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PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells

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Objectives: The proprotein convertase subtillisin/kexin type 9 (PCSK9) regulates cholesterol metabolism via degradation of low-density lipoprotein receptor (LDLr). Although PCSK9 is abundantly expressed in the intestine, limited data are available on its functions. The present study aims at determining whether PCSK9 plays important roles in cholesterol homeostasis and lipid transport in the gut.

Methods and results: Caco-2/15 cells were used allowing the exploration of the PCSK9 secretory route through the apical and basolateral compartments corresponding to intestinal lumen and serosal circulation, respectively. The output of PCSK9 occurred through the basolateral membrane, a site characterized by the location of LDLr. Co-immunoprecipitation studies indicated an association between PCSK9 and LDLr. Addition of purified recombinant wild type and D374Y gain-of function PCSK9 proteins to the basolateral medium was followed by a decrease in LDLr concomitantly with the accumulation of both forms of PCSK9. Furthermore, the latter caused a significant enhancement in cholesterol uptake also evidenced by a raised protein expression of cholesterol transporters NPC1L1 and CD36 without changes in SR-BI, ABCA1, and ABCG5/G8. Moreover, exogenous PCSK9 altered the activity of HMG-CoA reductase and acylcoenzyme A: cholesterol acyltransferase, and was able to enhance chylomicron secretion by positively modulating lipids and apolipoprotein B-48 biogenesis. Importantly, PCSK9 silencing led to opposite findings, which validate our data on the role of PCSK9 in lipid transport and metabolism. Moreover, PCSK9-mediated changes persisted despite LDLr knockdown.

Conclusions: These findings indicate that, in addition to its effect on LDLr, PCSK9 modulates cholesterol transport and metabolism, as well as production of apo B-containing lipoproteins in intestinal cells.

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1. Introduction

PCSK9 is a member of the proteinase K subfamily of subtilisin-related serine endoproteases [1] and plays a role in the regulation of LDLr [2], thereby influencing LDL-cholesterol concentrations [3]. The physiological relevance of PCSK9 was revealed from the identification of point mutations in *PCSK9* that cause hyper- and hypocholesterolemia as a consequence of gain- and loss-of-function alleles, respectively [4]. The way PCSK9 works within the cell is via the intramolecular autocatalytic cleavage of its ∼75-kDa precursor in the endoplasmic reticulum, resulting in 14-kDa prodomain and ∼65-kDa catalytic fragments [1]. The cleaved prodomain

Abbreviations: ABC, ATP binding cassette transporter; ACAT, acylcoenzyme A: cholesterol acyltransferase; Ab, antibody; Apo, apolipoprotein; CD36, cluster determinant 36/fatty acid translocase; CE, cholesteryl ester; CM, chylomicrons; FA, fatty acid; FC, free cholesterol; HMG-CoA-R, 3-Hydroxy-3-Methylglutaryl-coenzyme A-reductase; LDL, low-density lipoprotein; LDLr, LDL receptor; NPC1L1, Niemann-Pick C1 Like 1; PCSK9, proprotein convertase subtillisin/kexin type 9; SR-B1, Scavenger Receptor class B type 1; SREBP1/2, sterol regulatory element binding transcription factor 1/2; TG, triglycerides; VLDL, very-low-density lipoprotein.

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remains non-covalently associated to the catalytic domain forming a complex that is transported to the Golgi apparatus and is subsequently secreted [1]. PCSK9 circulates in the blood and binds the extracellular domain of the LDLr to produce post-translational down-regulation. Interestingly, secreted PCSK9 promotes LDLr degradation independently of its proteolytic activity [5].

Much attention is being paid to the potential benefits of lowering intestinal lipid absorption in view of the impact on postprandial lipoprotein metabolism and atherosclerosis [6]. Despite valuable advances, additional studies are clearly warranted to understand the complex molecular mechanisms that orchestrate cholesterol homeostasis in the small intestine. A tremendous opportunity is offered by the recent discovery of PCSK9, which displays various functions in addition to hepatic LDLr disruption, including liver regeneration, neuronal differentiation/apoptosis and cortical neurogenesis [7]. Even though PCSK9 is expressed mainly in the liver and intestine [1], studies have mostly been restricted to the liver [7]. However, maintaining cholesterol homeostasis in the body requires accurate metabolic cross-talk between hepatic and intestinal processes to adequately cope with large fluctuations in dietary cholesterol intake [8], whereas imbalance may lead to elevated LDL-cholesterol levels and increased risks for cardiovascular disease [9]. Notably, the intestine plays a key role in cholesterol balance in animals and humans [10], constitutes the only site for absorption of dietary sterols, quantitatively represents the single most active location for cholesterogenesis [11], and remains the second important organ for the uptake and degradation of circulating LDL [12].

