FEBS Letters 582 (2008) 949–955

Activation of the farnesoid X receptor represses PCSK9 expression in human hepatocytes

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Received 22 December 2007; revised 12 February 2008; accepted 17 February 2008

Available online 25 February 2008

Edited by Robert Barouki

Abstract **The purpose of this study was to determine whether bile acids (BAs) modulate hepatic pro-protein convertase subtilisin/kexin 9 (PCSK9) gene expression. Immortalized human hepatocytes were treated with various BAs. Chenodeoxycholic acid (CDCA) treatment specifically decreased both PCSK9 mRNA and protein contents. Moreover, activation of the BA-activated farnesoid X receptor (FXR) by its synthetic specific agonist GW4064 also decreased PCSK9 expression. Of functional relevance, coadministration of CDCA counteracted the statin-induced PCSK9 expression, leading to a potentiation of LDL receptor activity. This study suggests that a transcriptional repression of PCSK9 by CDCA or FXR agonists may potentiate the hypolipidemic effect of statins.**

Keywords: PCSK9; Bile acid; FXR; Statin; LDL-cholesterol

1. Introduction

Pro-protein convertase subtilisin/kexin 9 (PCSK9) has recently emerged as a central player in the regulation of cholesterol homeostasis [1]. In addition to mutations affecting the LDL-receptor (LDLr) and apolipoprotein B, "gain-of-function" PCSK9 mutations lead to an increase of plasma LDL-cholesterol (LDL-c) levels and premature atherosclerosis [1,2]. In contrast, "loss-of-function" mutations are associated with low levels of LDL-c and confer protection against cardiovascular disease [3]. PCSK9 is primarily expressed in the liver and the intestine. PCSK9 inhibits the LDLr activity in a post-transcriptional manner [1]. Recent data suggest that, once cleaved, secreted PCSK9 acts as a chaperone and promotes the intracellular degradation of the LDLr by interfering with its recycling to the plasma membrane [4,5].

Various positive and negative regulatory pathways of PCSK9 have been identified. The hypocholesterolemic drugs statins were shown to increase PCSK9 expression, a pathway which exerts a break on their efficiency [6,7]. In accordance with this negative feedback pathway, PCSK9-deficient mice [8] and patients bearing non sense mutations for PCSK9 [9] are more responsive to statins. Our laboratory characterized the PCSK9 promoter and showed that PCSK9 is also up-regulated by insulin as well as by the Liver X Receptor agonist T0901317 via SREBP-1c [10]. In an opposite way, fenofibrate decreases hepatic PCSK9 expression in a PPARα-dependent manner [11].

Bile acids (BAs) are liver-synthesized cholesterol-derivatives that represent the major route for removal of excess cholesterol from the body. Besides their role as detergents, it has now been clearly demonstrated that BAs also exert signalling activities and regulate gene expression in a variety of tissues, including liver and intestine, at least partly through the activation of the farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of ligand-activated transcription factors [12,13]. In addition to FXR, BAs can also activate other nuclear receptors such as PXR (pregnane X receptor), CAR (constitutive androstane receptor) and vitamin D receptor [14,15].

Here, we investigated whether BAs can modulate the expression of PCSK9. We found that chenodeoxycholic acid (CDCA) specifically represses PCSK9 expression in immortalized human hepatocytes, thereby potentiating the activity of the LDLr in response to statins.

2. Materials and methods

2.1. Chemicals

BAs (CDCA, UDCA, DCA, CA, LA), pravastatin, rifampicin and actinomycin D were purchased from Sigma (France). GW4064 was kindly provided by Genfit (Loos, France).

2.2. Cell culture

Immortalized human hepatocytes were cultured on collagen-coated flasks in William's E medium in the presence of a 10% fetal calf serum (FCS). HepG2 cells were cultured in DMEM containing 10% FCS and 1% glutamine. The cells were exposed to various treatments in the

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Abbreviations: BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FXR, farnesoid X receptor; IHH, immortalized human hepatocytes; LA, lithocholic acid; LDL-c, low density lipoprotein cholesterol; LDLr, low density lipoprotein receptor; PCSK9, pro-protein convertase subtilisin/kexin 9; PXR, pregnane X receptor; UDCA, ursodeoxycholic acid

0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.038

presence of a 5% lipoprotein deficient serum (LPDS) unless notified. For PCSK9 secretion analysis, cells were incubated in 1 ml of medium without FCS and LPDS. Treatment toxicity was assessed by quantification of lactate dehydrogenase activity in cell medium using the Roche cytotoxicity detection kit (Roche Diagnostics, Indianapolis, USA).

