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Cholesterol efflux from THP-1 macrophages is impaired by the fatty acid component from lipoprotein hydrolysis by lipoprotein lipase

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ABSTRACT

Lipoprotein lipase (LPL) is an extracellular lipase that primarily hydrolyzes triglycerides within circulating lipoproteins. Macrophage LPL contributes to atherogenesis, but the mechanisms behind it are poorly understood. We hypothesized that the products of lipoprotein hydrolysis generated by LPL promote atherogenesis by inhibiting the cholesterol efflux ability by macrophages. To test this hypothesis, we treated human THP-1 macrophages with total lipoproteins that were hydrolyzed by LPL and we found significantly reduced transcript levels for the cholesterol transporters ATP binding cassette transporter A1 (ABCA1), ABCG1, and scavenger receptor BI. These decreases were likely due to significant reductions for the nuclear receptors liver-X-receptor-α, peroxisome proliferator activated receptor (PPAR)-α, and PPAR-γ. We prepared a mixture of free fatty acids (FFA) that represented the ratios of FFA species within lipoprotein hydrolysis products, and we found that the FFA mixture also significantly reduced cholesterol transporters and nuclear receptors. Finally, we tested the efflux of cholesterol from THP-1 macrophages to apolipoprotein A-I, and we found that the treatment of THP-1 macrophages with the FFA mixture significantly attenuated cholesterol efflux. Overall, these data show that the FFA component of lipoprotein hydrolysis products generated by LPL may promote atherogenesis by inhibiting cholesterol efflux, which partially explains the pro-atherogenic role of macrophage LPL.

1. Introduction

Lipoprotein lipase (LPL) is an extracellular lipase that preferentially hydrolyzes triglycerides from triglyceride-enriched lipoproteins within the bloodstream [1]. LPL is expressed in a number of tissues, including heart, skeletal muscle, adipose, spleen, mammary glands, lung, and macrophages [2,3]. LPL is anchored to cell surfaces via heparan sulfate proteoglycans [4,5], as well as the glycerophosphatidylinositol high-density lipoprotein (HDL) binding protein 1 [6]. At the cell surface, LPL can capture lipoproteins independently of hydrolytic activity [7,8], thus bringing lipoproteins and lipid hydrolysis products in close proximity to various cell surface molecules that are associated with lipoprotein metabolism.

Macrophage LPL was first suggested to be pro-atherogenic in nature by Zilversmit [9], who observed that the arterial LPL content in cholesterol fed rabbits positively correlated with the amount of aortic cholesterol and the rate of plasma cholesteryl ester influx to the artery. Several studies have since confirmed a link between macrophage LPL expression and atherogenesis. For example, Babaev et al. [10] reported that the injection of homozygous LPL deficient fetal hepatic cells in irradiated C57BL/6 mice resulted in a significant decrease of atherosclerotic lesion size versus mice injected with wild type hepatic cells, and Wilson et al. [11] showed that the macrophage-specific overexpression of human LPL in male apolipoprotein (apo) E-null mice significantly increased aortic lesion size versus control mice. In both rabbits and mice with balloon-injured carotid arteries, the localized adenoviral expression of LPL at the site of injury resulted in a marked lipid accumulation [12,13], which suggests that the locally liberated hydrolysis products by LPL might augment atherosclerotic lesion progression.

The accumulation of lipid together with the overexpression of LPL in atherosclerotic lesions suggest that LPL may negatively influence the anti-atherogenic process of cholesterol efflux from macrophages, which is the first step in reverse cholesterol transport *in vivo* [14]. Thus, we hypothesized that the hydrolysis products that are generated by LPL from total lipoproteins lead to

Abbreviations: A/A, antibiotic/antimycotic; ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; ACCA, acetyl-CoA carboxylase A; apo, apolipoprotein; FAF-BSA, fatty acid free bovine serum albumin; FAS, fatty acid synthase; FFA, free fatty acid; HDL, high-density lipoprotein; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate-13-acetate; PPAR, peroxisome proliferator activated receptor; SCD-1, stearoyl-CoA desaturase 1; SR-BI, scavenger receptor class BI; THL, tetrahydrolipstatin.

