Proprotein Convertase Subtilisin Kexin Type 9 Promotes Intestinal Overproduction of Triglyceride-Rich Apolipoprotein B Lipoproteins Through Both Low-Density Lipoprotein Receptor–Dependent and –Independent Mechanisms

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Background—Proprotein convertase subtilisin kexin type 9 (PCSK9) promotes the degradation of the low-density lipoprotein (LDL) receptor (LDLR), and its deficiency in humans results in low plasma LDL cholesterol and protection against coronary heart disease. Recent evidence indicates that PCSK9 also modulates the metabolism of triglyceride-rich apolipoprotein B (apoB) lipoproteins, another important coronary heart disease risk factor. Here, we studied the effects of physiological levels of PCSK9 on intestinal triglyceride-rich apoB lipoprotein production and elucidated for the first time the cellular and molecular mechanisms involved.

Methods and Results—Treatment of human enterocytes (CaCo-2 cells) with recombinant human PCSK9 (10 μg/mL for 24 hours) increased cellular and secreted apoB48 and apoB100 by 40% to 55% each (P<0.01 versus untreated cells), whereas short-term deletion of PCSK9 expression reversed this effect. PCSK9 stimulation of apoB was due to a 1.5-fold increase in apoB mRNA (P<0.01) and to enhanced apoB protein stability through both LDLR-dependent and LDLR-independent mechanisms. PCSK9 decreased LDLR protein (P<0.01) and increased cellular apoB stability via activation of microsomal triglyceride transfer protein. PCSK9 also increased levels of the lipid-generating enzymes FAS, SCD, and DGAT2 (P<0.05). In mice, human PCSK9 at physiological levels increased intestinal microsomal triglyceride transfer protein levels and activity regardless of LDLR expression.

Conclusions—PCSK9 markedly increases intestinal triglyceride-rich apoB production through mechanisms mediated in part by transcriptional effects on apoB, microsomal triglyceride transfer protein, and lipogenic genes and in part by posttranscriptional effects on the LDLR and microsomal triglyceride transfer protein. These findings indicate that targeted PCSK9-based therapies may also be effective in the management of postprandial hypertriglyceridemia.

Key Words: apolipoproteins • lipids • molecular biology • pathophysiology • receptors, lipoprotein • risk factors

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted serine protease that plays an important role in the development of atherosclerotic coronary heart disease in humans. Gain-of-function mutations in PCSK9 are associated with autosomal-dominant hypercholesterolemia and sharply increased risk of coronary artery disease,1 whereas loss-of-function mutations are accompanied by remarkably large reductions in coronary heart disease risk.2 These findings have generated intense investigations in PCSK9 biology, which have attributed potential proatherosclerotic properties to PCSK9, linked to its effect on serum low-density lipoprotein (LDL) cholesterol levels,3 raised by PCSK9-mediated degradation of hepatic LDL receptors (LDLRs),4 which then causes a secondary increase in plasma PCSK9 levels.5

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It has recently emerged, however, that PCSK9 also affects the metabolism of triglyceride-rich apolipoprotein (apo) B–containing lipoproteins (TRLs).5,6 Triglyceride and apoB levels significantly and independently associate with coronary heart disease risk.7 Furthermore, TRL remnant particles drive cholesterol accumulation in arterial macrophages and cause progression of coronary artery disease.8

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Relatively few investigations have targeted the regulatory role of PCSK9 on TRL. Most of them have focused on the effect of PCSK9 on TRL metabolism at the hepatic level and have identified PCSK9 as a trigger of TRL production. The intestine is another major source of TRL, accounting for a significant portion of plasma lipids in the form of postprandial chylomicrons, with apoB48 as their characteristic apolipoprotein. It must be noted that PCSK9 expression in the intestine is quantitatively second only to hepatic expression.

Here, we investigated for the first time the comprehensive cellular, molecular, and physiological mechanisms through which PCSK9 causes intestinal TRL overproduction. Our results reveal that PCSK9 regulation of intestinal TRL metabolism is imparted at both transcriptional (lipid and apolipoprotein biosynthesis) and posttranscriptional (TRL assembly) levels. Furthermore, we show that the effects of human PCSK9 (hPCSK9) on intestinal apoB production are mediated through both LDLR-dependent and -independent means and involve microsomal triglyceride transfer protein (MTP). Finally, we show that acute PCSK9 knockdown substantially reduces enterocyte TRL production via downregulation of key protein mediators of the cellular TRL production pathway.

Methods

Cell Culture

Cultured CaCo-2 cells were obtained from American Type Culture Collection (Manassas, VA). CaCo-2 cells were first grown for 3 days in Eagle’s minimal essential medium (EMEM) specialty medium (American Type Culture Collection) in 20% FBS supplemented with 1% penicillin-streptomycin at 37°C and 5% CO2 until they reached 80% to 90% confluence. Induction of cell polarization is necessary for CaCo-2 cells to resemble human intestinal enterocytes in vivo. To induce polarization, CaCo-2 cells were maintained in culture in dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 14 days in specialized, collagen-treated trans-well plates with 0.4-μm pore-size polycarbonate filters (CoStar, Washington, DC).