2.3. Western blots

Proteins were analysed by Western blot as described elsewhere [10], using a polyclonal rabbit IgG directed against the CRSRHLAGASQELQ peptide (Neosystem, Strasbourg, France), an epitope of the C-terminal domain of human and mouse PCSK9, or with the monoclonal anti β-actin AC-15 antibody (Sigma). For secretion analysis, proteins from 400 μl of cell culture media were precipitated with acetone.

2.4. RNA extraction and real time PCR

RNA extraction and real time quantitative PCR (Q-PCR) was performed as previously described [10], using the following primers:

LDLR: AAGGCTGTCCCCCCAAGA forward, CGAACTGCCGAGAGATGCA reverse; ***PCSK9***: ACGTGGCTGGCATTGCA forward, AAGTGGATCAGTCTCTGCCTCAA reverse; ***18S***: AAGTCCCTGCCCTTTGTACACA forward, CGATCCGAGGGCCTCACTA reverse; ***UGT2B4***: CAACCAGTGAAGCCCCTTGA forward, GAAGGTGCTTGGCTCCTTTATG reverse; ***SHP***: CTCTTCCTGCTTGGGTTGGC forward, GCACATCGGGGTTGAAGAGG reverse; ***CYP3A4***: CTCTTCACCGTGACCCAAAGTACT forward, AGCAAACCTCATGCCAATGC reverse.

2.5. Isolation and radiolabelling of LDL

Human LDL (d 1.019–1.063 g/ml) was isolated from plasma of healthy normolipaemic fasting donors by isopycnic preparative ultracentrifugation using a discontinuous KBr density gradient [16]. Isolated LDL was dialysed at least 36 h at 4 °C against PBS pH 7.4. Radiolabelling procedure was performed according to the iodogen® method modified by Fraker et al. [17].

2.6. Binding of ^{125}I-labelled LDL

IHH cells were incubated for 4 h at 4 °C with 10 μg/ml of ^{125}I-labelled LDL in 250 μl of William's LPDS 5% containing 4% fatty acid-free BSA and 50 mM Hepes, pH 7.4. Non-specific binding was determined by the addition of 0.5 mg/ml unlabelled LDL. At the end of the incubation period, the cells were washed three times with 1 ml of D-PBS containing 1% BSA then three times with 1 ml of D-PBS.

