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Urokinase-type plasminogen activator (uPA) decreases hepatic SR-BI expression and impairs HDL-mediated reverse cholesterol transport

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ABSTRACT

Objectives: The aim of the present study was to investigate the effect of urokinase-type plasminogen activator (uPA) on the expression of the scavenger receptor class B type I (SR-BI) in hepatocytes, and its impact on the removal of HDL-cholesteryl ester (CE) in the liver.
Methods and results: Huh7 hepatoma cell lines were incubated with increasing concentrations of uPA. uPA dose-dependently decreased SR-BI protein expression, as determined by flow cytometry (FACS) and by Western blot assays, and down-regulated SR-BI gene expression. Functionally, uPA decreased both the cellular binding of HDL to Huh7 hepatocytes, and the selective uptake of CE from HDL, as determined by several methods including BODIPY staining, cellular cholesterol determination and chasing radio-labeled CE transfer from HDL to the cells. These results were further confirmed using primary rat hepatocyes. The effect of uPA on hepatic SR-BI expression was mediated via binding to the uPA receptor (uPAR). In vivo, SR-BI protein and gene expressions were found to be increased in hepatocytes derived from the uPAR-KO mice compared to C57Bl/6 mice, and in parallel HDL-cholesterol levels in plasma derived from uPAR-KO mice were decreased. Moreover, deficiency of uPAR significantly accelerated the plasma decay of injected HDL-[^{3}H]CE.
Conclusions: The results of this study suggest that uPA decreases the removal of HDL-CE in the liver via suppression of the hepatic SR-BI expression. Impaired reverse cholesterol transport (RCT) may result in atherogenic dysfunctional HDL metabolism and may contribute to atherosclerosis development.

1. Introduction

Atherosclerosis is the major cause of coronary artery disease and stroke. There is a strong inverse relationship between plasma levels of high density lipoproteins (HDL)-cholesterol and atherosclerosis [1], and this was attributed primarily to the capacity of HDL particles to transport cholesterol from peripheral tissues to the liver where it can be excreted into bile, an overall process known as reverse cholesterol transport (RCT) [2]. The cholesterol in HDL is transported into hepatocytes through the scavenger receptor class B type I (SR-BI) [3], a well-characterized HDL receptor highly expressed in the liver and in steroidogenic tissues [4]. Expression of SR-BI in hepatocytes is critical in controlling plasma levels of HDL-cholesterol, as well as the overall rate of RCT [5]. Overexpression of SR-BI in the liver reduces HDL levels [6], increases reverse cholesterol transport [7] and hepatic cholesterol concentration [8], and decreases susceptibility to atherosclerosis [9]. By contrast, SR-BI deficiency results in a significant increase in plasma HDL-cholesterol and increased atherosclerotic lesions in multiple mouse models [10,11].

uPA is a serine protease, and together with its membrane-associated receptor uPAR, is part of the uPA/uPAR system, which is an important component of the fibrinolytic system. In addition, it plays an important role in the pathogenesis of vascular disease [12,13]. There is a strong correlation between uPA/uPAR expression in macrophages and atherosclerotic lesion development. Overexpression of uPA in macrophages was shown to accelerate atherosclerosis [14]. We have recently shown that uPA has an impact on macrophage atherogenicity [12], by increasing macrophage cholesterol biosynthesis [15], or by promoting oxidative stress [16] via intracellular signaling from the uPA/uPAR interaction [17]. Moreover, we have demonstrated that uPA regulates gene expression also in the liver, via mechanism involving the inhibition of peroxisome-proliferator-activated receptor gamma (PPARγ) transcriptional activity [18]. However, the effects of uPA/uPAR

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system on the expression of genes involved in lipoprotein metabolism in the liver were not studied. Since the liver is a key organ in the regulation of cholesterol metabolism [19], and because hepatic SR-BI plays a key role in HDL-mediated RCT, we have investigated in this study whether uPA modulates hepatic SR-BI expression and consequently impairs HDL metabolism.

2. Materials and methods

2.1. Chemicals

Phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) (heat-inactivated at 56 °C for 30 min), penicillin, streptomycin, L-glutamine, and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). Total RNA isolation kit was from Epicentre Biotechnologies, Madison, WI, USA.

2.2. Antibodies

Monoclonal anti-human uPAR (CD87) and mouse IgG1 isotype control antibodies were from R&D Systems, Inc. Primary rabbit polyclonal anti-SR-BI antibody was from Novus Biologicals, LLC. Secondary goat-anti rabbit FITC-conjugated antibody from Jackson ImmunoResearch Laboratories, Inc., and monoclonal anti-β-actin antibody from Sigma (St. Louis, MO, USA).

