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Energy sensing factors PGC-1α and SIRT1 modulate PXR expression and function

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ABSTRACT

The pregnane X receptor (PXR), a xenobiotic-sensing nuclear receptor plays a major role in regulation of drug metabolism but also modulates hepatic energy metabolism. PXR interacts with and represses several important transcription factors and coactivators regulating key enzymes in energy metabolism. Much less is known about how energy sensing cellular factors regulate PXR function. In this study we have investigated the effect of two major regulators of hepatic energy homeostasis, the transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α) and the NAD-dependent deacetylase protein, sirtuin 1 (SIRT1) on PXR expression and function. Fasting induces PXR expression in liver. Furthermore, glucagon and PGC-1α overexpression upregulate PXR expression level in mouse primary hepatocytes suggesting that PGC-1α, in addition to coactivation of PXR, also transcriptionally regulates PXR gene. Knockdown of peroxisome proliferator-activated receptor α by siRNA attenuates PGC-1α mediated induction of PXR mRNA. PGC-1α overexpression alone has no effect on cytochrome P450 (CYP) 3A11 expression but potentiates induction by pregnenolone-16α-carbonitrile (PCN). Pyruvate, a nutrient signal activating SIRT1 abolishes synergistic induction of CYP3A11 by PCN and PGC-1α. Knockdown of SIRT1 prevented this effect of pyruvate. Downregulation of CYP7A1 by PCN was not affected by PGC-1α or pyruvate. Mammalian two hydrid assays indicate that pyruvate and SIRT1 interfere with interaction of PXR and PGC-1α. This may be mediated by well established PGC-1α deacetylation by SIRT1. However, we show by immunoprecipitation that SIRT1 also interacts with PXR. Thus we show that two fasting activated pathways PGC-1α and SIRT1 differentially modify PXR expression and function.

1. Introduction

Pregnane X receptor (PXR, NR1I2) is a xenobiotic-sensing nuclear receptor which after activation by broad variety of species specific xenobiotic ligands upregulates the transcription of target genes encoding drug metabolizing enzymes and drug transporters [1]. Ligand activated PXR forms a heterodimer with another nuclear receptor, the retinoid X receptor and subsequently interacts with DNA binding motifs constituted of different direct and everted repeats of hexameric AGGTCA-like sequences. The transactivation function of PXR is mediated through recruitment of coactivators such as peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α) [2].

In addition to drug metabolism, PXR has been found to regulate hepatic energy metabolism. Ligand activated PXR represses key transcription factors and coactivators controlling gluconeogenesis, fatty acid oxidation and ketogenesis through protein–protein interactions [3]. Xenobiotic-sensing pathways regulate energy metabolism, reciprocally energy homeostasis affects drug metabolism [4]. For example, both fasting and streptozotocin-induced diabetes induces CYP3A11 expression [5]. However, the mechanisms mediating the effects of energy homeostasis on drug metabolism are not well understood.

Coactivator PGC-1α is an essential regulator of liver energy metabolism. The basal hepatic expression of PGC-1α is relatively low in healthy, fed conditions, but is readily upregulated by fasting and diabetes mainly through altered insulin–glucagon balance [6]. Increased PGC-1α activates fatty acid oxidation and gluconeogenesis by facilitating expression of key enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G-6-Pase). PGC-1α cannot bind to DNA itself but functions through interaction with a number of transcriptional factors such as peroxisome proliferator-activated receptor alpha (PPARα), hepatocyte nuclear factor 4 alpha (HNF4α) or PXR.

Abbreviations: PXR, pregnane X receptor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1 alpha; SIRT1, sirtuin 1; CYP, cytochrome P450; PCN, pregnenolone-16α-carbonitrile; PPARα, peroxisome proliferator-activated receptor alpha; DMSO, dimethyl sulfoxide; HNF4α, hepatocyte nuclear factor 4 alpha.

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Coactivation by PGC-1α is further modulated by diverse posttranslational modifications, including phosphorylation and acetylation. Sirtuin 1 (SIRT1) is a NAD-dependent deacetylase which targets and deacetylates PGC-1α enhancing its ability to coactivate gluconeogenic genes [7]. SIRT1, similarly to PGC-1α, is activated by starvation in hepatocytes, however, the mechanism is fundamentally different. The NAD^+/NADH ratio is proportional to pyruvate concentration and rises during fasting. Higher levels of NAD^+ induce expression of SIRT1 protein and also directly affect SIRT1 enzyme activity [8]. In addition to PGC-1α, SIRT1 targets a number of other proteins including several nuclear receptors [9]. The SIRT1 mediated deacetylation may have either positive or negative effects on transcription depending on the target transcription factor and even promoter context [8].

In the current study we have investigated the role of energy sensing factors PGC-1α and SIRT1 in control of PXR mediated transcriptional regulation. We show that PGC-1α stimulates PXR expression and transactivation. However, transactivation is attenuated by SIRT1.

2. Materials and methods

2.1. Chemicals

Pregnenolone-16α-carbonitrile (PCN), rifampicin, pyruvate and glucagon were purchased from Sigma–Aldrich (Sigma–Aldrich Co., St. Louis, MO, USA)

2.2. Animals and animal treatment

The studies were carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by the local ethics committees for laboratory animal welfare at the University of Oulu and the Semmelweis University. All animals were housed in a temperature-controlled (22 °C ± 2 °C) and humidity-controlled (60% ± 5%) room with a 12 h light (7:00 am to 7:00 pm) and 12 h dark (7:00 pm to 7:00 am) cycle in standard cages. Standard rodent chow and water were provided ad libitum. In fasting experiment rats were deprived of food for 24 and 48 h with free access to water, were anesthetized with pentobarbital sodium (60 mg/kg body weight IP) and livers were excised rapidly and frozen in liquid nitrogen and stored at −80 °C.

