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Activation of the Nuclear Receptor PXR Decreases Plasma LDL-Cholesterol Levels and Induces Hepatic Steatosis in LDL Receptor Knockout Mice

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Abstract: To investigate the potential for pregnane X receptor (PXR) ligands as antiatherosclerotic drugs, we have determined the effect of PXR activation on lipid metabolism in an established atherosclerotic mouse model. LDL receptor knockout mice were treated with the PXR agonist PCN. PCN induced a striking 66% decrease in plasma LDL-cholesterol levels. PCN did not affect the cholesterol levels of high-density lipoprotein (HDL) or very-low-density lipoprotein (VLDL). VLDL-triglyceride levels were 2.2-fold increased by PCN, resulting in the presence of triglyceride-rich VLDL particles. This coincided with a 60% decreased hepatic lipase (HL)-mediated plasma lipolysis rate, which could be attributed to a decrease in the hepatic mRNA expression level of both HL (−31%) and its cofactor apolipoprotein A4 (−62%). In the liver, PCN induced a significant increase in the level of triglycerides (+65%) and phospholipids (+72%), a hallmark of hepatic steatosis, leading to a marked increase in Oil red O neutral lipid staining. A similar effect was noticed in ApoE knockout mice. Our studies show that activation of the nuclear receptor PXR by PCN leads to an inhibition of the plasma HL-mediated lipolysis rate, which is associated with a decrease in plasma LDL-cholesterol levels and induction of hepatic steatosis in LDL receptor knockout mice.

Keywords: Pregnane X receptor; cholesterol; triglycerides; liver; atherosclerosis; lipolysis; hepatic lipase

Introduction

The nuclear receptor pregnane X receptor (PXR; NR1I2) is highly expressed in liver, where it plays a major role in the metabolism of several endogenous substrates (i.e., steroids) and multiple foreign chemicals (i.e., drugs).[1] Upon its activation by xenobiotics, PXR forms obligate heterodimers with the nuclear receptor retinoic X receptor (RXR). The PXR/RXR complex is subsequently able to bind a variety of nuclear receptor response elements in the DNA leading to changes in the expression of PXR target genes. Established PXR targets include phase I and II detoxification enzymes like glutathione S-transferase α4 (GSTA4), cytochrome P450 3A4 (CYP3A4), and CYP3A11 and ATP-binding cassette (ABC) transporter proteins such as ABCC2 (MRP2), and ABCC3 (MRP3).[2] However, activation of PXR has also been associated with changes in the expression of several key genes involved in cholesterol, bile acid, and fatty acid metabolism,[2−4] suggesting an additional physiological function for PXR in lipid metabolism.

Interestingly, Masson et al. showed that the presence of PXR in mice inhibits the cholic acid-mediated decrease in the plasma levels of high-density lipoprotein (HDL).[5] HDL is considered to be a potent antiatherogenic factor, since it can mediate reverse cholesterol transport, the transport of

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(1) Kliewer, S. A.; Willson, T. M. Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J. Lipid Res.* **2002**, *43*, 359–364.

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(excess) cholesterol from peripheral tissues to the liver for subsequent excretion from the body.[6] This opens up the possibility that ligand-induced activation of PXR may be beneficial in the treatment of atherosclerosis, the primary cause of cardiovascular disease mortality and morbidity. To gain insight into the possible use of PXR ligands as antiatherosclerotic drugs, in the current study, we have determined the *in vivo* effect of PXR activation by the widely used PXR agonist 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) on lipid metabolism in an established atherosclerotic mouse model, the LDL receptor knockout mice, while for additional studies also the ApoE knockout mice was used.

Experimental Section

Animals. Homozygous LDL receptor knockout mice and apolipoprotein E (ApoE) knockout mice were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. LDL receptor knockout mice were fed a semisynthetic Western-type diet containing 15% (wt/wt) cacao butter and 0.25% (wt/wt) cholesterol (Diet W, Special Diet Services, Witham, U.K.) for 2 weeks. On days 11−13 mice were injected intraperitoneally once daily with 100 μL of 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN; 80 mg/kg/day; Sigma) or solvent control between 9:00 and 10:00 a.m. ApoE knockout mice were similarly injected for three consecutive days with PCN while being fed a regular chow diet containing 4.3% fat and no cholesterol (RM3, Special Diet Services, Witham, U.K.). Twenty-four hours after the last injection, blood was drawn for lipid analyses. Subsequently, a whole-body perfusion was performed using phosphate-buffered saline (4 °C, 100 mmHg) for 10 min. After perfusion, livers were excised and frozen in liquid nitrogen and stored at −80 °C. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of the Leiden University.

