha, plays a central role in hepatic lipogenesis and cholesterol synthesis in response to high a fat diet (Lin et al., 2005b). Table 1 gives an overview of transcription factors known to interact with PGC-1α and -1β.

Table 1 Transcription factors known to interact with PGC1 coactivators; adapted from Lin et al. 2005

Transcription factor	**PGC-1α**	**PGC-1β**	**Function**	**References**
NRF-1	+	+	mitochondrial genes	(Wu et al., 1999)
NRF-2	+	ND	mitochondrial genes	(Mootha et al., 2003)
ERR α , β , γ	+	+	mitochondrial genes	(Schreiber et al., 2003, Mootha et al., 2004)
PPAR α , β , γ	+	+/-	fatty-acid oxidation	(Wang et al., 2003, Puigserver et al., 1998)
LXR α , β	+	+	lipoprotein secretion	(Lin et al., 2005b)
ER α , β	+	+	?	(Kressler et al., 2002)
GR	+	-	gluconeogenesis	(Yoon et al., 2001, Kressler et al., 2002)
FXR	+	ND	triglyceride metabolism	(Zhang et al., 2004b)
PXR	+	ND	unknown	(Bhalla et al., 2004)
CAR	+	ND	fasting response, unknown	(Shiraki et al., 2003)
FOXO1	+	-	gluconeogenesis	(Puigserver et al., 2003)
HNF4 α	+	+	gluconeogenesis	(Yoon et al., 2001) (Lin et al., 2002)
SREBP1a, 1c, 2	–	+	lipogenesis/ lipoprotein secretion	(Lin et al., 2005b)

1.5 Comparative Genomics as a Novel Tool to Identify Regulatory Elements

In the past it was predicted that most of the DNA within the human genome does not have a function and that most if not all function is contained within protein coding regions. Initial comparison of human and rodent genomes (Waterston et al., 2002) (Gibbs et al., 2004) indicate that about 5% or more of the bases within the genome are under purifying selection. However, protein coding genes are believed to account for only about 1.5% of the human genome, which may leave around 3.5% of the bases in the genome that are thought to be functional, but not to code for proteins. This conserved non-coding sequence has been the subject of intense interest. It is now believed that it consists of structural elements, non-coding RNAs and of regulatory elements involved in gene expression (Dermitzakis et al., 2005).

If the assumption holds true that regulatory regions are conserved in evolution, they could be identified by cross species sequence comparison. In the last years several alignments programs, based on global alignment, such as AVID (Bray et al., 2003) and LAGAN (Brudno et al., 2003), or local alignment algorithms, such as BLASTZ (Schwartz et al., 2003), were created to specifically align long genomic sequences. One of the first reports that successfully applied this approach for the identification of regulatory regions was from Loots et al. who characterized a longe-range regulator of interleukin 4, 13, and 5 (Loots et al., 2000) within 1 megabase of sequences analyzed. Now it is generally well established that non-coding conserved regions can represent functional regulatory elements (Frazer et al., 2004b). And the method of so-called phylogenetic footprinting, which filters long genomic sequences for segments conserved across species, has become an attractive approach to reduce the number of false-positive predictions reported by conventional search algorithms for transcription factor binding sites (Lenhard et al., 2003).

2 Aim of the Studies

A number of factors can provoke attacks of hepatic porphyria. Among those, drugs have been shown to directly upregulate ALAS1 transcription via the nuclear hormone receptors CAR and PXR. We hypothesized that other triggering factors, such as fasting and endogenous ligands of nuclear receptors also induce ALAS1 directly at the transcriptional level. The goal of this thesis was to elucidate the transcriptional network regulating ALAS1 in order to get insight into the pathogenesis of acute porphyric crises.

During these studies we addressed the following questions:

1) What are the molecular mediators of the fasting response of ALAS1? In particular, does PGC-1α, known to mediate the adaptive response to fasting in the liver, have an effect on hepatic heme synthesis?

2) Can we identify new regulatory regions of ALAS1 by using a comparative genomic approach?

3) Do additional nuclear hormone receptors involved in detoxification, such as FXR, regulate ALAS1?

4) ALAS1 is expressed ubiquitously, however only in liver ALAS1 is inducible at the transcriptional level. What determines this liver specific inducibility of ALAS1? Does the master regulator of hepatic gene expression, HNF4α, play a role in basal as well as drug induced expression of ALAS1?

3 Heme Synthesis in Human Liver is Regulated by Bile Acids via the Farnesoid X Receptor FXR

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3.1 Abstract

δ-amino levulinic acid synthase 1 (ALAS1) is the rate limiting enzyme of heme synthesis in liver and is highly regulated to adapt to the metabolic demand of the hepatocytes. In the present study we describe that human hepatic ALAS1 is a new direct target of the bile acid activated nuclear receptor farnesoid X receptor (FXR). Our experiments in cultures of primary human hepatocytes reveal that ALAS1 transcripts and activity is increased upon stimulation by the most potent natural ligand of FXR, chenodeoxycholic acid (CDCA). Treatment of human liver slices with CDCA resulted in a similar increase in ALAS1 expression. The synthetic FXR agonist GW4064 was able to induce ALAS1 expression as well as activity in cultures of primary human hepatocytes. Moreover, by overexpression of a constitutively active form of FXR, the response of ALAS1 mRNA to GW4064 was significantly increased. In agreement with this, we identified and characterized a FXR response element in the 5'-flanking region of human ALAS1. The region was able to trigger a 5 fold increase in luciferase activity upon CDCA treatment. Site directed mutagenesis demonstrated the functionality of the IR1 element, and binding of FXR/RXR heterodimers was evidenced in gel shift experiments. Together these data strongly support a role of bile acid activated FXR in the regulation of ALAS1 and consequently of hepatic heme and porphyrin synthesis. In addition, our findings suggest that endogenous bile acids may precipitate neuropsychiatric attacks in patients with acute hepatic porphyrias.

3.2 Introduction

Synthesis of heme is indispensable for life and takes place in every cell of the body except mature erythrocytes. The bulk of heme is synthesized in bone marrow for the production of hemoglobin and in liver, where it is incorporated into various heme proteins, such as cytochromes P450, catalases, peroxidases and respiratory cytochromes. The pathway of heme synthesis consists of 8 enzymatic steps and is initiated by the condensation of glycine and succinyl CoA to δ-aminolevulinic acid (ALA), a reaction catalyzed by the enzyme δ-aminolevulinic acid synthase (ALAS). There are two isoforms of this enzyme encoded by different genes on separate chromosomes, erythrocyte specific ALAS2, expressed in erythroid cells only, and the ubiquitously expressed ALAS1 (Thunell, 2000).

Since either excess or deficiency of heme is detrimental to the cell, heme synthesis needs to be tightly controlled. In non-erythroid cells the rate of synthesis is controlled at its first step. Accordingly, ALAS1 is highly regulated in different cellular contexts to ensure adequate levels of intracellular heme. A negative feedback is exerted by a regulatory heme pool by several mechanisms including i) inhibition of the transfer of ALAS1 precursor protein into the mitochondrial matrix, ii) reduction of ALAS1mRNA stability and iii) direct inhibition of ALAS1 transcription (Thunell, 2000, May et al., 1995, Roberts et al., 2005) (Kolluri et al., 2005).

Hereditary defects of heme synthesis lead to rare metabolic diseases referred to as porphyrias (Anderson et al., 2005, Kauppinen, 2005). Partial defects of all enzymes of the synthesis pathway except ALAS1 are known. Depending on the location of the enzymatic defect within the pathway, different patterns of accumulation and excretion of intermediates and accordingly different clinical symptoms are observed. Photosensitivity due to accumulation of porphyrins in skin is the main clinical manifestation of some porphyrias. Inducible, acute hepatic porphyrias are characterized by intermittent attacks of neuropsychiatric dysfunction precipiated by a number of well-known stimuli, such as drugs, alcohol, sex steroids or fasting. These attacks are always associated with increased hepatic ALAS1 activity reflected by increased levels of the porphyrin precursors ALA and PBG in plasma and urine. Acute attacks are treated by withdrawal of the causative agent, high carbohydrate load and most efficiently by intravenous administration of hemin. If dehydration and imbalance of electrolytes ensue, attacks can be life threatening and require intensive care.

It is still under debate if increased ALA concentration in the nervous system or a neuronal heme deficiency account for symptoms in acute attacks (Sengupta et al., 2005, Soonawalla et al., 2004) (Meyer et al., 1998). However, progress has been made in understanding the molecular mechanisms of ALAS1 regulation in the liver. In the last years, members of the nuclear hormone receptor family of transcription factors, the xenosenors constitutive androstane receptor (CAR) and pregnane X receptor (PXR), were identified to mediate induction of ALAS1 by inducer drugs. We characterized drug

response elements in the far upstream 5'-flanking region of chicken, mouse and human ALAS1, demonstrating direct transcriptional upregulation of ALAS1 by these precipitating agents (Fraser et al., 2002, Fraser et al., 2003, Podvinec et al., 2004). Recently peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a coactivator involved in mitochondrial biogenesis and energy homeostasis, was shown to be the master regulator of the fasting response of ALAS1 by acting via an insulin sensitive FOXO1 site in the promoter (Handschin et al., 2005).

For many years bile acids were mostly known as important products of cholesterol metabolism, acting as detergents necessary for the intestinal absorption of nutrients. More recently new biological functions of bile acids as signaling molecules in particular in lipid and glucose homeostasis have been discovered (for a recent review (Claudel et al., 2005)). Bile acid signaling in the liver is mostly mediated via the farnesoid X receptor (FXR), a member of the nuclear receptor family of ligand activated transcription factors. The most potent natural ligand of FXR is the primary bile acid chenodeoxycholic acid (CDCA). In addition lithocholic acid (LCA), deoxycholic acid (DCA), as well as their taurine and glycine conjugates, have been shown to directly bind to FXR (Makishima et al., 1999, Parks et al., 1999). FXR heterodimerizes with the retinoic X receptor RXR and binds to consensus sequences, most commonly an IR1 (inverted hexameric nucleotide repeat separated by one nucleotide) in the flanking region of its target genes. FXR plays a critical role in bile acid homeostasis. Short heterodimer partner (SHP) is a one of the most studied direct targets of FXR and represses *de novo* synthesis via downregulation of CYP7A1 and CYP8B1, the two rate limiting enzymes in bile acid synthesis (Goodwin et al., 2000). Simultaneously FXR enhances the metabolism and excretion of bile acids. The enzymes induced by FXR, namely cytochrome P450 CYP3A4 (Gnerre et al., 2004), UDP-glucuronosyltransferases (Barbier et al., 2003), sulfotransferases (Song et al., 2001), or the transport proteins, such as MRP2 (Kast et al., 2002), also are induced by drugs via CAR and PXR, two transcription factors critical for the elimination of xenobiotics in the liver (Handschin and Meyer, 2003). Thus the liver evolved redundant pathways in the detoxification of endogenous and exogenous compounds (Guo et al., 2003). Low or near physiological concentrations of primary bile acids induce drug metabolizing enzymes and transport proteins via FXR, whereas toxic concentration of bile acids in particular the secondary bile acid LCA and exogenous compounds activate CAR and PXR and thereby their target genes (Staudinger et al., 2001, Xie et al., 2000).

One crucial enzyme for detoxification and therefore coregulated with cytochromes P450 via CAR and PXR is ALAS1, ensuring enough heme for newly synthesized apocytochromes. Since bile acids induce heme proteins such as cytochromes P450, we hypothesized that they also must affect heme synthesis.

Several lines of evidence indeed suggest a connection between bile acid homeostasis and the regulation of hepatic heme synthesis. The bile acid precursors 5β-cholestan-3α,7α-diol and 5β-cholestan-3α,7α,12α-triol were shown in the past to induce ALAS1 activity in chicken embryo hepatocytes (Javitt et al., 1973). In hepatobiliary diseases accompanied by cholestasis increased urinary excretion of porphyrins is a common feature. It has already been suggested that increased porphyrin synthesis contributes to this phenomenon (Gibson et al., 2000) (Rocchi et al., 2005)

In the present study, we investigated the effect of bile acids on the rate-limiting enzyme of heme synthesis, ALAS1, in two human liver culture systems, primary culture of human hepatocytes and human liver slices. We identified the bile acid activated nuclear receptor FXR as a novel regulator of ALAS1 thereby connecting bile acid signaling and hepatic heme synthesis.

3.3 Materials and Methods

Chemicals

All chemicals were purchased from Sigma (Buchs, Switzerland) unless stated otherwise. GW4064 was kindly provided by Dr T. M. Willson (GlaxoSmithKline, Research Triangle Park, North Carolina, USA). Mammalian expression plasmid for human FXR was previously described (Gnerre et al., 2004), pcDNA3 human PGC-1α was a kind gift from Dr. A. Kralli (The Scripps Research Institute, La Jolla, California, USA).

Animal experiments

C57/Bl6 wild-type animals were from a colony maintained at the Biozentrum as described (Gnerre et al., 2004). Animals were maintained on standard laboratory chow and were allowed food and water ad libitum. Ten to 16-week-old female mice (n = 4-5) were injected i.p. with vehicle alone (corn oil with 5% DMSO) or GW4064 40 mg/kg. After 16 h, animals were killed, liver tissue samples solubilized in 1 ml TRIzol TM reagent (Invitrogen) and total RNA was extracted. For the cholic acid feeding experiment, female animals (n=5) were either on standard laboratory chow or fed a 1% cholic acid diet for 1 week. Animals were killed, liver tissue samples solubilized in 1 ml TRIzol TM reagent (Invitrogen) and total RNA was extracted.

Isolation and culture of primary human hepatocytes

Liver tissue wedges (50-200mg) were obtained from consented patients undergoing surgical resections in the clinic of visceral surgery (Berne, Switzerland). Tissue was perfused via two cannulea with buffers heated to 37°C at a rate of 100 ml/min. Firstly PBS containing 10mM HEPES for 5 minutes followed by 500 ml of PBS HEPES

containing 2mM EGTA and the 500ml of the initial buffer to remove EGTA. The enzyme solution (0.05% collagenase, 0.02% dispase, 0.017% hyaluronidase and 0.02% DNase, in HBSS containing 5mM CaCl2) was then recirculated through the liver wedge for a period of 7-12 minutes until it was sufficiently softened. The tissue was mechanically disrupted in DMEM containing 10% fetal calf serum, filtered through 50-micron sterile gauze and washed twice in DMEM and kept on ice in suspension. Hepatocytes were subsequently seeded on rat-tail collagen coated plastic dishes (25μg/cm2, BD Biosciences, Basel, Switzerland) at a density of 300'000 cells per well in 12-well plates in Dulbecco's Minimum Essential Medium (Gibco BRL, Basel, Switzerland) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL), 1μM dexamethasone (Sigma, Buchs, Switzerland), 50U/ml penicillin and 50μg/ml streptomycin (both from Gibco BRL). After overnight culture, the medium was replaced with serum free Williams'E medium (Invitrogen, Basel, Switzerland) supplemented with 100nM hydrocortisone, 0.5x ITS (Insulin, Transferrin, Selenium; Sigma), 50U/ml penicillin and 50μg/ml streptomycin. Cells were kept for 24h under these conditions unless stated otherwise and subsequently exposed to chemicals as indicated in the figure legends.

Preparation and culture of human liver slices

Human liver slices were prepared as previously described (Elferink et al., 2004) and cultured individually in 6-well plates in 3.2 ml William's medium E (Gibco, Auckland, NZ) supplemented with D-glucose (25 mM) and 50 µg/ml gentamicin, under continuous shaking at 37°C in a humidified atmosphere saturated with 5% carbogen. Slices were transferred to fresh medium and incubated in the presence or absence of 10 or 100 µM CDCA for 8, 16 and 24 hours. At the end of the incubation time, slices were frozen in liquid nitrogen and stored at -80C° until RNA was prepared.

Isolation and culture of primary mouse hepatocytes

For the preparation of mouse hepatocytes animals were anaesthetized with Ketamine/Xylazine (Sigma, Buchs, Switzerland). The hepatic portal vein was canulated and perfused with HEPES-EGTA (pH 7.4) at a flow rate of 8ml/min for 5 minutes. The liver was then perfused with collagenase (type 2, Worthington, Lakewood NJ, USA) for 6 minutes. The perfusion solutions were continuously gassed with carbogen. The livers were excised into a Petri dish filled with Leibowitz' L-15 medium (Sigma, Buchs, Switzerland) supplemented with 10% newborn calf serum (Invitrogen, Basel, Switzerland). Cells were filtered through a nylon mesh and centrifuged at 50xg at 4°C for 5 minutes three times. After determination of viability, cells were plated at a density of 400'000 cells/well (12 well plate) and were allowed to attach for 2 hours in William's E medium without phenol red (Invitrogen, Basel, Switzerland), 10 % FCS, 4µg/ml insulin, 200µM glutamine and and 1% penicillin/streptomycin (50 IU/ml) on collagen-coated dishes. Medium was replaced with serum free Williams'E medium as described for human hepatocytes and exposed to chemicals 24h later as indicated in the figure legends.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated with the Trizol TM reagent (Invitrogen). 1.2 μg total RNA from primary cells or 1 μg of mouse liver total RNA were reverse transcribed with the Moloney murine leukaemia virus reverse transcriptase (Promega, Catalys AG, Wallisellen, Switzerland) using either random hexamers p(dN)6 (Roche Diagnostics, Rotkreuz, Switzerland) or oligo-(dT)15 N primers (Promega), respectively. PCR was performed with the qPCR TM Mastermix Plus (Eurogentec GmbH, Köln, Germany). Primers and probes were optimized as indicated in Table 1. Transcript levels were quantified with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Boston, Massachusetts, USA) according to the manufacturer's protocol. Briefly, relative transcript levels in induced cells or livers and untreated controls were determined using the relative quantification method measuring ΔΔCt (comparative threshold cycle method). The levels of either 18S or cyclophilin and GAPDH were used for the normalization of the human and mouse genes, respectively.

Transfection of Primary Human Hepatocytes

After overnight plating, cells were transfected with plasmid DNA using Effectene transfection reagent (Qiagen, Hombrechtikon, Switzerland) following the manufacturers recommendations. Transfection mixes contained 1μg of plasmid DNA (prepared using Endo Free® Plasmid Maxi Kit, Qiagen), 8μl Enhancer Buffer and 10μl Effectene per well to be transfected. 36h later cells were exposed to reagents for 10h followed by lysis and RNA preparation.

ALAS activity assay

ALAS activity was assayed as described (Sinclair et al., 1999) with the following modifications. Primary human hepatocytes were plated as described above except that 600'000 cells per well in 6-well plates were used. After culturing in serum free conditions for 12h, cells were induced overnight with either vehicle (0.15% DMSO), 250μM 4,6 dioxoheptanoic acid alone or together with 50μM CDCA, 1μM GW4064 or 500μM phenobarbital. Induction medium was replaced by 700μl prewarmed glycine buffer (35mM Tris-HCl, pH 7.4, 30mM Na2HPO4, 8.5mM Na citrate 2H20, 8mM MgCl2, 0.2mM pyridoxalphosphate, 10μM dioxoheptanoic acid, 5mM EDTA and 50mM glycine) and plates were incubated for additional 2h at 37°C. The assay was stopped by trichloracetic acid (5% final) and supernatant was taken for subsequent analysis of ALA production according to (Sinclair et al., 1999). Cells were lysed in standard lysis buffer for protein determination using the Bradford method. Data are given as nmol ALA produced per mg protein per hour.

In silico analysis of human ALAS1 flanking region

For cross-species sequence comparison a total of 66.7kb genomic sequence ranging from the 5'- to the 3'-neighboring annotated gene of human ALAS1 was used and included 43.7kb 5'-flanking, 16.3kb genic and 6.9kb 3'-flanking sequence. Multispecies

(human, chimpanzee mouse, rat and dog) sequence alignments produced by MULTIZ (Blanchette et al., 2004), which are based on pairwise sequence alignments performed by BLASTZ with subsequent chaining and netting, were retrieved from the Santacruz genome browser (genome.ucsc.edu). A total of 16 conserved blocks of non-coding sequences located from -27kb to + 19kb from the transcriptional start site as reported by MULTIZ were then individually scanned for putative IR1 elements using NubiScan algorithm with the provided FXR specific matrix based on 38 known binding sites from the literature and a score threshold of >0.62 (www.nubiscan.unibas.ch). The MULTIZ alignment shown in Fig. 8 includes sequences of human, chimpanzee, rhesus, rabbit, mouse, rat, dog and cow assemblies and was retrieved from genome.ucsc.edu.

Plasmid Construction

Fragments of human ALAS1 flanking region were PCR amplified using the previously isolated PAC clone harboring ALAS1 gene as template (Podvinec et al., 2004) and Pwo DNA Polymerase (Roche Diagnostics, Rotkreuz, Switzerland). Primers were designed using Primer 3 software (primer3_www.cgi v 02) with the following parameters: 24nt in length, 50% GC content, Tm 60°C, and restrictions sites added as indicated in Table 1. The fragments were cloned into pGL3 tk luciferase vector using standard cloning procedures. The 1.3kb promoter was cloned into pGL3 basic. Subfragments of the 795bp long -14kb ALAS1 construct were generated by restriction digest with AatII and PvuII (New England Biolabs, Bioconcept, Allschwill, Switzerland). The 175bp derived mutated construct was generated by PCR using complementary oligonucelotides mutated in the IR1 site (see Table 1) and Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA). The products were digested with DpnI (New England Biolabs) to remove the parental DNA template and selected for constructs containing mutations. Plasmid DNA was prepared using the Qiagen sytem. All constructs were verified by sequencing.

Transactivation assay

Transactivation assays were carried out in CV-1 (monkey kidney) cells as previously described (Gnerre et al., 2004) with minor modifications. Briefly, cells were expanded for 3 days in DMEM-F12 without phenol red (Gibco BRL) and supplemented with 10% charcoal treated FBS. Subsequently, cells were plated onto 96- well dishes at a density of 25 000 cells per well and grown overnight. Cells were transiently transfected in OptiMemI (Invitrogen) using 1μl of LipofectAMINE reagent (Invitrogen) per well. Transfection mixes contained 8 ng receptor expression vector, 20ng coactivator expression vector, 20ng reporter vector and 60ng pRSV-βGal to a total of 108ng DNA per well. After 24h, cells were exposed to drugs or vehicle in DMEM-F12 supplemented with 10% charcoal stripped delipidated FBS (Sigma). 24h later, cell extracts were prepared using 200μl of passive lysis buffer (Promega) and 10μl of the supernatants were assayed for luciferase activities using the luciferase assay kit (Promega) and a Wallac 1420 Multilabel Counter. β-galactosidase activities were measured as previously described (Podvinec et al., 2004). Luciferase levels were normalized against

β-galactosidase values to compensate for variation in transfection efficiency. Data are presented as mean +/- standard deviation. All transfections were repeated at least two to four times in triplicates or quadruplicates. Statistical significance is defined in two-tailed students t-test as $p < 0.05$ or $p < 0.01$ as indicated in the figures.

Electromobility shift assay

Human FXR and human RXRα were synthesized using the TNT T7 –coupled reticulocytes system (Promega). Double-stranded oligonucleotide probes were obtained by hybridizing single-stranded complementary oligonucleotides (Operon, Köln). Dimers with the sense sequences shown in Table I were labeled with [γ-32P] ATP using T4 polynucleotide kinase (NEB) and cleaned of unincorporated nucleotide using Sephadex Nick columns (Amersham, GE Healthcare, Otelfingen, Switzerland). 3μl of *in vitro* translated nuclear receptors were incubated on ice for 30min with 2-5 fmol of γ-32P-end-labeled dimerized oligonucleotide and 1μg of poly(dI)poly(dC) in 10 mM HEPES-KOH, pH 7.9, 10% glycerol, 50mM KCl, 1mM MgCl2, 0.25mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride. For competition assays, a 25-200-fold excess of unlabeled dimerized oligonucleotides was added. For supershift experiments, 1μl of antibody against FXR (Santa Cruz Biotec. sc-13063 X) or 0.5 μl of monoclonal anti-mouse-RXR rabbit antibody (kindly provided by P. Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Université Louis Pasteur, Illkirch, France) was added to the reaction mix. Reactions were analyzed by electrophoresis through 5% polyacrylamide gels in 0.25× Tris-borate EDTA buffer at 120V for 2 h.

3.4 Results

Bile acids induce ALAS1 mRNA in cultured primary human hepatocytes

To study whether hepatic heme synthesis is regulated by bile acids, cultures of primary human hepatocytes were exposed to the primary bile acid CDCA, and analyzed for the expression levels of ALAS1. After 8h incubation of the cells in the presence of 50μM CDCA, ALAS1 transcripts increased 4.5-fold, an increase similar to the effect of the classical ALAS1 inducer phenobarbital (PB) at 500μM (Fig. 1A). Combination of PB and CDCA resulted in an additive effect as compared to single treatment.

We then examined if other bile acids also were able to increase ALAS1 transcripts. Therefore cultured primary human hepatocytes were challenged with a set of different bile acids and again analyzed for the expression levels of ALAS1. In order the check for an intact bile acid signaling cascade, mRNA levels of SHP, a known bile acid responsive gene, were measured. As illustrated in Fig. 1B, the primary bile acids CDCA, cholic acid (CA) and ursodeoxycholic acid (UDCA; all at 50μM) and the secondary bile acid LCA (25μM) increased SHP as well as ALAS1 mRNA levels to various degrees. We conclude that endogenous substrates such as bile acids are able to increase ALAS1 to the same degree as inducer drugs.

Bile acids increase ALAS1 expression in human liver slices

To test if our findings in primary culture are reproduced in a system where the tissue architecture of the liver is intact, we examined the effect of bile acids on gene expression in precision cut human liver slices. Human liver slices are a well established *ex vivo* model for human physiology (Elferink et al., 2004). Slices of three different donors were incubated for time intervals of 8, 16 and 24hrs at the low or physiological concentration of 10μM and at the toxic concentration of 100μM CDCA. Again SHP expression was measured as a positive control and found to be increased in all 3 donors at both concentrations tested (data not shown). As depicted in Fig. 2, all three donors also showed an increase in ALAS1 mRNA expression upon CDCA treatment. The degree of induction and the time course of the response expectedly differed from one individual to another. Maximal induction was seen after 16h (donor A) or 24h (donor B and C). An increase in ALAS1 transcripts could be observed already at 10μM CDCA (donor A and B, 16 and 24h and donor C, 24h) suggesting physiological relevance of our findings.

The FXR agonist GW4064 and overexpression of constitutively active FXR increase ALAS1 mRNA

Since bile acids in addition to their potential to activate FXR may also affect alternative pathways, including activation of PXR, a known regulator of ALAS1, we used the synthetic agonist of FXR, GW4064, to test the specificity of the response. Primary human hepatocytes were cultured with increasing concentrations of GW4064. Indeed, GW4064 induced ALAS1 mRNA levels in a dose dependent manner (Fig. 3A). To further confirm the role of FXR in this pathway, primary human hepatocyte cultures were transiently transfected with a constitutively active form of FXR (VP16 huFXR), and subsequently challenged with GW4064 to achieve a maximal induction. As illustrated in Fig. 3B, VP16 huFXR alone or in combination with GW4064 significantly increased ALAS1 mRNA expression as well as the expression of the known FXR targets SHP and bile salt export pump (data not shown). These data further document the role of FXR in inducing ALAS1.

