Review

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The unique role of proprotein convertase subtilisin/kexin 9 in cholesterol homeostasis

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The LDL receptor (LDLR) plays an essential role in the regulation of plasma (LDL) cholesterol concentrations by virtue of its ability to clear plasma LDL. Down-regulation of the LDLR by proprotein convertase subtilisin/kexin 9 (PCSK9) has recently emerged as a regulatory mechanism that controls plasma LDL cholesterol concentrations. Studies in which PCSK9 is over-expressed in mice, have demonstrated that PCSK9, by enhancing hepatic LDLR degradation, decreases the availability of the LDLR for LDL uptake, resulting in increased plasma LDL cholesterol levels. However, PCSK9 has also recently been shown to mediate down-regulation of surface receptors other than the LDLR, suggesting that it may have much broader roles than initially thought.

Keywords: clathrin-mediated endocytosis, LDL uptake, LDL receptor, PCSK9, plasma LDL cholesterol.

Abbreviations: LDL, low density lipoprotein; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin 9; SREBP, sterol regulatory element binding protein; VLDL, very LDL.

Introduction

The liver has a central role in lipoprotein metabolism, in terms of both synthesis and catabolism. The liver produces and secretes very LDL (VLDL), the triglyceride-rich lipoprotein that contains a single copy of apolipoprotein B100 (apoB-100) and varying amounts of apolipoprotein E (apoE) and/or apoC [1]. In peripheral tissues (e.g. adipose tissue and muscle), VLDL particles are converted to triglyceride-poor, apoE-rich VLDL remnants as a result of hydrolysis of triglycerides by the action of lipoprotein lipase. Upon entering the liver, VLDL remnants are rapidly cleared by hepatocytes and those particles that escape clearance by the liver undergo further hydrolysis to produce the cholesterol-rich LDL, with apoB-100 as the only protein constituent [2]. The liver also plays a key role in the catabolism of other lipoprotein classes. Chylomicron remnants, apoE-containing lipoprotein particles formed after partial hydrolysis of intestinally derived chylomicrons, are rapidly and almost exclusively taken up by hepatocytes [3], and HDL particles, deliver excess cholesterol from peripheral cells to the liver, in a process termed 'reverse cholesterol transport' [4]. The principal endocytic receptor that mediates the clearance of LDL is the LDL receptor (LDLR), the founding member of the LDLR family. In addition to the LDLR, the LDLR family contains at least six other receptors including the LDL receptor-related protein 1 (LRP1), the VLDL receptor (VLDLR), and apolipoprotein E receptor-2 (apoER2) [5]. The LDLR is expressed in most cell types including hepatocytes, lymphocytes and fibroblasts. The hepatic LDLR also serves as a clearance receptor for VLDL remnants and chylomicron remnants, but it shares these ligands with at least two other receptors, namely LRP1 and cell surface heparin sulphate proteoglycans, which are abundantly expressed in

hepatocytes [6–8]. In this review, we summarize early clinical studies and review and discuss studies on the structure, biosynthetic regulation, transcriptional control and functions of PCSK9.

Identification of PCSK9 as a novel gene for autosomal dominant hypercholesterolemia

Hypercholesterolemia results from elevated levels of plasma LDL cholesterol, which are determined by various genetic and environmental factors. Individuals with hypercholesterolemia are at increased risk of developing atherosclerosis. Individuals with mutations in the LDLR gene suffer from familial hypercholesterolemia (FH), the most common form of autosomal dominant hypercholesterolemia, which clinically manifests as markedly elevated levels of serum LDL cholesterol, xanthomas in tendons, and premature cardiovascular disease. Mutations in the gene encoding apoB-100 cause autosomal dominant familial defective apoB-100 due to the inability of apoB-100 mutants to interact with the LDLR [9, 10].

Proprotein convertase subtilisin/kexin 9 (PCSK9) was identified in a screen of genes that were up-regulated in apoptotic neural cells and named neural-apoptosis-regulated convertase 1 (NARC-1) [see 11, 12], and, in parallel, through its genetic linkage with autosomal dominant hypercholesterolemia [13]. Following reports that two missense PCSK9 mutations that result in amino acid substitutions (S127R and F216L) are associated with elevated levels of plasma LDL cholesterol, PCSK9 was identified as the third gene involved in autosomal dominant hypercholesterolemia [13]. The importance of PCSK9 in cholesterol homeostasis was further highlighted by identifying another missense PCSK9 variant (D374Y), which causes severe hypercholesterolemia [14, 15].

