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**REGULATORY CROSSTALK BETWEEN DRUG  
METABOLISM AND LIPID HOMEOSTASIS: CAR AND PXR  
INCREASE INSIG-1 EXPRESSION**

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**Nonstandard abbreviations used :** CAR, constitutive androstane receptor; CYP, cytochromes P450; ER, endoplasmatic reticulum, HA, hemagglutinin; PB, Phenobarbital; PCN, pregnenolone-16 $\alpha$ -carbonitrile; PXR, pregnane x receptor; TCPOBOP, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene; AICAR, 5-aminoimidazole-4-carboxamide riboside

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## ABSTRACT

Activation of PXR (pregnane x receptor) and CAR (constitutive androstane receptor) by xenobiotic inducers of cytochromes P450 is part of a pleiotropic response that includes liver hypertrophy, tumor promotion, effects on lipid homeostasis and energy metabolism. Here we describe an acute response to CAR and PXR activators that is associated with induction of Insig-1, a protein with antilipogenic properties. We first observed that activation of CAR and PXR in mouse liver results in activation of Insig-1 along with reduced protein levels of the active form of sterol regulatory element binding protein 1 (Srebp-1). Studies in mice deficient in CAR and PXR revealed that the effect on triglycerides involves these two nuclear receptors. Finally, we identified a functional binding site for CAR and PXR in the Insig-1 gene by *in vivo*, *in vitro* and *in silico* genomic analysis. Our experiments suggest that activation of Insig-1 by drugs leads to reduced levels of active Srebp-1 and consequently to reduced target gene expression including the genes responsible for triglyceride synthesis. The reduction in nuclear Srebp-1 by drugs is not observed when Insig-1 expression is repressed by siRNA. In addition, we observed that Insig-1 is also a target of AMP-activated kinase (AMPK), the hepatic activity of which is increased by activators of CAR and PXR, and which is known to cause a reduction of triglycerides. The fact that drugs which serve as CAR or PXR ligands induce Insig-1 might have clinical consequences and explains alterations in lipid levels after drug therapy.

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Induction of cytochromes P450 (CYPs), other drug-metabolizing enzymes and drug transporters by their own substrates and other chemicals is an adaptive response of the liver to prevent accumulation of toxic xenobiotics and endobiotics. Xenosensors that mediate this response are the nuclear receptors pregnane X receptor (PXR) and constitutive active/androstane receptor (CAR; *for a review see* (Handschin and Meyer, 2003)). PXR and CAR form heterodimers with the retinoid X receptor (RXR) and bind to specific DNA sequences in the regulatory region of target genes. PXR and CAR induce an overlapping set of genes involved in metabolism and transport of drugs (Maglich et al., 2002) but also genes involved in the regulation of steroids, bile acids, eicosanoids and genes involved in cholesterol and bile acid homeostasis (Huang et al., 2003; Staudinger et al., 2001).

Insig-1 and Insig-2 are proteins of endoplasmatic reticulum (ER) membrane and play an important role in the control of triglyceride and cholesterol biosynthesis (Yabe et al., 2002; Yang et al., 2002). The two isoforms bind in a sterol-dependent fashion to another ER membrane protein, sterol regulatory element binding protein (Srebp) cleavage-activating protein, or Scap, a transport protein needed for escort and subsequent activation of Srebp transcription factors (Hua et al., 1996). When Insig proteins are activated by sterols, insulin or other stimuli, they retain the Scap-Srebp complex in the ER membrane thereby preventing Srebp-dependent target gene expression. Srebp's are a group of basic helix loop helix transcription factors, which activate an array of genes involved in the synthesis of cholesterol and triglycerides. While Srebp-2 is mainly involved in cholesterol biosynthesis, Srebp-1a and Srebp1c mainly activate genes involved in fatty acid and triglyceride synthesis (Shimano, 2001).

A decrease in hepatic and/or serum lipids, in particular triglycerides, has been observed in rodents after treatment with inducers of xenobiotic metabolism many years ago (Bjondahl, 1978; Hall et al., 1990; Venkatesan et al., 1994). More recently, known inducers of human drug metabolism such as the commonly used anti-retroviral drug efavirenz or the barbiturate phenobarbital (PB) also have been shown to inhibit lipogenesis (Hadri et al., 2004; Kiyosawa et al., 2004). The molecular mechanism of the effect of inducers on triglycerides has not been explained.

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In the present study we show that nuclear receptors CAR and PXR transcriptionally activate Insig-1 by binding to an enhancer sequence of the Insig-1 gene. Our results explain the negative effect of drugs and xenobiotics on hepatic lipids *in vivo* and show that CAR and PXR not only play a role in the catabolism of various endogenous and exogenous compounds but also directly affect lipogenic pathways by activating Insig-1. Moreover, as Insig-1 has recently been found to be a possible drug target for the treatment of diabetes (Nakagawa et al., 2006), this study contributes to the understanding of the regulation of this gene and possibly to the development of new therapies against dislipidemia.

