Dual mechanisms for the fibrate-mediated repression of Proprotein Convertase Subtilisin/Kexin type 9.
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Running title: fibrates repress PCSK9 and increase PC5/6A and furin expressions.

Pro-protein convertase subtilisin/kexin type 9 (PCSK9) is associated with familial autosomal dominant hypercholesterolemia and is a natural inhibitor of the LDL receptor (LDLr). PCSK9 is degraded by other proprotein convertases: PC5/6A and furin. Both PCSK9 and the LDLr are up-regulated by the hypocholesterolemic statins. Thus, inhibitors or repressors of PCSK9 should amplify their beneficial effects. In the present study we showed that PPARα activation counteracts PCSK9 induction by statins, by repressing PCSK9 promoter activity and by increasing PC5/6A and furin expression.

Quantification of mRNA and protein levels showed that various fibrates decreased PCSK9 and increased PC5/6A and furin expressions. Fenofibric acid (FA) reduced PCSK9 protein content in immortalized human hepatocytes (IHH) as well as its cellular secretion. FA suppressed PCSK9 induction by statins, or by the Liver X Receptor agonist TO901317. PCSK9 repression is occurring at the promoter level. We showed that PC5/6A and furin fibrate-mediated upregulation is PPARα dependent. As a functional test, we observed that FA increased by 30% the effect of pravastatin on the LDLr activity in vitro.

In conclusion, fibrates simultaneously decreased PCSK9 expression while increasing PC5/6A and furin expression indicating a broad action of PPARα activation in proprotein convertase-mediated lipid homeostasis. Moreover, this study validates the functional relevance of a combined therapy associating PCSK9 repressors and statins.

Pro-protein convertase subtilisin/kexin type 9 (PCSK9) is associated to autosomal dominant hypercholesterolemia (1) and is a natural inhibitor of the LDL receptor (LDLr) (2). Its autocatalytic activity is necessary to its processing and for the mature form to reach the LDLr and induce its degradation (3).This catalytic activity is not involved in the LDLr degradation per se. Indeed, cellular or secreted mature PCSK9 probably acts as a chaperone and prevents the LDLr from being recycled by binding to its EGFA domain (4-10). Accordingly, hepatic transient overexpression of wild type PCSK9 in mice (11-14), or elevated circulating levels in parabiotic mice (15), decrease the LDLr expression, and lead to hypercholesterolemia. It has been shown that the pro-protein convertase furin and to a lesser extent PC5/6A cleave PCSK9 into a secreted, truncated, inactive form (16). Interestingly, some “gain of function” mutations don’t necessarily induce a higher affinity of PCSK9 for the LDLr
but decrease PCSK9’s sensitivity to this degradation (4). In contrast, PCSK9-deficiency lowers plasmatic cholesterol concentration and confers protection against cardiovascular disease (17-20). Thus, treatments inhibiting PCSK9 synthesis, processing or binding to the LDLr, could be useful for hypercholesterolemic patients who do not reach therapeutic goals. In particular, these inhibitors could be added to statins to amplify their effect on the LDLr activity. Surprisingly, statins also increase PCSK9 expression via SREBP-2, a pathway which exerts a break on their efficiency (21-23). This negative feedback has been shown in vivo in mice deficient for PCSK9, and is supported by studies in patients bearing non sense mutations for PCSK9 and highly responsive to statins (19;24). Various positive and negative regulatory pathways of PCSK9 gene have been identified. PCSK9 is up-regulated in mice by the agonist of the Liver X Receptor (LXR), TO901317 (23). We recently demonstrated that PCSK9 is also up-regulated by insulin as well as the LXR agonist TO901317 via SREBP-1c which binds a response element located at -336bp from the ATG in the promoter region (25). However, we showed that fenofibrate, a ligand for the peroxisome proliferators activated receptor alpha (PPARα), decreases PCSK9 mRNA and protein quantity in the liver of wild type mice, but not in PPARα-/- mice (26). Fenofibrate is commonly used in clinical practice for its hypotriglyceridemic effects. Although the benefit of fenofibrate used as a monotherapy is debated (27), a significant decrease of the LDL-C has been observed in patients treated with an association of fenofibrate and simvastatin (28). Furthermore, the “epidemic” of combined hyperlipidemia related to the insulin-resistant states makes the association of statins with other drugs targeting triglycerides and HDL levels- as fenofibrate does- desirable (29).