Human uPA and amino-terminal fragment (ATF)-uPA were from American Diagnostica Inc.

2.3. Cells

2.3.1. Human hepatoma cell line HuH7

The human hepatoma cell line HuH7 was cultivated in DMEM medium supplemented with 10% (v/v) heat-inactivated FCS, 100,000 U/L penicillin, 100 mg/L streptomycin, 100 μg/mL pyruvate and 2 mM glutamine, at 37 °C in a humidified incubator (5% CO_2, 95% air). Sub-confluent cells were incubated with uPA for 18 h in medium containing 0.2% bovine serum albumin (BSA) without serum.

2.3.2. Primary rat hepatocytes

Primary hepatocytes were isolated from Male Lewis rat weighing 180–200 g, by a two-step collagenase perfusion method as previously described [20]. Hepatocyte viability was detected by trypan blue exclusion assay. Cells were used only when viability exceeded 90% after isolation. Cells were washed twice with PBS to remove any non-adherent cells and were incubated with uPA as described. Final cell density was 1×10^6 cell/mL.

2.4. SR-BI mRNA expression

RNA was extracted from cells using MasterPure™ RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). cDNA was prepared using Verso™ cDNA kit (Thermo Scientific, Epsom, UK). Primers and probes for genes were designed by Primer Design, Southampton, UK. Using ABsolute Blue QPCR ROX mix (Thermo Scientific), expression was determined by quantitative real-time PCR with Rotor-Gene 6000 amplification detection system. To normalize the data obtained for SR-BI mRNA expression, the amount of β-actin mRNA was measured by quantitative PCR as an internal standard in all treatments.

2.5. SR-BI protein expression

2.5.1. Flow cytometry

Cells were washed with PBS (×2) and incubated with anti-rabbit SR-BI antibodies (1:100) followed by a secondary incubation with goat-anti-rabbit antibody (1:200) conjugated to fluorescein isothiocyanate (FITC). Measurement of cellular fluorescence was determined by FACS. Ten thousand events were registered for each experiment. Cellular fluorescence was quantitated by mean fluorescence intensity (MFI).

2.5.2. Immunoblotting

Cells were lysed in 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5 mM NP-40, and TM protease inhibitor cocktail. Following centrifugation, the supernatant was diluted 1:1 in electrophoresis sample buffer containing 20% (v/v) glycerol, 4% (w/v) SDS 250 mM Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, and 0.5 mg/mL bromophenol blue. The protein sample was analyzed by SDS-PAGE (10% acrylamide) followed by immunoblotting with secondary goat anti-rabbit-HRP antibody.

2.6. Lipoproteins preparation and labeling

HDL and LDL were prepared using expired pooled plasma from healthy blood bank donors (Rambam Hospital Helsinki Committee number #0572-10-RMB) by discontinuous density gradient ultracentrifugation [21], and dialyzed against saline-Na EDTA (1 mmol/L). The protein content was determined with the Folin's phenol reagent [22].

2.7. Lipoprotein oxidation

For oxidation, LDL and HDL were diluted in phosphate-buffered saline (PBS) to 1 mg of protein/mL and dialyzed overnight against PBS at 4 °C to remove the EDTA. Oxidation of the lipoproteins was carried out at 37 °C under air in a shaking water bath. HDL and LDL (1 mg protein/mL) were incubated for 4 h at 37 °C with 35 μmol/L of freshly prepared $CuSO_4$. Oxidation was terminated by refrigeration at 4 °C. The oxidation extent of Ox-HDL and Ox-LDL was determined by the TBARS assay [23].

2.8. Lipoprotein labeling

The lipoproteins were conjugated to FITC for cellular uptake studies as previously described [24].

HDL was radiolabeled in its cholesterol moiety with [^{3}H]cholesteryl oleate (Perkin Elmer Life Sciences) as previously described [25].

2.9. Cellular lipoprotein binding

Huh7 cells were incubated at 37 °C for 3 h with FITC-conjugated lipoproteins (HDL, Ox-HDL, LDL, and Ox-LDL). Lipoprotein cell association was determined by FACS. Ten thousand events were registered for each experiment.

2.10. FACS analysis of cellular neutral lipids

Cells were stained for 20 min on ice with BODIPY 493/503 (3 μg/mL BODIPY for 5×10^7 cells, D3922; Invitrogen, Carlsbad, California). Stained cells were further washed and analyzed by FACS.