2.3. Mouse primary hepatocytes and cell culturing

Hepatocytes were isolated from male DBA/2 (OlaHsd) mice (Center for Experimental Animals, University of Oulu, Finland) aged 8–10 weeks. Livers were perfused with collagenase solution (Worthington Biochemical Co., Lakewood, NJ, USA) as described previously [10]. After filtration and centrifugation, the isolated hepatocytes were dispersed in William's medium E (Sigma–Aldrich Co., St. Louis, MO, USA) containing dexamethasone (Sigma–Aldrich Co., St. Louis, MO, USA), 20 ng/ml, ITS (insulin 5 mg/l, transferrin 5 mg/l, sodium selenate 5 μg/l) (Sigma–Aldrich Co., St. Louis, MO, USA), gentamicin (Invitrogen, Carlsbad, CA, USA) 50 μg/ml, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at a density of 1×10^6 cells/well in six-well plates and 3×10^5 cells/well in twelve-well plates (FALCON Polystyrene Cell Culture Dish, BD Biosciences, San Jose, CA, USA). The cultures were maintained at 37 °C in a humidified incubator for 1–2 h, after which non-attached cells were discarded by aspiration, and the medium was replaced by serum-free William's E medium. The cultures were maintained overnight before further treatments and serum-free William's E medium was used in all experiments.

HepG2 and COS-1 cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal calf serum, 100 U/ml of penicillin and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 4500 mg/ml glucose. DMEM supplemented with 1 mM pyruvate and lower 1000 mg/ml glucose (Invitrogen, Carlsbad, CA, USA) was used in mammalian two hydrid assays in indicated cases.

2.4. Plasmids

Mouse PGC-1α expression vector (pcDNA3-mPGC1α) was kindly provided by Dr. J.K. Kemper (University of Illinois at Urbana-Champaign, Urbana; Illinois, USA). The human FLAG-hPXR was a generous gift from Dr. Masahiko Negishi (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, USA). pCRUzHA-SIRT1WT and pCRUzHA-SIRT1G261A were obtained from Addgene (www.addgene.org) (Addgene plasmids 10962 and 10963) and have been described previously [11].

2.5. siRNA transfection

Mouse primary hepatocytes were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol using the following siRNAs (Sigma–Aldrich Co., St. Louis, MO, USA): siSIRT1 5′-AAAUGUCUCCACGAACAGC(dT)(dT); siPPARα: 5′-GAUCGGAGCUGCAAGAUUC(dT)(dT); Scramble 5′-AAGCUUCAUAAGGCGCAUAGC(dT)(dT) [12–14].

2.6. Virus construction and infection

PGC-1α adenoviruses containing mouse wild type (WT) and NR binding domain mutant (L2L3M) were prepared and propagated as previously described [15,16]. Cells were infected with PGC-1α WT, L2L3M or a control adenovirus expressing either green fluorescent protein (GFP-Ad) or LacZ-Ad at MOI 1. The infected cultures were maintained for various time points as indicated before collection or further treatments. Transduction efficiency of the hepatocytes with the used amounts of adenovirus is near 100% based on the observation of the cells infected with the GFP-Ad virus.

2.7. Mammalian two-hybrid assay

GAL4-mPXR and VP16-PGC1α vectors were constructed by fusing the complete sequence of mouse PGC-1α and the ligand binding domain of mouse PXR (371–1297 bp) in frame with VP16 or GAL4 domains of pBIND and pACT plasmids for Two-Hybrid Assay (Promega, Madison, Wisconsin, USA). HepG2 cells were co-transfected with both plasmids and treated with PCN and pyruvate as indicated. Low glucose was used to further activate SIRT1. In addition, a combination of negative controls was used to normalize samples against the background signal. Cells were collected after 48 h and luciferase activity was measured.

2.8. Protein Isolation, immunoprecipitation and antibodies

Nuclear protein fractions of mouse primary hepatocytes were prepared as described [17]. FLAG-tagged proteins from COS-1 cells were isolated according to the manufacturer's protocol using ANTI-FLAG M2 affinity gel (Sigma–Aldrich Co., St. Louis, MO, USA). Proteins were separated on 10% (mouse primary hepatocytes) or 12% (COS-1) SDS-polyacrylamide gel, transferred on to a polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA, USA) and incubated with appropriate antibodies. The following antibodies were used: mouse anti-HA-Tag(6E2) antibody (1:500 dilution, Cell Signaling Technology, Danvers, MA, USA); mouse anti-FLAG-Tag antibody (1:500 dilution, Abcam, Cambridge, UK),

HRP-goat anti-mouse IgG (1:20,000 dilution, Zymed), mouse anti-acetylated lysine IgG2a antibody (1:250 dilution, Cell Signaling Technology, cat.# 9681). Anti-PXR (1:500, cat. #616901, BioLegend, San Diego, CA, USA), anti-SIRT1 (1:750, cat. #D60E1 Cell Signaling Technology, Inc), HRP-goat anti-rabbit IgG (1:20,000, Santa Cruz Biotechnology Inc. CA, USA). The immunoreactive bands were visualized by Chemiluminescent Peroxidase Substrate (CPS) 1 reaction (Sigma–Aldrich Co., St. Louis, MO, USA) using LAS-3000 detection system (FujiFilm, Tokyo, Japan).