FXR activation increases ALAS1 activity

Having shown that activation of FXR increases ALAS1 transcripts we investigated in a next step whether the activity of the enzyme also was increased. ALAS1 activity was assayed after exposure of cultures with the drugs indicated in Fig. 4 in the presence of 4,6-dioxoheptanoic acid (DHA), an inhibitor of aminolevulinic acid dehydratase, to prevent further usage of ALA. Phenobarbital, as well as natural and synthetic FXR agonists (50μM CDCA and 1μM GW4046), increased the activity of ALAS1 (expressed as nmole ALA produced per mg protein per hour) to similar levels. These data clearly show that not only mRNA levels of ALAS1 increase but also the corresponding protein and its activity.

Identification of a FXR responsive sequence in the 5'-flanking region of ALAS1

Having established that ALAS1 mRNA as well as activity was induced upon FXR activation, we searched for putative FXR response elements in human ALAS1 gene. Therefore a combined *in silico* approach was chosen; a total of 67kb intergenic human ALAS1 sequence was filtered by cross species sequences comparison to retain only sequences, which are conserved between different species. Multispecies sequence alignments preformed by MULTIZ (Blanchette et al., 2004) resulted in the identification of a total of 16 blocks of conserved non-genic sequences, which then were individually scanned for putative FXR binding sites using NuibScan algorithm, specifically designed to detect nuclear receptors binding sites (Podvinec et al., 2002). Two conserved non-genic sequences were found to contain high scoring FXR response elements (score > 0.62), located at minus 14kb from the transcriptional start site and within intron 10 respectively (called -14kb ALAS1 and ALAS1 intron). In order to see if the identified regions were able to confer a response to FXR, these regions were cloned in front of a thymidine kinase driven luciferase reporter construct for subsequent testing in CV-1 transactivation assays. Since FXR response elements mostly have been characterized within the proximal promoter region, a construct encompassing 1.3kb of human ALAS1 promoter was included in the analysis, although no putative FXRE was identified by NubiScan within the threshold used.

As illustrated in Fig. 5B, CDCA increased the reporter activity of the -14kb ALAS1 in the presence of exogenous FXR. In contrast, the promoter construct as well as the construct spanning intron 10 were unaffected by CDCA treatment, indicating that the region at minus 14kb was able to confer a response to bile acids. To check if the response of the -14kb ALAS1 fragment to CDCA was FXR specific, the fragment was co-transfected with expression constructs of either FXR or PXR, in the absence or presence of PGC-1α, a coactivator also shown to co-activate various nuclear receptors including FXR (Zhang et al., 2004b). Again CDCA only augmented reporter gene activity of the -14kb ALAS1 fragment when FXR was present. This increase was FXR dependent, since the co-transfection of PXR had no effect on CDCA response of the fragment. As expected, the CDCA induction of reporter activity via FXR was further increased by PGC-1α up to 5-fold (Fig. 5C).

In order to experimentally identify the exact sequences conferring the FXR response, the -14kb ALAS1 construct was digested and the resulting fragments were cloned into a reporter vector as illustrated in Fig. 6A. Subsequent testing in transactivation assays clearly showed (Fig. 6A), that a 175bp middle fragment (D) retained the response to CDCA in the presence of FXR with or without PGC-1α, while there was no significant response in the flanking fragments A and C. This middle region contains the IR1 element initially reported by NUBIScan algorithm, showing a high similarity to known FXR bindings sites. The first halfsite is composed of a perfect nuclear receptor hexamer AGGTCA; the other halfsite is more degenerate with the sequence AGCCCC (Fig. 6). To demonstrate the importance of the identified IR1 in the FXR response, we

performed site directed mutagenesis as indicated. The response of the mutated 175bp fragment was robustly reduced as compared to the wildtype construct when stimulated with CDCA in the presence of FXR (Fig. 6C left panel). Moreover the activation of the 175bp fragment by the constitutively active FXR (VP16 huFXR) was significantly reduced by the mutation (Fig. 6C right panel). These data clearly demonstrate that the identified region confers a specific response to FXR. We now describe another distal enhancer, in addition to the known CAR and PXR binding sites located at minus 21kb and minus 16kb (Podvinec et al., 2004), regulating ALAS1 transcription.

The FXR/RXR heterodimer binds to the IR1 in the ALAS1 enhancer

To assess whether an FXR-RXR heterodimer is able to bind to the newly identified IR1 element, electromobility shift assays were performed using *in vitro* translated FXR and RXR together with 32P-labeled double stranded oligonucleotides derived from the responsive sequence (Table 1) and a perfect IR1 as a positive control. A specific DNA protein complex was formed when FXR and RXR were present in the binding reaction (Fig. 7: *lane 4* and *lane 15*). Successful competition of the binding to the radiolabeled probe was achieved by adding cold oligonucleotide dimers of either human ALAS1 IR1 or the perfect IR1 in increasing doses (25-200 fold excess) to the binding reaction (*lane 5-8* and *lane 10-13*). In contrast, no competition was observed, when the mutated IR1 was used as a competitor (*lane 9*) demonstrating specificity of the binding. The addition of an antibody against FXR abolished the formation of a DNA protein complex at the ALAS1 IR1 and the perfect IR1 (*lane 14* and *lane16* in 7A and *lane 3* in 7B), suggesting that this antibody targets a region in FXR essential for DNA binding or heterodimer formation. In contrast, an anti-RXR antibody successfully super-shifted the DNA protein complex (*lane 2* in B). Taken together, these data indicate that the FXR/RXR specifically binds to the identified ALAS1 IR1.

FXR activation of ALAS1 is 'human specific' and does not occur in mice

To determine if FXR dependent induction of ALAS1 also occurs in rodents, C57Bl/6 mice were treated with the FXR selective agonist GW4064. In livers form treated animals GW4064 significantly repressed not only CYP7a1 (data not shown), a well studied and characterized gene negatively regulated by FXR via SHP, but also ALAS1 transcript levels (Fig. 8A). This inhibitory effect on ALAS1 was also seen when mice were fed a 1% cholic acid diet for 1 week (Fig. 8B). To exclude that the dissimilar findings in human liver culture systems and *in vivo* in mice were due to differences in experimental conditions, primary mouse hepatocytes were incubated with 50µM CDCA and analyzed for ALAS1 expression levels 8h later. Again, known FXR target gene SHP was markedly induced by CDCA treatment, while mouse ALAS1 transcript was significantly repressed (Fig. 8C). Taken together, these data show that both natural and synthetic FXR ligands affect hepatic ALAS1 levels in a species specific manner. Comparison of the homologous region of our newly identified human FXR response

element in the mouse genome demonstrated, that no putative IR1 element is present in mice (Fig. 8D). Even though the IR1 element is flanked by nucleotides showing high cross species sequence identity, the element itself is not conserved. The lack of conservation of a FXR response element therefore reflects the above described species specific regulation at the genomic nucleotide level.

3.5 Discussion

In the present study we identify human hepatic ALAS1 as a novel direct target of FXR. Both natural and synthetic agonists of FXR, CDCA and GW4064, were able to increase ALAS1 transcripts as well as activity in cultures of primary human hepatocytes. Treatment of human liver slices with near physiological concentration of CDCA also resulted in an increase in ALAS1 transcript levels. Moreover, by overexpression of a constitutively active form of FXR in primary human hepatocytes, the response of ALAS1 mRNA to GW4064 was significantly increased. In agreement with this, we identified and characterized in reporter assays a FXR response element in the 5'flanking region of human ALAS1. Site directed mutagenesis experiments demonstrated the functionality of the IR1 element, and binding of FXR/RXR heterodimer was shown in gel shift experiments. These data suggest a role of bile acid activated FXR in the regulation of ALAS1 and consequently of hepatic heme and porphyrin synthesis.

What is the physiological explanation for bile acids stimulating ALAS1? The rate limiting enzyme of heme synthesis is subject to various stimuli to adapt to ever changing demands of the cell for heme. More than 80% of hepatic heme is needed for incorporation into cytochromes P450, enzymes essential for detoxifying drugs and xenobiotics but also responsible for the synthesis and metabolism of endogenous substances, such as steroid hormones, cholesterol and bile acids (Guengerich, 2004). Cytochrome P450 CYP3A4 accounts for the metabolism of more than 50% of prescribed drugs, but is also a prerequisite for the hydroxylation and therefore detoxification of endogenous bile acids. The corresponding gene has been identified as a direct target of FXR only recently (Gnerre et al., 2004). This finding together with the identification of other FXR target genes involved in the elimination and metabolism of bile acids, such as UDP-glucuronosyltransferases (Barbier et al., 2003), sulfotransferases (Song et al., 2001), or transport proteins, such as MRP2 (Kast et al., 2002), illustrate the central role of FXR in coordinating detoxification processes of potentially toxic bile acids. Since functional CYP3A4 requires heme as a prosthetic group, a sufficient supply of the hepatocytes with heme needs to be guaranteed, whenever bile acids accumulate. We now propose a model of coordinated induction of

apocytochrome CYP3A4 and heme synthesis conducted by FXR. Bile acid activated FXR simultaneously and directly stimulates the transcription of apoprotein as well as ALAS1 to ensure adequate amount of heme for incorporation into newly formed CYP3A4. A similar coordinated response upon drug treatment has been suggested for CAR and PXR (Podvinec et al., 2004); activated by drugs, CAR and PXR bind to response elements in the flanking region of members of the CYP3As and 2B families as well as to ALAS1 drug response elements. Hence ALAS1 is not only an essential accessory enzyme for detoxification of exogenous drugs but also of potentially toxic endogenous bile acids.

Substantial crosstalk between the xenosensors CAR and PXR and bile acid receptor FXR has been established in the last years (Guo et al., 2003, Handschin and Meyer, 2005, Zhang et al., 2004a). First, PXR itself is activated by bile acid precursors (Goodwin et al., 2003a) as well as by the secondary bile acid LCA and its metabolites (Staudinger et al., 2001, Krasowski et al., 2005). However, unlike FXR, PXR is not efficiently activated by primary bile acids (Krasowski et al., 2005). Second, the three nuclear receptors all repress bile acid synthesis, although via different mechanisms (Wang et al., 2002, Miao et al., 2006, Goodwin et al., 2000), and they share a common set of target genes involved in detoxification and elimination of toxic compounds from the body as mentioned above. And third, PXR has been found to be a direct target of FXR, further supporting the connection between these factors (Jung, D et al, submitted). Together, these findings lead to the model of a multifactorial and redundant detoxification system the liver has evolved to adapt to potentially toxic compounds. FXR activated by low or near physiological concentrations of primary bile acids represents the first line of defense, whereas toxic concentrations of bile acids in particular the secondary bile acid LCA push the response to a maximal level via CAR and PXR. We now add ALAS1 to the target genes, which are concomitantly regulated by CAR and PXR as well as FXR.

Although overlapping substrate specificity for FXR and PXR have been reported, a pharmacological discrimination between the two nuclear receptors is possible. The induction of ALAS1 by a FXR specific pathway is underlined by the following data. First, low concentrations of CDCA, 10μM and 50μM, induced ALAS1 transcripts in human liver slices and cultures of primary hepatocytes, respectively. It is well established that these concentrations are unable to activate PXR. Despite earlier reports, where high concentration of CDCA (100μM) were found to activate PXR (Staudinger et al., 2001), CDCA was not found to have any potential to activate PXR in a recent detailed study (Krasowski et al., 2005). Second the specific FXR agonist GW4064 induced ALAS1, an effect, which was further augmented in the presence of exogenous FXR. In conclusion, these results strongly indicate an independent role of FXR in regulating ALAS1.

The increase in ALAS1 mRNA upon FXR activation in two independent human experimental systems was not observed in mice. In contrast, FXR activation by GW4064 treatment, or by a 1% cholic acid diet for 1 week resulted in a significant

repression of hepatic ALAS1 transcripts in mice. This finding most likely was not due to the different experimental conditions between human (primary cells and liver slices) and rodents (*in vivo*), since treatment of primary mouse hepatocytes by CDCA also resulted in a suppression of murine ALAS1 transcripts. The mechanism underlying this species specific effect at this time remains purely speculative. Interestingly, the identified human IR1, even though flanked by sequences showing high cross species sequence identity, is not conserved in rodents, but rather seems to be a primate invention. This finding is consistent with the previous observations of the fast turnover of transcription regulatory sites during evolution (Emberly et al., 2003). Major differences between human and rodents in cholesterol and bile acids homeostasis are a well-established feature; the most prominent example is CYP7a1, which is positively regulated by cholesterol precursors via liver x receptor (LXR) in rodents but not humans (Chiang et al., 2001). Human specific targets of FXR have been previously described and include hepatic lipase (Sirvent et al., 2004), α-crystallin (Lee et al., 2005) and peroxisome proliferative receptor α (Pineda Torra et al., 2003). There is a rapidly growing number of pathways regulated by FXR (for a recent review: (Claudel et al., 2005) (Houten et al., 2006), and the list is far from being complete. Future progress in this field should shed light into the species-specific response reported herein.

Interestingly, our findings are consistent with a well known, although only incompletely understood clinical observation: Increased urinary porpyhrin excretion in humans is commonly seen in hepatobiliary diseases accompanied by cholestasis (Rocchi et al., 2005, Doss M, 1972) (Gibson et al., 2000). Impaired biliary excretion of porphyrins in these conditions is one obvious explanation. In humans the main bile acid retained in cholestasis is CDCA. We now identified CDCA as a potent inducer of human ALAS1, which may imply that increased porphyrin synthesis in cholestatic conditions contributes to the phenomenon of elevated urinary porphyrin excretion as it has previously been suggested (Rocchi et al., 2005, Doss M, 1972). Analysis of porphyrin precursors in the urine of cholestatic patients could test this hypothesis.

The clinical implications of the data reported here are the situations where ALAS1 is more sensitive to induction, as in acute hepatic porphyrias. Due to a relative heme deficiency, the negative feedback exerted by heme on ALAS1 is reduced, and synthesis is rapidly and excessively stimulated whenever ALAS1 is activated. Known triggering factors include drugs that induce P450 hemeproteins, alcohol, fasting, sex steroids and all forms of stress. However the triggering events of most acute attacks remain obscure. It has long been assumed that ALAS1 is only indirectly upregulated via a diminishing regulatory heme pool upon heme consuming stimuli (Thunell, 2000, May et al., 1995). However, in our previous studies we established that several precipitating factors directly upregulate ALAS1 at the transcriptional level. Phenobarbital type drugs stimulate ALAS1 via xenosensing nuclear receptors PXR and CAR acting through two distal enhancer elements (Podvinec et al., 2004). The coactivator PGC-1α drives the fasting response via one FOXO1 site (Handschin et al., 2005) probably in concert with a c-AMP response (Giono et al., 2001) within the proximal promoter of ALAS1. With the

identification of a novel enhancer element conferring a FXR specific response of ALAS1, we here describe an additional mechanism having the potential to trigger acute porphyric attacks.

3.6 Figures

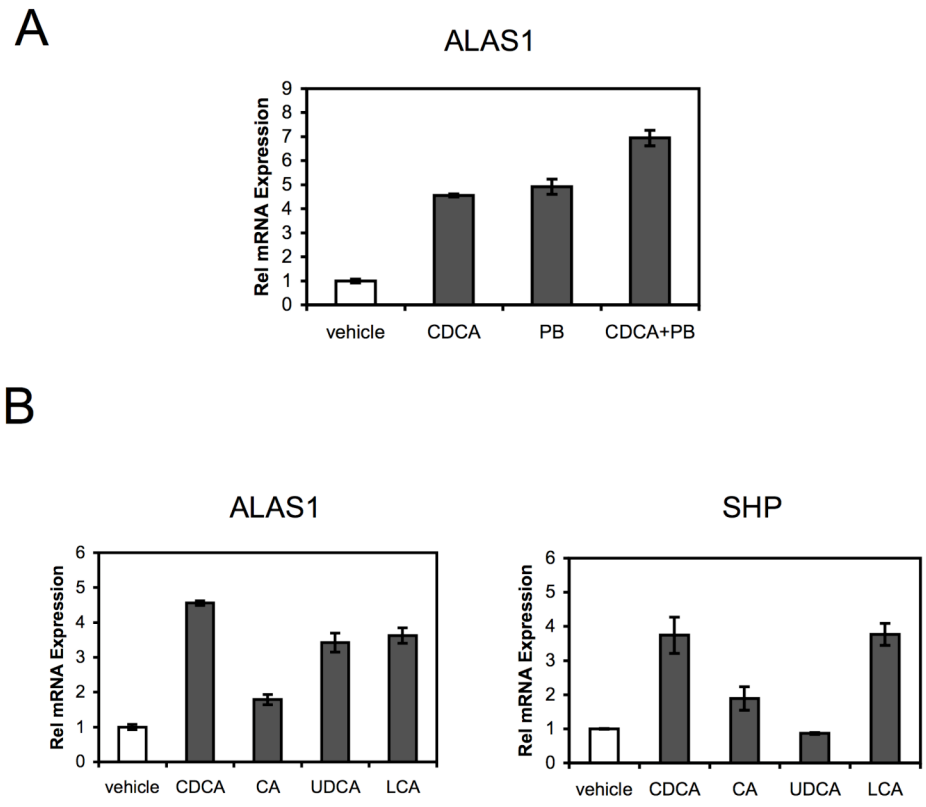

Figure 1: Bile acids induce ALAS1 mRNA level in cultured primary human hepatocytes.

Cultures of primary human hepatocytes were prepared and plated as described under materials and methods. After 24h incubation in serum free conditions, cells were challenged in (A) with vehicle (DMSO 0.1%), 50µM chenodeoxycholic acid (CDCA), 500µM phenobarbital (PB) or CDCA and PB in combination and in (B) with the primary bile acids CDCA, cholic acid (CA), ursodeoxycholic acid (UDCA; all at 50µM) and the secondary bile acid 25µM lithocholic acid (LCA). 8h later, RNA was isolated and ALAS1 mRNA expression relative to 18S was determined. A) ALAS1 mRNA expression is increased by its classical inducer PB as well as by the primary bile acid CDCA. The combination of both further increases the response. B) Different primary bile acids CDCA, CA, UDCA and the secondary bile acid LCA all induced ALAS1 and SHP mRNA expressions to various degrees. Values are given as mean +/- SD of duplicates with vehicle set at 1. The data presented are representative of the results from 2 different donors.

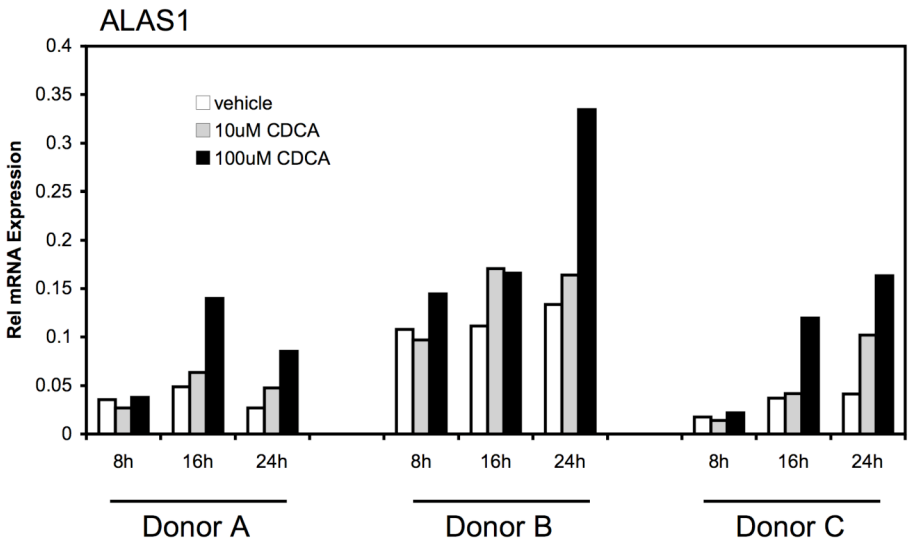

Figure 2: Chenodeoxycholic acid induces ALAS1 mRNA expression in human liver slices

Human liver slices were prepared as described under materials and methods. Slices of three different donors A, B and C were incubated with either vehicle (0.1% DMSO), 10µM and 100µM chenodeoxycholic acid (CDCA). After 8h, 16h and 24h treatment, RNA was extracted and expression levels for ALAS1 relative to cyclophilin were determined.

ALAS1 mRNA expression was increased upon CDCA treatment in slices of all three donors tested. Individual variety with respect to basal ALAS1 mRNA expression, the time course of response and the fold increase upon CDCA treatment are illustrated. Highest ALAS1 mRNA levels were achieved upon 100µM CDCA in donor A after 16h, in donor B and C after 24h. The low and near physiological concentration of 10µM CDCA was sufficient to increase ALAS1 transcripts in donor A (16h and 24h), donor B (16h and 24h) and donor C (24h). Values are given as means of PCR duplicates for each donor separately.

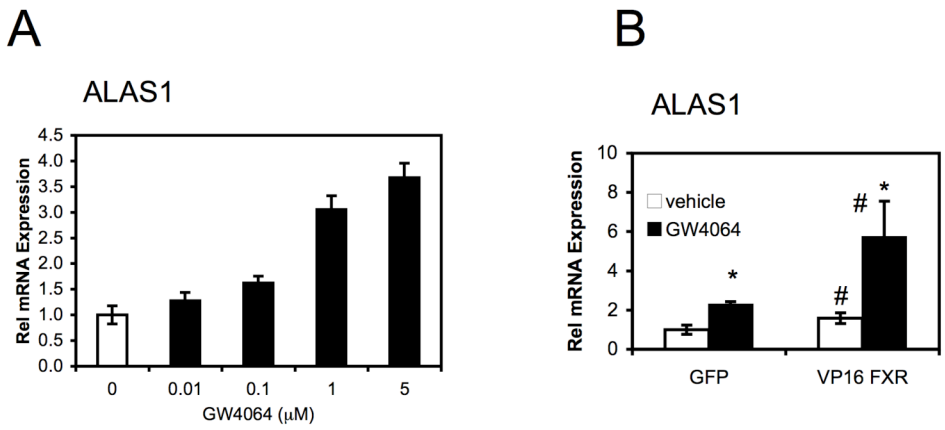

Figure 3: Activation of FXR induces ALAS1 mRNA expression in cultures of primary human hepatocytes.

*Cultures of primary human hepatocytes were plated and treated as in Fig. 1, except that the specific FXR agonist GW4064 was used from 10nM to 5μM. A) GW4064 induces ALAS1 in a dose dependent manner. Values are given as mean +/- SD of duplicate samples. The data presented is representative of the results from 2 different donors. B) After overnight plating, primary human hepatoytes were transfected with VP16 human FXR or a control plasmid. 36h later cells were induced with either vehicle or 1μM GW4064 for 8h when RNA was isolated and ALAS1 mRNA expression relative to cyclophilin was determined. Overexpression of VP16 FXR increased the mRNA level of ALAS1 and further increased the induction of ALAS1 by 1μM GW4064. Values are given as mean +/- SD of triplicate samples. * and ** indicate $p<0.05$ and $p<0.01$ respectively VP16 FXR versus vector control. # indicates $p<0.01$ GW4064 versus vehicle control.*

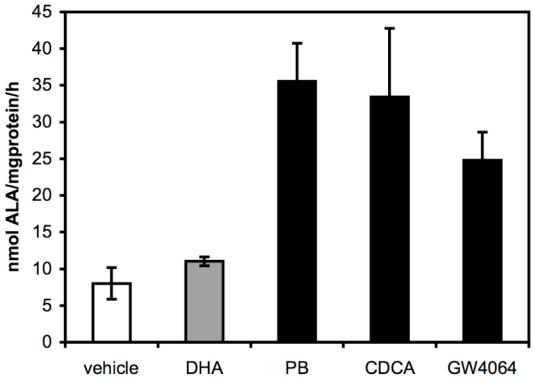

Figure 4: FXR activation increases ALAS activity in cultures of primary human hepatocytes

Cultures of primary human hepatocytes were prepared and plated as described under materials and methods. After 12h incubation in serum free medium, cells were exposed overnight to vehicle (0.15% DMSO), 250µM 4,6-dioxoheptanoic acid (DHA) alone or together with 50µM chenodeoxycholic acid (CDCA), 1µM GW4064 or 500µM Phenobarbital (PB). ALAS activity was assayed in culture plates for 2hrs, and the supernatants were prepared and analyzed for ALA production as described under materials and methods. Values are expressed as nmol ALA produced per mg protein per hour and given as mean +/- SD of duplicate samples. The data presented are representative of the results from 2 different donors.

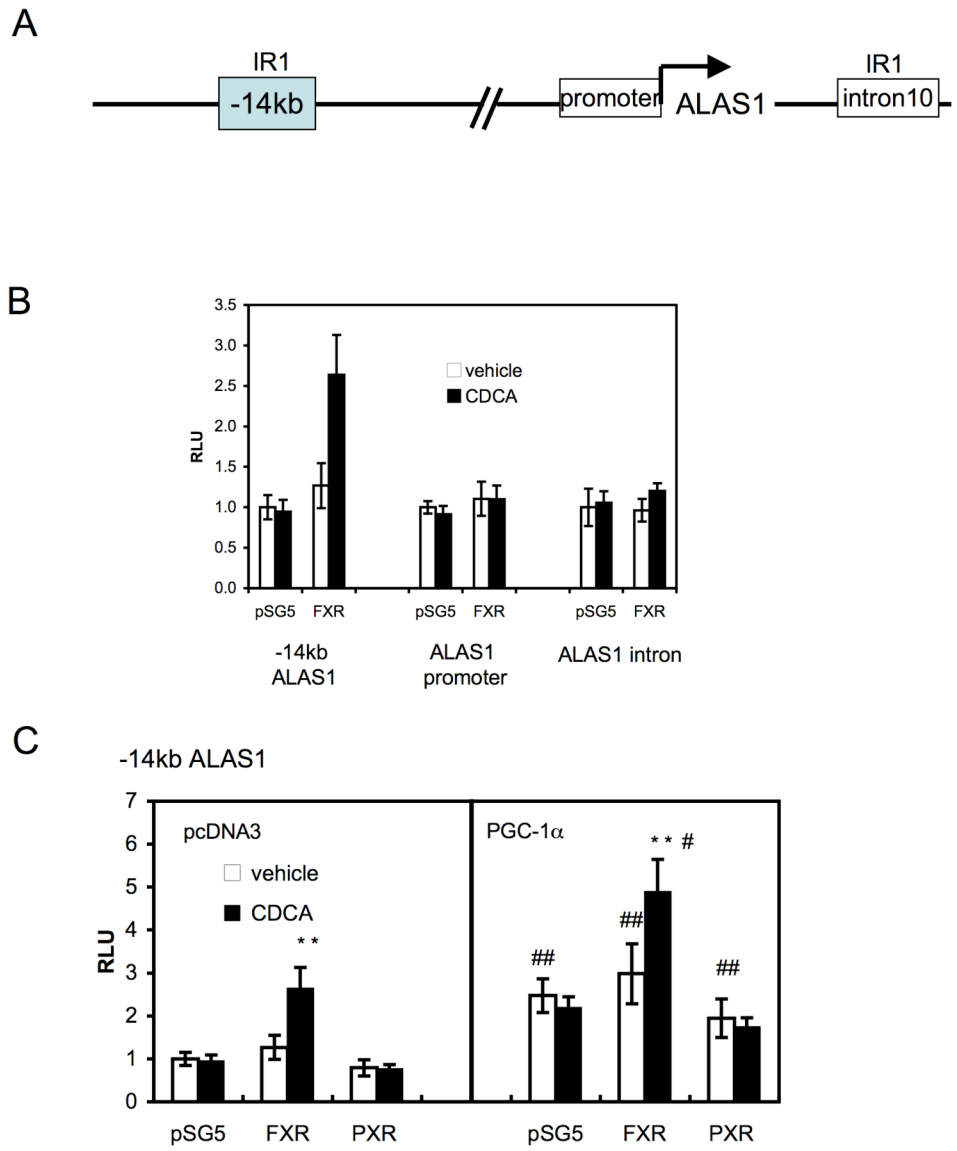

Figure 5: Identification of a FXR Response Element (FXRE) in the 5'-flanking region of human ALAS1

A) Schematic representation of human ALAS1 gene. NubiScan identified two high scoring IR1 elements within cross species conserved non-genic sequences, located at minus 14kb and within intron 10 respectively. The 1.3kb spanning promoter construct used in (B) is shown in white box.