Mutations in the PCSK9 gene may also cause autosomal dominant hypocholesterolemia. In studies of American subjects, it was found that two nonsense mutations in the PCSK9 gene (Y142X and C697X) were significantly more common amongst individuals of African descent (combined prevalence 2%), whereas a missense mutation (R46L) was more common amongst individuals of European descent (prevalence 3.6%). It was further shown that individuals heterozygous for one of these mutations have significantly lower plasma levels of LDL cholesterol than normal and are protected from cardiovascular disease [16, 17], due to decreased circulating PCSK9 levels [18]. These data, which show that a modest, life-long reduction in plasma LDL cholesterol levels is associated with a dramatic reduction in the risk of cardiovascular disease, highlight the importance of lowering plasma LDL cholesterol levels from an early age. Studies in two African populations have also revealed a high prevalence of the C697X mutation in Nigerian and Zimbabwean subjects [16, 19]. Additional missense mutations that cause hypocholesterolemia (G106R, L253F, A443T) or hypercholesterolemia (R215H) have been found in other studies [20–22]. These clinical findings suggested that the PCSK9 missense mutations cause hypercholesterolemia through a ‘gain-of-function’ mechanism, whereas nonsense and missense variants of PCSK9 associated with hypocholesterolemia represent ‘loss-of-function’ mutations.

How is PCSK9 involved in cholesterol homeostasis?

In vivo evidence that PCSK9 regulates plasma cholesterol levels has come from studies using different approaches to over-express or knock down PCSK9. Adenoviral-directed over-expression of PCSK9 in mice was found to be associated with a dramatic decrease in hepatic LDLR levels and increased plasma levels of LDL cholesterol [23–25], without any apparent effect on LDLR mRNA levels [24, 25]. The latter finding suggested that the ability of PCSK9 to decrease the hepatic LDLR occurs via a posttranslational process. Similar results have been observed in transgenic mice over-expressing PCSK9 in liver [24, 25]. Additionally, using parabiosis, which involves the connection of the bloodstream of a transgenic mouse with that of a normal mouse thereby establishing a common circulatory system, it was shown that PCSK9 produced in transgenic mice can down-regulate the LDLR protein in the livers of normal mice [26]. Conversely, PCSK9 knockout mice were found to have a marked up-regulation of hepatic LDLR levels and a significant reduction in plasma LDL

cholesterol levels [27, 28], due to increased LDLR-mediated clearance of plasma LDL by the liver [27]. Finally, knocking down hepatic PCSK9 expression with small interfering RNAs significantly increased hepatic LDLR levels and reduced plasma LDL cholesterol levels in mice, rats, and cynomolgus monkeys [29]. Similar results have been obtained in mice treated with an antisense oligonucleotide directed against PCSK9 mRNA [30]. Taken together, these results suggest that the involvement of PCSK9 in cholesterol homeostasis reflects its influence on hepatic LDLR levels.

In accordance with this notion is the observation that plasma cholesterol profiles in compound LDLR/PCSK9-deficient mice are similar to those in LDLR-deficient mice, indicating that PCSK9 deficiency causes no additional effects on total and LDL cholesterol levels beyond the increases found in LDLR-deficient mice. This finding strongly suggests that PCSK9 impacts plasma cholesterol levels exclusively via its effect on the LDLR [28].

Consistent with the notion that PCSK9 is able to down-regulate the LDLR, transient transfection of several cell lines, including hepatoma cell lines such as HepG2 and HuH7 cells and the human embryo kidney cell line HEK293, showed that overexpression of either wild-type or gain-of-function mutants of PCSK9 results in decreased cellular LDLR levels [24, 25, 31, 32]. By contrast, knockdown of PCSK9 expression with small interfering RNAs enhances the ability of cells to bind and take up LDL [23, 31], most likely due to a reduced PCSK9-mediated degradation of the LDLR. Together, these results provided clear *in vitro* evidence that the LDLR is the direct target of PCSK9 action.

Regulation of the biosynthesis and secretion of PCSK9

Proprotein convertase subtilisin/kexin 9 is a serine protease belonging to the proprotein convertase family of serine proteases characterized by a three-domain structure and a catalytic triad (consisting of Asp, His, and Ser residues) involved with substrate binding and catalysis. This family of processing proteases comprises nine known members in mammals, of which two representatives are furin and PC5/6. Proteolytic processing mediated by these enzymes provides a posttranslational means of regulating the function of many soluble and membrane proteins including a wide variety of proteins that are synthesized as proproteins [33].