2.9. RNA preparation and quantitative PCR

Total RNA was isolated using Tri-Reagent (Sigma–Aldrich Co., St. Louis, MO, USA) according to the manufacturer's protocol for monolayer cells. One μg of each RNA sample was transcribed to produce cDNA using p(dN)6 random primers (Roche, Basel, Switzerland) and M-MLV reverse transcriptase RT (Promega, Madison, Wisconsin, USA). RT negative samples were prepared similarly but without reverse transcriptase enzyme. FastStart universal SYBR green master mix containing passive reference dye ROX (Roche, Basel, Switzerland) was used for real-time quantitative PCR (qPCR). Fluorescence values of the qPCR products were corrected with fluorescence signals of ROX. The RNA levels of genes of interest were normalized against the 18S control levels using the comparative CT (ΔΔCT) method. All standard primers were manufactured by Sigma–Aldrich Co. (St. Louis, MO, USA) and were as follows: (Forward/Reverse, presented in 5′ to 3′ orientation) CYP3A11:AAGCATTGAGGAGGATCAC/CCATGTCGAATTTCCATAAACC; PXR: GATGGAGGTCTTCAAATCTGCC/GGCCCTTCTGAAAAACCCCT; SIRT1 GCTGACGACTTCGACGACG/TCGGTCAACAGGAGGTTGTCT 18S: CGCCGCTAGAGGTGAAATTC/CCAGTCGGCATCGTTTATGG; PPARα: AGAGCCCCATCTGTCCTCTC/ACTGGTAGTCTGCAAAACCAAA;

G-6-Pase: CATCAATCTCCTCTGGGTGG/TGCTGTAGTAGTCGGTGTCC. Primer sequences of PXR, SIRT1 and PPARα were obtained from PrimerBank [18]. CYP7A1 (Mm00484152_m1) probe was purchased from Applied Biosystems (Carlsbad, CA, USA).

3. Results

3.1. PGC-1α upregulates PXR expression

To study the effect of metabolic state on PXR gene expression, mRNA levels were measured in the livers of rats starved for 24 and 48 h. PXR expression was induced 1.9 and 1.6 fold by 24 or 48 h fasting, respectively (Fig. 1a). Increased secretion of glucagon is one of the hallmarks of fasting. Therefore we investigated whether glucagon can affect expression of PXR in mouse primary hepatocytes. First however, as a control, we measured expression of a well established glucagon inducible gene: glucose-6-phosphatase (G-6-Pase). As expected G-6-Pase was efficiently induced by glucagon (Fig. 1b). PXR was modestly, but significantly induced 3 and 24 h after the hormone treatment (Fig. 1b). Furthermore, PXR protein level was elevated by glucagon at 48 and 72 h after treatment (Fig. 1c). Fasting and glucagon strongly induce hepatic expression of coactivator PGC-1α [19] which in turn regulates central metabolic pathways. We studied whether upregulation of this coactivator would affect PXR expression. Mouse primary hepatocytes were transduced with PGC-1α-Ad or control GFP-Ad virus and temporal mRNA changes were measured up to 72 h by quantitative RT-PCR. Expression of G-6-Pase, an established PGC-1α target gene, was measured as a control. As expected, G-6-Pase was strongly induced by PGC-1α-Ad (Fig. 1d). PXR mRNA was elevated by PGC-1α-Ad at all time points compared to GFP-Ad, reaching a 3 fold induction after 72 h (Fig. 1d). Also PXR protein level was elevated at 72 h after treatment with PGC-1α-Ad (Fig. 1e).

PGC-1α is not able to directly bind to DNA, but interacts with several nuclear receptors and other transcription factors. To further characterize the mechanism of PXR regulation by PGC-1α we transduced hepatocytes with L2L3M mutant of PGC-1α-Ad that lacks functional nuclear receptor interaction domain [16]. The L2L3M mutant was unable to induce expression of PXR, suggesting that the effect of PGC-1α is mediated by a nuclear receptor (Fig. 2a). PPARα expression is induced by PGC-1α and mediates many effects of fasting [20]. Moreover PPARα regulates PXR gene expression [21]. Therefore, we examined involvement of PPARα in PGC-1α mediated regulation of PXR by using PPARα knockdown. PPARα siRNA downregulated expression of PPARα mRNA by 40% in control cells transducted with LacZ-Ad (Fig. 2b). Consequently, an established PPARα target gene lipoprotein lipase was reduced by 63% (data not shown). As expected PPARα and PXR were induced by PGC-1α-Ad (Fig. 2b). This response was efficiently reduced by PPARα knockdown indicating involvement of PPARα in PGC-1α mediated upregulation of PXR (Fig. 2b).

3.2. Pyruvate attenuates PXR coactivation by PGC-1α

We next studied the effect of increased PGC-1α expression level on PXR mediated gene regulation. We transduced mouse primary hepatocytes with PGC-1α-Ad to specifically increase PGC-1α expression. Furthermore, the cells were treated with the PXR ligand PCN to activate PXR. PXR mRNA was induced two fold by PGC-1α-Ad, but was not affected by PCN (data not shown). PXR target gene *Cyp3a11* was induced 7 fold by PCN alone while PGC-1α-Ad alone had an insignificant effect (Fig. 3). GFP-Ad had no effect (data not shown). However, PCN and PGC-1α-Ad had strong synergistic effect and combined treatment induced CYP3A11 mRNA more than 15 fold (Fig. 3).