Plasma Lipids. Plasma concentrations of total cholesterol and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The distribution over the different lipoproteins in plasma was analyzed by fractionation of 30 μL of serum of each mouse using a Superose 6 column (3.2 × 300 mm, Smart-system, Pharmacia). Total cholesterol, triglyceride, and phospholipid content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics).

***In Vivo* VLDL-Triglyceride Secretion.** Mice were injected intravenously with 500 mg of Triton WR-1339 (Sigma) per kg body weight as a 15 g/dL solution in 0.9% NaCl. Previous studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions.[7] Blood samples (50 μL) were taken at 0, 1, 2, 3, and 4 h after Triton WR-1339 injection. Plasma triglycerides were analyzed enzymatically. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as mg/mL/h.

Post-Heparin Plasma Lipolysis. To determine the plasma lipoprotein lipase (LPL) and hepatic lipase (HL) activity, blood was drawn at 20 min after an intravenous bolus injection of heparin (100 U/kg). The lipolytic activity of the post-heparin plasma was measured by using a radiolabeled triolein emulsion, as described by Zechner.[8] In brief, the substrate consisted of a radiolabeled triolein emulsion prepared by sonication of a mixture of 50 mCi glycerol-tri[9,10(n)-3 H]oleate, 4 mg of unlabeled glycerol trioleate, 0.1 mol/L Tris-HCl (pH 8.6), 0.1% Triton X-100, 2% BSA, and 2 mL of heat-inactivated human serum (a source of ApoC2, an LPL activator). Subsequently, 10 μL plasma was added to 0.2 mL of substrate and incubated for 30 min at 37 °C. The reaction was stopped by addition of 3.25 mL of a mixture of chloroform−methanol−*n*-heptane (1:1.28:1.37, v/v/v) and 1 mL of 0.1 mol/L $K_2CO_3-H_3BO_3$ (pH 10.5). Free fatty acids (FFAs) were extracted by vortexing this mixture for 15 s; phases were separated by centrifugation at 3,000 rpm at 4 °C for 20 min, and 1 mL of the upper phase was counted for radioactivity. The lipolytic activity was calculated from the amount of FFAs released per milliliter per minute. The lipolytic activity was determined in the presence or absence of 1 mol/L NaCl to differentiate between LPL and HL activity. LPL activity was calculated as the portion of total lipase activity inhibited by 1 mol/L NaCl.

Quantitative Real-Time PCR. Quantitative gene expression analysis on liver was performed as described.[9] In short, total RNA was isolated according to Chomczynski and

(2) Eloranta, J. J.; Kullak-Ublick, G. A. Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism. *Arch. Biochem. Biophys.* **2005**, *433*, 397–412.

(3) Handschin, C.; Meyer, U. A. Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. *Arch. Biochem. Biophys.* **2005**, *433*, 387–396.

(4) Moreau, A.; Vilarem, M. J.; Maurel, P.; Pascussi, J. M. Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Mol. Pharmaceutics* **2008**, *5*, 35–41.

(5) Masson, D.; Lagrost, L.; Athias, A.; Gambert, P.; Brimer-Cline, C.; Lan, L.; Schuetz, J. D.; Schuetz, E. G.; Assem, M. Expression of the pregnane X receptor in mice antagonizes the cholic acid-mediated changes in plasma lipoprotein profile. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 2164–2169.

(6) Van Eck, M.; Pennings, M.; Hoekstra, M.; Out, R.; Van Berkel, T. J. Scavenger receptor BI and ATP-binding cassette transporter A1 in reverse cholesterol transport and atherosclerosis. *Curr. Opin. Lipidol.* **2005**, *16*, 307–15.

(7) Aalto-Setälä, K.; Fisher, E. A.; Chen, X.; Chajek-Shaul, T.; Hayek, T.; Zechner, R.; Walsh, A.; Ramakrishnan, R.; Ginsberg, H. N.; Breslow, J. L. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. *J. Clin. Invest.* **1992**, *90*, 1889–1900.

(8) Zechner, R. Rapid and simple isolation procedure for lipoprotein lipase from human milk. *Biochim. Biophys. Acta* **1990**, *1044*, 20–25.

(9) Hoekstra, M.; Kruijt, J. K.; Van Eck, M.; Van Berkel, T. J. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J. Biol. Chem.* **2003**, *278*, 25448–25453.

