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Hepatic overexpression of the prodomain of furin lessens progression of atherosclerosis and reduces vascular remodeling in response to injury

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ABSTRACT

Objective: Atherosclerosis is a complex disease, involving elevated LDL-c, lipid accumulation in the blood vessel wall, foam cell formation and vascular dysfunction. Lowering plasma LDL-c is the cornerstone of current management of cardiovascular disease. However, new approaches which reduce plasma LDL-c and lessen the pathological vascular remodeling occurring in the disease should also have therapeutic value. Previously, we found that overexpression of profurin, the 83-amino acid prodomain of the proprotein convertase furin, lowered plasma HDL levels in wild-type mice. The question that remained was whether it had effects on apolipoprotein B (ApoB)-containing lipoproteins.
Methods: Adenovirus mediated overexpression of hepatic profurin in *Ldlr*$^{-/-}$mice and wild-type mice were used to evaluate effects of profurin on ApoB-containing lipoproteins, atherosclerosis and vascular remodeling.
Results: Hepatic profurin overexpression resulted in a significant reduction in atherosclerotic lesion development in *Ldlr*$^{-/-}$mice and a robust reduction in plasma LDL-c. Metabolic studies revealed lower secretion of ApoB and triglycerides in VLDL particles. Mechanistic studies showed that in the presence of profurin, hepatic ApoB, mainly ApoB100, was degraded by proteasomes. There was no effect on ApoB mRNA expression. Importantly, short-term hepatic profurin overexpression did not result in hepatic lipid accumulation. Blood vessel wall thickening caused by either wire-induced femoral artery injury or common carotid artery ligation was reduced. Profurin expression inhibited proliferation and migration in vascular smooth muscle cells *in vitro*.
Conclusion: These results indicate that a profurin-based therapy has the potential to treat atherosclerosis by improving metabolic lipid profiles and reducing both atherosclerotic lesion development and pathological vascular remodeling.

1. Introduction

Proprotein convertases (PCSKs) constitute a family of calcium-dependent serine endopeptidases that are conserved from bacteria to mammals. Of the nine members, all except two are known as the "typical proprotein convertases (TPCs)" and activate (or inactivate, depending on the substrate) peptides/proproteins by cleaving after the final basic residue of the peptide (R/K)-Xn-(R/K) [1]. PCSK9 and S1P are atypical in that they cleave following non-basic residues [2].

Hepatic PCSK9 and S1P have established roles in plasma lipid metabolism [3]. However, the roles of the other hepatic TPCs in lipid metabolism are unclear. Because all except PCSK1, 2 and 4 are expressed in the liver and there is considerable overlap in their substrates, it is difficult to study their individual roles in lipid metabolism [2]. The situation is further complicated in that global knockout of each TPC is lethal [2].

The TPCs, including those expressed in the liver, are synthesized as inactive zymogens in the endoplasmic reticulum. Each contains an N-terminal prodomain that is presumed to act as both a specific intramolecular chaperone and an inhibitor of the convertase's

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proteolytic activity. Profurin, an 83-amino-acid peptide, is the prodomain of furin (PCSK3) and is generated during the passage of furin from the endoplasmic reticulum to the Golgi. Endogenous profurin undergoes rapid intracellular degradation after assisting in furin maturation [4]. However, when over expressed, profurin is one of the most potent TPC catalytic site inhibitors and has been shown by several groups, including ours [5,6], to inhibit the activity of multiple TPCs both *in vitro* and *in vivo*. Previously, we found that hepatic overexpression of profurin dramatically reduced plasma HDL levels [6]. However, the effect of profurin overexpression on lipid metabolism beyond that on HDL remained to be determined.

There are few studies directly demonstrating a role for the TPCs in atherosclerosis. The most compelling comes from the observation that furin and PCSK5 are expressed in human atherosclerotic lesions and that their expression is up-regulated following vascular injury *in vivo* in animal models [7]. It was therefore of interest to determine whether profurin, the TPCs' inhibitor, had any significant impact on vascular disease.

Here we describe the effects of overexpression of profurin on apolipoprotein B (ApoB) containing lipoproteins, atherosclerosis, and vascular remodeling in response to injury. We find that hepatic profurin overexpression *in vivo* robustly lowers plasma LDL-c levels and is vasculoprotective.

2. Methods

Adenoviral Vectors: Adenovirus encoding human profurin (Ad-profurin) and control adenovirus without a transgenic expression cassette (Ad-null) were generated as previously described [8].

Mice and Diets: Wild-type (WT) and *Ldlr*$^{-/-}$ mice (8-week-old females) on a C57BL/6 J background were purchased from Jackson Laboratory (Bar Harbor, ME). All procedures were conducted in conformity with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center. Plasma samples were collected on the third day after virus injection. Mice were fed a chow diet unless otherwise stated in the text. For the time course study of Fig. 4, *Ldlr*$^{-/-}$ mice were injected with Ad-null or Ad-profurin (10^{11} viral particles/mouse) via the tail vein ($n = 4$ in each group). Plasma samples were collected on days 0, 3, 5, 14 and 28 after injection. For the atherosclerosis study of Fig. 1, *Ldlr*$^{-/-}$ mice were fed a Western diet (TD88137; Harlan Teklad, Madison, WI) for 4 weeks. They were then divided into two groups ($n = 11$ in each group), injected with Ad-null or Ad-profurin (10^{11} particles/mouse) via the tail vein (day 0) and subsequently fed the Western diet for another 4 weeks (Day 28). Finally, they were sacrificed after 4 h of fasting. Plasma and liver samples were collected, and atherosclerotic lesions were analyzed as described [9]. Experimental details regarding diet and sample timing for other experiments are given in the text and Fig. legends.