Proprotein convertase subtilisin/kexin 9 is synthesized in the endoplasmic reticulum (ER) as a precursor consisting of 692 amino acids including a signal peptide (residues 1–30), which is cleaved during the translocation process in the ER, an N-terminal pro-domain (residues 31–152) followed by a catalytic domain (residues 153–449) and a cysteine and histidine rich C-terminal domain (450–692). The pro-domain, which serves as an intramolecular chaperone to ensure the proper folding of the catalytic domain in the ER, is cleaved by auto-processing [12, 34, 35]. The autocleavage event is thought to occur co-translationally [36], probably as soon as the catalytic domain of PCSK9 reaches the active state in the ER. Typically, pro-domains of proprotein convertases remain attached to their catalytic domains following auto-processing in order to prevent premature enzymatic activity. When the mature/pro-domain complexes traffic to the Golgi apparatus, cleavage at a second site within these pro-domains generates active proteases by dissociating pro-domains from the catalytic domains. However, PCSK9 lacks such a second cleavage site and the cleaved pro-domain remains associated with the catalytic domain through noncovalent interactions, preventing potential substrates from accessing the active site of PCSK9. As a result, PCSK9 is secreted from cells as a ~75-kDa pro-domain-containing, enzymatically inactive form [12, 35, 37].

Tissue expression of PCSK9 mRNA

Quantitative reverse transcriptase polymerase chain reaction analysis of several tissues from rat and mouse have shown significant PCSK9 mRNA expression in the liver and lower expression in other organs such as intestine, kidney, lung, spleen, testis and thymus [12, 28]. Determination of the contribution of the

liver to plasma PCSK9 levels in PCSK9 liver-specific knockout mice demonstrated that these mice have virtually undetectable circulating PCSK9, as is the case for knockout mice totally lacking PCSK9. This indicates that most of PCSK9 in plasma is hepatic of origin [28].

Transcriptional regulation of PCSK9

In mice fed cholesterol-enriched diet and in transgenic mice over-expressing nuclear forms of human sterol regulatory element binding protein-1a (SREBP-1a) or SREBP-2, hepatic PCSK9 was shown to be coordinately regulated with genes encoding proteins involved in cholesterol homeostasis [38, 39]. SREBPs are transcriptional factors that are synthesized as membrane-associated precursors. There are three SREBPs: SREBP-1a, SREBP-1c, and SREBP-2. Like SREBP-1a and SREBP-1c, SREBP-2 is synthesized as a precursor that becomes part of a ternary complex containing SREBP cleavage-activating protein (SCAP) and the ER-retention protein insulin-inducible gene (Insig). In the presence of high levels of sterols (largely derived from LDL-associated cholesterol) this complex is retained in the ER and maintains an inactive pool of SREBP-2. However, when cellular sterol levels are low, the SCAP–SREBP-2 complex is transported to the Golgi apparatus, where SREBP-2 undergoes proteolytic processing to produce the active SREBP-2 which is released into the cytosol. Following this activation, SREBP-2 migrates to the nucleus, where it mediates transcriptional activation of genes involved in cholesterol metabolism including 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, the enzyme catalysing the first step in conversion of acetyl-CoA to cholesterol, HMG CoA reductase, the rate-limiting enzyme that catalyses the conversion of HMG CoA to mevalonate, and the LDLR [40–42].

Like many other sterol-responsive genes, the promoter region of PCSK9 contains a sterol regulatory element (SRE), the primary binding sequence for SREBP-2, [43, 44], which together with its adjacent upstream nucleotide sequence govern the sterol-dependent regulation of PCSK9 transcription [44]. PCSK9 is highly responsive to changes in cellular sterol status. Treatment of primary human hepatocytes and HepG2 cells with statins, cholesterol-lowering drugs that inhibit HMG CoA reductase, up-regulates PCSK9 and LDLR mRNA levels [43]. Statin treatment also increases circulating levels of PCSK9 in humans [45, 46]. Depletion of sterols by incubating HepG2 cells in lipoprotein deficient medium results in increased mRNA and protein levels of both PCSK9 and LDLR either with or without additional statin treatment, whereas incubation of cells in the presence of suppressive sterols (25-hydroxycholesterol) down-regulates PCSK9 and LDLR mRNA and protein levels. Moreover, these changes correlate with the abundance of nuclear form of SREBP-2 [44], indicating that cellular sterol status is an important regulator of PCSK9 gene expression and that the nuclear form of SREBP-2 accounts for the increase in PCSK9 mRNA expression in HepG2 cells.

These findings are consistent with animal studies, which have shown that re-feeding with a cholesterol-rich diet exerts a suppressive effect on the expression of PCSK9 and nuclear SREBP-2 in livers of fasted mice, whereas LDLR protein and mRNA are maintained, indicating that the primary mode of sterol-dependent regulation of PCSK9 *in vivo* is via the SREBP-2 pathway [44].

A recent *in vitro* study revealed that Caco-2/15 cells, a human intestinal epithelial cell line, respond similarly to sterol addition and removal in terms of repression and induction of PCSK9, LDLR and nuclear SREBP-2 [47], indicating that the regulation of PCSK9 gene expression in nonhepatic cells also occurs via the SREBP-2-mediated pathway.