Table 1. Primers Used for Real-Time PCR

gene	Pubmed accession	forward primer	reverse primer
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCC3	NM029600	TCCCACTTTTCGGAGACAGTAAC	ACTGAGGACCTTGAAGTCTTGGA
ApoA4	NM007468	CAGCTGACCCCATACATCCAG	TCATCGAGGTGTGCAGGTTG
ACC1	NM133360	AGAATCTCCTGGTGACAATGCTTATT	GCTCTGTGAGGATATTTAGCAGCTC
FOXO1	NM019739	GCTGGGTGTCAGGCTAAGAG	GCATCTTTGGACTGCTCCTC
β-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
cyclophilin	AK010338	CCATTTCAAGAAGCAGCGTTT	ATTTTGTCTTAACTGGTGGT
CYP3A11	NM007818	GGATGAGATCGATGAGGCTCTG	CCAGGTATTCCATCTCCATCACA
CD36	NM007643	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
GAPDH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
GSTA2	NM008182	CAGCAGCCTCCCCAATGT	CTTGAAAACCTTCCTTGCTTCTTC
GSTA4	NM010357	GCTTTTCTCGTTGGCAACCA	CTGAGTTCTTCCACCATCAAAATG
HL	NM008280	CAGCCTGGGAGCGCAC	CAATCTTGTTCTTCCCGTCCA
SCD1	NM009127	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA
PPARα	NM011144	TGAACAAAGACGGGATG	TCAAACTTGGGTTCCATGAT
PPARγ	NM011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT
PEPCK	NM011044	TTGAACTGACAGACTCGCCCT	GATATGCCCATCCGAGTCATG
PGC-1α	NM008904	CCCGATCACCATATTCCAGG	GTAGTGGCTTGATTCATAGTAGTAACAGGA
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC
FAS	NM007988	GGCATCATTGGGCACTCCTT	GCTGCAAGCACAGCCTCTCT
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
LRP1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA
PPARγ	NM011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG

Sacchi[10] and reverse transcribed using RevertAid reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in Table 1, which were validated for identical efficiencies (slope = −3.3 for a plot of Ct versus log ng of cDNA). Hypoxanthine guanine phosphoribosyl transferase (HPRT), β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acidic ribosomal phosphoprotein P0 (36B4), and cyclophilin were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of HPRT, β-actin, GAPDH, 36B4 and cyclophilin (Ct housekeeping) and raising 2 to the power of this difference. The average Ct of five housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes.

Tissue Lipid Composition and Histology. Lipids from liver were extracted using the method of Bligh and Dyer.[11] After dissolving the lipids in 1% Triton X-100, contents of phospholipids, triglycerides, cholesterol were determined as described above and expressed as micrograms per milligram of protein. Seven micrometer cryosections were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with hematoxylin (Sigma) for nuclei and Oil red O (Sigma) for lipid visualization.

Data Analysis. Statistical analysis was performed using Graphpad Instat software (San Diego, USA, http://www.graphpad.com). Normality testing of the experimental groups was performed using the method Kolmogorov and Smirnov (Graphpad Instat). The significance of differences was calculated using a two-tailed Student's *t* test. Probability values less than 0.05 were considered significant.

Results

In the current study we investigated the potential of PXR ligands as antiatherosclerotic drugs. For this purpose, we treated LDL receptor knockout mice that were fed a Western-type diet containing 0.25% cholesterol and 15% fat, a widely used atherosclerotic mouse model,[12] with the potent synthetic PXR ligand 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN[3]) for 3 days and subsequently determined the effects on lipid metabolism. The mRNA expression of the PXR target genes GSTA2, GSTA4, ABCC3, and CYP3A11 was significantly stimulated in livers of PCN-treated mice as compared to solvent controls (Figure 1), indicating that PCN induced efficient activation of the nuclear receptor PXR in the LDL receptor knockout mice *in vivo*.

(10) Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **1987**, *162*, 156–159.

(11) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

(12) Ishibashi, S.; Goldstein, J. L.; Brown, M. S.; Herz, J.; Burns, D. K. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.* **1994**, *93*, 1885–1893.

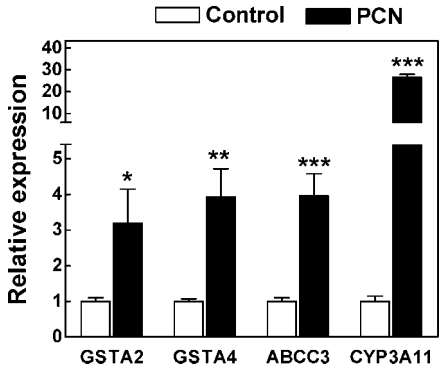

Figure 1. The effect of the PXR agonist PCN on the hepatic relative mRNA expression level of established PXR target genes in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control-treated mice.

