Activation of nuclear receptor PXR impairs glucose tolerance and dysregulates GLUT2 expression and subcellular localization in liver

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Abstract

Pregnane X receptor (PXR) is a nuclear receptor that senses chemical environment and is activated by numerous clinically used drugs and environmental contaminants. Previous studies have indicated that several drugs known to activate PXR appear to induce glucose intolerance. We now aimed to reveal the role of PXR in drug-induced glucose intolerance and characterize the mechanisms involved. We used PXR knockout mice model to investigate the significance of this nuclear receptor in the regulation of glucose tolerance. PXR ligand pregnenolone-16α-carbonitrile (PCN) impaired glucose tolerance in the wildtype mice but not in the PXR knockout mice. Furthermore, DNA microarray and bioinformatics analysis of differentially expressed genes and glucose metabolism relevant pathways in PCN treated primary hepatocytes indicated that PXR regulates genes involved in glucose uptake. PCN decreased the expression of glucose transporter 2 (GLUT2) in mouse liver and in the wildtype mouse hepatocytes but not in the PXR knockout cells. Data mining of published chromatin immunoprecipitation-sequencing results indicate that Glut2 gene is a direct PXR target. Furthermore, PCN induced internalization of GLUT2 protein from the plasma membrane to the cytosol in the liver in vivo and repressed glucose uptake in the primary hepatocytes. Our results indicate that the activation of PXR impairs glucose tolerance and thus PXR represents a novel diabetogenic pathway. PXR activation dysregulates GLUT2 function by two different mechanisms. These findings may partly explain the diabetogenic effects of medications and environmental contaminants.

Keywords: PXR, GLUT2, GCK, impaired glucose tolerance, diabetes
1. **INTRODUCTION**

Pregnane X receptor (PXR, systematic name NR1I2) is a nuclear receptor that plays a major role in sensing the chemical environment [1]. Originally, PXR was recognized to regulate drug-metabolizing enzymes and drug transporters, i.e. mechanisms involved in the detoxification of xenobiotics, with a major role in the drug-drug interactions. Subsequently, this xenobiotic receptor was found to possess much broader regulatory functions and cumulating evidence during the last ten years has established PXR as a regulator of hepatic glucose and lipid metabolism [2,3].

PXR is activated by several clinically used drugs known to induce hyperglycemia, including rifampicin, several statins, phenytoin and some antiretroviral drugs [4]. However, the causal relationship between PXR activation and hyperglycemia has not been established in most cases. We recently reported that treatment with rifampicin and pregnenolone-16α-carbonitrile (PCN), the human and rat PXR ligands, respectively, induce impaired glucose tolerance (IGT) in humans and rats [5]. Besides being PXR ligands, these compounds are structurally and functionally unrelated strongly suggesting involvement of PXR in the observed detrimental effects on glucose tolerance. Furthermore, a PXR ligand atorvastatin increased area under the concentration curve (AUC) value in glucose tolerance test in the high-fat diet (HFD) fed rats, but PXR-nonactivating pravastatin did not have a similar effect [6]. Finally, in a clinical study investigating the effects of the PXR activating herbal remedy St. John’s wort, IGT was found to persist six weeks after treatment [7]. Thus, strong pharmacological evidence suggests that PXR activation impairs glucose tolerance. However, direct evidence indicating involvement of PXR in the drug-induced IGT has been thus far lacking.
Mechanisms behind PXR mediated IGT are still unclear. PXR is mainly expressed in the liver and intestine and therefore these tissues are the main candidates to mediate the effects of PXR activation. Liver is one of the key organs in glucose metabolism and it plays an important role in maintenance of blood glucose level within the narrow normal range. We have previously reported that PXR ligand PCN represses glucose transporter 2 (GLUT2, SLC2A2) and glucokinase (GCK; EC 2.7.1.2) in rat liver and mouse hepatocytes [5]. GLUT2 is the major hepatic glucose transporter facilitating the glucose influx when plasma glucose is high and therefore acts as a part of the hepatic glucose sensing system [8]. Phosphorylation of glucose to glucose-6-phosphate by GCK is the first step in glucose utilization, and similar to GLUT2, GCK facilitates glucose uptake during the postprandial increase of plasma glucose [9]. Disturbance in the hepatic glucose sensing and utilization mechanisms may potentially explain PXR induced IGT.

In the current study we applied in vivo and in vitro mouse models to investigate the role of PXR in the regulation of glucose tolerance. Using PXR knockout mice we establish that this nuclear receptor is indispensable for PCN induced glucose intolerance. Furthermore, we show that PXR activation disturbs hepatic glucose transporter GLUT2 function by two distinct mechanism: by affecting GLUT2 expression and by changing GLUT2 subcellular localization.
2. MATERIALS AND METHODS

2.1. Materials

The chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Animals

The animal experiments were approved by the National Animal Experiment Board, Finland (License number ESAVI/6357/04.10.07/2014) following EU legislation (Directive 2010/63/EU). PXR knockout mice (9100-M, C57BL/6-Nr1i2tm3Arte) were purchased from Taconic Biosciences (Ejby, Denmark). Wildtype C57BL/6 mice (WT) were bred in Laboratory Animal Centre, University of Oulu, Finland. The mice were kept in plastic cages with free access to tap water and regular chow in a room with a controlled 40% humidity and a temperature of 22 °C. A controlled 12-hour environmental light cycle was maintained.

2.3. PCN treatment and oral glucose tolerance test (OGTT) in mice

8-weeks old male C57BL/6 or 8-10 weeks old male PXR knockout mice, weight 23-31 g, were treated with pregnenolone-16α-carbonitrile (PCN) 50 mg/kg (Sigma Aldrich, St. Louis, MO, USA) dissolved in DMSO/corn oil, or with DMSO/corn oil as vehicle control i.p. once a day for four days. The used PCN concentration and treatment length was selected based on literature and our previous experience. Blood sample was taken from vena saphena after 4 h fast and the mice were given glucose 2g/kg by oral gavage. Then the mice, 9-10 per group, were anesthetized using fentanyl citrate and midazolam (Hameln Pharmaceuticals GmbH, Hameln, Germany), and blood samples were taken at 15, 30, 60, 90 and 120 minutes from vena saphena. Blood glucose
concentrations were measured using Precision Xceed Blood Glucose & Ketone Monitoring System (Abbott Laboratories, Abbot Park, IL, USA).