Unless otherwise indicated, all studies were performed at least 3 times. Optimally polarized confluent CaCo-2 cells in 1% FBS–Dulbecco modified Eagle medium were treated for 24 hours with recombinant hPCSK9 (R&D Systems, Minneapolis, MN) reconstituted in Millipore H20. PCSK9 was mostly used at the concentration of 10 μg/mL for 24 hours, although many experiments included different doses (0, 5, 7.5, 10, and 12 μg/mL) and different times (0, 2, 4, 8, 12, and 24 hours).

Mice

C57BL/6 (wild-type [WT]) and LDLR−/− mice were purchased from The Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME) and housed at Vanderbilt University Medical Center. hPCSK9 transgenic mice were generated in our laboratory as previously described. All animal experiments were carried out in compliance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

PCSK9 siRNA Studies

Four siRNAs targeting hPCSK9 (Qiagen, Gaithersburg, MD) were transfected into CaCo-2 cells at a final concentration of 15 μmol/L with the HiPerFect transfection reagent (Qiagen) at 0.5% final volume. The siRNA that produced the maximum decrease in PCSK9 mRNA and protein expression (70% mRNA knockdown 48 hours after transfection) was chosen for further experiments. As a negative control, CaCo-2 cells were transfected with a scrambled siRNA control vector containing a nonsense mRNA sequence (Qiagen). As a positive control, CaCo-2 cells were transfected with siRNA targeting and silencing the constitutively expressed GAPDH gene.

Immunoprecipitation and Western Blots

Cell lysates, collected with radiolymphoprecipitation assay buffer (50 mmol/L Tris, 150 mmol/L sodium chloride, 1% NP-40, 12 mmol/L sodium deoxycholate, 3.5 mmol/L SDS, pH 7.4), protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada), and media, were immunoprecipitated for apoB48, apoB100, LDLR, MTP, Niemann-Pick C1-like 1 (NPC1L1), PCSK9, GAPDH, β-actin, and albumin with Catch-and-Release immunoprecipitation columns and kits (Millipore, Billerica, MA). Immunoprecipitates were subjected to SDS-PAGE, transferred onto nitrocellulose membranes (BioRad, Hercules, CA), and immunoblotted with the use of antibodies against apoB48 and apoB100 (human) (Santa Cruz Biotechnology, Santa Cruz, CA), PCSK9 (Cayman Chemicals, Ann Arbor, MI), LDLR ( Fitzgerald International, Acton, MA), MTP (Santa Cruz Biotechnology, Santa Cruz, CA), NPC1L1 (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (Sigma-Aldrich, St. Louis, MO), β-actin (Sigma-Aldrich, St. Louis, MO), and albumin (Santa Cruz Biotechnology). Horseradish peroxidase–conjugated antibodies (BioRad) were used as secondary antibodies. Immunoreactive bands were visualized with a chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA). The blots were exposed to KODAK Biomax films, and the signal was quantified by densitometry with Quantity One version 4.6.7 software (BioRad).

Samples of proximal small intestine and large intestine were lysed with modified radiolymphonprecipitation assay buffer (Sigma). Samples (50 μg) were loaded onto NuPage 4% to 12% Bis-Tris gels (Life Technologies, Carlsbad, CA) for electrophoresis, and the size-separated proteins were transferred to nitrocellulose membranes. Primary antibodies toward MTP (a kind gift of Larry Swift, PhD, Vanderbilt University), β-actin (Abcam, Cambridge, MA), and GAPDH (Novus Biologicals, Littleton, CO) and horseradish peroxidase–conjugated secondary antibodies (Sigma) were used to detect target proteins. Signal was detected by use of a mixture of luminol, p-coumaric acid, and hydrogen peroxide in 100 mmol/L Tris (pH 8.5). Intensity quantification of the bands was obtained with ImageJ software and normalized to β-actin or GAPDH.

Semi-quantitative Polymerase Chain Reaction

Total RNA was isolated from the proximal part of the small intestine and distal part of the large intestine with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and processed for reverse transcription with the iSCRIPT cDNA synthesis (BioRad). cDNA was amplified with the Platinum PCR MasterMix (Invitrogen Life Technologies, Carlsbad, CA) and primers for MTP (forward primer, TGGTACAGGAAAGCTGTGCAG; reverse primer, GAAAGAAAACATCTCCTCCAGG) and β-actin (forward primer, AGGGAAATCGTGCGTGACAT; reverse primer, AGGGAAATCGTGCGTGACAT; reverse primer, CGTTGCAAAFGTGATGACC) and loaded onto a 4% agarose gel.

Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA was isolated from cell lysates (RNasy Mini Kit, Qiagen) and used as templates for cDNA synthesis (QuantiTech Reverse Transcription Kit, Qiagen). Quantitative real-time reverse transcriptase (RT)–polymerase chain reaction (PCR) was performed with an Applied Biosystems 7300 Real Time PCR system, according to the manufacturer’s instructions (Applied Biosystems Life Technologies, Carlsbad, CA), and with the SYBR green master kit (Qiagen). Primers for RT-PCR succinate dehydrogenase (SDH), APOB, MTP, Sterol Regulatory Element Binding protein (SREBP) 1, SREBP2, 3-hydroxy-3-methylglutaryl-Coenzyme (HMG-CoA) reductase, HMG-CoA synthase, squalene synthase (SS), LDLR, PCSK9, fatty acid synthase (FAS), steroyl-CoA desaturase (SCD), diglyceride acyltransferase 1 (DGAT1), and DGAT2 were purchased (the proprietary sequences are not available) from Qiagen. The values reported for each mRNA were corrected to SDH mRNA values.