Activation of PXR did not affect plasma cholesterol levels of the antiatherogenic lipoprotein high-density lipoprotein (HDL) or the pro-atherogenic lipoprotein very-low-density lipoprotein (VLDL; Figure 2A). In contrast, PCN induced a marked 66% ($P < 0.001$; Figure 2A) decrease in the plasma level of cholesterol associated with the pro-atherogenic lipoprotein low-density lipoprotein (LDL), resulting in a significantly lower (−32%; $P = 0.029$) pro-/anti-atherogenic lipoprotein−cholesterol ratio (atherogenic index) in PCN-treated mice as compared to controls (Figure 2B). A similar decrease in the level of LDL-phospholipids was observed upon PCN-treatment (−63%; $P < 0.001$; Figure 2C), suggesting that the amount but not cholesterol content of the LDL particles was decreased. PCN treatment increased the level of triglycerides associated with VLDL (+120%; $P = 0.037$) without affecting LDL- and HDL-triglyceride levels (Figure 2D). As a result, the VLDL particles in plasma were significantly enriched in triglycerides in the PCN-treated mice as compared to controls (24.4 ± 0.1% vs 11.7 ± 0.7% of total lipid; $P < 0.001$).

The metabolism of LDL consists of several steps including (1) the synthesis and secretion of triglyceride-rich VLDL particles by the liver, (2) the conversion of VLDL to cholesteryl ester-rich LDL via lipolysis of the triglycerides by hepatic lipase (HL) and lipoprotein lipase (LPL), and (3) the clearance of LDL from plasma by receptors located on hepatocytes. To delineate the mechanism behind the rapid decrease in plasma LDL-cholesterol levels, we determined the effect of PXR activation on the synthesis of VLDL-particles by the liver. As shown in Figure 3, PCN did not affect the VLDL-triglyceride secretion rate, which indicates that the production of VLDL particles by the liver was unchanged in response to PXR activation. PCN induced a significant decrease in the total post-heparin plasma lipolytic activity (−41%; $P = 0.003$), which could be fully attributed to a decrease in the activity of HL (−60%; $P < 0.001$), but not LPL (Figure 4A). The decrease in HL activity coincided with a significant decrease in the mRNA expression levels of both HL (−31%; $P = 0.008$) and its cofactor apolipoprotein A4 (ApoA4; −62%; $P = 0.012$) in livers of PCN-treated mice (Figure 4B). It is thus anticipated that the metabolism of the triglyceride-rich VLDL particles to cholesteryl ester-rich LDL particles was inhibited as a result of PXR activation by PCN. In wild-type mice, LDL is cleared from the blood circulation essentially via the action of the LDL receptor on hepatocytes.[13] However, the LDL receptor-related protein 1 (LRP1) and scavenger receptor class B type I (SR-BI) mediate the uptake of LDL in mice that lack functional LDL receptors.[14,15] The hepatic expression of LRP1 was unaffected by PCN, while a significant 60% decrease ($P = 0.002$) in SR-BI mRNA expression levels was detected in livers of PCN-treated mice (Figure 5). This suggests that the hepatic clearance of LDL was probably decreased in response to PXR activation in LDL receptor knockout mice.

Receptor-mediated uptake of cholesteryl esters is a major contributor to the intrahepatic regulatory free cholesterol pool. As the availability of the receptors (i.e., SR-BI) on liver cells as well as the substrate (i.e., LDL) was markedly decreased by PCN, the effect of PXR activation on hepatic lipid levels was determined. Oil red O neutral lipid staining showed extreme lipid accumulation in livers from PCN-treated mice (Figure 6A). Lipid analyses indicated that livers of mice exposed to PCN were highly enriched in triglycerides (+65%; $P = 0.028$) and phospholipids (+72%; $P = 0.002$), but not cholesterol, as compared to livers from controls (Figure 6B). To investigate the mechanism behind the PCN-induced hepatic triglyceride accumulation, we determined the effect of PCN treatment on relative mRNA expression levels of genes involved in the uptake, *de novo* synthesis, and metabolism of fatty acids. Hepatic CD36 expression was unaffected by PCN exposure (Figure 7A), suggesting that the uptake of fatty acids was not changed by PXR activation. In addition, no change was observed in the mRNA expression of acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD1) (Figure 7A), which mediate the synthesis of malonyl-CoA, long-chain saturated fatty acids and monosaturated fatty acids, respectively. The mRNA expression of the lipogenic transcription factors sterol regulatory element binding protein 1 (SREBP-1) and forkhead box O1 (OXO1) was also not significantly changed upon PXR activation (Figure 7B). PCN did stimulate the expression of the lipogenic transcription factor peroxisome proliferators-activated receptor γ (PPARγ; $P = 0.004$) and its coactivator PPARgamma coactivator 1α (PGC-1α)

(13) Ishibashi, S.; Brown, M. S.; Goldstein, J. L.; Gerard, R. D.; Hammer, R. E.; Herz, J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **1993**, *92*, 883–893.