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impaired cholesterol efflux. To test this hypothesis, we used human THP-1 macrophages to study the effects of lipoprotein hydrolysis products on the cholesterol transporters ATP binding cassette A1 (ABCA1), ABCG1, and the scavenger receptor BI (SR-BI), as well as select nuclear receptors that modulate the expression of these cholesterol transporters. Furthermore, we tested the free fatty acid (FFA) component of the hydrolysis products on the nuclear receptors and cholesterol transporters, and how the FFA component influenced apoA-I mediated cholesterol efflux.

2. Materials and methods

2.1. Cell culture

HEK-293 cells (ATCC, Manassas, VA, USA) were cultured in 100-mm dishes to 90% confluency and transiently transfected using Lipofectamine™ (Invitrogen, Burlington, ON, Canada) with either 5.85 μg of an empty pcDNA3 mammalian expression vector (mock), or with 5.85 μg of pcDNA3 containing the cDNA for human LPL [GenBank: NM_000237] (pcDNA3·LPL), exactly as we previously described [15]. Heparinized media containing no LPL (from pcDNA3 transfected cells) or LPL (from pcDNA3·LPL transfected cells) was obtained and processed exactly as we previously described [15]. Media were divided in aliquots and stored at −80 °C until needed. The expression of LPL was verified by immunoblot analyses of media (Supplemental Fig. 1A), as we described previously [15], using a 1:1000 dilution of a polyclonal antibody against LPL (#sc-32885, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a 1:1000 dilution of a horseradish peroxidase-conjugated anti-rabbit secondary antibody (#SA1-200, Pierce Biotechnology, Rockford, IL, USA), and ECL™ Prime (GE Healthcare, Baie d'Ufre, QC, Canada), according to manufacturer's instructions. Catalytic activity within media was verified using a resorufin ester assay, as previously described [16] (Supplemental Fig. 1B).

THP-1 cells (ATCC) were cultured and 9.65×10^5 cells/well in 6-well plates were differentiated into macrophages over 48 h with 100 nM phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO, USA) also exactly as we previously described [15]. After 48 h of differentiation with PMA, cells were washed three times with RPMI-1640 (HyClone, South Logan, UT, USA) containing 0.2% w/v fatty acid free bovine serum albumin (FAF-BSA) (Sigma), then cells were cultured for 24 h with RPMI containing 0.2% w/v FAF-BSA, 1% v/v antibiotic/antimycotic (A/A) (HyClone), and 100 nM PMA. After 24 h, cells were washed three times with RPMI containing 0.2% w/v FAF-BSA, then cells were cultured for 1 h with RPMI containing 0.2% w/v FAF-BSA, 1% v/v A/A, 100 nM PMA, and 25 μg/ml of the lipase inhibitor tetrahydrolipstatin (THL) – a concentration shown to inhibit LPL activity by at least 95% [17]. Cells were subsequently used for incubations with lipoprotein hydrolysis products or a FFA mixture (described below).