Relative quantification of MTP mRNA from small and large intestine samples was performed with the ABI Prism 7700 Sequence...
Detection System (Applied Biosystems Life Technologies) with the use of TaqMan gene expression assays (Applied Biosystems Life Technologies). Expression levels were calculated with the ΔΔCT method and normalized to 18S rRNA levels.

**Oil Red O and Hematoxylin Staining**

Cells were stained with Oil Red O to examine the total amount of neutral lipid accumulation in the cells, as previously described.\(^{16}\)

**Cell Viability**

Cell viability was determined with 0.4% Trypan blue (Sigma-Aldrich, Oakville, ON, Canada) staining and calculated from the following formula: Percent cell viability equals the number of unstained (living) cells divided by the total number of cells times 100.\(^{16}\)

**MTP Activity Assay**

Cell monolayers were washed twice with cold PBS and once with 5 mL buffer (1 mmol/L Tris-HCl, pH 7.6, 1 mmol/L EGTA, and 1 mmol/L MgCl\(_2\)) at 4°C. Cells were then incubated for 2 minutes at room temperature in 5 mL of cold buffer. The buffer was aspirated, and 0.5 mL of the same buffer was added to cells. Small pieces (50–70 mg) of proximal small intestine were collected and homogenized in 0.5 mL of the same buffer. Cells and tissues were scraped, collected, vortexed, and centrifuged (SW55 Ti rotor, 90,000 rpm, 4°C, 1 hour, depending on sample size), and supernatants were tested for MTP activity with a kit (Chylos Inc, Woodbury, NY), as previously described.\(^{17}\) The triglyceride transfer activity of MTP was measured as percent lipid transfer per hour per 1 mg protein.

**Statistical Analyses**

Data (Figures 1–4) were analyzed by use of Wilcoxon rank-sum tests, and a Bonferroni correction was applied for multiple pairwise comparisons. The in vivo data (Figures 5 and 6 and Figures I and II in the online-only Data Supplement) were analyzed with \(t\) tests or ANOVA, as appropriate, with the Bonferroni test for post hoc comparisons. All results are presented as mean±SEM. Asterisks indicate statistically significant differences (*\(P<0.05\) and **\(P<0.01\)) compared with respective controls.

**Results**

**PCSK9 Directly Stimulates ApoB Cellular Protein Expression and Secretion in Human Enterocytes**

Confluent, polarized CaCo-2 cells in 1% FBS–Dulbecco modified Eagle medium were treated for 24 hours with recombinant hPCSK9. Of note, CaCo-2 cells secreted negligible amounts of hPCSK9 relative to the 10-\(\mu\)g/mL concentration of recombinant PCSK9 added to the media during the experiments. The identity of recombinant hPCSK9 was confirmed by immunoblot (Figure 1A).

The addition of physiological levels of hPCSK9 (10 \(\mu\)g/mL)\(^{18}\) to optimally polarized human enterocytes (CaCo-2 cells) resulted in an \(\approx\)50% increase in apoB cellular protein expression and a similar increase in secreted apoB compared with untreated CaCo-2 cells (Figure 1B and 1C). This stimulatory effect of PCSK9 was rapid, occurring at 24 hours after treatment, and prolonged, lasting at least 48 hours.

PCSK9 treatment (10 \(\mu\)g/mL for 24 hours) markedly stimulated enterocyte production and secretion of both apoB48 (Figure 1B) and apoB100 (Figure 1C) proteins. Whereas intestinal human TRL contains only the apoB48 isoform, CaCo-2 cells secrete TRL containing both apoB48 and apoB100,\(^{19}\) allowing us to characterize the effect of PCSK9 on both apoB proteins. We found quantitatively similar stimulatory effects on both forms of apoB.

We next performed dose-response and time-course experiments to assess enterocyte apoB secretion (Figure 1D). The maximum stimulatory effect of PCSK9 on both apoB48 and apoB100 secretion was seen with 10 \(\mu\)g/mL PCSK9, and no further stimulation was obtained with 12.5 \(\mu\)g/mL. Moreover, a significant increase in secreted apoB was observed in PCSK9-treated enterocytes as early as 4 hours after PCSK9 treatment (10 \(\mu\)g/mL), an effect that persisted for 24 hours, the time point at which the maximal cumulative effect of PCSK9 on apoB secretion was observed (Figure 1E).

**Quantification of the Contribution of PCSK9 to Enterocyte ApoB Production**

PCSK9 siRNA treatment reduced PCSK9 mRNA levels by 70% (\(P<0.01\)) compared with CaCo-2 cells transfected with a scrambled negative control vector. Treatment with the PCSK9 siRNA also caused a 40% reduction in the secretion of both apoB48 and apoB100 (Figure 2).

