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APP, APLP2 and LRP1 interact with PCSK9 but are not required for PCSK9-mediated degradation of the LDLR *in vivo*

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that post-transcriptionally regulates the levels of hepatic low-density lipoprotein receptors (LDLRs). PCSK9 binds to the extracellular domain of the LDLR, and the PCSK9-LDLR complex is internalized through canonical clathrin-dependent endocytosis and then delivered to lysosomes for degradation. The mechanism by which PCSK9 blocks recycling of the LDLR has not been fully defined. Previous reports showed that amyloid precursor-like protein 2 (APLP2) interacts with PCSK9, but its role in PCSK9-mediated LDLR degradation remains controversial. Here we found that amyloid precursor protein (APP), APLP2 and LDL receptor-related protein 1 (LRP1) interact with PCSK9. To test whether any of these proteins are required for PCSK9-mediated LDLR degradation, we examined the effects of disrupting these proteins in mice. Infusion of PCSK9 into *App*$^{-/-}$, *Aplp2*$^{-/-}$, *Aplp2*-depleted *App*$^{-/-}$, or liver-specific *Lrp1*$^{-/-}$ mice resulted in similar reductions in the levels of hepatic LDLR as seen in wild-type (WT) mice. Infusion of PCSK9 into WT mice also had no effect on the levels of hepatic APP, APLP2 or LRP1. Thus, APP, APLP2 and LRP1 are not required for PCSK9-mediated LDLR degradation and are not regulated by PCSK9 *in vivo*.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily [1]. Gain-of-function mutations in *PCSK9*, including D374Y and S127R, cause hypercholesterolemia [2–4], and loss-of-function mutations result in lower plasma LDL-cholesterol in humans [5]. Similarly, overexpression of PCSK9 in mice causes a dramatic increase in plasma LDL-cholesterol levels [6–8].

Most circulating LDL is removed *via* LDLRs in the liver [9]. After LDL binds the LDLR on the cell surface, the LDL:LDLR complex is internalized through clathrin-dependent endocytosis [10]. In the acidic milieu of the endosome, LDLR undergoes a conformational change and dissociates from the LDL particle [11]. The receptor is then recycled back to the cell surface, and LDL is delivered to the lysosome. Each LDLR undergoes multiple rounds of internalization and recycling [12]. PCSK9 reroutes LDLRs from the recycling pathway to lysosomes for degradation [13].

The mechanism whereby PCSK9 promotes degradation of LDLRs has not been fully elucidated. PCSK9 is a 692-amino-acid glycoprotein that contains a signal sequence followed by a prodomain, a catalytic domain, and a cysteine- and histidine-rich C terminal domain [1]. PCSK9 undergoes autocatalytic cleavage in the endoplasmic reticulum that is required for PCSK9 secretion [6]. After cleavage, the prodomain remains tightly associated with the catalytic domain and blankets the catalytic site as the protein traverses the secretory pathway and is secreted into the blood [1,7,14]. PCSK9 binds the epidermal growth factor-like repeat (EGF)-A in the extracellular domain of the LDLR, and the PCSK9:LDLR complex is internalized by receptor-mediated endocytosis [15]. The C-terminal domain of PCSK9 is not required for LDLR binding at the cell surface, although it is required for LDLR degradation [15–17].

After the PCSK9:LDLR complex is internalized by clathrin-dependent endocytosis, it is also delivered to the endosomal compartment [13]. In contrast to LDL, the acidic environment of the endosome increases the affinity of PCSK9 for the LDLR by 50- to 150-fold [15,18]. Subsequently, the PCSK9:LDLR complex is transported to lysosomes for degradation by a process that remains to be defined [13,15,19–22]. One hypothesis is that PCSK9 interacts with other protein(s) that target the PCSK9:LDLR complex to the lysosome for degradation [13].