B) The -14kb ALAS1 construct confers a FXR response. CV1 cells were co-transfected with the ALAS1 -14kb, 1.3kb ALAS1 promoter or the ALAS1 intron construct, together with expression plasmids of huFXR or vector control. 24h later, cells were treated with vehicle (0.1% DMSO) or 50μM CDCA and analyzed for luciferase light units relative to β-galactosidase after 24h. CDCA in the presence of FXR increased the reporter activity of the -14kb ALAS1 construct but not 1.3kb promoter nor ALAS1 intron.

*C) PGC1α coactivates CDCA response to FXR at the -14kb ALAS1. CV1 cells were co-transfected with the -14kb ALAS1 together with expression plasmids of huFXR or huPXR with or without huPGC1α. 24h later, cells were treated with vehicle (DMSO 0.1%) or 50μM CDCA and analyzed for luciferase light units relative to β-galactosidase 24h later. CDCA increased reporter activity of the -14kb ALAS1 only in the presence of FXR, and not PXR. Co-transfection of FXR with PGC1α resulted in maximal response. Values are given as mean +/- SD. All transfections were performed in at least 3 to 4 different experiments in duplicates or quadruplicates. ** p<0.01 indicates CDCA versus vehicle control. # and ## indicate p<0.05 and p<0.01 respectively.*

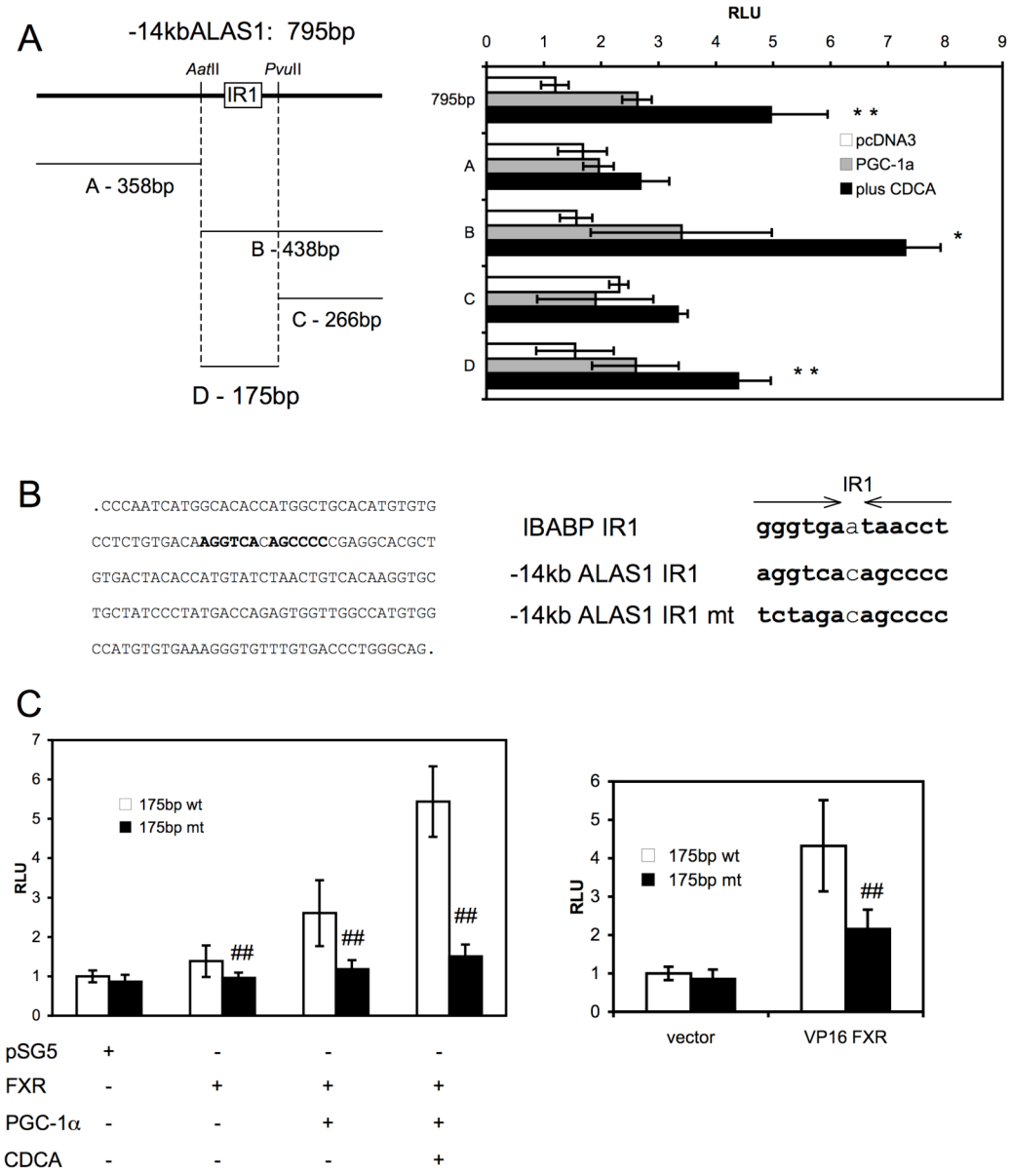

Figure 6: Characterization of a functional IR1 within the 795bp FXRE

A) Subcloning of the -14kb ALAS1 construct. CV1 cells were co-transfected with the parental 795bp construct and the subfragments A, B, C and D as illustrated together with expression plasmids of huFXR with or without huPGC1α. 24h later, cells were treated with vehicle (DMSO 0.1%) or 50μM CDCA and analyzed for luciferase light units relative to β-galactosidase 24h later. The 175bp middle fragment D retained the response to CDCA and PGC-1α in the presence of FXR, while there was no significant response observed in the flanking fragment A and C.

B) Sequence of 175bp fragment with the identified IR1 element shown in bold. The ALAS1 IR1 shows high similarity to the known FXR response element in the ileal bile acid binding protein (IBABP) promoter.

*C) Site directed mutagenesis of the IR1. CV1 cells were transfected with wildtype or mutated 175bp fragment together with expression constructs for FXR and PGC1α and subsequently stimulated with CDCA as in (A) (left panel) or CV1 cells were co-transfected with the constitutively active VP16 FXR (right panel). Mutation of halfsite 1 to TCTAGA resulted in a significant loss of the FXR response to CDCA and PGC1α (left panel) as well as to VP16 huFXR (right panel). Values are given as mean +/- SD. All transfections were performed in at least 2 to 4 different experiments. * and ** indicate p<0.05 and p<0.01 respectively CDCA versus vehicle control. ## indicates p<0.01 wildtype versus mutated fragment.*

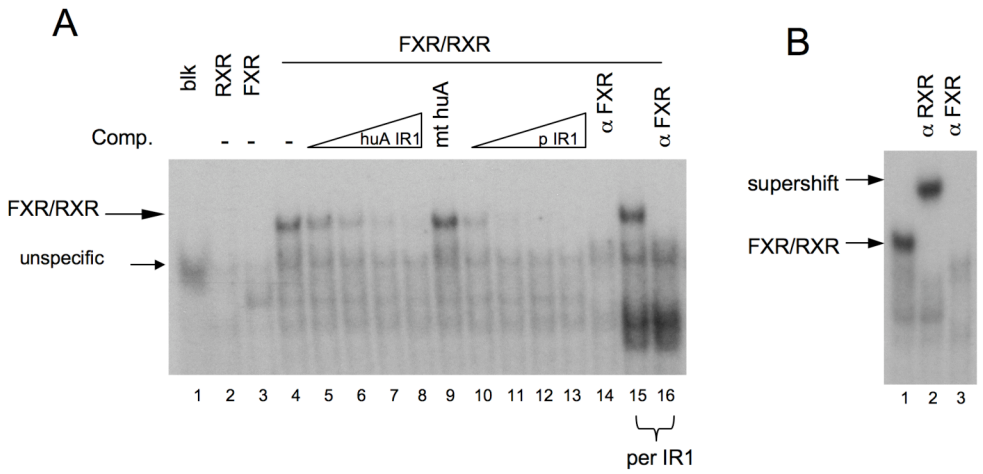

Figure 7: FXR/RXR heterodimer binds to the IR1 element of human ALAS1

EMSA assays were performed using the radiolabeled probes of human ALAS1 (huA IR1) (lane 1-14 in A and lane 1-3 in B) or a perfect IR1 (p IR1) (lane 15-16). The sequences of the wildtype and mutated probes are listed in Table 1.

A) FXR/RXR heterodimer binds to huA IR1 with high affinity (lane 4). The protein DNA complex disappears with increasing doses of competing wildtype unlabeled probe (lane 5-8), or unlabeled perfect IR1 (lane 10-13), but not with mutated huA IR1 (lane 9). An antibody against human FXR blocks the formation of a FXR/RXR DNA complex at huA IR1 and at p IR1 (lane 14 and 16 in A and lane 3 in B)

B). The FXR/RXR complex is successfully supershifted by an antibody against RXR (lane 2 in B).

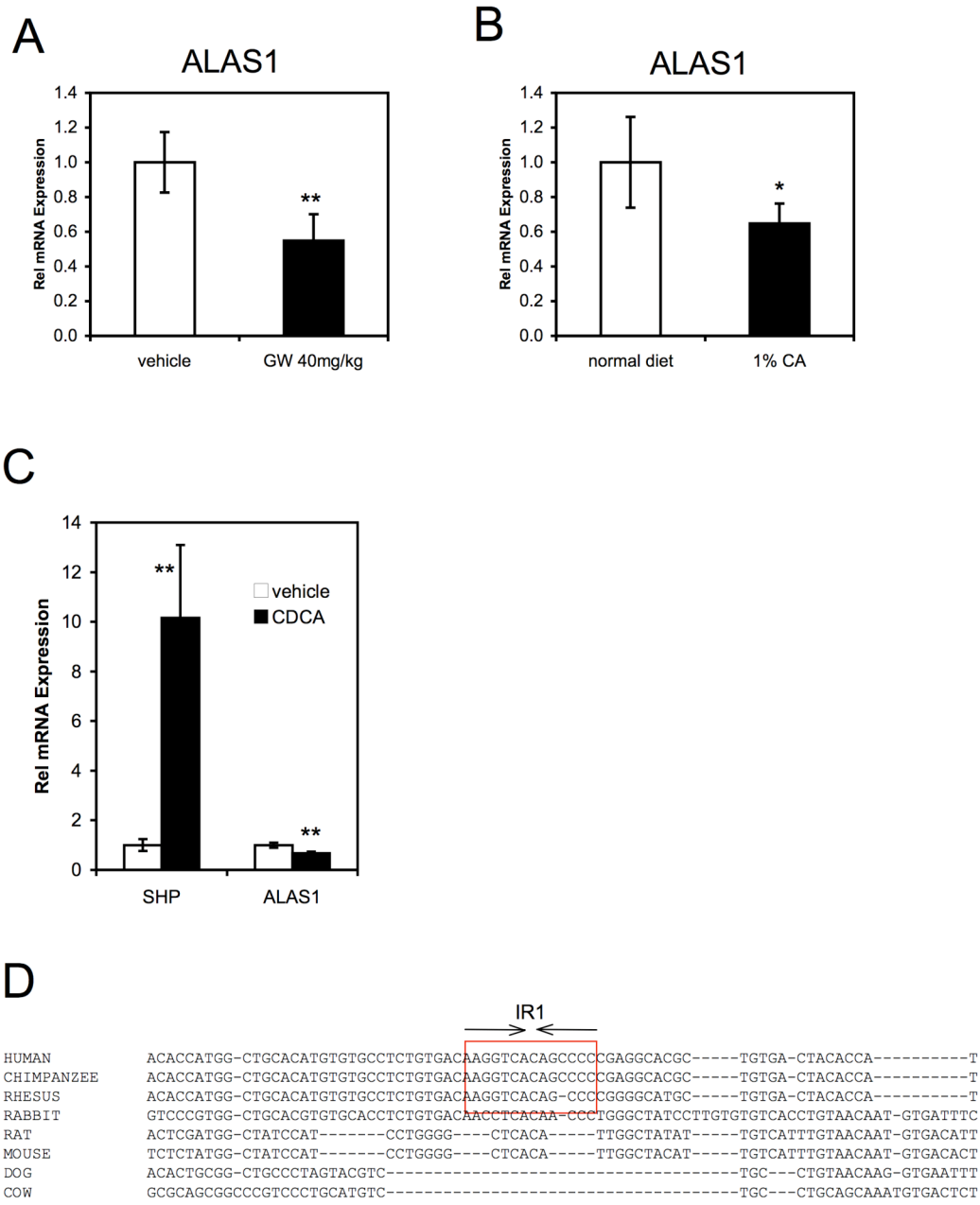

Figure 8: FXR activation of ALAS1 is 'human specific' and does not occur in mice

FXR activation by GW4064 or by cholic acid feeding represses hepatic ALAS1 in mice.

A) C57Bl/6 mice were injected with 40mg/kg GW4064 (i.p.), sacrificed 16hrs later, when the livers were excised and analyzed for ALAS1 mRNA expression relative to GAPDH.

*B) C57Bl/6 mice were fed normal chow or a 1% cholic acid diet for 1 week. Relative mRNA levels for hepatic ALAS1 were determined as in (A). n = 4-5 animals per group. * and ** indicate $p<0.05$ and $p<0.01$ resp. treated versus untreated control.*

*C) Primary mouse hepatocytes were isolated as described under material and methods. After culturing the cells 24h in serum free conditions, cultures of primary mouse hepatocytes were incubated with either vehicle or 50µM CDCA for 8h, when RNA was extracted and mRNA levels of SHP and ALAS1 relative to GAPDH were determined. Values are given as mean +/- SD of triplicate samples. ** indicates p<0.01 CDCA versus vehicle control.*

D) Multispecies (human, chimpanzee, rhesus, rabbit, mouse, rat, dog and cow) sequence alignment produced by MULTIZ covering the characterized human IR1 element was retrieved from Santa Cruz Genome Browser. The IR1 element is conserved only in primate species and not present in mice.

Table 1: Sequences used for real time PCR, cloning, EMSA and mutagenesis. Only the sense strands are shown for oligonucleotides used in EMSA assays.

Gene/name	Primers	Purpose
h18S	5'-AGTCCCTGCCCTTTGTACACA-3' F 5'-CGATCCGAGGGCCTCACTA-3' R 5'-CGCCCGTCGCTACTACCGATTGG-3' Probe	RT-PCR, Taqman
hCyclophilin	5'-GGAGATGGCACAGGAGGAA-3' F 5'-GCCCGTAGTGCTTCAGCTT-3' R	RT-PCR, SYBR
hALAS1	5'-ATGATGCCAGGCTGTGAGATTT-3' F 5'-GCTGTTTCGAATCCCTTGGA-3' R 5'-TCTGATTCTGGGAACCATGCCTCCA-3' Probe	RT-PCR, Taqman
hSHP	5'-CTTCAATGCTGTCTGGAGTCCTT -3' 5'-GCACCAGGGTTCCAGGACTT-3'	RT-PCR, SYBR
hBSEP	5'-CAGTTCCCTCAACCAGAACATG-3' F 5'-TTTGATCATTTCGCTCTCGATG-3' R	RT-PCR, SYBR
mGAPDH	5'-CCAGAACATCATCCCTGCATC-3' F 5'-GGTCCTCAGTGTAGCCCAAGAT-3' R 5'-CCGCCTGGAGAAACCTGCCAAGTATG-3' Probe	RT-PCR, Taqman
mALAS1	5'-GGCCTCCCGGTCATCC-3' F 5'-TGTTCTTAGCAGCATCGGCA-3' R 5'-CTGTCCGAGTCACATCATCCCTGTGC-3' Probe	RT-PCR, Taqman
mSHP	5'-TGAGCTGGGTCCCAAGGA-3' F 5'-AGGGCTCCAAGACTTCACACA-3' R	RT-PCR, SYBR
1.3kb prom ALAS1	5'- cgGGTACCGAGCTCTCAGACCAAAGCCC -3' F 5'- ccgCTCGAGCAAGTCGAGAAGTCCAAACG -3' R	Cloning
-14kb ALAS1	5'-cgGGTACCGGTGGAGAATCTGAGGTCCA -3' F 5'-ccgCTCGAGCTCTTCCTTGACCACCACCT-3' R	Cloning
Intron10 ALAS1	5'- cgGGTACCAATGGCCTGTCTCTGATTGG -3' F 5'- ccgCTCGAGGCCTCTGAAGGGCTTCAATTA -3' R	Cloning
Mut IR1	5'-CATGTGTGCCTCTGTGACATCTAGACAGCCCCCGAGGCACGC-3'	Mutagenesis
hA IR1	5'-TGTGACAAGGTCACAGCCCCCGAGGCA-3'	EMSA
hA mt IR1	5'-TGTGACATCTAGACAGCCCCCGAGGCA-3'	EMSA
Perfect IR1	5'-GCTTTTAGGTCAATGACCTAGCCCTC-3'	EMSA

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Nutritional Regulation of Hepatic Heme Biosynthesis and Porphyria through PGC-1α

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Summary

Inducible hepatic porphyrias are inherited genetic disorders of enzymes of heme biosynthesis. The main clinical manifestations are acute attacks of neuropsychiatric symptoms frequently precipitated by drugs, hormones, or fasting, associated with increased urinary excretion of δ-aminolevulinic acid (ALA). Acute attacks are treated by heme infusion and glucose administration, but the mechanisms underlying the precipitating effects of fasting and the beneficial effects of glucose are unknown. We show that the rate-limiting enzyme in hepatic heme biosynthesis, 5-aminolevulinate synthase (ALAS-1), is regulated by the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Elevation of PGC-1α in mice via adenoviral vectors increases the levels of heme precursors in vivo as observed in acute attacks. The induction of ALAS-1 by fasting is lost in liver-specific *PGC-1α* knockout animals, as is the ability of porphyrogenic drugs to dysregulate heme biosynthesis. These data show that PGC-1α links nutritional status to heme biosynthesis and acute hepatic porphyria.

Introduction

The heme biosynthetic pathway in eukaryotic cells is comprised of eight enzymatic steps; the first and the last three enzymes are located in the mitochondria, while the rest are in the cytoplasm (Figure 1A). Eighty to ninety percent of total heme in mammals is synthesized in erythroid cells for incorporation into hemoglobin. Regulation of heme biosynthesis in these cells involves the erythroid-specific aminolevulinate synthase (*ALAS*) gene *ALAS-2*. In contrast, the housekeeping *ALAS* gene *ALAS-1*, also called *ALAS-N* or *ALAS-H*, is ubiquitously expressed, given that all nucleated cells must synthesize heme for respiratory cytochromes. The bulk of the nonerythroid-synthesized heme is produced in the liver for various heme proteins, in particular microsomal cytochromes P450. Because either a deficiency or an excess of heme is toxic to the cell, hepatic heme production has to be tightly controlled, mostly via its rate-limiting step ALAS-1. Accordingly, hepatic *ALAS-1* is highly regulated in different contexts to ensure adequate levels of intracellular heme (May et al., 1995). Inherited mutations in all genes encoding for heme biosynthetic enzymes have been described, except for *ALAS-1*, and the resulting diseases are referred to as porphyrias (Elder, 1998). Depending on the specific enzymatic defect, different patterns of overproduction, accumulation, and excretion of intermediates of heme synthesis are observed.

The main clinical manifestations of porphyrias are intermittent attacks of neuropsychiatric dysfunction and/or sensitivity of the skin to sunlight. The neuropsychiatric syndrome occurs only in those porphyrias in which there is intermittent induction of hepatic *ALAS-1* and consequent increased urinary excretion of δ-aminolevulinic acid (ALA). Attacks are characteristically precipitated by drugs such as barbiturates, fasting, and hormones and result in abdominal pain, tachycardia, peripheral motor neuropathies, psychosis, and other mental disturbances (Elder, 1998; Thadani et al., 2000; Thunell, 2000). Inducible hepatic porphyrias are caused by rare defects in δ-aminolevulinic acid dehydratase (*ALAD*), porphobilinogen deaminase (*PBGD*, also known as hydroxymethylbilane synthase), coproporphyrinogen oxidase, and protoporphyrinogen oxidase. The classical names for the corresponding diseases are ALAD deficiency, acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria.

Although not definitively proven, historic personalities thought to have suffered from porphyria include King George III (Macalpine and Hunter, 1966), Friedrich Wilhelm I of Prussia (Macalpine et al., 1968; Pierach and Jennewein, 1999), and Vincent van Gogh (Bonkovsky et al., 1992; Loftus and Arnold, 1991). Thus, the psychoses arising from their disease potentially influenced the course of the American war for independence and/or the creative genius of van Gogh. Acute attacks of inducible hepatic porphyria are treated by discontinuing exposure to the precipitating agents, heme infusions, and high carbohydrate load. The carbohydrates are typically given as concentrated glucose infusion. Heme directly represses its own biosynthesis in a negative feedback loop (May et al., 1995). In contrast, the underlying mechanisms of the beneficial effects of carbohydrates are not understood.

Proliferator-activated receptor γ coactivator 1α (PGC-1α) is a coactivator of nuclear receptors and other transcription factors (Puigserver and Spiegelman, 2003). PGC-1α controls mitochondrial biogenesis and oxidative metabolism in many tissues, including brown adipose tissue, skeletal muscle, heart, and liver (Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999; Yoon et al., 2001). In the liver, PGC-1α is induced during fasting, when the liver ceases using glucose as an energy supply and changes to the β-oxidation of fatty

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[3]This paper is dedicated to the memory of our dear colleague and friend, Stanley J. Korsmeyer.

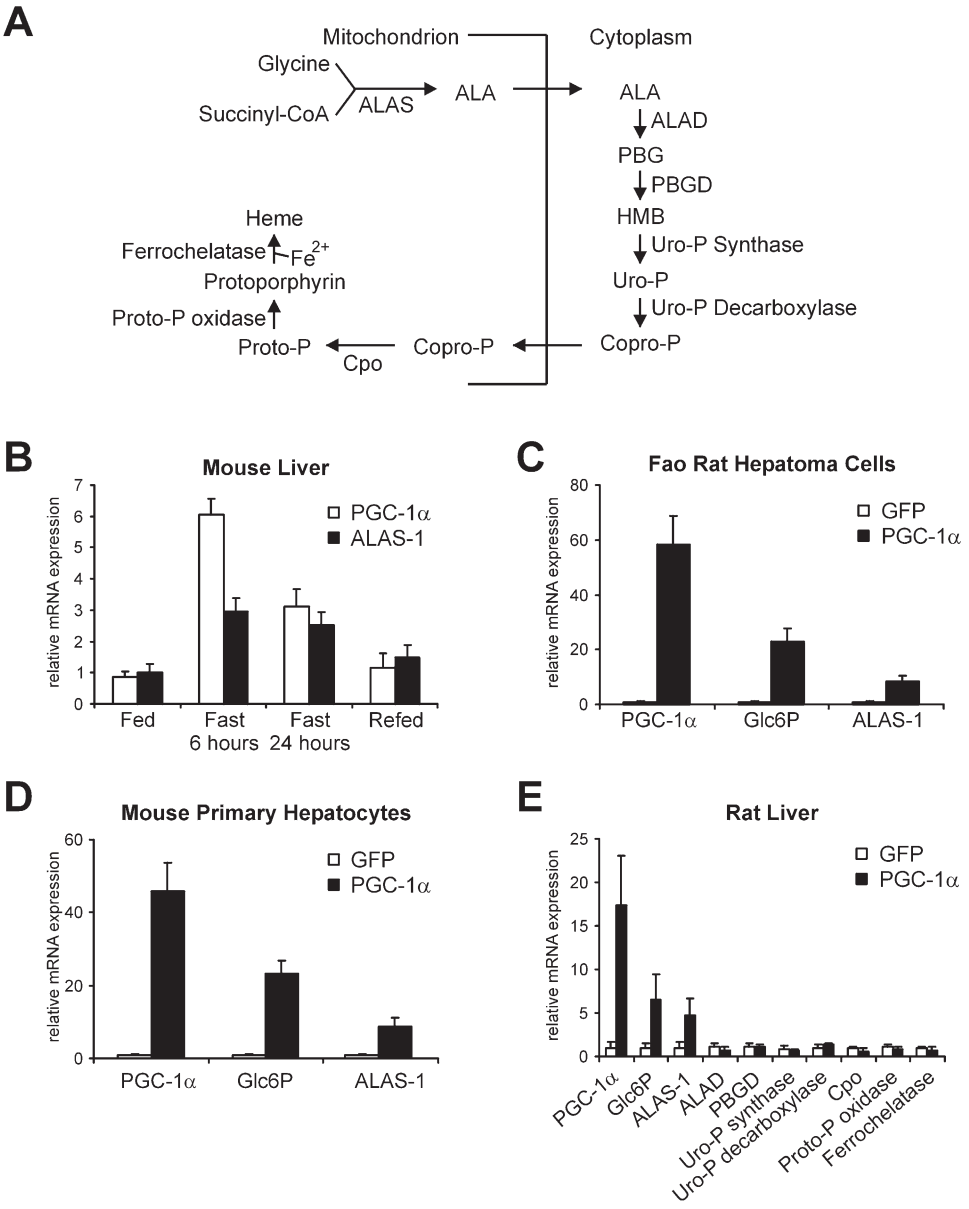

Figure 1. *ALAS-1* Expression Is Activated by PGC-1α in Hepatocytes and Liver In Vivo

(A) Heme biosynthesis pathway. ALA, 5-aminolevulinic acid; ALAD, ALA dehydratase; PBG, porphobilinogen; PBGD, PBG deaminase; HMB, hydroxymethylbiline; Uro-P, uroporphyrinogen; Copro-P, coproporphyrinogen; Cpo, coproporphyrinogen oxidase; Proto-P, protoporphyrinogen.

(B) ALAS-1 and PGC-1α mRNAs are coinduced in fasting. Mice were fasted for 6 hr and 24 hr, respectively, and hepatic levels of PGC-1α and ALAS-1 were compared to those of fed and refed animals by semiquantitative PCR.

(C and D) Adenoviral PGC-1α increases *ALAS-1* gene expression in cell culture. Fao rat hepatoma cells (C) and mouse primary hepatocytes (D) were infected with adenovirus encoding GFP or PGC-1α, respectively, and relative mRNA levels of PGC-1α, glucose-6-phosphatase (Glc6P), and ALAS-1 were determined by semiquantitative PCR 24 hr after infection.