Nutritional factors have been shown to regulate expression of PCSK9 *in vivo*. Hepatic PCSK9 mRNA and protein levels in mice are significantly decreased by fasting and return to normal levels after re-feeding with a carbohydrate-rich diet [44, 48]. LDLR mRNA and protein levels remain unchanged during fasting, whereas they are slightly reduced in carbohydrate-re-fed mice [44], suggesting that the expression of PCSK9, but not LDLR, is responsive to changes in carbohydrate intake. This is consistent with the

observation that hepatic PCSK9 expression levels in mice and in rodent primary hepatocytes increase in response to treatment with insulin [48] and there are data suggesting that SREBP-1c is involved in mediating insulin's actions in the liver. These include the presence of a response element for SREBP-1c in the PCSK9 promoter [48] and increased nuclear translocation of SREBP-1c in response to re-feeding [44].

Proprotein convertase subtilisin/kexin 9 and LDLR have also been shown to exhibit divergent regulation in response to glucagon [49], diabetes [50], bile acids [51], lipid-lowering drugs such as fenofibrate [52, 53], and berberine [54] and inflammation [55].

Posttranscriptional regulation of PCSK9

During its transport along the biosynthetic pathway, PCSK9 is modified by several posttranslational events: N-linked glycosylation at Asn533, sulfation at Tyr38, and phosphorylation at Ser47 and Ser688 [31, 56, 57]. These posttranslational modifications might be regulated in a cell type- and tissue type-dependent manner. Whether these modifications are associated with specific functions is not yet known, although the phosphorylation of Ser47 appears to reduce the susceptibility of the pro-domain to proteolysis [57]. PCSK9 activity may also be regulated via proteolytic inactivation in the Golgi apparatus. The catalytic domain of PCSK9 comprises a recognition motif for cleavage by furin/PC5A, $RFHR^{218}$ ↓QA, which generates a soluble protein (~53 kDa) [56]. This PCSK9 form does not possess functionality and allows for a subsequent cleavage at the plasma membrane by ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motif) in a PC5A-dependent manner to produce a ~34 kDa fragment [58]. Both of these inactive forms are detected in human and mouse plasma [56, 58], suggesting that this regulated processing might be involved in determining the amount of active circulating PCSK9.

Pathways involved in PCSK9-mediated degradation of the LDLR

Early over-expression studies implicated both the secretory and endocytic pathways in PCSK9-mediated degradation of LDLR [25, 31, 32, 59]. Subsequent studies; however, demonstrated that extracellularly provided PCSK9 is sufficient to down-regulate the cellular LDLR. Treatment of HepG2 cells with conditioned media from PCSK9-transfected cells was shown to significantly reduce cellular LDLR levels and reduce the ability of cells to take up LDL [60]. Similar results have subsequently been obtained by others [61, 62]. In accordance with these findings, treatment of HepG2 cells with purified PCSK9 was shown to dose-dependently and significantly decrease cellular LDLR levels [26].

Several lines of evidence have suggested that PCSK9-mediated LDLR degradation is dependent on the endocytosis of PCSK9. First, knockdown of clathrin heavy chain by RNA interference markedly reduced LDLR degradation in cells over-expressing PCSK9, suggesting that PCSK9-mediated degradation of LDLR requires clathrin-mediated endocytosis [63]. The adaptor protein autosomal recessive hypercholesterolemia (ARH) is necessary for clathrin-mediated endocytosis of the LDLR in hepatocytes and lymphocytes but not in other cells such as fibroblasts [64, 65]. Several studies have addressed the role of ARH in the PCSK9-mediated degradation of the LDLR and contradictory results have been obtained. A study with ARH-deficient mice that over-expressed PCSK9 suggested that ARH is not required for PCSK9-mediated degradation of the LDLR [25]. However, data from two subsequent *in vitro* studies strongly suggested that LDLR-mediated endocytosis of PCSK9 via an ARH-dependent route is a prerequisite for subsequent LDLR degradation [26, 62], although the involvement of an additional, but minor, clathrin-independent pathway in the internalization of PCSK9 has also been suggested [66, 67].

Secondly, exogenously added PCSK9 has been shown to co-localize with markers of late endocytic compartments [26, 56] and PCSK9-induced loss of the LDLR appears to occur via the lysosomal pathway, as suggested by the inhibitory effects of acidotropic agents such as NH_4Cl and chloroquine [26, 31, 32, 63, 67], but not by inhibitors of lysosomal cysteine and aspartyl proteases [32]. Thirdly, biochemical analysis of PCSK9 degradation have shown that radiolabeled

PCSK9 is internalized and degraded with kinetics typical of receptor-mediated endocytosis [68, 69].

