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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

to β-actin using previously described calculations [20], and all normalized data are presented as a percent of control treatments.

2.5. Cholesterol efflux

Lyophilized apoA-I (Sigma), used as an acceptor of cholesterol, was resuspended in deionized water and salt-exchanged for PBS using a PD-10 desalting column (GE Healthcare) equilibrated with PBS. The protein concentration was measured using the absorbance at 280 nm and the molar absorption coefficient of 1.23 ml × mg^{-1} × cm^{-1} [21]. Cholesterol efflux assays were performed as similarly described [22]. Briefly, THP-1 cells in 12-well plates (4.63 × 10^5 cells/well) were differentiated into macrophages using 100 nM PMA over 48 h. After 48 h, media were replaced with 500 μl RPMI-1640 containing 1% v/v FBS, 1% v/v A/A, 100 nM PMA, and 1 μCi/ml [^{3}H] cholesterol (PerkinElmer, Waltham, MA, USA); cells were cultured for 24 h at 37 °C to load cells with labelled cholesterol. After 24 h, cells were washed three times using 750 μl RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A. The cells were then cultured with 500 μl RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A for 5 h at 37 °C. After 5 h, media were replaced with 500 μl RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, 1% v/v A/A, and 25 μg/ml THL; cells were then cultured for 1 h at 37 °C. After 1 h, media were replaced with RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, 1% v/v A/A, 25 μg/ml THL, and ±0.68 mM FFA mixture (as described above); cells were then cultured for 18 h at 37 °C. After 18 h, cells were washed three times with 750 μl RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A. Following washes, media were replaced with 500 μl RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A ± 25 μg/ml apoA-I; cells were then incubated for 6 h at 37 °C. After 6 h, media were collected and cells were lysed using 1 ml 0.2 M NaOH for 30 min; [^{3}H] cholesterol from media and cell lysates was quantified by scintillation counting. The amount of [^{3}H] cholesterol effluxed was calculated as a percentage of [^{3}H] cholesterol effluxed into the medium per amount of total cell and medium [^{3}H] cholesterol. Background efflux (in the absence of apoA-I) was subtracted from efflux data to apoA-I to obtain apoA-I specific efflux.

2.6. Statistical analyses

Where indicated, Student's *t*-tests were performed. All data represent the mean ± standard deviation (SD).

3. Results and discussion

We prepared total lipoprotein hydrolysis products that were generated by LPL, and we subjected human THP-1 macrophages to the products over 18 h. The LPL hydrolysis reactions were carried out in the presence of 0.2% FAF-BSA, which is a concentration that would limit lipoprotein hydrolysis in our study because FFAs inhibit lipases [23,24], but a concentration of albumin that may be observed within the aortic intima [25]. The concentration of total lipoproteins hydrolyzed by LPL was 2.0 mM based on phospholipid pre-hydrolysis and within the normophysiological range [26]. From those reactions, we added 0.9 mM to THP-1 macrophages based on phospholipid pre-hydrolysis, which corresponds to 0.68 mM FFA from LPL hydrolysis – a comparable concentration to that observed in fasted human sera [27].

We show that total lipoprotein hydrolysis products that are generated by LPL led to significant reductions within THP-1 macrophages in the expression of the transcripts for the cholesterol efflux transporters ABCA1 and ABCG1, as well as the bi-directional cholesterol transporter SR-BI (Fig. 1A).

The upstream regulation of the expression of ABCA1, ABCG1, and SR-BI is through common nuclear receptors, including LXR-α, PPAR-α, and PPAR-γ. The endogenous ligands for PPARs include fatty acids and eicosanoids [28]. Once activated, PPARs will heterodimerize with the retinoid X receptor, and following this they activate the transcripts of target genes by binding to PPAR response elements in the promoter regions of target genes [29]. Of note, PPARs are a target for LXR-α [30]. Both PPAR-α and PPAR-γ have been implicated as critical modulators of cholesterol efflux within macrophages by regulating the expression of the cholesterol transporters ABCA1, ABCG1, and SR-BI through either a LXR-α dependent or independent pathway [31–34]. We found that the total lipoprotein hydrolysis products that are generated by LPL significantly reduce the expression of LXR-α, PPAR-α, and PPAR-γ (Fig. 1B), which likely explains the reduced expression of ABCA1, ABCG1, and SR-BI. We also found that the reduction of these nuclear transporters led to a significant reduction in additional downstream targets, including ACCA, FAS, SCD-1, and interestingly LPL (Supplemental Fig. 2). While more study is necessary to understand the reduction of LPL and transcripts associated with lipid synthesis, we speculate that these events might be part of an initial protective mechanism by macrophages to prevent lipid accumulation and foam cell formation.