Here we show that various fibrates repressed PCSK9 expression in immortalized human hepatocytes (IHH). We show for the first time that fibrates also increase the synthesis of PC5/6A and furin in a PPARα dependent fashion suggesting a dual mechanism of action on PCSK9 both at the transcriptional and protein level. Fenofibric acid (FA), the active form of fenofibrate, reduced the protein content of PCSK9 in IHH as well as its cellular secretion, and prevented its accumulation due to statins or the LXR agonist TO901317. These repressive effects were reproduced at PCSK9 promoter’s level. To test the functional relevance of these findings, we verified that FA amplified the effect of pravastatin on the LDLr activity.

**Methods**

**Chemicals.**

Fenofibric acid was purchased from ABCR (Karlsruhe, Germany); other chemicals and drugs came from Sigma (saint Quentin Fallavier, France). Drugs were dissolved in DMSO, also used in controls.

**Cell culture**

Human immortalized hepatocytes were described elsewhere (30). They were cultured on collagen-coated flasks in William’s E medium in the presence of a 10% FCS. The day before the experiment, the cells were plated in 6-wells plates at 1.10⁶ cells/well. The cells were exposed to various treatments in the presence of a 5% lipoprotein deficient serum (LPDS) unless notified. Media and drugs were renewed after 24hrs of culture. When the secretion was to be analysed, cells were incubated in 1ml of medium without serum.

**Western blots**

Proteins from 300μl of cell culture media were precipitated with acetone. Cell were scrapped and homogenized in 1X PBS, 1% Triton X-100. 75μg proteins were analysed by western blot as described elsewhere (25), using a polyclonal rabbit IgG directed against the CRSRLAGASQELQ peptide (Neosystem, Strasbourg, France), an epitope of the C-terminal domain of human PCSK9, or with a polyclonal anti-PC5 antibody (abcam, Cambridge UK), a polyclonal anti-furin (abcam) and with the monoclonal anti β-actin AC-15 antibody (Sigma).

**Real time PCR**

Real time PCR analysis were performed as described elsewhere (25) with SYBR green PCR Master Mix (Applied Biosystems, Courtaboeuf, France). Oligonucleotides used for real time PCR are described as “supplemental methods”. Because primers targeting PC5 detect both PC5/6A and 6B we refer to these mRNA as PC5.
Promoter analysis
We previously identified PCSK9 proximal promoter (25). Briefly, a long (-4bp to -1460 bp) and short (-4bp to -1027bp) versions of the proximal promoter (wt) were inserted into PGL3 basic vector and versions mutated (mut) for a SREBP response located at -336 bp were generated. We used long or short hPCSK9wt or hPCSK9mut (0.150 \( \mu \)g per well) together with pRL-CMV renilla (5ng/well) in HepG2 cells and long hPCSK9wt (0.5 \( \mu \)g per well) with TK-renilla (10ng/well), in IHH cells.

The day before the experiment, HEPG 2 cells were plated at a density of 300,000 cells per well and IHH cells at a density of 350 000 cells per well, out of 12-well plates, in DMEM glucose 1g/l (HepG2) or William’s medium E (IHH), SVF 10%, 10 \( \mu \)g/ml of streptomycin, 100u/ml penicillin. The transfection was performed with lipofectamine-2000, according to the manufacturer instruction (Invitrogen, Cergy Pontoise, France), and cells were maintained in the same culture medium without serum for 20 hrs. The analysis was performed with the Dual-Luciferase Reporter Assay System (Promega, Charbonnières, France). TK-renilla was a generous gifts from K.E. Berge. IHH cells were a generous gift from H. Moshage and D. Bernuau.

SiRNAs
The following pre-designed SiRNAs were used as duplexes for PCSK9-knockdown experiments: sense sequence: GGUCUUGGAUGCAAAGUCAdTdT and antisense sequence UGACUUUGCAUCCAGACCdTdT. Non-targeting SiRNA or “negative SiRNA”were used as a control (Eurogentec, liege, Belgium). Transfections were carried out with lipofectamine-2000 (Invitrogen) over 7hrs, with a final concentration of 200 nM SiRNA in 6 well-plates according to the manufacturer’s recommended procedures. The cells were then incubated in fresh medium and grown without serum for 48 hours to increase the basal activity of the LDLr.

For PPAR\( \alpha \) knockdown, a set of duplex SiRNA was used (On target plus SiRNA smart pool, Dharmacon, USA) under the same conditions as above. After transfection, cells were left in fresh medium with LPDS for 24hrs and exposed to vehicle or FA for 8hrs.