Fourth, analysis of the LDLR structural requirements for PCSK9 binding has demonstrated that the interaction between the LDLR and PCSK9 is mediated by the epidermal growth factor (EGF)-like repeat A (EGF-A) of the LDLR [70], which interacts with the catalytic domain of PCSK9 [71]. Synthetic or recombinant LDLR-derived peptides representing the EGF-A alone or the tandem EGF-like repeats A and B (EGF-AB) have been shown to interact with PCSK9 and to be effective in reducing the ability of PCSK9 (secreted or exogenously added) to degrade LDLR or to lower LDL uptake in HepG2 cells [62, 68, 72, 73]. However, the most compelling evidence comes from the observations that the EGF-AB peptide containing the FH-associated H306Y mutation can not only block the effects of secreted PCSK9 on LDLR levels but also completely block the endocytosis of exogenously added PCSK9 [68], clearly indicating that PCSK9 is endocytosed in a receptor-dependent manner. The H306Y mutation in which the mutant residue is located in the EGF-A domain exhibits increased binding affinity for PCSK9 due to the formation of a hydrogen bond with Asp374 of PCSK9 at neutral pH, explaining why this mutation is able to cause FH [68, 71]. Finally, antibody neutralization of PCSK9 has been shown to abolish PCSK9-induced LDLR degradation and restore effective LDL uptake [74], and a polyclonal antibody raised against LDLR is capable of blocking the PCSK9–LDLR interaction [73].

The mechanism of action of PCSK9 to induce LDLR degradation

Another issue concerning the effect of PCSK9 has been whether PCSK9 itself or an enzyme closely regulated by PCSK9 is responsible for the degradation of the LDLR [25, 31, 32, 62]. However, recent findings have shown that catalytically inactive PCSK9 can promote degradation of the LDLR as efficiently as the wild-type protein (in cells or mice) [75–78], indicating that the catalytic activity is not required and that it is a structural action of PCSK9 that mediates its effect.

Thus, despite some initial controversy, it is now believed that PCSK9–LDLR complexes formed at the plasma membrane enter the cell through a clathrin/ARH-mediated endocytosis and that this process is a prerequisite for subsequent receptor degradation [26, 63, 68] (Fig. 1). Moreover, the function of

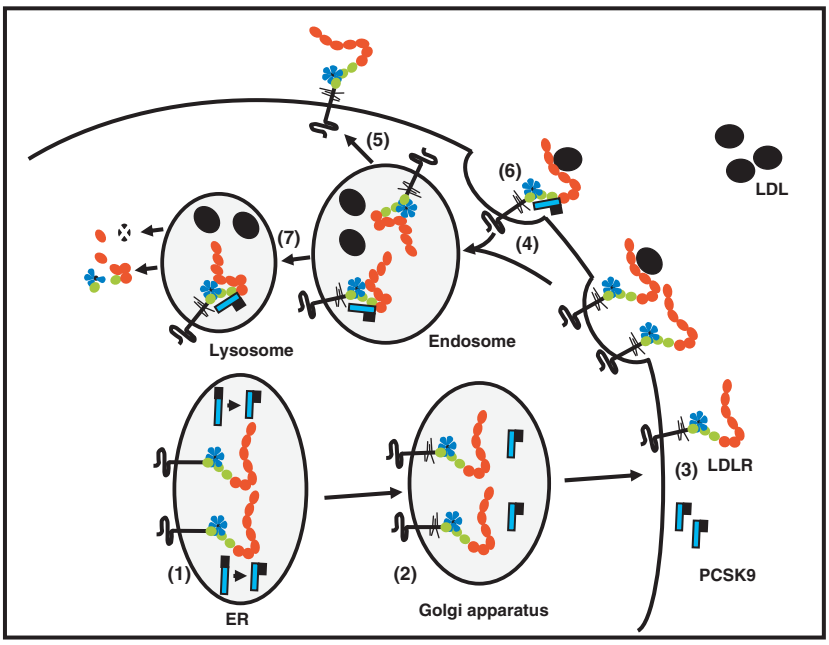

Fig. 1 Model of LDLR down-regulation by PCSK9: the LDLR is processed by the biosynthetic/secretory pathway (1,2) and delivered to the plasma membrane (3), where it undergoes ARH-dependent, clathrin-mediated endocytosis (4) whether or not bound to LDL. In the absence of PCSK9, the LDLR is recycled, not degraded (5). PCSK9 is made by hepatocytes and secreted into the circulation, where it interacts with the cell surface LDLR (6). The LDLR is internalized in the presence of PCSK9 by the same route (4), but PCSK9 prevents LDLR recycling from endosomes leading to lysosomal degradation of the LDLR (7).

PCSK9 does not involve enzymatic activity. However, the signal that identifies PCSK9-bound LDLR for postendocytic degradation still has to be elucidated.

Role of hepatic LDLR in PCSK9 catabolism

The aforementioned *in vivo* studies suggest that the hepatic uptake of PCSK9 may be mediated mainly by the LDLR. As is often the case; however, the situation is not so simple.

