Hepatic PCSK9 Expression Is Regulated by Nutritional Status via Insulin and Sterol Regulatory Element-binding Protein 1c

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Familial autosomal dominant hypercholesterolemia is associated with high risk for cardiovascular accidents and is related to mutations in the low density lipoprotein receptor or its ligand apolipoprotein B (apoB). Mutations in a third gene, proprotein convertase subtilisin kexin 9 (PCSK9), were recently associated to this disease. PCSK9 acts as a natural inhibitor of the low density lipoprotein receptor pathway, and both genes are regulated by depletion of cholesterol cell content and statins, via sterol regulatory element-binding protein (SREBP). Here we investigated the regulation of PCSK9 gene expression during nutritional changes. We showed that PCSK9 mRNA quantity is decreased by 73% in mice after 24 h of fasting, leading to a 2-fold decrease in protein level. In contrast PCSK9 expression was restored upon high carbohydrate refeeding. PCSK9 mRNA increased by 4–5-fold in presence of insulin in rodent primary hepatocytes, whereas glucose had no effect. Moreover, insulin up-regulated hepatic PCSK9 expression in vivo during a hyperinsulinemic-euglycemic clamp in mice. Adenoviral mediated overexpression of a dominant or negative form of SREBP-1c confirmed the implication of this transcription factor in insulin-mediated stimulation of PCSK9 expression. Liver X receptor agonist T0901317 also regulated PCSK9 expression via this same pathway (a 2-fold increase in PCSK9 mRNA of primary hepatocytes cultured for 24 h in presence of 1 μM T0901317). As our last investigation, we isolated PCSK9 proximal promoter and verified the functionality of a SREBP-1c responsive element located from 335 bp to 355 bp upstream of the ATG. Together, these results show that PCSK9 expression is regulated by nutritional status and insulinemia.

Autosomal dominant hypercholesterolemia is associated with mutations in genes involved in the regulation of LDL\(^2\) homeostasis. The most common and severe form of monogenic hypercholesterolemia is familial hypercholesterolemia, caused by mutations in the LDL receptor (LDLR) (1). Familial hypercholesterolemia is characterized by elevated plasma LDL-cholesterol levels and premature cardiovascular disease.

Another form of this disease, familial defective apolipoprotein (apo)B100, is caused by mutations in the LDLr binding domain of ApoB100 (1). ApoB100 is synthesized by the liver and is the major protein component of very low density lipoprotein and LDL. Recently, proprotein convertase subtilisin kexin 9 (PCSK9) has been identified as the third gene involved in autosomal dominant hypercholesterolemia (5). Proprotein convertases are proteolytic enzymes that cleave their substrate, producing a biologically active molecule (2). We showed that patients mutated in PCSK9 promodarn (mutation S127R) have decreased LDL catabolism and increased very low density lipoprotein, intermediary density lipoprotein, and LDL production (3). Studies on knock-out mice and plasma lipid profiles of patients with nonsense mutations, showed that PCSK9 deficiency results in low circulating LDL-cholesterol concentrations (4–6). Although it is established that PCSK9 impairs the LDLr pathway by increasing the degradation of the receptor, its effect on apoB production is still controversial (7–10).

LDLR and PCSK9 share a common regulatory pathway. Indeed, the transcription of both genes is activated by cholesterol cell content depletion, via the sterol regulatory element-binding protein (SREBP) (11–14). In mice, PCSK9 is down-regulated by dietary cholesterol, and moderately up-regulated by the liver X receptor (LXR) agonist T0901317. PCSK9 expression is higher in livers of transgenic mice overexpressing truncated nuclear forms of SREBP-1a and SREBP-2 (13, 15). However, in vitro, data obtained in HepG2 cells did not support a direct regulation of PCSK9 by LXR and SREBP-1 (11). Therefore, although it is admitted that PCSK9 is regulated by SREBP2 and cholesterol, the existence of a regulation by SREBP1 isosforms is unclear.