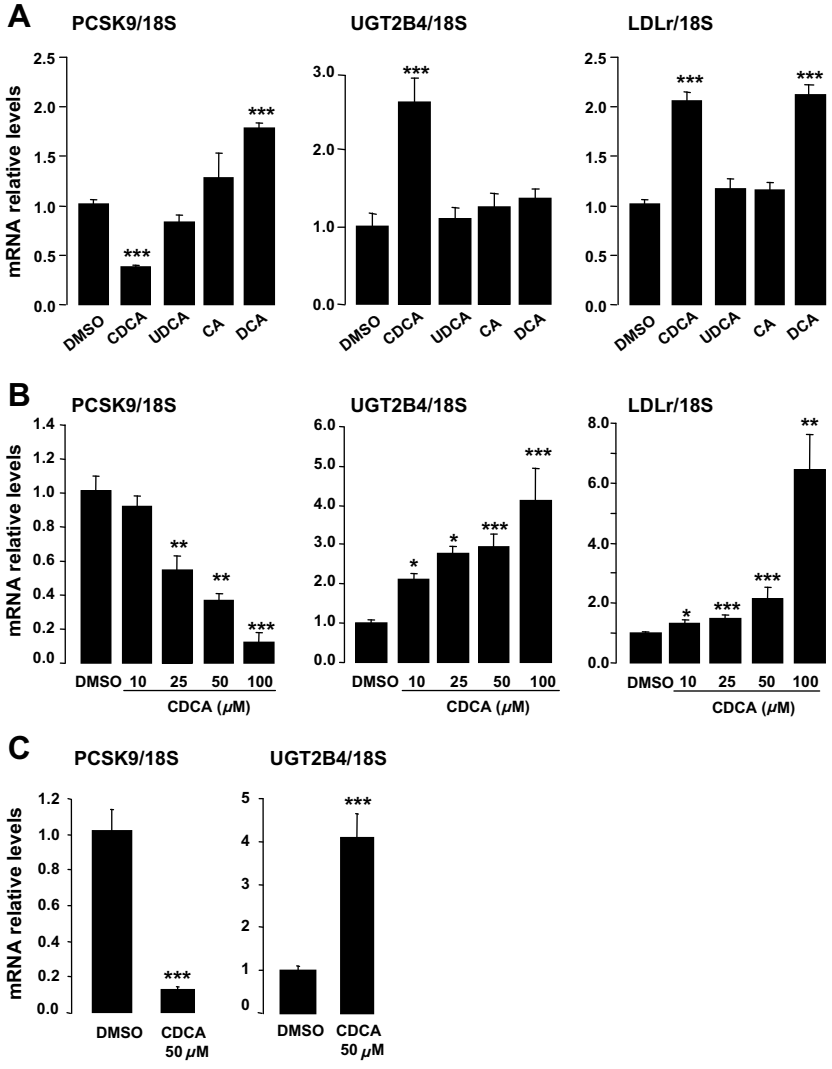

Fig. 1. CDCA reduces PCSK9 mRNA levels in human hepatocytes cell lines. PCSK9, UGT2B4 and LDLr mRNA contents were measured by Q-PCR. (A) IHH, cells were incubated for 48 h with 50 μM CDCA, CA, UDCA, DCA or vehicle (DMSO). (B) IHH cells were treated with increasing concentrations (as indicated) of CDCA for 48 h. (C) HepG2 cells were treated with 50 μM CDCA for 48 h. Values are normalized relative to 18S mRNA and are expressed (means ± S.E.M.; $n = 5$) relative to those of vehicle-treated cells, which are arbitrarily set at 1. Statistically significant differences compared to vehicle-treated cells are indicated ($^{***}P < 0.001$; $^{**}P < 0.01$; and $^{*}P < 0.05$).

The cells were solubilized in 1 ml of 1 M NaOH, the protein content was determined using BSA as a standard, and the radioactivity was measured (1480 Wizard 3″ Automatic Gamma Counter, Wallac, Waltham, Massachusetts, USA). The measured radioactivity was normalized per milligram of cell protein, the specific binding was calculated by subtracting the non-specific binding of ^{125}I-labelled LDL from the total binding.

2.7. Statistics

Results are representative of at least two independent experiments, with at least triplicates in each experiment. Statistical significance was analyzed using an unpaired Student's *t*-test. The values of $P < 0.05$ were considered significant.

3. Results

3.1. Repression of PCSK9 by chenodeoxycholic acid in human hepatocytes

To investigate the effects of BAs on PCSK9 gene expression, IHH cells were treated for 48 h with a variety of primary (CDCA, CA, UDCA) and secondary (LA, DCA) BAs at the concentration of 50 μM (Fig. 1A). Excepted for the LA, these treatments did not affect the cell toxicity assessed by the level of the lactate dehydrogenase activity in cell medium (*data not shown*). CDCA was found to decrease PCSK9 mRNA