**2.4. PCN treatment of mice for RNA and protein measurements and for immunohistochemistry**

8-weeks old, male C57BL/6 mice, weight 18-26 g, were treated with PCN 50 mg/kg dissolved in DMSO/corn oil, or with DMSO/corn oil as a vehicle control i.p. once a day for four days. The mice were fasted for 4 hours (except for 12 hours in the experiment in which the protein lysates were extracted), after which some mice were given glucose 2g/kg by oral gavage. One hour after glucose administration the mice, 5-8 per group, were sacrificed by CO$_2$ inhalation and the tissues were harvested and frozen in liquid nitrogen for isolation of RNA or protein or fixed in formalin solution for immunohistochemistry.

**2.5. Isolation of mouse primary hepatocytes**

Mouse primary hepatocytes were isolated from untreated 8-week-old male C57BL/6 or PXR$^{-/-}$ mice. For perfusion, the mouse was anesthetized with medetomidine (Orion Pharma, Espoo, Finland) and ketamine (Hameln Pharmaceuticals GmbH, Hameln, Germany). The abdomen was opened longitudinally and *vena cava* was exposed. The catheter was inserted into *superior vena cava* close to the heart and a ligature was placed around *inferior vena cava*. Liver portal vein was cut open and the pump was immediately turned on for perfusion. The liver was perfused (flow rate 2.5 ml/min) with 35 ml of preperfusion solution (Hank’s Balanced Salt Solution (Gibco 14175, Thermo Fisher Scientific, Waltham, MA, USA) with 0.0375 % NaHCO$_3$, 10 mM HEPES, 0.5 M EDTA, pH 7.4) and with 35 ml perfusion solution (William’s E medium (Sigma Aldrich, St. Louis,
MO, USA) with 10 mM HEPES, pH 7.4, and collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA) 80 U/ml) as well. The solutions used for perfusion were circulated through oxygen atmosphere and +37 °C water bath. After perfusion, the liver was removed and placed on a petri dish containing 30 ml plating medium (William’s E with 10% FBS, supplemented with dexamethasone 10 ng/ml, gentamycin 10 µg/ml and insulin-transferrin medium supplement [containing 5 mg/L insulin, 5 mg/L transferrin and 5 µg/L sodium selenite]). The hepatocytes were released gently by using comb and filtrated through 100 µm cell strainer (Falcon, BD, Franklin Lakes, NJ, USA). The cells were centrifuged at 50 x g for 2 min, and seeded on cell culture dishes (2 x 10^6 cell per 6-well for RNA isolation and 1.2 x 10^5 cells per 24-well for glucose uptake measurements). The cells were plated in plating medium. After 3 hours the hepatocytes were attached and the medium was changed to maintenance medium (Plating medium without FBS). The hepatocytes were incubated overnight at +37 °C with 5 % CO₂ and used for experiments. The cultures were always made in triplicate if not otherwise stated.

2.6. Glucose withdrawal experiments

Mouse primary hepatocytes were first cultured in glucose free DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS for 18 hours and then supplemented with 1 g/L glucose for additional 6 hours. Furthermore, the cells were treated with 10 µM PCN (dissolved in DMSO) for the whole 24-hour period or exposed to the vehicle control (6 per group). Additional control groups without PCN were incubated in DMEM containing 10 % FBS and 1 g/L glucose, or glucose free DMEM (with 10 % FBS) for the entire 24-hour experiment (3 per group). After the experiment the cells were collected and used for RNA isolation.
2.7. RNA extraction and quantitative PCR

Total RNA was isolated from mice liver tissue or from mice primary hepatocytes using RNAzol (Sigma Aldrich, St. Louis, MO, USA). Complementary DNA was made from 1 µg RNA using random hexamer primer (Roche, Basel, Switzerland) and reverse transcriptase enzyme (Thermo Fisher Scientific, Waltham, MA, USA). cDNAs were diluted 1:10 (for target genes) and 1:1000 (for 18S control), and 2 µl of cDNA dilution was used for 20 µl reaction. Quantitative PCR (QPCR) was performed in duplicates with Fast start universal SYBR green master mix (Roche, Basel, Switzerland) and Applied Biosystem 7300 Real time PCR instrument (Applied Biosystems, Foster City, CA, USA). The primer sequences are presented in Table 1. The fluorescence values of the QPCR products were corrected with the fluorescence signals of the passive reference dye (ROX). The RNA levels of target genes were normalized against the 18S control levels using the comparative CT (ΔΔCT) method.

2.8. Immunoblotting

Total protein was isolated from PCN-treated or vehicle-treated mouse liver (7 per group). Liver samples were homogenized in lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, pH 7.5) with Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), 1 mM DTT and 50 mM NaF using TissueLyzer LT (Qiagen, Hilden, Germany) for 3 minutes. The homogenate was centrifuged 16 000 x g for 20 minutes at +4 °C and the protein concentration was determined from the supernatant. 30 µg of total protein samples were analyzed using immunoblotting with rabbit polyclonal anti-GLUT2 antibody.
2.9. **Immunohistological analysis**

To assess the expression of GLUT2 and GCK in mouse livers, the samples were fixed in neutral buffered 10% formalin solution and embedded in paraffin. Before application of the primary antibodies, the 5 µm thick sections of samples were heated in citrate buffer, pH 6.0 for 15 minutes. Rabbit polyclonal antibodies in dilution 1:100 for GLUT2 (07-1402, Merk Millipore Darmstadt, Germany) and for GCK (ac37796, Abcam, Cambridge, UK) were used. The chromogen used was 3,3’-diaminobenzidine (DAB) (Dako, Glostrup, Denmark). Negative control stainings were carried out by substituting the primary antibodies with nonimmune rabbit serum.

