Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the subtilases that promotes the internalization and degradation of LDL receptor in liver and thereby controls the level of LDL cholesterol in plasma. Here, we show that the expression of PCSK9 in HepG2 cells is completely dependent on the absence or presence of sterols. The minimal promoter region of the PCSK9 gene contains a sterol-regulatory element (SRE), which makes the transcription of PCSK9 dependent on sterols. Expression of nuclear forms of sterol-regulatory element binding protein-1 (SREBP-1) and SREBP-2 dramatically increased the promoter activity of PCSK9. In vitro-translated nuclear forms of SREBPs showed interactions with SRE, whereas mutations in SRE abolished their binding. In vivo studies in mice showed that Pcsk9 protein and mRNA were decreased significantly by fasting and increased by refeeding. However, supplementation with 2% cholesterol in the diet prevented the increase in Pcsk9. The amounts of Pcsk9 mRNA in livers of refeed mice showed correlated regulation by the changes in the nuclear form of Srebp-2. In summary, it is suggested that the expression of PCSK9 is regulated by sterol at the transcriptional level in HepG2 cells and that both SREBP-1 and SREBP-2 can transcriptionally activate PCSK9 via SRE in its proximal promoter region in vitro. However, in vivo, it is suggested that the sterol-dependent regulation of PCSK9 is mediated predominantly by SREBP-2.

Supplementary key words low density lipoprotein receptor • hypercholesterolemia • transcriptional regulation • lovastatin

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily of subtilisin serine proteases of which the gain-of-function mutations cause hypercholesterolemia (1–3). PCSK9 was independently identified as one of the genes that are regulated by sterol-regulatory element binding proteins (SREBPs) (4, 5). The SREBPs are members of the basic helix-loop-helix leucine zipper family of transcription factors that regulate the expression of the target genes by binding to the sterol-regulatory elements (SREs) in their promoter regions (6). Using microarrays hybridized with RNA from livers of mice that either overexpressed nuclear forms of human SREBPs (transgenic model) or lacked SREBP-activating protein (knockout model), PCSK9 was identified as a SREBP target gene. Soon after the first cloning of this gene, with its relationship to neural apoptosis and liver regeneration, studies focused on its relationship with the regulation of cholesterol in plasma. Subsequently, the loss-of-function mutations of PCSK9 have been reported to decrease LDL cholesterol level (7–9) and reduce the risk of coronary heart disease (10). The definite evidence for a role of PCSK9 in LDL metabolism was revealed by a set of in vivo animal experiments. Adenovirus-mediated overexpression of PCSK9 reduced the amount of low density lipoprotein receptor (LDLR) in livers posttranscriptionally (11, 12), whereas the amount of LDLR increased significantly in livers of Pcsk9 knockout mice (13). The mechanism by which PCSK9 reduces LDLR suggests that secreted PCSK9 in plasma interacts directly with LDLR protein on the cell surface (14) and functions as a chaperone to prevent

Abbreviations: DLPS, delipidated serum; EMSA, electrophoretic mobility shift assay; LDLR, low density lipoprotein receptor; Ni-NTA, nickel-nitrilotriacetic acid; PCSK9, proprotein convertase subtilisin/kexin type 9; SRE, sterol-regulatory element; SREBP, sterol-regulatory element binding protein.

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LDLR recycling and/or to target LDLR for lysosomal degradation (15).

Although several studies have focused on the importance of the posttranslational regulation of PCSK9, less is known about the transcriptional regulation of PCSK9, despite its identification as a gene regulated by SREBPs. Dubuc et al. (16) reported that the transcription of PCSK9 was increased by statins, most likely through SREBP-2 activation in primary human hepatocytes, and proposed the importance of conserved SRE in the promoter region of mouse, rat, and human PCSK9 genes. Costet et al. (17) reported that insulin increased PCSK9 via SREBP-1c in primary mouse and rat hepatocytes, as well as in vivo, during hyperinsulinemic-euglycemic clamp procedures performed on mice. However, the critical changes in the nuclear forms of both SREBP-1 and SREBP-2 proteins in coordination with the changes in PCSK9 protein, mRNA, and promoter activity have not been described yet. In this report, we show that the expression of PCSK9 is dependent on the absence or presence of sterols via SRE in the minimal promoter region of the human PCSK9 gene by both SREBP-1 and SREBP-2 in HepG2 cells. Finally, we propose that the predominant transcriptional regulator of PCSK9 by cholesterol is SREBP-2 in vivo.