SREBP-1a, -1c, and -2, are synthesized as transmembrane precursors in the endoplasmic reticulum (16). When cellular sterols are low, SREBP-1a and SREBP-2 are cleaved into a mature, truncated, peptide that is directed to the nucleus, where it activates its target genes. SREBP-1a and -1c are products from another gene than SREBP-2 and correspond to two different mRNA species. In the liver, SREBP-1c is the main SREBP-1 and therefore seems the most relevant physiologically (17). SREBP-2 preferentially activates genes involved in cholesterol biosynthesis, whereas SREBP-1c and -1a preferentially activate genes involved in fatty acid biosynthesis or carbohydrate metabolism, including fatty acid synthetase, acetyl-CoA carboxylase, or glucokinase (GK) (18–23). GK converts glucose into glucose 6-phosphate, and therefore SREBP-1c is thought to have a permissive action on glucose-dependent gene regulation (24).

Contrary to what has been observed in vitro, cholesterol cell content does not affect SREBP-1c in vivo (25). SREBP-1c transcription is regulated by insulin and liver X receptor agonists but not glucose (18, 26–30). LXR is involved in many steps of reverse cholesterol transport, macrophage cholesterol efflux, fecal cholesterol excretion, and bile acid synthesis. Therefore it constitutes a promising therapeutic target (31).
However, LXRs activate directly, or indirectly via SREBP-1c, the genes of fatty acid synthesis and promote hypertriglyceridemia (27, 32, 33). Interestingly, insulin also increases the LDLr transcription via SREBP-1c (34, 35). These data, together with the ability of T0901317 to activate PCSK9 in vivo, which could be an indirect effect mediated by SREBP-1c, prompted us to investigate whether insulin could activate PCSK9 transcription via SREBP-1c.

Here we show that fasting decreases PCSK9 expression and that insulin increases PCSK9 via SREBP-1c in primary mouse and rat hepatocytes, as well as in vivo, during hyperinsulinemic-euglycemic clamps performed on mice. The non-steroidal LXR agonist T0901317 increased PCSK9 mRNA quantity in primary hepatocytes through the same pathway. As our last investigation, we demonstrated the functionality of a SREBP-1c response element spanning from −355 bp to −335 bp of the PCSK9 proximal promoter. These results suggest that PCSK9 might play a role in insulin-related pathologies like type 2 diabetes mellitus and the metabolic syndrome.

**MATERIALS AND METHODS**

**Western Blots**—Liver pieces were homogenized in 1× phosphate-buffered saline containing 0.25% sodium-deoxycholate, 1% Triton X-100, and a protease inhibitor mixture (Roche Diagnostic). 60 μg of total or 40 μg of cytoplasmic and 40 μg of nuclear lysate proteins (Pierce Biotechnology), were resolved on NuPAGE 4–12% BisTris gels in MES-SDS buffer (Invitrogen) under reducing conditions, as described elsewhere (8). Proteins were transferred onto a Protran nitrocellulose membrane (Schleicher & Shuell), probed with a polyclonal goat IgG directed against the extracellular domain of the mouse LDLr (R&D Systems, Minneapolis, MN) or polyclonal rabbit IgG directed against the C-terminal domain of human and mouse PCSK9, or polyclonal rabbit anti-SREBP-1 or anti-SREBP-2 (Santa Cruz Biotechnology) or with the monoclonal anti-β-actin AC-15 antibody (Sigma). Immunoreactive bands were revealed using the ECL plus kit (Amerham Biosciences).

**Real Time PCR**—Total RNA was isolated using the RNeasy kit and QiaShredder mini columns, as well as Rnase free DNase I (Qiagen, Courtaboeuf, France). First strand cDNA was synthesized with random primers using a Superscript II reverse transcriptase reagent kit (Invitrogen). Real time PCR analysis was performed on the 7000 Sequence Detection System with SYBR green PCR Master Mix (Applied Biosystems, Courtaboeuf, France). Oligonucleotides were designed using Primer Express software (Applied Biosystem). Sequences were: mouse PCSK9, 5’-CCTCTCTTGGTGGTGGCAATGCAAGTTG forward and 5’-CCTCTTGAGAACCAATGGCGGTAA and 5’-CCCCCAGAGGCTTTGA; rat LDLr, 5’-GGCGTGAGCGGTAAGCCTTTAC and 5’-ATGCGAGGCAATGGCGGTAA reverse; mouse/rat SREBP-1c, 5’-CCCTTTGGGTCGAGTGCTGAGT forward and 5’-CACTCTTTGGTGGTGGGCAATGCAAG fund; mouse acetyl-CoA carboxylase, 5’-CACCTTTGGGTCGAGTGCTGAG forward and 5’-CACCTTTGGGTCGAGTGCTGAGT reverse; mouse/rat ATP binding cassette transporter G5 (ABCG5), 5’-CTGCCAGCCTTTGAGAATTGTGCAAC and 5’-CTGCCAGCCTTTGAGAATTGTGCAAC reverse; mouse/rat SREBP-1c, 5’-GGAGCGCTGAGGCTCAGTTGTTTGGG forward and 5’-CCAATAGGCCAGGGAGA-