(E) PGC-1α induces ALAS-1 transcript levels in vivo. Male Wistar rats were tail-vein injected with adenovirus encoding for GFP and PGC-1α, respectively. Five days postinjection, animals were sacrificed, and hepatic mRNAs were analyzed for changes in expression using semiquantitative PCR. Data in (B)–(E) are represented as mean ± standard deviation.

acids. This increase in fatty-acid β-oxidation and elevation of hepatic gluconeogenesis are both under control of PGC-1α (Herzig et al., 2001; Yoon et al., 2001).

Thus, because of the key role of PGC-1α in liver energy homeostasis and the finding that many PGC-1α targets are heme proteins, we investigated the role of PGC-1α in the regulation of hepatic heme biosynthesis by nutrition. We found that PGC-1α is an important factor controlling the expression of *ALAS-1* in the fasted and fed liver. Moreover, we showed that hepatic PGC-

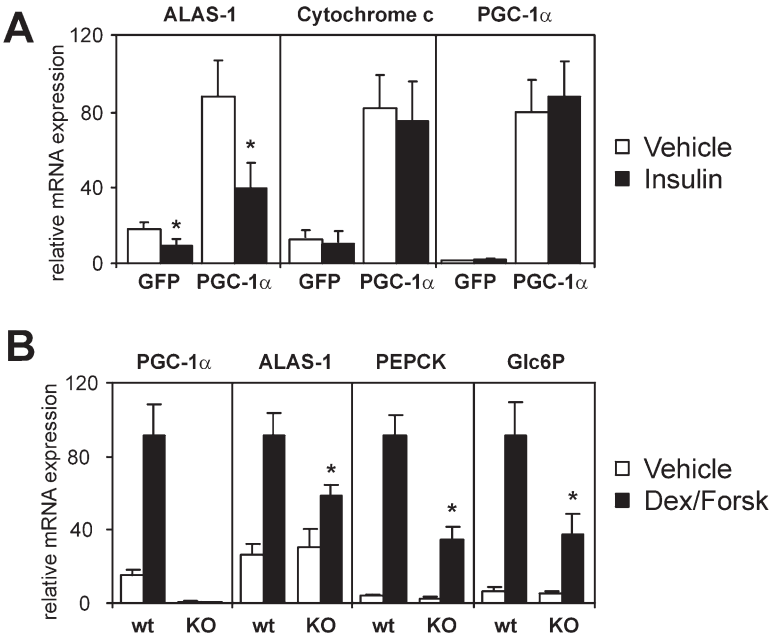

Figure 2. *ALAS-1* Expression Is Regulated by PGC-1α and Insulin

(A) Insulin represses PGC-1α-mediated induction of *ALAS-1*. H2.35 SV-40-transformed hepatocyte cells were infected with adenoviral GFP and PGC-1α for 24 hr and subsequently treated with vehicle (PBS) or 10 nM insulin for 12 hr before relative ALAS-1, cytochrome c, and PGC-1α mRNA levels were determined. *p < 0.05 between vehicle- and insulin-treated cells.

(B) *ALAS-1* induction by dexamethasone and forskolin is partially dependent on PGC-1α. Mouse primary hepatocyte cultures were established from wild-type and *PGC-1α* total knockout animals. These cells were subsequently treated with dexamethasone (Dex, 1 μM) and forskolin (Forsk, 0.2 μM) for 3 hr, and relative mRNA levels were determined by semiquantitative PCR. Data in (A) and (B) are represented as mean ± standard deviation. *p < 0.05 between wild-type and knockout cells in Student's t test.

1α is a major determinant of the severity of acute porphyric attacks in mouse models of chemical porphyria.

Results

Hepatic *PGC-1α* and *ALAS-1* Are Coregulated in Fasting and Feeding

Since fasting can be a powerful stimulus to induce an acute porphyric attack and the liver is central to the fasting response in mammals, the metabolic status of the liver should be crucial for the regulation of heme biosynthesis. The transcriptional coactivator PGC-1α has been described as a key factor in the control of hepatic gluconeogenesis in the fasted liver (Herzig et al., 2001; Yoon et al., 2001). Interestingly, *ALAS-1* and *PGC-1α* are coregulated in fasted mice, with increased mRNA levels (Figure 1B). To test the relationship between the regulation of these two genes, Fao rat hepatoma cells, mouse primary hepatocytes, and rat liver in vivo were infected with adenovirus expressing PGC-1α. In all of these systems, ectopic expression of PGC-1α increased ALAS-1 transcript levels in a manner similar to that of glucose-6-phosphatase (*Glc6P*), a PGC-1α target gene involved in gluconeogenesis (Yoon et al., 2001) (Figures 1C–1E). In contrast to *ALAS-1*, none of the other seven genes of the heme biosynthetic pathway were induced by PGC-1α in rat liver (Figure 1E).

Insulin and Glucagon Regulation of *ALAS-1* Involves PGC-1α

Regulation of ALAS-1 in fasting and feeding is mediated by the counterregulatory hormones insulin and glucagon (Scassa et al., 1998; Varone et al., 1999). Insulin treatment of primary mouse hepatocytes reduces basal levels of ALAS-1 mRNA (Figure 2A). Furthermore, PGC-1α-induced ALAS-1 transcript levels are reduced by insulin, suggesting that PGC-1α is in the pathway of the insulin regulation of *ALAS-1* (Figure 2A). Primary hepatocytes from wild-type and *PGC-1α* knockout mice (Lin et al., 2004) were used to elucidate the function of PGC-1α in the *ALAS-1* induction in fasting, with dexamethasone and forskolin representing the effects of glucocorticoids and glucagon that are elevated when blood glucose levels are low. Induction of ALAS-1 mRNA by these agents was reduced in the *PGC-1α* knockout hepatocytes as compared to wild-type cells (Figure 2B). Similarly, the response of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*PEPCK*) and *Glc6P* to these hormones was blunted. These findings imply that PGC-1α is involved in the fasting/feeding regulation of all three genes.

Insulin Repression of *ALAS-1* Is Mediated by FOXO1 and PGC-1α

In the *ALAS-1* promoter, two binding sites for the nuclear respiratory factor-1 (NRF-1) have been identified (Braidotti et al., 1993). NRF-1 is a transcription factor that increases expression of nuclear-encoded mitochondrial genes (Virbasius and Scarpulla, 1994) and is known to be potently coactivated by PGC-1α (Wu et al., 1999). Thus, NRF-1 is a potential binding partner by which PGC-1α controls *ALAS-1* expression. In addition to the NRF-1 site, an insulin-responsive element (IRE) has been defined in the *ALAS-1* promoter (Scassa et al., 2001, 2004), but the identity of transcription factors binding to this element has remained elusive (Figure 3A).

Chromatin immunoprecipitation assays in mouse hepatoma cells illustrate that PGC-1α is recruited to both the NRF-1 and the IRE regions (Figure 3B). Moreover, PGC-1α recruitment to the IRE region is sensitive

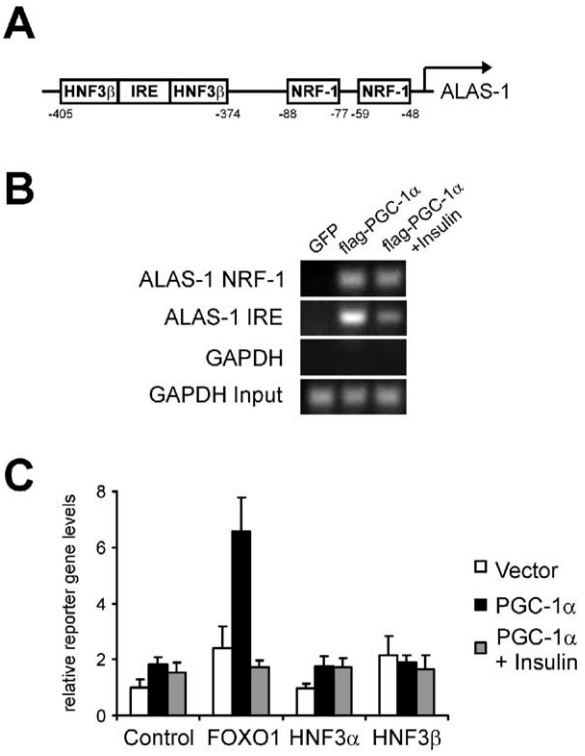

Figure 3. PGC-1α Regulates *ALAS-1* Expression via NRF-1 and FOXO1

(A) Structure of the *ALAS-1* promoter.

(B) PGC-1α binds to the NRF-1 and the IRE sites on the *ALAS-1* promoter. H2.35 cells were infected with adenoviral GFP or FLAG-tagged PGC-1α. Cells were treated with 10 nM insulin for 12 hr before cells were harvested and chromatin immunoprecipitation was performed using an anti-FLAG antibody.

(C) PGC-1α coactivates FOXO1 on the *ALAS-1* promoter. H.35 cells were transfected with *ALAS-1*-promoter construct and expression plasmids for FOXO1, HNF3α (FOXA1), HNF3β (FOXA2), and PGC-1α. After transfection, cells were treated with 10 nM insulin for 12 hr before reporter-gene levels were determined. Data are represented as mean ± standard deviation.

to insulin, in contrast to PGC-1α binding to the NRF-1 site (Figure 3B). Sequence comparison of the *ALAS-1* IRE to those of the gluconeogenic genes *PEPCK* and *Glc6P* revealed high sequence conservation between these sites. In the flanking regions of the gluconeogenic genes, PGC-1α binds to FOXO1 at these elements. After insulin exposure, FOXO1 is phosphorylated, its binding to PGC-1α disrupted, and, subsequently, FOXO1 is exported from the nucleus (Puigserver et al., 2003). Thus, FOXO1 is a plausible candidate to bind to the *ALAS-1* promoter.

Recently, Scassa and coworkers described the hepatocyte nuclear factor 3β (HNF3β, alternatively called FOXA2) to bind to sites adjacent to the *ALAS-1* IRE (Scassa et al., 2004) (Figure 3A). They found that integrity of the IRE and not of the HNF3β sites is obligatory for insulin regulation of *ALAS-1* and that the regulation of *ALAS-1* by HNF3β cannot account for the repression of *ALAS-1* by insulin (Scassa et al., 2004). We thus tested the ability of PGC-1α to coactivate the different Forkhead box family members FOXO1, hepatocyte nuclear factor 3α (HNF3α, FOXA1), and HNF3β on the *ALAS-1* promoter. As described by Scassa et al., HNF3β increased reporter-gene levels controlled by the *ALAS-1* promoter, whereas HNF3α had no effect (Figure 3C). However, of the three transcription factors, PGC-1α only coactivated FOXO1 in this context. In addition, merely the FOXO1-PGC-1α-mediated induction of the *ALAS-1* promoter was repressed by insulin (Figure 3C).

To further characterize the role for FOXO1 in the regulation of the *ALAS-1* promoter, we showed direct physical interaction of FOXO1 with the DNA probes containing the *ALAS-1* IRE (Figure 4A, lanes 4–7) and NRF-1 with the NRF-1 site (Figure 4A, lanes 1–3) in electrophoretic mobility shift assays. Site-directed mutagenesis of the FOXO1 site abolished binding of FOXO1 to this element (Figure 4A, lanes 3 and 7, respectively). The specificity of the FOXO1-IRE complex was confirmed by using an anti-FOXO1 antibody that resulted in a supershift (Figure 4A, lane 6). Functionally, PGC-1α coactivates both NRF-1 and FOXO1 in reporter-gene assays using the *ALAS-1* promoter in mouse H2.35 SV-40-transformed hepatocytes (Figure 4B). Mutagenesis of the NRF-1 or the IRE sites reduced the ability of PGC-1α to augment the activity from the *ALAS-1* promoter stimulated by NRF-1 and FOXO1, respectively. Moreover, an *ALAS-1*-promoter allele with a combined mutation of both the NRF-1 and IRE sites is completely insensitive to PGC-1α, strongly suggesting that NRF-1 and FOXO1 are the major binding partners of PGC-1α in the *ALAS-1* promoter.

As shown in Figure 4C, insulin represses induction of *ALAS-1*-promoter-driven reporter-gene expression by either FOXO1 alone or in combination with PGC-1α. In contrast, a nonphosphorylatable mutant of FOXO1 with three alanines in place of the serine/threonine residues targeted by Akt kinase (termed FOXO1 3A) prevented repression by insulin. Similarly, insulin is unable to inhibit PGC-1α coactivation of FOXO1 3A. This suggests that the insulin repression of the *ALAS-1* promoter is controlled by the FOXO1-PGC-1α interaction.

Liver-Specific *PGC-1α* Knockout Animals Have a Blunted Induction of *ALAS-1* in Fasting

To investigate whether PGC-1α is a key mediator of the metabolic regulation of *ALAS-1* in an in vivo setting, we first examined animals with a total knockout of *PGC-1α* (Lin et al., 2004) under fasting and feeding conditions. Unfortunately, regulation of *ALAS-1* is masked by systemic effects of the whole-body knockout of *PGC-1α*, similar to what has previously been described for the gluconeogenic genes (Lin et al., 2004): ALAS-1 mRNA is constitutively induced to fasted levels in the total knockout animals, even in the fed state (Figure 5A). It is unclear whether the same compensatory mechanisms (elevation of C/EBPβ) account for the constitutive expression of the gluconeogenic genes and *ALAS-1* (Lin et al., 2004). Thus, in order to dissect systemic effects of the total knockout from the liver phenotype, we had to generate liver-specific *PGC-1α* knockout animals by crossing mice with a floxed *PGC-1α* allele to transgenic

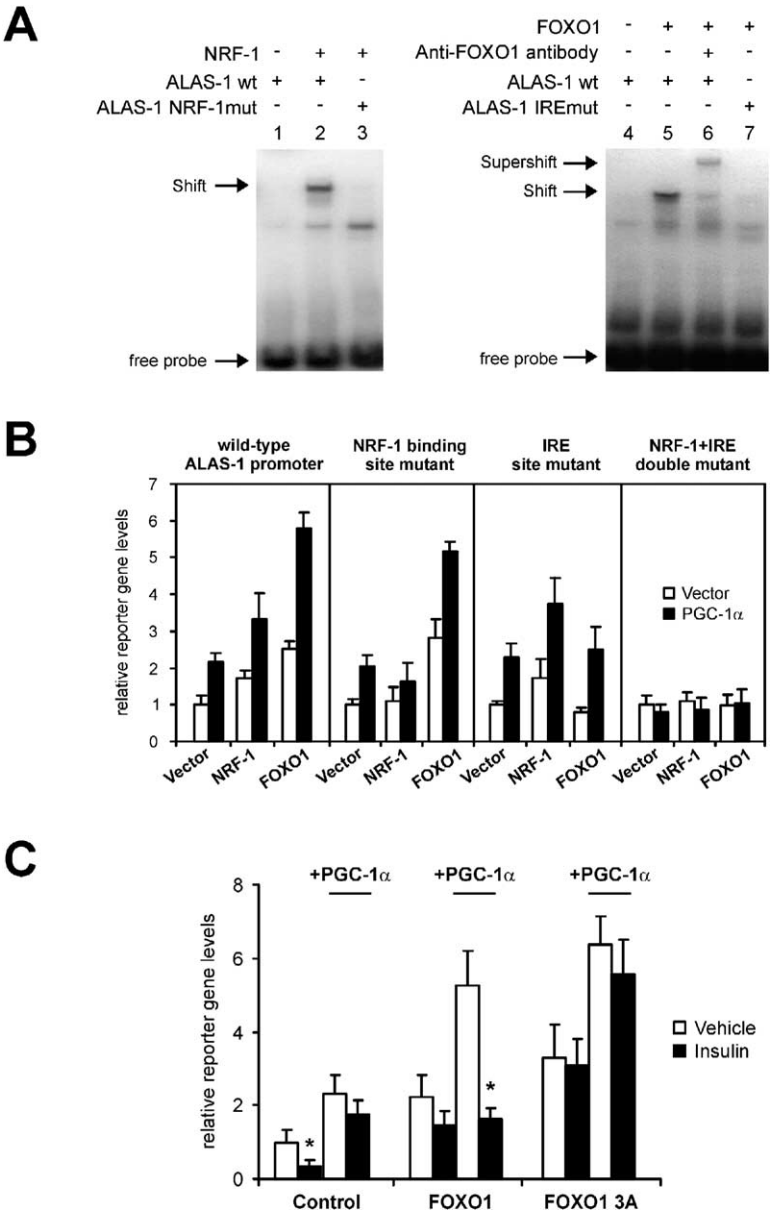

Figure 4. FOXO1-PGC-1α Mediate the Repression of the *ALAS-1* Promoter by Insulin

(A) FOXO1 binds to the ALAS-1 promoter. Electrophoretic mobility shift assays were performed using radiolabeled wild-type and mutated *ALAS-1* promoter as probe together with in vitro-transcribed/translated NRF-1 and FOXO1, respectively.

(B) PGC-1α induction of the *ALAS-1* promoter is mediated by NRF-1 and FOXO1. H2.35 cells were transfected with wild-type and *ALAS-1*-promoter constructs with mutations in the NRF-1 and the IRE sites together with expression plasmids for PGC-1α, NRF-1, and FOXO1. Reporter-gene levels were determined 16 hr after transfection.

(C) Insulin regulation of the *ALAS-1* promoter is inhibited by a mutant FOXO1 allele. H2.35 cells were transfected with a reporter-gene plasmid driven by the wild-type *ALAS-1* promoter together with expression plasmids for PGC-1α, FOXO1, and FOXO1 3A, a FOXO1 allele that is not phosphorylated by insulin signaling. After transfection, cells were treated with 10 nM insulin for 12 hr before reporter-gene levels were determined. Data in (B) and (C) are represented as mean ± standard deviation. *p < 0.05 between vehicle- and insulin-treated cells in Student's t test.

animals expressing cre recombinase under the control of the *albumin* promoter (Figure 5B). Tissue-specific ablation of *PGC-1α* in the liver was verified by mRNA analysis (Figure 5C). PGC-1α levels in the heart, skeletal muscle (SKM), and brown adipose tissue (BAT) were unaltered. Moreover, liver-specific knockout of *PGC-1α* did not change the expression of the closely related family member PGC-1β in liver, heart, skeletal muscle, or brown adipose tissue.

In these mice, basal levels of ALAS-1, PEPCK, and Glc6P were not elevated, as was seen in the total knockout (Figure 5D). Indeed, ALAS-1 basal expression was significantly lower in the liver-specific *PGC-1α* knockout as compared to wild-type animals. Moreover, the induction of *ALAS-1* and the gluconeogenic genes after 16 hr of fasting was severely blunted in the liver-specific knockout animals compared to wild-type mice (Figure 5D). These findings indicate a requirement for PGC-1α in the fasting/feeding regulation of the gluconeogenic genes and, furthermore, imply a key role for PGC-1α in the dietary control of *ALAS-1* transcription. Patients suffering from porphyric attacks are given a high carbohydrate load. Thus, in addition to refeeding, fasted mice were i.p. injected with a bolus of glucose (1 g/kg) or glucose (1 g/kg) and insulin (1.0 U/kg) 30 min, 60 min, or 120 min before mice were sacrificed and hepatic PGC-1α and ALAS-1 mRNA levels were determined (Figure 5E). Glucose loading reduces ALAS-1 transcript levels 30 min after injection. The combination of glucose and insulin is even more potent in inhibiting fasting-mediated induction of *PGC-1α* and *ALAS-1*, supporting the hypothesis that at least part of the bene-

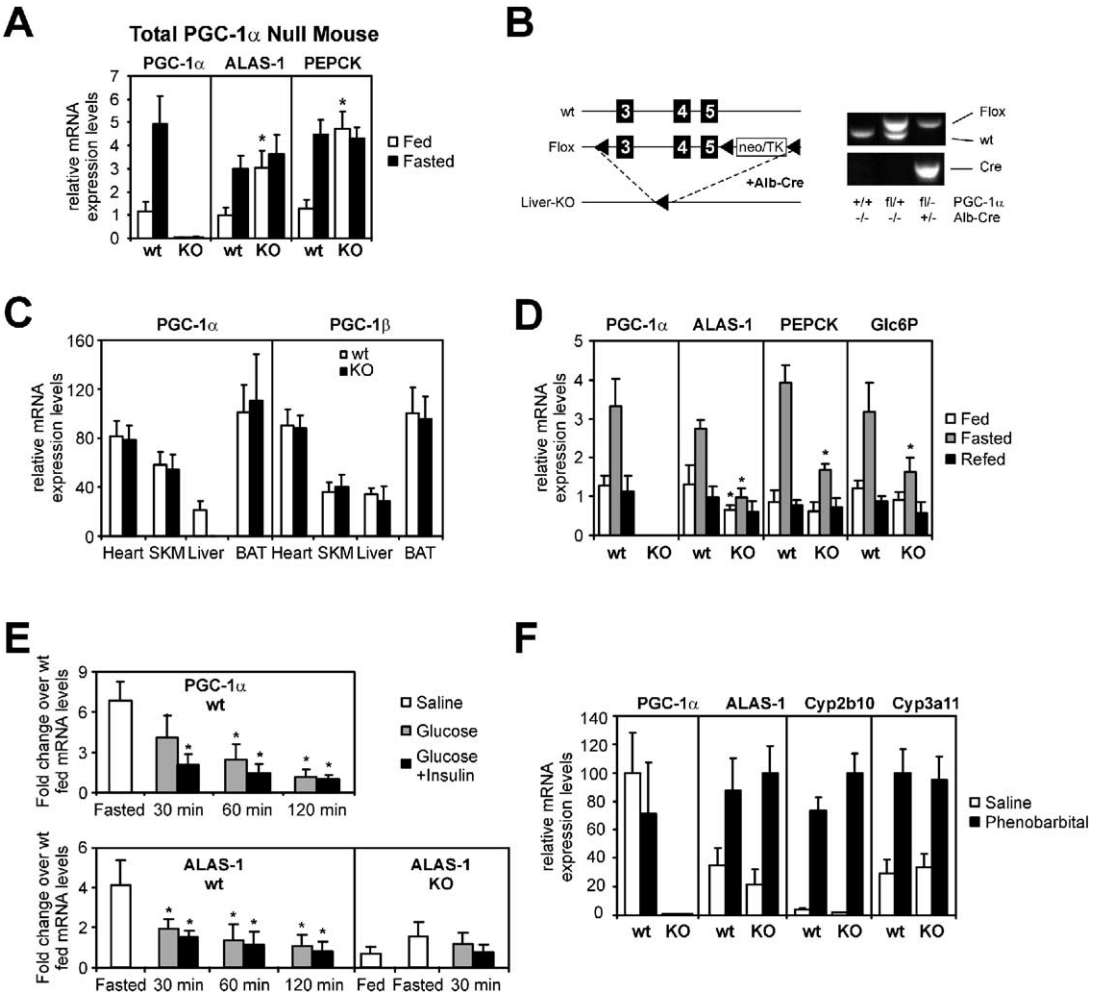

Figure 5. Fasting-Mediated Induction of *ALAS-1* Is Drastically Reduced in a Liver-Specific *PGC-1α* Knockout Model

(A) Hepatic ALAS-1 is constitutively increased in fasting and feeding in *PGC-1α* total knockout mice. *PGC-1α* total knockout animals were fasted for 12 hr before relative mRNA levels for PGC-1α, ALAS-1, and phosphoenolpyruvate carboxykinase (PEPCK) were determined.

(B) Generation of liver-specific *PGC-1α* knockout animals. Mice with a floxed *PGC-1α* allele were crossed with animals that transgenically express cre recombinase under the control of the *albumin* promoter.

(C) Hepatic expression of *PGC-1α* is absent in the liver-specific knockout animals. Different tissues were harvested from wild-type and knockout mice, and relative PGC-1α and PGC-1β levels were determined by semiquantitative PCR. SKM, skeletal muscle; BAT, brown adipose tissue.

(D) Absence of PGC-1α abolishes *ALAS-1* induction in the liver by fasting. Wild-type and liver-specific *PGC-1α* knockout mice were fasted for 12 hr, and relative transcript levels for PGC-1α, ALAS-1, PEPCK, and glucose-6-phosphatase (Glc6P) were determined by semiquantitative PCR.

(E) Glucose reverses fasting-mediated induction of *PGC-1α* and *ALAS-1*. Wild-type and liver-specific *PGC-1α* knockout animals were fasted for 6 hr and subsequently injected with vehicle, glucose, or glucose and insulin. After 30, 60, or 120 min, respectively, mice were sacrificed, livers were harvested, and relative PGC-1α and ALAS-1 expression levels were determined.

(F) Phenobarbital induces *ALAS-1* independent of PGC-1α. Wild-type and liver-specific *PGC-1α* knockout animals were injected i.p. with vehicle (saline) or phenobarbital (100 mg/kg). Sixteen hours after injection, livers were harvested, and relative mRNA levels of PGC-1α, ALAS-1, and cytochrome P450 2b10 and 3a11 (Cyp2b10 and Cyp3a11, respectively) were determined by semiquantitative PCR. Data in (A) and (C)–(F) are represented as mean ± standard deviation. *$p < 0.05$ between wild-type and knockout animals in Student's t test.

ficial effect of glucose in acute porphyric attacks is mediated by the glucose-triggered increase of plasma insulin. ALAS-1 mRNA is not regulated by fasting in the knockout mice, and, thus, no effect of glucose and/or insulin on ALAS-1 transcript levels could be observed.

Taken together, these data strongly suggest that PGC-1α may be involved in fasting-induced acute porphyric attacks. Apart from fasting, certain drugs strongly regulate *ALAS-1* levels and, therefore, are able to precipitate porphyric attacks (Elder et al., 1997; Thadani et al., 2000). There are two different classes of chemicals that perturb heme homeostasis: first, drugs that increase heme biosynthesis by inducing *ALAS-1*, and second, compounds that block different steps of heme biosynthesis and thus generate various heme intermediates. Representative for the first class of drugs,

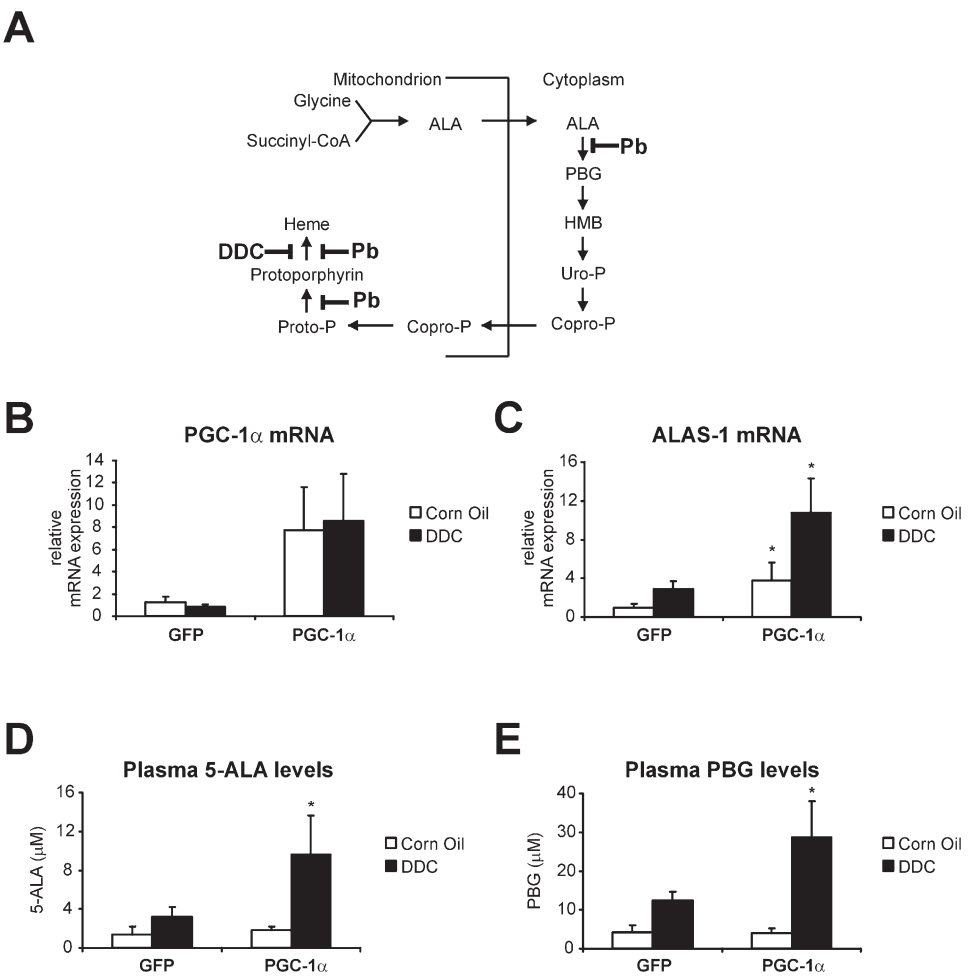

Figure 6. Ectopic Expression of PGC-1α Elicits an Acute Porphyric Attack

(A) Lead (Pb) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) inhibit heme biosynthesis at different steps.