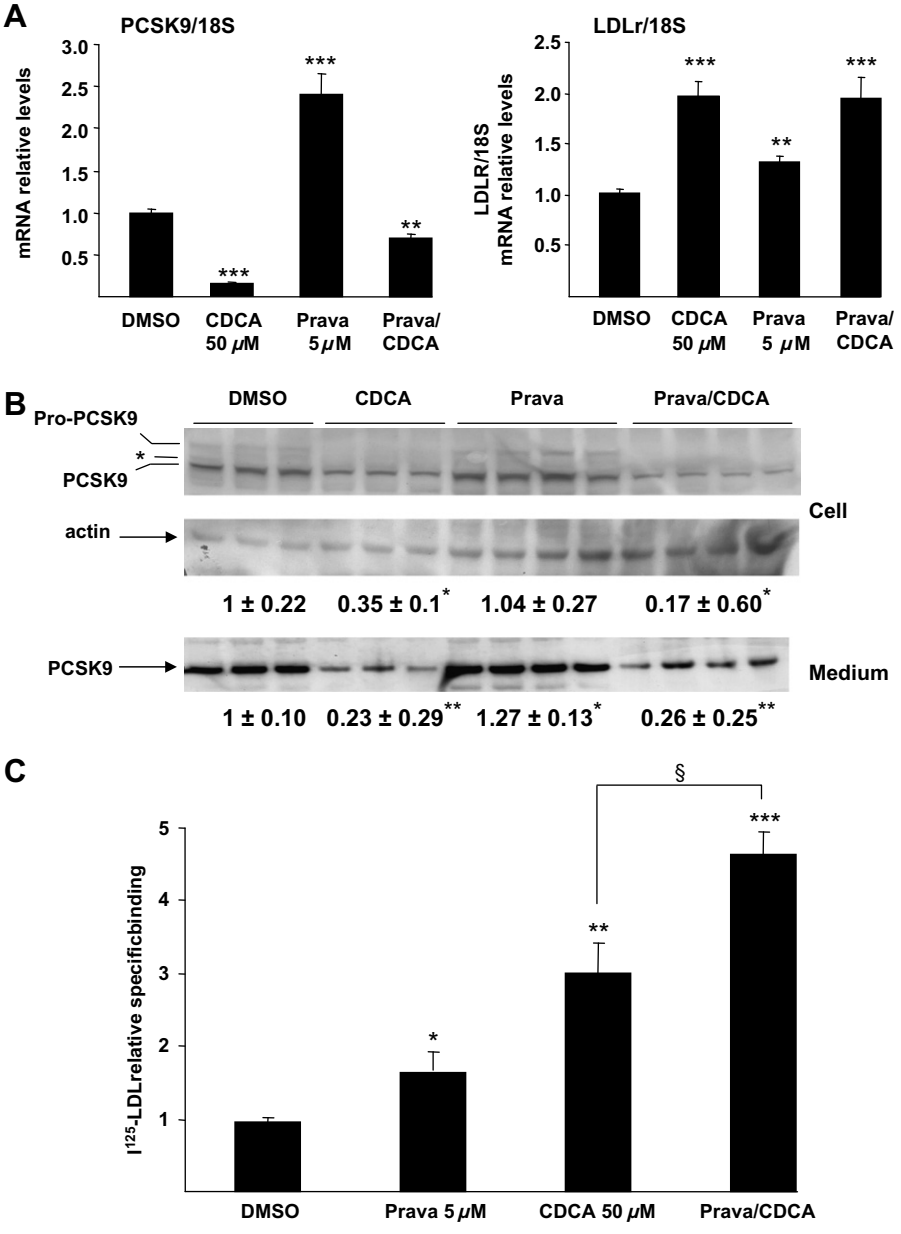

Fig. 2. CDCA amplifies the effect of pravastatin on LDLr activity. IHH cells were pre-treated with 50 μM CDCA or vehicle (DMSO) for 24 h. Then, 5 μM pravastatin was added, or not, to each condition for additional 24 h (DMSO, Prava, CDCA, CDCA+Prava). (A) PCSK9 and LDLr mRNA contents were measured by Q-PCR, as described in Fig. 1. Values are means ± S.E.M. ($n = 6$). (B) Cell lysates and media were collected and their protein content analyzed by Western blot and quantified using Image J 1.37v (Wayne Rasband, N.I.H., USA). Values represent the means ± S.E.M. ($n = 3$–4). This blot is representative of two independent experiments. ∗ indicates a non-specific band. (C) LDLr activity was quantified by measuring the specific binding of I^{125}-labelled LDL for 4 h at 4 °C. Statistically significant differences compared to DMSO ($^{***}P < 0.001$; $^{**}P < 0.01$; and $^{*}P < 0.05$) or between combined treatment CDCA/pravastatin with CDCA ($^{§}P < 0.05$) are indicated.