SIRT1 is known to modify PGC-1α mediated coactivation [7]. Pyruvate, an intermediate compound of glucose metabolism, is elevated by fasting and induces SIRT1 protein expression [7]. Therefore we investigated whether pyruvate affects PXR mediated gene expression and coactivation by PGC-1α. Pyruvate did not affect CYP3A11 induction by PCN alone. In contrast, it attenuated the synergistic effect of PGC-1α-Ad and PCN (Fig. 3). PXR mRNA level was not affected by pyruvate (data not shown). In contrast, PGC-1α expression was induced four fold by pyruvate (data not shown), what is in agreement with another report [22]. Therefore, attenuation of CYP3A11 expression by pyruvate cannot be explained by lower levels of PGC-1α or PXR. Altogether, these data suggest that pyruvate inhibits PGC-1α ability to coactivate PXR.

Ligand activated PXR represses expression of several genes. Mechanisms of PXR mediated gene repression have been studied most extensively for *Cyp7a1* gene [2,23]. Therefore we investigated if SIRT1 activation by pyruvate affects repression *Cyp7a1* by ligand activated PXR. CYP7A1 mRNA was significantly downregulated by PCN with and without PGC-1α-Ad (Fig. 3). Pyruvate had no effect of CYP7A1 downregulation by PCN indicating that repression and induction by PXR are mechanistically different. However, in PGC-1α-Ad samples without PCN expression of CYP7A1 was downregulated by pyruvate, which is in agreement with other reports on CYP7A1 regulation [24].

3.3. SIRT1 modifies PXR coactivation by PGC-1α

Cellular metabolism is broadly affected by pyruvate levels, and not all of its effects are mediated by SIRT1. This prompted us to investigate whether the modulation of PXR coactivation by PGC-1α involves SIRT1. Treatment of primary hepatocytes with 1 mM pyruvate elevated SIRT1 protein level about two fold, while

