Statins Upregulate PCSK9, the Gene Encoding the Proprotein Convertase Neural Apoptosis-Regulated Convertase-1 Implicated in Familial Hypercholesterolemia

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Objective—Neural apoptosis-regulated convertase (NARC)-1 is the newest member of the proprotein convertase family implicated in the cleavage of a variety of protein precursors. The NARC-1 gene, PCSK9, has been identified recently as the third locus implicated in autosomal dominant hypercholesterolemia (ADH). The 2 other known genes implicated in ADH encode the low-density lipoprotein receptor and apolipoprotein B. As an approach toward the elucidation of the physiological role(s) of NARC-1, we studied its transcriptional regulation.

Methods and Results—Using quantitative RT-PCR, we assessed NARC-1 regulation under conditions known to regulate genes involved in cholesterol metabolism in HepG2 cells and in human primary hepatocytes. We found that NARC-1 expression was strongly induced by statins in a dose-dependent manner and that this induction was efficiently reversed by mevalonate. NARC-1 mRNA level was increased by cholesterol depletion but insensitive to liver X receptor activation. Human, mouse, and rat PCSK9 promoters contain 2 typical conserved motifs for cholesterol regulation: a sterol regulatory element (SRE) and an Sp1 site.

Conclusions—PCSK9 regulation is typical of that of the genes implicated in lipoprotein metabolism. In vivo, PCSK9 is probably a target of SRE-binding protein (SREBP)-2. (Arterioscler Thromb Vasc Biol. 2004;24:1454-1459.)

Key Words: cholesterol ■ QPCR ■ SRE ■ HepG2 ■ primary hepatocytes

Neural apoptosis-regulated convertase (NARC)-1 is a serine proteinase belonging to the proteinase K subfamily of subtilases. Its acronym reflects the fact that its mRNA was upregulated when apoptosis was induced in neuronal primary cultures and that it is similar to 8 other subtilase-like proteinases, called proprotein convertases (PCs; Millenium Pharmaceuticals, patent No. WO 01/57081 A2). PCs are involved in the processing (and activation) of precursors of hormones, receptors, surface glycoproteins, etc, which transit through the secretory pathway.1–3 Seven of them, PC1/3, PC2, furin, PC4, PACE4, PC5/6, and PC7/LPC, recognize basic sites and belong to the kexin subfamily. The eighth member, SKI-1/S1P,4,5 is classified in the pyrolysin subfamily of subtilases. It has been involved in the processing of endoplasmic reticulum (ER)–anchored transcription factors such as sterol regulatory element (SRE)–anchored transcription factors such as sterol regulatory element (SRE)-binding proteins (SREBPs) and thus plays a key role in cholesterol homeostasis.4,6 When cellular cholesterol is low, SREBPs are recruited to the Golgi apparatus, where SKI-1/S1P cleaves in their luminal loop. A second cleavage by the metalloprotease S2P in their first transmembrane domain liberates the cytosolic N-terminal region that goes to the nucleus and activates target genes. SREBP-1c, the isoform that is dominant in liver, regulates the lipogenic pathway, whereas SREBP-2 preferentially activates genes of cholesterol metabolism.7

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NARC-1 is highly expressed in embryonic liver.8 It then decreases in the adult liver but significantly increases after hepatectomy.8 The transcript is also detected transiently in specific areas such as the telencephalon, skin, kidney, intestine, and cerebellum. It has been hypothesized that NARC-1 may be expressed preferentially in progenitor cells and play a role in hepatic and neuronal differentiation.8 In human, the NARC-1 gene, PCSK9, is ≈22-kb long and comprises 12 exons encoding a 692-aa protein. Located on chromosome 1p32, PCSK9 was identified recently as the third locus involved in autosomal dominant hypercholesterolemia (ADH),8 characterized by high levels of low-density lipoprotein (LDL) cholesterol, xanthomas, and a high frequency of coronary artery diseases. The majority of familial hypercholesterolemia cases are attributable to mutations in the genes encoding the LDL receptor (LDLR) and apolipoprotein B.
Quantitative RT-PCR (QPCR) was used to measure expression of genes involved in cholesterol metabolism. Regulation under various conditions known to modulate the NARC-1 substrate(s) and physiological function(s) are still unknown. Thus, we decided to check for NARC-1 with abnormally high levels of LDL-cholesterol. LPDS was prepared by ultracentrifugation as described and stored at 4°C. Several French families, 2 exonic NARC-1 mutations, S127R and F216L, were associated with haplotypes segregating with the disease. This work was confirmed recently by the identification of a new PCSK9 mutation, D374Y, in a large Utah kindred and 2 Japanese polymorphisms, intron 1C(-161)T and I474V, all associated with abnormally high levels of LDL-cholesterol.