## **MATERIALS & METHODS**

### **Animals**

C57BL/6 mice were maintained in a 12-hour light/12-hour dark cycle and had free access to food and drinking water. 9-11 week old male animals received an i.p. injection of either 100 mg/Kg phenobarbital (PB, Sigma, Buchs, Switzerland), 10 mg/Kg 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP; Bayer, Leverkusen, FRG) or 40 mg/Kg pregnenolone-16 $\alpha$ -carbonitrile (PCN; Sigma, Buchs, Switzerland) in a 5% DMSO-corn oil solution i.p. or with vehicle 10h before dissection. Animals were sacrificed by exposure to CO<sub>2</sub>, blood was collected by heart puncture, livers excised and snap-frozen in liquid nitrogen and stored at -80°C until use.

### **Analysis of Triglycerides and Cholesterol**

50-100 mg of liver was used for each preparation. After weight determination, liver samples were put in an ethanol: ether (3:1, v/v) mixture in Fastprep tubes (Lysing matrix D, Qbiogene, Basel, Switzerland). Livers were homogenized on the Fastprep instrument for 40s at position 6,5 and evaporated to complete dryness on a Speed-vac evaporator. Samples were redissolved in 1ml isopropanol and tissue remnants spun down for 5 minutes at 14000 xg. 600  $\mu$ l of the supernatant was mixed with 400  $\mu$ l of water and lipids were determined using the esterase/oxidase kit for cholesterol determination and the L- $\alpha$ -glycerol phosphate oxidase kit for the determination of triglycerides (Roche Diagnostics, Rotkreuz, Switzerland).

### **RT PCR Analysis**

RNA from cells and tissues was isolated using Tri-Reagent (Sigma, Buchs, Switzerland). One microgram total RNA was reverse-transcribed with MMLV reverse transcriptase (Roche Molecular Biochemicals, Rotkreuz, Switzerland). PCR was performed using the TaqMan PCR Core Reagent Kit (PE Applied Biosystems, Rotkreuz, Switzerland) and the transcript level quantitated with an ABI

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PRISM 7700 Sequence Detection System (PE Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's protocol. Briefly, relative transcript levels were determined using the relative quantitation method by measuring the  $\Delta C_t$  between the gene of interest and the internal control GAPDH. Primers and fluorescent probes used in these PCRs are listed in Table 1.

### **Reporter Gene Assays**

Culture and transfection of CV-1 cells with Lipofectamine Transfection Reagent (Invitrogen, Carlsbad, USA) was performed as previously published (Handschin et al., 2000). Expression vectors encoding mouse PXR and mouse CAR as well as the beta-galactosidase vector used for signal normalization have been described (Handschin et al., 2002). For construction of hemagglutinin-tagged vp16 fusion proteins nuclear receptor sequences were amplified from expression plasmids and subcloned into pcDNA3/vp16-HA (a kind gift from Dr. Dieter Kressler, Biozentrum, University of Basel, Switzerland). Reporter vectors were based on PGL3-LUC (Promega, Wallisellen, Switzerland). Genomic DNA from the murine *Insig1* enhancer region was amplified using PCR primers carrying restriction sites suitable for direct subcloning into the reporter vector.

### **Preparation of Primary Hepatocytes**

For the preparation of mouse hepatocytes animals were anaesthetized with Ketamine/Xylazine (Sigma, Buchs, Switzerland). The portal vein was cannulated and perfused with HEPES-EGTA (pH 7.4) for 5 minutes and then with collagenase (type 2, Worthington, Lakewood NJ, USA) for 6 minutes. The livers were excised, cells were filtered through a nylon mesh and centrifuged at 50 xg 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 1% penicillin/streptomycin (50 IU/ml) on collagen-coated dishes. Induction experiments were performed

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in the same medium without FCS and with reduced insulin (2 µg/ml) but with the addition of 1 µM hydrocortisone.

Primary human hepatocytes in suspension were allowed to attach on collagen-coated plates in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (50 IU/ml) and 1 µM dexamethasone overnight before start of the experiments. For induction cells were cultured in DMEM without serum but supplemented with insulin-transferrine-selenium mixture (Sigma, Buchs, Switzerland) and 1µM hydrocortisone

### **Production of recombinant adenovirus particles**

Expression cassettes of interest were PCR-amplified using vector-specific primers with attB1/attB2-Gateway extensions for subsequent cloning into pDONR221 (Invitrogen, Carlsbad, CA, USA). For pcDNA3 constructs (Vp16-PXR, Vp16-CAR) the following primers were used: TTAGGGTTAGGCGTTTTGCGC (Fwd), TCAGAAGCCATAGAGCCCAC (Rev). Entry clones carrying the PCR products were used for cloning into pAd-DEST (Invitrogen, Carlsbad, CA, USA). PacI-digested plasmids were transfected into HEK293A cells and adenovirus particles produced and processed according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Functionality of PXR- and CAR-expressing adenoviruses was assessed in reporter gene assays in CV-1 cells using PXR- and CAR-responsive reporter vectors. We also tested the recombinant proteins in mouse hepatocytes by measuring mRNA expression of the target genes Cyp3a11 and Cyp2b10, respectively (data not shown).The adenovirus particles encoding recombinant forms of AMPK have been described (20)