**Stimulation of Enterocyte ApoB Production by PCSK9 Occurs at the Transcriptional Level on Cellular Apolipoprotein and Lipid Biosynthesis**

Whether enterocyte apoB production by PCSK9 is regulated at the transcriptional level was assessed via real-time RT-PCR analyses. Our results show a significant 1.5-fold increase in apoB mRNA levels in PCSK9-treated cells (10 \(\mu\)g/mL for 24 hours; Figure 3A) and a converse 50% decrease in apoB mRNA in cells transfected with PCSK9 siRNA (for 48 hours; Figure 3A) compared with control untreated cells, demonstrating the specificity of the apoB mRNA effect by PCSK9.

Because intracellular neutral lipids inhibit cellular apoB protein degradation and enhance apoB protein stability,\(^{20}\) we determined whether an increase in cellular neutral lipids contributes to the enhanced cellular apoB protein expression and secretion with PCSK9. We therefore performed Oil Red O/hematoxylin staining of CaCo-2 cells. The results showed a clear increase in enterocyte neutral lipid content in PCSK9-treated (10 \(\mu\)g/mL for 24 hours) cells versus control untreated cells (Figure 3B) and a slight increase in cellular neutral content in enterocytes treated with PCSK9 siRNA (for 48 hours; Figure 3C).

To study whether the PCSK9-mediated increase in enterocyte lipid content is attributable to increased cellular de novo lipogenesis, we measured the expression levels of \(SREBP1\) (fatty acid and triglyceride synthesis) and \(SREBP2\) (cholesterol synthesis and uptake) target genes. The results showed that PCSK9 treatment (10 \(\mu\)g/mL for 24 hours) caused a 1.5- to 2-fold increase in mRNA levels of \(SREBP1\) target genes such as \(FAS\), \(SCD\), and \(DGAT2\) (Figure 3D). There was no change, however, in \(SREBP1\) or \(DGAT1\) expression. In addition, no differences were observed in the mRNA levels of \(SREBP2\) or \(SREBP2\) target genes, \(HMGR\), \(HMGC\), \(SS\), \(LDLR\), or \(PCSK9\) (Table I in the online-only Data Supplement).
Figure 1. Direct stimulatory effect of proprotein convertase subtilisin kexin type 9 (PCSK9) on apolipoprotein (apo) B expression and secretion in human enterocytes. A, The human recombinant human PCSK9 used in all experiments was characterized via immunoblotting. A 2-μg quantity of recombinant human PCSK9 was immunoblotted with 10% SDS gels and a polyclonal antibody against human PCSK9. The results confirmed the expected 62-kDa size of recombinant human PCSK9. B, PCSK9 treatment (10 μg/mL for 24 hours) significantly stimulated the cellular expression and secretion of apoB48 protein (by 40%–50%) in optimally polarized CaCo-2 cells compared with control untreated cells maintained in 1% FBS. C, PCSK9 (10 μg/mL for 24 hours) markedly stimulated the cellular expression and secretion of apoB100 protein (by 40%–50%) in CaCo-2 cells compared with control untreated cells maintained in 1% FBS. D, The stimulatory effect of PCSK9 (for 24 hours) on apoB48 and apoB100 secretion in CaCo-2 cells (vs control untreated cells maintained in 1% FBS) occurred in a dose-responsive manner in the 0- to 12.5-μg/mL concentration range of PCSK9. The maximum stimulatory effect was reached at 10 μg/mL PCSK9. E, Time course effects of PCSK9-treated CaCo-2 cells (10 μg/mL) showed an enhancement in both apoB48 and apoB100 secretion as early as 4 hours after treatment (vs control untreated cells) and continued to the 24-hour time frame of the study.
Treatment with PCSK9 siRNA (for 48 hours) showed no change in mRNA levels of SREBP1 or SREBP1 target genes, with the exception of a slight increase in the expression of DGAT2 (Figure 3D). Despite no effects on SREBP2 mRNA levels, PCSK9 siRNA produced significant reductions in the SREBP2 target genes involved in cholesterol biosynthesis, HMGCR, HMGCS, and S5 (Figure 3E). However, there was no change in SREBP2 targets involved in cholesterol uptake, LDLR and PCSK9 (Table II in the online-only Data Supplement).

MTP is crucial for the transfer of neutral lipids to apoB and a key regulator of intracellular apoB degradation and stability.20 Enterocytes treated with PCSK9 (10 μg/mL for 24 hours) showed a nearly 2-fold increase in MTP mRNA levels (Figure 3A), whereas PCSK9 siRNA–treated enterocytes (for 48 hours) demonstrated a 20% reduction in MTP mRNA (Figure 3A).

Key Protein Mediators Through Which PCSK9 Stimulates Enteroocyte ApoB Production

Treatment of enterocytes with PCSK9 (10 μg/mL for 24 hours) markedly (2.5-fold) increased cellular levels of the active, 62-kDa form of PCSK9. This suggests that after entry into the cell, a substantial amount of exogenous PCSK9 does not undergo lysosomal degradation (Figure 4A).

PCSK9 reduced enterocyte LDLR protein expression by 50%, whereas PCSK9 gene knockdown (via PCSK9 siRNA for 48 hours) raised LDLR protein levels in enterocytes by 50% (Figure 4B).