2.10. **Glucose uptake assay**

Protocol 1: The glucose uptake in mouse primary hepatocytes was measured by using commercial colorimetric glucose uptake assay kit (136955, Abcam, Cambridge, UK) according to the kit protocol. First, mouse primary hepatocytes were isolated and cultured overnight on 24-well plates as described above. Day after, the cells were washed with PBS and cultured overnight in DMEM with 1 g/L glucose, without FBS and with 10 µM PCN (dissolved in DMSO) or DMSO as a vehicle.
control. Next day, the cells were washed with PBS and starved for glucose in Krebs-Ringer-Phosphate-Hepes buffer (20 mM HEPES, 5 mM \( \text{KH}_2\text{PO}_4 \), 1 mM \( \text{MgSO}_4 \), 1 mM \( \text{CaCl} \), 136 mM \( \text{NaCl} \), 4.7 mM \( \text{KCl} \), pH 7.4) with 2 % BSA for 40 min at +37 °C. Control cells were lysed at this point. Treated cells were stimulated with DMEM (no FBS, no glucose) with or without insulin (1 \( \mu \text{M} \)) for 20 min at +37 °C to activate glucose transporters. Next, 1 mM 2-deoxyglucose (2-DG) was added to the cells for 20 min at +37 °C. The cells were washed with PBS and lysed. The enzymatic assay was continued according to the kit protocol. The absorbance at 412 nm was read 20 min after addition of the reaction mixture.

Protocol 2: The glucose uptake in mouse primary hepatocytes was verified with IRDye800-labeled 2-DG. The cells were treated similarly as in the protocol 1, but IRDye800-labeled 2DG (LI-COR, Lincoln, NE, USA) was used and the amount taken up by the hepatocytes was quantified using Odyssey infrared scanner (LI-COR, Lincoln, NE, USA). The assays in both protocols were done using three independent biological replicates.

2.11. Gene expression profiling and pathway analysis of differentially expressed genes

Gene expression profiling was done by using Affymetrix Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) as previously described [5]. Microarray data can be accessed at the NCBI Gene Expression Omnibus (GEO), with the accession number GSE106293. Confirmatory QPCR analyses of selected differentially expressed genes were generally in line with the expression profiling [5]. Data were analyzed with Ingenuity Pathways Analysis (IPA) (Qiagen, Hilden, Germany) to determine canonical pathways, functional networks and upstream regulators associated with differentially expressed genes between PCN-treated and control hepatocytes.
Fischer's exact test [a -Log(P-value)<0.05] was used to calculate whether there is a an over-representation of significantly up- or downregulated genes and a particular functional annotation compared to what is expected by chance alone.

2.12. Statistical analyses

Statistical and AUC analysis was performed using GraphPad Prism Software (GraphPad Software Inc., La Jolla, CA, USA). Unless stated otherwise, the comparison of means between two groups was done by Student’s t-test, whereas multiple groups were compared by one-way ANOVA followed by Dunnett’s post hoc test. Differences were considered significant at $p < 0.05$. 
3. RESULTS

3.1. PCN treatment impairs glucose tolerance in PXR dependent manner

Our previous studies have shown that PXR ligands induce glucose intolerance in rats and humans [5]. We now investigated if similar effect could be observed in mice. Mice were treated with i.p. injections of mouse PXR ligand PCN (50 mg/kg) or vehicle only for four days after which the animals were subjected to OGTT following 4-hour fasting. PCN treatment did not affect fasting glucose levels (Fig. 1a). In contrast, during OGTT PCN-treated mice displayed significant impairment of glucose tolerance (Fig 1a). Plasma glucose levels were elevated at the 30-, 60- and 90-min time points. Furthermore, both the total and the incremental AUC (0-120 min) were significantly increased in the PCN treated group indicating that the PXR ligand induces glucose intolerance in mice similar to the previous findings in humans and rats [5].

To further investigate the involvement of PXR in PCN-induced glucose intolerance we performed experiments in PXR knockout mice. Similar to the experiment in wildtype mice described above, PXR\(^{-/-}\) mice were treated with PCN or vehicle and subjected to OGTT. There was no difference in the fasting glucose level. Furthermore, there was no difference in glucose concentrations in any of time point during OGTT or in the total or incremental AUC values (Fig. 1b). These results indicate that PXR is crucial for the effect of PCN on glucose tolerance.

We also compared the glucose tolerance of the control groups of the wildtype and the PXR knockout mice. There was no difference in the plasma glucose level after 4h fasting. Interestingly, the PXR\(^{-/-}\) mice had significantly higher glucose levels at the 30-, 60- and 90-min time points.
during the OGTT and also the total AUC (0-120 min) was increased compared with the PXR$^{+/+}$ animals. However, there was no statistically significant difference in the incremental AUC (Fig. 1c).

### 3.2. PCN treatment represses hepatic genes involved in glucose sensing

In order to understand the mechanisms involved in PCN-induced IGT we explored the glucose metabolism-related gene pathways affected by PCN treatment in mouse primary hepatocytes. We performed DNA microarray and pathway analyses of differentially expressed genes in mouse hepatocytes treated with 10 µM PCN for 12 h in comparison to vehicle (DMSO)-treated cells.

PCN upregulated 95 genes and downregulated 174 genes (threshold set at 1.5-fold) compared to control cells (GEO dataset GSE106293). None of the ten most upregulated genes are related to glucose metabolism. In contrast, among the ten most downregulated genes there are *Glut2* (*Slc2a2*), *Gck* and *Pdk2* (pyruvate dehydrogenase kinase 2; EC 2.7.11.2) (Table 2). We have already previously reported the observed repression of these three genes by PCN [5].

We analyzed the canonical pathways affected by PCN treatment using Ingenuity Pathway Analysis. Among the top 20 pathways affected (Fig. 2a) there were two glucose metabolism related pathways affected; “Maturity Onset Diabetes of Young (MODY) Signaling” and “Ketogenesis” with three (*Glut2*, *Gck*, *Aldob* (aldolase B fructose-bisphosphate)) and two (*Hmgcs2* (3-hydroxy-3-methylglutaryl-CoA synthase 2), *Bdh1* (3-hydroxybutyrate Dehydrogenase 1)) genes affected, respectively. Interestingly, the top molecular network of the differentially expressed genes was “Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry” suggesting that
glucose-related pathways are among the major targets of PXR activation (Fig. 2b). Again, Glut2 and Gck were among the central members of this network.