**Primary Culture of Hepatocytes and Adenoviral Infection**—At 9:00 a.m., hepatocytes were isolated from the liver of fed male rats or mice by the collagenase method (36), modified as described elsewhere (24, 37). Briefly, livers were perfused with Hanks’ balanced salt solution and washed at a rate of 5 ml/min using the inferior vena cava before collagenase (0.025%) was added. Dead cells were eliminated through a density gradient using Percoll, and viable cells were plated at a density of 7.5×10^4/ml on collagen-coated plates. Cells were given a time span of 2 h to attach in Williams’ medium E with Glutamax (Invitrogen) 10% fetal bovine serum, 10 μg/ml of streptomycin, 100 units/ml penicillin, 100 μg/ml dexamethasone, and 100 μg/ml insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). After attachment, adenosinereaction was performed for 3 h when indicated. Cultures were washed twice with 1× phosphate-buffered saline and maintained in Dulbecco’s modified Eagle’s medium, 10 μg/ml streptomycin, 100 units/ml penicillin, and 100 nM dexamethasone overnight. Both adenosinereaction vectors containing the transcriptionally active N-terminal fragment (amino acids 1–403) of SREBP-1c (AdSREBP-1c, 6 plaque-forming units/cell) and the dominant negative form of SREBP-1 (AdSREBP-1cDN, 15 plaque-forming units/cell) were generous gifts from Dr. Foufelle (INSERM U465, Paris, France) and have been described previously (30) (18). Sham control vector (Adnl) was generated by the Vector Core of the University Hospital of Nantes (38). T0901317 (Sigma) was dissolved with MeSO3.

**Animal Studies**—All studies were approved by the institutional review boards for the care and use of experimental animals. C57BL/6 mice were housed with a 12 h light/12 h dark cycle. Before initiation of diet studies, the mice were maintained on a standard laboratory chow diet (UR Ao3, Villemoison sur Orge, France). For the fasting and refeeding experiments, 8–12 week-old female mice were divided into three groups: non-fasted, fasted, and refed. The non-fasted group was fed ad libitum with the standard chow diet and killed at 9:00 a.m. The fasted group was fasted 24 h before sacrifice (from 9:00 a.m. to 9:00 a.m.), and the refed group was subjected to an additional refeeding with a high carbohydrate/low fat diet (Harlan Teklad TD88122, Madison, Wisconsin) for 6 or 24 h. The high carbohydrate diet contained 48% sucrose, 16% corn starch, 22% casein, 5.5% cellulose, 1% corn oil, 4% mineral, and 1% vitamin.

**Blood and Tissue Sampling**—Plasma glucose concentrations were determined using AccuCheck active. Plasma insulin levels were measured using a solid phase two-site enzyme immunoassay (Mercodia AB, Sweden). The mice were sacrificed, and their livers were removed and immediately frozen in liquid N2 and then stored at −80 °C until further analysis.

**Hyperinsulinemic-Euglycemic Clamp**—C57BL/6 mice were equipped with a permanent catheter in the right atrium via the jugular vein, and allowed to recover for 4 days before the hyperinsulinemic euglycemic clamp experiment was performed. The mice were infused for 6 h with two solutions after a 9-h fasting period. The first solution contained 40 μg/ml somatostatin (UCB, Breda, Netherlands), 200 mg/ml glucose, and insulin (Actrapid). This solution contained insulin (Actrapid), 100 μg/ml somatostatin, and lead to an insulin infusion rate of 10 million units/kg/min. This solution was infused at a constant flow rate of 0.135 ml/h. The second solution contained 300 mg/ml glucose, and its infusion rate was adjusted according to measured blood glucose levels to maintain euglycemic conditions. A second infusion of 100 mg/ml glucose was started after 2 h. The infusion rate was adjusted to maintain euglycemic conditions.