We next investigated whether mechanisms independent of the LDLR were involved in the PCSK9-mediated increase in enterocyte apoB TRL production. We assessed MTP protein levels (Figure 4C) and MTP lipid transfer activity (Figure 4D) and found that both were significantly enhanced, by 30% to 40%, in enterocytes by PCSK9 treatment compared with control untreated cells. Conversely, PCSK9 siRNA inhibition in enterocytes (for 48 hours) significantly lowered MTP protein levels and activity.

Furthermore, we determined enterocyte levels of NPC1L1, a critical transporter of cholesterol across the intestinal lumen that affects the overall cellular lipid content.21 Enteroocyte NPC1L1 protein levels were increased by PCSK9 treatment, whereas PCSK9 siRNA significantly downregulated NPC1L1 levels (Figure 4E).

PCSK9 Affects the TRL Secretion Machinery In Vivo in an LDLR-Dependent and -Independent Fashion

Human PCSK9 expression in mice increases serum cholesterol via both hepatic LDLR reduction and increased TRL secretion.5 To test the effect of PCSK9 on the intestine in vivo, we used transgenic mice expressing PCSK9 in multiple tissues, including the small and large intestines.5 Intestinal samples from hPCSK9 transgenic, WT, and murine PCSK9 knockout (mPCSK9−/−) mice were tested for LDLR levels.22

PCSK9 expression significantly reduced LDLR levels in the small intestine (54%), whereas the absence of mPCSK9 increased LDLR levels by nearly 70% (Figure I in the online-only Data Supplement). In contrast, PCSK9 overexpression or deletion did not affect LDLR levels in the large intestine (Figure II in the online-only Data Supplement). We also tested the in vivo effect of PCSK9 expression on MTP, the rate-limiting enzyme for TRL particle secretion20,23 in the presence or absence of LDLR. Expression of hPCSK9 significantly increased MTP mRNA (3.8-fold; Figure 5A and 5B) and protein (2-fold; Figure 5C, lanes 1–3) levels compared with WT mice. In agreement with canonical knowledge that fat absorption and assembly of chylomicrons occur in the small intestine,24 MTP protein was not detected in the large intestine (Figure 5C, lanes 4–6). Human PCSK9 expression resulted in a significant (32%) increase in MTP activity (Figure 5D), but the absence of mPCSK9 did not alter MTP at any level, mRNA, protein, or activity.

The LDLR-independent effect of PCSK9 on TRL secretion was further tested in mice expressing hPCSK9 but not LDLR. Figure 6A and 6B shows that MTP mRNA levels were increased 3.9-fold in LDLR−/− mice expressing hPCSK9 compared with...
Figure 3. Proprotein convertase subtilisin kexin type 9 (PCSK9)–induced changes in expression levels of genes involved in the control of enterocyte lipid and lipoprotein biosynthesis. A, The mRNA levels of APOB and MTP genes were assessed by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) in CaCo-2 cells treated with 10 μg/mL PCSK9 for 24 hours and in cells transfected with PCSK9 siRNA for 48 hours and compared with control cells. PCSK9 treatment resulted in a significant 1.5-fold increase in cellular apolipoprotein (apo) B mRNA content vs untreated control cells. PCSK9 also caused a similar 1.5-fold increase in cellular microsomal triglyceride transfer protein (MTP) mRNA content. PCSK9 siRNA treatment resulted in a significant 50% decline in cellular apoB mRNA content vs control cells treated with a scrambled siRNA control vector. PCSK9 also caused a 20% decrease in cellular MTP mRNA content. B, The lipid content of CaCo-2 cells treated with PCSK9 (10 μg/mL for 24 hours) was assessed with Oil Red O/hematoxylin staining. PCSK9-treated CaCo-2 cells showed a markedly greater cellular neutral lipid content (triglycerides and cholesteryl esters) than untreated control cells. C, The lipid content of CaCo-2 cells treated with PCSK9 siRNA (for 48 hours) was assessed with Oil Red O/hematoxylin staining. PCSK9–treated CaCo-2 cells showed a markedly greater cellular neutral lipid content (triglycerides and cholesteryl esters) compared with control cells treated with a scrambled siRNA control vector. D, The mRNA levels of target genes in the SREBP1 intracellular lipid biosynthesis pathway were assessed in CaCo-2 cells treated with PCSK9 (10 μg/mL for 24 hours) and in cells transfected with PCSK9 siRNA (for 48 hours) via real-time RT-PCR. The mRNA levels of target genes in the SREBP1 pathway involved in cellular fatty acid biosynthesis (FAS and SCD) and triglyceride biosynthesis (DGAT2) were significantly greater in enterocytes treated with PCSK9 than in control untreated cells. DGAT2 mRNA levels, but not other SREBP1 target genes, were slightly higher in PCSK9 siRNA–transfected enterocytes compared with control cells treated with a scrambled siRNA control vector. E, The mRNA levels of target genes in the SREBP2 cholesterol biosynthesis pathway were assessed in CaCo-2 cells treated with PCSK9 siRNA (for 48 hours) via real-time RT-PCR. The mRNA levels of HMGCR, HMGCS, and SS, but not other SREBP2 target genes, were significantly reduced in PCSK9 siRNA–transfected enterocytes compared with control cells treated with a scrambled siRNA control vector.
control LDLR−/− mice, with protein expression also showing a similar trend (Figure 6C). As expected, MTP was not expressed in the large intestine of LDLR−/− mice (Figure 6B). Finally, MTP activity was increased by 37% (P<0.05) in the small intestine of LDLR−/− mice expressing hPCSK9 (Figure 6D).