The NARC-1 substrate(s) and physiological function(s) are still unknown. Thus, we decided to check for NARC-1 involvement in cholesterol homeostasis by studying its regulation under various conditions known to modulate the expression of genes involved in cholesterol metabolism. Quantitative RT-PCR (QPCR) was used to measure NARC-1 mRNA levels in the human hepatic cell line HepG2, or human primary hepatocytes. Here we report that statins, which inhibit 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase, a key enzyme in cholesterol synthesis, increased NARC-1 expression, most likely through SREBP-2 activation. In contrast, NARC-1 expression was not affected by liver X receptor (LXR) stimulation. In agreement, the NARC-1 promoter shows the typical association of conserved Sp1 and SRE-1 sites but no LXR response element.

Methods

Materials
Lovastatin and simvastatin were kindly provided by Merck Frosst (Montreal, Canada). Cerivastatin, atorvastatin, and pitavastatin were a generous gift from Bayer (Toronto, Canada), Parke-Davis (Ann Harbor, MI) and Kowa (Tokyo, Japan), respectively. Stock solutions containing 10 mmol/L cerivastatin and atorvastatin (both in water), lovastatin and simvastatin (both in ethanol), and pitavastatin (in dimethyl sulfoxide) were stored at −20°C until use. Compactin (mevastatin), mevalonolactone, 25-hydroxycholesterol, 22(R)-hydroxycholesterol, 9-cis-retinoic acid, and cholesterol were purchased from Sigma.

Cell Culture
The human hepatoma HepG2 cells were grown in DMEM containing 100 μmol/L nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS or lipoprotein-deficient serum (LPDS, in a humidified atmosphere (5% CO₂, 37°C). All culture reagents were from Gibco/BRL (Invitrogen; Grand Island, NY). LPDS was prepared by ultracentrifugation as described and stored at −20°C. Typically, 10⁶ cells per well were seeded in 6-well plates and grown to 70% to 80% confluence. Primary hepatocytes were isolated from patients undergoing hepatectomy and were a generous gift from Dr Marc Bilodeau with agreement of the institutional review committee of St-Luc Hospital (Montreal, Canada) and informed consent of the patients. These cells were grown in William’s E medium with the same additives. Before seeding, the plates were coated with a thin layer of rat tail collagen type-I (BD Biosciences) at 5 μg/cm².

Secreted ApoB-100
After the required incubation, the medium was collected and centrifuged 10 minutes at 1500 rpm to remove cell debris. Secreted apoB-100 levels in the medium were measured by a sandwich ELISA as described, using a goat human apoB-48/100 antibody (Biodesign; Saco, ME).

Intracellular Lipids
Intracellular lipids were extracted as described. Total cholesterol and triglycerides were determined by enzymatic methods (Roche Diagnostics). The remaining cells were digested in 2 mL 0.1N NaOH, and total proteins were quantified.

RNA Preparation and cDNA Synthesis
Cells were washed 3× with PBS and directly incubated with Trizol reagent (Life Technologies). Total RNA was extracted according to the recommendations of the manufacturer and resuspended in ~30 μL of water. Isolated RNA integrity was electrophoretically verified by ethidium bromide staining and optical density (OD), with an OD₂₆₀/OD₂₈₀ average absorption ratio of 1.8 to 2.0. Typically, 250 to 600 ng of total RNA were used for cDNA synthesis in a total volume of 20 μL using SuperScript II reverse transcriptase, 25 μg/mL oligo(dT)₃-₁₅, 0.5 mmol/L 2’- deoxynucleoside 5’-triphosphates, and 40 U of RNaseOUT, all products from Life Technologies, and used according to the recommendations of the manufacturer.