We next used Ingenuity Pathway Analysis to identify putative upstream regulators involved in the PCN affected gene regulation (Table 3). Among the predicted upstream regulators, there are several transcription factors with known connection to the regulation of hepatic energy metabolism; and among these forkhead box protein A2 (FOXA2), hepatocyte nuclear factor 4 alpha (HNF4A), peroxisome proliferator activated receptor gamma (PPARG), forkhead box protein O1 (FOXO1) and hepatocyte nuclear factor 1 homeobox A (HNF1A) are associated with the regulation of Glut2 and/or Gck genes. The most consistent regulatory networks associated with the PCN-affected genes were related to concentration of D-glucose (consistency score -5.307) (Fig. 2c) as well as hypoglycemia and synthesis of D-glucose (consistency score 1.789) (Fig. 2d). Top regulators of genes related to these functions are predicted to be HNF1A and CCAAT/enhancer binding protein beta (CEBPB).

3.3. PCN regulates GLUT2 and GCK through a PXR mediated mechanism

According to the pathway analysis the regulation of Glut2 and Gck may be involved in the PCN-mediated IGT. We therefore investigated the effect of PCN on these genes in mouse liver in vivo. Mice were treated for four days with PCN or vehicle only. After 5 hours fasting the mice were killed and the liver samples were collected. As a control, we measured the expression of a well-established PXR target gene cytochrome P450 3a11 (Cyp3a11). CYP3A11 mRNA was induced 6-fold by PCN as expected. Furthermore, both GLUT2 and GCK mRNAs were strongly repressed by PCN and the treatment decreased the expression 90 % and 66 %, respectively (Fig. 3a). There
was a tendency for lower PDK2 mRNA in the PCN-treated mouse livers, however, the difference was not statistically significant.

Genes involved in glucose metabolism are often regulated by nutritional status and this may potentially affect the response to PXR activation. Therefore, we next investigated if PCN can regulate GLUT2 and GCK expression during the prandial condition. Similar to the previous experiment, mice were treated with PCN or vehicle for four days. In the morning of the fifth day the animals were first fasted for four hours and then given an intragastric glucose dose through oral gavage (2g/kg, a dose similar to the OGTT). One hour after glucose administration the mice were sacrificed and the liver samples were collected. CYP3A11 mRNA was again induced by PCN, however, compared to the fasting mice the effect was much stronger, up to 23-fold (Fig. 3b). GLUT2 mRNA was repressed by 58 % in the livers of the mice treated with glucose. However, compared with the 90 % depression in the fasting mice the effect was less pronounced (Fig 3b). GCK repression did not reach statistical significance in the glucose treated mice.

We also measured GLUT2 expression in the intestinal samples (ileum) of these mice. There was a tendency for GLUT2 mRNA to be lower in the intestinal samples of the PCN + glucose treated mice, however, the effect was not significant ($p = 0.0567$) (Fig 3c). In addition, the CYP3A11 induction in the intestinal samples was not statistically significant (Fig 3c). There were only four samples per group in the intestinal analyses, which may explain the lack of significant effects.

Furthermore, we studied the effect of PCN on GLUT2 protein expression in liver lysates by immunoblotting. After four-day PCN administration, the mice were fasted for 12 hours and then
treated with intragastric glucose (2g/kg). 1 hour after glucose dose, the mice were killed and the livers were collected. PCN significantly reduced GLUT2 protein level by 75% (Fig. 3d). In contrast, we did not observe a significant effect on GCK protein (data not shown).

We next aimed to confirm the role of PXR in the regulation of GLUT2 and GCK by PCN. Primary hepatocytes were extracted from WT and PXR knockout mice livers and treated with PCN for 24 and 48 h. CYP3A11 was strongly induced in the WT hepatocytes in the both time points, but was not affected in the PXR\(^{-/-}\) mice (Fig 4a,b). Furthermore, as expected, GLUT2 and GCK mRNAs were repressed in the WT hepatocytes by PCN after 24 and 48 h. No response could be observed in the PXR\(^{-/-}\) hepatocytes in either time point (Fig. 4a,b). These results indicate that GLUT2 and GCK repression by PCN requires PXR.

We also compared the mRNA expression GLUT2, GCK, PDK2 and CYP3A11 in the vehicle treated WT and the PXR\(^{-/-}\) hepatocytes. GLUT2 and CYP3A11 expression was lower in the PXR\(^{-/-}\) hepatocytes, while the levels of GCK and PDK2 were increased (Fig. 4c).

Cui et al. investigated PXR genome-wide DNA-binding sites with chromatin immunoprecipitation (ChIP)-sequencing in the mouse livers treated with PCN or corn oil vehicle as control [10]. We analyzed this published data for Glut2 gene. Interestingly, Glut2 appears to be a direct target for PXR and there are several PXR binding sites present within or nearby the Glut2 gene (Table 4). Two binding sites in the introns 4 and 8 were bound by PXR both in the control and PCN treated livers. Two additional sites, one in the intron 8 and another at the -2 kb upstream region, were observed after PCN treatment. Also Gck has three PXR binding sites in the intron 1 [10].
3.4. **PCN affects glucose-mediated regulation of GLUT2**

Our results indicate that PCN may affect GLUT2 expression both under fasting and fed conditions. Furthermore, Glut2 gene is known to be directly regulated by glucose concentration [11]. We therefore investigated if PCN may affect glucose-mediated regulation of Glut2 gene. Wildtype primary hepatocytes were either cultured in media containing 1 g/L glucose or in glucose free media for 24 h. Glucose withdrawal decreased GLUT2 mRNA expression by 70 % compared to the cells cultured in the presence of glucose (Fig. 5a). In the cells first subjected to glucose deprivation for 18 h, the restoration of the glucose level back to the 1 g/L for 6 h partially recovered the GLUT2 expression. However, treatment with PCN prevented the recovery effect (Fig. 5a). GCK was not affected by glucose deprivation. Furthermore, PCN had no effect on GCK expression under these conditions indicating that the regulatory mechanisms of GLUT2 and GCK are different (Fig. 5b).