**Promoter Analysis**—The PCSK9 proximal promoter was identified using Genomatix software. We arbitrarily annotated the first base of the...
translated ATG as +1. A human genomic fragment spanning −4 to −1460 bp was amplified by PCR and cloned into TopoTA vector (Invitrogen) using the following oligonucleotides, forward 5′-TTCTTGCTCCAGGGGAGCTTGTAGT and reverse 5′-GGCCAGGGAGAGGTGGTGGCAG. The fragment was transferred to PGL3 basic vector between KpnI and XhoI sites. A mutated version was produced using QuikChange II site-directed mutagenesis kit (Stratagene), and the following oligonucleotides: 5′-GGCTCTGCGGCGCCGCAATTGCGCAACACAGCAG reverse (mutations are indicated by bold italicized characters). Short versions, spanning from −4 bp to −1027 bp, were produced by enzymatic digestion, using Sac-1. The constructs (long/short hPCSK9wt and long/short hPCSK9mut) were verified by sequencing.

Transfections—The day before the experiment, HEK293 cells were plated at a density of 350,000 cells/well, out of 24-well plates, in Dulbecco’s modified Eagle’s medium glucose 4.5 g/liter, SVF 10%, 10 μg/ml of streptomycin, 100 units/ml penicillin. The transfection was performed with Lipofectamine 2000, according to the manufacturer’s instruction (Invitrogen), and cells were maintained in the same culture medium without serum for 20 h. We used hPCSK9wt or hPCSK9mut (0.150 μg/well) together with pRL-CMV/renilla (5 ng/well) and/or expression vectors for mature transcriptionally active SREBP-1c (7.5 ng/well), generous gift from H. Vidal and B. Spiegelman. The analysis was performed with the dual-luciferase reporter assay system (Promega).

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were performed as described elsewhere (39). SREBP-1c expression vector was translated in vitro using TNT Coupled Reticulocyte Lysate Systems (Promega). Oligonucleotides containing the wild type (wtSRE) or mutated SRE (mutSRE) were wtSRE, 5′-ATGGGGCTCTGGTGCTTTGATG and reverse 5′-GAGAGGTTGCTGTCCTGGCGAG. The fragment was transferred using Klenow large fragment of DNA polymerase I (Invitrogen). The probes (40,000 cpm) were separately incubated with in vitro translated ATGGGGCTCTGGTGCTTTGATG forward and 5′-GAGAGGTTGCTGTCCTGGCGAG reverse (mutations are indicated by bold italicized characters). Short versions, spanning from −4 bp to −1027 bp, were produced by enzymatic digestion, using Sac-1. The constructs (long/short hPCSK9wt and long/short hPCSK9mut) were verified by sequencing.

RESULTS

Hepatic PCSK9 Expression Is Decreased by Fasting and Restored upon Refeeding—To determine whether PCSK9 expression is modulated by glycemia and/or insulinemia, C57BL/6 female mice were subjected to a 24-h fasting period followed by refeeding with a carbohydrate-rich diet for 6 and 24 h; their glycemia and insulinemia were determined at each time point, and PCSK9 mRNA and protein levels were analyzed afterward (Fig. 1).