### **Immunoblotting, Gel-Mobility-Shift Assay & Chromatin Immunoprecipitation**

For Western blot analysis of SREBP1, liver proteins were extracted from 100-200 mg of frozen tissue in 1 ml ice-cold buffer (50 mM Tris-HCl pH 7.4, 100mM KCl, 1mM EDTA pH 8.0, 10mM beta-Mercaptoethanol, 5mM DTT, 0.1% (v/v) Triton X-100, 0.1% (v/v) NP 40, 1 tablet/50ml buffer



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Protease Inhibitor Cocktail (Roche Diagnostics, Rotkreuz, Switzerland)) in a 5 ml polystyrene tube using a Polytron Rotor-Stator Homogenizer. The homogenate was centrifuged for 30 min at 10000 rpm at 4°C and 50 µg of protein was loaded on a 10% SDS-gel. SREBP1 isoforms were detected using mouse anti-SREBP1 monoclonal antibody (Anti-SREBP1 monoclonal #557036, Clone IgG-2A4BD, Pharmingen, Allschwil, Switzerland).

Transcription factors were synthesized *in vitro* by using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Wallisellen, Switzerland) according to the manufacturer's instructions. Probes were labeled with the Klenow fragment of DNA polymerase in the presence of radiolabeled [  $\gamma$ -<sup>32</sup>P]ATP, and the probe was purified over a Biospin 6 chromatography column. A volume of labelled oligonucleotide corresponding to 100,000 cpm was used for each reaction in 10 mM Tris•HCl, pH 8.0/40 mM KCl/0.05% Nonidet P-40/6% (vol/vol) glycerol/1 mM DTT containing 0.2 µg of poly(dI-dC)•poly(dI-dC) and 2.5 µl of the *in vitro* synthesized proteins as described previously (Handschin et al.). The mix was incubated for 20 min at room temperature and subsequently electrophoresed on a 6% polyacrylamide gel in 0.5× Tris/borate/EDTA buffer (1x TBE buffer is composed of 0.9 M Tris-Borate, 0.002 M EDTA, pH 8.3) followed by autoradiography at 70°C. Oligos used for EMSAs were obtained as follows: For the mouse Insig-1 DR4 the following oligonucleotide were annealed and labelled using polynucleotide kinase: CCTGAGGGTCAACAGAGGACACCTAG (Fwd) and CTAGGTGTCCTCTGTTGACCCCTCAGG (Rev).

Chromatin Immunoprecipitation was performed using the EZ-Chip™ kit from Upstate (Charlottesville, USA). Primary mouse hepatocytes were infected with adenoviral particles encoding HA-tagged vp16-mouse CAR or mouse PXR, respectively. After 24 hours cells were harvested and samples processed according to the manufacturers recommendations. For immunoprecipitation hemagglutinin (HA) antibody (monoclonal *HA.11* clone 16B12 mouse IgG1 MMS-101P) from Covance (Princeton, USA) was used.

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**Targeting of Insig-1 in primary mouse hepatocytes by siRNA**

For the transfection of primary mouse hepatocytes, Dharmafect1 transfection reagent (#T2001-01, Dharmacon, Chicago, USA) was used. 100nM siRNAs (siGenome SmartPool mINSIG1 #M-060068-00; siControl Nontargeting siRNAPool#2 #D-001206-14-05; Dharmacon, Chicago, USA) and 14 $\mu$ l Dharmafect were used according to the manufacturer's instructions. 6h after transfection medium was removed and replaced with fresh medium without serum. 24h later, medium was replace by medium containing 500 $\mu$ M phenobarbital and mRNAs and Srebp-1 protein analyzed after 24h as described.

## RESULTS

Mice were injected with drugs known to activate nuclear receptors PXR and CAR, namely phenobarbital (PB, activator of both PXR and CAR), pregnenolone-16 $\alpha$ -carbonitrile (PCN, activator of PXR) and 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, activator of CAR; Fig. 1). After 10 hours of exposure, liver samples were analyzed for hepatic triglycerides and cholesterol (Fig. 1, top panel). All three drugs caused a substantial drop in triglycerides, whereas cholesterol levels were less affected. PB and TCPOBOP at the doses applied resulted in a 49% or 67% decrease in triglycerides, respectively, and a 28% or 33% decrease in cholesterol. Serum analysis of PB-treated animals revealed no significant change in triglycerides or cholesterol at 10 hours (Fig. 1, top panel, right). RT-PCR analysis of these livers showed marked induction of *Insig-1* mRNA (Fig. 1, middle panel). The mRNA levels of *Insig-2* were not significantly induced by drug treatment (data not shown) and mRNA levels of *Srebp* genes remained unaffected by drug treatment as well. Accordingly, Hmg-CoA reductase (*Hmgcr*), a target gene of *Srebp-2* (Horton et al., 1998) was unchanged while Stearoyl-CoA desaturase 1 (*Scd-1*), which is a target gene of *Srebp-1* (Shimano et al., 1999), was reduced after drug treatment. Again, TCPOBOP showed strongest effects (Fig. 1, middle panel). Whether the reduction in hepatic triglycerides was due to reduced nuclear expression of *Srebp-1* was tested by immunoblotting of liver protein extracts from drug-treated animals using an antibody which can discriminate between the inactive precursor form of *Srebp1* and the activated nuclear (mature) form of *Srebp-1* (Fig. 2A). The graph shows a reduction in nuclear content of *Srebp-1* in drug-treated mice with strongest effects by PB and TCPOBOP. A time course experiment in primary mouse hepatocytes revealed that the time-dependent induction profile of *Insig-1* mRNA paralleled the one of the classic CAR- and PXR-inducible gene *Cyp2b10* (Fig. 2B).