LDLr activity.
After the incubation period time cells were washed two times in ice-cold PBS and then treated with 20\( \mu \)g/ml \( ^{125} \)LDL without or with 500\( \mu \)g/ml unlabeled LDL in William’s E medium, 5% LPDS, 4% BSA, 50mM HEPES, for 4hrs at 4°C. Cells were washed three times in PBS containing 1% BSA and rinsed three times with PBS. Cells were lysed with NaOH 1N during 30mns and radioactivity counted. \( ^{125} \)LDL was corrected for cellular protein. Specific binding was calculated by substracting the normalized \( ^{125} \)LDL radioctivity in the presence of excess unlabeled LDL from radioactivity in its absence.

Statistics:
Each experiment is representative of at least two independent experiments with a minimum of triplicates per condition. All values are reported as means ± sd. Statistical significance was analyzed using a Student’s unpaired \( t \) test. The values of p< 0.05 were considered significant.

Results
PCSK9 expression is repressed by various PPAR\( \alpha \) ligands in human immortalized hepatocytes.
To assess whether PCSK9 repression by fenofibrate could be extended to other PPAR\( \alpha \) agonists and to the human gene, we exposed IHH cells for 24hrs to Wy14643 (100\( \mu \)M) or to 250 \( \mu \)M of FA, clofibrate and gemfibrozil (fig. 1A). Cells were cultured with LPDS 5%, in order to reduce the amount of natural PPAR\( \alpha \) ligands associated to lipoproteins. As expected, PPAR\( \alpha \) itself responded positively to the treatments (respectively +1000%, p<0.05, +400%, p<0.01, +377%, p<0.01, +300% p<0.01). Each agonists induced a dramatic decrease of PCSK9 mRNA quantities, (-75% for clofibrate and -95% for FA, p<0.05), down to almost undetectable levels for Wy14643 and gemfibrozil.

Next we characterized FA-dependent PCSK9 repression in IHH. We observed a dramatic decrease of the quantity of PCSK9 mRNA within 12hrs of treatment at the dose of 250\( \mu \)M (86%, p<0.05), with a concomitant 181% (p<0.05) increase of the quantity of PPAR\( \alpha \) mRNA, as expected. Increasing doses of FA resulted in a dose dependent decrease of PCSK9 mRNA quantity in IHH exposed to FA.
for 24 hrs, with an EC50 of 200 μM (p<0.05) (fig. 1C). PPARα was responsive to a 100μM FA (+100%, p<0.01) and doses above.

FA also repressed PCSK9 in the other human cell line HepG2 (supplemental data, -40% after 24 hrs of exposure to 250μM FA, p<0.01).

FA prevents the induction of PCSK9 in response to statins and LXR agonist TO901317.

To determine whether PCSK9 up-regulation by statins could be affected by FA, we exposed IHH to pravastatin 10 μM (PV) and/or FA 250 μM over 24 hrs (fig. 2A). As a positive control, FA treatment increased the quantity of Carnitine Palmitoyl Transferase-I (CPT-1) mRNA (+219%, p<0.001) (31). Pravastatin didn’t interfere with this response. Pravastatin increased the quantity of PCSK9 mRNA, (+96%, p<0.01). Similar results were obtained with lovastatin 10μM (data not shown). As published, the increase of the quantity of the LDLr mRNA was weaker in response to pravastatin than for PCSK9 (21) (+26%, p<0.05). FA decreased the quantity of PCSK9 mRNA (-90%, p<0.001). Interestingly, FA counteracted the statin-induced expression of PCSK9 (-90% for PV + FA vs. control, p<0.001). FA increased the quantity of LDLr mRNA (+44% p<0.05 and +59%) but didn’t impair its response to pravastatin (+44%, p<0.05). We recently showed that PCSK9 is up-regulated by the LXR agonist TO901317 via SREBP-1c, in murine cells (25). We verified these results in IHH cells and investigated the effect of FA on this pathway (fig. 2B). After 48 hrs of exposure, the quantity of CPT-1 mRNA augmented by 163% in presence of FA (p<0.05), showing the integrity of the PPARα-related pathway. The effect of FA on PCSK9 mRNA was still important (-76% compared with control, p<0.01). TO901317 1μM also increased PCSK9 mRNA (+151%, p<0.05) in these human cells. Interestingly, FA suppressed the LXR agonist dependent induction of PCSK9. However, FA did not suppress SREBP-1c induction in response to TO901317 (fig. 2B). The LDLr responded positively to FA and TO901317 (+116% and +149% respectively, p<0.01,) and when both compounds were added together, an amplification of their effect was observed (+308% compared with control, p<0.01, and +89%, p<0.05 when compared with FA alone).

FA impairs the stimulation of PCSK9 secretion and activity by pravastatin.