**Discussion**

The controversy about whether variations in PCSK9 concentrations affect TRL levels in humans was settled with recent studies showing significant associations between serum PCSK9 levels and the levels of circulating TRL markers in
a broad spectrum of the population. In the present study, we have further shown, for the first time, that physiological levels of hPCSK9 directly stimulate intestinal TRL apoB48 and apoB100 production by 50%. Given the role that intestinal TRL production in humans plays in determining serum lipid levels after a meal, this magnitude of increase in intestinal TRL apoB production by PCSK9 should be a significant contributor to postprandial lipids. In addition, this level of TRL apoB stimulation can influence fasting triglycerides in conditions of dysregulated hepatic TRL production, characteristic of individuals with insulin resistance and metabolic syndrome. Furthermore, because 1 apoB molecule is

![Figure 5. Microsomal triglyceride transfer protein (MTP) levels and activity in the small intestine in murine (mPCSK9−/−), wild-type (WT), and human proprotein convertase subtilisin kexin type 9 (hPCSK9) transgenic (tg) mice. A, Semiquantitative polymerase chain reaction (PCR) results showing mRNA levels of MTP and β-actin in the large intestine. B, Relative mRNA expression of MTP evaluated by real-time reverse transcriptase–polymerase-PCR. Expression levels were calculated with the ΔΔCT method. Each sample was measured in duplicate with 6 mice in each group (n=6) and normalized to 18S rRNA levels. C, Quantitative analysis of MTP protein levels normalized to β-actin in the small intestine, with the indicated number of mice. Inset, Representative immunoblot of MTP and β-actin in the small and large intestines. Lane 1, mPCSK9−/− small intestine; lane 2, WT small intestine; lane 3, hPCSK9 tg small intestine; lane 4, mPCSK9−/− large intestine; lane 5, WT large intestine; and lane 6, hPCSK9 tg large intestine. D, MTP activity in the small intestine. MTP activity was measured in duplicate with 3 mice in each group (n=3) and normalized to total protein content. *P<0.05, **P<0.01, 1-way ANOVA.](http://circ.ahajournals.org/)

![Figure 6. Microsomal triglyceride transfer protein (MTP) levels and activity in the small intestine of wild-type (WT) and human proprotein convertase subtilisin kexin type 9 (hPCSK9) transgenic (tg) mice on an LDLR−/− background. A, Semiquantitative polymerase chain reaction (PCR) results showing mRNA levels of MTP and β-actin in the small intestine. B, Relative mRNA expression of MTP evaluated by real-time reverse transcriptase–PCR. Expression levels were calculated with the ΔΔCT method. Each sample was measured in duplicate with the indicated number of mice, and mRNA levels were normalized to 18S rRNA levels. C, Quantitative analysis of MTP protein levels normalized to GAPDH in the small intestine, with the indicated number of mice. Inset, Representative immunoblot of MTP and β-actin in the small and large intestines. Lane 1, mPCSK9−/− small intestine; lane 2, WT small intestine; lane 3, hPCSK9 tg small intestine; lane 4, mPCSK9−/− large intestine; lane 5, WT large intestine; and lane 6, hPCSK9 tg large intestine. D, MTP activity in the small intestine. MTP activity was measured in duplicate with 3 mice in each group (n=3) and normalized to total protein content. *P<0.05, **P<0.01, 1-way ANOVA.](http://circ.ahajournals.org/)
present per TRL particle, this level of increase in TRL apoB by PCSK9 suggests a potential doubling in the levels of pro-atherogenic TRL intestinal remnant particles in the circulation after a meal.

We further characterized the dynamics of PCSK9-induced stimulation of intestinal TRL and found that it was rapid and prolonged, affecting both cellular and secreted apoB levels. More specifically, the enhancement in enterocyte apoB by extracellular PCSK9 was observed as early as 4 hours after treatment and persisted for 24 hours. Furthermore, PCSK9 increased both cellular and secreted apoB protein levels by similar extents, which indicates that the apoB synthesized in the enterocyte in response to PCSK9 induction is stable and preserved throughout the secretory pathway, without significant degradation.

Importantly, we identified, for the first time, the cellular and molecular mechanisms through which PCSK9 drives intestinal TRL overproduction. First, we showed that PCSK9 stimulates enterocyte synthesis of both the major protein and lipid components of TRL particles, apoB and triglycerides, by altering cellular processes at both the transcriptional and posttranscriptional levels. Moreover, the PCSK9-mediated increase in intestinal TRL production in vitro and in vivo was attributed to both LDLR-dependent and -independent mechanisms, involving both transcriptional effects on apoB, MTP, and lipogenic genes and posttranscriptional effects on MTP.

We show here that a portion of the PCSK9 that enters the cell from the extracellular compartment remains intact, does not undergo lysosomal degradation, and is able to alter intracellular processes. This may explain how extracellular PCSK9 selectively activates transcriptional and posttranscriptional processes. The observation that a portion of internalized PCSK9 is not degraded has recently been reported by us after finding hPCSK9 in liver of mice 4 hours after injection of exogenous hPCSK9. Similarly, a recent study with human fibroblasts has documented that a portion of internalized PCSK9 is diverted from the lysosomal degradation pathway to endocytic recycling compartments. It is unclear whether this mechanism involves dissociation of PCSK9 from LDLR or if it is a route for the coupled recycling for both protein partners.