To define the role for PXR and CAR in the activation of *Insig1* and the subsequent drop in hepatic triglycerides we applied PB to mice deficient in these two receptors (Zhang et al., 2004), Fig. 3). Fig. 3 shows that two typical target genes of PXR and CAR, *Cyp2b10* and *Cyp3a11*, expectedly were not

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inducible by PB in PXR/CAR null mice. Blunted induction of *Insig-1* mRNA in these animals after PB treatment as well as an unchanged triglyceride profile compared to wild type animals was observed (Fig. 3, lower panels). These data support a PXR/CAR-dependent mechanism for the triglyceride-lowering effect of inducer compounds.

We therefore designed experiments to identify functional binding sites for the PXR and CAR in the regulatory region of *Insig-1*. Two 3kb fragments of genomic DNA from the 5'-flanking region of the *Insig1* gene were cloned into a luciferase reporter vector and activity was assessed in transactivation assays in CV-1 cells (Fig. 4A). The first DNA element spanned the transcriptional start site including the proximal promoter of *Insig1* to -3044 bp and showed no activation after drug treatment. The second large DNA stretch was overlapping with the first one and ended at -6252bp. There was slight (as compared to empty control luciferase vector) activation of reporter gene transcription after drug treatment. This DNA element was cut into smaller pieces and activity assessed until a 760 bp fragment revealed a robust response to PXR and CAR. Within this fragment a DR-4 type drug response element was identified using the Nubiscan algorithm (Podvinec et al., 2002). This element responded well to PXR and CAR and specificity was assessed using a mutated version of the DR-4 element, which resulted in a decreased response to drugs (Fig. 4A). The same DR-4 element was used in electromobility shift assays together with *in vitro* translated PXR, CAR and their heterodimeric partner, RXR (Fig. 4B). A strong band was observed when CAR and RXR were incubated with the oligonucleotide carrying the DR-4 element and less intensive binding appeared when PXR was used. Both nuclear receptor complexes were super-shifted by co-incubation with an anti-RXR antibody. Functionality of this element was tested *in vivo* by chromatin immunoprecipitation in primary mouse hepatocytes (Fig. 4C). Cells were infected with adenovirus particles expressing HA-tagged versions of both CAR and PXR. The amplified PCR product corresponds to the 157bp region in the murine *Insig-1* promoter where the designated DR-4 element is located. In Fig 5B a reporter gene analysis using the *Insig-1* DR-4 with inducers of mouse PXR and CAR, respectively, was performed and

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revealed a 4-fold induction after PCN-treatment and a 7.2-fold induction after TCBOBOP-treatment (Fig. 5A).

A role for Insig-1 in mediating the repressive effects of inducer drugs was established by siRNA-mediated inhibition of Insig-1 expression and concurrent treatment with PB (Fig. 5B). Primary mouse hepatocytes were transfected with either unspecific control siRNAs or siRNA targeting Insig-1. After 48h, mRNA analysis revealed markedly reduced Insig-1 expression (Fig. 5B, left panel). Immunoblot analysis of Srebp-1 protein levels in siRNA transfected cultures treated with or without PB was performed (Fig. 5B, right panel). The results reveal reduced nuclear expression of Srebp-1 in cells transfected with control siRNA and treated with PB. In cells where Insig-1 expression was reduced by siRNA, Srebp-1 protein levels in the nucleus remained unaffected (Fig. 5B, right panel).

To reveal a potential role of this mechanism in humans we tested the inducibility of human Insig1 by PB in primary human hepatocytes (Fig. 6A). Treatment for 50 hours with PB resulted in a significant induction of Insig-1 in cultures of two different donors and this was paralleled by a reduction in Srebp1c expression. Induction of CYP2B6 and CYP3A4 served as positive controls.

As recently reported, induction of drug metabolizing enzymes requires activation of AMP-activated kinase (Rencurel et al., 2006; Rencurel et al., 2005). We therefore wanted to test whether this kinase, alone or in combination with drug, can regulate transcription of Insig1 (Fig. 6B). Primary human hepatocytes were infected with control virus (expressing beta-galactosidase) or different versions of AMPK: a dominant negative construct (kinase dead, KD), the alpha-1 or the alpha-2 subunit of AMPK. While the dominant negative version repressed expression of Insig1, both subunits induced Insig1 with stronger effects seen using the alpha-1 subunit. Furthermore, exposure to PB enhanced these effects (Fig. 6B).