Next we verified whether FA impairs PCSK9 protein accumulation in response to pravastatin treatment (fig. 3B). A western blot was performed with proteins from cells or the culture medium after they were exposed to FA 250μM or pravastatin 10μM for 48 hrs in the absence of serum. Within the cells, pravastatin increased pro-PCSK9 and PCSK9 total content by 47% (p<0.05). FA decreased PCSK9 expression by 65% (p<0.05) and no change was observed compared with control when both drugs were added together. In the medium, pravastatin increased PCSK9 content by 45% (vs control p<0.05). FA diminished PCSK9 quantities by 68% (vs control p<0.01). Interestingly, the repressing effect of FA on PCSK9 protein content was maintained when both drugs were added (-63% p<0.01).

PC5 and furin expression are increased by fibrates in a PPARα dependent fashion

Because PC5/6A and furin degrades PCSK9 (16), we decided to test whether PPARα agonists would increase their expression in IHH, concomitantly with the analysis described above. A 24 hr-long treatment with Wy14643 (100μM), gemfibrozil (250μM) and fenofibric acid (250μM) increased PC5 mRNA quantity by 600% (p<0.001), 370% (p<0.001), 116% (p<0.05) respectively and furin expression was raised by 275% (p<0.05), 990% (p<0.001), 160% (p<0.001) respectively (fig. 1A). The same tendency was observed with clofibrate although not statistically significant. At a dose of 250μM, the kinetic of induction showed a maximal effect of FA after 12 hrs of exposures for both genes (+200% for PC5 and +400% for furin, p<0.05, fig.1B). In a 24 hrs-long treatment and dose dependent study, a plateau was observed for PC5/6A starting with the lowest dose of FA tested, 100μM (+120%, p<0.05) (fig 1C). Interestingly a dose-dependent effect was observed for furin.

Similar results were observed in HepG2 cells (Supplemental data). After 24hrs of exposure to 250μM, furin mRNA levels were increased by 340% (p<0.001) and PC5 by 100% (p<0.001).
We verified that these changes in PC5 and furin expression were also present at the protein level (fig. 3A). A western blot analysis showed that upon FA treatment (250 μM for 48 hrs), both mature PC5/6A isoform and furin protein quantities were increased by 130% and 70% respectively (p<0.001). PCSK9 content were reduced by 60% (p<0.001).

Next we verified the effect of statins and the LXR agonist on furin and PC5 expression. No effect of pravastatin or T0901317 was detected on PC5 nor furin expressions or FA-dependent repression at the mRNA level (fig. 2A and 2B).

We checked whether PPARα is involved in the response of PC5 and furin to fibrate (fig. 4A). Cells were transfected with non targeting SiRNA or a pool of PPARα-targeting SiRNAs and cultured for 24 hrs in medium containing 5% LPDS and then exposed to FA for 12 hrs. This resulted in the knockdown of 80% PPARα mRNA (p<0.001). FA alone increased by 100% PPARα mRNA quantity but, as desired, this short treatment was unable to overcome the effect of the SiRNAs on PPARα target gene CPT-1. Indeed FA alone increased the mRNA quantity by 81% (p<0.05) but the upregulation was lost in presence of the SiRNAs. A very similar profile was observed for PC5 and furin strongly suggesting that they constitute genuine PPARα target genes. We observed an important and reproducible decrease of PCSK9 expression in cells depleted in PPARα (-70%, p<0.001) and no additional effect of FA with this knockdown. Because SREBPs are major regulators of PCSK9 we checked their expression under these conditions. Both SREBP-1c and SREBP-2 were significantly downregulated in PPARα knockdowned cells (respectively -75% p<0.001 and -30%, p<0.05, p = 0.06 for SREBP1a), suggesting that this might be the cause of PCSK9 mRNA fall down. Their expression levels were not affected by FA alone with or without SiPPARα.