(14) Huszar, D.; Varban, M. L.; Rinninger, F.; Feeley, R.; Arai, T.; Fairchild-Huntress, V.; Donovan, M. J.; Tall, A. R. Increased LDL cholesterol and atherosclerosis in LDL receptor-deficient mice with attenuated expression of scavenger receptor B1. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 1068–1073.

(15) Rohlmann, A.; Gotthardt, M.; Hammer, R. E.; Herz, J. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J. Clin. Invest.* **1998**, *101*, 689–695.

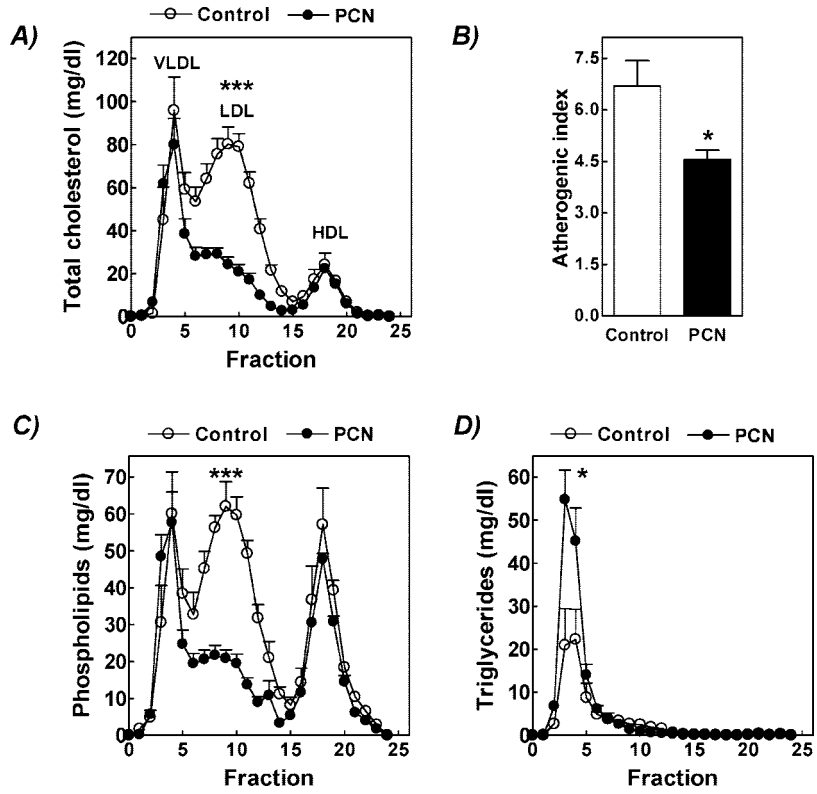

Figure 2. The effect of the PXR agonist PCN on the plasma lipoprotein (A) total cholesterol, (C) phospholipid, and (D) triglyceride distribution and atherogenic index (B). Fractions 2−5 represent VLDL; fractions 6−14 represent LDL; fractions 15−21 represent HDL. The distribution over the different lipoproteins in PCN-treated (closed circles) and control-treated (open circles) mice is shown. Values represent means + SEM of 5 mice. $*P < 0.05$, $***P < 0.001$ vs control-treated mice.

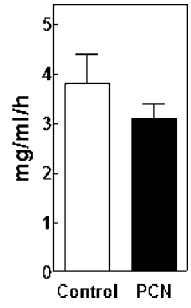

Figure 3. The effect of the PXR agonist PCN on the hepatic VLDL-triglyceride secretion rate in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice.

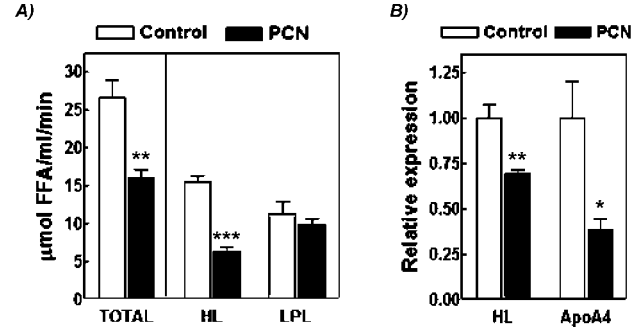

Figure 4. The effect of the PXR agonist PCN on (A) total, HL-, and LPL-mediated post-heparin plasma lipolysis rate and (B) the hepatic relative mRNA expression level of hepatic lipase (HL) and apolipoprotein A4 (ApoA4) in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs control-treated mice.