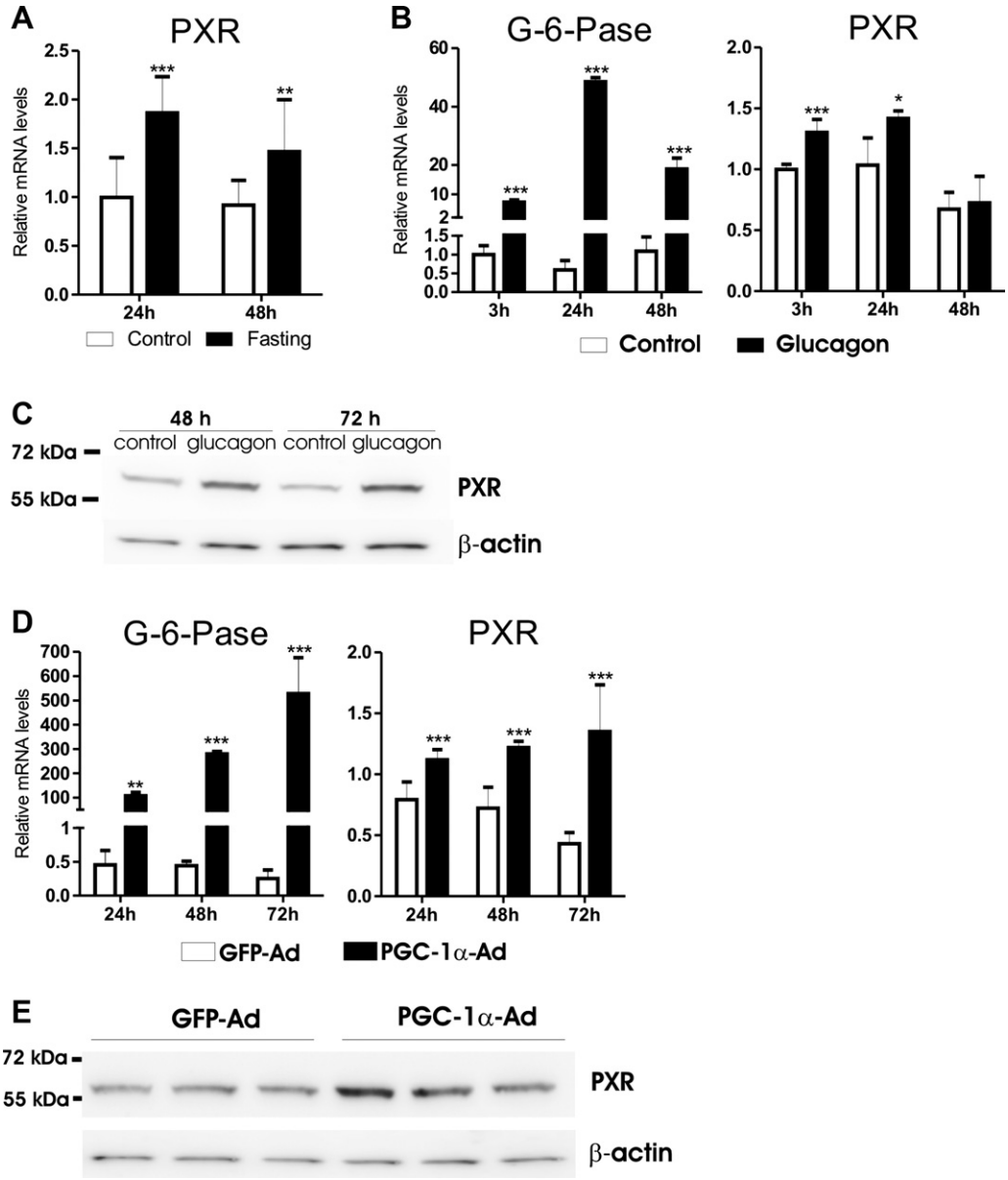

Fig. 1. Fasting, glucagon and PGC-1α-Ad induce PXR mRNA expression. (A) Male Wistar rats were fasted for 24 or 48 h and sacrificed. Livers were isolated immediately and snap frozen in liquid nitrogen. Random liver sections of minimum 80 mg were used for mRNA isolation and subsequent QPCR analysis. Values are presented relative to the mean of control sample (24 h, Fed) and represent means + SD, n is 4–8 per point, Student two tailed t-test $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Mouse primary hepatocytes were treated with 5 μg/ml of porcine glucagon and collected at indicated time points. mRNA levels were measured with QPCR. Values represent means + SD, $n = 3$, Student two tailed t-test $^{*}P < 0.05$; $^{***}P < 0.001$. (C) Immunoblotting of PXR in nuclear protein fraction of mouse primary hepatocytes treated with 5 μg/ml of glucagon for 48 and 72 h. β-actin was detected as a loading control. (D) Mouse primary hepatocytes were transduced with PGC-1α-Ad or control GFP-Ad adenoviruses (MOI = 1) or left untreated and collected at the indicated time points. mRNA was measured with QPCR. Data are represented as fold change compared to untreated controls at a given time point. Bars representing untreated control samples are not shown. Values represent means + SD, $n = 4$, (ANOVA, Bonferroni post-test) and indicate that $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Immunoblotting of PXR in nuclear protein fraction of mouse primary hepatocytes transduced with PGC-1α-Ad or control GFP-Ad (MOI = 1) adenoviruses for 72 h. β-actin was detected as a loading control.

SIRT1 siRNA abolished this effect (Fig. 4a). The previous experiment indicated that pyruvate specifically interferes with the induction of CYP3A11 by PXR in the presence of elevated PGC-1α. Therefore primary hepatocytes were transduced with PGC-1α-Ad. In addition the cells were treated with siRNA targeting SIRT1 (Fig. 4b). As before, treatment with PCN efficiently induced CYP3A11, while pyruvate reduced this response (Fig. 4b). PXR expression was not affected by SIRT1 siRNA (data not shown). SIRT1 knockdown prevented the inhibitory effect of pyruvate on CYP3A11 induction by PCN in presence of PGC-1α-Ad indicating a mechanism involving SIRT1. Downregulation of CYP7A1 by PCN was not affected by pyruvate or SIRT1 siRNA (Fig. 4b.).

3.4. SIRT1 interferes with the interaction of PXR and PGC-1α and forms complexes with PXR

We used mammalian two-hybrid assay to study whether pyruvate and SIRT1 interfere with the direct interaction between PXR and PGC-1α. Sequences of ligand binding domain of mouse PXR and full lenght PGC-1α were cloned into two-hybrid assay vectors to generate fusion proteins with the DNA-binding domain of GAL4 and the activation domain of VP16 (GAL4-mPXR and VP16-PGC1α, respectively). HepG2 cells were cotransfected with both fusion plasmids and treated with PCN alone or in combination with pyruvate. Low glucose was used to further activate SIRT1 [7]. PCN strongly stimulated luciferase activity indicating ligand dependent

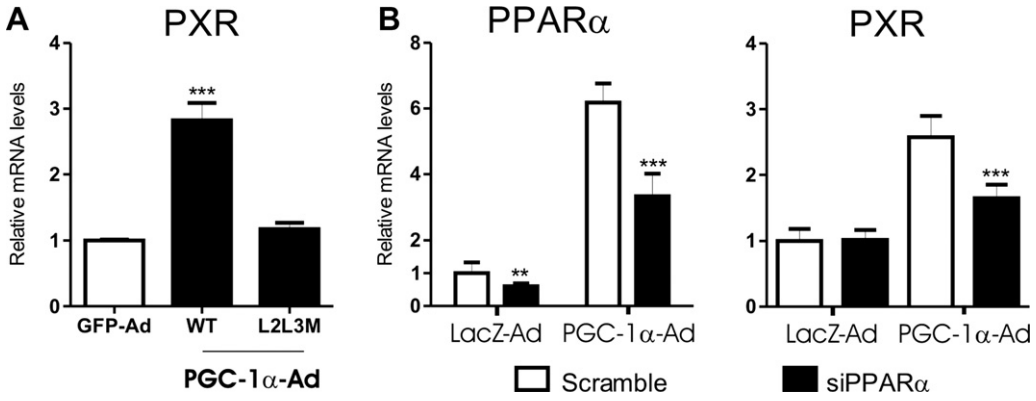

Fig. 2. Induction of PXR gene expression by PGC-1α-Ad involves PPARα. (A) Mouse primary hepatocytes were transduced with wild type (WT) or L2L3 M mutant of PGC-1α-Ad or GFP-Ad at MOI = 1. Cells were collected after 48 h and mRNA expression was analyzed with QPCR. Data are represented as fold change compared to GFP-Ad controls. * depicts statistical significance compared to GFP-Ad samples. Values represent means + SD, $n = 4$, ANOVA, Bonferroni post-test, *** $P < 0.001$ vs. GFP-Ad. (B) Mouse primary hepatocytes were transfected with 150 pmol/μl of control (Scramble) or PPARα siRNA 5 h before transduction with PGC-1α-Ad or LacZ-Ad and collected after 48 h. mRNA levels were measured with QPCR. Values are represented relative to LacZ-Ad control sample (Scramble) and represent means + SD, $n = 3$. ** $P < 0.01$, *** $P < 0.001$, siPPARα vs. scramble, Student two tailed t-test.