3.5. **PCN affects GLUT2 subcellular localization**

In the muscle glucose transporter 4 (GLUT4) shuttling between plasma membrane and cytosolic compartment is regulated by insulin [12]. In contrast, there is little evidence of such subcellular regulation of GLUT2 in the liver. We investigated the subcellular localization of GLUT2 in the mouse liver and the effect of PCN treatment on that by immunohistochemistry. After fasting, the GLUT2 staining was concentrated to the plasma membrane in control animals (4 out of 4) (Fig. 6a). In contrast, in the PCN-treated mice the staining was mainly cytoplasmic (3 out of the 4 animals), suggesting internalization of the protein. However, some membrane positivity could still
be seen mainly around the central veins (Fig. 6b). In one of the PCN-treated mice, the staining was still mostly concentrated to plasma membranes.

When fed with glucose, both control and PCN groups (2 out of 3 mice in the both groups) showed the same staining pattern as in the fasted animals, i.e. membranous staining in controls and cytoplasmic staining in PCN treated mice (data not shown). The remaining two animals, one in each group, had variable staining in different areas. Similarly to the fasting mice, the membranous staining was more distinct around the central veins.

In fasting mice, GCK staining was granular and concentrated mostly close to the plasma membrane, however, not as distinctly to the plasma membrane itself as GLUT2. The GCK staining was more intense and cytoplasmic around the central veins (Fig. 6c). In the PCN-treated mice, the staining was evenly distributed and consistently cytoplasmic throughout the liver lobule (Fig. 6d). In glucose-fed mice the GCK staining was evenly cytoplasmic in all the animals (data not shown).

3.6. **PCN downregulates glucose uptake in hepatocytes**

Functional significance of PCN treatment on hepatic glucose uptake was investigated in primary hepatocytes with two different methods. In the first approach the uptake of 2-deoxyglucose (2-DG) was measured with a method based on the measurement of NADPH generation as described in the methods section. PCN efficiently inhibited the insulin-stimulated glucose uptake (Fig. 7a). We also used IRDye800-labeled 2-DG and measured the intracellular 2-DG directly. This second approach further confirmed that PCN reduced the insulin-mediated glucose uptake (Fig. 7b).
4. DISCUSSION

Type 2 diabetes is one of the most important global health challenges [13]. In addition to the well-established risk factors for type 2 diabetes, i.e. obesity, sedentary lifestyle and unhealthy nutrition, increasing evidence suggests that the risk of diabetes is linked to the exposure to certain chemical substances, including some clinically used drugs. The recent discovery of the increased risk of new-onset diabetes among patients receiving statins has drawn considerable attention [14,15]. However, actually several classes of drugs including glucocorticoids, thiazide diuretics, β-blockers and antiretroviral drugs are known to induce dysglycemia and suspected to promote progression of type 2 diabetes [16-18]. In addition to drugs, some endocrine disrupting environmental contaminants, have also been associated with diabetes risk [19-21]. The mechanisms behind drug- or other chemical-induced diabetes are mostly unclear, and probably not common for all the prodiabetic compounds. Understanding the underlying disease-mechanisms would be of critical importance for prediction of the harmful metabolic effects and for the effective precaution measures.

In the present study we provide conclusive evidence, using PXR knockout mouse model, that PXR activation per se impairs glucose tolerance and thus establish a novel, molecular prodiabetic mechanism. Since several PXR activating pharmaceuticals are used in long term drug therapies, this may represent a potential risk for metabolic adverse drug reaction. Furthermore, Stage et al. observed that after 21-day treatment with St. John’s wort, the unfavorable influence on glucose tolerance was sustained for or even further increased six weeks after the cessation of the therapy, suggesting that even a short term treatment may have long lasting metabolic effects [7]. In addition
to therapeutic drugs, a number of environmental contaminants, including many pesticides, plasticizers and flame retardants, are known to activate PXR [4,22-24]. Many of these chemicals are experimentally or epidemiologically associated with incidence of type 2 diabetes [25]. It is tempting to speculate that PXR could play a significant role in these putative endocrine disrupting, diabetogenic effects; however, the mechanisms are still mostly unknown.

PXR activation affects several hepatic genes involved in regulation of glucose homeostasis. Originally, it was reported that PXR activation represses the gluconeogenic genes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (Pck1) in mouse hepatocytes [26,27]. Indeed, we also observed in the current study that Pck1 was repressed by 1.8 fold in mouse hepatocytes after PCN treatment. However, the suppression of gluconeogenic genes may not explain PXR-mediated IGT, because the suppression of gluconeogenesis should improve glucose tolerance. Our bioinformatics evaluation of the PCN affected pathways in hepatocytes revealed that the most significant canonical pathways and molecular networks of the differentially expressed genes were related to glucose balance. These pathways and networks consistently involved Glut2 and Gck, genes involved in hepatic glucose sensing and uptake. GLUT2 and GCK have also been reported to be repressed by rifampicin in HepG2 cells [6]. In this study, both GLUT2 and GCK mRNAs were found to be repressed by PCN in PXR-dependent manner in primary hepatocytes. Both genes were also repressed in mouse liver in vivo after PCN treatment. However, while GLUT2 was repressed in both the fasting and the prandial condition simulated with oral glucose dose, GCK was repressed with statistical significance only during fasting. Furthermore, at the protein level we could detect significant repression only for GLUT2.
The PXR knockout itself also affected GLUT2 and GCK mRNA expression in primary hepatocytes. While GCK and the control gene CYP3A11 were regulated to the opposite direction compared to the PCN treatment, GLUT2 was repressed by both PCN and the PXR knockout. The mechanisms and significance of this finding require further study in the future.