After 24 h of fasting, PCSK9 mRNA levels dropped dramatically, to almost undetectable levels. In contrast, PCSK9 expression was restored by refeeding, showing appreciable levels (27% of control fed mice) as soon as 6 h after refeeding; mRNA levels subsequently returned to their non-fasted state after 24 h of refeeding. Accordingly, PCSK9 protein (pro- and mature peptide) was decreased by 2.25-fold (p < 0.05) after the fasting period and restored to control value after 24 h of refeeding. Although the SREBP-1c cytosolic content was dramatically altered by fasting, no change was observed for the nuclear form. However, a significant increase was observed after refeeding (2.2-fold for nSREBP-1c versus “non-fasted”, p < 0.001). The LDLr protein quantity did not change during fasting or refeeding, accordingly with what has been published (40).
PCSK9 mRNA Accumulates in the Presence of Insulin in Rodent Hepatocytes—To assess whether insulin and/or glucose mediates the increased PCSK9 expression in response to CHO refeeding, we chose the primary rodent hepatocyte model (Fig. 2, A and B). When mouse cells were cultured in the presence of physiological (5 mM) or high concentrations (25 mM) of glucose, no change was observed in PCSK9 mRNA levels (Fig. 2A, left panel). Our positive control, acetyl-CoA carboxylase, responded to the treatment by a 5-fold increase of its mRNA levels (∗, p < 0.05) (18), suggesting that in vitro PCSK9 is not regulated by glucose. As expected, both SREBP-1c and LDLr mRNA expression did not vary in response to high glucose concentrations (25 mM) (data not shown). When using a constant glucose concentration (5 mM), PCSK9 mRNA levels increased by 2.3-fold (∗, p < 0.01) in the presence of 100 nM insulin compared with cells cultured without insulin (Fig. 2B, left panel). Similar results were obtained using high glucose concentration (25 mM) (data not shown). As expected, mRNA levels for GK, which is regulated by insulin via SREBP-1c, increased by 2.8-fold (∗, p < 0.001) (18). Accordingly, SREBP-1c mRNA increased in presence of insulin (2-fold, p < 0.05). A similar profile was obtained for the LDLr (Fig. 2B, right panel). To verify that PCSK9 response to insulin is not species-specific we cultured rat primary hepatocytes in similar conditions (Fig. 2C). We detected a comparable 4-fold increase in PCSK9 and SREBP-1c mRNA levels (∗, p < 0.05 and **, p < 0.001, respectively) but no difference for SREBP-2.

Next, we verified that insulin stimulates PCSK9 expression in vivo by performing a 6-h long hyperinsulinemic-euglycemic clamp (Fig. 2D). Interestingly, PCSK9 mRNA levels were significantly increased at the end of the clamp (2-fold, p < 0.001) as well as our positive control GK (4-fold, p < 0.05), confirming that, in vivo, PCSK9 is up-regulated by insulin. Although not statistically significant, there was a tendency toward decreasing amounts of SREBP-1c mRNAs and no change in SREBP-2 mRNA levels.

SREBP-1c Mediates PCSK9 Insulin-dependent Activation—Because it is known that SREBP-1c mediates insulin action on gene transcription, we analyzed PCSK9 expression in response to insulin in primary cultures of rat hepatocytes infected by an adenovirus expressing an

FIGURE 2. Insulin activates PCSK9 expression in vitro and in vivo. Mouse primary hepatocytes were exposed for 24 h to glucose at physiological (5 mM) or high concentrations (25 mM) (A) or were cultured for 24 h in the absence or presence of insulin 100 nM (glucose 5 mM) (B). RNA levels were determined by real time PCR analysis using primers for PCSK9, acetyl-CoA carboxylase (glucose-responsive gene), GK (insulin-responsive gene), LDLr, and SREBP-1c and standardized relative to cyclophilin expression (means ± S.E.). *, p < 0.05 and **, p < 0.01 25 mM versus 5 mM glucose and 100 nM versus no insulin. Rat primary hepatocytes were cultured in presence or absence of 100 nM insulin for 24 h (C). RNA levels were determined by real time PCR analysis using primers for PCSK9, SREBP-2, and SREBP-1c and standardized relative to cyclophilin expression (means ± S.E.). *, p < 0.05 100 nM insulin versus no insulin. Hyperinsulinemic-euglycemic clamp effects of insulin infusion on the mRNA levels of PCSK9 and GK in the livers of C57Bl/6 mice are shown (D). Animals were sacrificed at the end of a 6-h hyperinsulinemic euglycemic clamp with saline (basal) or insulin. Total RNAs from livers were subjected to real time PCR quantification. Values are normalized relative to 36B4 mRNA and are expressed (means ± S.E.) relative to those of control mice, which are arbitrarily set at 1,* p < 0.05 and ***, p < 0.001 after versus before 6 h of insulin infusion (n = 4–6 mice/group).
PCSK9 Is Regulated by Insulin