MATERIALS AND METHODS

General methods and supplies

DNA manipulations were performed using standard molecular biology techniques (18). Delipidized serum (DIDS) was prepared from FBS as described by Hannah et al. (19). Protein concentrations were determined using a BCA Kit (Pierce, Rockford, IL). Cell culture medium and reagents were obtained from Invitrogen (Carlsbad, CA). Nickel-nitritotriacetic acid (Ni-NTA) Sepharose was obtained from GE Healthcare Bio-Science (Piscataway, NJ). N-Acetyl-leucine-leucine-norleucinal was obtained from Merck Biosciences (Calbiochem, San Diego, CA). Lovastatin was kindly provided by Merck and Co., Inc. (Rahway, NJ). Sodium mouninate was prepared from the mouninate acidamide (Sigma-Aldrich Co., St. Louis, MO) as follows. To prepare 1.0 M sodium mouninate, 5 g of mouninate acidamide was dissolved in distilled water, then 4 ml of 10 N NaOH was added dropwise and stirred for 30 min at room temperature. The pH of the solution was adjusted by 0.5 N HCl to pH 7.5, then the final volume was brought to 38.4 ml with distilled water. The stock solution was filter-sterilized, divided into aliquots, and stored at –20°C until use. Other reagents not specified were obtained from Sigma.

The following antibodies were used in the current studies: anti-HSV from Merck Biosciences (Novagen, Madison, WI), and anti-GAPDH and anti-cAMP-response element binding protein from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. The polyclonal antibodies against human SREBP-1 (#932), which cross-reactivity to mouse Srebpl-1, mouse Srebpl-2 (#841), bovine ldlr (#3143), and receptor-associated protein (#649), were kindly provided by Jay Horton (Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas).

Construction of promoter-reporter plasmids

We arbitrarily annotated the first base of the translated ATG as +1, as described by Costet et al. (17). The human PCSK9 promoter fragment spanning −2,112 to −94 was amplified by PCR from the human genomic DNA isolated from leukocytes using the following primers: 5’-AAGAGGGAATTTAGGGATAGG-3’ and 5’-ACTGTGCAGAGCTGAACTTGC-3’. The amplified DNA product was gel-purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA) and cloned using the pcR2.1-TOPO TA cloning kit (Invitrogen). After the integrity of the amplified DNA sequences was verified by DNA sequencing, this plasmid was used as the template for the PCR amplification of deletion fragments (for details, see supplementary methods). Briefly, the promoter fragment for each deletion clone (D1–D7) was amplified by PCR with the respective primers, gel-purified, and cloned using the pcR2.1-TOPO TA cloning kit (Invitrogen). After the orientation of the promoter fragments inserted in pcR2.1 vector was verified by DNA sequencing, the promoter fragments were subcloned into pGL3-Basic vector (Promega, Madison, WI) to produce deletion clones of PCSK9 promoter-reporter constructs. The SRE mutant (SRE-Mut) and Sp1 mutants (Sp1-Mut1–Sp1-Mut5) were generated from construct D4 using the QuickChange site-directed mutagenesis kit (Stratagene) and the respective oligonucleotides as described in the supplementary methods. The integrity of promoter-reporter plasmid sequences was confirmed by DNA sequencing.

Construction of expression plasmids

The bacterial expression plasmids that were used for the production of antibodies against human PCSK9 (amino acids 153–692) or mouse Pcsk9 (amino acids 156–694) were constructed as follows. Human PCSK9 cDNA was amplified by PCR using pcMV-PCSK9-FLAG (12) as the template with the following primers: 5’-ACCATGGAATTTACGATCCGTAAGAATCCTG-3’ and 5’-CCGCTCTCAGAGAGCTGAACTTGC-3’. The amplified DNA was digested with EcoRI and XhoI, then ligated into the pET-21a(+) vector (Novagen). Mouse Pcsk9 cDNA was amplified by PCR using the template cDNA prepared from livers of fasted/refed mice with the following primers: 5’-CTGCAAGATCCCATGGGAACCTG-3’ and 5’-TCTCTCAGAGAGCTGAACTTGC-3’. The amplified DNA was gel-purified and ligated into pcR2.1-Topo cloning vector (Invitrogen). The plasmid with cDNA in the correct orientation was selected, and the cDNA was subcloned into pET-21a(+) vector after digestion with EcoRI and HindIII. The other expression plasmids used in this experiment are described in the supplementary methods. The following plasmids were constructed: the plasmids that were used in cotransfection experiments, encoding HSV epitope-tagged nuclear forms of human SREBP-1a (pTK-HSV-nBP1a), SREBP-1c (pTK-HSV-nBP1c), and SREBP-2 (pTK-HSV-nBP2), the expression of which is under the control of the thymidine kinase promoter; the plasmids that were used as templates for the in vitro translation of nuclear forms of SREBPs (pCMV-nBP1a and pCMV-nBP2); the bacterial expression plasmid used for the production of antibodies against human SREBP-2, pET-nhBP2-AHLH, encoding the nuclear form of human SREBP-2 (amino acids 14–330) lacking its helix-loop-helix domain. The integrity of each plasmid was confirmed by DNA sequencing.