**FA abolishes PCSK9’s proximal promoter response to statins.**

We showed that PCSK9 proximal promoter contains a response element for SREBP-1c located at -336bp from the ATG (25) and it is known that in vivo and in HepG2 cells, PCSK9 is regulated by statins, and SREBP-2 (21-23). First, we verified whether PCSK9 response to statins was mediated by the proximal promoter. To reproduce the experimental conditions of the literature quoted above, we chose lovastatin and HepG2 cells as a model (fig. 4B). We transfected the cells with either a long version or a short version of PCSK9 proximal promoter (long wt, short wt), or with the equivalent constructs mutated for the SREBP response element (long mut, short mut). After transfections, cells were cultured in presence of lovastatin (10μM), in presence or not of the cholesterol precursor mevalonolactone 2.5mM (mevalonate). As we previously showed (25), the short version of the promoter reproducibly exhibited a higher activity than the long version (p= 0.07, ns), suggesting that a repressor element might exist between -1460bp and -1027bp. Lovastatin increased the wild type promoter activity by 450% (p<0.001) for the long version and by 512% (p= 0.012) for the short version, showing that there is probably no response element involved in this pathway between -1460bp and -1027 bp. The activation by lovastatin was significantly reduced by 66% with the concomitant addition of mevalonate (long wt “lovastatine” versus “lovastatine + mevalonate” p< 0.001), confirming that the pathway involved is indeed cholesterol-related. Mutating the SREBP response element impaired severely the activity of the promoter itself suggesting that it is a critical element for PCSK9 expression (long wt vs long mut: -65%, p< 0.001, short wt vs short mut: -68%, p<0.05). The mutated versions of the promoter were still responsive to statins but to a lesser extent than for the wild type version (183% increase for long mut vs long wt p<0.01, and +118% fold increase for short mut vs short wt), suggesting that this element is the major site accountable for the effect of lovastatine.

Next, we verified whether FA would repress the activation of PCSK9’s promoter by statins (fig. 4C). To make a parallel with the data of the present paper, we extended our results to IHH and showed that the activity of the long version of the promoter is increased by 260% in response to pravastatine 10μM (p<0.001) and reduced by 47% by FA alone (p<0.001). Interestingly, the concomitant addition of FA 250μM to pravastatin suppressed the activation of the promoter, suggesting that this is the mechanism underlying PCSK9’s repression by PPARα ligands.
FA amplifies the effect of pravastatins on the LDLr activity.

PCSK9 down-regulation by FA should result in a better efficacy of statins on LDLr activity. First we validated that our cellular model was appropriate to evaluate interactions between the LDLr pathway and PCSK9. Indeed, we observed that PCSK9 knockdown (-40 to -60% cellular and secreted protein content depending on experiments) by SiRNA resulted in a 200% increase in LDLr activity (fig. 5A). Next, we verified our hypothesis by incubating IHH cells in presence of FA 250 \( \mu \)M for 24 hrs followed by a 24hr treatment with pravastatin alone or in combination (5 \( \mu \)M) (fig. 5B). Cells were washed and exposed to \( ^{125} \)I-LDL as described in Methods, at 4°C. As expected, pravastatin increased the amount of specifically bound \( ^{125} \)I-LDL by 500% (p<0.01). FA increased the binding by 170% (p< 0.05). In agreement with our hypothesis, when both drugs were added together, an additional 30% increase in LDLr activity was observed compared with pravastatin alone (varying from 18 to 46% in a total of 5 independent experiments, data not shown).

Discussion

PCSK9 is a natural post-transcriptional inhibitor of the LDLr pathway, and its deficiency results in very low LDL-C levels and a protection against cardiovascular disease (2). Here we show that various fibrates, ligands for PPAR\( \alpha \), repressed PCSK9 expression in immortalized human hepatocytes (IHH). Fibrates also increased the expression of PC5/6A and furin in a PPAR\( \alpha \) dependent fashion. These pro-protein convertases degrade PCSK9, suggesting the existence of a dual mechanism of repression of PCSK9 targeting both the mRNA synthesis and the protein degradation. Fenofibric acid, the active form of fenofibrate, prevented PCSK9 accumulation within the cell or in the media due to statins or the LXR agonist TO901317. These repressive effects were reproduced at the promoter level. As a functional test of the relevance of our findings, we showed that FA amplified the effect of pravastatin on the LDLr activity.

PPAR\( \alpha \) is an essential actor of lipoprotein metabolism. It displays normolipidemic properties by governing the expression of many genes involved in fatty acid catabolism and transport, reverse cholesterol transport via HDL, as well as enzymes involved in the remodelling of lipoproteins (32). Its activity in peripheral tissues has also been involved in the pleiotropic effects of statins (33;34). Our data support a broader role for PPAR\( \alpha \) in LDL metabolism (fig. 6). However, it is striking that one would expect a more potent effect of fibrates on LDL-C in humans, considering the intensity of the repression on PCSK9 we observed and the relative 28% decrease of LDL-C observed in patients heterozygotes for non sense mutations (18). Furthermore, we observed that FA alone also increases the LDLr activity and it’s mRNA cellular quantity, a trait that was previously found for gemfibrozil (35).