levels (−59%, $P < 0.001$). In contrast, DCA increased PCSK9 gene expression (+76%, $P < 0.001$), whereas CA and UDCA had no effect. Since CDCA is the more potent natural agonist of FXR [18], the expression of the FXR target-gene UGT2B4 [19] was measured under the same conditions. As expected, CDCA was the sole BA which significantly increased UGT2B4 mRNA levels (Fig. 1A). The effect of BAs treatment on LDLr expression was also investigated in IHH cells. In accordance with previous results in HepG2 cells [20], both CDCA and DCA increased LDLr mRNA levels (+104%, $P < 0.001$ and +109%, $P < 0.001$, respectively) (Fig. 1A). Notably, a strong induction of LDLr mRNA levels was observed for the higher dose of CDCA (+544% with 100 μM, $P < 0.001$) (Fig. 1B). Conversely, a significant and dose-dependent repression of PCSK9 mRNA levels was observed in IHH cells treated with various doses of CDCA for 48 h (Fig. 1B). Moreover, this repression of PCSK9 also occurred in HepG2 cells treated with 50 μM CDCA (−87%, $P < 0.001$) (Fig. 1C). Taken together, these results demonstrate that CDCA is a new repressor of PCSK9 gene expression in human hepatocytes.

3.2. CDCA amplifies the effect of pravastatin on LDLr activity

To assess whether PCSK9 upregulation by statins could be affected by CDCA, we first exposed IHH cells to 50 μM CDCA or DMSO during 24 h before adding or not 5 μM pravastatin for an additional 24 h. As expected [6], 24 h treatment with pravastatin alone increased PCSK9 mRNA levels (+141%, $P < 0.001$). Interestingly, pretreatment with CDCA abolished the induction of PCSK9 gene expression in response to pravastatin. The statin only slightly increased LDLr gene expression (+32%, $P < 0.01$), as described elsewhere [6]. The combination of both drugs did not further increase LDLr mRNA levels compared to CDCA treatment alone (Fig. 2A). In accordance to the mRNA variations, CDCA strongly reduced the quantity of both intracellular and secreted PCSK9 protein content, even in the presence of pravastatin (Fig. 2B). To further investigate whether CDCA functionally influences LDLr activity, IHH cells were treated for 48 h with 50 μM CDCA and the surface binding of ^{125}I-labelled human LDL was assayed (Fig. 2C). Both pravastatin and CDCA increased the LDLr activity (+72%, $P < 0.05$ and +204%, $P < 0.001$ *vs.* DMSO, respectively). Interestingly, when a combined treatment with CDCA and pravastatin was performed, a significant 55% ($P < 0.05$) additional increase in LDLr activity was observed compared with CDCA alone. Altogether these data suggest that CDCA potentiates the effect of pravastatin on LDLr activity by preventing the concomitant induction of PCSK9 expression.

3.3. CDCA-mediated repression of PCSK9 gene expression occurs at the transcriptional level

In order to verify the possibility that CDCA reduced PCSK9 mRNA quantity by a process involving mRNA stabilization, IHH cells were treated for 24 h with either vehicle or 50 μM CDCA before the transcriptional inhibitor actinomycin D (5 μg/ml) was added to the medium during the indicated times. As observed in Fig. 1, CDCA treatment for 24 h decreased PCSK9 mRNA levels while it concomitantly increased the expression of SHP, a direct FXR target gene [21]. PCSK9 mRNA half-life was greater than 9 h and was not altered by CDCA. Under the same experimental conditions, the half-life of SHP mRNA was less than 3 h and seemed unaltered by CDCA pre-treatment (Fig. 3). These results suggest that CDCA acts at the transcriptional level to modulate PCSK9 gene expression.

3.4. Activation of FXR by the synthetic FXR ligand GW4064 represses PCSK9 expression and increases LDLr activity

In order to test whether the regulation of PCSK9 expression by CDCA depends on FXR activation, IHH cells were treated by the synthetic FXR agonist GW4064 (Fig. 4A). A dose-dependent down-regulation of PCSK9 mRNA levels was observed with GW4064 compared to vehicle-treated cells. Moreover, treatment with high doses of GW4064 ($\geqslant 2.5$ μM) also led to a significant increase of LDLr mRNA levels. Importantly, a similar repression of PCSK9 in response to 5 μM GW4064 was observed in HepG2 cells (−88%, $P < 0.001$) (Fig. 4B). In accordance with mRNA variations, treatment with 1 μM GW4064 for 48 h decreased both intracellular and secreted PCSK9 protein content (Fig. 4C).

