Low Levels of Low-Density Lipoprotein-C Associated With Proprotein Convertase Subtilisin Kexin 9 Inhibition Do Not Increase the Risk of Hemorrhagic Transformation

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Background and Purpose—Low levels of low-density lipoprotein-cholesterol (LDL-C) are suspected to be associated with a risk of hemorrhagic transformation after ischemic stroke. We assessed the risk of hemorrhagic transformation after cerebral ischemia/reperfusion in mice with low levels of LDL-C resulting from proprotein convertase subtilisin kexin 9 (PCSK9) deficiency.

Methods—PCSK9−/− and PCSK9+/+ mice were fed with a high-fat/high-cholesterol (21%/0.15%) diet for 1 month. Plasma lipids were measured using colorimetric assays. PCSK9−/− and PCSK9+/+ mice (n=15 per group) were subjected to a 4-hour intraluminal occlusion of the middle cerebral artery followed by 20 hours of reperfusion. Spontaneous hemorrhagic transformation was assessed by quantification of hemoglobin in ischemic tissue. In vitro, a cell model of blood–brain barrier was used to test endothelial barrier integrity in response to decreasing concentrations of LDL-C from 1 to 0.25g/L in ischemia/reperfusion conditions.

Results—PCSK9−/− mice had lower LDL-C, high-density lipoprotein-cholesterol, and total cholesterol levels than PCSK9+/+ mice before and after 1 month on the high-fat/high-cholesterol diet. Hemoglobin concentration in ischemic cerebral tissue was not different between PCSK9−/− and PCSK9+/+ mice (31.5 [18.9–60.1] and 32.8 [14.7–69.9] ng/mg protein, respectively; P=0.81). Infarct volume was also similar in both groups (P=0.66). Incubation of human cerebral endothelial cells with decreasing concentrations of LDL-C under ischemia/reperfusion conditions did not alter blood–brain barrier permeability.

Conclusions—Low levels of LDL-C did not increase the risk of hemorrhagic transformation after cerebral ischemia/reperfusion in mice. Our observations suggest that PCSK9 inhibition, leading to LDL-C lowering, should not increase hemorrhagic complications after acute ischemic stroke. (Stroke. 2014;45:3086-3088.)

Key Words: hemorrhage ◼ low-density lipoprotein cholesterol ◼ mice ◼ PCSK9 protein ◼ stroke

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a natural inhibitor of the low-density lipoprotein (LDL) receptor pathway. PCSK9 binds to the LDL receptor at the cell surface and targets it for lysosomal degradation after endocytosis.1 As a result, heterozygous carriers of PCSK9 loss-of-function mutations have naturally low LDL-C levels and a reduced incidence of cardiovascular events.2 Pharmacological inhibition of PCSK9, especially when used in combination with statins, reduces LDL-C to low levels (≈0.25g/L).3 A relationship between low LDL-C levels and the risk of hemorrhagic transformation (HT) after ischemic stroke has been suggested.4,5 HT occurs in 10% to 40% of patients with ischemic stroke4 and is predictive of the worst clinical outcomes.6 Our aim was to test whether LDL-C lowering with chronic PCSK9 inhibition increases the risk of HT in acute ischemic stroke. We assessed this risk in a mouse model with low LDL-C levels because of PCSK9 deficiency.

Methods

Our in vivo protocol was approved by the Animal Ethics Committee of Inserm/Diderot University-Paris (2012–2015/698-0098). PCSK9-knockout (PCSK9−/−) and control mice (PCSK9+/+) were purchased from Jackson Laboratory, were fed with a high-fat/high-cholesterol (21%/0.15%) diet for 1 month. Plasma lipids were measured using colorimetric assays on an Olympus AU400 chemistry analyzer. Mice (male; 10 weeks old; 25 g) were anesthetized with isoflurane, and their body temperature was maintained at 37°C with a heating pad. Cerebral ischemia/reperfusion was induced by a 4-hour intraluminal middle cerebral artery occlusion (MCAO) by introducing a 7-0 silicon-coated monofilament (Doccol Corporation) through the right common carotid to decrease cerebral blood flow at the bifurcation of the right MCA and the right internal carotid. After removing the monofilament, reperfusion was allowed for the next 20 hours. Mice