by 60−70% (Figure 7B). In contrast, the expression of the nuclear receptor PPARα was 70% decreased by PCN ($P < 0.001$; Figure 7C). The expression of the PPARα target gene phosphoenolpyruvate carboxylase (PEPCK) was similarly decreased by 70% ($P = 0.029$; Figure 7C), indicating that the observed decrease in PPARα expression was also associated with a decreased PPARα signaling in livers of PCN-treated mice. In animals, PEPCK facilitates the rate-limiting step in hepatic gluconeogenesis, namely the conversion of oxaloacetate into phosphoenolpyruvate. The marked decrease in the expression of PEPCK therefore suggests that less substrate (i.e., oxaloacetate) is used for the synthesis of glucose, resulting in an increased availability of substrate for the synthesis of acetyl-CoA, an important precursor for fatty acid synthesis. Although the mRNA expression of key genes involved in fatty acid synthesis is not changed (Figure 7A), we anticipate that the fatty acid synthesis rate in liver will be increased upon PXR activation due to the increased substrate availability.

ApoE knockout mice are another established atherosclerosis mouse model. In these mice, the clearance of VLDL

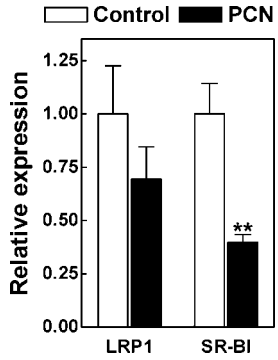

Figure 5. The effect of the PXR agonist PCN on the hepatic relative mRNA expression level of the LDL receptor-related protein 1 (LRP1) and scavenger receptor BI (SR-BI) in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice. $^{**}P < 0.01$ vs control-treated mice.

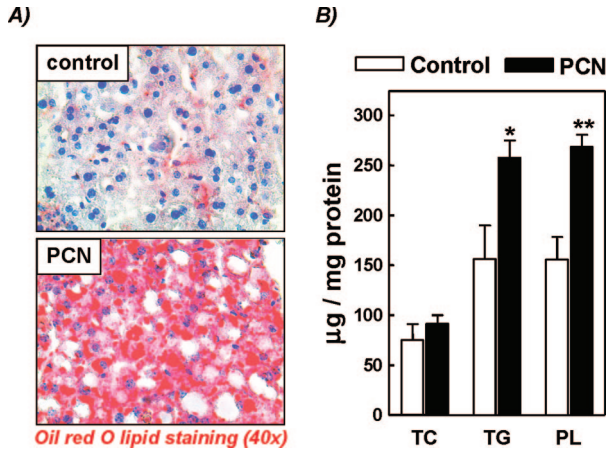

Figure 6. (A) Cryosections of livers from atherogenic diet-fed LDL receptor knockout mice were stained with Oil red O (red) for neutral lipids and counterstained with hematoxylin (blue) for nuclei. Note the striking increase in lipid staining as compared to control-treated mice in livers of PCN-treated mice. (B) The effect of the PXR agonist PCN on hepatic total cholesterol (TC), triglyceride (TG), and phospholipids (PL) levels in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control-treated mice.

and IDL particles is impaired resulting in a strongly elevated plasma VLDL-cholesterol level and the formation of atherosclerotic lesion already on a regular chow diet.[16] To exclude that the changes of lipid levels observed in the LDL receptor knockout mice on the Western-type diet were mouse model- or diet-dependent, we also determined the effect of three day PCN treatment in ApoE knockout mice that were fed a regular chow diet containing 4.3% fat and no cholesterol. Similarly as what was detected in the LDL receptor knockout mice, plasma triglyceride levels were significantly increased by PCN in ApoE knockout mice that exhibit functional LDL receptor expression (Figure 8A). Furthermore, PCN exposure also stimulated the deposition of lipid in livers of ApoE knockout mice (Figure 8B). It thus seems that the effects of PXR activation on plasma and hepatic lipid levels do not depend on either the type of diet (chow/Western type) or the type of atherosclerosis mouse model (LDL receptor knockout/ApoE knockout) used.