Next, we assessed whether the repression of PCSK9 by BAs can be mediated by the activation of PXR. IHH cells were treated with rifampicin, a potent PXR ligand [22] (Fig. 4D), PXR activation led to a dose-dependent increase of PCSK9 gene expression, thus excluding its participation in the CDCA-mediated repression of PCSK9. While rifampicin

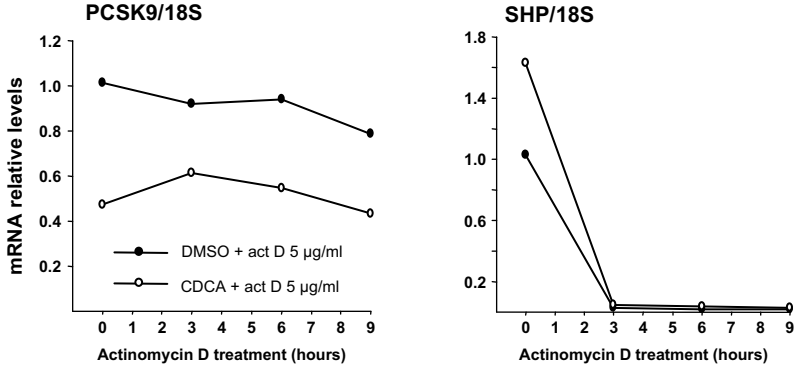

Fig. 3. CDCA regulates PCSK9 expression at the transcriptional level. The half-life of PCSK9 mRNA is unaffected by CDCA. IHH cells were cultured for 24 h in the presence or absence of 50 μM CDCA. Actinomycin D (5 μg/ml) was then added to all dishes, and RNA was isolated after the indicated time. PCSK9 and SHP mRNA contents were measured by Q-PCR as described in Fig. 1. Values are means ± S.E.M. ($n = 6$). Statistically significant differences compared to DMSO ($^{***}P < 0.001$).