Antibody preparation

The antibodies against human PCSK9, mouse Pcsk9, and human SREBP-2 were prepared using recombinant proteins according to standard procedures. The recombinant proteins were expressed in Escherichia coli BL21 by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside and purified by affinity chromatography with Ni-NTA Sepharose (GE Healthcare Bio-Science) under denaturing conditions according to the manufacturer’s
instructions. The proteins were further purified by electrophoresis on 10% SDS-polyacrylamide gels, and the bands corresponding to the size of each protein were cut and homogenized with a minimum volume of 0.9% (w/v) NaCl using a Polytron homogenizer. The amounts corresponding to 500 μg of purified proteins were injected at least seven times at 3-week intervals into male New Zealand White rabbits. The specificity of SREBP-2 antiserum to discriminate SREBP-1a and SREBP-2 was verified by immunoblotting using the in vitro-translated nuclear forms of SREBP-1a and SREBP-2 (described above), which have no 6-His epitope.

Cell culture, transient transfection, and reporter gene assay

HepG2 (American Type Culture Collection number HB-8065) cells were maintained in medium A (DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FBS. Transfection of DNA into HepG2 cells in suspension was carried out using Lipofectamine Plus Reagent (Invitrogen) according to the method described by Notarangelo et al. (20) with minor modifications. Briefly, plasmids were complexed using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer’s instructions in Opti-MEM (Invitrogen). While the DNA-Lipofectamine complex was prepared, HepG2 cells were trypsinized and suspended in medium A supplemented with 10% FBS. The DNA-Lipofectamine complex was mixed with 2 × 10⁵ cells per well/12-well plate in 0.75 ml of medium A supplemented with 10% FBS and rocked for 40 min at 37°C in the tube. The mixtures were plated on 12-well plates and cultured overnight at 37°C under a humidified atmosphere of 5% CO₂.

For the treatment of cells with or without sterols, on day 1, cells were washed twice with PBS and changed to medium A supplemented with 10% DLPS, 1 mM Iovastatin, and 50 μM sodium melenolate in the absence or presence of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% (v/v) ethanol. On day 2, cells were washed twice with PBS, harvested, and analyzed for luciferase activity using the dual-luciferase assay system with passive lysis buffer (Promega) according to the manufacturer’s instructions. The firefly luciferase activity was normalized to the renilla luciferase activity and the amounts of protein in the lysate.

Immunoblot analysis

HepG2 cells were set up and treated as described in the figure legends. After treatment, cells were washed twice with PBS and lysed with 100 μl of NUN buffer (21) containing 0.33 M NaCl, 1.1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.6), and proteinase inhibitors (1 mM DTT, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM PMSF, 2 μg/ml aprotinin, and 50 μg/ml N-acetyl-leucine-leucine-norleucine) by adding directly onto the plate. Cell lysates were harvested and further vortexed at room temperature for 10 min for the complete liberation of proteins. Lysate were cleared by centrifugation at 20,000 × g for 15 min at 4°C, and the supernatants were collected as whole cell lysate. For immunoblot analyses of mouse liver proteins, the membrane and nuclear fractions were prepared as described (12) except for the following modification. The membrane pellets were resuspended in NUN buffer, vortexed for 10 min at room temperature, and centrifuged at 20,000 × g for 15 min at 4°C, and the supernatants were collected as the membrane fractions. After quantitation of proteins, aliquots of proteins were subjected to 8% SDS-PAGE and transferred onto nitrocellulose ECL membranes (GE Healthcare), and immunoblot analyses were performed using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce).