Fenofibrate activates lipases and promotes LDL remodelling into particles with optimal binding capacities for the LDLr (36). The overall limited effect of fibrates on LDL-C suggests the existence of a negative feedback pathway similar to PCSK9 up-regulation by statins. If this hypothesis is true, identifying this pathway could help with improving PPAR\( \alpha \) agonists and making them more efficient toward the LDL-C. This is supported by the observation that significant reductions of LDL-C, following fibrate administration, were still reported in clinical trials. This was the case for gemfibrozil in the Helsinki Heart study (37). Although less efficient than statins (38), the decrease of plasma LDL-C levels observed with fenofibrate in short term studies reached up to 30% (39). In the Fenofibrate Intervention and Event Lowering in Diabetes study (FIELD), the decrease compared to the control group was 12 % after four months of treatment and was only 5.8 % after five years. But the results are questionable because of a strong drop in treatment with statins that contributed to the decrease of LDL-C concentrations in both placebo and fenofibrate groups (27). However, our data suggests that fenofibrate could be the most useful in combination with statins. In patients with combined dyslipidemia, co-administration of fenofibrate with fluvastatin or simvastatin resulted in an additional decrease of 10 % of LDL-C compared to the monotherapy (28;40;41). A large cardiovascular endpoint trial, ACCORD (Action to Control Cardiovascular Risk in Diabetes), is currently in progress and will evaluate the beneficial effect of such a combined therapy (http://www.accordtrial.org/public/index.cfm). Large differences in lipid profiles were observed in patients carriers of PCSK9 non sense
mutations, suggesting a complex pathway of regulations (18;42). It is unknown whether variations of the individual responses to the combined treatment correspond to PCSK9 mutations or polymorphisms, and whether the repression of PCSK9 is sustainable. Evaluating the amount of circulating PCSK9 in patients before and after treatment by fibrates would be needed to test these hypotheses and verify our results in vivo in humans.

Here we show for the first time that PC5/6A and furin are positively regulated by fibrates in a PPARα dependent way. Although it would be very informative to show the relative contribution from each pathway to the fibrate-mediated inhibition of PCSK9 (transcriptional or enzymatic), we have not been able to successfully knockdown PC5 and furin at the same time and conclude.

PC5/6A and furin are known for cleaving lipoprotein lipase and endothelial lipase (43). Interestingly, while this manuscript was under review, Jin et al. showed that hepatic cleavage and inhibition of the endothelial synthase by furin and PC5/6A modulate HDL metabolism (44). In particular their inhibition resulted in an increase of HDL-C levels. We propose that the regulation of both these genes by fibrates and PPARα contributes to the beneficial effect of these drugs on HDL-C (fig. 6) (37;38).

Although our findings were confirmed in the human HepG2 cells (supplemental data), we found no regulation of PC5 or furin in vivo in mice (data not shown) after a treatment by fenofibrate, suggesting strong species specificity. We designed oligonucleotides annealing to the junction of human exon 7 and 8, thus targeting both PC5/6A and PC5/6B transcripts. Both transcripts are present in the liver (45;46). PC5/6B encodes a larger protein with a transmembrane domain and is unable to cleave PCSK9, contrary to the shorter, soluble PC5/6A (16). Our western blots confirm that PC5/6A isoform is upregulated by fenofibric acid.

We were surprised to observe that PCSK9 expression fell in response to the knockdown of PPARα since we could expect the opposite. No additional decrease was observed in presence of fenofibric acid, in agreement with a role for PPARα. It is also reminiscent of what we observed in vivo in mice at the mRNA level but it does illustrates the complexity of the regulatory pathways at play, in particular when they result in a repressive effect (26). We propose that at least in vitro the downregulation of SREBPs is responsible for these effects although more work would be needed to investigate the abundance of their nuclear forms under these conditions.

LXR is a very promising therapeutic target because of its anti-atherogenic properties. However, in vivo, LXR agonists also exhibit hypertriglyceridemic and lipogenic properties (47). Whether PCSK9, which acts on VLDL production and triglyceridemia under specific pathophysiological conditions, is involved in this process is unknown (26;48). Here we showed that the LXR agonist TO901317 is an activator of PCSK9 in human cells. As published recently, it also induced LDLr transcription (49). Interestingly, we found an additive effect of both drugs on the LDLr regulation. This suggests that LXR agonists might potentiate the effect of PCSK9 inhibitors on the LDLr activity.