LXR Agonist T0901317 Increases PCSK9 Expression via SREBP-1c—It was shown that T0901317 moderately increases PCSK9 expression in vivo, and it is known that LXR activates SREBP-1c transcription (13, 27). Therefore, we exposed rat primary hepatocytes to T0901317 for 24 h in the presence, or absence, of AdSREBP-1c-DN (Fig. 4). Compared with control, T0901317 1 mM increased by 19-fold (p < 0.001) SREBP-1c whereas LXR activation had no effect on SREBP-2 mRNA levels (data not shown). Interestingly, T0901317 treatment increased PCSK9 mRNA levels by 2-fold PCSK9 (p < 0.001). The PCSK9 response was abolished in the presence of AdSREBP-1c-DN, showing that T0901317 activates PCSK9 via SREBP-1c. It is known that ATP binding cassette transporter G5 (ABCG5) is a direct target of LXRa; therefore we analyzed its expression in the same set of experiments (41). As expected for a direct LXR target, T0901317 increased ABCG5 mRNA quantity 6.3-fold (p < 0.01), and this response was conserved in the presence of AdSREBP-1c-DN.

The PCSK9 Proximal Promoter Contains a Functional SRE—To decipher the molecular mechanisms underlying PCSK9 regulation by insulin, we first performed a computer-based analysis of the 5′-extremity of the gene (Fig. 5A). The gene does not exhibit any TATA box, therefore we chose to note the first translated base (A of ATG) as +1. As noted by

active nuclear truncated SREBP-1c protein (AdSREBP-1cDP), a dominant negative form (AdSREBP-1cDN) (Fig. 3), or a sham control adenovirus (Adnul). The upper panel describes the relative endogenous versus adenoviral SREBP-1 and SREBP-2 cell content. Although it does not seem that SREBP-2 protein levels are affected, real time PCR analysis showed that Ad SREBP-1c-DN and AdSREBP-1c-DP constructs decreased by 2-fold SREBP-2 mRNA levels (data not shown). We verified the quality of our experiments by measuring GK and LDLr mRNA relative quantities for each condition. In accordance with what has been described in the literature, GK mRNA increased by 4.4-fold (p < 0.001) when cells were infected with AdSREBP-1cDP, even in the absence of insulin (18). However, the response to insulin (4-fold accumulation, p < 0.05) was abolished by overexpressing AdSREBP-1c-DN. LDLr mRNA levels presented a very similar profile. PCSK9 mRNA relative quantity was 2.3-fold higher in cells infected with AdSREBP-1cDP (p < 0.05), which is less than with insulin alone (4.3-fold accumulation, p < 0.05). PCSK9 response to insulin was diminished by 68% when cells were infected by AdSREBP-1cDN, resulting in no statistically significant difference with the control situation (Fig. 3B, first bar). Interestingly, in the presence of AdSREBP-1cDN, PCSK9 constitutive levels were lower, but the gene remained responsive to insulin (2-fold mRNA accumulation, p < 0.01). The adenoviral infection by itself, estimated with cells exposed to Adnul, did not prevent the mRNA accumulations in response to insulin for each genes. These results demonstrate that insulin activates PCSK9 mainly via SREBP-1c.
Dubuc et al. (11), PCSK9 proximal promoter contains an SRE that is conserved among rats, humans, and mice (Fig. 5A). Using Genomatix software, we also detected an E-box spanning from −4 bp to −1460 bp or −4 bp to −1027 bp of the PCSK9 promoter (PCSK9wt) and the mutated versions (PCSK9mut) were inserted into PGL3-luciferase coding vector. Matinspector revealed SREBP1/2 binding site spanning −335 bp to −355 bp on the non-coding strand, noted in italicized characters. Bases mutated to produce hPCSK9mut or the oligonucleotides mutSRE are indicated with bold characters, and the corresponding mutated bases are noted above the sequence. B, HEK293 cells were transfected with PGL3 empty vector and long or short PCSK9wt and PCSK9mut in the presence (+) or absence (−) of an expression vector for a mature SREBP-1c. Results express -fold activation (mean ± S.E) relative to PCSK9wt activity arbitrarily set at 1 (***, p < 0.001) or relative to short PCSK9wt (∥∥, p < 0.01). C, EMSA were performed with the P32-labeled, cold wild type, or mutated oligonucleotides as described under "Material and Methods." Wild type oligonucleotides were incubated with in vitro translated SREBP-1c (lanes 1–4), in the presence of an excess of cold wild type (lane 2) or mutated (lane 3) oligonucleotide. An arrow indicates the shifted complex formed between SREBP-1c and P32-labeled wild type oligonucleotides in lane 1. In lane 4 the mix was pre-incubated with the anti-SREBP1 antibody, and the resulting shift is indicated with a hatched arrow. In lane 5, P32-labeled, mutated oligonucleotides replaced wild type oligonucleotides.