The liver is very efficient in taking up PCSK9 from the circulation; by 15 min postinjection, up to 90% of the intravenously injected radio-iodinated PCSK9 in mice is recovered in the liver, indicating a circulating half-life of PCSK9 of ~5 min. The experiments using LDLR-deficient mice revealed, however, that PCSK9 is still cleared efficiently with a half-life of 15 min and that the liver accounts for ~60% of the total clearance of PCSK9 [75].

These data suggest that clearance of PCSK9 from the circulation is largely mediated by the liver and that most of PCSK9 taken up by the liver is removed through LDLR-mediated endocytosis leading to down-regulation of the hepatic LDLR. However, there is considerable residual uptake of PCSK9 in the absence of LDLR, suggesting the involvement of LDLR-independent mechanisms in the clearance of circulating PCSK9 by the liver. Currently, it is unclear what accounts for this residual uptake. The LDLR-independent hepatic clearance of circulating PCSK9 is too rapid to be explained by fluid-phase endocytosis, suggesting that additional hepatic receptor(s) also function in the clearance of circulating PCSK9. It is also possible that PCSK9 in part may associate with another plasma protein and be taken up as a complex by a scavenger receptor that does not interact directly with PCSK9. Clearly, further work is needed to understand the catabolism of PCSK9 by the liver.

Effect of PCSK9 on LDLR levels in extraheaptic tissues

As the LDLR is expressed in most cell types the question arises as to whether circulating PCSK9 also affects LDLR levels in other tissues. In a recent study, it was shown that intravenous administration of PCSK9 (30 μg) to mice, in addition to inducing a marked reduction in hepatic LDLR levels, PCSK9 also reduced LDLR levels in adipose tissue, lung and kidney, though less so than in the liver [78], suggesting that circulating PCSK9 may also have effects on the LDLR in tissues other than liver.

The LDLR is highly enriched in the adrenal glands [79]. However, LDLR levels in the adrenal glands appear to be unaffected by continuous infusion (6 h) of PCSK9 (32 $\mu g\ h^{-1}$) into mice [75]. This is consistent with the observation that transgenic over-expression of PCSK9 in kidneys of mice has no effect on adrenal LDLR levels, despite marked decrease in hepatic LDLR levels [80]. On the other hand, PCSK9 knockout mice have adrenal LDLR levels 1.4-fold greater than wild-type mice, but the increase is less compared with LDLR levels in the liver (2.8-fold increase) [75]. The adrenal glands do not express PCSK9 [75], so any effects of PCSK9 on the adrenal LDLR would be expected to be due to circulating PCSK9. It seems, therefore, paradoxical that high levels of circulating PCSK9 do not affect LDLR levels in the adrenals but their LDLR levels are increased in the absence of PCSK9.

No data regarding the down-regulation of the intestinal LDLR by PCSK9 are currently available. A recent study has, however, reported that PCSK9 deficiency in mice increased LDLR protein levels in both the liver and small intestine [81].

Given the differential effects of PCSK9 on LDLR levels in different tissues, it is possible that postsecretory mechanisms regulate the activity of circulating PCSK9. The recent finding that PCSK9 binds to cell surface Annexin-2A may point to Annexin-2A as an inhibitor of PCSK9 activity [82]. Notably, the expression level of cell surface Annexin-A2 in the liver is low, as compared to other tissues. It is therefore possible that cell surface Annexin-A2 may limit PCSK9 action in extra-hepatic tissues, for example by sequestering PCSK9 from LDLR binding.

Role of PCSK9 in down-regulation of other receptors

Apart from its role in the down-regulation of LDLR, PCSK9 has been recently implicated in the down-regulation of other members of the LDLR family. It has been shown that incubation of cells expressing either VLDLR or apoER2 with conditioned media containing PCSK9 decreases cellular levels of these receptors in a Dab1-dependent manner [83]. In agreement with these observations, data from an *in vitro* alphascreen receptor binding assay have indicated specific interactions between PCSK9 and VLDLR and apoER2 [73]. These findings contrast with a previous study using a cell surface binding assay, in which no specific interaction between PCSK9 and the VLDLR was observed [70]. However, LRP1, another member of the LDLR family, has also been shown to bind PCSK9, albeit with low affinity [26]. However, experiments with HepG2 cells over-expressing the S127R variant of PCSK9 suggested that PCSK9 may lack the ability to regulate LRP1 levels [31]. Finally, a recent study using HuH7 cells demonstrated that PCSK9 is able to mediate down-regulation of CD81, a cell surface receptor that has been shown to be required for the infection of cells by hepatitis C virus. PCSK9 prevents infection of HuH7 cells by hepatitis C virus by inducing a strong down-regulation of CD81. CD81 down-regulation by PCSK9 occurs whether or not LDLR is present, suggesting that this function of PCSK9 is independent of its ability to down-regulate LDLR. Furthermore and in agreement with this conclusion, PCSK9 knockout and double PCSK9/LDLR knockout mice display elevated CD81 protein expression in the liver [84].