(B and C) Ectopic PGC-1α expression increases ALAS-1 levels and heme intermediates. Adenoviral GFP or PGC-1α was injected into the tail veins of wild-type animals, which were fasted 4 days later and treated with corn oil and DDC (10 mg/kg) for 24 hr. Transcript levels of PGC-1α (B) and ALAS-1 (C) were determined after the mice were sacrificed 24 hr later.

(D and E) Plasma of tail-vein-injected animals was collected, and 5-aminolevulinic acid (ALA) levels (D) and porphobilinogen (PBG) levels (E) were determined. Data in (B)–(E) are represented as mean ± standard deviation. *$p < 0.05$ between GFP- and PGC-1α-infected animals in Student's t test.

we chose the barbiturate phenobarbital (PB), a classical drug that precipitates porphyric attacks in patients. As shown in Figure 5F, no significant difference in PB induction of *ALAS-1* and the prototypical PB-target genes cytochromes P450 *Cyp2b10* and *Cyp3a11*, two microsomal cytochromes P450 with a heme moiety as prosthetic group, was observed between wild-type and liver-specific *PGC-1α* knockout animals. These data indicate that the role of PGC-1α in *ALAS-1* regulation does not extend universally to all other mechanisms, such as the induction by barbiturate drugs (Fraser et al., 2002, 2003; Podvinec et al., 2004).

Elevated Expression of PGC-1α Causes Acute Attacks in Chemical Porphyria

The consequence of *ALAS-1* regulation by PGC-1α in fasting and feeding for acute porphyric attacks was subsequently tested using proporphyrogenic drugs that are known to function by disruption of the pathway of hepatic heme biosynthesis. Two members of this class of chemicals are lead (Pb) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Figure 6A). Lead intoxication produces symptoms resembling those of acute hepatic porphyria (May et al., 1995). Because of its ability to replace other ions such as zinc and to block thiol groups, lead inhibits several enzymes in heme biosynthesis, most importantly ALAD. Another drug widely used to induce porphyria in systems that lack the genetic predisposition for this disorder, DDC, causes accumulation of N-methyl protoporphyrin, a potent inhibitor of ferrochelatase (De Matteis et al., 1973). In gain-of-function experiments, mice were injected i.v. with adenoviral GFP and PGC-1α. These animals were subsequently fasted and treated with vehicle (corn oil) or DDC for 24 hr. DDC did not change adenoviral ex-

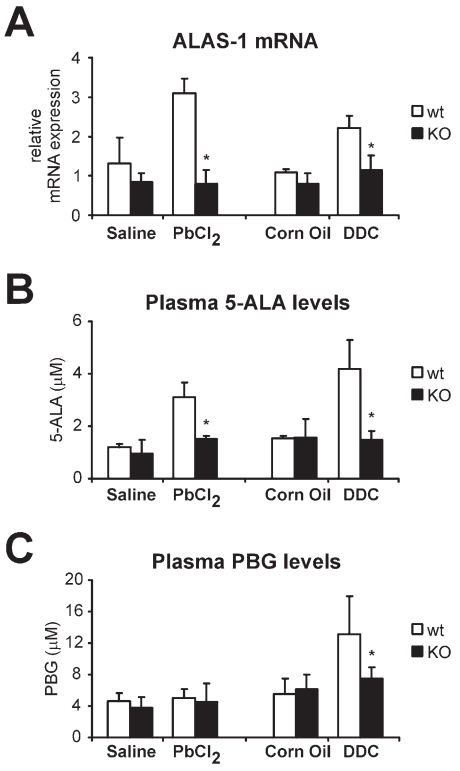

Figure 7. Liver-Specific *PGC-1α* Knockout Animals Are Protected from Chemical Porphyria

Lack of increase in ALAS-1 mRNA and heme biosynthesis intermediates in liver-specific *PGC-1α* knockout animals. Fasted wild-type and knockout animals were i.p. injected with saline and lead chloride ($PbCl_2$, 20 mg/kg) or corn oil and DDC (10 mg/kg) for 24 hr. After animals were sacrificed, ALAS-1 mRNA levels (A), ALA plasma levels (B), and PBG plasma levels (C) were determined. Data are represented as mean ± standard deviation. *$p < 0.05$ between wild-type and knockout animals in Student's t test.

pression of PGC-1α mRNA (Figure 6B). In contrast, ALAS-1 transcript levels were elevated 10-fold in animals that received both PGC-1α adenovirus and DDC (Figure 6C). This high induction of *ALAS-1* was reflected in the dramatically increased levels of the heme precursors 5-ALA and PBG in their plasma (Figures 6D and 6E), to 9 μM and 27 μM, respectively. Importantly, these are levels that are seen in acute attacks in mouse models of porphyria. Thus, in wild-type animals with chemical porphyria, elevation of PGC-1α expression in the liver results in accumulation of heme precursors comparable to that classically observed in drug-precipitated acute attacks in genetic mouse models of porphyria (Lindberg et al., 1996, 1999).

Liver-Specific *PGC-1α* Knockout Animals Are Protected from Chemical Porphyria

The requirement for PGC-1α in fasting-induced porphyria was tested in the liver-specific *PGC-1α* knockout animals. In fasted wild-type animals treated with lead chloride and DDC for 24 hr, increased ALAS-1 mRNA levels were observed as compared to treatment with their vehicles saline and corn oil, respectively (Figure 7A). In contrast, neither lead chloride nor DDC changed endogenous PGC-1α levels (data not shown). As a consequence of the *ALAS-1* induction and the chemical block in the biosynthetic pathway, 5-ALA accumulates in plasma after lead and DDC treatment (Figure 7B). Since lead and DDC inhibit heme biosynthesis at different steps (Figure 6A), only DDC elevates PBG levels (Figure 7C). Thus, as reported, blocking of heme biosynthesis with porphyrogenic drugs results in a state of latent porphyria in wild-type mice, comparable to the status of patients between attacks, which is characterized by moderately elevated levels of 5-ALA and PBG (De Matteis, 1973). In an actual acute attack, 5-ALA and PBG levels rise to those observed in the gain-of-function experiment shown in Figure 6D and 6E. Strikingly, lead and DDC completely fail to induce either ALAS-1 mRNA (Figure 7A) or plasma 5-ALA (Figure 7B) or PBG levels (Figure 7C) in the liver-specific *PGC-1α* knockout mouse. These data indicate that PGC-1α is absolutely required for animals treated with porphyrogenic drugs to enter a state of latent porphyria.

Discussion

While the biochemical consequences of mutations in the heme biosynthetic pathway are well known, the molecular mechanisms underlying the nutritional regulation of hepatic porphyrias have been poorly understood. Specifically, questions regarding how fasting can precipitate porphyric attacks and why glucose infusions provide therapeutic benefit have remained unanswered. The results presented here provide a clear-cut mechanism, deduced from biochemical and genetic evidence: the transcriptional coactivator PGC-1α is induced in the liver in fasting and potently turns on expression of the *ALAS-1* gene in hepatocytes and in liver in vivo. The induction of PGC-1α in fasting has previously been shown to be a consequence of glucagon action and the transcription factor cAMP element binding protein (CREB), which binds directly to the *PGC-1α* promoter (Herzig et al., 2001). In addition, CREB can also directly activate the *ALAS-1* promoter (Varone et al., 1999).

PGC-1α activates the *ALAS-1* promoter by coactivating NRF-1 and FOXO1, both of which directly bind to the *ALAS-1* promoter (Figure 6). The ability of PGC-1α to positively regulate the *ALAS-1* gene and the requirement for PGC-1α in the fasting induction of *ALAS-1* together provide a direct explanation for how fasting can provoke an acute attack in an individual with a mutation in the pathway of heme biosynthesis that results in hepatic porphyria. Indeed, adenoviral expression of PGC-1α in mice with chemical inhibition of enzymes of heme biosynthesis results in significantly elevated porphyrin precursor levels reminiscent of acute porphyric attacks. In contrast, the excess production of heme intermediates by porphyrogenic drugs is lost in the liver-specific *PGC-1α* knockout.

The therapeutic effect of glucose on acute hepatic porphyria is well documented (Robert et al., 1994). Moreover, it has been established that *ALAS-1* transcription is inhibited by the insulin pathway involving Akt (Kappas et al., 1995; Scassa et al., 2001). Our data

for the first time illustrate likely mechanisms by which glucose and the subsequent elevation of insulin, which occurs in vivo in response to glucose, can ameliorate an acute porphyric attack. First, increased levels of insulin will certainly blunt the expression of PGC-1α. Glucagon is important in *PGC-1α* expression, and rising blood glucose dampens glucagon secretion (Herzig et al., 2001; Yoon et al., 2001). Second, insulin has been shown to activate the protein kinase Akt in the liver, and Akt in turn phosphorylates FOXO1 (Brunet et al., 1999; Nakae et al., 2001). Phosphorylation of FOXO1 results in disruption of its binding to PGC-1α and its export from the nucleus (Puigserver et al., 2003), thus inhibiting PGC-1α action. Increased blood glucose levels would therefore be expected to alter the PGC-1α modulation of *ALAS-1* gene expression by two related but independent mechanisms. This hypothesis is supported by the reduced ALAS-1 mRNA levels in fed mice that have constitutively elevated PGC-1α levels after tail-vein injection of adenoviral vectors (see Figure S1 in the Supplemental Data available with this article online). Thus, despite high PGC-1α levels, ALAS-1 mRNA transcription can be reduced by insulin-triggered nuclear exclusion of FOXO1. Moreover, like insulin, glucose has been shown to have an inhibitory effect on *ALAS-1* transcription in cell culture (Canepa et al., 1984; Giger and Meyer, 1981). Under our experimental conditions, glucose alone did not significantly repress basal or PGC-1α-induced ALAS-1 mRNA levels (Figure S2). However, insulin and glucose together were more efficient than insulin in reducing *ALAS-1* expression. Thus, in addition to its effect on insulin secretion, glucose could directly affect *ALAS-1* transcription. Candidate pathways include the AMP-activated protein kinase (AMPK), protein phosphatase 2A (PP2A), and carbohydrate-response element binding protein (ChREBP) (Kawaguchi et al., 2002; Yamashita et al., 2001), although an effect of this signaling cascade on *ALAS-1* remains to be shown.

Agents that elevate hepatic PGC-1α levels are therefore potentially dangerous for patients with hepatic porphyrias. Accordingly, drugs and foods that induce PGC-1α in the liver should be avoided. Unfortunately, because of the therapeutic high carbohydrate intake, patients with hepatic porphyrias are prone to weight gain. Losing excess weight is very difficult for some of these patients because of fasting-induced acute attacks. Hopefully, our findings described here might lead to the development of more specific treatments for these patients.

Experimental Procedures

RNA Isolation and Analysis

Total RNA was isolated from liver or cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. For semiquantitative real-time PCR analysis, 1 μg of total RNA was treated with RNase-free DNase and subsequently reverse transcribed with random hexamer primers (Roche Applied Science). Relative mRNA abundance normalized to 18S rRNA levels was determined with the ΔΔCt method after amplification using a iCycler iQ real-time PCR detection system (Bio-Rad) and SYBRGreen (Bio-Rad). Data are represented as mean ± standard deviation. Significance is defined as $p < 0.05$ in Student's t test.

Animal Experiments

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Animals were fed standard rodent chow and housed in a controlled environment with 12 hr light and dark cycles. For fasting experiments, mice were deprived of food for the indicated amount of time before animals were sacrificed. Drugs (phenobarbital, 100 mg/kg; $PbCl_2$, 20 mg/kg; DDC, 10 mg/kg) were injected i.p., and livers and blood were harvested after 16 or 24 hr. All groups consisted of at least three to six mice. Glucose (1 g/kg) and insulin (1.0 U/kg) were injected i.p. into mice that were fasted for 6 hr. Data are represented as mean ± standard deviation. Significance is defined as $p < 0.05$ in Student's t test.

Generation of Liver-Specific *PGC-1α* Knockout Animals

Generation of animals with floxed *PGC-1α* alleles has been described (Lin et al., 2004). These mice were crossed with mice that transgenically express cre recombinase under the control of the rat *albumin* promoter (Jackson Laboratory, strain B6.Cg-Tg(Alb-cre) 21MGn/J) to obtain liver-specific *PGC-1α* knockout mice.

Adenoviral Infection

Cultured cells were infected with adenovirus as published (Yoon et al., 2001). Twenty-four to forty-eight hours after infection, cells were harvested. Male Wistar rats were transduced with purified adenovirus via tail-vein injection as described (Yoon et al., 2001). Mice were tail-vein injected with 0.2 OD of cesium-chloride-gradient-purified adenovirus. Five days later, mice were sacrificed and livers and plasma were harvested. Data are represented as mean ± standard deviation. Significance is defined as $p < 0.05$ in Student's t test.

Cell Culture (Fao, H2.35, Primary Hepatocytes), Transfection, and Reporter-Gene Assays

Fao rat hepatoma cells were cultured in RPMI medium with 10% fetal calf serum. H2.35 mouse SV-40-transformed hepatocyte cells were kept in DMEM supplemented with 4% fetal calf serum and 0.2 μM dexamethasone. Primary mouse hepatocytes were isolated and cultured as described (Lin et al., 2004). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For reporter-gene assays, cells were harvested 48 hr after transfection. Luciferase levels were determined and normalized to β-galactosidase expression as published (Iniguez-Lluhi et al., 1997). For treatment with glucose (33 mM), cells were cultured in glucose-free DMEM (Mediatech). Data are represented as mean ± standard deviation. Significance is defined as $p < 0.05$ in Student's t test.

Cloning of Promoter Constructs and Site-Directed Mutagenesis

The rat *ALAS-1* promoter (Braidotti et al., 1993) was amplified by PCR and cloned into the pGL3 basic luciferase reporter-gene vector (Promega). Site-directed mutagenesis was performed using overlapping primers. All constructs were verified by sequencing.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed as described (Handschin et al., 2003). Briefly, wild-type and mutant *ALAS-1*-promoter fragments were radiolabeled and used as probes together with in vitro-transcribed/translated proteins. Protein-DNA complexes were subsequently separated by polyacrylamide gel electrophoresis. FOXO1 antibody was purchased from Santa Cruz Biotechnology.

Chromatin Immunoprecipitation

Experiments were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate) following the manufacturer's protocol. H2.35 cells were infected with adenoviral GFP and FLAG-tagged PGC-1α for 24 hr and treated with vehicle or 10 nM insulin for 12 hr before cells were harvested, DNA-protein complexes crosslinked, and immunoprecipitation reactions performed using anti-FLAG beads (Sigma). After reverse crosslinking, DNA was purified by phenol/chloroform extraction and ethanol precipitation, and relative levels were subsequently analyzed by PCR.

Determination of ALA and PBG Plasma Levels

When animals were sacrificed, blood was harvested by cardiac puncture. Blood plasma was purified by centrifugation in heparin

tubes (Becton Dickinson) and treated as described (Mendez et al., 1999). ALA and PBG levels in the plasma were subsequently analyzed by sequential ion-exchange chromatography using columns from the ALA/PBG by Column Test Kit (Bio-Rad) using a modified protocol (Davis and Andelman, 1967). PBG and ALA were absorbed on anion- and cation-exchange resins, respectively. Following elution and conversion to pyrrole in the case of ALA, Ehrlich's reagent was added, and PBG and ALA levels were determined colorimetrically. Data are represented as mean ± standard deviation. Significance is defined as $p < 0.05$ in Student's t test.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/4/505/DC1/.

Acknowledgments

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Supplemental Data

Nutritional Regulation of Hepatic Heme

Biosynthesis and Porphyria through PGC-1α

Christoph Handschin, Jiandie Lin, James Rhee, Anne-Kathrin Peyer, Sherry Chin, Pei-Hsuan Wu, Urs A. Meyer, and Bruce M. Spiegelman

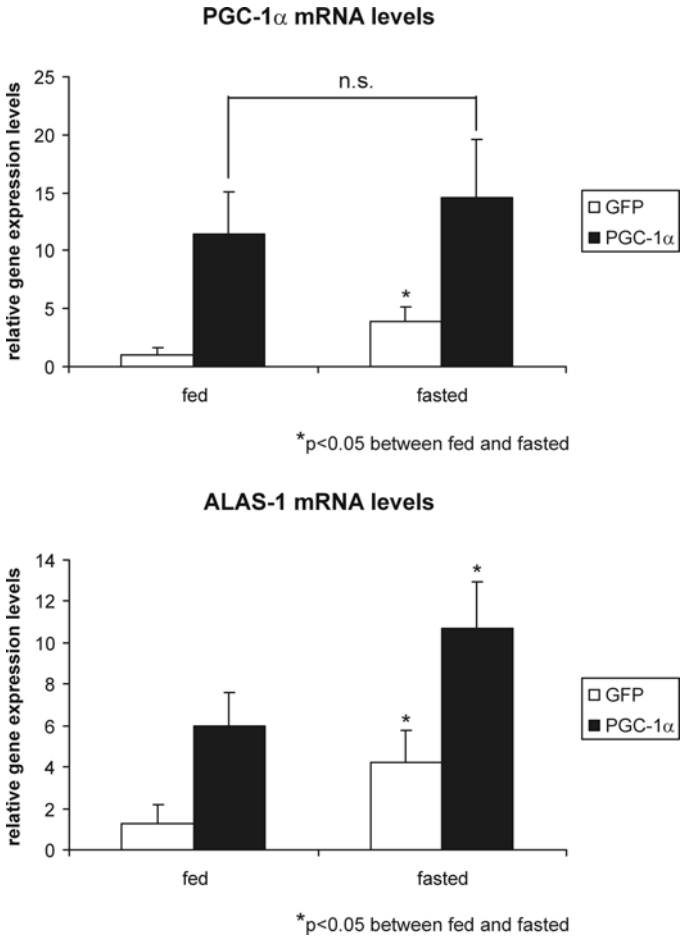

Figure S1. Feeding Reduces ALAS-1 Levels Despite Constitutively High PGC-1α Expression

Adenoviral vectors for GFP and PGC-1α were tail-vein injected into animals. After four days later, mice were fasted overnight and hepatic PGC-1α and ALAS-1 expression compared to fed control animals. Despite constant adenoviral production of PGC-1α, ALAS-1 is significantly lower in the fed vs. the fasted state. Data are represented as mean +/- standard deviation. n.s., non-significant; *p<0.05 between fasted and fed samples in Student's t-test.

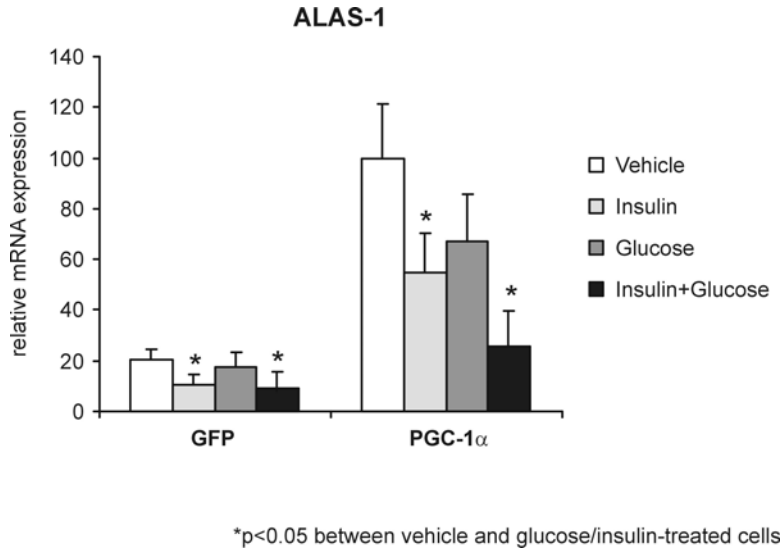

Figure S2. Distinct Effects of Glucose and Insulin on H2.35 Mouse Hepatoma Cells

H2.35 cells were infected with adenoviral GFP and PGC-1α and cultured in glucose-free medium for 24 hours. Cells were subsequently treated with 33 mM glucose and 10 nM insulin for 12 hours before RNA was isolated and relative expression of ALAS-1 determined. Although glucose alone did not significantly inhibit basal or PGC-1α-induced ALAS-1 levels, the combination of insulin and glucose was more potent than insulin by itself at repressing ALAS-1 transcription. Data are represented as mean +/- standard deviation. *p<0.05 between vehicle and glucose/insulin-treated cells in Student's t-test.

5 Identification of Multiple HNF4α Response Elements by Cross Species Sequence Comparison

5.1 Comparative Genomic Analysis of ALAS1

To identify additional putative regulatory regions of human ALAS1 a total of 66.7kb genomic sequence of ALAS1 was analyzed by cross-species sequence comparison. As illustrated in Fig.1 this region spans the entire intergenic region ranging from the 5'-neighboring to the 3'- neighboring gene.

At the time when the analysis was initiated, human and mouse sequences covering the above region were available. Annotation of rat ALAS1 gene was missing in the assembly, however part of the 5'-flanking region could be identified based on mouse – rat synteny and cross species sequence comparison. Sequences used for the analysis are schematically represented in Fig. 1 and were all obtained from the University of California Santa Cruz Genome Browser (UCSC) (http://genome.ucsc.edu).

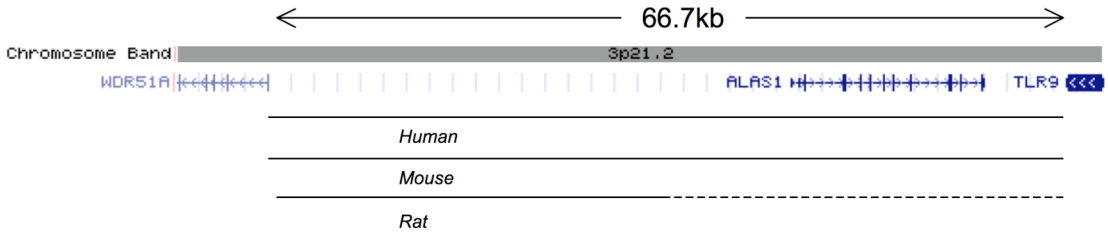

Figure 1 Overview of human ALAS1 genomic locus

The human ALAS1 gene is located on chromosome 3. The gene consists of 2 uncoding and 10 coding exons and spans 16.8kb. ALAS1 is flanked by WDR51A (WD repeat domain 51A) and TLR9 (Toll like receptor 9) at the 5'- and 3'- end respectively. This intergenic region spans a total of 66.7kb which was used for the cross species sequence analysis. Corresponding mouse and rat sequences used for the multispecies alignment are indicated. Note that part of the rat sequence is missing in the assembly (dashed line). (from http://genome.ucsc.edu)

First a human and mouse sequence comparison was performed using global alignment algorithm AVID through the VISTA portal with parameters set to at least 70% sequence identity over 100bp (Frazer et al., 2004a). This analysis resulted in the identification of a total of 30 segments of non-coding sequences, whose location with respect to ALAS1 transcriptional start site are indicated in Fig. 2. Since global alignment algorithms miss rearrangement events likely to have happened during genomic evolution, we analyzed

in parallel our region of interest with the local alignment algorithm now mostly used for aligning long genomic sequences, BLASTZ (Schwartz et al., 2003). This algorithm is the basis for the multispecies sequence alignments, called MULTIZ, which can be accessed through the UCSC genome browser. Therefore 3 species (human, mouse and rat) sequence alignments performed by MULTIZ were retrieved from the UCSC browser. (http://genome.ucsc.edu). Blocks of conserved non-coding sequences reported by MULTIZ were again plotted along the genomic locus of ALAS1 (Fig. 2).

First, the results of the two alignments plotted in Fig.2 illustrates that blocks of conserved non-genic sequences are found along the entire genomic locus of ALAS1, within the 5'-flanking region, in the introns and in the 3'-flanking region. Secondly, all the regions reported by VISTA within the parameters used were present in the MULTIZ alignment, but not vive versa. Of note, the blocks of conserved non-genic regions reported by MULTIZ are longer, which reflects the fact that the BLASTZ algorithm is less stringent in allowing high scoring sequence matches to be extended to both sites. According to the location of conserved blocks of non-genic sequences reported by both algorithms we defined a total of 16 Conserved Non-Genic fragments (named CNGs), which were PCR amplified and cloned into a reporter vector for subsequent testing in cell-based transactivation assays. The location of the constructs with respect to human ALAS1 genomic locus and their naming, which will be used in the work below, is illustrated in Fig. 2.

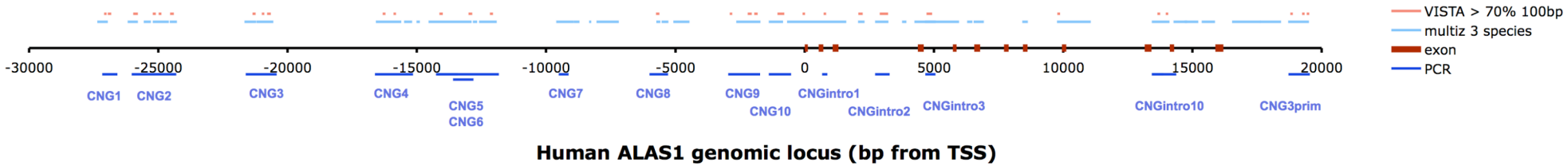

Figure 2 Multispecies sequence comparison of human ALAS1

Overview of conserved non-coding sequences in human ALAS1 gene. Transcriptional start site (TSS) is set to 0. Location of exons are depicted in red. Results of human - mouse comparison performed by VISTA (> 70% sequence identity over at least 100bps) are shown in pink. Conserved non-coding blocks of multispecies alignment (human, mouse, rat) as reported by 'MULTIZ' are shown in light blue. Accordingly, Conserved Non Genic Sequences (CNGs) were PCR amplified for subsequent testing in cell based transactivation assays (dark blue).