Data mining of previously published ChIP-sequencing data [10] indicates that *Glut2* gene is a direct target for nuclear receptor PXR in mouse liver. PXR binds several regions within or near the *Glut2* gene. Two sites in the introns 4 and 8 were occupied independent from the ligand treatment, while two other sites, in the 5’ upstream region and in the intron 8 were bound only after PCN treatment. Thus repression of *Glut2* gene appears to be associated with increased PXR binding. At this moment it is poorly understood how increased PXR DNA-binding results in gene repression. Potentially this could involve recruitment of corepressors in context of the repressed genes or competition for DNA-binding sites with some activating factors. Interestingly, according to the results of Cui et al. about 30% of the PXR-repressed genes are direct PXR targets [10]. Thus far, the most established PXR mediated repression mechanisms have involved protein-protein interactions [28]. For example, PXR activation represses carnitine palmitoyltransferase 1a (*Cpt1a*) and 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (*Hmgcs2*) expression through interaction and repression of FOXA2 [29]. Also HNF4A signaling is repressed by PXR, although not through direct interaction, but through competition for a common coactivator PPAR coactivator 1 alpha (PGC-1α) resulting in repression of cytochrome P450 7a1 (*Cyp7a1*) [30]. FOXA2 and HNF4A are both direct transcriptional activators of the *Glut2* gene in liver [31] and they were among the predicted upstream regulators of the differential expressed genes after PCN treatment. Thus, also these established protein-protein interaction mediated mechanisms could in theory play a role in
the Glut2 repression. However, the multiple PXR binding sites in the Glut2 gene and the observed significant changes in the PXR binding activity after PCN treatment would primarily point to the role of direct PXR regulation [10].

Interestingly, we observed that PCN treatment regulated, in addition to the expression level, the GLUT2 protein subcellular localization. In the livers of vehicle-treated mice GLUT2 was very distinctly detected at the plasma membrane in accordance with its function as a cell membrane transporter [32]. However, PCN treatment induced a very drastic change and staining was detected mainly at the cytoplasm suggesting internalization of the protein. Alternatively, the relocation could also be due to impaired/disrupted sorting of the protein. In any case, the displacement can be expected to reduce the GLUT2-mediated transport function. While insulin-induced subcellular trafficking is a very well-established character of GLUT4 regulation, there is less evidence of such regulation of GLUT2 [12,33]. In polarized enterocytes, the GLUT2 concentration in apical membrane is regulated by intracellular trafficking [33]. Furthermore, dynamic basal to apical translocation has been observed in cultured, polarized, kidney epithelial cell line in response to glucose [34]. There are also some reports describing internalization of GLUT2 in liver in response to feeding mediated by interaction with insulin receptor [35,36]. In our study, however, one hour oral glucose treatment did not have marked effect on hepatic GLUT2 localization.

The findings of the current study indicate that PXR activation affects GLUT2 function in at least two ways, i.e. by regulating the level of mRNA expression and by interfering with the subcellular trafficking. This mode of dual regulation suggests major importance for this regulatory pathway. GLUT2 is the main hepatic glucose transporter and it facilitates two-way transport of the glucose
across the cell membrane [32]. However, studies in mice with liver-specific GLUT2 knockout indicated that the loss of GLUT2 suppresses hepatic glucose uptake, but not output [8] indicating crucial role especially in the postprandial phase.

We observed that PCN treatment of mouse primary hepatocytes decreased glucose uptake suggesting that PXR mediated GLUT2 repression and the internalization of GLUT2 from the plasma membrane have functional consequences on hepatic glucose metabolism. Liver-specific GLUT2 knockout did not cause immediate glucose intolerance in mice despite the reduced glucose uptake; however, glucose tolerance was impaired gradually because of the decrease in glucose-stimulated insulin secretion indicating a role for hepatic GLUT2 as a glucose sensor and a regulator of the whole body glucose homeostasis [8]. Therefore, the PCN-induced reduction in hepatic glucose uptake observed in this study can hardly solely explain the impairment of the whole-body glucose tolerance. However, it remains possible that the reduction in hepatic glucose uptake disturbs hepatic glucose sensing and signaling to the other metabolic tissues. In support of this concept of inter-tissue cross talk, PXR knockout mice fed high-fat diet have improved insulin sensitivity compared with the wildtype mice, not only in liver, but also in muscle and white adipose tissues although these extrahepatic tissues do not express PXR [37].

In our current study, the effect of PCN on GCK expression was less consistent than on GLUT2. However, Ling et al. observed PCN-induced repression of GCK protein in HFD treated rat livers [6]. Furthermore, in PXR-humanized mouse model GCK induction by HFD was abolished [38,39] suggesting that PXR indeed interferes with the GCK regulation. Thus, in addition to GLUT2, also
the repression of GCK may contribute to the reduced glucose uptake in liver and subsequently to the IGT after PCN treatment.

In conclusion, we show in this study that the activation of nuclear receptor PXR impairs glucose tolerance and therefore propose PXR activation as a novel diabetogenic pathway. PXR may thus be involved in the metabolic side effects of clinically used drugs as well as in the endocrine-disturbing effects of environmental contaminants. Furthermore, PXR dysregulates hepatic glucose sensing by decreasing the GLUT2 expression and changing the subcellular localization of it.

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Conflicts of interest: None
References


<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc2a2 (Glut2)</td>
<td>TCAGAAGACAAAGATCACCGGA</td>
<td>GCTGGTGTGACTGTAAGTGGG</td>
</tr>
<tr>
<td>Gck (1)</td>
<td>ATGGCTGTGGATACTACAAGGA</td>
<td>TTCAGGCCACCGGTCCATCT</td>
</tr>
<tr>
<td>Gck (2)</td>
<td>GACCCGGAACTTGGACAAAG</td>
<td>AATGCCATACGACCAGAGGTG</td>
</tr>
<tr>
<td>Pdk2</td>
<td>AGGGGCACCCAAGTACATC</td>
<td>TGCCGGAGGAAGTGAATGAC</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>GAAGCATTGAGGAGGATCAC</td>
<td>GTCCCATATCGGTAGAGGAG</td>
</tr>
<tr>
<td>18S</td>
<td>CGCCGCTAGAGGTGAATTC</td>
<td>CCAGTCGGGCATCGTTATGG</td>
</tr>
</tbody>
</table>