2.2. Lipoprotein hydrolysis products and incubation with THP-1 macrophages

Total lipoproteins ($d < 1.21$ g/ml) were isolated from freshly isolated pooled human plasma from overnight fasted normolipidemic subjects (approval #11-109 by the Human Investigation Committee of Memorial University of Newfoundland) using KBr density gradient ultracentrifugation as previously described [18]. Lipoproteins were dialyzed against phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4 °C, with replacement of the PBS every 6 h. After dialysis, the phospholipid content was measured using a commercial kit (Wako Diagnostics, Richmond, VA, USA), then lipoproteins were stored under $N_{2(g)}$ at 4 °C and used within 2 weeks of isolation. Lipoprotein hydrolysis was carried out as previously described [15,19]. Briefly, total lipoproteins (from a diluted stock of 3.9 mM by phospholipid) were gently mixed with an equal volume of heparinized media without or with LPL from transfected HEK-293 cells (prepared as described above), plus FAF-BSA to a final concentration of 0.2% w/v. The mixture was incubated for 4 h at 37 °C. Total FFA generated by heparinized media without or with LPL was quantified using a commercial kit (Wako Diagnostics). For all total lipoprotein/LPL media mixtures and all total lipoprotein/mock media mixtures, the total FFA generated was within our previously reported ranges of 1.64–1.73 nmol/μl, and 0.01–0.13 nmol/μl, respectively [15].

THP-1 macrophages that were pre-treated with THL (as described above) were incubated for 18 h with 1 ml/well of RPMI containing 0.2% w/v FAF-BSA, 1% v/v A/A, 100 nM PMA, 25 μg/ml THL, and hydrolysis products generated by mock media or LPL containing media (as described above) from total lipoproteins at 0.84 mM based on pre-hydrolysis phospholipid, which corresponded to 0.68 mM FFA following hydrolysis by LPL and 0.04 mM FFA following hydrolysis by mock media. After 18 h, cells were processed for RNA isolation and assessment (described below).

2.3. Free fatty acid mixture and incubation with THP-1 macrophages

A FFA mixture that represented the FFA liberated from total lipoproteins by LPL was prepared as similarly described [15]. Myristate, palmitoleate, palmitate, linoleate, oleate, stearate, arachidonate, and docosahexaenoate (all from Nu-Chek Prep, Elysian, MN, USA) were dissolved in high performance liquid chromatography grade methanol to a concentration of 10 mg/ml and stored at −20 °C under $N_{2(g)}$ until needed. To prepare 1 ml media with 0.68 mM fatty acids for cell culture, 18.6 nmol myristate, 23.7 palmitoleate, 275.0 nmol palmitate, 70.0 nmol linoleate, 241.8 oleate, 45.4 stearate, 0.9 nmol arachidonate, and 0.4 nmol docosahexaenoate were removed from stock solutions and methanol was evaporated at 35 °C under $N_{2(g)}$; fatty acids were resuspended in 10 μl dimethylsulfoxide. The fatty acid/dimethylsulfoxide mixture or 10 μl dimethylsulfoxide (as vehicle control) was added at a rate of 1 μl/min to 990 μl RPMI containing 0.2% w/v FAF-BSA, 1% v/v A/A, 100 nM PMA, and 25 μg/ml THL while continuously vortexing. THP-1 macrophages that were pre-treated with THL (as described above) were incubated for 18 h with 1 ml/well of the RPMI/fatty acid mixture or the RPMI/dimethylsulfoxide mixture. After 18 h, media were collected for assessing lactose dehydrogenase activity using a commercial kit (Pierce Biotechnology) to test for cell death, and cells were processed for RNA isolation and assessment (described below).

2.4. Real-time PCR analyses

Total RNA from treated THP-1 macrophage cells was extracted using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada), and isolated RNA was stored at −80 °C until needed. cDNA was synthesized from the isolated RNA using the iScript RT Supermix (Bio-Rad, Mississauga, Ontario, Canada), according to manufacturer's instructions. Quantitative real-time PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad) per manufacturer's instructions, and primers against β-actin, LXR-α, PPAR-α, PPAR-γ, ABCA1, ABCG1, SR-BI, acetyl-CoA carboxylase A (ACCA), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and LPL (Integrated DNA Technologies, Coralville, IA, USA) on a Mastercycler ep realplex (Eppendorf, Mississauga, ON, Canada) real-time PCR system. Primer sequences and their efficiencies, calculated as previously described [20], are listed in Supplemental Table 1. Real-time PCR conditions were 1 cycle of 95 °C for 3 min; and 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 20 s. All data were normalized