Discussion

Inhibition of cholesterol synthesis in the liver by statins leads to a decrease in plasma LDL-cholesterol levels and is currently the most widely used therapy in patients prone to cardiovascular disease. However, CVD can not be prevented in ~70% of the patients treated with statins,[17] indicating that additional therapies to treat atherosclerosis are still needed. Drugs that bind to and regulate the activity of nuclear receptors are estimated to make up 10−15% of the pharmaceuticals currently on the market.[18] Nuclear receptor modulators may possibly also be useful as drugs for the treatment of atherosclerosis and cardiovascular disease. In the current study, we have therefore evaluated the potential for ligands of the nuclear receptor PXR as antiatherosclerotic drugs. Treatment of LDL receptor knockout mice with the PXR agonist PCN was associated with a marked improvement of the plasma lipoprotein−cholesterol profile, as PCN treatment induced a decrease in the plasma LDL-cholesterol level without changing VLDL- or HDL-cholesterol levels, leading to a significantly decreased atherogenic index of the plasma. Based upon this finding, it can be suggested that activation of PXR may be beneficial in the treatment of atherosclerosis. However, the positive change in plasma cholesterol levels coincided with an increase in triglycerides in liver and plasma. Accumulation of triglycerides in the liver is a pathological hallmark of hepatic steatosis development and nonalcoholic fatty liver disease (NAFLD).[19] Nonalcoholic steatohepatitis (NASH), a chronic inflammatory liver disease, is the most severe form of NAFLD, which can ultimately progress into cirrhosis, liver failure, and death.[20] The rise in liver triglycerides upon PCN treatment in LDL receptor knockout mice, and also observed in ApoE knockout mice, is probably due to the increase in the mRNA expression of the nuclear receptor PPARγ, which is a direct regulatory

(16) Zhang, S. H.; Reddick, R. L.; Piedrahita, J. A.; Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **1992**, *258*, 468–471.

(17) Winkler, K.; Ablethauser, C. B.; Gimpelewicz, C.; Bortolini, M.; Isaacsohn, J. L. Risk reduction and tolerability of fluvastatin in patients with the metabolic syndrome: a pooled analysis of thirty clinical trials. *Clin. Ther.* **2007**, *29*, 1987–2000.

(18) Pearce, K. H.; Iannone, M. A.; Simmons, C. A.; Gray, J. G. Discovery of novel nuclear receptor modulating ligands: an integral role for peptide interaction profiling. *Drug Discovery Today* **2004**, *9*, 741–751.

(19) Mensink, R. P.; Plat, J.; Schrauwen, P. Diet and nonalcoholic fatty liver disease. *Curr. Opin. Lipidol.* **2008**, *19*, 25–29.

(20) McCullough, A. J. Pathophysiology of nonalcoholic steatohepatitis. *J. Clin. Gastroenterol.* **2006**, *40*, S17–S29.

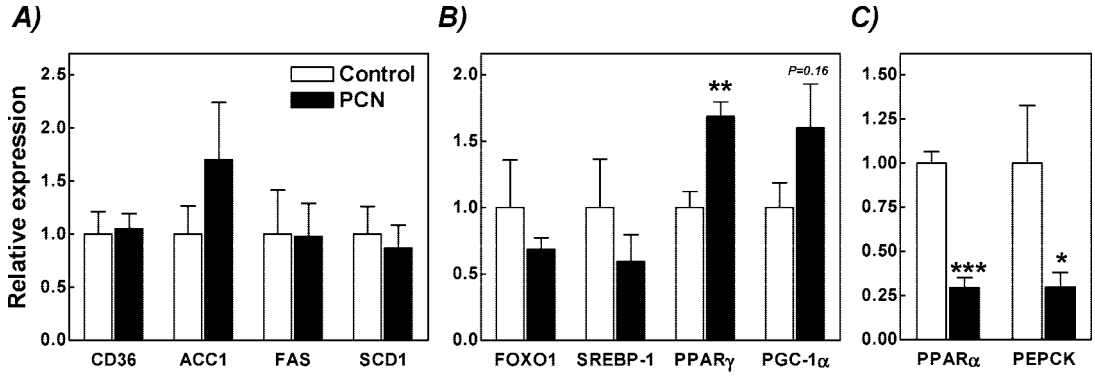

Figure 7. The effect of the PXR agonist PCN on the hepatic relative mRNA expression level of (A) genes involved in *de novo* synthesis of fatty acids, (B) lipogenic transcription factors, and (C) the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) and phosphenolpyruvate carboxylase (PEPCK) in atherogenic diet-fed LDL receptor knockout mice. Values represent means + SEM of 5 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control-treated mice.

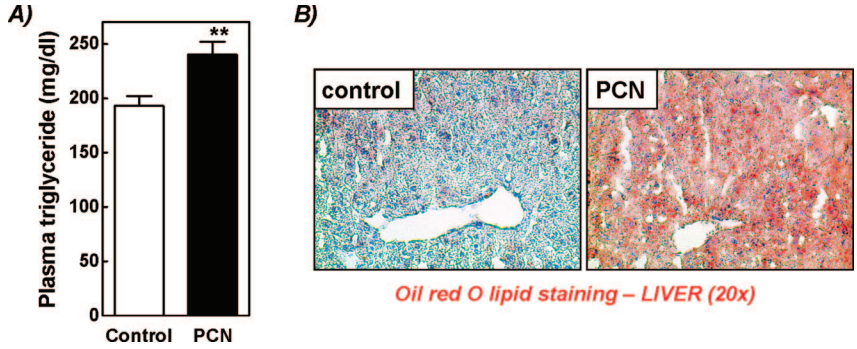

Figure 8. (A) The effect of the PXR agonist PCN on plasma triglyceride level in chow-fed ApoE knockout mice. Values represent means + SEM of 7 mice. $^{**}P < 0.01$ vs control-treated mice. (B) Cryosections of livers from chow-fed ApoE knockout mice were stained with Oil red O (red) for neutral lipids and counterstained with hematoxylin (blue) for nuclei. Note the marked increase in lipid staining as compared to control-treated mice in livers of PCN-treated mice.