At the transcriptional level, PCSK9 caused a significant increase in enterocyte apoB mRNA levels, an effect not observed with apoB regulators such as insulin and fatty acids. Indeed, prior studies have suggested that apoB is solely regulated posttranscriptionally. In contrast, we demonstrate here a strong transcriptional effect of PCSK9 on apoB.

Processes involved in posttranscriptional apoB stability and degradation were also enhanced by PCSK9. Because intracellular neutral lipids inhibit apoB protein degradation and enhance apoB protein stability, the increase in enterocyte neutral lipid content that we observed with PCSK9 treatment explains in part the PCSK9-mediated increase in cellular apoB. An increase in de novo cellular triglyceride biosynthesis by PCSK9 can account for this increase in enterocyte neutral lipids because PCSK9 stimulated the expression of target genes in the SREBP1 fatty acid/triglyceride biosynthesis pathway (FAS, SCD, and DGAT2). PCSK9, however, did not alter the expression of genes in the SREBP2 cholesterol biosynthesis/uptake pathway.

Although de novo cellular cholesterol biosynthesis mediated by SREBP2 was not enhanced by PCSK9, PCSK9 increased cellular levels of NPC1L1 (by 30%–40%), critical in mediating intestinal cholesterol transport, absorption, and incorporation into enterocytes. With respect to potential mechanisms through which PCSK9 might have increased NPC1L1 protein levels, NPC1L1 levels are regulated by the transcription factors SREBP2 and peroxisome proliferator-activated receptor-α. Peroxisome proliferator-activated receptor-α, in turn, is activated by FAS. Because PCSK9 treatment significantly increased FAS mRNA expression, peroxisome proliferator-activated receptor-α-mediated upregulation of NPC1L1 is the likely mechanism by which PCSK9 increased enterocyte NPC1L1 protein and specifically needs to be tested in future studies.

MTP, an endoplasmic reticulum apoB chaperone critical for the transfer of lipids to apoB, also contributed to the enhanced apoB stability induced by PCSK9. PCSK9 treatment significantly increased MTP mRNA, protein, and lipid transfer activity. The PCSK9-mediated 30% to 40% increase in MTP activity occurred rapidly, within 24 hours, for a protein with a half-life of 4 days. Such a rapid effect can be explained by a stimulatory effect on the de novo MTP protein synthesis.

In addition to the above LDLR-independent regulators of apoB production, the LDLR is well known to affect hepatic apoB stability. PCSK9 also influences intestinal apoB production by reducing enterocyte LDLR levels in vitro by 50%.

In vivo hPCSK9 overexpression reduced LDLR levels and increased MTP expression and activity in the small intestine, both known to affect TRL secretion rates. The increased MTP levels and activity persisted in the absence of LDLR in mice expressing hPCSK9. Surprisingly, deletion of PCSK9 in mice did not significantly affect either MTP expression or activity, suggesting a specific effect of hPCSK9 and demonstrating the human relevance of our findings. In fact, neither deletion nor transgenic expression of murine PCSK9 affects cholesterol levels in mice lacking LDLR, whereas transgenic expression of hPCSK9 increases serum TRL and TRL secretion in LDLR−/− mice. An important implication of these prior studies, combined with our in vivo results above, is that human and murine PCSK9 appears to behave differently in the absence of the LDLR.

Of note, acute siRNA-induced inhibition of PCSK9 gene expression markedly reduced (by 50%) intestinal TRL apoB protein and mRNA levels and downregulated the production of several enzymes involved in the enterocyte TRL synthesis and assembly process: MTP, NPC1L1, and cholesterol biosynthetic enzymes. The decline in the expression of multiple cholesterol biosynthetic enzymes in the cellular SREBP2 pathway that we observed was an unanticipated benefit of PCSK9 inhibition. PCSK9 knockdown also raised enterocyte LDLR levels by 50%, a significant albeit lesser effect than what we observed in hepatocytes. These results further highlight the key role of PCSK9 as an essential regulator of intestinal TRL production.

As a side effect, PCSK9 siRNA treatment of enterocytes also resulted in a very mild increase in cellular neutral lipid content. This is probably attributable to the counterregulatory
effect of PCSK9 siRNA in inducing a small increase in DGAT2 mRNA levels as a result of SREBP1 activation.

In the context of the rapid development and success to date of PCSK9 as a drug target, it is critical to unravel the pleiotropic effects of PCSK9 on lipoprotein metabolism. Our results suggest that in addition to its established role as a mediator of hepatic LDL metabolism, PCSK9 is an essential regulator of intestinal TRL metabolism. Thus, our findings prove that the targeted effects of PCSK9 in mediating cellular lipid and lipoprotein metabolism are broad and not limited to the LDL apoB-degrading action of PCSK9, as initially thought. Moreover, the results demonstrate the potential of PCSK9 as a highly attractive therapeutic drug target also for mitigating TRL production.