Physiological functions of PCSK9

The best understood function of PCSK9 is the down-regulation of the hepatic LDLR. However, in view of recent studies implicating PCSK9 in the down-regulation of receptors other than LDLR, PCSK9 should perhaps be viewed as a multifunctional secreted protein. The physiological importance of PCSK9-mediated down-regulation of the hepatic LDLR remains unclear, but might represent a mechanism to prevent re-uptake of newly secreted VLDL by hepatocytes leading to a shunt of VLDL particles towards peripheral tissues [75]. The significance of PCSK9 up-regulation in apoptotic nerve cells, in which it was discovered, is unknown.

However, several observations suggest that the functional absence of PCSK9 is of no significant consequence. PCSK9-deficient mice have no apparent abnormal phenotype other than decreased plasma cholesterol levels [27]. PCSK9-deficient mice do not exhibit gross defects in the central nervous system [83], despite the fact that PCSK9 expression is developmentally regulated [12] and is expressed in the brain early postnatally [83]. Thus, PCSK9 does not appear essential for normal mouse development or functional redundancy can compensate for the loss of PCSK9 in brain. Moreover, database searches have indicated that bovine may lack functional PCSK9 [21]. Finally, three adult individuals with loss-of-function mutations in both PCSK9 alleles have been identified. One mutation, an in-frame amino acid deletion (ΔR97), which prevents autocatalytic cleavage and secretion of PCSK9, has been identified in compound heterozygosity with the Y142X mutation. The subject, an African-American woman, has a total lack of circulating PCSK9 and is reported to be healthy [18]. An ostensibly healthy Zimbabwean woman homozygote for the C679X mutation has been shown to have a very low LDL cholesterol concentration [19]. The third individual (reported as African-American) is compound heterozygous for Y142X and C697X mutations [85]. The latter study has shown that low LDL cholesterol concentrations associated with loss-of-function mutations in PCSK9 do not increase the individual susceptibility to develop cancer [85].

Does PCSK9 play a role in VLDL secretion?

There has been some controversy as to whether PCSK9 may play a role in VLDL secretion. In a clinical study, it has been found that the hepatic secretion of apoB-100 is increased in patients with the S127R gain-of-function mutation of PCSK9 under fasting conditions, suggesting that the S127R mutation induces hypercholesterolemia by increasing hepatic VLDL production [86]. In line with these observations,

LDLR-deficient mice over-expressing wild-type human PCSK9 have been shown to have markedly elevated plasma levels of both VLDL and LDL [31]. Increased secretion of apoB-100 has also been observed in cultured rat hepatoma cells transfected with gene constructs containing PCSK9 gain-of-function variants [59]. These observations led to the suggestion that PCSK9 may play a role in the regulation of the hepatic production and secretion of VLDL [59, 86]. However, results from subsequent studies have indicated that PCSK9 does not play a direct role in the regulation of apoB-100 production and VLDL secretion. Studies have shown that the hepatic triglyceride content is increased in conditions that cause reduced hepatic VLDL secretion. Thus, one would expect that the hepatic triglyceride content would be increased in individuals with loss-of-function PCSK9 mutations who have low LDL cholesterol levels. However, this has not been demonstrated [22]. This observation is in accordance with findings of studies showing that PCSK9 over-expression or deletion in primary mouse hepatocytes has little impact on the rate of apoB-100 (and apoB-48) secretion [25, 27]. Moreover, plasma VLDL cholesterol levels are not increased in mice that over-express PCSK9 in a LDLR knockout background [25], indicating that in the absence of any interactions between PCSK9 and the LDLR, PCSK9 does not influence VLDL secretion. This is consistent with the observation that compound LDLR/PCSK9 deficiency does not further increase total cholesterol levels above those observed in LDLR-deficient mice [28]. On the basis of these observations, PCSK9 may not play a direct role in the secretion of apoB-100 by the liver.