Quantitative real-time PCR

Total RNA was prepared from HepG2 cells or livers of mice using an RNaseasy Mini kit (Qiagen) or Trizol reagent (Invitrogen), respectively. Removal of DNA from RNA was achieved with RNase-free DNase (Qiagen) for RNA from cells or a DNA-free kit (Ambion, Austin, TX) for RNA from tissues. cDNA was synthesized from 2 μg of DNase-treated total RNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed as described previously (12) using the RG-3000 Rotor-Gene (Corbett Research, Sydney, Australia). All reactions were done in triplicate, and the relative amounts of all mRNAs were calculated by the comparative cycle-time method (22). Cyclophilin mRNA was used as the invariant control. Specific primers for each gene were described previously (16, 23). The sequences of primers for human PCSK9, and cyclophilin were as follows: human PCSK9, 5'-GGGACGTTGCCAGCTGTIT3'-3' and 5'-CGGTAGGTAGCCGGATGTG3'-3'; human cyclophilin, 5'-GGA-TGGCGACAGAGGAAA-3' and 5'-CCGTAGTGTCTACAGTT-GAAGTGT3'-3'.

Electrophoretic mobility shift assay

Nuclear forms of human SREBP-1a and SREBP-2 were generated from pCMV-nBP1a and pCMV-nBP2 (detailed procedures are described in the supplementary methods) using the TNT Quick Coupled Transcription/Translation system (Promega) according to the manufacturer’s instructions. The DNA probe was double-stranded oligonucleotides derived from the wild-type sequence found between −360 and −327 relative to the first ATG codon in the PCSK9 promoter sequence. Sequences of the wild-type and the mutant SRE probe are shown in Fig. 5A below. The DNA probe was prepared as follows. The 3'-biotinylated oligonucleotide was hybridized to a 3-fold molar excess of the unlabeled complementary strand in 50 mM Tris-Cl, pH 7.9, 50 mM NaCl, and 10 mM MgCl₂ by incubation at 65°C for 10 min and cooling at room temperature for 30 min. Binding reactions were performed in a total volume of 20 μl using a LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce) according to the manufacturer’s instructions, with minor modifications. Two hundred femtomoles of labeled probes and 1 μl of intron transcription/translated protein were complexed by incubation at room temperature for 30 min. The DNA-protein complexes were subjected to electrophoresis on a 6% TBE Gel Retardation Gel (Invitrogen) in 0.5× TBE buffer (80 V, 40 min at room temperature), transferred onto the positively charged nylon membrane (GE Healthcare), UV cross-linked, and visualized by a Chemiluminescent Nucleic Acid Detection Module (Pierce). For the antibody supershift assay, 1 μl of anti-human SREBP or nonimmune rabbit serum was added to the reaction after the formation of the DNA-protein complex and incubated at room temperature for another 15 min before electrophoresis. For the oligonucleotide competition assay, the competing unlabeled double-stranded oligonucleotide was added to the reaction with the biotin-labeled probe at 0.2- to 25-fold molar excesses.

Mice and diet

Eight-week-old male C57B/6 mice purchased from Japan SLC, Inc. (Shizuoka Prefecture, Japan) were maintained on 12 h dark/12 h light cycles and fed a chow diet (Rodent Diet from SCF Co., Inchon, Korea) that contained 5.9% (w/w) crude fat and <0.06% (w/w) cholesterol. The nonfasted group was fed a chow diet ad libitum, the fasted group was fasted for 24 h, and the refed groups were fasted for 24 h and then refed the indicated diets for 24 h before euthanasia. The starting times for the experiments were staggered so that all mice were euthanized at the same time.
which was at the end of the dark cycle. The high-sucrose diet was prepared as described by Yang, Wang, and Chen (24). A chow diet supplemented with 2% cholesterol was prepared as described (25). The powdered diets were reformed into pellets with minimal amounts of distilled water. After treatment, livers were collected and stored at −80°C until later use. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Kwandong University College of Medicine.