5.2 Multiple CNGs respond to PGC1α - HNF4α activation

In order to see if the CNGs contained any regulatory sequences, we first screened the CNG constructs in CV1 transactivation assays for a response to the versatile coactivator PGC-1α that is known to co-activate a large number of transcription factors from inside and outside the nuclear hormone receptor family. Therefore CV1 cells were co-transfected with empty tk luc vector, a 1.3kb long promoter fragment or the various CNGs in the presence or absence of PGC-1α. As illustrated in Fig. 3 a majority of the CNGs, namely CNG1, 3, 4, 6, 7, 9, intron10 and 3 prim showed a significant increase in reporter gene activity in response to PGC-1α as compared to the empty vector.

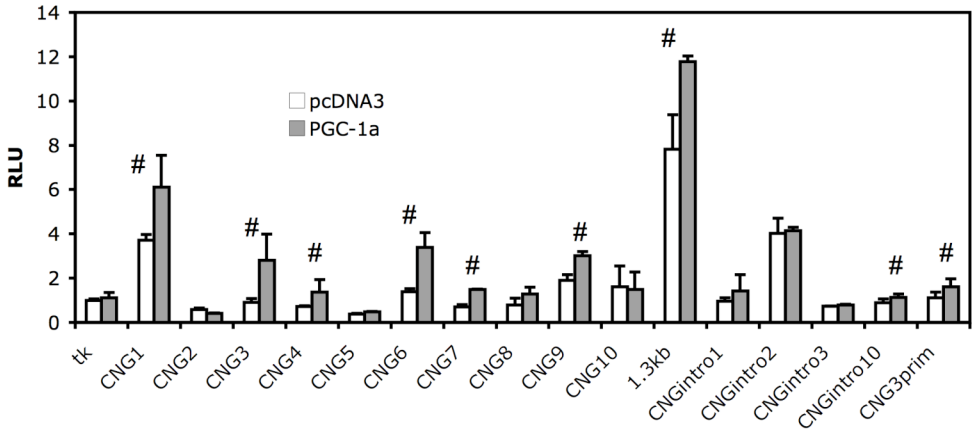

Figure 3 Transactivation Screen of CNGs with PGC1α

CV1 cells were co-transfected with either empty tk-luc vector, a 1.3kb long promoter construct or the various CNG constructs along with PGC1α to screen for potential regulatory sequence. Co-transfection of PGC1α significantly increased the reporter activity of multiple CNGs including the 1.3kb promoter. Data presented result from at least 2 independent transfections performed in duplicates. # indicates p < 0.05 PGC1α vs vector control.

The data suggest that the above mentioned constructs contained response sequences for transactivators, which are present in CV1 cells and which are stimulated by PGC-1α in a ligand independent manner. We therefore conclude that ALAS1 gene contains a multitude of enhancer modules spread over the entire genomic locus.

One of the nuclear hormone receptors potently activated by PGC-1α is HNF4α. This liver enriched transcription factor is an important regulator of hepatic gene expression and has been shown to be essential for the full response of different cytochromes P450s to xenobiotics (Chen et al., 2005, Ferguson et al., 2005, Tirona et al., 2003). We hypothesized that this factor also played a role in ALAS1 regulation and hence was supposed to be responsible for the PGC-1α response seen in various CNGs.

Therefore a subset of the above PGC-1α responsive CNGs were co-transfected into CV1 cells together with HNF4α, PGC-1α or a combination of both. CNG number 10,

although no significant increase in reporter activity could be observed in response to PGC-1α, was included in the analysis, since this region contained high scoring DR1 elements (see below). As illustrated in Fig. 4 co-transfection of HNF4α resulted in a significant increase in reporter gene activity in the absence or presence of PGC-1α in CNG1, 3, 6, 10, intron10 and 3prim. Indeed, reporter activity of CNG10 significantly increased in the presence of HNF4α. Of note the clear increase in activity in the 1.3kb promoter construct exerted by PGC-1α was abolished in the presence of HNF4α. As described in chapter 4, the ALAS1 promoter regions contains a NRF1 and FOXO1 site, which are efficiently co-activated by PGC-1α. We therefore conclude that co-transfected HNF4α competes with PGC-1α binding to those sites resulting in a loss of stimulatory effect of PGC-1α on the promoter construct.

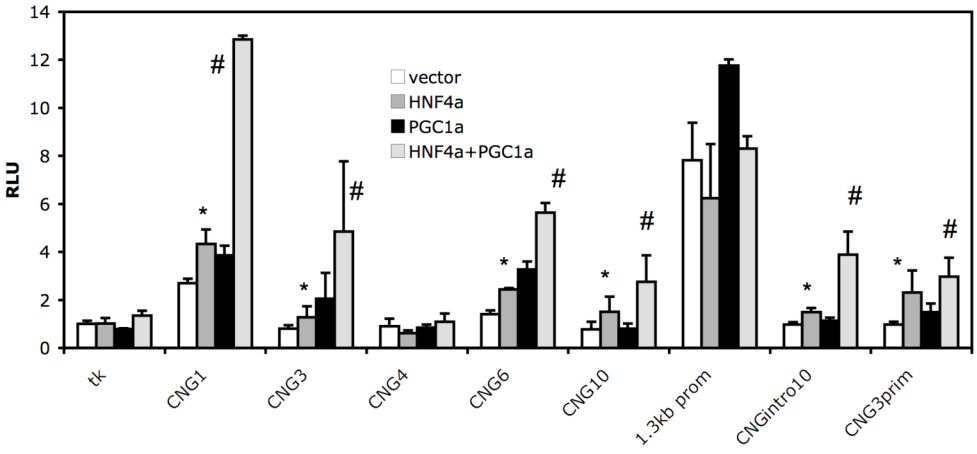

Figure 4 Multiple CNGs respond to HNF4α activation

*CV1 cells were co-transfected with either empty tk-luc vector, a 1.3kb long promoter construct or the indicated CNG constructs along with HNF4α, PGC1α or a combination of both. HNF4α alone or in combination with PGC1α increases reporter activity of CNG1, 3, 6, 10, inro10 and 3prim. Note that PGC-1α co-activation of the promoter is inhibited in the presence of HNF4α. Data presented results from at least 3 independent transfections performed in duplicates. * indicates p < 0.05 HNF4a vs vector control. # indicates p < 0.05 HNF4α + PGC1α vs PGC 1α alone.*

CNG3 and CNG4 located at minus 21kb and minus 16kb respectively harbor the CAR and PXR binding sites previously identified in our laboratory (Podvinec et al., 2004) and CNG6 the newly characterized FXR response element IR1. Functional HNF4α bindings sites in close proximity of CAR and PXR binding sites have been described and shown to be necessary for full response to PXR and CAR specific activation. In our experiments, CNG3 is efficiently activated by HNF4α - PGC1α, suggesting a HN4α binding site in close proximity to the drug response elements. In contrast, reporter activity of CNG4 did not significantly increase in the presence of HNF4α. However since binding of HNF4α to this region could be demonstrated in chromatin immunoprecipitation experiments described below, we suppose that repressive

sequences within the full length 1453bp CNG4 construct mask a response to HNF4α activation.

In summary we conclude that HNF4α responsive sequences are present at various regions within the human ALAS1 genomic locus.

5.2.1 Identification of DR1 elements

In order to identify the exact binding sites within the HNF4α responsive CNGs we either pursued a classical subcloning strategy or searched the regions with two different web-based algorithms, NubiScan (Podvinec et al., 2002) and Cluster Buster (Frith et al., 2003) designed to detect transcription factor binding sites.

5.2.1.1 Characterization of a functional HNF4α RE within CNG1 at minus 27kb

Classical subcloning procedure was applied to identify the exact sequences conferring the response to HNF4α within the CNG showing the highest response in transactivation assays, which was the 595bp long CNG1 located at minus 27kb from the transcriptional start site.

As illustrated in Fig. 5, a 247bp subclone A retained the response to HN4α as well as PGC1α, whereas sublcone B and C did not show any significant increase in reporter gene activity. The NubiScan algorithm, which has been specifically designed to identify nuclear hormone receptor binding sites, identified one DR1 element within the 247bp construct showing high similarity to the HNF4α consensus sequence. To demonstrate the importance of the identified DR1 in the HNF4α response, we performed site directed mutagenesis of one or both halfsites. Mutation of either the first or both halfsites resulted in a significant loss of the response to HNF4α in the presence or absence of PGC1α (right panel). These data clearly demonstrate the functionality of the identified DR1 that is located at minus 27kb from the transcriptional start site.

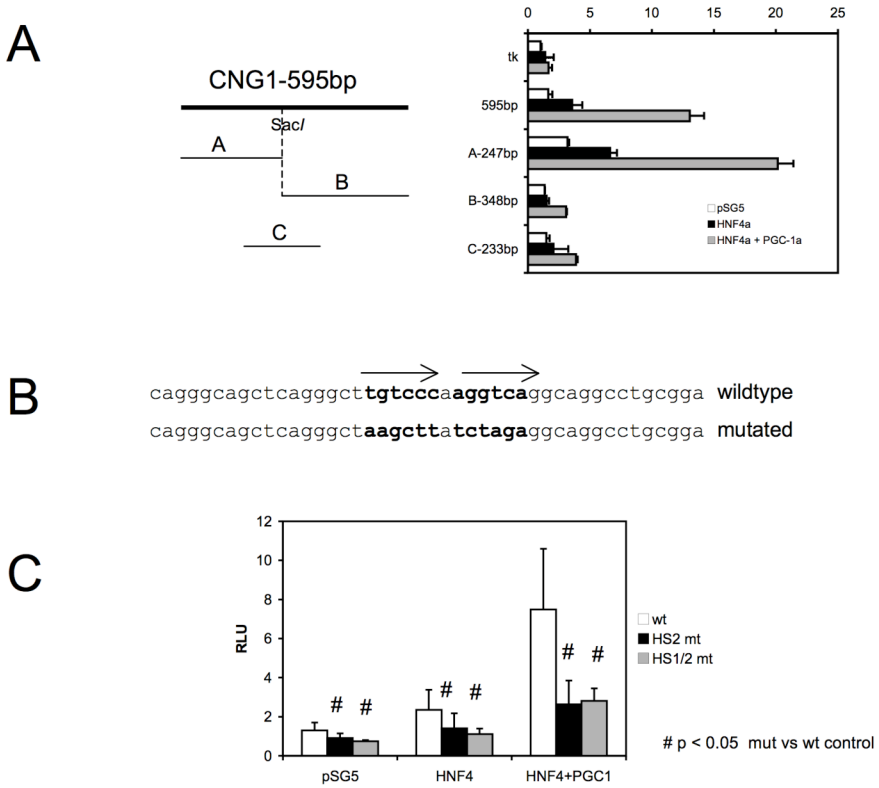

Figure 5 Characterization of a functional DR1 element within CNG1

A) Subcloning of the 595bp CNG1 construct results in a 247bp fragment A retaining the response to HNF4α and PGC1α in CV1 transactivation assays.

B) NubiScan identifies one DR1 element within subclone A.

C) Site directed mutagenesis of one or both halfsites (as indicated in B) results in a significant loss of the response to HNF4α and PGC1α activation.

5.2.1.2 In *silico* search for HNF4α binding sites

We next searched the additional HNF4α responsive CNGs in their entire length for putative HNF4α binding sites using NubiScan algorithm. This algorithm developed by M. Podvinec relies on the combination of nucleotide distribution weight matrices of single hexamer halfsites, which reflects the binding characteristic of nuclear receptors and indeed results in more specific prediction of functional sites than conventional search tools (Podvinec et al., 2002). The highest scoring DR1 elements within each CNG reported by NubiScan are listed in Table 1.

In parallel, we applied an additional web based *in silico* approach, Cluster Buster (Frith et al., 2003), which has been specifically designed to find clusters of pre-specified motifs in long nucleotide sequences. This approach has been already successfully used in identifying HNF4α bindings sites (Wiwi and Waxman, 2005).

We therefore scanned the entire 66.7kb genomic locus of human ALAS1 for clusters of HNF4α consensus sites using Cluster Buster (http://zlab.bu.edu/cluster-buster/) using the HNF4α binding site matrix defined by the TransFac data base. The algorithm reported two regions containing multiple high scoring HNF4α consensus sites located at minus 9kb and minus 1.5kb from the transcriptional start site respectively. Interestingly, both regions lie within CNGs previously identified by multi species sequence comparison: CNG7 and CNG10. Hence these regions contain clusters of putative HNF4α bindings sites and in addition show a high level of sequence identity across species. Together these findings strongly support a regulatory function of these regions.

Table 1: Overview of the DR1 elements within different CNGs

CNG	Sequence of DR1 element	Strand +/-	Identification method	NubiScan Score
CNG1	CAGGGCT**TGTCCCA****AGGTCA**GGCAGGC	+	Subcloning, NubiScan	0.795
CNG3	ATGTGTA**TGCGCT**G**TGCCCC**GTCATGC	-	NubiScan	0.808
CNG4	GAGGGCC**AGGACA**G**GGGACT**CAGGGGC	+	NubiScan	0.837
CNG6	AGACACT**AGGCCCT****AAGAAC**TTCCAAA	-	NubiScan	0.801
CNG7	GACTCAA**TGGCCTT****TGGTCT**CACTTCC	-	Cluster Buster NubiScan	0.861
CNG10-1	TGCACTT**GGGTCCA****AGTCCA**AGTGCTG	+	Cluster Buster NubiScan	0.804
CNG10-2	ATCATTC**TGGACTT****TGTCCC**TCTTGTT	-	Cluster Buster NubiScan	0.860
CNGintro10	CCTCTCC**GGAGCA**C**TGACCT**TAACAGG	-	NubiScan	0.724
CNG3prim	TGAGCAG**AGTCCTT****TGACCT**CGCCTCC	-	NubiScan	0.914

5.3 HNF4α binds to newly identified DR1 elements in EMSAs

Having identified multiple novel DR1 elements in the flanking region of human ALAS1 by a combined *in silico* and *in vitro* approach, we next assessed whether HNF4α protein was able to bind to the DR1 elements performing electromobility shift assays. The following DR1 elements were selected for the binding assay: CNG1 DR1, whose functionality was shown by site directed mutagenesis; the highest scoring DR1 elements identified by both NubiScan and Cluster buster: CNG7 DR1, CNG10-1 and CNG10-2; and the overall highest scoring element identified by NubiScan (0.9) within CNG3prim.

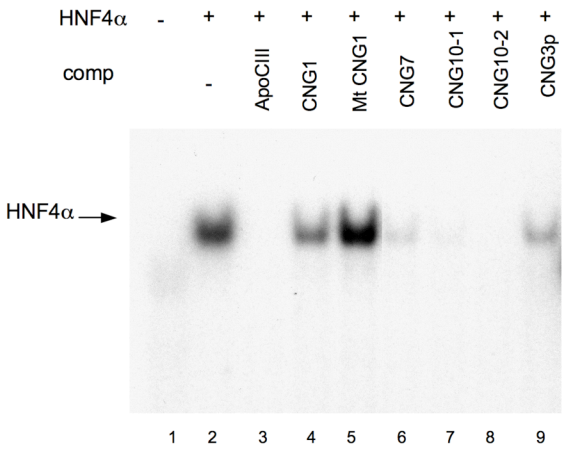

Figure 6 HNF4α binds to the identified DR1 elements

Competition EMSAs were performed using the radiolabeled oligonucleotide dimers of the known HNF4α response element in the ApoCIII promoter together with cold oligo dimers of the identified DR1 elements. A specific protein DNA complex was formed at the ApoCIII element in the absence of any competing oligos (lane 2). Unlabeled ApoCIII element (lane 3) competitively inhibited the formation of a DNA protein complex. CNG1 DR1 moderately inhibited the binding, whereas the mutated fragment had no effect. Efficient competition was observed with the DR1 elements within CNG7 (lane 6), both DR1 elements within CNG10 (lanes 7 and 8), as well as the DR1 element within CNG3prim (lane 9).

For competition EMSAs a known HNF4α response element from the Apolipoprotein CIII promoter (Barbier et al., 2005) was ^{32}P-labeled and incubated with *in vitro* synthesized HNF4α. As illustrated in Fig. 5, a specific protein-DNA complex was formed in the presence of HNF4α (*lane 2*). The formation of this complex could be competitively inhibited by addition of a 100-fold excess of unlabeled oligodimers. We next wanted to test, if unlabeled oligodimers of our newly identified DR1 elements also were able to compete with the binding. In fact, 100-fold excess of wildtype CNG1 DR1 element moderately competed for HNF4α binding, whereas the mutated fragment showed no competition. Efficient competition was seen with both DR1 elements within CNG10 (*lane7,8*), and to a lower degree with the DR1 elements within CNG7 and CNG3prim. Based on these *in vitro* binding assays we conclude that HNF4α is able to bind to our newly identified DR1 elements in human ALAS1 flanking region with low (CNG1), moderate (CNG7 and CNG3prim) or high affinity (CNG10-1 and CNG10-2).

5.4 HNF4α binds to various regions in native chromatin

To assess whether HNF4α was indeed binding to the various responsive sequences within the human ALAS1 gene in a native chromatin context, we performed chromatin immunoprecipitation (ChIP) in cultured G2F cells using an antibody against HNF4α. ChIP experiments demonstrate the presence of proteins within a specified region of a target locus in vivo. Essentially, DNA-protein and protein-protein interactions within the chromatin architecture are cross-linked by formaldehyde. This is followed by lysis of the

cells and isolation of the nuclei. Chromatin is sheared by sonication into a discrete size (200 - 1000 bp), which is then subjected to precipitation using antibodies against the protein of interest. A critical prerequisite thus is the availability of a specific antibody. After extensive washing, the DNA is extracted from the immunoprecipitates and used in PCR for identifying the genomic region of interest. In the experiment shown in Fig. 7 an antibody against HNF4α and a negative control IgG was used for precipitation of chromatin. Precipitated and purified DNA was then amplified by quantitative RT-PCR using primer pairs spanning the DR1 elements listed in Table 2. The location of the amplified regions with respect to human ALAS1 gene is illustrated in Fig. 7. The data are expressed as relative enrichment of our target regions as compared to a negative control region located in GAPDH flanking region. As illustrated in Fig.7 an enrichment of DNA in the HNF4α precipitate could be observed for CNG1, CNG3, CNG4, CNG7, CNG10 located in the 5'-flanking region, for the CNG in intron 10 (CNGintro) as well as for the CNG located in the 3'-flanking region (CNG3prim). To demonstrate the ability of our ChIP experiment to distinguish between specific regions of the ALAS1 locus, we tested a region within coding exon 12 lacking any putative cis-regulatory element for HNF4α as a negative control. Indeed, no enrichment was seen in the HNF4α precipitate when part of coding exon 12 was amplified.

These data clearly demonstrate that HNF4α is bound to at least 7 distinct regions within the genomic locus of human ALAS1 ranging from minus 27kb to plus 19kb from the transcriptional start site.

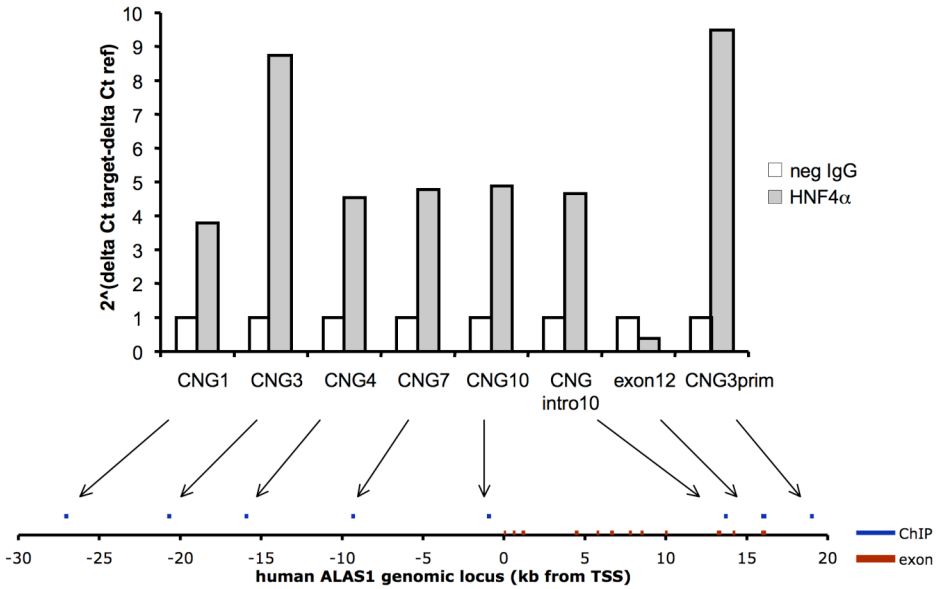

Figure 7 *HNF4α binds within various CNGs in native chromatin*
Chromatin Immunoprecipitation assays were performed in cultured G2F cells using an antibody against HNF4α or a negative control IgG. Precipitated DNA was amplified by RT PCR with primers pairs spanning the regions indicated in blue. An enrichment of DNA in the HNF4α precipitate could be observed for the regions within CNG1, 3, 4, 7, 10, intron10 as well as 3prim, but not with primers spanning a region within coding exon 12.

5.5 HNF4α knock-down affects drug induction

The identification of multiple HNF4a responsive sequences together with the finding that HNF4α is indeed able to bind to various regions in ALAS1 genomic locus in native chromatin, suggest a role of HNF4α in basal expression and or regulation of ALAS1. To address this question we specifically targeted HNF4α by siRNA in cultures of primary human hepatocytes.

5.5.1 Transient HNF4α knock-down in primary human hepatocytes

Since hepatoma cell lines such as HepG2 and Huh7 do not show a significant and reproducible response of ALAS1 to classical inducer drugs such as phenobarbital or rifampicin, we performed a HNF4α knock-down experiment in a system, where drug induction of ALAS1 is preserved; i.e. cultures of primary human hepatocytes. Therefore primary cells were transfected with 10nM and 25nM of a pool of four different HNF4α specific siRNAs using HiPerfect transfection reagent as described under supplemental materials and methods. As seen in Fig.8, by transfecting 10nM and 25nM HNF4α specific siRNA pool, HNF4α mRNA level decreased to 50% and 30% respectively as compared with cells transfected with non-targeting control siRNA. Basal levels of ALAS1 significantly decreased down to 76% as compared to siRNA control when 25nM of HNF4α specific siRNA pool was used. No significant change could be observed at the lower concentration of siRNA pool. Maximal induction of ALAS1 by classical PXR agonist rifampicin was slightly but significantly reduced at both concentrations of HNF4α specific siRNA pool when compared to the non targeting siRNA control. These data indeed point towards a role of HNF4α in basal as well as drug induced expression of ALAS1, which will be discussed in Chapter 6.3.

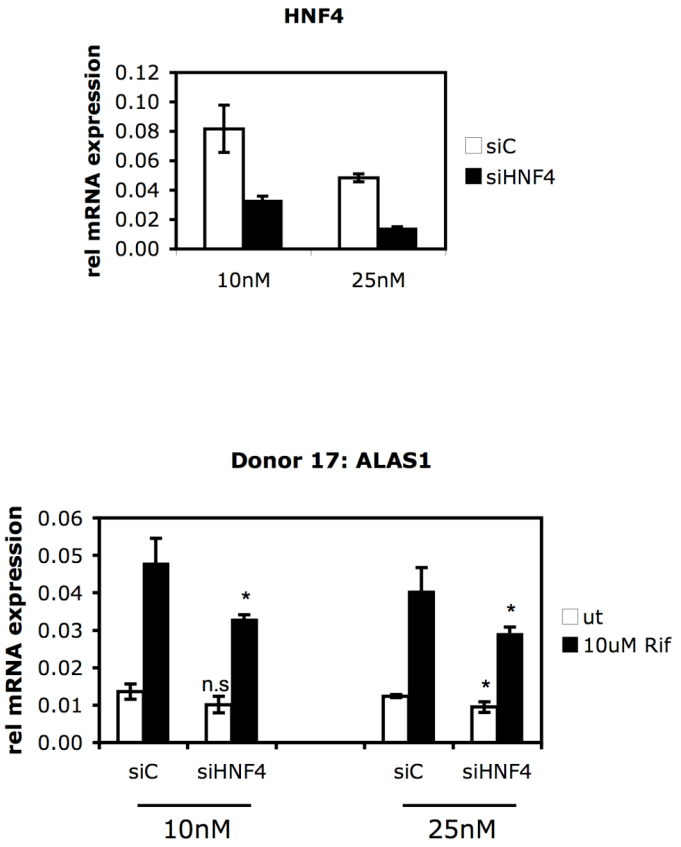

Figure 8 Transient knock-down of HNF4α in cultures of primary human hepatocytes

A) Transfection of cultures of primary human hepatoytes with siRNA targeting HNF4α dose-dependently reduces HNF4α mRNA levels.

*B) Basal expression of ALAS1 was significantly reduced when 25nM of siRNA was used. Maximal induction of ALAS1 my rifampicin is slightly but significantly reduced at both siRNA concentrations (10nM and 25nM) tested. The experiment was performed in triplicates in one single donor. * $p < 0.05$ siHNF4α vs siC control*

5.6 Appendix: Extended multispecies sequence alignment

In the present work, we were able to identify various regulatory regions within human ALAS1 based on a 3 species sequence alignment (human, mouse and rat). Clearly, addition of other species to the alignment will increase the power of the technique, and will allow to further define the exact boundaries of the conserved segments (Thomas et al., 2003). With the availability of the chimpanzee, dog and chicken genome in 2005, we were able to complement the 3 species sequence alignment with these additional vertebrate species. The results will be briefly discussed below.

Comparison of chicken to mammalian species resulted in the alignment of all coding exons, but only in one segment of conserved non-genic sequences. Obviously the evolutionary distance is too far, to detect regulatory regions with the algorithms

available. This finding is in agreement with (Hillier et al., 2004), who report that only 30% of known regulatory regions, which were conserved between human and rodents, were also found in the chicken genome.

The identified DR1 element within CNG10 (called CNG10-2) lies within the above mentioned region, which is evolutionary conserved not only in mammals but also in birds. In fact, this 88bp long stretch of conserved regions is the only human non-coding segment, which could be aligned to corresponding chicken sequence, suggesting high functional constraint of this region (Fig 10). Deletion of this region within a natural promoter construct will be required to decipher the importance of the element in basal or induced promoter activity. Using this stretch of DNA as a bait in one hybrid assays is a way to identify nuclear proteins bound to the region.

CNG10-2-DR1

```
Human      GCAAAGTACCTGGACAC--ACCCTGGTCACCATCATT---CTGGACTTTGTCCCTCTTGTTAATGCTCAGCTGGGAAAGG
Chimpanzee GCAAAGTACCTGGACAC--ACCCTGGTCACCATCATT---CTGGACTTTGTCCCTCTTGTTAATGCTCAGCTGGGAAAGG
Mouse      G-GAAGTACCTGGACAC--GCCTCCATCTTTATCATT---CTGGACTTCGTTCTTCTTCTTGATGCCCAGCTAGGAAAGG
Dog        GGGAAGTACCTGGACAT--ATCTTGGTCACTATCACT---CTGGACTTTAGCCCTTGTACTATTGCCCAACTGGGTACGG
Chicken    GCAGAGGAACTGAGCCCCAGCCCTATGCATCACCACTCTGCTGTGCGTTGTTCCCCTGACCCAAGTGCAGCTGGGTGAGG
           * * ** * ***  *      *     *  *  ** *   **   * *    *          *  ** ** **   **
```

Figure 9: 5 species MULTIZ alignment containing the DR1 element within CNG10.