PCSK9 as a therapeutic target in hypercholesterolemia

Current therapies with statins result in limited responses in terms of the reduction in plasma LDL cholesterol levels (40–50%) and reduction in risk for cardiovascular disease [87]. As already stated above, statins induce, via the SREBP-2-mediated pathway, simultaneous up-regulation of both LDLR and PCSK9. The reason why statin therapy does not lead to a more favourable outcome might therefore be that the increased number of LDLR is attenuated by an increased synthesis and secretion of PCSK9, thereby reducing the overall net reduction in plasma LDL cholesterol levels. The observation that several adult individuals lacking PCSK9 are healthy suggests that PCSK9 might be a novel therapeutic target for the treatment of hypercholesterolemia and several strategies to block the synthesis and activity of PCSK9 have been developed. These include antisense oligonucleotides, RNA interference and neutralizing antibodies. Information obtained from PCSK9 knockdown animals has demonstrated that inhibition of PCSK9 expression is an effective strategy to decrease plasma LDL cholesterol levels [29, 30]. Recently, a human monoclonal antibody against human PCSK9, mAb1, has been developed and shown to bind to PCSK9 and prevent its interaction with the LDLR. Significant reductions in total plasma cholesterol levels in mice and plasma LDL cholesterol levels in non-human primates were observed. Also, combination of mAb1 with a statin had an additive effect on LDLR up-regulation in HepG2 cells compared with statin treatment alone, suggesting that inhibition of PCSK9 activity can enhance therapeutic efficacy of statins [88]. These findings have raised the possibility that knockdown of PCSK9 or inhibition of its interaction with the cell surface LDLR could be used as a monotherapy for treatment of severe hypercholesterolemia in FH patients who do not respond to stains or in combination with statins in patients who cannot be treated adequately with statins alone. However, these agents have to be administered by injection which may limit their therapeutic usefulness. Clinical trials are needed to asses the utility of targeting PCSK9 in humans.

Other options for blocking interaction between PCSK9 and LDLR include soluble peptide fragments of the LDLR (e.g. the EGF-AB domain containing the H306Y mutation) and small molecule PCSK9 inhibitors. Small molecules have not been pursued as possible PCSK9 inhibitors, probably because PCSK9–LDLR interaction, like most protein–protein interactions, involves relatively large protein surface. Targeting circulating PCSK9 by small molecule inhibitors is unlikely to represent an option because the

mechanism of action of PCSK9 in reducing cellular LDLR does not involve proteolytic activity. However, small cell-permeable molecules targeting the catalytic site of PCSK9 pro-enzyme could theoretically inhibit the auto-processing of PCSK9, thereby promoting its degradation in the ER. However, cross-reactivity associated with such inhibitors raises concern that PCSK9 pro-enzyme inhibition could co-inhibit other proprotein convertases.

Conclusion

Evidence from human mutations in the PCSK9 gene has highlighted the importance of PCSK9 in cholesterol homeostasis. Experiments, in which PCSK9 either is knocked out or over-expressed in mice, have confirmed a key role of PCSK9 in regulating plasma LDL cholesterol concentrations. Several lines of evidence suggest that PCSK9 acts via a nonproteolytic mechanism to enhance degradation of the hepatic LDLR through a clathrin/ARH-mediated pathway and that this is the main mechanism whereby PCSK9 brings about its effect on plasma LDL cholesterol concentrations. Subsequent studies have also shown that PCSK9 is involved in regulating the levels of several other cell surface receptors, implying a broad physiological role for PCSK9.

Proprotein convertase subtilisin/kexin 9, like the LDLR, is a SREBP-2 target gene that is regulated by the feedback mechanism that maintains homeostatic control of cellular cholesterol levels. The role of PCSK9 in cholesterol homeostasis is unique as it places an additional control on cholesterol homeostasis at the level of posttranslational regulation of the LDLR.

Despite advances in the understanding of PCSK9's role in cholesterol homeostasis, several questions about its established and putative functions remain. For instance, although PCSK9 seems to be developmentally regulated, it is unclear why it is dispensable for the normal development of mammalian organisms. Another question is how exactly does the binding of PCSK9 to LDLR impact LDLR membrane trafficking? Characterizing the molecular mechanisms of PCSK9-mediated down-regulation of the LDLR in hepatocytes will be one of the most important challenges within the field of PCSK9 biology. Given that there is a substantial LDLR-independent uptake of PCSK9 in the liver, the existence of another receptor (or receptors) for PCSK9 cannot be excluded. The importance of alternative receptors in mediating the uptake of PCSK9 must be addressed in future studies.

Note added in proof

Seidah and colleagues (Poirier, S., *et al.* Dissection of the endogenous cellular pathways of PCSK9-induced LDLR degradation: evidence for an intracellular route. J. Biol. Chem. (2009, July 27, Epub ahead of print)) recently reported a set of experiments that led them to propose that some of the effects of PCSK9 on the LDLR are driven from its ability to act in the intracellular space of the cells in which it is synthesized (HepG2 cell line, endogenously expressing PCSK9). Silencing expression of ARH, which mediates LDLR internalization via the clathrin pathway, or blocking dynamin function, which is necessary for multiple endocytic pathways including clathrin-mediated endocytosis, had no or little effect on total LDLR levels in these cells. Notably, silencing expression of both clathrin light chain isoforms, which has been shown to block trans-Golgi-to-late endosome/lysosomal trafficking but not clathrin-mediated endocytosis, significantly increased LDLR protein levels in HepG2 cells, but not in a PCSK9 negative cell line (HEK293 cells). These results suggest that PCSK9 may be able to down-regulate the LDLR by more than one pathway.

Conflict of interest statement

No conflict of interest was declared.

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