To our knowledge this study is the first formal demonstration that PCSK9 promoter activity is activated by statins, although it was already known that statins and SREBP-2 increase PCSK9 expression (21-23). The decrease of activity and the lower sensitivity to statins consequent to the mutation within the SRE located at -336bp strongly suggest that this sequence mediates the promoter response to statins, as it does with insulin and SREBP-1c (25). It would be interesting to genotype individuals with abnormal LDL-C levels for this region of the gene. Although our experiments suggest that PCSK9, fibrate-dependent repression is occurring transcriptionally via the inhibition of the promoter activity, the exact mechanism is still to be determined. It has been shown in rat hepatocytes that clofibrate and Wy14682 decrease the amount of nuclear active SREBP-2 (50). The hypothesis that this down-regulation is responsible for PCSK9 repression would be difficult to reconcile with the concomitant induction of the LDLr we observe.

It has recently been shown that PCSK9 probably acts as a molecular chaperone on the LDLr by binding to its extra-cellular domain(4;5;10). Several strategies have been proposed or discussed in the literature to inhibit PCSK9, including inhibitors of its catalytic activity or peptides preventing its binding to the LDLr as well as antisense oligonucleotides to shut down its synthesis (2;51). Our results suggest that PCSK9 expression could be successfully repressed at the mRNA level, a strategy that could be useful for patients
suffering from mutations that considerably enhance the affinity of PCSK9 for the LDLr and therefore make patients less responsive to statins, like the D374Y (52).

In conclusion, various fibrates repressed human PCSK9 expression in a transcriptional fashion, and increased PC5/6A and furin expression, in a PPARα dependent fashion. By defining PPARα as being a simultaneous regulator of three proprotein convertases, this study identifies a new class of targets for this nuclear receptor and reinforce their role in lipid homeostasis. Based on the recent findings form Jin et al. we also propose that part of the beneficial effects of fibrates on HDL-C is mediated by this regulatory pathway. Moreover, this study supports the functional relevance of a combined therapy associating PCSK9 repressors and statins.

References


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ABBREVIATIONS:

LXR (Liver X Receptor), PCSK9 (Proprotein Convertase Subtilisin Kexin Type 9), SREBP (Sterol Regulatory Element Binding protein), PC5/6A (proprotein Convertase 5/6A), LDLr (Low Density Lipoprotein receptor), HDL-C (HDL-cholesterol), LDL-C (LDL-cholesterol), Immortalized Human Hepatocytes (IHH), LPDS (lipoprotein Deficient Serum), PPARα (Peroxisome Proliferators Activated Receptor alpha), Carnitine Palmitoyl Transferase-1 (CPT-1), FA (fenofibric acid), PV (pravastatin).
FIGURE LEGENDS

Figure 1: Fibrates reduce PCSK9 and increase PC5 and furin mRNA quantities.
A) Immortalized human hepatocytes (IHH) were exposed for 24hrs to various agonists of PPARα: Wy14643 100μM (W) or 250 μM of FA (FA), clofibrate (Cl), gemfibrozil (G). B) IHH were exposed from 0 to 48hrs to FA 250 μM in 5% LPDS or C) to increasing doses of FA for 24 hrs. PPARα, PCSK9, PC5, furin, PPARα and 18s mRNA levels were quantified by real time PCR and mRNA relative levels are presented . Bars represent the mean ± SD, *p<0.05, ** p<0.01, ***p<0.001.

Figure 2: FA impairs PCSK9 mRNA accumulation in presence of statins or TO901317.
A) IHH cells were exposed for 24hrs to FA 250 μM, and/or pravastatin 10μM in presence of 5% LPDS. Gene specific expression was measured by real time PCR analysis. B) A similar experiment was run for 48hrs using the Liver X Receptor agonist T0901317 1μM. Bars represent the mean ± SD, *p<0.05, ** p<0.01, &: p< 0.05 when compared with FA alone.

Figure 3: FA impairs pravastatin-mediated increase of PCSK9 content in cells and media.
A) IHH cells were exposed for 48 hrs to FA 250 μM in William’s E medium. Cell lysates were collected and their protein content analysed by western blot using antibodies raised against PC5, furin or PCSK9. The bands detected correspond to the mature form of PC5/6A (65KDa) and furin (95 KDa) or to both proPCSK9 and PCSK9. B) IHH cells were exposed for 48hrs to FA 250 μM, and/or pravastatin 10μM (PV) in the absence of serum. Results represent the mean ± SD, *p<0.05, ** p<0.01, ***p<0.001. < indicates a non specific band.