target of PXR[21] and has been shown to induce lipogenesis in the liver.[22] Furthermore, the marked 70% decrease in the expression PPARα and PEPCK which is associated with enhanced substrate availability for *de novo* synthesis of fatty acids is expected to lead to an increased hepatic fatty acid synthesis rate, explaining the increased liver lipid content upon PCN treatment. Plasma triglycerides have been identified as an independent risk factor for the development of atherosclerosis.[23] The rise in plasma triglycerides upon PXR activation may diminish the potential beneficial effect of the decrease in plasma LDL-cholesterol levels on atherosclerotic lesion development. The decrease in LDL-cholesterol and the rise in VLDL-triglycerides upon PCN treatment are likely due to an inhibition of the hepatic lipase (HL) activity in plasma, resulting from a decrease in the hepatic expression of both HL and its cofactor ApoA4. Importantly, LDL receptor knockout mice that lack functional HL are susceptible to spontaneous atherosclerotic lesion development.[24,25] Although PCN treatment greatly lowers plasma LDL-cholesterol levels, we anticipate that the observed increase in liver and plasma triglycerides may hamper the clinical application of drugs that activate PXR.

The semisynthetic drug rifampicin is a potent activator of human PXR *in vitro*.[26] Rifampicin is used in the clinic for the treatment of tuberculosis and inactive meningitis. Hyperlipidemia, as characterized by highly increased blood cholesterol

(21) Zhou, J.; Zhai, Y.; Mu, Y.; Gong, H.; Uppal, H.; Toma, D.; Ren, S.; Evans, R. M.; Xie, W. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J. Biol. Chem.* **2006**, *281*, 15013–15020.
(22) Yu, S.; Matsusue, K.; Kashireddy, P.; Cao, W. Q.; Yeldandi, V.; Yeldandi, A. V.; Rao, M. S.; Gonzalez, F. J.; Reddy, J. K. Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. *J. Biol. Chem.* **2003**, *278*, 498–505.
(23) Carmena, R.; Duriez, P.; Fruchart, J. C. Atherogenic lipoprotein particles in atherosclerosis. *Circulation* **2004**, *109*, III2–7.
(24) Barcat, D.; Amadio, A.; Palos-Pinto, A.; Daret, D.; Benlian, P.; Darmon, M.; Bérard, A. M. Combined hyperlipidemia/hyperalphalipoproteinemia associated with premature spontaneous atherosclerosis in mice lacking hepatic lipase and low density lipoprotein receptor. *Atherosclerosis* **2006**, *188*, 347–355.
(25) Freeman, L.; Amar, M. J.; Shamburek, R.; Paigen, B., Jr.; Santamarina-Fojo, S.; González-Navarro, H. Lipolytic and ligand-binding functions of hepatic lipase protect against atherosclerosis in LDL receptor-deficient mice. *J. Lipid Res.* **2007**, *48*, 104–113.
(26) Teng, S.; Jekerle, V.; Piquette-Miller, M. Induction of ABCC3 (MRP3) by pregnane X receptor activators. *Drug Metab. Dispos.* **2003**, *31*, 1296–1299.

(740 vs 240 mg/dL) and fasting plasma triglyceride levels (4165 vs 120 mg/dL) due to excess chylomicrons/VLDL, has been detected as a serious side effect in a patient receiving rifampicin for 3 months for the treatment of pulmonary tuberculosis.[27] No data are currently present on the effect of rifampicin treatment on lipid levels in patients with familial hypercholesterolemia and homozygous LDL receptor deficiency. However, based upon the aforementioned findings in the tuberculosis patient, we are confident that our present data regarding the effect of PXR activation on plasma and liver lipid levels in mice are relevant for the human situation.

(27) Khogali, A. M.; Chazan, B. I.; Metcalf, V. J.; Ramsay, J. H. Hyperlipidaemia as a complication of rifampicin treatment. *Tubercle* **1974**, *55*, 231–233.

In conclusion, our studies show that activation of the nuclear receptor PXR by PCN leads to an inhibition of the plasma HL-mediated lipolysis rate, which is associated with a decrease in plasma LDL-cholesterol levels and induction of hepatic steatosis.

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