Gck (1): Used for *in vivo* samples (liver tissue)
Gck (2): Used for *in vitro* samples (primary hepatocytes)
Table 2. The 10 most up- and downregulated genes in PCN treated mouse primary hepatocytes compared to control.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp3a25</td>
<td>Cytochrome P450 Family 3 Subfamily A Member 25</td>
<td>7.8</td>
</tr>
<tr>
<td>Thbs1</td>
<td>Thrombospondin 1</td>
<td>4.9</td>
</tr>
<tr>
<td>LOC666781</td>
<td>Tenascin C</td>
<td>2.9</td>
</tr>
<tr>
<td>Tnc</td>
<td>ATP Binding Cassette Subfamily B Member 1A</td>
<td>2.6</td>
</tr>
<tr>
<td>Abcb1a</td>
<td>Glutathione S-Transferase Mu 3</td>
<td>2.9</td>
</tr>
<tr>
<td>Gstm3</td>
<td>Cytochrome P450 Family 2 Subfamily C Member 55</td>
<td>2.3</td>
</tr>
<tr>
<td>4931406C07Rik</td>
<td>RIKEN cDNA 4931406C07</td>
<td>2.2</td>
</tr>
<tr>
<td>Krt20</td>
<td>Keratin 20</td>
<td>2.2</td>
</tr>
<tr>
<td>Vpreb1</td>
<td>Pre-B Lymphocyte 1</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cyp26a1</td>
<td>Cytochrome P450 Family 26 Subfamily A Member 1</td>
<td>-7.4</td>
</tr>
<tr>
<td>C730036D15Rik</td>
<td>RIKEN cDNA C730036D15</td>
<td>-3.4</td>
</tr>
<tr>
<td>Dhrs3</td>
<td>Dehydrogenase/Reductase 3</td>
<td>-2.9</td>
</tr>
<tr>
<td>Akr1c19</td>
<td>Aldo-keto reductase family 1, member C19</td>
<td>-2.8</td>
</tr>
<tr>
<td>Slc2a2 (Glut2)</td>
<td>Solute Carrier Family 2 Member 2 (Glucose Transporter Type 2)</td>
<td>-2.7</td>
</tr>
<tr>
<td>Gck</td>
<td>Glucokinase</td>
<td>-2.4</td>
</tr>
<tr>
<td>Pdk2</td>
<td>Pyruvate Dehydrogenase Kinase 2</td>
<td>-2.4</td>
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<td>1700016C15Rik</td>
<td>RIKEN cDNA 1700016C15</td>
<td>-2.3</td>
</tr>
<tr>
<td>LOC100042793</td>
<td></td>
<td>-2.3</td>
</tr>
<tr>
<td>Baat</td>
<td>Bile Acid-CoA:Amino Acid N-Acyltransferase</td>
<td>-2.3</td>
</tr>
</tbody>
</table>
Table 3. Top ten upstream regulators of differentially expressed genes in PCN treated mouse primary hepatocytes compared to control cells.

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>p-value</th>
<th>Genes (n)</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXA2</td>
<td>1.35E-07</td>
<td>13</td>
<td>ABCB1↑, ADH1C↓, ALDOB↓, CEBPA↓, GCK↓, Hmga2↑, HMGCS2↓, HPGD↓, IL33↑, ONECUT1↓, PCK1↓, PDHX↑, SLC2A2↓</td>
</tr>
<tr>
<td>ACOX1</td>
<td>4.04E-07</td>
<td>12</td>
<td>BAAT↓, CYP26A1↓, CYP2C8↑, Cyp3a25 (includes others)↑, GADD45A↑, HAL↓, MGL↓, Orm1 (includes others)↓, RAD51B↓, RGS2↓, SELENBP1↓, SLC37A4↓</td>
</tr>
<tr>
<td>LEP</td>
<td>5.88E-07</td>
<td>17</td>
<td>ALDOB↓, Cyp2a12/Cyp2a22↓, CYP2Cl8↓, CYP2C8↑, CYP3A5↑, E2F3↑, FAAH↓, FMO5↓, GADD45A↑, GCK↓, Gstm3↑, KLB↓, PCK1↓, SLC2A2↓, SMPD2↓, STEAP4↓, TIMP1↑</td>
</tr>
<tr>
<td>MGEA5</td>
<td>9.07E-07</td>
<td>18</td>
<td>ACAD10↓, AHNAK2↓, CAV1↑, CEBPA↓, CNOT3↓, CREB5↑, FGFR4↓, GADD45A↑, GDF11↑, GPT2↓, GSN↑, HPGD↓, MGL↓, TFDP1↓, THBS1↑, TIMP1↑, TNC↑, Tnfrsf22/Tnfrsf23↑</td>
</tr>
<tr>
<td>RORC</td>
<td>2.04E-06</td>
<td>11</td>
<td>ABCG5↓, AKR1D1↑, CYP2C8↑, Cyp3a25 (includes others)↑, CYP3A5↑, HPGD↓, Keg1↓, PCK1↓, RDH16↓, SELENBP1↓, ZBTB16↓</td>
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<tr>
<td>SMARCB1</td>
<td>3.68E-06</td>
<td>9</td>
<td>A2M↓, ABCB1↑, COL18A1↓, CYP2C8↑, Cyp3a25 (includes others)↑, GSN↑, PCK1↓, PKFB1↓, SLC37A4↓</td>
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<tr>
<td>POR</td>
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<td>12</td>
<td>BDH1↓, C11orf54, Ces2a↑, CSAD↓, CYP26A1↓, CYP2C18↑, CYP2C8↑, GCK↓, Gstm3↓, PLA2G7↑, RAD51B↓, UBD↓</td>
</tr>
<tr>
<td>HNF4A</td>
<td>6.69E-06</td>
<td>17</td>
<td>ABCG5↓, ALDOB↓, ARG1↓, BAAT↓, BDH1↓, CEBPA↓, Cyp2a12/Cyp2a22↓, CYP3A5↑, FMO1↓, GGT6↓, GSN↑, ONECUT1↓, PCK1↓, SLC2A2↓, Sprt2h↑, TDO2↓, TNC↑</td>
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<tr>
<td>PPARG</td>
<td>1.31E-05</td>
<td>17</td>
<td>Abcb1b↑, ADRB2↓, BDH1↓, CAV1↑, CEBPA↓, CYP26A1↓, DHRS3↓, GABARAPL2↓, GCK↓, HMGCS2↓, KLB↓, KRT20↓, MGL↓, PCK1↓, PF4↑, PLIN5↓, SLC2A2↓</td>
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<td>FOXO1</td>
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<td>14</td>
<td>ALDOB↓, AQP9↓, ATP6V0A1↓, GADD45A↑, GCK↓, HAL↓, IRF5↑, ITGB6↑, PCK1↓, RPIA↑, TAT↓, TDO2↓, THBS1↑, VPREB1↑</td>
</tr>
</tbody>
</table>

↑ upregulated and ↓ downregulated by PCN
Table 4. PXR binding to the *Slc2a2 (Glut2)* gene. Original data from Cui et al. [10].