## DISCUSSION

The experiments described here reveal a novel molecular mechanism by which drugs that induce drug-metabolizing enzymes and drug transporters can acutely regulate hepatic triglyceride levels. While several CYPs and other enzymes involved in metabolism and transport of xenochemicals have been known to be targets of nuclear receptors CAR and PXR, the upregulation of Insig-1 by the same receptors after drug treatment is new and adds a potentially clinically important aspect to the present understanding on how the liver reacts to accumulating lipophilic compounds such as PB. This report shows that the drug metabolizing process also includes direct regulation of hepatic lipid biosynthesis by induction of an important regulatory protein as is Insig-1. We present evidence for a functional DR-4 binding site for CAR and PXR in the upstream promoter region of Insig-1 (Fig. 4). Binding of the xenobiotic receptors to this DR-4 site can account for the induction of Insig-1, which results in the reduced expression of the activated nuclear form of Srebp-1 and the substantial reduction in hepatic triglycerides after only 10 hours of treatment (Fig. 1,2). The fact that Insig-1 has been observed in expression studies to be induced early after treatment with the CAR ligand TCPOBOP (Locker et al., 2003) and that overexpression of Insig-1 in livers of mice has been shown to cause a drop in triglyceride levels with smaller effects on cholesterol (Engelking et al., 2004; Takaishi et al., 2004) made Insig-1 an interesting candidate gene for transcriptional regulation by CAR and PXR. We first established that in CAR/PXR double knockout mice PB had lost its effect on triglycerides and there was no induction of Insig-1 (Fig. 3). This strongly supported the idea of a CAR/PXR-mediated transcriptional activation of the Insig-1 gene. Moreover, the induction of Insig1 mRNA appeared early after addition of drug (Fig. 2B) making a rapid decrease in activated nuclear form of Srebp-1 protein levels a plausible scenario (Fig. 2A). The more pronounced effects on triglycerides seen with the CAR activators TCPOBOP and PB compared to the PXR ligand PCN is in line with the more pronounced activation of the Insig1 promoter by CAR (Fig. 4A, 5B), with the higher affinity of CAR to bind to the DR-4 element (Fig. 4B) as well as with the stronger enrichment in CAR-immunoprecipitated

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samples of this fragment (Fig. 4C). A role for Insig-1 in mediating these effects was established in mouse hepatocytes with siRNA-reduced Insig-1 expression (Fig. 5B). While the repressive effect of PB on nuclear protein levels of Srebp-1 was evident, in cells with repressed Insig-1 expression this effect was not detectable, strongly supporting the concept that PXR/CAR-activated Insig-1 is responsible for reduced Srebp-1 and thereby for lowered hepatic triglycerides. Moreover, the fact that these effects could be reproduced in human hepatocytes supports the concept of a general mechanism by which drugs affect hepatic lipid biosynthesis (Fig. 6A). Insig-2, the other member of the Insig family of Srebp-regulating genes was not affected by PXR/CAR inducers and a potential role of this gene in linking drug treatment to reduced triglyceride levels was not pursued. It cannot be ruled out of course, that under different conditions, this gene may also play a role in mediating lipid synthesis due to a xenobiotic challenge.

An effect of PXR on lipogenic genes has recently been described by Nakamura *et al.* (Nakamura *et al.*, 2007). In strong support of our data they observed downregulation of lipogenic genes by the PXR-specific activator PCN and this effect was not seen in PXR<sup>-/-</sup> mice. Interestingly, Nakamura and colleagues furthermore observed an induction of Scd-1 in PCN-treated animals that were fasted for 24h. This suggests an additional level of regulation of lipogenesis by xenobiotics in the fasted state.

Another new finding of our study is the role of AMPK in the induction of Insig1 (Fig. 6B). AMPK is considered a metabolic master-switch sensing cellular energy levels and regulating glucose transport and gluconeogenesis. It is activated in response to metabolic stress signals that deplete cellular ATP and stimulate fatty acid oxidation (Kahn *et al.*, 2005). It was recently shown that CAR-dependent induction of CYP2B by PB requires activation of AMPK (Rencurel *et al.*, 2006). Blattler *et al.* (Blattler *et al.*, 2007) demonstrated that PB interferes with mitochondrial function and activates the AMPK upstream kinase LKB1 which then mediates the activation cascade of AMPK to CAR. Interestingly, AMPK, either via activation by AICAR (5-aminoimidazole-4-carboxamide riboside) or via adenoviral overexpression of its catalytic subunit, also has been shown to reduce Srebp-1c expression (Foretz *et al.*, 2005; Zhou *et al.*, 2001). As these observations lacked a mechanistic

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explanation, the activation of Insig-1 by AMPK shown here may indicate a signaling pathway leading to repression of Srebp-1c. In line with observations by Rencurel *et al.* (2006), AMPK alone seems capable of regulating expression of CAR/PXR target genes and addition of a nuclear receptor activator leads to a synergistic effect on gene transcription. However, the detailed interplay between PB, nuclear receptors and AMPK in the induction of Insig-1 clearly requires further investigation.

Also, the data presented here account for the immediate physiologic response of the liver to a xenobiotic challenge. Chronic treatment with drugs leading to constant activation of PXR and/or CAR may lead to diverse adaptive gene-regulations to maintain lipid homeostasis (Kiyosawa *et al.*, 2004; Zhou *et al.*, 2006).