The identified DR1 response element (halfsites shown in bold) at minus 1.5kb from the transcriptional start site is conserved from mammals to birds. MULTIZ alignment from http://genome.ucsc.edu

We next retrieved multispecies alignment of human, chimpanzee, mouse, rat and dog and compared blocks of conserved segments with the results from our initial 3 species alignment with the 5 species alignment as illustrated in Fig. 10. Comparison shows that addition of the above mentioned species to the alignment did not markedly change our initial results in defining conserved non-genic blocks. Due to the short evolutionary distance within primate species, conservation occurs independent of functional constraint, and therefore sequence comparison does not suit our purposes in defining functional elements. Segments, which initially were reported to align to mouse and rat sequences aligned also to dog sequence, demonstrating the higher degree of relationship of dogs to human.

It has been suggested that comparison of such distantly related species as human and mouse was ideally for identification of regulators sites, since conservation most likely resulted from functional constraint. At the time of writing, sequences of additional species were included in the conservation track from UCSC browser. These species include rhesus macaque, rabbit, cow, elephant and opossum with additional ones to follow in the near future.

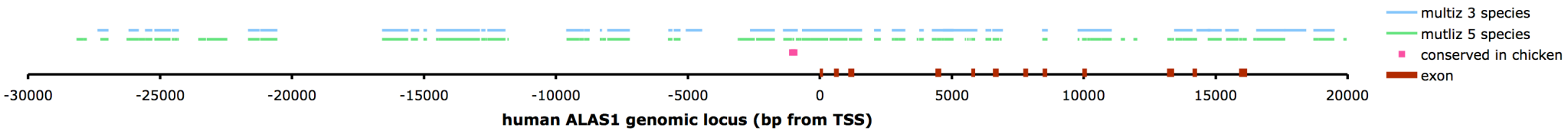

Figure 10 Comparison of 3 and 5 species MULTIZ alignment

Conserved blocks of non-coding sequences as reported by 3 species (human, mouse, rat, 2004) alignment and by 5 species (human, chimpanzee, mouse, rat and dog, 2005) alignment are plotted along human ALAS1 genomic locus as in Fig. 2.. Addition of chimpanzee and dog to the multispecies alignment does not markedly redefine blocks of conserved non-genic sequences. The absence of a conserved block at minus 24kb and at minus 28kb in the 3 species alignment results from missing sequence data in the assembly in 2004. Only one non-coding segments was found to be aligned to chicken (shown in pink). Of note is that this regions contains one of the identified DR1 elements. For explanation, see text.

5.7 Supplemental Material and Methods

5.7.1 In silico analysis of human ALAS1 genomic locus

5.7.1.1 Cross species sequence comparison

All genomic sequences for the alignment were retrieved from the University of Santacruz Genome Browser (http://genome.ucsc.edu). Human mouse pairwise sequence alignment was performed by AVID through the VISTA portal with parameters set to at least 70% sequence identity over at least 100bp (http://genome.lbl.gov/vista/index.shtml). Multispecies sequence alignments (human, mouse, rat and human, chimpanzee, mouse, rat and dog) were retrieved from the UCSC genome browser conservation track as described below.

5.7.1.2 Conservation track at UCSC Genome Browser

University of Santacruz Genome Browser contains the reference sequence and working drafts of a large number of genomes. Genome annotations not only include assembly data, sequence composition, genes and gene predictions, mRNA and expressed sequence tag evidence but also comparative genomics data (for a recent overview (Hinrichs et al., 2006)). The browser provides genome wide multispecies alignments. UCSC generates pairwise sequence alignment using BLASTZ, and subsequently produces best-in genome alignments by chaining and netting as described in (Kent et al., 2003). Multiple pairwise sequence alignments are then combined into multispecies sequence alignments by a program called MULTIZ (Blanchette et al., 2004).

5.7.1.3 *In silico* search for transcription factor binding sites

CNGs showing a HNF4α response in transactivation assays were scanned by NubiScan for putative HNF4α response elements using the provided HNF4α matrix (based on a collection of 18 HNF4α bindings sites from literature) and a score threshold of 0.6 (www.nubiscan.unibas.ch; (Podvinec et al., 2002)).

In parallel 66.7kb genomic human ALAS1 sequence was analyzed using the web-based program Cluster Buster (zlab.bu.edu/cluster-buster) to identify clustered DNA binding motifs in the entire sequence using the HNF4α binding site matrix defined by the TransFac data base and the default cluster finding parameters (cluster score treshold of 5) (Frith et al., 2003).

5.7.2 Plasmid Construction

Fragments of human ALAS1 flanking region were PCR amplified using the previously isolated PAC clone harboring ALAS1 gene as template (Podvinec et al., 2004) and *Pwo* DNA Polymerase (Roche Diagnostics, Rotkreuz, Switzerland). Primers were designed using Primer 3 software (primer3_www.cgi v 02) with the following

parameters: 24nt in length, 50% GC content, Tm 60°C, and restrictions sites added as indicated in Table 2. All fragments were cloned into pGL3 tk luciferase vector (Podvinec et al., 2004). Subfragments of the CNG1 construct were generated using standard cloning procedures. The mutated 247bp construct was generated by PCR using complementary oligonucelotides mutated in the DR1 site (see Table 2) and Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA). The products were digested with Dpn*I* (New England Biolabs) to remove the parental DNA template and selected for constructs containing mutations. Plasmid DNA was prepared using the Qiagen sytem. All constructs were verified by sequencing.

5.7.3 CV1 transactivation assay

Transactivation assays were carried out in CV-1 (monkey kidney) cells, which were maintained in high glucose (4500mg/l) Dulbecco's Minimum Essential Medium (Gibco BRL, Basel, Switzerland) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL), 50U/ml penicillin and 50μg/ml streptomycin (Gibco BRL). Prior to transfection, cells were expanded for 3 days in DMEM-F12 without phenol red (Gibco BRL) and supplemented with 10% charcoal treated FBS. Subsequently, cells were plated onto 96- well dishes at a density of 25 000 cells per well and grown overnight. Cells were transiently transfected in OptiMeml (Invitrogen) using 1μl of LipofectAMINE reagent (Invitrogen) per well. Transfection mixes contained 8 ng receptor expression vector, 20ng coactivator expression vector, 20ng reporter vector and 60ng pRSV-βGal to a total of 108ng DNA per well. After 24h, medium was changed to DMEM-F12 supplemented with 10% charcoal stripped delipidated FBS (Sigma). 24h later, cell extracts were prepared using 200μl of passive lysis buffer (Promega) and 10μl of the supernatants were assayed for luciferase activities using the luciferase assay kit (Promega) and a Wallac 1420 Multilabel Counter. β-galactosidase activities were measured as previously described (Podvinec et al., 2004). Luciferase levels were normalized against β-galactosidase values to compensate for variation in transfection efficiency. Data are presented as mean +/- standard deviation. Statistical significance is defined in two-tailed students t-test as $p < 0.05$ or $p < 0.01$ as indicated in the figures.

5.7.4 G2F Human Hepatoma Cell line

Generation of G2F cell line, a subclone of HepG2, has been described (Rencurel et al., 2005). G2F cells were maintained in Dulbecco's Minimum Essential Medium (Gibco BRL, Basel, Switzerland) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL), 1μM dexamethasone (Sigma, Buchs, Switzerland), 50U/ml penicillin and 50μg/ml streptomycin (Gibco BRL). Cells were passaged every 2 to 3 days.

5.7.5 Chromatin Immunoprecipitation

ChIP analysis was performed using the ChIP-IT kit, according to the manufacturer's instructions (Active Motif, Carlsbad, CA). G2F cells were grown in 150-mm dishes to 70-80% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine

serum. After culturing the cells 24h in serum free condition, medium was changed to fixing medium containing 1% formaldehyde, and the cultures were incubated for 10 min at room temperature. The cells were washed twice with ice-cold PBS and treated with glycine to stop fixation. The cells were scraped in PBS containing protease inhibitors, collected by centrifugation, lysed in SDS-lysis buffer supplemented with protease inhibitors, and sonicated for 8 cycles (10-s pulse and 30-s rest on ice). The sonication conditions were optimized by procedures as described in the manufacturer's instructions and examined by agarose gel electrophoresis to determine generation of DNA fragments between 200 and 1000 base pairs in length. Sheared chromatin was immunocleared with protein G-agarose slurry for 1 h at 4 °C. A portion of the precleared chromatin was stored and labeled as "input DNA." The remaining chromatin was immunoprecipitated with rabbit polyclonal HNF4 antibody (sc-8987) and normal rabbit IgG supplied with the ChIP-IT kit as a negative control. After overnight incubation, the immunoprecipitates were washed sequentially with wash buffers for 2 min each. Protein-DNA complexes were eluted from the antibody with freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3). Formaldehyde cross-links were reversed by addition of 200 mM NaCl and heating at 65 °C for 4 h in the presence of RNase A. DNA was purified using proteinase K treatment and purification columns provided by the kit. RT-PCR was performed using 1:10-diluted input DNA and 3 µl of immunoprecipitated DNA from a 100-µl DNA extraction with the optimized primer pairs as indicated in Table 2.

5.7.6 Transfection of Primary Human Hepatocytes with siRNA

Primary human hepatocytes were plated at a density of 15000 cells/ well into collagen coated 24-well plates in DMEM supplemented with 10%FBS, 1µM dexamethasone and penicillin/streptomycin. Cells were transfected with 10 and 25nM siRNA against HNF4α (SMARTpool Dharmacon) and corresponding amount of siControl (Qiagen, non-silencing control, Alexa 488 Fluor labeled) using HiPerfect (Qiagen) according to the manufacturers fast-forward protocol. Briefly, siRNA was diluted in 100µl DMEM (w/o additives) per well to a final concentration of 10 or 25nM and subsequently 3µl of HiPerfect transfection reagent per well was added to the diluted siRNA. After a 5-10min incubation at R/T, the siRNA/HiPerfect mixture was then added dropwise to the cells. After overnight incubation of the cells in FBS containing DMEM, medium was replaced by serum free Williams E supplemented with 0.5x ITS, hydrocortisone (Sigma) and 50U/ml penicillin and 50µg/ml streptomycin (Gibco BRL). Cells were induced 38h after transfection and plating and harvested 10h later. RNA was isolated and relative expression levels of ALAS1 were determined as described in chapter 3.

Table 2: Primers used for cloning, mutagenesis, EMSAs and ChIP RT-PCR

Name	**Sequence (from 5' to 3')**	**Purpose**
CNG 1 -fw	ggGGTACCAGCCCCATCAATGCTATCAG	Cloning
CNG 1 -rv	ccgCTCGAGTAAAGCAGGGAAAGGGAAGG	Cloning
CNG2 -fw	atgGCTAGCCTATTGCACTAGGCCCACCT	Cloning
CNG2 -rv	ccgCTCGAGGGTTTGGCTACTTTGGGACA	Cloning
CNG3 –fw	cgGGTACCCAGCTGAGGATCCCTGTTGT	Cloning
CNG3 –rv	ccgCTCGAGCCTACCCCCACCACTCTTTT	Cloning
CNG4 –fw	cgGGTACCACCACAGGCCTAGAACATGG	Cloning
CNG4 –rv	ccgCTCGAGCTGCTGTTGGACCACACTTG	Cloning
CNG5 –fw	atgGCTAGCCAAGCTCTGGAAGGACTTGG	Cloning
CNG5 –rv	ccgCTCGAGTGGTGTAGCTGGTGGGAAG	Cloning
CNG6 –fw	cgGGTACCGGTGGAGAATCTGAGGTCCA	Cloning
CNG6 –rv	ccgCTCGAGCTCTTCCTTGACCACCACCT	Cloning
CNG7 –fw	cgGGTACCGAAAAGGCTTCCCCAAGATA	Cloning
CNG7 –rv	ccgCTCGAGGGAGCTGGAGTCAGGATGTA	Cloning
CNG8 –fw	cgGGTACCGATTGAGACCCTCCTGGCTA	Cloning
CNG8 –rv	ccgCTCGAGGGGTGTCAGTGCCCTTCTAT	Cloning
CNG9 –fw	atgGCTAGCAAAAGTCCCCAGCGTCATC	Cloning
CNG9 –rv	ccgCTCGAGGCCAAAGCAGAAAGAACTGG	Cloning
CNG10-fw	agtGGTACCTGTTTCCCAATCTTTCTCTA	Cloning
CNG10-rv	atgGCTAGCCTCAGCTGTGGCACTTTCTG	Cloning
CNGintro1 –fw	cgGGTACCAGAGTCTTCCCTGCCTGGAT	Cloning
CNGintro1 –rv	ccgCTCGAGCGGGTCACCAACTTCTTCAT	Cloning
CNGintro2 –fw	cgGGTACCGGGTGTGGGCTTGTAAGAAA	Cloning
CNGintro2 –rv	ccgCTCGAGACAAAGAGGCTTGCTGTTGC	Cloning
CNGintro3 –fw	cgGGTACCGGCCCTCAGATTTGTGTATG	Cloning
CNGintro3 –rv	ccgCTCGAGGGGGAGCCTTGAGTTCTCTG	Cloning
CNGintro10 –fw	cgGGTACCAATGGCCTGTCTCTGATTGG	Cloning
CNGintro10 –rv	ccgCTCGAGGCCTCTGAAGGGCTTCAATTA	Cloning

Name	Sequence (from 5' to 3')	Purpose
CNG3prim –fw	cgGGTACCCCACCCACAATGACTTTTCC	Cloning
CNG3prim –rv	ccgCTCGAGCTTTGGCCTCCCAGAGTGTT	Cloning
C1 mt1	AGCTCAGGGCTTGTCCCATCTAGAGGCAGGCCTGCGGATGAG	Mutagenesis
C1 mt2	GCAGGGCAGCTCAGGGCTAAGCTTATCTAGAGGCAGGCCTGC	Mutagenesis
ApoCIII-DR1	CAGCAGGTGACCTTTGCCCAGCGCCC	EMSA
C1-DR1-wt	CAGGGCTTGTCCCAAGGTCAGGCAGGC	EMSA
C1 DR1-mt	CAGGGCTTGTCCCATCTAGAGGCAGGC	EMSA
C6-IR1	TGTGACAAGGTCACAGCCCCCGAGGCA	EMSA
C6-IR1-mt	TGTGACATCTAGACAGCCCCCGAGGCA	EMSA
C7-DR1	GACTCAATGGCCTTTGGTCTCACTTCC	EMSA
C10-1-DR1	TGCACTTGGGTCCAAGTCCAAGTGCTG	EMSA
C10-2-DR1	ATCATTCTGGACTTTGTCCCTCTTGTT	EMSA
CNG3p-DR1	TGAGCAGAGTCCTTTGACCTCGCCTCC	EMSA
ChIP-CNG1-fw	GCTATCAGTTTCCCCTGCCTAC	ChIP
ChIP-CNG1-rv	GACACTAATGAAGAGCTCATGGAA	ChIP
ChIP-CNG3-fw	ACCGTGTGTCCATGTTTATGTGT	ChIP
ChIP-CNG3-rv	ACACTTTGACTCTTGGCCTCTACT	ChIP
ChIP-CNG4-fw	ACAGGCCTAAGCTAAACCTTTCC	ChIP
ChIP-CNG4-rv	GAGCACTCTGTGTTCTTGATGC	ChIP
ChIP-CNG7-fw	ATGACAATTCTAAGGCAGGTGAAT	ChIP
ChIP-CNG7-rv	ATCCACCTCCTTGTCAAGACC	ChIP
ChIP-CNG10-fw	AAATGCAAAGTACCTGGACACAC	ChIP
ChIP-CNG10-rv	AACACATAAAGTGTAGGGCTTGG	ChIP
ChIP-intro10-fw	CTGACTTCACCAAGAGAAGAAAGC	ChIP
ChIP-intro10-rv	CAACTGTGACATCAACAGTGCTAC	ChIP
ChIP-3prim-fw	AGACTGAGACAGCTGAAGCAAAAC	ChIP
ChIP-3prim-rv	CCTGCCTAGGTGTATGGTTTTATC	ChIP
ChIP-ex12-fw	GAGAATCTGCTAGTCACATGGAAG	ChIP
ChIP-ex12-rv	GTAGCCAATAGCAGAAAGAACCAC	ChIP

6 Overall Discussion

6.1 Bile acids positively regulate human ALAS1

In the present work (Chapter 3) we identified and characterized human ALAS1 as a novel direct target of bile acid activated nuclear receptor FXR. Both natural and synthetic agonist of FXR increase ALAS1 mRNA as well as activity in cultures of primary human hepatocytes. The most abundant primary bile acid in human bile, chenodeoxycholic acid (CDCA), was able to increase ALAS1 mRNA at near physiological concentration in human liver slices, a system where the architecture of human liver still is intact. In agreement with this we identified and characterized a FXR response element in the 5'-flanking region of human ALAS1, which triggers the response to CDCA in reporter gene assays and is able to bind FXR/RXR heterodimer in gel shift assays.

The activation of ALAS1 by bile acids can be fitted in the physiological context of bile acid metabolism. Bile acids are the major product of cholesterol metabolism. In addition to their physiological role in dietary fat absorption new biological functions of bile acids as signaling molecules have been discovered, in particular in lipid and glucose homeostasis (Claudel et al., 2005). Since accumulation of bile acids causes liver cell damage and eventually cirrhosis, their level needs to be tightly controlled. One major governor of this process is FXR, representing the physiological bile acid sensor (Wang et al., 1999). FXR not only represses *de novo* bile acid synthesis but also switches on a detoxification machinery consisting of metabolizing enzymes and biliary efflux transport proteins. The enzymes induced by FXR include UDP-glucuronosyltransferases (Barbier et al., 2003), sulfotransferases (Song et al., 2001), or the transport proteins, such as MRP2 (Kast et al., 2002) as well as cytochrome P450 CYP3A4 (Gnerre et al., 2004). Since a functional heme protein such as CYP3A4 requires heme as a prosthetic group, a coordinated supply with heme is required. Our results presented in Chapter 3 provide a model how apocytochrome and heme synthesis is activated via the coordinate action of FXR on CYP3A4 and ALAS1, the rate limiting enzyme of heme synthesis.

In addition to FXR, the xenosensing nuclear receptors CAR and PXR, have been shown to protect the liver from bile acid induced toxicity (Stedman et al., 2005). The three nuclear receptors all share a common set of target genes involved in detoxification processes (see above). PXR is activated by high concentrations of bile acids and by toxic secondary bile acids, whereas CAR does not seem to be directly activated bile acids (Staudinger et al., 2001). Studies in mice lacking the two xensoensing receptors however have demonstrated that both receptors are required for an efficient removal of bile acids from the body (Stedman et al., 2005). Again, in response to toxic levels of bile acids ALAS1 is directly targeted by CAR and PXR to ensure adequate supply of heme for bile acid hydroxylating CYP3A4 (Podvinec et al., 2004, Fraser et al., 2003). Together, these findings demonstrate that ALAS1 is a crucial

accessory enzyme for detoxification of endogenous as well as exogenous compounds. Direct targeting of ALAS1 by CAR, PXR as well as FXR is in agreement with the model of a multifactorial and redundant detoxification system the liver has evolved to adapt to potentially toxic compounds (Eloranta and Kullak-Ublick, 2005).

Interestingly, our findings that bile acids increase ALAS1 in human liver goes in line with a well known, although only incompletely understood clinical observation: Increased urinary porphyrin excretion in humans is commonly seen in hepatobiliary diseases accompanied by cholestasis (Rocchi et al., 2005, Doss M, 1972) (Gibson et al., 2000). Of course, impaired bile flux in cholestatic conditions affects biliary excretion of lipohilic porpyhrins, such as coproporhyins. An increased active synthesis of porphyrins in response to cholestatic conditions has already been suggested by several authors (Rocchi et al., 2005, Doss M, 1972). The molecular mechanism has not been studied so far. The present work now identifies CDCA as a potent inducer of ALAS1 in cultures of primary human hepatocytes as well as in human liver slices. Since the main bile acid retained in cholestasis in human is CDCA, direct activation of ALAS1 and subsequently heme synthesis by increased CDCA levels may contribute to the above mentioned clinical phenomenon. A detailed analysis of porphyrins and their precursors in the urine of cholestatic patients could test this hypothesis.

Our functional data clearly demonstrate that hepatic ALAS1 is differently regulated in humans and in mice. Treatment of mice with synthetic FXR agonist GW4064 as well as feeding a diet rich in cholic acid results in a repression of hepatic ALAS1 mRNA. The mechanism underlying this species specific effect remains purely speculative so far. Interestingly, the repression of murine ALAS1 was absent in FXR -/- mice (data not shown). We therefore hypothesize, that a FXR SHP dependent pathway might be responsible for the repression of ALAS1 by bile acids in mice. SHP has been shown to repress a variety of transcription factors from inside (Bae et al., 2004) and outside (Yamagata et al., 2004) the family of nuclear hormone receptors. Putative targets of SHP repression on murine ALAS1 include the CAR response element located at minus 16kb (Fraser et al., 2003) or the newly characterized FOXO1 site in the promoter (Chapter 4). Further studies, such as overexpression of SHP in mouse hepatocytes, should provide insight into this pathway. Of note, in agreement with our functional data, the IR1 element in human ALAS1 flanking region is not conserved in rodents, but rather seems to be primate invention. Major differences between human and rodents in cholesterol and bile acids homeostasis are a well-established feature; i.e mice cope with excess cholesterol in the diet by stimulation of its conversion into bile acids via a liver X receptor (LXR) dependent mechanism, which is not present in humans (Goodwin et al., 2003b).

Human specific targets of FXR have been previously described and include hepatic lipase (Sirvent et al., 2004), α-crystallin (Lee et al., 2005) and peroxisome proliferative receptor α (Pineda Torra et al., 2003). There is a rapidly growing number of pathways regulated by bile acids, dependent and independent from FXR (for a recent review:

(Claudel et al., 2005) (Houten et al., 2006), and the list is far from being complete. Future progress in this field should shed light into the species-specific response reported herein.

Bile acids, apart from activating FXR and all its target genes in liver, serve also as signaling molecules with endocrine functions (Houten et al., 2006). Circulating bile acids, acting via a FXR independent pathway, were recently shown to robustly increase PGC-1α expression and mitchondrial activity and oxidative phosphorylation in peripheral tissue (Watanabe et al., 2006). ALAS1 is a well established marker of mitochondrial activity in skeletal muscle, and coregulated with genes involved in oxidative phosphorylation via the action of PGC-1α ((Patti et al., 2003) and Chapter 4). These findings suggest that ALAS1 may well be target of bile acid signaling in extrahepatic tissues. Moreover we hypothesize that such an FXR independent activation of ALAS1 in extrahepatic tissue is also present in rodents.

With the identification of ALAS1 as a direct target of bile acid signaling in human liver, we add another link between bile acid signaling and mitochondrial function.

Our findings may have clinical implications in cases of hypersensitivity of ALAS1 to external stimuli, such as it is the case in acute hepatic porphyrias. Due to a relative heme deficiency, the strong negative feedback exerted by heme controlling ALAS1 is reduced, and porphyrin synthesis is excessively stimulated without control whenever ALAS1 is activated. Known triggering factors include drugs, alcohol, fasting, sex steroids and all forms of stress, however most of the triggering events remain obscure (von und zu Fraunberg et al., 2005). It has long been assumed, that ALAS1 was indirectly upregulated via a diminishing regulatory heme pool upon heme consuming stimuli. Previous work in our laboratory however revealed that drugs directly upregulate ALAS1 at the transcriptional level via the xenosensing nuclear hormone receptors CAR and PXR (Podvinec et al., 2004, Fraser et al., 2003). We now propose a similar inducing effect on ALAS1 mediated via bile acid activated FXR. Hence our data imply that elevated levels of bile acids, such as in cholestatic conditions, may have the potential to trigger acute attacks or worsen the course of the disease in porphyric patients. Due to the limited number of patients, we are not aware of such a phenomenon neither in practice nor in literature. However, disturbed bile acid homeostasis may well account for the high number of attacks, whose triggering events are unexplained. Therefore analysis of bile acids in the serum of patients suffering from an acute attack is required, in order to test this hypothesis.

6.2 Nutritional Regulation of ALAS1

The clinical characteristics of acute hepatic porpyhrias since long have suggested that hepatic heme synthesis is controlled by the nutritional status. Caloric restriction, such as fasting, is one of the well established factors, which may trigger acute neuropsychiatric attacks in susceptible patients. On the other hand, high carbohydrate

load administrated as glucose infusion at the onset of an attack was beneficial to some individuals (Thunell et al., 2000). However the molecular basis of these counteracting effects has remained obscure. We now demonstrate that both responses are under the control of PPARγ coactivator alpha, PGC-1α.

PGC-1α regulates mitochondrial biogenesis and oxidative metabolism. Its expression in liver is low in the fed state, but it strongly increases upon fasting and subsequently turns on the entire fasting response of the liver, including glucoenogenesis, ketogenesis and fatty acid oxidation (Lin et al., 2005a). Our data demonstrate that hepatic heme synthesis is also targeted by PGC-1α via direct transcriptional upregulation of ALAS1. Fasting experiments in mice showed that ALAS1 transcripts are increased in a similar manner than to gluconeogenic enzymes, phosphoenolpyruvate carboxykinase PEPCK and glucose 6-phosphatase, which are under control of PGC-1α. Overexpression of PGC-1α in mouse hepatocytes and in liver *in vivo* resulted in a robust induction of ALAS1 expression levels, suggesting a direct effect of this coactivator on ALAS1. In agreement with this, we found that PGC-1α activates ALAS1 via coactivation of nuclear respiratory factor 1 (NRF1) and forkhead transcription factor FOXO1, both of which directly bind to ALAS1 promoter. The formation of a PGC-1α complex at both distinct sites in the promoter could also be verified in a native chromatin context. Importantly, the fasting response of ALAS1 was blunted in liver specific PGC-1α knock-out animals. Taken together these data allow to set-up a likely mechanism how fasting can trigger an acute attack in susceptible patients. Indeed, in an animal model of acute hepatic porphyria, induced by the administration of inhibitors of heme synthesis, fasting and concomitant overexpression of PGC-1α leads to the accumulation of porphyrin precursors in the blood of the mice, reflecting the biochemical characteristics of an acute attack in humans. Demonstrating the specificity of the PGC-1α effect, the excess accumulation of porphyrin precursors was absent in fasted liver specific PGC-1α knock-out animals. In conclusion, these findings point towards a master regulatory role of PGC-1α in the fasting response of ALAS1.