PGC-1α recruitment by PXR. Pyruvate efficiently abrogated PCN induction, implying that interaction between PGC-1α and ligand activated PXR was interrupted (Fig. 5a). Consequently we studied direct effect of SIRT1 on PGC-1α and PXR interaction. HepG2 cells were introduced with GAL-mPXR and VP16-PGC-1α fusion plasmids and treated with PCN or as controls with DMSO. In addition the cells were cotransfected with expression vectors bearing sequences of either wild type or mutated SIRT1 (SIRT1WT and SIRT1-G261A, respectively). SIRT1-G261A poorly interacts with PGC-1α [11]. Neither the wild type nor the mutant SIRT1 plasmid had any effect on luciferase activity on DMSO treated samples. In contrast, SIRT1WT attenuated luciferase signal in PCN treated cells. SIRT1-G261A did not differ significantly from the control (Fig. 5b). This data indicate that SIRT1 interferes with PXR coactivation by PGC-1α by modulating the interaction between the two factors.

Because of the well documented interaction between SIRT1 and PGC-1α, it is reasonable to assume that deacetylation of PGC-1α is at least partially responsible for the observed phenomenon. However, PXR could also be targeted. Therefore we examined whether SIRT1 interacts with PXR. COS-1 cells were transfected with FLAG tagged human PXR alone or in combination with HA tagged SIRT1. Proteins were immunoprecipitated with anti-FLAG antibody and subjected to Western blotting. Anti-FLAG immunoreactive protein, corresponding in size with PXR, was detected in all samples transfected with FLAG-PXR. Furthermore, in samples cotransfected with HA-SIRT1 we could detect anti-HA reactive band corresponding in size with SIRT1 (Fig. 5c). This indicates that SIRT1 is able to interact with PXR. No acetylation of PXR was detected with anti-acetylated lysine antibody.

4. Discussion

Research during the recent few years has revealed originally unsuspected, broad roles of the xenobiotic-sensing receptor PXR beyond drug metabolism and transport. Several reports have shown that PXR is also involved in regulation of energy homeostasis. PXR ligands reduce expression of key enzymes in gluconeogenesis phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, increase fatty acid uptake by inducing Cd36 in hepatocytes and repress β-oxidation related genes [25–28]. Our current work suggests that there is a reciprocal relationship and nutrient state affects the expression of PXR as well as its interaction with coactivators.

Thus far only one microarray study has suggested that fasting promotes PXR expression in mouse liver, but the phenomenon has not been studied in detail [29]. Our experiments confirm that fasting indeed induces PXR expression. Moreover, we utilized rats in fasting experiments and thus show that the induction is not species specific. Glucagon plays a major role in maintaining normal

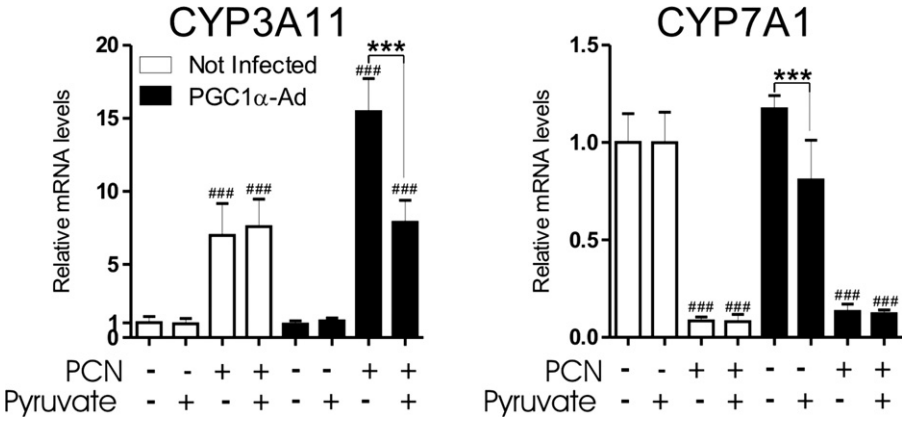

Fig. 3. Effect of PCN, PGC-1α-Ad and pyruvate on CYP3A11 and CYP7A1 mRNA expression. (A) Mouse primary hepatocytes were transduced with PGC-1α-Ad at MOI = 1 for 24 h and treated for additional 12 h with 10 μM PCN in medium supplemented with 1 mM pyruvate as indicated. Cells were collected and mRNA levels were measured with QPCR. * represents statistically significant values between control and pyruvate treated cells, # indicates statistical significance between PCN and as control DMSO treated samples. Values represent means + SD, $n = 3$, ANOVA, Bonferroni post-test, *** $P < 0.001$, ### $P < 0.001$.