RESULTS

Time-course expression of PCSK9 in the presence or absence of sterols in HepG2 cells

Several reports have shown that PCSK9 is regulated by sterols at the transcriptional level; however, the endogenous PCSK9 protein itself has not been verified to be regulated accordingly. To determine whether PCSK9 expression is modulated by the depletion of sterols, HepG2 cells were grown in medium supplemented with 10% DLPS for the indicated times up to 24 h. After incubation, the cells were changed to a fresh medium supplemented with 10% DLPS, 1 µM lovastatin, and 50 µM sodium mevalonate in the presence or absence sterols for the indicated times. Figure 1A shows that the depletion of sterols induces PCSK9 in a time-dependent manner (lanes 1–5). The basal level of PCSK9 was barely detectable when HepG2 cells were grown in medium supplemented with 10% FBS (Fig. 1A, lane 1). Depletion of sterols gradually increased the expression of PCSK9 by 12 h, and the increase in PCSK9 was attenuated by 24 h (Fig. 1A, lane 6), supposedly because of the partial restoration of sterols synthesized endogenously in the cells. When 1 µM lovastatin and 50 µM sodium mevalonate were supplemented to the cells, which is a condition to suppress the endogenous synthesis of sterols, PCSK9 was further increased by 24 h (Fig. 1A, lanes 7–12). When the sterols were added to the cells, the increase in PCSK9 proteins was completely blocked, and PCSK9 was almost undetectable by 24 h of sterol treatment (Fig. 1A, lanes 13–18). These findings suggest that the expression of PCSK9 is regulated by sterols that are supplied endogenously or exogenously in HepG2 cells.

The expression of LDLR followed exactly the same pattern as that of PCSK9 when the cells were grown in the absence of sterols (Fig. 1A, lanes 1–12), whereas the expression of LDLR was restored after 12 h of sterol supplementation with lovastatin (Fig. 1A, lanes 17, 18). Nuclear forms of SREBP-2 showed similar changes to that of PCSK9 in HepG2 cells. Accordingly, the membrane forms of SREBP-2 showed reciprocal changes to its nuclear forms. However, it was shown that the changes in the nuclear form of SREBP-2 preceded the changes in PCSK9 or LDLR by depletion of sterols. Depletion of sterols even for 2 h resulted in increases in the amount of the nuclear form of SREBP-2 (Fig. 1A, lane 2). They reached the highest level by 8 h and remained constant by 12 h (Fig. 1A, lanes 4, 5). Similar to the changes in PCSK9 and LDLR, nuclear forms of SREBP-2 were decreased by 24 h (Fig. 1A, lane 6). Lova-
sterol and mevalonate significantly increased the nuclear form of SREBP-2 by 24 h in the absence of sterol (Fig. 1A, lanes 7–12), whereas in the presence of sterol the expression of nuclear forms of SREBP-2 vanished completely by 4 h (Fig. 1A, lanes 15–18).

To determine whether the changes in PCSK9 protein by sterols accompany the change in PCSK9 mRNA, total RNA from the cells for each condition was isolated and analyzed for the expression of each gene by real-time PCR (Fig. 1B). The amount of PCSK9 mRNA increased gradually for 24 h by sterol depletion (Fig. 1B). When the cells were changed to the fresh medium supplemented with lovastatin and mevalonate in the absence of sterols, PCSK9 mRNA decreased slightly for 1 h, then increased by 4–24 h. The addition of sterols completely blocked the increase in PCSK9 mRNA by lovastatin. Lovastatin also increased LDLR mRNA in the absence of sterols. However, LDLR mRNA was restored by 24 h after its initial decrease by the presence of sterols. These changes in LDLR mRNA corresponded to those in LDLR protein in HepG2 cells. The changes in SREBP-2 mRNA were not as obvious as those in PCSK9 or LDLR mRNA when the cells were grown in the absence of sterols without lovastatin. However, the evident changes in SREBP-2 mRNA were observed when the cells were grown with lovastatin and mevalonate. These findings suggest that the expression of PCSK9 is regulated by sterols and that this regulation is achieved at the transcription level possibly mediated by SREBP-2.

Lovastatin increases the expression of PCSK9, but mevalonate reverses the increase partially at high concentration

To verify the effects of the inhibition of endogenous cholesterol synthesis on PCSK9 expression, HepG2 cells were grown in medium supplemented with varying amounts of lovastatin or sodium mevalonate (Fig. 2). The addition of lovastatin increased both the proteins and mRNAs for PCSK9, LDLR, and the nuclear form of SREBP-2 in a concentration-dependent manner (Fig. 2A, lanes 1–6). These changes by lovastatin were reversed by 10 mM sodium mevalonate (Fig. 2A, lane 12). The precursor form of SREBP-2 protein showed the reciprocal changes to nuclear forms. Together, these results strongly suggest that the expression of PCSK9 is regulated by sterols that are supplied either endogenously or exogenously, and the nuclear form of SREBP-2 is a key regulatory factor for this regulation.