How can then the beneficial effect of high carbohydrate load on porphyric symptoms be explained? As a matter of fact insulin levels *in vivo* rise upon nutrition. A repressive effect of insulin on ALAS1 expression has been already described in hepatoma cell lines (Scassa et al., 2001). Our data now provide a mechanistic explanation for this phenomenon. We show that the strong stimulatory effect of PGC-1α on FOXO1 activity at the promoter is sensitive to insulin. It is well known that FOXO1 is a direct target of the insulin-signaling pathway in liver (Nakae et al., 2001). Insulin activates Akt, a key protein kinase downstream of the insulin receptor, which in turn phosphorylates FOXO1. This then leads to nuclear exclusion of this transcription factor and subsequent loss of activation of its target genes. As demonstrated in chromatin immunoprecipitation experiments, recruitment of PGC-1α to the FOXO1 site in ALAS1 promoter is abolished in the presence of insulin. In contrast to the wildtype protein, a mutated form, which cannot be phosphorylated by Akt anymore, stimulates ALAS1 promoter activity in either the presence or absence of insulin. These finding clearly suggest that the repressive

effect of insulin on ALAS1 promoter activity requires a functional FOXO1 protein. So far, multiple ways are known, how insulin dampens ALAS1 promoter. The insulin counteracting hormone glucagon has been shown to stimulate ALAS1 via an axis involving protein kinase A (PKA) and activation of cAMP response element binding protein CREB in the promoter of ALAS1 (Giono et al., 2001). The same pathway also controls PGC-1α expression (Herzig et al., 2001). Hence, insulin, by counteracting this stimulatory effect of glucagon, represses ALAS1 as well as PGC-1α expression. And, as described above, insulin disturbs the co-activation of FOXO1 by PGC-1α. In addition, it is likely that HNF3beta, whose binding sites are flanking the newly identified FOXO1 site in ALAS1 promoter as described by Scassa et al. (Scassa et al., 2004), contributes to the nutritional regulation. HNF3beta (alternatively called FOXA2) like FOXO1 is induced in the fasted state and its activity is repressed by insulin signaling through phosphorylation and nuclear exclusion (Wolfrum et al., 2004). Altogether, this results in a potent repression of ALAS1 in response to high carbohydrate load.

Like insulin, glucose has been shown to exert a inhibitory effect on ALAS1 (Giger and Meyer, 1981). However, the exact mechanisms of this glucose effect are not fully resolved. It may well involve additional pathways, as will be briefly discussed below.

Changes in cellular energy status activate a number of signaling cascades. Among those is the AMP activated kinase AMPK, which serves as the major energy sensor of the cell (Kahn et al., 2005). Activated by any kind of cellular stress, such as hypoxia, osmotic stress or energy depletion, AMPK switches off all ATP consuming processes, while it switches on ATP producing pathways including oxidative phosphorylation. In muscle, it has been previously shown that AMPK targets ALAS1 via its NRF1 site in the promoter (Bergeron et al., 2001). Therefore we hypothesized that activation of AMPK during caloric restriction in liver would contribute to the fasting response mediated by PGC-1α. However, the increase in ALAS1 transcripts upon fasting was boosted in the liver of mice lacking the two catalytic isoforms of AMPK (liver specific α1/α2 knock-out mice; personnel communication, Benoit Viollet, Institut Cochin, Paris). This rather suggests an inhibitory effect of AMPK on hepatic ALAS1. Interestingly, it has been shown that AMPK activation during fasting dampens gluconeogenesis, in order to prevent excess ATP consumption. The detailed mechanism has been resolved only recently and involves phosphorylation and nuclear exclusion of coactivator TORC2 (transducer of regulated CREB activity 2) and consequently reduced expression of CREB target genes (Koo et al., 2005). Since CREB is also involved in the regulation of promoter activity of ALAS1, the above described repressive effect of AMPK could also apply for ALAS1 and might explain the in vivo findings. In summary the effect of AMPK on ALAS1 remains to be unraveled.

Our results further suggest that the master regulatory role of PGC-1α is specific to the nutritional regulation of ALAS1 and does not apply to all inducing effects, such as drug induction. Although PGC-1α potently and dose dependently co-activated CAR and PXR at the murine ALAS1 drug response element in transactivation assays (data not

shown), drug induction in liver specific PGC-1α knock out animals was not altered. This finding suggests that additional co-activating factors are involved in the response to drugs *in vivo*. Together our data demonstrate that different inducing effects of ALAS1, such as fasting and drug induction, follow independent and separate pathways.

6.3 Role of HNF4α in the regulation of ALAS1

In chapter 5 we describe the identification of multiple HNF4a responsive sequences in ALAS1 gene. The presence of HNF4α in least seven distinct locations could be confirmed in a native chromatin context by chromatin immunoprecipitation assays. In the following we will discuss the putative role HNF4α in basal expression of ALAS1 as well as in response to drugs.

Such a high number of regions within human ALAS1 genomic locus binding HNF4α suggest a role of HNF4α in basal expression and or hepatic regulation of ALAS1. Indeed HNF4α is recognized as the major regulator of the hepatic phenotype (Watt et al., 2003). The importance of HNF4α in hepatic gene expression is highlighted by a recent analysis combining chromatin immunoprecipitation with promoter microarrays (Odom et al., 2004). The authors demonstrated that hundreds of RNA Polymerase II bound promoters are also bound by HNF4α in human primary hepatocytes. The promoter array targeted regions spanning from minus 700 base pairs upstream to 500bp downstream of the transcriptional start site of 13000 human genes. However, our analysis of ALAS1 reveals that the HNF4α response element with the closest proximity to the transcriptional start site confirmed by chromatin immunoprecipitation is located at minus 1.5kb (CNG10-2), explaining, why ALAS1 gene is not included in their huge data set of putative HNF4α target genes.

In cultures of primary human hepatocytes we demonstrate that ALAS1 expression is reduced, when HNF4α expression was downregulated to 30% by siRNA specifically targeting HNF4α. Moreover, lowering of HNF4α levels significantly diminished maximal induction of ALAS1 by the classical PXR activator rifampicin. These data indeed suggest a role of HNF4α in basal expression as well as in drug induction of ALAS1. The central role of HNF4α in drug mediated induction of cytochromes P450 is a well recognized feature. Conditional HNF4α knock-out in fetal hepatocytes results in reduced expression of PXR and its target gene CYP3a11 (Kamiya et al., 2003). In liver specific HNF4 knock-out mice, hepatic CAR expression is drastically reduced (Hayhurst et al., 2001). And, as it has been reported only recently, mice (α7 only mice) lacking HNF4α1, the predominant HNF4α isoform in liver, show reduced CAR expression as well as complete loss of 2b10 induction by typical CAR inducer TCPOBOP (Briancon and Weiss, 2006). Moreover HNF4α response elements have been characterized in various human cytochromes P450 genes and been shown to be essential for maximal CAR and PXR mediated drug induction (Chen et al., 2005, Ferguson et al., 2005, Tirona et al., 2003). We therefore suggest that ALAS1 is controlled by HNF4α in a comparable manner to cytochromes P450s. How the different

HNF4α response elements identified so far in this study cooperate for full CAR/PXR type activation will be addressed in ongoing studies. Therefore chimeric constructs, consisting of the distal enhancer elements fused to natural ALAS1 promoter containing the proximal HNF4α response element at minus 1.5kb will be generated. Site directed mutagenesis of the proximal or distal HNF4α response element will then give insight into their specific contribution to the full drug response. And a more efficient transient knock-down of HNF4α is demanded such as by adenovrial siRNA transfer in primary cultures, to further decipher the contribution of HNF4α in various induction processes of ALAS1.

In chapter 4, we highlight the regulatory role of PGC-1α in the fasting response of ALAS1. The importance of HNF4α in the induction of gluconeogenic enzymes such as PEPCK during fasting is also well established. Rhee et al. demonstrated that the fasting response on gluconeogenic enzymes was lost in HNF4α liver specific knock-out animals (Rhee et al., 2003). Since ALAS1 has been shown to be co-regulated with gluconeogenic enzymes through PGC-1α, we investigated if the fasting response of ALAS1 also was affected in the absence of HNF4α. A first analysis of liver samples from fasted liver specific HNF4α knock-out animals (Hayhurst et al., 2001) and their controls, which were kindly provided by Insook Kim and Frank Gonzalez, did not point towards a dominant role of HNF4α in the fasting response (data not shown). Although not significantly, it seemed that ALAS1, unlike PEPCK, was still induced upon fasting in HNF4α knock-out animals. As reported by Rhee et al. (Rhee et al., 2003), liver specific HNF4α knock-out animals show a striking increase in PGC-1α expression. Apparently PGC-1α compensates for the lack of HNF-4α, a phenomenon, which remains unexplained so far. Unlike gluconeogenic enzymes, ALAS1 is additionally controlled by NRF1, involved in mitochondrial biogenesis and also potently co-activated by PGC-1α. We propose that downstream of PGC-1α multiple and redundant pathways converge on ALAS1, including FOXO1, NRF1 and most probably HNF-4α. The excess of PGC-1α, which is observed in these animals, masks a potential contribution of HNF4α in the fasting response, further highlighting the dominant role of PGC-1α.

Although ALAS1 is ubiquitously expressed, its regulation in liver differs from other cells in the body. Liver, and to a minor degree kidney and intestine, are the only organs where ALAS1 transcription is highly inducible by hormonal and exogenous substances (Thunell, 2000). In fact, these are the organs, where HNF4α is expressed. It has been postulated that HNF4α functions as major regulator of chromatin remodeling in early development (Li et al., 2000). The remodeling of the chromatin, which is largely transcriptional inactive, is a prerequisite for the binding of transcription factors to their target sequences in order to induce gene expression. Hyperacetylation of histones 3 and 4, is one of the processes, which correlates with transcriptional activity of the specific region (Eberharter and Becker, 2002). In order to study the hepatocyte specific chromatin organization Rollini et al. transferred the entire human chromosome 14 from fibroblasts to hepatoma cells. Importantly, the chromatin reorganization into a hepatocyte specific pattern including the activation of hepatic genes failed to occur in

cells deficient of HNF1α and HNF4α, but could be rescued by transfection of one or the other factor (Rollini and Fournier, 1999). Furthermore, HNF4α, by recruiting powerful histone acetylases, was absolutely required to induce hyperacetylation and consequently the activity of the hepatocyte specific serpin locus (Baxter et al., 2005). Together these finding point towards a superior function of HNF4α in chromatin organization.

Based on these finding, we hypothesize, that binding of HNF4α to various enhancer modules within the ALAS1 genomic locus, promotes hyperacetylation of those regions and therefore is a prerequisite for other transcriptional regulators to access their target sequences. Hence, HNF4α tissue expression together with its coordinative role in chromatin remodeling processes would determine the inducibility of ALAS1. However this hypothesis so far remains purely speculative. One first approach to address this question, would be to compare the acetylation pattern of ALAS1 genomic locus in hepatic (HNF4α competent) and non hepatic tissue (HNF4α deficient).

In conclusion, our data provide first evidence that HNF4α plays a role in basal as well as drug induced expression of ALAS1.

6.4 The mystery of conserved non-genic sequences of ALAS1

In the present study we applied the *in silico* method of phylogenetic footprinting to locate novel regulatory regions of ALAS1. This approach uses cross-species correlation and is based on the assumption that sequence conservation also in non-coding regions results from functional constraint. Conserved non-genic sequences thus most likely reflect regulatory sites (Dermitzakis et al., 2005). The method became possible only with the availability of whole genome sequences. Phylogenetic footprinting can be regarded as a filtering method, retaining conserved stretches of sequence of a few 100bp within a large genomic region. Using such a filter, the high number of false positive bindings sites reported by conventional binding site search algorithms can be reduced (Lenhard et al., 2003). However, there are some critical points to mention.

The evolutionary distance of the species one chooses for comparison determines the stringency. Obviously, comparing such closely related species like human and chimpanzee does not only retain functional regions. On the other extreme of the evolutionary tree, only a few experimentally verified regulatory regions are also conserved in chicken. Nevertheless, alignment of human with chicken or even further distantly related *fugu rubripes* sequences has been helpful in identifying crucial regulatory sites fundamental for development (Boffelli et al., 2004). It hence seems eminent that we found one single 88bp region in the ALAS1 gene conserved between

human and chicken (See chapter 5) and could suggest a functional role by binding of HNF4α.

The ideal candidate for comparison with the human genome has been regarded to be mouse, evolutionary separated by 75 million years (Nardone et al., 2004). Conservation of sequences between the two species may result from functional constraint as illustrated by Loots et al (Loots et al., 2000) .

It was then a big surprise in the field of comparative genomics when Rubin et al reported, that transgenic mice, with megabase deletions of gene deserts harboring hundreds of human-mouse conserved non-genic regions, did not show any overt phenotype (Nobrega et al., 2004). Although this indicates that not all conserved gene deserts in mammalian genome are functional, it is not clear if it is possible to detect all kind of phenotypic effects. Still, these results indicate that we should be careful about our interpretation of selective constrain solely based on human-mouse conservation.

Addition of further species to the alignment will certainly improve the predictive accuracy of phylogenetic footprinting (Thomas et al., 2003). And further improvement of this method can be achieved by integrating additional filtering criteria. It is now well established that several transcription factors act in concert in so-called enhancer modules, which consist of clusters of transcription factor binding sites. Therefore on can rather look for modules (clusters) than for single sites (Frith et al., 2003).

At the time the present study was initiated, human, mouse and part of rat sequence covering ALAS1 gene were available. With the search criterion of more than 70% sequence identity over at least 100bp we identified a total of 30 CNGs within ALAS1. Accordingly a total of 16 regions, partly consisting of multiple CNGs were PCR amplified and tested in cell based reporter gene assays. Based on a positive response to the versatile coactivator PGC-1α, eight CNGs were found to contain putative regulatory sequences. Within the framework of the study, the specific regulatory factor together with its binding site could be identified as FXR within CNG6, and HNF4α within CNG1, CNG10 and CNG3prim.

There is still a number of putative regulatory regions within the ALAS1 gene, whose function waits to be explored. Summing up the *in silico* and experimental evidence, our analysis suggests that ALAS1 is highly regulated at the transcriptional level.

7 Closing remarks and outlook

For many years it has been assumed that acute porphyric attacks are provoked by a depletion of regulatory heme pool upon heme consuming stimuli and subsequent stimulation of heme synthesis. Previous work in our laboratory however pointed towards a direct transcriptional upregulation of ALAS1 by inducer drugs, which led us to the hypothesis that other triggering factors, such as fasting or the response to endogenous nuclear receptor ligands, influence ALAS1 at the transcriptional level.

During these studies additional triggering events were identified to directly act on ALAS1 transcription. The versatile coactivator PGC-1α was found to be the master regulator of the nutritional response. PGC-1α acts via two NRF1 sites and a insulin sensitive FOXO1 site within ALAS1 promoter. Fasting response of ALAS1 was blunted in liver specific PGC-1α knock-out animals. Together, our data provide a likely mechanism how fasting is able to provoke an acute porphyric attack, and how a high carbohydrate load may alleviate the symptoms.

Cross species sequence analysis led to the identification of numerous conserved non-genic sequences within ALAS1 gene suggesting the presence of multiple regulatory regions. Subsequent screening of the CNGs to nuclear hormone receptor response in transactivation assays resulted in the identification of two additional nuclear receptors regulating ALAS1, FXR and HNF4α. Our data show that ALAS1 is targeted by bile acid activated nuclear receptor FXR in human, but not in mice. These findings suggest that elevated bile acids, such as in cholestasis, have the potential to trigger acute attacks in porphyric patients. Measuring serum bile acids in individuals suffering acute attacks will answer the questions, if elevated bile acids are causative agents in the still high number of attacks, whose triggering events remain obscure.

Our studies provide first evidence that the master regulator of hepatic phenotype, HNF4α, is involved in basal and drug induced expression of ALAS1. The presence of multiple distinct regions within ALAS1 gene responding to HNF4α activation and binding HNF4α *in vivo*, led us hypothesize that HNF4α may play a superior role in chromatin organization of ALAS1 locus. In this model, HNF4α would provide an open chromatin structure for other *cis*-acting factors to access their binding sites as it has been postulated for the liver specific serpin locus (Baxter et al., 2005) (see Fig. 2). Tissue distribution of HNF4α would determine inducibility of ALAS1, and therefore explain the liver specific regulation of ALAS1.

Different triggering events follow independent and separate pathways. PGC-1α is clearly required to drive the fasting response of ALAS1, but it does not seem to be necessary for drug induction *in vivo*. How nutrition and drugs differently affect ALAS1 transcription is highlighted in the two models below.

Studying the metabolic response of the liver to fasting and caloric restriction is a rapidly growing field, and the list of molecular mediators of the response is constantly

increasing. Indeed the complex promoter region of ALAS1 provides the basis of multiple mechanism to take place. The model below (Fig. 1) summarizes the known (solid line) and some hypothetical (dashed line) pathways, which converge on ALAS1 promoter in response to nutritional regulation.

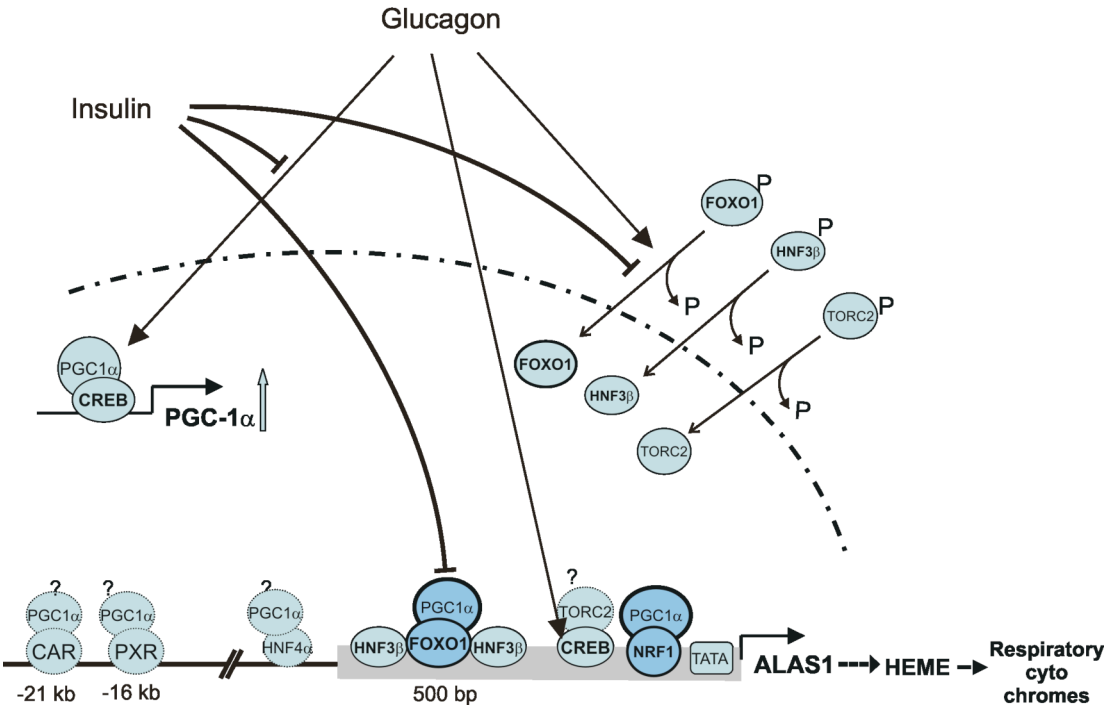

Figure 1: Nutritional regulation of ALAS1 within its promoter

NRF1 is critical for basal promoter activity and potently co-activated by PGC-1α (Braidotti et al., 1993). In the fasted liver glucagon-triggered increase in cAMP levels directly stimulate ALAS1 transcription via the cAMP-responsive element binding protein (CREB), which in parallel binds to the PGC-1α promoter (Yoon et al., 2001, Giono et al., 2001). The two members of the family of forkhead transcription factors, FOXO1 and HNF3beta, are sequestered in the cytoplasm under feeding conditions, however dephosphorylated in the fasted state and transported to the nucleus, where they also bind to ALAS1 promoter (Scassa et al., 2004, Handschin et al., 2005). FOXO1 activity in ALAS1 promoter is specifically enhanced by PGC-1α. In addition transducer of regulated CREB activity 2 (TORC2) is also known to undergo nuclear translocation in response to fasting where it enhances CREB dependent gene transcription (Conkright et al., 2003). We assume that it also acts on ALAS1 promoter. Expression levels of nuclear receptors, CAR, PXR and HNF4α are all known to be increased in the fasted state (Maglich et al., 2004). They are likely to contribute to the overall fasting response. Upon feeding, increased insulin levels result in phosphorylation and nuclear exclusion of FOXO1 and disruption of its interaction with PGC-1α (Puigserver et al., 2003). Insulin also counteracts HNF3beta and TORC2 accumulation in the nucleus. Altogether, activation of these pathways upon feeding results in a robust inhibition of ALAS1 transcription.

Whereas the nutritional response of ALAS1 clearly is regulated via critical sites within ALAS1 proximal promoter, multiple distal enhancer regions mediate the response to exogenous and endogenous substances, including bile acids. Figure 2 summarizes our

current knowledge about the multitude of regulatory regions spread over ALAS1 genomic locus.

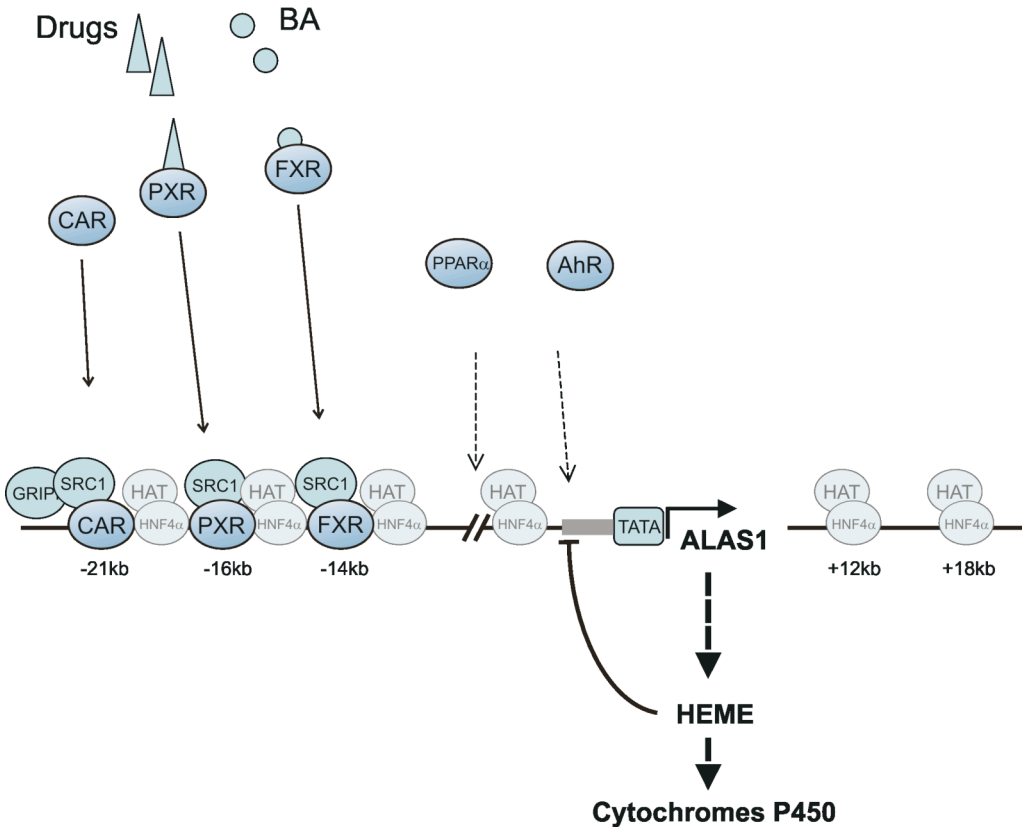

Figure 2: Xeno-and endobiotic response of ALAS1 via multiple distal enhancers.
We assume that HNF4α bound to distinct regulatory regions within ALAS1 gene recruits histone acetyltransferases (HAT), such as CREB binding protein CBP, resulting in a open (permissive) chromatin structure for other cis-acting factors to access their binding sites (Baxter et al., 2005). CAR and PXR activated by xenobiotics induce ALAS1 transcription via the two distal enhancer elements located at minus 21kb and minus 16kb respectively. Bile acid activated FXR acts via the newly identified enhancer located at minus 14kb. Upon ligand binding, the nuclear receptors recruit coactivators, such as SRC1 and GRIP1, with chromatin-modifying features, resulting in an increase RNA polymerase II mediated transcription. PPARα and AhR ligands are also known to induce ALAS1 mRNA. We assume, corresponding response elements to be present within ALAS1 gene. Through the direct transcriptional upregulation of ALAS1, synthesis of heme for the incorporation into apocytochromes is guaranteed. Heme strongly represses its own synthesis acting on ALAS1 via multiple mechanisms, including direct transcriptional inhibition. Peroxisome proliferator activated receptor α PPARα; Aryl hydrocarbon receptor AhR.

So far all events directly stimulating ALAS1 transcription are known to elicit a demand for newly synthesized heme. In the fasted liver, respiratory cytochromes involved in oxidative phosphorylation are required to restore cellular ATP levels. Ligands for the above described nuclear hormone receptors as well as for aryl hydrocarbon receptor (AhR) are known to induce hemeproteins cytochromes P450. In this context, direct

transcriptional upregulation of ALAS1 by endogenous, such as fasting, and environmental challenges, i.e. exposure to xenobiotics, provides an early and fast mechanism to increase heme synthesis according to the current metabolic demand for hemeproteins. However, as soon as cellular heme levels reach a critical level, a robust negative feedback of heme acting on ALAS1 comes into play, including a reduction in ALAS1 mRNA stability (Roberts et al., 2005) and direct inhibition of ALAS1 transcription. The presence of a heme repressive sequence in chicken ALAS1 promoter has been postulated (Kolluri et al., 2005). In addition heme regulates its own catabolism via stimulation of heme oxygenase 1. Together these mechanisms ensure a fine tuning of cellular hepatic heme levels according to the current metabolic need.

There is still a number of putative regulatory regions within ALAS1 gene whose function waits to be explored. This finding appears in different light within the recently discovered central role of heme synthesis pathway in the maintenance of circadian oscillation in liver (Kaasik and Lee, 2004). During the last years it has become clear that peripheral organs, such as liver or kidney, are able to self sustain circadian gene expression (Schibler and Naef, 2005). This is achieved through a system of multiple feedback loops, consisting of positively and negatively acting transcription factors. Among those are the heme-binding PAS domain proteins, NPAS2 and Per2, whose transcriptional activity is enhanced by heme and repressed by carbon monoxide, the degradation product of heme. In addition, ALAS1 transcripts in liver oscillate highly in a circadian pattern. NPAS2 is required to maintain this circadian expression of hepatic ALAS1 and was found to activate a 5kb murine ALAS1 promoter construct (Kaasik and Lee, 2004). Together these findings suggest that the peripheral oscillation is not only dependent on a cycle of heme anabolism and catabolism but itself influences heme synthesis via transcriptional regulation of ALAS1.

Moreover, triple knock-out mice with targeted disruption of the three proline- and acid-rich (PAR) basic leucine zipper (bZip) transcription factors - albumin-D-site-binding protein (DBP), hepatic leukaemia factor (HLF) and thyrotroph embryonic factor (TEF) - which are output regulators of the peripheral clock, show reduced transcript levels of hepatic ALAS1 (Gachon et al., 2004). These data further demonstrate that ALAS1 is under the control of peripheral oscillators, implicating that various cis-regulatory elements for these transcription factors are present within ALAS1 gene.

For the diverse regulatory pathways, multiple regulatory regions within ALAS1 gene are likely to coexist. The multitude of conserved non-genic sequences we located and were able to experimentally support may well reflect the complex transcriptional regulation of ALAS1. Insight into the transcriptional regulation of ALAS1 will finally provide novel pharmacological approaches to alleviate symptoms of patients suffering from acute porphyric attacks.

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