Lipid Analysis: For blood sampling, mice were fasted for 4 h and bled from the retro-orbital plexus under isoflurane anesthesia using heparinized microcapillary tubes. Blood was centrifuged at 10,000 × *g* for 10 min at 4 °C and plasma was separated and used for the analysis and/or stored at −70 °C. Liver lipid was extracted using the Folch method [10]. Total triglyceride, cholesterol, phospholipids and HDL cholesterol were determined using commercially available kits (Wako Pure Chemical Industries Ltd, Richmond, VA.). ApoB-containing lipoprotein cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Lipoprotein profiles were obtained from equal amounts of pooled plasma samples of the mice constituting experimental groups by fast-protein liquid chromatography (FPLC) as described [11].

Atherosclerotic Lesion Measurement: Mice were fasted for 4 h and then sacrificed under anesthesia. The aorta was dissected *in situ* from the ascending aorta to the iliac bifurcation and the arch photographed. An aortic root assay and an *en face* assay were performed as previously described [9]. Briefly, the heart was removed with the ascending aorta cut halfway between the aortic root and the brachiocephalic artery. The aortic root was sectioned horizontally into 10 μm-thick slices which were stained with hematoxylin and eosin. Total intimal lesion area and acellular/anuclear areas (negative for hematoxylin-positive nuclei) per total cross section area were quantified by taking the average of 6 sections spaced 30 μm apart, beginning at the base of the aortic root. The results were expressed as the average for the 6 sections. For the *en face* assay, the dissected aorta from the arch to the iliac bifurcation was opened longitudinally, fixed between glass slides and stained with Oil Red O. The *en face* lesion area of the aorta was quantified relative to total surface area as described [9]. Images were viewed and captured with a Nikon Labophot 2 microscope equipped with a SPOT RT3 color video camera attached to a computerized imaging system with Image−Pro Plus version 6.0 software (Media Cybernetics Inc., Rockville, MD). Macrophage and trichrome staining were carried out as previously reported [12].

Femoral Artery Injury and Flow Dependent Vascular Remodeling: Adenoviral vectors (10^{11} particles/mouse) were intravenously injected immediately prior to artery injury or ligation. Femoral artery injury and left common carotid artery ligation were performed as previously reported [13]. To induce a denuding endothelial injury, the inside of the left femoral artery of mice was repeatedly scraped (5−7 times) by the end of a straight wire (0.38 mm in diameter, No. C-SF-15-15, Cook, Inc.) inserted into the artery to systematically disrupt the inner lumen of the vessel [13]. To generate flow dependent vascular remodeling, the distal left common carotid artery (LC) artery and its bifurcation into the external and internal carotid were exposed using blunt dissection. 8−0 nylon sutures (USSC Sutures) were used to ligate the LC artery, just proximal to the external/internal carotid artery bifurcation. Incisions were closed (5−0 suture) and mice were left to recover. After 5 weeks of flow reduction induced by LC ligation and wire injury, mice were anesthetized, exsanguinated, and perfused via the left ventricle with physiologic saline. Mice were subsequently perfusion fixed with neutral buffered formalin. Left femoral arteries and both right and left common carotid arteries and were carefully excised and post-fixed either overnight for morphometric studies or immediately embedded in frozen medium for cryotome processing. Morphometric analysis of the carotid arteries was performed using video microscopy as described [13]. Wall thickness was quantified at 4 regions 90° apart in one location and compared.

Western Blot Analysis: SDS-PAGE employed a 4−20% gradient gel with 0.1 μl of mouse plasma applied per well. The separated proteins were electroblotted to a PVDF membrane and Western blot analysis for mouse ApoB and ApoE and carried out as described [14] using anti-ApoB and anti-ApoE antibodies (Abs) sc-12332 and sc-31283, respectively (Santa Cruz Biotechnology, Dallas, TX). The anti-profurin antibody was kindly provided by Dr. Nabil G. Seidah and the anti-microsomal triacylglyceride transfer protein (MTP) antibody was a gift from Dr. M. Mahmood Hussain. Liver homogenates or cell lysates were prepared in RIPA buffer with added protease inhibitor cocktail (Sigma, St. Louis, MO). Protein was quantified using the BCA kit (Fisher Scientific, Pittsburgh, PA) and equal amounts of protein (50 μg) were loaded on a 4−20% gel. Anti-profurin and anti-actin Abs were used as previously described [10]. Blots were incubated with appropriate horseradish peroxidase-conjugated secondary Abs (Jackson ImmunoResearch, West Grove, PA) followed by detection with a SuperSignal West Pico kit (Fisher Scientific, Pittsburgh, PA). The blots were scanned using a ScanMaker 5950 scanner (MicroTek, Santa Fe Springs, CA) and the intensity of each band measured by Image-Pro Plus version 6.0.