<table>
<thead>
<tr>
<th>Binding sites detected in control and PCN treated mice livers</th>
<th>Treatment</th>
<th>Chr3 Start</th>
<th>End</th>
<th>Length</th>
<th>Peak Value</th>
<th>Location in the <em>Slc2a2</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>28 613 316</td>
<td>28 613 533</td>
<td>217</td>
<td>35</td>
<td>Intron 4</td>
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<tr>
<td>PCN</td>
<td>PCN</td>
<td>28 613 284</td>
<td>28 613 533</td>
<td>249</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>28 621 636</td>
<td>28 621 789</td>
<td>153</td>
<td>42</td>
<td>Intron 8</td>
</tr>
<tr>
<td>PCN</td>
<td>PCN</td>
<td>28 621 572</td>
<td>28 621 789</td>
<td>217</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Binding sites detected in PCN treated mice livers only</td>
<td>PCN</td>
<td>28 594 596</td>
<td>28 594 781</td>
<td>185</td>
<td>61</td>
<td>2 kb upstream</td>
</tr>
<tr>
<td>PCN</td>
<td>PCN</td>
<td>28 624 260</td>
<td>28 624 477</td>
<td>217</td>
<td>55</td>
<td>Intron 8</td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1.** Effect of 4-day PCN treatment on OGTT in A) wildtype mice, B) PXR knockout mice. C) Comparison of glucose tolerance in the control wildtype and the control PXR knockout mice. AUC, Area under the curve (min×mmol/L); IAUC, Incremental area under the curve (min×mmol/L). Values represent means ±SD; *p<0.05.

**Figure 2.** The functional analysis of differentially expressed genes (a significant 1.5-fold upregulation or downregulation compared to controls) by PCN in mouse primary hepatocytes generated by Ingenuity Pathway Analysis (IPA). A) The most significant canonical pathways associated with PCN-regulated gene sets (negative log of P-value calculated using Fisher’s exact test). (B) Carbohydrate metabolism, molecular transport and small molecule biochemistry network was identified as a top molecular network affected by PCN. Green color means downregulation and red color upregulation of gene expression. The intensity of the color indicates the degree of upregulation or downregulation. Genes in uncolored nodes were not differentially expressed in our datasets and were integrated into the computationally generated networks by the IPA knowledge database because they are connected to this network. (C-D) The most consistent regulatory networks generated for the functions of differentially expressed genes by PCN in mouse primary hepatocytes are related to glucose homeostasis. Upstream regulator analysis of differentially expressed genes was generated by IPA Analysis. Green color means downregulation of gene expression. Blue and orange mean predicted inhibition and activation, respectively. Solid and dashed lines indicate direct and indirect interactions, respectively. Yellow lines indicate that dataset findings are inconsistent based on previously reported interactions in the literature, and
grey line indicates that the effect was not predicted. Genes are represented as various shapes that represent the functional class of the gene product. (SLC2A2 = GLUT2)

**Figure 3.** Effect of 4-day PCN treatment on relative mRNA levels of GLUT2, GCK, PDK2, CYP3A11 in mouse liver **A)** after 5h of fasting, **B)** after fasting for 4h and subsequent i.g. glucose (2 g/kg) for 1 h. **C)** Effect of 4-day PCN treatment on relative mRNA levels of GLUT2 and CYP3A11 in mouse intestine (ileum) after fasting for 4h and subsequent i.g. glucose (2 g/kg) for 1 h. **D)** Effect of 4-day PCN treatment of GLUT2 protein level in mouse liver lysates after fasting for 12h and subsequent i.g. glucose (2 g/kg) for 1h. β-actin was detected as loading control. The values represent mean ±SD; * p<0.05. For the liver samples n = 5-8, for the intestinal samples n =4.

**Figure 4.** Effect of 24 h **(A)** and 48 h **(B)** PCN (10 µM) treatment on mRNA expression in wildtype and PXR knockout mouse primary hepatocytes. Both the wildtype and the knockout control group values have been normalized to one. **C)** Effect of PXR knockout on gene expression in primary hepatocytes. The vehicle treated samples after 24 h treatment were compared. The values represent mean ±SD. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

**Figure 5.** Effect of PCN (10 µM) and glucose (1 g/L) on **A)** GLUT2 and **B)** GCK mRNA levels in cultured mouse primary hepatocytes. The cells were maintained either in glucose containing or glucose free media for 24 h, or were first kept 18 h in glucose free media after which glucose was reintroduced to the cells for the remaining six hours, as indicated. The values represent mean ±SD. *** p<0.001, **** p<0.0001 (compared to the glucose containing control); ## p<0.01.
Figure 6. Immunohistochemical detection of GLUT2 and GCK in mouse liver. A) GLUT in the liver of a control mouse, B) GLUT in the liver of a PCN treated mouse, C) GCK in the liver of a control mouse, D) GCK in the liver of a PCN treated mouse. Central vein (C) and portal area (P) are indicated in each picture.

Figure 7. Effect of 24 h PCN (10 µM) treatment on glucose uptake in mouse primary hepatocytes. A) Relative uptake of unlabeled 2-deoxyglucose (2-DG) B) Relative uptake of IRDye 800-labeled 2-DG. The values represent mean ±SD. * $p<0.05$, **** $p<0.0001$ (compared to untreated control); # $p<0.05$, ### $p<0.001$. 