The biology of PCSK9 inhibition: some unanswered questions

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Cell biology of PCSK9

The serine protease PCSK9 is a secreted inhibitor of the LDL receptor (LDLR), mainly expressed by the liver. The PCSK9 precursor undergoes intra-molecular autocatalytic processing in the endoplasmic reticulum (ER). The resulting heterodimer is then routed towards the secretory pathway. Following secretion, PCSK9 binds to the EGF-A domain of the LDLR at the cell surface and is internalized together with the receptor by endocytosis. The affinity between LDLR and PCSK9 increases as a result of acidic conditions in endosomes.[1]

The interaction between PCSK9 and the receptor locks the LDLR in an open conformation, which precludes normal recycling of the LDLR to the plasma membrane and targets it for endo-lysosomal degradation[2] (*Figure 1*). GRP94 an ER-resident protein expressed in hepatocytes binds to PCSK9 and thereby prevents an early degradation of the LDLR within the secretory pathway.[3] Thus, PCSK9 is major circulating inhibitor of LDLR expression and function. As such, it has become a prime therapeutic target for lowering LDL cholesterol (LDL-C).[4]

Its unique mode of action has prompted the development of circulating PCSK9 inhibitors such as the two fully human anti-PCSK9 monoclonal antibodies Praluent® and Repatha®, both recently approved for clinical use to treat severe or statin-resistant hypercholesterolaemia.

Heterogeneity of circulating PCSK9: does it matter?

A significant proportion of PCSK9 circulating in the plasma is bound to apoB-containing lipoproteins.[5–8] Up to 40% of total PCSK9 in human plasma can be found associated with LDL, with a K_d of 160–320 nM.[5] The interaction of PCSK9 to LDL is a common event for PCSK9, however, the stoichiometry of the interaction suggests that it is a rare event for LDL, with only one in 500–1000 LDL particles carrying one PCSK9 molecule.[9]

Interestingly, although PCSK9 can bind apoB within hepatocytes,[10] it is not found associated with very low-density lipoproteins (VLDLs),[11] suggesting that the association between PCSK9 and

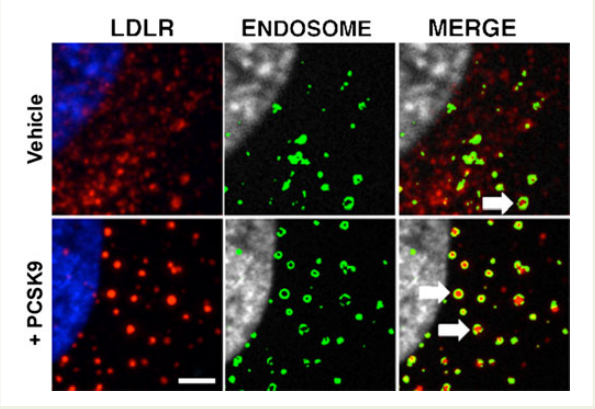

Figure 1 PCSK9 targets the low-density lipoprotein receptor toward endo-lysosomal degradation. Human dermal fibroblasts were grown in serum depleted culture conditions in the presence of 10 μg/mL mevastatin to maximally upregulate low-density lipoprotein receptor expression. Recombinant PCSK9 (300 ng/mL) or vehicle control was added to the culture medium for 10 min. Fibroblasts were then fixed, permeabilized, and visualized by confocal microscopy using fluorescent antibodies for the low-density lipoprotein receptor (clone C7 at 4 μg/mL [in red]), and Rab5 (PA3-915 at 1 μg/mL [in green]) a specific marker of endolysosomes. Nuclei were counterstained with DAPI (in blue). Arrows indicate the colocalization of low-density lipoprotein receptor within endo-lysosomes. Scale 1 μm.

apoB-containing lipoproteins occurs in the plasma and requires VLDL lipolysis to LDL. PCSK9 does not bind to chylomicrons or remnant lipoproteins (Tavori *et al.*, unpublished observation). It remains to be seen whether PCSK9 associates with lipoprotein (a) [Lp(a)].

The *in vivo* relevance of PCSK9 association with LDL was first shown in patients undergoing lipoprotein apheresis (LA), a dialysis procedure that removes apoB-containing lipoproteins from plasma in a matter of few hours. In addition to the >70% reduction in LDL, LA also reduces plasma PCSK9 levels by slightly >50%.[7–9,12,13] This is mainly, but not exclusively, due to the removal of apoB-bound PCSK9.[9] The combined loss of PCSK9 and LDL during apheresis may thus synergize to keep LDL-C levels down between LA treatments.