Sterol-dependent regulation of the promoter activity of the PCSK9 gene

To identify the location of functionally essential DNA sequences that mediate the sterol-dependence of the PCSK9 promoter, we amplified the 5′ flanking region from −2,112 to −94 of the PCSK9 gene. This amplified DNA was used as a template for the construction of promoter-reporter constructs using pGL3-Basic reporter vector to generate constructs D1–D7 (Fig. 3A). Sequence analysis (Fig. 3B) revealed the presence of SRE at −346 and several Sp1 binding sites in the proximal region of the PCSK9 promoter, as described previously (16, 17). The SRE site, 5′-GTGGCGTGAT-3′ (5′-ATCACGCCA-3′ in the complementary strand), bears the identical sequences to SRE found in the LDLR promoter, except for a single nucleotide at position 6 (based on the complementary sequences), the replacement of which in the LDLR promoter did not affect the basal transcription or sterol-dependent regulation of the LDLR promoter (26–28). The binding sites for Sp1, whose supportive function has been reported to be important in the SREBP-mediated transcriptional regulation of many genes (29–31), were found close to the SRE
site in the PCSK9 promoter. As shown in Fig. 3C, the constructs D1–D5 showed gradual increases in the promoter activities when the cells were grown in sterol-depleted conditions. The transcriptional activity of each construct was decreased by ~95% by the presence of sterols. Deletion in construct D6 dramatically decreased the promoter activity in the absence of sterols, and the sterol-mediated suppression of the transcription was attenuated from 95% to 23%. Mutation of SRE in the D4 construct (SRE-Mut) dramatically decreased the transcriptional activity of the PCSK9 promoter in the absence of sterols; consequently, its sterol-dependent suppression of transcription was attenuated (from 95% to 41%). These results suggest that the SRE site is important for the sterol-dependent regulation of the PCSK9 promoter. However, the sterol-dependent regulation of the PCSK9 promoter was preserved in all Sp1 mutants. As a result of these findings, it is suggested that the SRE site and the adjacent upstream nucleotides are important for the transcriptional regulation of the PCSK9 promoter by sterol, whereas the Sp1 binding sites are not critically required for this regulation.

Regulation of the promoter activity of PCSK9 by overexpression of SREBPs

To determine whether the SRE in the PCSK9 promoter is activated by SREBPs, the wild-type construct D4 or the mutant form (SRE-Mut) was cotransfected into HepG2 cells with pTK-HSV-nBP1a, pTK-HSV-nBP1c, or pTK-HSV-nBP2, and luciferase activity was measured (Fig. 4A). The
expression of nuclear forms of SREBPs was directed by the thymidine kinase promoter to control the amounts of expressed SREBPs in a dose-dependent manner (32). The amounts of cotransfected expression plasmids were determined to be in a range below the amounts showing the saturation effect of activation (data not shown). The amounts of expressed proteins were verified to be similar to each other by immunoblot analysis (Fig. 4B). As expected, the expression of nuclear forms of SREBPs dramatically increased the transcription of the PCSK9 promoter in construct D4. The expression of the nuclear form of SREBP-1c, the activation domain of which is shorter than that in SREBP-1a, activated the wild-type PCSK9 promoter much less than SREBP-1a or SREBP-2. The upregulation of PCSK9 promoter activity by SREBPs was abolished by the mutation of SRE. These results suggest that the function of SRE in the regulation of PCSK9 promoter activity is mediated by both SREBP-1 and SREBP-2 in HepG2 cells.

Recombinant SREBPs bind to SRE in the PCSK9 promoter

To determine whether the SREBPs can bind directly to the SRE in the PCSK9 promoter, we used in vitro-translated nuclear forms of SREBP-1a and SREBP-2 to perform EMSA. Figure 5A shows the wild-type and mutated sequences of the 3′ biotinylated probes used in EMSA. Figure 5B shows that SREBP-1a and SREBP-2 can bind to the probes (lanes 2, 11), although the migration-retarded signals by SREBP-2 always appeared weaker and much less clear than those produced by SREBP-1a. The mutant SRE probe showed no migration-retarded signals (Fig. 5B, lanes 3, 12), and unlabeled wild-type oligonucleotide effectively competed for the binding of SREBPs to the probe (Fig. 5B, lanes 5–7, 13–16). The retarded bands were supershifted by the addition of antibodies against SREBP-1 or SREBP-2 (Fig. 5B, lanes 8, 17), whereas the formation of the shifted complex was unaffected by the addition of the nonimmune serum (Fig. 5B, lanes 9, 18). These results strongly suggest that SRE in the PCSK9 promoter specifically binds both SREBP-1a and SREBP-2 in vitro.