Quantitative RT-PCR
In a typical experiment, each cDNA sample was submitted to 2 polymerase chain reaction (PCR) amplifications: one for the normalizing ribosomal protein S14 gene and the other for the gene of interest, each in triplicate. Each reaction was in a final volume of 25 μL using the QuantiTec SYBR green PCR master mix from Qiagen, cDNA dilutions that gave threshold cycle (Ct) values for both amplifications, and primers for S14 or the chosen target gene (Table). All primers (Life Technologies) were designed using Primer3 software to produce amplicons that overlapped exon splicing junctions to avoid genomic DNA amplification. Oligonucleotide Cts were optimized for each amplification. The PCR program comprised a polymerase activation step (15 minutes at 95°C) followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 30 seconds at 72°C. The Mx4000 system from Stratagene was used to perform and analyze the QPCR reactions, using S14 amplifications as normalizers and control samples as calibrators. Excel software was used for SD and Student test calculations. The data shown correspond to representative experiments.

Results
NARC-1 Is Upregulated by Statins
Because NARC-1 seemed to be involved in cholesterol metabolism and that patients responded well to statin treatment, we studied the effect of 5 different statins on NARC-1.
expression. HepG2 cells were treated with 1 μmol/L cerivastatin, atorvastatin, simvastatin, or pitavastatin. Expression of the LDLR gene, which is known to be upregulated by statins, was used as a positive control. Statin treatment significantly increased PCSK9 expression by a factor of ≈3 for cerivastatin and 1.5 for the other statins (Figure 1A). This increase was confirmed when HepG2 cells were treated with increasing Cts of statins. The dose-dependent response induced by atorvastatin is shown in Figure 1B. PCSK9 expression was upregulated ≈7.5-fold by 10 μmol/L atorvastatin, whereas under the same conditions, LDLR expression increased by only 2.5-fold. By inhibiting HMG-CoA reductase, an enzyme of the cholesterol synthesis pathway, statins induce a cellular depletion in cholesterol. In contrast to NARC-1 and LDLR, SKI-1 that cleaves SREBPs was not regulated at the transcriptional level by statin treatment (Figure 1B).

NARC-1 Is Upregulated by Cholesterol Depletion

To verify whether NARC-1 mRNA upregulation was attributable to inhibition of cholesterol synthesis or to another effect of statins, we quantified PCSK9 expression in the presence or absence of sterols, a mixture of cholesterol and 25-hydroxycholesterol (Figure 2). In both HepG2 cells and human hepatocytes in primary culture, NARC-1 mRNA level was upregulated, albeit to a higher extent in HepG2 cells (5 versus a 2-fold increase in primary hepatocytes). In HepG2 cells, LDLR and SREBP-2 mRNA levels were also upregulated (2-fold), whereas that of SREBP-1 remained unaffected, as reported previously. As expected, in the absence of exogenous sterols, the intracellular cholesterol and the levels of apoB in the medium were reduced, whereas intracellular triglycerides were increased (Figure 2, inset). These data suggested that statin-induced upregulation of NARC-1 was mediated by the cholesterol-lowering effect of statins.

Statin-Induced Upregulation of NARC-1 Was Reversed by Addition of Mevalonate

The above hypothesis was verified by treating HepG2 cells or primary hepatocytes with atorvastatin in the absence or presence of mevalonate (Figure 3). Mevalonate was expected to prevent the cholesterol depletion caused by statin because it is the product of the reaction catalyzed by HMG-CoA reductase. Analysis of NARC-1 and LDLR expression levels showed that addition of mevalonate efficiently reversed NARC-1 and LDLR upregulations. As in Figure 2, NARC-1 upregulation was higher than that of LDLR (2-fold). Interestingly, NARC-1 downregulation by addition of mevalonate was also more drastic than that of LDLR. It is important to note that in HepG2 cells and hepatocytes at 1 μmol/L statin, mevalonate lowers NARC-1 (but not LDLR) level below that of the control, suggesting that PCSK9 is regulated more tightly by cholesterol than the LDLR gene.