Another line of evidence underpinning PCSK9 association with LDL comes from studies of the two common PCSK9 forms: (i) the intact 62 + 13 kDa heterodimer, and (ii) the furin-cleaved 55 + 13 kDa heterodimer, a product of cleavage of the intact PCSK9 by furin in the circulation or at the surface of hepatocytes.[9,14–16] Most of apoB-bound PCSK9 is intact (62 + 13 kDa), and may therefore be the most physiologically active form of PCSK9, since furin-cleaved PCSK9 is generally considered inactive.[10] But *in vitro* binding of intact PCSK9 to LDL appears to inhibit PCSK9 action.[11] The significance of PCSK9 binding to LDL and its effect on the distribution of PCSK9 molecular forms and function therefore require further investigation.

Given the heterogeneity of circulating PCSK9, it is not surprising that correlations between plasma PCSK9 and LDL-C levels in cohorts are so low.[17] The PCSK9 ELISAs developed in research laboratories or those commercially available lack the ability to discriminate between the various forms of PCSK9 present in the plasma, such as the gain-of-function and loss-of-function variants, the intact and furin-cleaved forms, the forms differentially modified post-translationally through sulfation or phosphorylation, and the lipoproteins-associated and apoB-free forms.

It is not known whether apoB-bound and apoB-free PCSK9 equally affect the LDL receptor. Thus, new methods to quantify plasma PCSK9 form are needed, especially in the light of recent data showing that total plasma PCSK9 levels do not predict CVD event.[18]

PCSK9 inhibitors lower Lp(a), but how?

Lp(a) consists of a unique protein homologous to plasminogen, apolipoprotein (a), that is covalently linked to the apoB100 moiety of an LDL size particle by a unique disulphide bond. Lp(a) is widely regarded as extremely atherogenic.[19] Both statins and PCSK9 inhibitors act by increasing the abundance of LDLR at the surface of hepatocytes, thus lowering LDL-C levels.[20] But unlike statins,[21] PCSK9 inhibitors also promote a uniform as-yet unexplained 25–30% reduction in circulating Lp(a) levels.[22–24]

The molecular and cellular pathways governing apo(a)/Lp(a) hepatic production and Lp(a) cellular uptake and degradation are not well understood.[19] The potential role of the LDLR in Lp(a) clearance remains extremely controversial.[25,26] For instance, hepatoma cell lines use the LDLR for Lp(a) clearance,[25] whereas primary hepatocytes (Lambert *et al.* unpublished observation) and *in vivo* studies indicate that the LDLR is not involved in Lp(a) clearance.[27] In addition, PCSK9 was shown to affect apoB-containing lipoprotein production,[28–30] and it was suggested that PCSK9 inhibition would thereby reduce Lp(a) levels.

Finally, if PCSK9 associates with Lp(a) in the circulation, this could provide a possible mechanism for the reduction in Lp(a) caused by PCSK9 monoclonal antibodies via target-mediated or reticuloendothelial clearance. There is currently a clear need to fully elucidate how, unlike statins, PCSK9 inhibitors reduce circulating levels of Lp(a).

Is PCSK9 inhibition safe for the brain?

Because cholesterol is an essential component of the developing and adult brain, it has been suggested that very-low LDL-C levels could impact on brain functions and cognition.[4] The FDA advised for neurocognitive impairments assessment in phase III clinical trials of Praluent® and Repatha®. A slight but not significant increase in neurocognitive troubles such as amnesia, memory loss, and confusional states was observed in patients treated with both drugs.[22,24]

Given that (i) brain cholesterol mainly originates from endogenous synthesis, (ii) people with loss-of-function PCSK9 mutations do not apparently exhibit cognitive impairments,[31] and (iii) PCSK9 inhibitors are monoclonal antibodies that should not cross the blood–brain barrier, a significant impact of PCSK9 inhibitors on brain health

or cognition remains extremely unlikely. By improving arterial health, PCSK9 inhibitors could even potentially ameliorate cognition and reduce dementia.

What else do not we know?

The recent discovery of GRP94, a PCSK9 inhibitory binding protein that prevents LDLR premature degradation in hepatocytes[3] clearly underlines the possibility that PCSK9 function may be physiologically regulated by additional protein partners intracellularly or in the plasma. Besides the potential relevance of the various circulating forms of PCSK9, and besides the elusive molecular pathway by which PCSK9 inhibitors reduce Lp(a), there are several unanswered questions pertaining to PCSK9 function and to PCSK9 inhibition.

One series of questions relate to the putative consequences of very low LDL levels achieved with PCSK9 inhibitors not only on brain function but also on the risk of haemorrhagic stroke, cancer, and new-onset diabetes.

A second series of questions relate to the potential adverse effects of a massive upregulation of LDLR in tissues such as pancreatic β cells and hepatocytes where the LDLR may serve as an entry route for viral infections.

Finally, the physiology of PCSK9 in extra hepatic tissues (brain, kidney, intestine, pancreas, and steroidogenic tissues) in adults and during development needs to be fully addressed. The comprehensive study of PCSK9 gain-of-function and loss-of-function mutations carriers beyond just cardiovascular health appears invaluable in that respect.

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