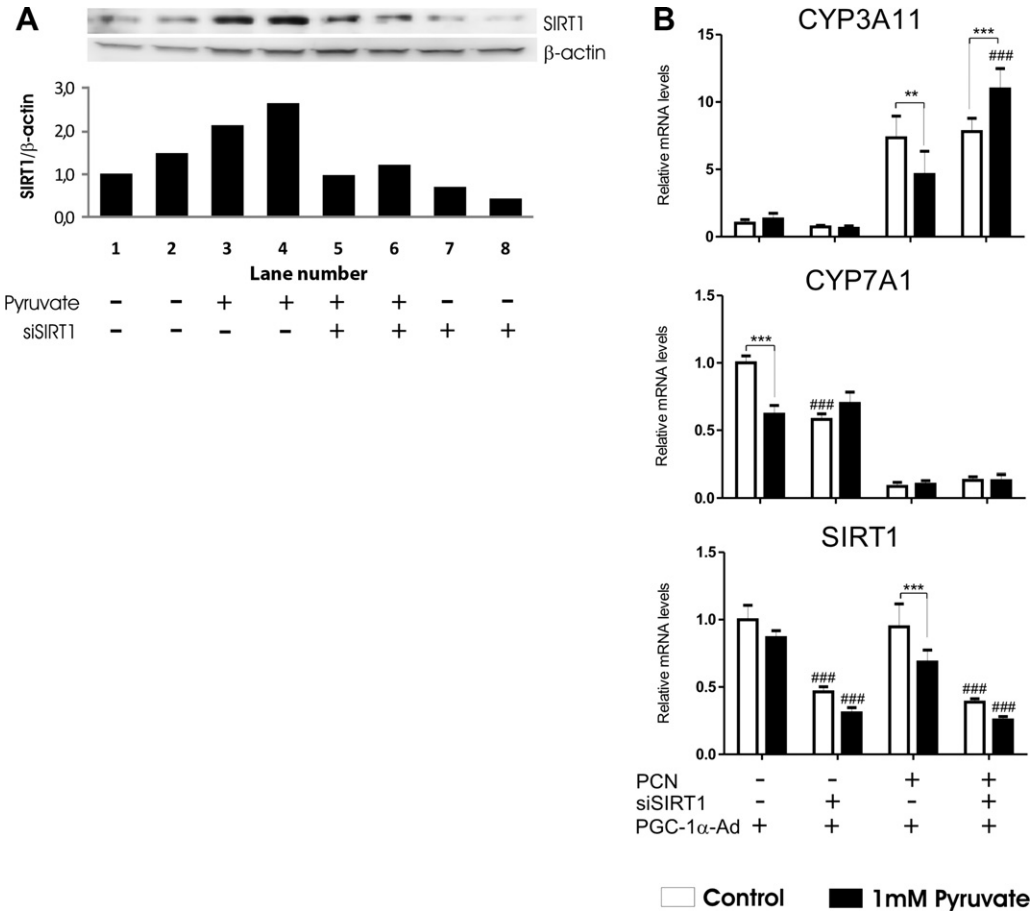

Fig. 4. The effect of pyruvate on CYP3A11 expression is mediated by SIRT1. (A) Immunoblotting of SIRT1 protein in nuclear fraction. Mouse primary hepatocytes were transfected with 150 pmol/μl of control (Scramble) or SIRT1 siRNA for 5 h before treatment with 1 mM pyruvate and collected 48 h later. β-actin was detected as a loading control. Ratio of SIRT1 signal to β-actin in corresponding lanes is shown. Values are normalized to lane number 1. (B) Mouse primary hepatocytes were transduced with PGC-1α-Ad before transfection with 150 pmol/μl of control (Scramble) or SIRT1 siRNA. After 24 h incubation cells were treated for additional 12 h with 10 μM PCN and 1 mM pyruvate as indicated. mRNA changes were measured with QPCR. * depicts statistical significance between control and pyruvate treated cells, # represents statistical significance between corresponding Scramble and siSIRT1 samples. Values represent means + SD, $n = 3$, ANOVA, Bonferroni post-test, ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$.

blood glucose concentrations under fasting condition and regulates numerous liver functions. Treatment of mouse primary hepatocytes with glucagon induced PXR indicating involvement of this hormonal pathway in regulation of PXR. Together, these results suggest that energy sensing factors are involved in transcriptional regulation of *Pxr* gene. Fasting through an altered insulin/glucagon ratio induces hepatic PGC-1α expression and PGC-1α in turn controls energy metabolism and other critical liver functions [19]. We hypothesized that PGC-1α could facilitate also *Pxr* transcription. Indeed transduction of hepatocytes with PGC-1α−Ad increased expression of PXR in a nuclear receptor dependent manner. Knocking down PPARα shows that the induction of PXR by PGC-1α is mediated at least partially by this nuclear receptor. Indeed, PPARα is known to regulate PXR expression [21]. The regulatory steps may involve both upregulation of PPARα expression by PGC-1α and furthermore PPARα coactivation by PGC-1α.

In addition to the induction of PXR expression by fasting, other energy metabolism related factors can also regulate PXR function. Indeed, coactivation of PXR by insulin regulated FOXO1 has been described to be one such factor [30]. PGC-1α is induced by fasting and coactivates PXR. We therefore suspected that PGC-1α could enhance PXR transactivation function upon starvation. In concordance with this hypothesis overexpression of PGC-1α potentiated CYP3A11 induction by PCN. However, we discovered that this simple relationship is complicated by posttranslational modifications.

Intracellular concentration of pyruvate, a key metabolite of glycolysis, increases during fasting. This leads to activation of SIRT1, subsequent deacetylation of PGC-1α and induction of gluconeogenic genes but repression of glycolytic genes [7]. On the other hand, PGC-1α mediated regulation of mitochondrial genes is not affected by SIRT1 [8]. Therefore SIRT1 can differently modulate various PGC-1α mediated processes. We investigated the effect of SIRT1 on PXR coactivation by PGC-1α. Pyruvate prevented synergistic induction of CYP3A11 by PGC-1α overexpression and PCN treatment, but had no effect on PCN treatment alone. This effect was lost by SIRT1 knockdown. Since PXR expression was not affected by SIRT1 this data indicates that SIRT1 attenuates PXR coactivation by PGC-1α. It is possible, that this effect, at least in part, is caused by reduced transcriptional activity of PGC-1α. Indeed it has been shown that SIRT1 reduces transcriptional activity of PGC-1α fused to GAL4 DNA binding domain [11]. However, our results with mammalian two-hybrid assay indicate that SIRT1 interferes with PXR interaction with PGC-1α.