Although the present studies were focused on the intestine, the potential for PCSK9 TRL-based therapies that we showed here likely also extends beyond the intestine to hepatic TRL metabolism because parallel TRL pathways are present in hepatocytes. Consistent with this notion, we recently showed that hPCSK9 expression also increases hepatic secretion of TRL in an LDLR-independent fashion in mice. A further potentially important advantage of PCSK9-based TRL therapies, compared with the currently available MTP inhibitor therapies, which also specifically target the TRL apoB production of both chylomicron and VLDL triacylglycerol.

Sources of Funding

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Disclosures

None.

References


CT, Thornberry NA. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci USA.* 2005;102:8132–8137.


### CLINICAL PERSPECTIVE

Proprotein convertase subtilisin kexin type 9 (PCSK9) raises plasma low-density lipoprotein (LDL)-C levels by increasing the degradation of hepatic LDL receptors, thereby reducing plasma LDL clearance. PCSK9 loss-of-function mutations in humans lead to lifelong low LDL-C levels and low coronary heart disease rates. A key question is whether the lipid effect of PCSK9 is exclusively one of lipoprotein clearance caused by the interaction with the LDL receptor, or if an influence on lipoprotein assembly is also at play. In the current study, we show that PCSK9 has a central role in driving production of triglyceride-rich lipoproteins by intestinal cells, and have identified the cellular and molecular mechanisms involved. We found that PCSK9 stimulates intestinal triglyceride-rich lipoprotein synthesis by increasing the activity of microsomal triglyceride transfer protein, the transporter of the lipid droplet to apolipoprotein (apo)B. PCSK9 also increased intracellular levels of apoB, triglycerides, and cholesterol via mechanisms active at both transcriptional and post-transcriptional levels. These findings indicate that targeted PCSK9-based therapies may also be effective in the management of postprandial hypertriglyceridemia, thus contributing to additional coronary heart disease risk reduction. This adds significantly to the already high enthusiasm for interventions aimed at inhibiting PCSK9. aimed at inhibiting PCSK9.
Proprotein Convertase Subtilisin Kexin Type 9 Promotes Intestinal Overproduction of Triglyceride-Rich Apolipoprotein B Lipoproteins Through Both Low-Density Lipoprotein Receptor–Dependent and –Independent Mechanisms

Shirya Rashid, Hagai Tavori, Patrick E. Brown, MacRae F. Linton, Jane He, Ilaria Giunzioni and Sergio Fazio

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In the article by Rashid et al, “Proprotein convertase subtilisin kexin type 9 promotes intestinal over-production of triglyceride-rich apolipoprotein B lipoproteins through both low-density lipoprotein receptor-dependent and -independent mechanisms”, which appeared in the July 29, 2014 issue of the journal (Circulation. 2014;130:9 431–441. DOI: 10.1161/CIRCULATIONAHA.113.006720), the authors stated that only few investigators have characterized the functional importance of PCSK9 in the small intestine and its regulatory role on the metabolism of triglyceride-rich lipoproteins. A paper from Le May et al published in the journal Arteriosclerosis, Thrombosis, and Vascular Biology in 2009 showed that PCSK9-deficient mice display reduced postprandial hyper-triglyceridemia and apoB levels following olive oil gavage, and that adenoviral PCSK9 overexpression or targeted ShRNA silencing of PCSK9, respectively, led to increase and reduction of apoB secretion. The authors consider the paper by Le May et al central to their story, and its omission from the Discussion and reference list was unintentional.

The authors apologize for the oversight.

Reference:

SUPPLEMENTAL MATERIAL
Supplementary Figure 1.

A

B

Relative LDLR expression (normalized to β-actin)

mPCSK9 -/-
n=2

WT
n=5

hPCSK9 tg
n=6

*
Supplementary Figure 2.

A

B

Relative LDLR expression (normalized to GAPDH)

mPCSK9 -/-

WT

hPCSK9 tg

n=3

n=9

n=9
### Supplementary Table 1.

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Supplementary Legends.

Supplementary Figure 1. LDLR Levels in the Small Intestine of mPCSK9 −/−, WT and hPCSK9 tg Mice. (A) Immunoblot showing LDLR and β-actin in the small intestine. Lane 1: mPCSK9 −/−; lane 2: WT; lane 3: hPCSK9 tg. (B) Quantitative analysis of LDLR protein levels normalized to β-actin in the small intestine. (One-Way ANOVA: *p<0.05, *** p<0.001).

Supplementary Figure 2. LDLR Levels in the Large Intestine of mPCSK9 −/−, WT and hPCSK9 tg Mice. (A) Immunoblot showing LDLR and GAPDH in the large intestine. Lane 1: mPCSK9 −/−; lane 2: WT; lane 3: hPCSK9 tg. (B) Quantitative analysis of LDLR protein levels normalized to GAPDH in the large intestine.

Supplemental Table 1. Fold Change in Gene Expression Levels in PCSK9 Treated CaCo-2 Cells (10 µg/mL, 24 Hours) from Control Untreated Levels. All Data in the Current Table Show Data with Expression Levels which are Not Significantly Different from Control Levels.

Supplemental Table 2. Fold Change in Gene Expression Levels in siPCSK9 Transfected CaCo-2 Cells (48 Hours) Versus Cells Transfected with a Negative Control Vector. All Data in the Current Table Show Data with Expression Levels which are Not Significantly Different from Control Levels.