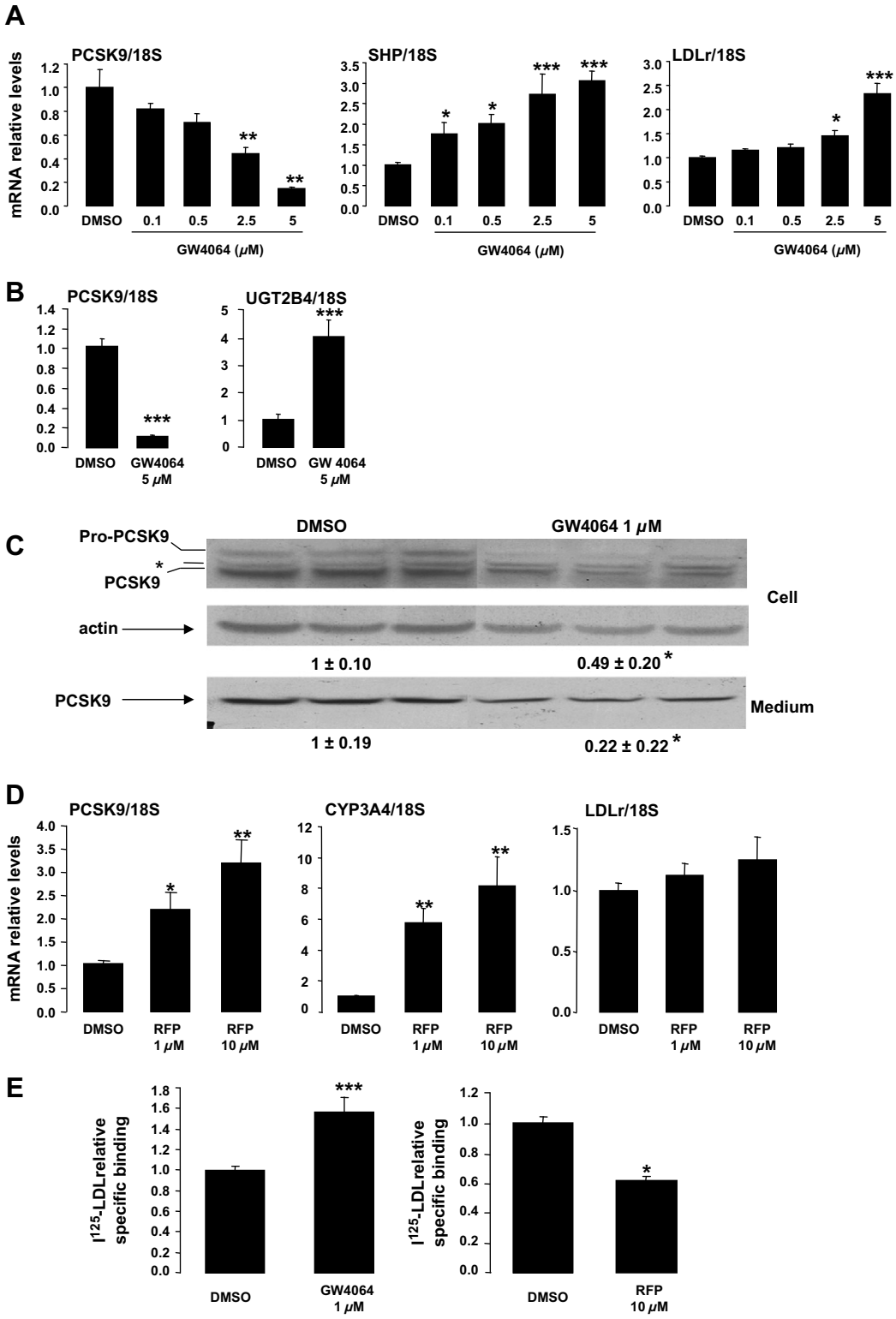

Fig. 4. FXR synthetic agonist GW 4064 represses PCSK9 expression and increases LDLr activity. IHH (A) and HepG2 (B) cells were treated with the indicated concentrations of the FXR synthetic agonist GW4064. PCSK9, SHP, LDLr and UGT2B4 mRNA contents were measured by Q-PCR as described in Fig. 1. Values are means ± S.E.M. ($n = 6$). (C) GW4064 increases PCSK9 protein content. IHH Cell lysates and media were collected and their protein content analysed by Western blot and quantified using Image J 1.37v (Wayne Rasband, N.I.H., USA). Values represent the means ± S.E.M. ($n = 3$). This blot is representative of two independent experiments. ∗ indicates a non-specific band. (D) IHH cells were treated with the indicated concentrations of the PXR agonist rifampicine (RFP). PCSK9, CYP3A4 and LDLr mRNA contents were measured by Q-PCR as described in Fig. 1. Values are means ± S.E.M. ($n = 6$). (E) Effects of GW4064 and RFP on LDLr activity. LDLr activity was quantified as described in Fig. 2B. Values are means ± S.E.M. ($n = 6$). Statistically significant differences between control DMSO and specific treatments are indicated ($^{***}P < 0.001$; $^{**}P < 0.01$; and $^{*}P < 0.05$).

strongly induced mRNA levels of the PXR target gene Cyp3A4, it did not alter LDLr mRNA levels.

Of functional relevance, 1 μM GW4064 significantly increased the LDLr activity (+60%, $P < 0.001$). In correlation with the PXR-mediated induction of PCSK9, LDLr activity was conversely reduced by 30% ($P < 0.05$) in response to 10 μM rifampicin (Fig. 4E). These results suggest that FXR activation may contribute to the CDCA-mediated repression of PCSK9 in IHH cells.

4. Discussion

Human genetic studies indicate that inhibiting PCSK9 is a very promising strategy to lower LDL-c [3]. One way to reach this goal is to decrease the synthesis of PCSK9. Recently, the administration of an anti-sense oligonucleotide inhibitor targeting PCSK9 in high-fat fed mice led to a 38% decrease of the plasma LDL-c concentrations [23]. An alternative strategy is to directly inhibit PCSK9 transcription. In the current study, we demonstrated for the first time that CDCA is a new repressor of PCSK9 gene expression in human hepatocytes. Of functional relevance, the down-regulation of PCSK9 mRNA levels by CDCA treatment is associated with an increased LDLr activity. More importantly, CDCA is able to counterbalance the positive regulation of PCSK9 by pravastatin and therefore potentiates its stimulating effect on the LDLr activity. CDCA increases LDLr mRNA levels [20], an effect that should also contribute to stimulate LDLr activity. In our cellular model, however, the potentiation of LDLr activity in response to CDCA and pravastatin combination treatment occurs in the absence of a further increase of LDLr mRNA levels. This finding validates the working hypothesis that coadministration of a PCSK9 inhibitor may amplify the hypolipidemic effect of statins.