Figure 4: Fenofibric acid regulates PCSK9, PC5 and furin transcriptionally.
A) IHH cells were transfected with non targeting SiRNA (Si negative) or SiRNA directed against PPARα (SiPPARα) and cultured as described in Material and Methods. Gene specific expression was measured by real time PCR analysis. Results represent the mean ± SD, *p<0.05, ** p<0.01.
B) HepG2 cells were transfected with a construct containing a long or short version of the human PCSK9 proximal promoter, wild type or mutated for the SREBP response element located at -336 bp from the ATG (wt, mut) (cf Methods). A) Cells were exposed to lovastatine 10μM and/or mevalonolactone 2.5mM. B) IHH cells were transfected with the long version of PCSK9 wild type promoter and immediately exposed to PV 10μM and/or fenofibric acid 250μM. Results represent the mean ± SD, *p<0.05, ** p<0.01, *** p<0.001.

**Figure 5: FA repress the LDLr activity and potentiate the statin-mediated binding of LDL.**

A) To validate IHH cells as an appropriate model, we first decreased PCSK9 expression using SiRNA targeting PCSK9 mRNA (SiPCSK9), compared with cells exposed to “negative” non targeting SiRNA (Si neg.). IHH were cultured for 48hrs without serum after tranfection and the binding of I$^{125}$-LDL was measured at 4°C as described in “Methods”. A western blot analysis shows PCSK9 expression levels within cells and in media. B) Similar conditions of cultures were used to study the effect of FA 250μM and PV 5μM. When used, FA was added to the cells for a pre-treatment of 24hrs to decrease PCSK9 expression and statins added or not for another 24hrs. Results represent the mean ± SD, & and *p<0.05, ** p<0.01, *** p<0.001.

**Figure 6: PPARα activation acts on pro–protein convertases PCSK9, PC5 and furin to promote LDL-clearance and increase HDL-C concentrations.**

PPARα activation promotes PCSK9 repression by inhibiting its transcription and increasing PC5 and furin expression. Fibrates also directly increases the synthesis of the LDLr and promote a remodelling of LDLs, reducing the number of dense pro-atherogenic particles and increasing the number of large LDLs with optimal binding capacities for the LDLr. Interestingly, PC5 and furin cleave the lipoprotein lipase (triglyceride lipase ) and degrade the phospholipase endothelial lipase (43), an enzyme known for hydrolysing phospholipids from HDLs but that also converts large LDL into smaller LDL with less affinity for the LDLr. This promotes higher plasmatic concentration of
HDL-C (44) and lower LDL-C. In humans, the relative low efficiency of PPARα on LDL clearance compared with statins suggests the existence of a negative feedback pathway.

**Supplemental data**

**FA decreases PCSK9 and increases PC5 and furin expressions in HepG2 cells.**

HepG2 cells were cultured in DMEM medium with 5% LPDS and exposed or not to FA for 24 hrs. PPARα, PCSK9, furin, PC5, and 18S mRNA levels were quantified by real time PCR. Results represent the mean ± SD, **p<0.01, ***p<0.001.
Figure 1

A

B

C

PPARα/18S

PCSK3/18S

PC5/18S

Furin/18S

PPARα/18S

PCSK3/18S

PC5/18S

Furin/18S

PPARα /18S

PCSK3/18S

PC5/18S

Furin/18S

0 100 150 200 250

FA μM

0 100 150 200 250

FA μM

0 100 150 200 250

FA μM

0 100 150 200 250

FA μM
Figure 2

A

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<th>PCSK9/18s</th>
<th>LDLr/18s</th>
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CPT-1/18s | PC5/18s | Furin/18s |

B

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LDLr/18S | PC5/18S | Furin/18S |
Figure 3

A

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<td>1 ± 0.02</td>
<td>2.3 ± 0.3***</td>
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<tr>
<td>Furin</td>
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<td>β-actin</td>
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<tr>
<td>1 ± 0.17</td>
<td>1.7 ± 0.2***</td>
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<td>β-actin</td>
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<tr>
<td>1 ± 0.14</td>
<td>0.42 ± 0.07***</td>
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B

Control → PV → FA → FA + PV → Cell

Pro-PCSK9 → PCSK9 → β-actin

1 ± 0.26 | 1.47 ± 0.19* | 0.35 ± 0.14** | 0.79 ± 0.39

Pro-PCSK9 → PCSK9 → β-actin

1 ± 0.16 | 1.45 ± 0.19* | 0.32 ± 0.08** | 0.37 ± 0.18**
Figure 4

A

B

C

Fold activation

CTRL  FA  PV  PV +

FA
Figure 6
Dual mechanisms for the fibrate-mediated repression of proprotein convertase
subtilisin/kexin type 9
Sanee Kourimate, Cédric Le May, Cédric Langhi, Anne Laure Jarnoux, Khadija
Ouguerram, Yassine Zaïr, Patrick Nguyen, Michel Krempf, Bertrand Cariou and
Philippe Costet

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