DISCUSSION

Here we show that the nutritional status modulates PCSK9 expression and that insulin increases PCSK9 expression via a pathway involving SREBP-1c. First, we showed that a 24-h fasting dramatically decreased hepatic PCSK9 mRNA and protein levels in fasted mice and that they were progressively restored by refeeding. Hyperinsulinemic-euglycemic clamp left on for 6 h resulted in a 2-fold increase in hepatic PCSK9 mRNA levels, confirming in vivo the insulin-dependent regulation of PCSK9. In primary rodent hepatocytes, PCSK9 was responsive only to insulin or to the overexpression of mature SREBP-1c but not to...
The response to insulin was also diminished by a dominant negative SREBP-1c. T0901317, an LXR agonist also increased PCSK9 mRNA quantity in rat hepatocytes via SREBP-1c. Finally, we identified the PCSK9 proximal promoter (spanning –4 to –1460 bp) and isolated a functional SREBP response element that SREBP-1c is able to bind to.

Our results indicate that PCSK9 seems responsive only to insulin but not glucose in vitro, in primary cultures of hepatocytes, or in vivo, during hyperinsulinemic-euglycemic clamps. Therefore, PCSK9 would be a new gene among the few others to present such a trait, together with GK and SREBP-1c (24, 29). In this work, the decrease of PCSK9 expression during fasting cannot be explained by diminished amounts of nuclear SREBP-1c and suggest that other factors are potentially involved in this process. The restoration of PCSK9 expression upon refeeding is accompanied by a significant increase in nuclear SREBP-1c, in agreement with previous publications (42, 43). Although we ca not exclude that the same factors might be involved in this restoration, this might illustrate that SREBP-1c is not involved in the constitutive expression of PCSK9 but only during a positive signaling by insulin. Because of new data on the acute effect of insulin on SREBP nuclear isoforms, we cannot rule out that SREBP-2 itself contributes in part to the PCSK9 response to insulin (44, 45). Furthermore, we observed that AdSREBP-1c DP was not as efficient as insulin alone to induce PCSK9 and that AdSREBP-1cDN did not fully suppress the response of PCSK9 to insulin. The repressing effect of adenoviral constructs on SREBP-2 mRNA (data not shown) could participate to the attenuated PCSK9 response to insulin in this model. In contrast, we were unable to detect any variation in SREBP2 protein content under these experimental conditions. Nevertheless, these findings raise the possibility that another factor might be involved in PCSK9 insulin-dependent regulation in these conditions and that it could have additional effect with SREBP-1c. SREBP-1c mRNA levels in mice submitted to hyperinsulinemic clamps were not increased, despite PCSK9 and GK up-regulation. This intriguing result is in accordance with other works in rat liver (46). One might speculate that in these conditions SREBP-1c activity might be enhanced.

It is known that the LDLr transcriptional activation by sterol deple- tion and statins is counteracted by a post-transcriptional feedback (47). PCSK9 is probably a major determinant of this effect (48). Notably, one could speculate that the dynamic of the response of LDLr and PCSK9 to insulin and sterol deprivation (e.g. the half-life of mRNAs and/or proteins) are different and that, in the end, PCSK9 shuts down the LDLr pathway. This interesting issue remains to be addressed. Interestingly, in vivo, the hepatic LDLr protein quantities did not change following fasting or refeeding (Fig. 1), nor did the mRNA level (data not shown). This suggests that, in vivo, the decrease in SREBP activity is compensated during fasting concerning the LDLr.