In conclusion, the results of our experiments suggest that the signaling pathways involved in mediating the effect of xenobiotics on detoxification also induce Insig-1, a gene regulating lipid biosynthesis and that this is associated with an acute lowering of triglyceride levels in the liver. As Insig-1 has recently been suggested as a possible drug target for the treatment of dislipidemic diseases including diabetes (Nakagawa *et al.*, 2006) the observation that CAR and PXR ligands or activators induce Insig-1 may have clinical consequences and explains the reported alterations in lipid levels after drug therapy.



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## FOOTNOTES

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## LEGENDS TO FIGURES

### Fig. 1

**Lipid and gene expression changes after drug application in mouse liver.** PB, TCPOBOP and PCN were i.p.-injected in mice and livers or serum samples analyzed after 10h. Upper panel, triglyceride (TG) and total cholesterol (CHOL) analysis in liver homogenates and blood samples. Middle and lower panel, RT-PCR analysis of RNAs from mouse livers. Bars indicate mean values from 6 animals per treatment group and standard deviations therefrom. \* $p < 0.05$ , \*\* $p < 0.01$ .

### Fig. 2

**Effect of drugs on Srebp1 protein levels.** A, Srebp-1 immunoblot protein analysis in mouse livers after 10h drug treatment. Mice were injected 100mg/Kg phenobarbital (PB), 10mg/Kg 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) or 40mg/Kg pregnenolone-16 $\alpha$ -carbonitrile (PCN) in a 5% DMSO-corn oil solution i.p. or with vehicle. Proteins were extracted from liver homogenates and analyzed using a monoclonal Srebp-1 antibody. \*Srebp-1 precursor form, \*\*Srebp-1 mature form. B, Time course of Cyp2b10 and Insig-1 mRNA induction in primary mouse hepatocytes exposed to 500 $\mu$ M PB.

### Fig. 3

**Role of CAR and PXR in the regulation of Insig1 and hepatic triglycerides.** Gene expression changes (RT-PCR) of CYPs (upper panel) and Insig1 in CAR/PXR-wild type and null mice 10h after injection of 100mg/Kg PB. Bars indicate mean values from 6 animals per treatment group and standard deviations therefrom. \*\* $p < 0.01$ . Lower panel right, triglyceride analysis in mouse liver homogenates of the same animals (see Materials & Methods for triglyceride analysis).

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**Fig. 4**

**Analysis of the Insig1 promoter for drug response elements.** A, Reporter gene assays using different genomic DNA sequences from the Insig1 promoter cloned into tk-Luc vector. At the bottom of the Fig. a Luc vector with a mutated Insig1 DR-4 was used.. Plasmids were cotransfected together with mouse CAR or PXR into CV-1 cells and luciferase activities determined 16h after addition of 1 $\mu$ M TCPOBOP and 10 $\mu$ M PCN, respectively. Cell lysates were analyzed for luciferase expression and fold activation of reporter fragment calculated relative to empty luciferase vector treated with drugs is indicated. The sequence of the identified DR-4 type drug response element as well as the mutated form used in the reporter assay is shown in a separate box. B, Gel shift assay using *in vitro* translated mouse CAR, PXR and RXR and a radiolabelled oligonucleotide encoding the putative Insig1 DR-4 element. The experimental conditions for each lane are indicated in the panel above the picture: “+” indicates that the corresponding receptor was added, while “-“ indicates that no receptor was added, \* position of shifted nuclear receptor heterodimer, \*\* position of anti-RXR supershifted nuclear receptor heterodimer. C, Association of CAR and PXR with the Insig1 DR-4 analyzed by chromatin immunoprecipitation in primary mouse hepatocytes using adenovirus encoding HA-tagged CAR and PXR, respectively. Lower panel shows a control experiment using the same lysates on the GAPDH promoter.

androstanol (AND), 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and pregnenolone-16 $\alpha$ -carbonitrile (PCN)

**Fig. 5**

**Induction of Insig1 DR-4 reporter gene by PXR and CAR inducers and lack of effect of Phenobarbital on SREBP-1 proteins when Insig-1 expression is suppressed by RNAi.** A, Effect of PXR, CAR and their ligands/activators on the Insig-1 DR-4 reporter gene. PXR was activated by over night treatment with 10 $\mu$ M pregnenolone-16 $\alpha$ -carbonitrile (PCN). Constitutive active CAR was

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repressed by the addition of 1 $\mu$ M androstanol and activated by addition of 10 $\mu$ M 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). Fold activations are calculated as relative reporter gene levels over non-drug-treated cells. B, primary mouse hepatocytes were transfected with either control siRNA or siRNA against Insig-1. mRNA analysis of Insig-1-siRNA effect after 48h is shown in the left panel, effect of PB treatment on Srebp-1 protein levels in cultures transfected with either control or Insig-1 siRNA is shown in the right panel. Asterisks indicate precursor (\*) and mature form (\*\*) of Srebp-1.

**Fig. 6**

**Induction of Insig1 by PB and AMPK in primary human hepatocytes.** A, Cells from two different donors were incubated with 500 $\mu$ M PB and mRNA analyzed 50h thereafter. B, AMPK and PB synergistically activate human INSIG-1. Primary human hepatocytes from a third donor were infected with adenoviral particles encoding either control gene (B-Gal), a dominant negative form of AMPK (KD), the alpha-1 subunit of AMPK (AMPK $\alpha$ 1) or the alpha-2 subunit of AMPK (AMPK $\alpha$ 2). \*p<0.05, \*\*p<0.01; n=3; p-values were calculated between either untreated or treated wells.