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were euthanized, and intravascular washout was performed by intra-cardiac perfusion of heparinized saline. The brain was removed and cut into 1-mm coronal slices using a brain matrix mold for evaluation of infarct volume and HT. HT was macroscopically scored on coronal brain slices by 3 independent observers blinded to the group status and confirmed by Masson trichrome staining. Hemoglobin was measured to assess HT in homogenates of ischemic brain tissue quantitatively (Hemoglobin Mouse ELISA Kit-Abcam). Infarct volume was determined using Image J software from coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride.7

In vitro, a cell model of blood–brain barrier, consisting of human cerebral microvascular endothelial cells/D3 donated by P.O. Couraud (Institut Cochin, Paris, France), was used to test the endothelial barrier integrity in response to decreasing concentrations of LDL-C. Blood–brain barrier integrity was measured as transendothelial electric resistance using the xCELLigence system (Roche, Basel, Switzerland) as follows: cells were seeded at 15×103 cells per well onto E-plates coated with collagen I. When transendothelial electric resistance reached a maximal plateau, the confluent cell monolayer was incubated with decreasing concentrations of LDL-C (isolated from human plasma), from 1 to 0.25 g/L, for ≥24 hours and subjected to oxygen-glucose deprivation for 4 hours. Glucose and oxygen were then resupplied to the cells for 20 hours to mimic reperfusion. Changes in transendothelial electric resistance are attributed to resistance variations because of modifications of paracellular junction tightness. Kinetics of transendothelial electric resistance were displayed as Cell Index (arbitrary units).

Data were analyzed using a Mann–Whitney test, and \( P \) values were 2-sided, with a significance level of 0.05. Results are expressed as medians (min–max).

Results

As anticipated,1 PCSK9−/− mice had lower LDL-C, high-density lipoprotein-cholesterol, and total cholesterol levels than PCSK9+/+ mice before (not shown) and after 1 month on the high-fat/high-cholesterol diet (Figure 1). Transient intraluminal MCAO was performed on 15 PCSK9−/− and 15 PCSK9+/+ mice. One mouse of each genotype died overnight after cerebral ischemia. Twenty-four hours after MCAO, hemoglobin concentration in ischemic cerebral tissue was not different between PCSK9−/− and PCSK9+/+ mice (31.5 [18.9–60.1] and 32.8 [14.7–69.9] ng/mg protein, respectively; \( P=0.81 \); Figure 2). Macroscopic and histological (Masson trichrome) qualitative evaluations of HT by blinded multiple-observer analysis confirmed this observation. Infarct volumes were also similar in PCSK9−/− and PSCK9+/+ mice (0.86 [0.70–1.07] and 0.90 [0.59–1.26] cm3, respectively; \( P=0.66 \)).

In vitro, the transendothelial electric resistance of the human cerebral microvascular endothelial cells/D3 monolayer was unaffected by decreasing LDL-C concentrations under oxygen-glucose deprivation conditions (Figure 3).

Discussion

There are some inherent limitations to the experimental models used in the present study. Notably, mice are primarily an high-density lipoprotein-cholesterol species, whereas humans are LDL predominates. We have limited this important bias by feeding the animals a high-fat/high-cholesterol diet to increase their LDL-C levels by 100%. Another important limitation is that PCSK9 gene invalidation does not mimic the transient pharmacological inhibition of circulating PCSK9 in vivo. PCSK9 knockout mice may, for instance, adapt to the permanent lack of PCSK9 in a way that may affect the cell response to ischemia. Unfortunately, specific antimus PC9 monoclonal inhibitors are not commercially available.
In addition, this experimental model does not account for the cumulative LDL-lowering effects resulting from the combined inhibition of PCSK9 by monoclonal antibodies and of hydroxymethylglutaryl-CoA reductase (HMGCoA) reductase by statins. Transient MCAO procedure is a clinically relevant model to study HT after cerebral ischemia/reperfusion that has been previously validated in rats. The blood–brain barrier human cerebral microvascular endothelial cells/D3 monolayer permeability model has also been validated in our laboratory and is appropriate to assess the functionality of brain endothelium. Thus, the low circulating lipid levels of PCSK9 knockout mice do not aggravate HT after MCAO. Likewise, low levels of LDL-C do not alter blood–brain barrier permeability. Concentrations of LDL-C from 1 to 0.25 mg/dL are in the same range as those observed in patients treated with PCSK9 inhibitors. Despite the inherent limitations mentioned above, our observations suggest that PCSK9 inhibition leading to LDL-C lowering should not increase hemorrhagic complications after acute ischemic stroke. The effects of PCSK9 inhibitors alone or in combination with statins on the risk of HT after ischemic stroke should, however, be carefully monitored in the large outcome clinical trials currently underway.

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References
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