NARC-1 Is Not Affected by LXR Induction

Although SREBP-2 is the prominent factor that regulates cholesterol synthesis and uptake, the transcription factor LXR plays a key role in cholesterol elimination. LXR is a nuclear hormone receptor that binds oxysterols and activates its target genes, such as CYP7A encoding the rate-limiting enzyme in the conversion of cholesterol to bile acids by dimerizing with retinoid X receptor (RXR). PCSK9 regulation was assessed in the absence or presence of 22(R)-hydroxycholesterol, 1 of...
the most potent oxysterols for LXR activation (Figure 4). Because LXR also plays a role in fatty acid metabolism through SREBP-1 upregulation, we measured both SREBP-1 and SREBP-2 expression levels as positive and negative controls, respectively. As expected, SREBP-2 mRNA remained stable whereas SREBP-1 mRNA was upregulated 3-fold in the presence of 22(R)-hydroxycholesterol, an effect comparable to the 2.5-fold increase found in HepG2 cells stimulated with a synthetic LXR agonist or in mice fed the same agonist. Under these conditions, NARC-1 expression level was not upregulated.

Comparative Analysis of Human, Mouse, and Rat PCSK9 Promoters

Regulation of NARC-1 mRNA levels by cholesterol was strongly in favor of a SREBP-2–mediated effect. The latter activates cholesterol biosynthetic genes by binding to SREs exhibiting adjacent sites for Sp1 or nuclear factor-Y (NF-Y) cofactors. Human, mouse, and rat promoters were analyzed using the MatInspector software, and the identified consensus binding motifs for SREBPs, Sp1, and NF-Y are represented schematically in Figure 5. Both mouse and rat sequences exhibit, in addition to an ATG codon aligned with that of the human sequence, an upstream ATG that extents their open reading frame of 13 and 55 codons, respectively. Whether these upstream ATGs are bona fide translation initiation sites remains to be determined. Only 2 sites, separated by ~75 bp, were conserved perfectly in the PCSK9 proximal promoter of the 3 species: an SRE (ATCACGCCAC) at −337, −227, and −218, and an Sp1 site (GGGAGGGGAGGAGG) at −430, −320, and −313 in human, mouse, and rat sequences, respectively. In the LDLR promoter, the orientation of the SRE-1 (−159; ATCACCCCCAC) and the most important Sp1 site (−144; GGGAGGGGAGGAGG) is inverted.
compared with that observed in PCSK9 promoter. As symbolized by the dotted line in Figure 5, PCSK9 SRE and the Sp1 site are comprised in an ≈200-nucleotide conserved area of the promoter (basically no gaps and 90% identity between human and mouse sequences). In the proximal region, aside from important gaps in mouse and rat sequences (equivalent to 37% of the human sequence), human and mouse promoters share only 66% identity. In the distal region, the identity is 58%. Two (human) and 1 (mouse and rat) other SREs with adjacent Sp1 and NF-Y sites were present further upstream in the PCSK9 promoter (data not shown) and may also contribute to gene regulation by sterols. Consistent with the absence of a significant increase of NARC-1 mRNA expression by LXR activation (Figure 4), no conserved response element was detected in the PCSK9 promoter.

Discussion

Our results showed that the NARC-1 gene, PCSK9, involved in familial hypercholesterolemia,9,11,12 is regulated as a typical cholesterogenic gene. We showed for the first time that NARC-1 mRNA expression was upregulated by statins (Figure 1) and cholesterol depletion (Figure 2), ≈2-fold more than that of LDLR. The statin-induced upregulation of PCSK9 was reversed quantitatively by addition of mevalonate (Figure 3). This indicated that the effect of statins was attributable to the inhibition of HMG-CoA reductase and not to the other effect(s) of pleiotropic statins.16,30 Therefore, NARC-1 upregulation was most likely a result of the cholesterol-lowering effect of the drug. This is the first study of NARC-1 regulation in human cells. The data obtained in HepG2 cells were similar to that observed in human hepatocytes in primary culture (Figures 2 and 3), thereby validating the studies in HepG2 cells.

Our in vitro results are in agreement with a recent study by Breslow et al, who identified PCSK9 as a putative family member of the genes involved in cholesterol homeostasis using a DNA microarray approach.31 They found an ≈2-fold decrease in NARC-1 mRNA levels in livers of mice fed a cholesterol-rich diet. In contrast, PCSK9 was highly upregulated in SREBP-2 transgenic mice.