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**Table 1: Primers and Probes used for RT-qPCR**

Gene	Forward	Reverse	Taqman-Probe
<i>Mouse:</i>			
Gapdh	CCAGAACATCATCCCTGCATC	GGTCCTCAGTGTAGCCCAAGAT	CCGCCTGGAGAAACCTGCCAAGTATG
Cyp2b10	CAATGTTTAGTGGAGGAAGTGGC	CACTGGAAGAGGAACGTGGG	CCCAGGGAGCCCCCTGGA
Cyp3a11	AGAACTTCTCCTTCCAGCCTTGTA	GAGGGAGACTCATGTCCAGTTA	CTAAAGGTTGTGCCACGGGATGCAGT
Srebp1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCAGCATA	CAGCTCATCAACAACCAAGACAGTGACTTCC
Scd-1*	CCGGAGACCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC	
Hmgcr*	CGAGGAAAGACTGTGGTTTGTG	CGTCAACCATAGCTTCCGTAGTT	
Insig-1*	TGCAGATCCAGCGGAATGT	CCAGGCGGAGGAGAAGATG	
<i>Human:</i>			
CYP2B6	ACATCGCCCTCCAGAGCTT	GTCGGAAAATCTCTGAATCTCATAGA	ACCGAGCCAAAATGCCATACACAGAGG
CYP3A4	CATTCCTCATCCCAATTCTTGAAGT	CCACTCGGTGCTTTTGTGTATCT	CGAGGCGACTTCTTTCATCCTTTTACAGATTTTC
INSIG-1*	AGCCCTACCCCAACACCT	ACCACCCCAACCGAGAAGA	
SREBP1c*	TCAGCGAGGCGCTTTGGAGCAG	CATGTCTTCGATGTCGGTCAG	
18s	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGCGCTCACTA	CGCCCGTCGCTACTACCGATTGG

(\*SYBR primers)