Amyloid precursor protein (APP) is a type I transmembrane protein that is ubiquitously expressed in humans and mice. Its cleavage by α- or β-secretase is a key regulatory process in the generation of the amyloid

Abbreviations: PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; APP, amyloid precursor protein; APLP2, amyloid precursor-like protein 2; LRP1, LDL receptor-related protein 1; DSP, dithiobis[succinimidylpropionate]; SDA, (succinimidyl 4,4-azipentanoate); WT, wild type

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β peptide (Aβ). The deposition of Aβ is assumed to be the first event in the pathogenesis of Alzheimer's disease (AD), while the physiological function of APP remains obscure [23]. Mice lacking APP alone have very subtle phenotypes, whereas APP and its mammalian homolog APLP2 double-knockout mice (*App*$^{-/-}$/*Aplp2*$^{-/-}$) are not viable, indicating that APP and APLP2 have partly redundant functions [24].

APP contains an endocytic motif of the NPXY class and cycles back and forth between the plasma membrane and endosome [25]. This motif is also found in other type I membrane proteins such as LDLR and LRP1 [26,27]. APLP2 is a ubiquitously expressed type I transmembrane protein. Its cytoplasmic tail contains two binding motifs of adaptor protein-2 (AP-2), NPXY and YXXØ, which are both required for APLP2 internalization [28]. Tuli et al. [29] reported that APLP2 interacts with the MHC class I molecule K(d) on the cell surface and targets it to the lysosome for degradation through a canonical clathrin-dependent pathway. PCSK9 was previously shown to physically interact with APLP2 in a pH-dependent manner, but the importance of this interaction for PCSK9-mediated LDLR degradation remains controversial [30,31].

In the present study, we tested the hypothesis that PCSK9 interacts with another protein(s) to target the PCSK9:LDLR complex to lysosomes. To identify proteins that interact with PCSK9, we used crosslinking and affinity purification in HuH7 cells treated with exogenous PCSK9. We found that PCSK9, when added to the medium of cells in culture or into the circulation in mice, interacts with APP, APLP2 and LRP1 in a specific and reproducible fashion. Next, we tested whether genetic inactivation of these proteins inhibited PCSK9-mediated LDLR degradation. Finally, we tested whether PCSK9 promoted the degradation of any of these receptors in a similar fashion to that seen for LDLR.

2. Material and methods

2.1. Materials

Cell culture medium and phosphate buffered saline (PBS) were obtained from Meditech, Inc. (Herndon, VA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). EDTA-free protein inhibitor cocktail was purchased from Roche Applied Science. The crosslinkers DSP (dithiobis[succinimidylpropionate]) and SDA (succinimidyl 4,4-azipentanoate) were obtained from Thermo Scientific. Formulated anti-amyloid precursor-like protein 2 (APLP2) siRNA was provided by Alnylam. All other chemicals and reagents were obtained from Sigma unless otherwise specified.

The following antibodies were used in the experiments described herein: 3143, a rabbit polyclonal antibody (Ab) against the terminal 14 residues of mouse LDLR that also cross-reacts with human LDLR [32]; 172C, home-made rabbit polyclonal anti-sera against full-length human PCSK9 [13]; and 377, an anti-LRP1 antibody produced in rabbit against the 85 kDa domain of mouse LRP1 [27]. Commercial antibodies were purchased to detect calnexin (StressGen), APP (Millipore) and APLP2 (EMD Biosciences).

2.2. Animals

Animal experiments were approved by the University of Texas Southwestern Medical Center Animal Care and Use Committee (IACUC) and were performed at UT Southwestern Medical Center in accordance with federal animal welfare policies and regulations. *App*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Aplp2*$^{-/-}$ mice were provided by Joachim Herz (UT Southwestern) [33], and liver-specific *Lrp1* KO mice were obtained from Michael Brown and Joseph Goldstein (UT Southwestern) [34]. PCSK9 transgenic (overexpression), PCSK9 knockout (KO) and age- and gender-matched wild-type (WT) mice were obtained from Jay Horton (UT Southwestern) [35].