Expression of PCSK9 is downregulated in liver of mice refed with cholesterol-supplemented diets

We finally determined the regulation of Pcsk9 in livers of mice that were subjected to 24 h of fasting followed by refeeding with a diet containing 2% (w/w) cholesterol for 24 h. In livers of mice, the antibody used in Fig. 6A did not pick up the proteins of the size corresponding to the precursor form of Pcsk9. It is supposed that the amount of the precursor form of Pcsk9 in vivo is too small to be detected by immunoblot analysis under the conditions applied in this experiment. Figure 6A shows that fasting the mice for 24 h abolished the expression of Pcsk9 protein (lane 2), and refeeding a high-carbohydrate diet or chow diet normalized the amount of Pcsk9 up to the level in livers of nonfasted mice (lanes 3, 5). This restoration of Pcsk9 expression was partially blocked by supplying cholesterol in a high-sucrose diet (Fig. 6A, lane 4) and was blocked more efficiently when mice were refed with a chow diet with cholesterol (Fig. 6A, lane 6). In contrast, the amount of Ldlr in livers of mice fasted for 24 h showed insignificant change compared with the amount in nonfasted mice (Fig. 6A, lane 2 vs. lane 1), whereas refeeding a high-sucrose diet or a chow diet without cholesterol slightly reduced the expression of Ldlr (Fig. 6A, lanes 3–5). However, refeeding the mice with a chow diet supplemented with cholesterol restored the amount of Ldlr to the level shown in the nonfasted mice (Fig. 6A, lane 6 vs. lane 1). These findings suggest that the amount of Ldlr in vivo follows the changes of Pcsk9 expression reciprocally when the mice are refed. The amounts of the nuclear form of Srebp-1c were upregulated when the mice were refed, regardless of the supplementation of cholesterol, whereas the nuclear form of Srebp-2 showed suppression by cholesterol (Fig. 6A, lanes 4, 6), which correlated with the changes of Pcsk9.

To determine the changes in Pcsk9 and Ldlr mRNA by fasting and refeeding, total RNA was isolated from the livers of mice and real-time PCR was performed (Fig. 6B).
The changes in Pcsk9 mRNA corresponded to those in Pcsk9 proteins: fasting dramatically decreased Pcsk9 mRNA; refeeding restored Pcsk9 mRNA; and supplementation of cholesterol partially blocked the restoration of Pcsk9 mRNA. Ldlr mRNA remained relatively unchanged by fasting and refeeding. Nevertheless, Ldlr mRNA was downregulated when the mice were refed a diet supplemented with cholesterol.

We next determined the changes in the other genes involved in fatty acid and cholesterol biosynthesis, the transcription of which is under the control of Srebps, to compare the effect of cholesterol on the transcription of each gene. The mRNA for HMG-CoA reductase (Hmgcr), one of the well-characterized Srebp-2 target genes (33), showed downregulation by supplementation of cholesterol. The changes of mRNAs for three lipogenic enzymes, acetyl-CoA carboxylase α (Acaca) (34), fatty acid synthase (Fasn) (35), and stearoyl-CoA desaturase-1 (Scd-1) (36), as Srebp-1 target genes were also determined. The mRNAs for Acaca, Fasn, and Scd-1 showed downregulation by fasting, whereas drastic increases in their mRNAs were observed by refeeding a high-sucrose diet. Most importantly, the transcription of these Srebp-1 target genes was not downregulated by cholesterol in livers of mice. Together, these data suggest that the regulation of Pcsk9 expression by cholesterol in vivo is achieved at the transcription level and that Srebp-2 predominantly mediates this regulation.

**DISCUSSION**

PCSK9 has been reported as one of the genes whose transcription is under the control of SREBPs (4, 5). Considering the function of PCSK9 in cholesterol metabolism, it is suggested that the major regulator of its expression is SREBP-2 (16), whereas experiments in mice that were fasted followed by refeeding with a carbohydrate-rich diet showed the involvement of SREBP-1 in the activation of PCSK9 expression (17). This regulation of PCSK9 transcription by SREBPs has been supported by the presence of SRE on the promoter region of the PCSK9 gene. However, most importantly, no clear-cut evidence for the regulation of endogenous PCSK9 proteins by sterols has been provided. In the present experiments, we produced the polyclonal antibody against PCSK9 and determined the regulation of endogenous PCSK9 expression by sterols andLovastatin. The expression PCSK9 protein has proven to be induced when HepG2 cells were deprived of sterols.