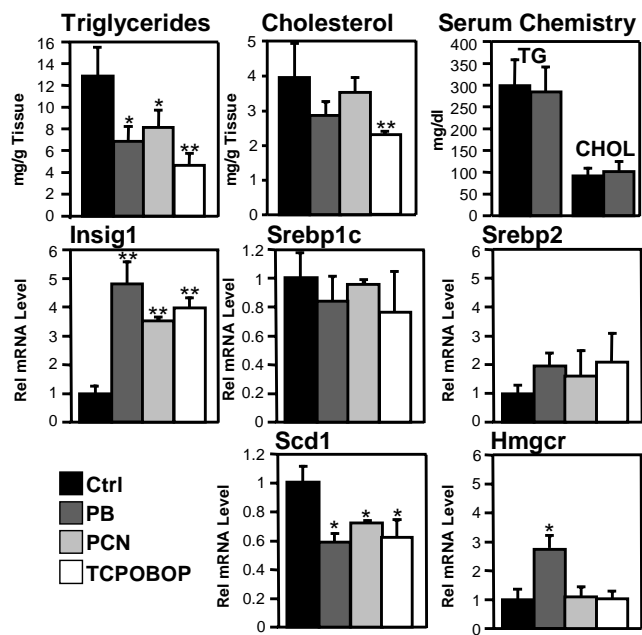


Figure 1

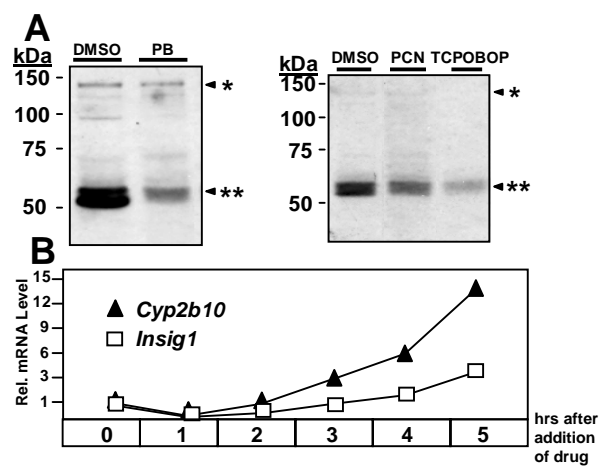


Figure 2

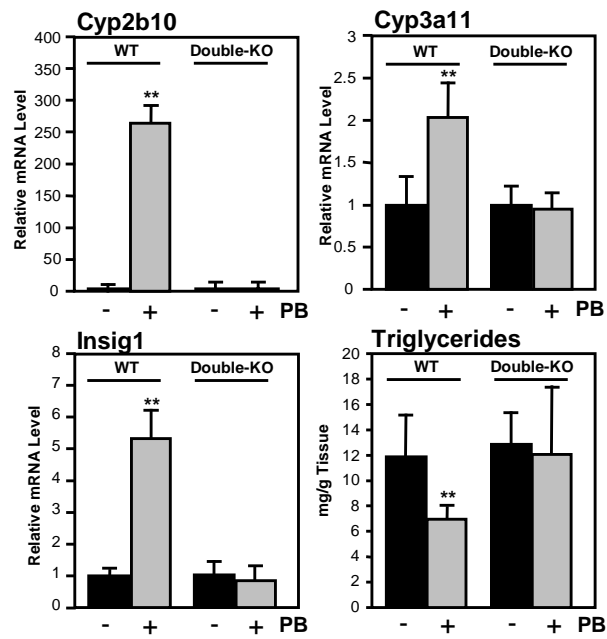


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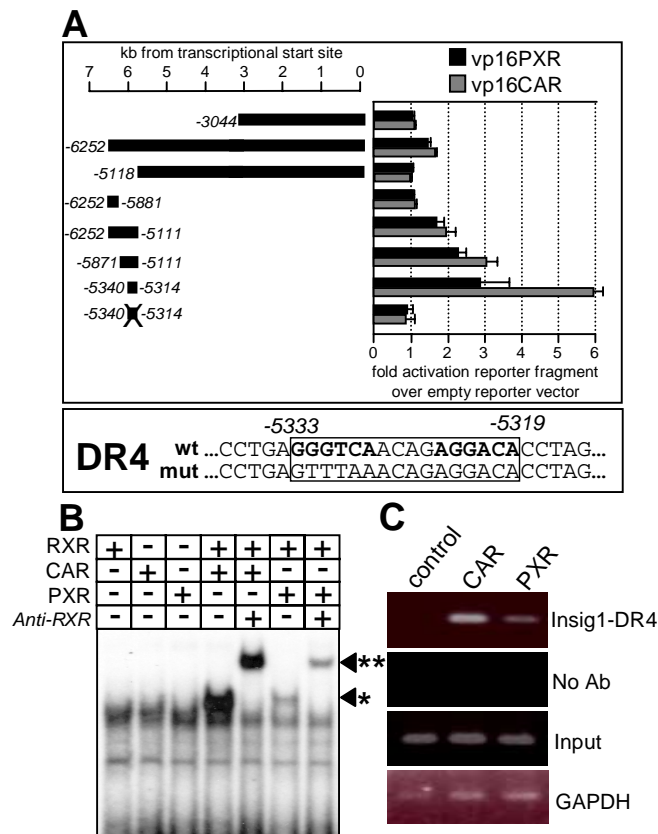


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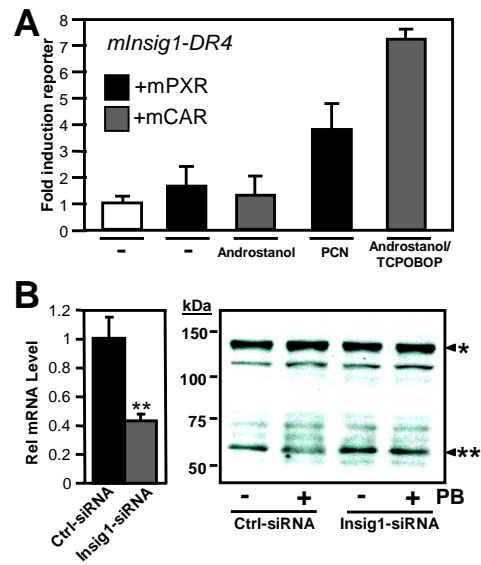


Figure 5

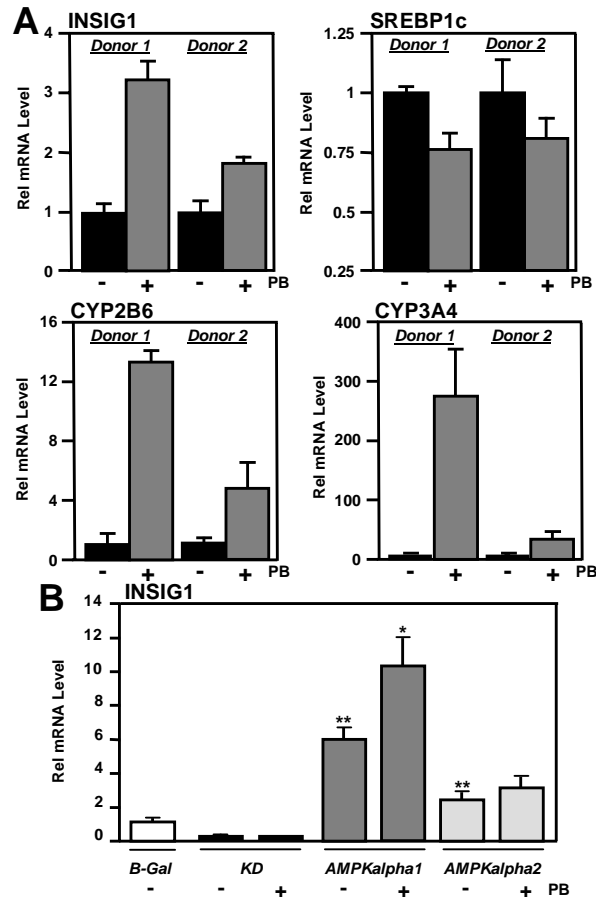


Figure 6