Amongst the BAs we tested, only the most potent FXR agonist CDCA repressed PCSK9 expression, indicating that the regulatory pathway might be specific for this nuclear receptor. In accordance with this hypothesis, we found that the specific synthetic agonist GW4064 reduces PCSK9 mRNA and protein contents. Moreover, we demonstrate for the first time that GW4064 increases the LDLr activity in vitro. On the other hand, a contribution of the other signalling pathways induced by the CDCA in the repression of PCSK9 was excluded. Activation of PXR leads indeed to an increased PCSK9 expression and a subsequent decrease of LDLr activity. In addition, treatment with DCA, a more potent ligand than CDCA for the G protein-coupled cell-surface BA receptor TGR5 [24], moderately increased PCSK9 expression. CDCA has been shown to increase LDLr mRNA stability by inducing mitogen activated protein (MAP) kinase signalling pathways, particularly extracellular-regulated kinases (ERK) 1/2 [20]. Inhibition of ERK1-2 signalling pathway by the specific inhibitor U0126 significantly reduced PCSK9 mRNA levels (*data not shown*). These results indicate that neither the activation of PXR, TGR5 nor ERK1–2 is involved in the CDCA-mediated repression of PCSK9.

To definitively confirm that CDCA-mediated repression of PCSK9 is FXR-dependent, a FXR-silencing gene experiment using siRNA was performed. Despite a highly significant decrease of FXR mRNA levels (−80%, $P = 0.001$) and the abolition of CDCA-induced SHP gene expression, the repression of PCSK9 still occurred in FXR siRNA transfected cells (Supplementary Fig. S1). Therefore, this RNAi mediated approach failed to abolish the repressive potency of the FXR pathway. Thus, in addition to FXR activation, it can not be formally excluded that additional BAs-mediated signalling pathways can govern CDCA-mediated PCSK9 repression.

In addition, we cannot use the FXR-deficient mouse model since the CDCA-mediated PCSK9 repression was not retrieved in primary mouse hepatocytes *(data not shown)*. Such a specie-specific regulation in response to FXR was already observed for many genes involved in lipid metabolism as PPARα [25], hepatic lipase [26] and syndecan-1 [27]. While actinomycin D experiments indicate that CDCA acts at the transcriptional level for repressing PCSK9, additional studies are needed to precisely assess the molecular mechanism by which CDCA and GW4064 repress PCSK9 expression.

Very recently, *Nilsonn* et al. demonstrated that a 3-weeks treatment with CDCA reduces LDLr mRNA levels in the liver of subjects who underwent a cholecystectomy for gallstone disease, while PCSK9 mRNA levels were not altered [28]. Conversely, a 3-weeks treatment with cholestyramine, a BA sequestrant, increased LDLr and PCSK9 mRNA levels by 65% and 70%, respectively. Although this study reinforces the hypothesis for a cross-talk between BAs and PCSK9, it remains difficult to reconcile these in vivo findings with our in vitro data, as well as with previously published results demonstrating that CDCA induces LDLr gene expression in vitro [20,29]. The reason for this discrepancy remains unclear. However, it should be noticed that CDCA treatment in these patients failed to modulate the expression of hepatic FXR-target genes such as ApoCIII and ApoAI [28].

FXR appears as a promising target to treat dyslipidemia [12]. CDCA has been shown to reduce triglyceride and cholesterol levels in the fructose fed hamster model of metabolic syndrome and combined dyslipidemia [30]. Similar results were recently reported with a new synthetic FXR agonist in several rodent models of dyslipidemia [31], and phase I studies in human are in progress (ClinicalTrials.gov Identifier: NCT00499629 and NCT00509756). Our present results suggest that part of the hypolipidemic effects of FXR agonists might be mediated by PCSK9 repression.

Acknowledgements: This work was supported by Grants from the Agence Nationale de la Recherche (No. PPV06217NSA and ANR-06-PHYSIO-027-01, Project R06510NS), Laboratoires Pierre Fabre, and the EU Grant Hepadip (No. 018734). C. Langhi is a recipient of a fellowship from the Nouvelle Société Française d'Athérosclérose. C Le May is supported by a Grant from the Fondation pour la Recherche Médicale. S. Kourimate is supported by a Grant from Région Pays de la Loire and Philippe Costet is titular of a Contrat d'interface INSERM – CHU de Nantes.

We gratefully acknowledge Lucie Arnaud and Anne-Laure Jarnoux for expert Technical Assistance. We thank Dr. K.E. Berge for providing us TK-Renilla plasmid.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.038.

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