Previously, our findings showed the discrete regulation of PCSK9 in the enterocyte from stimuli originating from the luminal route [13]. Experiments have now been designed in the present study to explore whether PCSK9 actively participates in the tight control of intra-enterocyte cholesterol homeostasis. More precisely, we investigated the influence of purified PCSK9 on LDL protein status, cholesterol transporters, key enzymes for cholesterol synthesis and esterification, as well as on lipid synthesis, apolipoprotein (apo) biogenesis and lipoprotein assembly in intestinal Caco-2/15 cells. Validation of the data was obtained with *PCSK9* or *LDLr* knockdown with a lentiviral vector containing an appropriate shRNA.

2. Materials and methods

2.1. Production of human wild-type and D374Y-PCSK9

Culture and transfection of HEK293T and HepG2 cells with constructs expressing either empty vector (Control), human wild type PCSK9 (WT-PCSK9) or human D374Y-PCSK9, a gain-of-function mutant, were carried out as described previously [14,15] except that they were myc-tagged. After 72 h, conditioned media were harvested, chromatographed, concentrated and stored at −80 °C.

2.2. Cell culture and treatment

The human epithelial colorectal adenocarcinoma Caco-2/15 cell line, a stable clone of the parent Caco-2 cells (American Type Culture Collection, Rockville, MD), was obtained from Dr. JF Beaulieu (Department of Cellular Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada). Caco-2/15 cells were grown at 37 °C with 5% CO_2 in minimum essential medium (MEM, GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% MEM nonessential amino acids (GIBCO BRL) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Flow, McLean, VA) as described previously [16]. At 21-day post-confluence, they were treated for 24 h with conditioned media containing human myc-tagged wild type PCSK9 (WT-PCSK9) or gain-of-function mutant (D374Y-PCSK9) obtained following transfection of HEK293T cells [14,15].

2.3. Caco-2/15 cell integrity

Cell integrity was estimated by viability, morphology and differentiation assays. Briefly, after treatment with WT-PCSK9 or D374Y-PCSK9, cellular differentiation was assessed by determination of villin and sucrase protein expression. Furthermore, monolayer intactness and physical barrier function were tested by evaluating morphology and transepithelial electric resistance. Finally, cell viability was evaluated with 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromid (MTT, Sigma). At the end of cell incubation, the medium was aspirated and replaced with an MTT solution (0.5 mg/mL) and cells were incubated for 2 h at 37 °C with 5% CO_2 to allow MTT oxidation by the succinate dehydrogenase enzyme in viable cells. The MTT solution was then aspirated and 500 μl of dimethyl sulfoxide was added to each well to dissolve the resulting blue formazan crystals. The absorbance was measured at 535 nm with DMSO as a blank.

2.4. PCSK9 or LDL receptor silencing in Caco-2/15 cell line

Exponentially growing 293FT packaging cell lines were transiently transfected with either pRS vector harboring sh (short hairpin) RNA expression cassette against PCSK9 (TR302581) or pRS vector harboring shRNA expression cassette against LDLr (TR311766) (Origene Technologies, Inc., Rockville, MD) to generate replication-deficient lentivirus that were used to infect Caco-2/15 cells. Fourty-eight hour post-infection, cells were grown in Eagle's minimal essential medium (EMEM) containing 2 μg/ml puromycin (Sigma−Aldrich, USA) for an additional 7 days to establish stable shRNA expressing Caco-2/15 cells. Thereafter, cells were cultivated until 10 days post-confluence and used only after the validation of suppression of the gene of interest by Western blot assay.

2.5. Antibodies against PCSK9

The PCSK9 and myc polyclonal antibodies (Abs) were prepared by HyperOmics Farma Inc. (Montreal, Quebec, Canada) and (Santa Cruz Biotechnology, CA), respectively. The specificity of the Abs was evaluated by various methods, which exclusively recognized PCSK9 among various proteins: Western blotting following the incubation of the Abs in the presence or absence of specific antigens, the omission of the primary PCSK9 Abs in Western blot and the identification of the PCSK9 sequences after immunoprecipitation and SDS-PAGE.

2.6. Protein expression analysis

To assess the presence and regulation of PCSK9, Caco-2/15 cells were homogenized and adequately prepared for Western blotting as described previously [17]. Briefly, Proteins were determined by immunoblotting 20 μg of total proteins from lysates and then were denatured in sample buffer containing SDS and β-mercaptoethanol, separated on a 4−20% gradient SDS-PAGE, and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked with defatted milk proteins followed by the addition of primary Abs directed against 1:2000 anti-villin (BD Biosciences); 1/3000 anti-Niemann-Pick C1 Like 1 (NPC1L1, Novus); 1/50,000 anti-Scavenger Receptor class B type 1 (SR-BI, Novus); 1/10,000 anti-3-Hydroxy-3-Methylglutaryl-coenzyme A-reductase (HMG-CoA-R, Upstate); 1/10,000 anti-phosphorylated (P)-HMG

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