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**Fig. 5.** Binding of SREBP-1a and SREBP-2 to the SRE site in the PCSK9 promoter. A: Nucleotide sequences of the sense strand for wild-type and mutant SRE probes. The locations of the SRE and mutated nucleotides are indicated with the arrow and dots, respectively. Each strand was synthesized with modification of 3’-biotinylation, and the double strand probes were produced by annealing with a 3-fold molar excess of the respective unlabeled antisense strands as described in Materials and Methods. B: Electrophoretic mobility shift assay using the wild-type (WT) or mutant (Mu) biotinylated probes with in vitro-translated nuclear human SREBP-1a (BP-1a) or SREBP-2 (BP-2) protein was performed as described in Materials and Methods. Protein Mo indicates the in vitro translation reaction using mock pcDNA3 vector. For competition assay (Competitor), competing unlabeled double-stranded oligonucleotides were added to the reaction with the biotin-labeled probe at 0.2- to 25-fold molar excess. For supershift assays, antiserum against SREBP-1 (BP-1) or SREBP-2 (BP-2) was incubated with the complex for an additional 15 min. Antibody Pre indicates the nonimmune rabbit serum. The arrow and arrowheads indicate the shifted and supershifted bands, respectively. The asterisks and double asterisks indicate the shifted bands produced by the nonspecific interaction of probes with proteins and free probes, respectively.
When the endogenous supply of sterols was blocked by lovastatin with a limited amount of mevalonate to supply nonsterol isoprenoid compounds (37, 38), the induction of PCSK9 was augmented. The increase in PCSK9 caused by the depletion of sterols was blocked completely by the addition of sterols in the culture medium. These changes in PCSK9 protein by sterols paralleled thoroughly the changes in its mRNA. The changes in the nuclear forms of SREBP-2 by sterols were similar to those of PCSK9, however, they preceded the changes in PCSK9 expression suggesting the possible involvement of SREBP-2 in this regulation. Lovastatin, which has been reported to increase the transcription of PCSK9 (16), has proven to increase the expression of PCSK9 protein and mRNA even at low concentrations (8–40 nM). This induction of PCSK9 by lovastatin was reversed by a very high concentration (10 mM) of mevalonate. These findings suggest that the regulation of PCSK9 expression is dependent on the presence or absence of sterols, which are supplied either exogenously or endogenously, and that this regulation is achieved at the transcription level.

The expression of LDLR showed similar changes to PCSK9 in HepG2 cells under the sterol-depleted conditions. Considering that the function of PCSK9 is to promote the degradation of LDLR (39), the parallel change in LDLR to PCSK9 by sterols in HepG2 cells should be interpreted as a paradox. One possible explanation for this paradox is that the sterol-depleted condition applied in this experiment is too stringent to show the reciprocal regulation of LDLR and PCSK9 proteins. When the cells are deprived of sterols, the increased expression of the nuclear form of SREBP-2 might induce the expression of both PCSK9 and LDLR to a level that overwhelms the reciprocal regulation of these proteins. This hypothesis could be supported by the finding that supplementation of sterols to the cells for 24 h resulted in the partial restoration of LDLR protein and mRNA, whereas the expression of PCSK9 and the nuclear form of SREBP-2 remained completely suppressed. Another possibility, that of lacking a component or machinery in HepG2 cells to coordinate the expression of these proteins, remains to be uncovered.

To clarify the transcriptional regulation of PCSK9 by SREBPs, we cloned the 5′ flanking region of human PCSK9 and determined its promoter activities. As reported previously by Dubuc et al. (16), the SRE site in the PCSK9 promoter has proven to be important in the sterol-dependent regulation of PCSK9 transcription in HepG2 cells. Mutations of the SRE site in the PCSK9 promoter dramatically...
lated the restoration of the decreased expression of SREBP-1 and -2 in livers of mice under the nutritional manipulations. Fast-observed in livers of fasted mice. Attenuation of the mobility-retarded bands produced by the tag sequence. The absence of the tag sequence to exclude the effect on the mobility-retarded bands produced by the tag sequence.

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