It is known that insulin and LXR agonists increase SREBP-1c mRNA and mature transcription factor (18, 27, 33, 49). It was recently shown that insulin potentiates the effect of T0901317. Indeed T0901317 increased SREBP-1c transcription and insulin consequently promoted more SREBP-1c precursor cleavage (44). In our hands, a 24-h exposure of primary hepatocytes to T0901317 resulted in a 2-fold increase in PCSK9 mRNA, in agreement with the Maxwell et al. (13) in vivo studies in mice. This regulation was abolished when cells expressed a dominant negative SREBP-1c. However, the response of a direct LXR target, ABCG5, was robust (6-fold) and was not affected by this dominant negative SREBP-1c. This demonstrates that LXR regulates PCSK9 indirectly via SREBP-1c. Dubuc et al. (11) recently showed that 22-hydroxycholesterol, a natural LXR agonist, does not increase PCSK9 mRNA levels in HepG2 cells, although it does increase SREBP1 mRNA. This discrepancy might be explained by the difference in the nature of the cells used (primary cell cultures versus a hepatoma cell line), although we cannot rule out a ligand-specific effect. Because LXR activates SREBP-1c both in HepG2 and murine cells and because the SRE we isolated (Fig. 5 and above) is conserved among these species, we do not favor the hypothesis for a species-dependent regulation (33). Whether the moderate response of PCSK9 to T0901317 has physiological consequences remains to be determined. Interestingly, SREBP-1c is involved in the hypertriglyceridemic properties of LXR agonists (32, 33). More investigation will be needed to determine the extent of the role of PCSK9 in these deleterious effects, in particular in PCSK9 knock-out mice (6).

Our study describes for the first time the PCSK9 promoter and shows that it contains a functional SRE spanning from –355 to –335 bp before the ATG. In addition, our results suggest that a repressor site is located in a region spanning from –1028 bp to –1460 bp. Whether the SRE is a genuine sterol response element or not, meaning an SREBP2 binding site, is still to be determined. Mutating this element reduced the SREBP-1c-dependent promoter activation by 2-fold. However, the remaining response to SREBP-1c, together with the impairing effect of the mutation on SREBP-1c binding on the SRE (Fig. 5c), suggests that another hidden response element might be present in the sequence. Indeed, at an equal concentration, no binding was detected between SREBP-1c and mutSRE, compared with wtSRE as showed with EMSA (Fig. 5c). However, an excess of cold mutSRE diminished SREBP-1c binding to wtSRE, showing that SREBP-1c itself might bind the mutated SRE only when it is present at high concentration. It is known that SREBP's themselves are weak activators and that they need co-regulatory transcription factors that bind nearby sequences. SP1 is the co-regulator for the LDLr (50), and CCAAT-binding factor/nuclear factor-Y (CBF/NFY) is the key co-regulatory factor for 3-hydroxymethylglutaryl-CoA synthase (51, 52). Finally, insulin-dependent activation of the fatty acid synthase promoter in primary hepatocytes is dependent upon SREBP and both SP1 and CBF/NFY (52). Both an SP1 and CBF/NFY sites have been proposed in the PCSK9 promoter based on a computerized analysis (11). It will be interesting to verify their functionality and respective contribution to PCSK9 activation by sterol deprivation and insulin in future studies.

PCSK9 up-regulation by LXR agonist and down-regulation by cholesterol cell content is typical of a gene involved in fatty acid synthesis (13). Its regulation by SREBP-1c also supports this hypothesis (16). Interestingly, ApoB synthesis and secretion in hepatocytes is repressed by acute exposure to insulin, whereas chronic insulin exposure and/or insulin resistance increases apoB secretion (reviewed in Ref. 53). The relationship of PCSK9 with apoB production is controversial and confused by mixed messages depending on the integrity of the protein and the nature of mutations. Our laboratory showed that PCSK9-S127R patients exhibit an overproduction of apoB, an effect confirmed on apoB100 in vitro by Sun et al. (54) who showed the specificity of this trait for certain mutations and not others (3). In fed mice or primary mouse hepatocytes, wild type PCSK9 overexpression did not lead to apoB overproduction (7–10). Our findings on PCSK9 insulin-dependent expression suggest that the nutritional status and especially insulin concentrations might be of importance when studying the link between PCSK9 and apoB production. Our laboratory is currently characterizing dramatic hypertriglyceridemia because of an increase in apoB production in mice overexpressing PCSK9 and submitted to a nutritional challenge. Thus, one can also speculate that PCSK9 may play a role in

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dyslipidemias occurring in insulin-related pathophysiologies, such as insulin-resistant and/or diabetic patients.

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