2.3. Purification of recombinant human PCSK9

C-terminal FLAG-tagged fusion proteins of WT PCSK9 (referred to as PCSK9 in this paper), PCSK9 containing the D374Y substitution (PCSK9-D374Y), the double substitution D374Y and S127R (PCSK9-D374Y and S127R), and the C-terminal domain (aa 425–692) of PCSK9 (PCSK9-C) were purified using anti-FLAG M2 beads (Sigma A2220) and size exclusion chromatography (Superdex 200 10/300 fast performance liquid chromatography (FPLC), GE Healthcare, Piscataway, NJ) [35].

2.4. Cell culture and crosslinking

For the crosslinking experiments, HuH7 cells were plated onto 60 dishes (150 mm; 1.5×10^6 cells/dish) in high-glucose DMEM (hDMEM) supplemented with 10% FCS and 100 units/ml penicillin G/streptomycin. After the cells reached confluence, the medium was replaced with hDMEM supplemented with 10% newborn calf lipoprotein-poor serum (NCLPPS) and 100 units/ml penicillin G/streptomycin. After 24 h, half of the cells (30 dishes) were treated with buffer only and the other half (30 dishes) were loaded with PCSK9 (10 μg/ml), PCSK9-D374Y (5 μg/ml), PSCK9-S127R and D374Y (5 μg/ml), or PSCK9-C (10 μg/ml) for 30 min at 37 °C. Cells were washed twice with PBS and subjected to crosslinking. Two different crosslinkers were used for these experiments: the homobifunctional crosslinker dithiobis[succinimidylpropionate] (DSP) and the heterobifunctional crosslinker succinimidyl 4,4-azipentanoate (SDA). DSP was first dissolved in DMSO (20 mM) and diluted to 1.5 mM in PBS for crosslinking at room temperature (RT) for 1 h (20 ml/15-cm dish). The reaction was terminated by adding 50 mM Tris-Cl for 30 min. SDA was dissolved in DMSO (20 mM), diluted to 1.5 mM in PBS and incubated with PCSK9 (1.2 μg/ml) for 30 min at RT. Free crosslinker was removed using Zeba Spin Desalting Columns (Thermo). SDA-conjugated PCSK9 (10 μg/ml) was added to HuH7 cells for 30 min at 37 °C. Cells were washed twice with PBS and exposed to 365 nm UV light for 15 min to activate the SDA crosslinker according to the manufacturer's instructions. For monensin treatment, cells were pretreated with monensin (30 μg/ml) for 15 min before PCSK9 was added.

After crosslinking, cells were washed twice with PBS and lysed in buffer containing Triton X-100 (1% *v/v*), 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail (Roche). Cell lysates were then incubated in 200 μl of anti-Flag M2 beads for 16 h at 4 °C and then washed with 20 ml of wash buffer (1% *v/v* Triton X-100, 50 mM Tris-Cl, pH 7.5, 450 mM NaCl, and 5 mM EDTA). Bound proteins were eluted with 500 μl of elution buffer (0.05% *v/v* Triton X-100, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA plus 200 ng/ml Flag peptide). The eluted sample was concentrated and loaded onto SDS-polyacrylamide gradient gels (4–12%) under non-reducing conditions. Bands containing PCSK9 were identified by aligning the gel with an immunoblot of the same material. Bands excised from the gel were analyzed by mass spectrometry. Proteins identified in the PCSK9-treated and control samples, or proteins that did not achieve a score over 150 using the computer program Mascot (Matrix Sciences) were not considered further.

For crosslinking in mouse liver, each mouse was first injected with 32 μg of PCSK9 in the tail vein. Five minutes later, mice were sacrificed and livers were washed and perfused with DSP (1.2 mM in PBS) for 30 min. The crosslinking reaction was stopped by perfusion with 50 mM Tris-Cl for another 15 min. Livers were harvested and proteins extracted and subjected to immunoprecipitation with anti-Flag M2 beads as described above.

2.5. Immunoblot analysis

Liver, brain (cerebellum) and adrenal glands were homogenized in RIPA buffer containing complete protease inhibitor cocktail (Roche). Protein concentrations in the supernatants were measured using a BCA