Interestingly, they also showed a slight increase (1.6-fold) in NARC-1 transcripts in liver from mice treated with an LXR agonist.31 The latter observation could not be confirmed in our study, a discrepancy that may reflect species-specific regulations or in vivo versus in vitro variations. In addition, genes for which expression is repressed by an excess of cholesterol and upregulated by SREBP-2 are usually not LXR targets. Finally, another DNA microarray study confirmed the above results and showed that in SREBP cleavage-activating protein−/− liver, in which SREBP activation cannot take place, NARC-1 messengers were reduced.32

Human, mouse, and rat NARC-1 promoters share 2 conserved sites for transcription factor binding: Sp1 and SRE. The NARC-1 SRE differs from the classical LDLR SRE-1 (ATCACCCCCAC) by 1 transversion at position 6 (C to G), shown not to affect SREBP-2 binding.33 It has been suggested that in LDLR promoter, the respective directional binding of SREBP-2 and Sp1 to their sites, which are head to head in repeats 2 and 3, allows the interaction of SREBP-2 with the N-terminal region of Sp1 and facilitates Sp1 recruitment28 (Figure 5). Because of the mirror image of SRE and Sp1 site disposition in the PCSK9 promoter, it is possible that the same interaction takes place. In vitro studies7 demonstrated that SREBP-2 binds efficiently to classical SRE, whereas SREBP-1c shows little binding. The absence of a conserved E-box, which is known to be preferred by SREBP-1c,7 is also in favor of NARC-1 regulation by SREBP-2. NARC-1 is particularly abundant in liver and small intestine.8 Further studies will define whether the sterol regulation of the PCSK9 requires tissue-specific factors, such as hepatocyte nuclear factor-4, which was shown to be essential for SREBP-2 activation of sterol Δ^5-isomerase.34

The absence of an LXR-mediated upregulation of NARC-1 is not in favor of its implication in cholesterol catabolism. Our data rather suggest that the enzyme is implicated in cholesterol biosynthesis or uptake. The fact that patients harboring a mutated PCSK9 have high plasma cholesterol levels reinforces a putative role of NARC-1 in LDL uptake. The autoprocessing site of NARC-1 has been identified recently35 (Benjannet et al, submitted) and further studies that will better define NARC-1 cleavage specificity should help in identifying NARC-1 substrate(s). The dominant character of PCSK9 mutations could be attributable either to a dominant-negative or gene-dosage effect. The S127R9 and D374Y11 mutations that have been associated with ADH partially and totally abrogated NARC-1 autocatalytic zymogen processing, respectively (Benjannet et al, submitted), supporting a correlation with the enzyme activity. However, we cannot exclude that these mutations generated dominant-negative forms of the enzyme that acquired novel deleterious properties, which may have no relation to the normal physiological function(s) of NARC-1, as reported for superoxide dismutase 1.36 Nevertheless, because the enzyme belongs to the PC family, it is tempting to hypothesize that an as yet unknown substrate(s) activated by NARC-1 is an essential actor in the cholesterol pathway. Identification of NARC-1 substrates may lead to elucidation of disease mechanism(s), and these substrates may constitute targets for new strategies to limit elevation of LDL particles and prevent morbidity and mortality from premature atherosclerosis.

In addition, and as reflected by its acronym, NARC-1 was shown to be upregulated by apoptosis induction in primary culture of neurons. Caspase 3, which has a pivotal role in apoptosis, was reported to generate active SREBP-37,38 By using reporter genes under control of the LDLR SRE and Sp1 sites, Higgins and Ioannoni38 showed that the 2 elements mediated a sterol-independent upregulation of the reporter genes very early in apoptosis induction. The physiological relevance of stimulation of SREBP targets in apoptotic cells remains unclear. It will be important to verify whether the identified PCSK9 SRE and Sp1 site also mediate the increased expression of NARC-1 observed during apoptosis induction. To date, we cannot exclude that NARC-1 may play an important role in cholesterol homeostasis and apoptosis, both of which may be related functionally.

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