PGC-1α is a well established target of SIRT1 deacetylase [7,11]. This relationship could explain the observed effect on PGC-1α-PXR interaction. However, we observed that SIRT1 directly interacts with PXR. This is not totally unexpected since many nuclear receptors have already been shown to be targeted by SIRT1. Actually, a very recent study by Biswas et al. reported deacetylation of PXR by SIRT1 [31]. They also suggested that deacetylation promotes PXR mediated lipogenesis. We did not detect acetylation

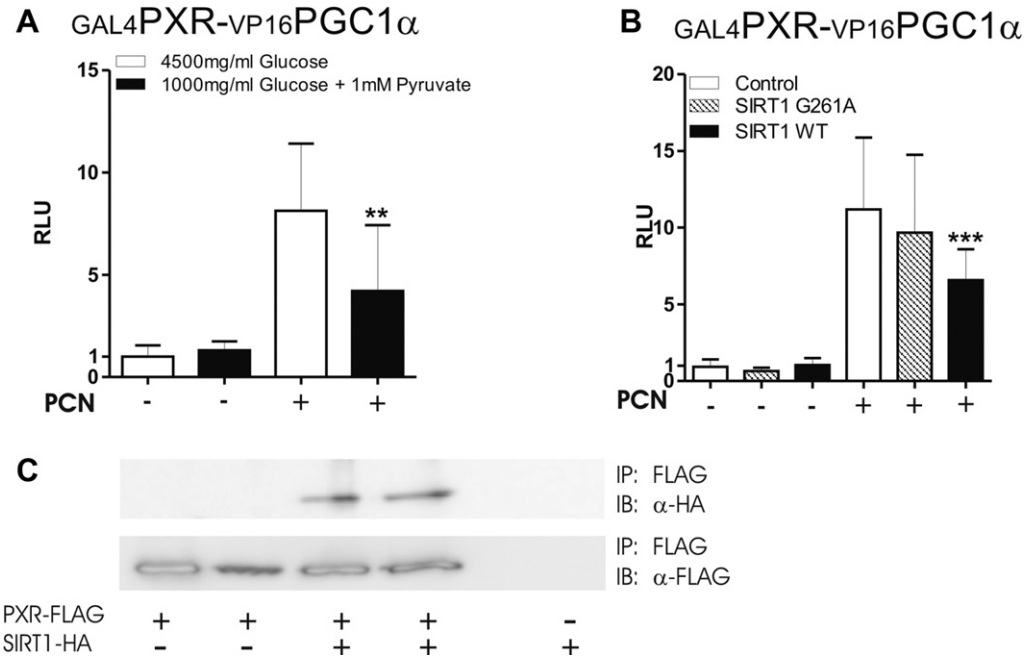

Fig. 5. Pyruvate and SIRT1 interfere with the interaction between PXR and PGC-1α. (A) Mammalian two-hybrid assay was performed in HepG2 cells. Cells were transfected with GAL4-mPXR and VP16-PGC-1α fusion plasmids for 48 h and treated with 10 μM PCN or vehicle (DMSO) only. 1 mM pyruvate and lower glucose content were used for activation of SIRT1. Values represent mean luciferase activity + SD, $n = 16$, Student two tailed t-test ** $P < 0.01$. B) HepG2 cells were cotransfected with GAL4-mPXR and VP16-PGC-1α fusion plasmids in addition to SIRT1 wild type (SIRT1 WT) or SIRT1 G261A mutant. Cells were treated with 10 μM PCN or vehicle (DMSO) only for 48 h, collected and luciferase activity was measured. Statistical significance to controls without SIRT1 vector is shown. Values represent mean luciferase activity + SD, $n = 16$, ANOVA, Bonferroni post-test *** $P < 0.01$ (Representative of two independent experiments) (C) SIRT1 directly interacts with PXR. COS-1 cells were transfected with FLAG-hPXR and HA-SIRT1 expression vectors, treated with 20 μM rifampicin and collected 48 h later. Proteins were immunoprecipitated with anti-FLAG antibody. Visualized bands correspond in size to PXR and SIRT1. Representative of two independent experiments.

of PXR protein in COS-1 cells. The reason for this discrepancy between our study and Biswas et al. [31] is unknown, but could be related to the different cell lines. Nevertheless, our current results support the notion that SIRT1 modulates PXR function; however, the effect may depend on the target genes.

Altogether, the mechanisms regulating PXR expression and function by fasting are rather complex and seemingly opposite to each other. While PGC-1α increases PXR expression and transactivation function, SIRT1 opposes PXR coactivation by PGC-1α. This crosstalk apparently restricts the effect of fasting on PXR mediated regulation of drug metabolism. In addition to drug metabolism, PXR is known to regulate pleiotropic cell functions [32]. It is apparent that selectivity of PXR mediated regulatory processes has to be carefully controlled and fine tuned in different physiological conditions. Crosstalk of PGC-1α and SIRT1 in regulation of PXR function may be part of that process.

Interestingly, downregulation of CYP7A1 by PCN was not affected by pyruvate. CYP7A1 downregulation has been suggested to be mediated by PXR interference of HNF4α coactivation by PGC-1α through competition for common coactivator [2]. However, later studies by Li and Chiang suggested that the HNF4α-PGC-1α interaction is rather blocked by PXR interaction with HNF4α [23]. Since pyruvate was able to decrease PXR and PGC-1α interaction but did not affect CYP7A1 downregulation by PCN these results suggest that the latter hypothesis is more plausible.

In conclusion, we show that energy state modulates PXR mediated signaling and regulation of drug metabolism. Furthermore, we identify PGC-1α and SIRT1 as two energy sensing factors involved in this process.

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