In an attempt to understand which components of the total lipoprotein hydrolysis products that are generated by LPL are responsible for reducing the expression of nuclear receptors that subsequently are lowering the expression of cholesterol transporters, we tested the effects of a 0.68 mM FFA mixture on the expression of nuclear receptors and cholesterol transporters within THP-1 macrophages; the FFA mixture tested represents our previously reported ratios of those liberated by LPL from total lipoproteins [15]. The FFAs in the mixture were not associated with any lipoprotein components, thus we were concerned with the possibility of the FFA mixture resulting in cell death during treatments; however, lactate dehydrogenase activities in the media from cells treated with the FFA mixture were comparable to the media from cells treated with the dimethylsulfoxide vehicle control (Supplemental Fig. 3), thus indicating no cell death by the FFA mixture. We found that the FFA mixture significantly reduced the expression of ABCA1, ABCG1, and SR-BI (Fig. 2A), and we found that the FFA mixture significantly reduced the expression of LXR-α and PPAR-γ, but not PPAR-α (Fig. 2B). The expression of the LXR-α and PPAR downstream target ACCA was significantly decreased by the FFA mixture, while the expression of FAS, SCD-1, and LPL were unaffected (Supplemental Fig. 4), likely due to no change to the expression of PPAR-α. These data suggest that the expression of LXR-α and PPAR-γ, but not PPAR-α, is inhibited by one or more of the FFA components that are liberated by LPL, thus likely leading to a subsequent reduction in ACCA and cholesterol efflux. Interestingly, Schoonjans et al. [35] have previously reported that the expression of LPL was increased through a tissue specific stimulation of either PPAR-α or PPAR-γ. We suspect that the expression of LPL in the THP-1 macrophage is modulated specifically by PPAR-α, and that it is through a non-FFA lipid component or a liberated protein following total lipoprotein hydrolysis by LPL.

Because the FFA component from total lipoprotein hydrolysis clearly reduced the expression of cholesterol transporters, we labelled THP-1 macrophages with [^{3}H] cholesterol and we tested the apoA-I mediated efflux of cholesterol from labelled cells treated without or with the 0.68 mM FFA mixture that represents our previously reported ratios of those liberated by LPL from total lipoproteins [15]. In agreement with the reduced expression of cholesterol transporters, we found that the apoA-I mediated efflux from macrophages treated with the FFA mixture was significantly reduced versus vehicle control treated macrophages (Fig. 3).

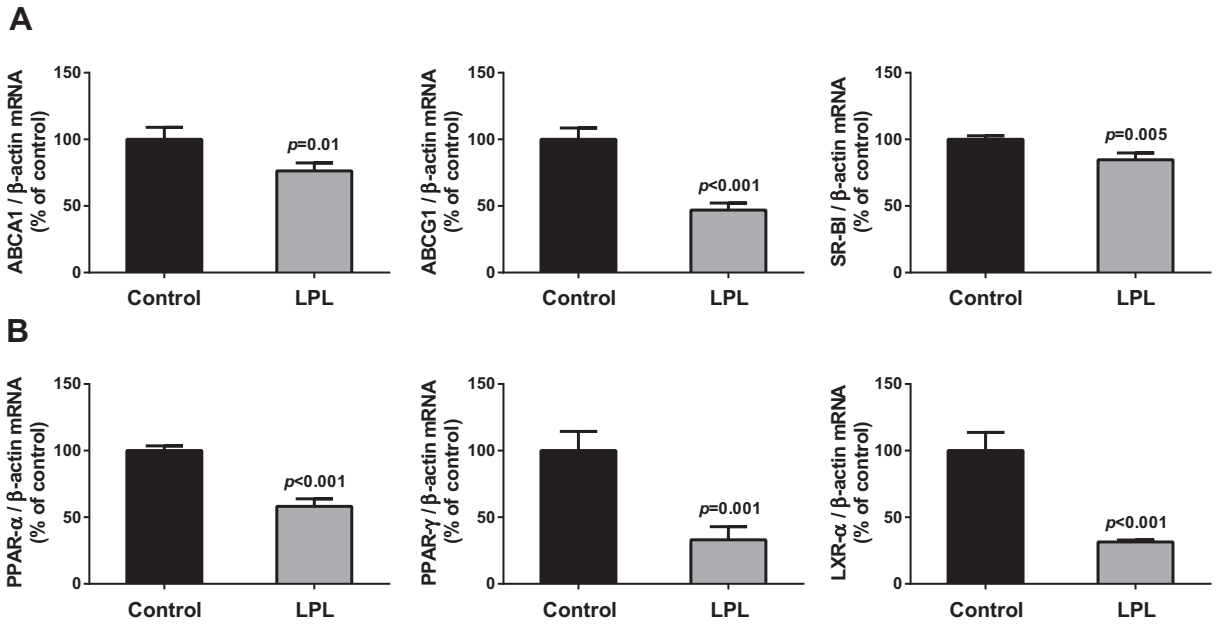

Fig. 1. Expression of cholesterol transporters and nuclear receptors in response to total lipoprotein hydrolysis products. Total lipoproteins ($d < 1.21$ g/ml, 2.0 mM by phospholipid) were hydrolyzed for 4 h at 37 °C by media from mock-transfected (control) or LPL-transfected (LPL) cells, as described under "Section 2". Hydrolysis products (control, 0.04 mM; LPL, 0.68 mM by FFA) were added to THP-1 macrophages for 18 h and RNA were isolated for real-time PCR analyses. (A) Real-time PCR analyses of ABCA1, ABCG1, and SR-BI. (B) Real-time PCR analyses of PPAR-α, PPAR-γ, and LXR-α. All data are means ± SD from triplicate experiments.

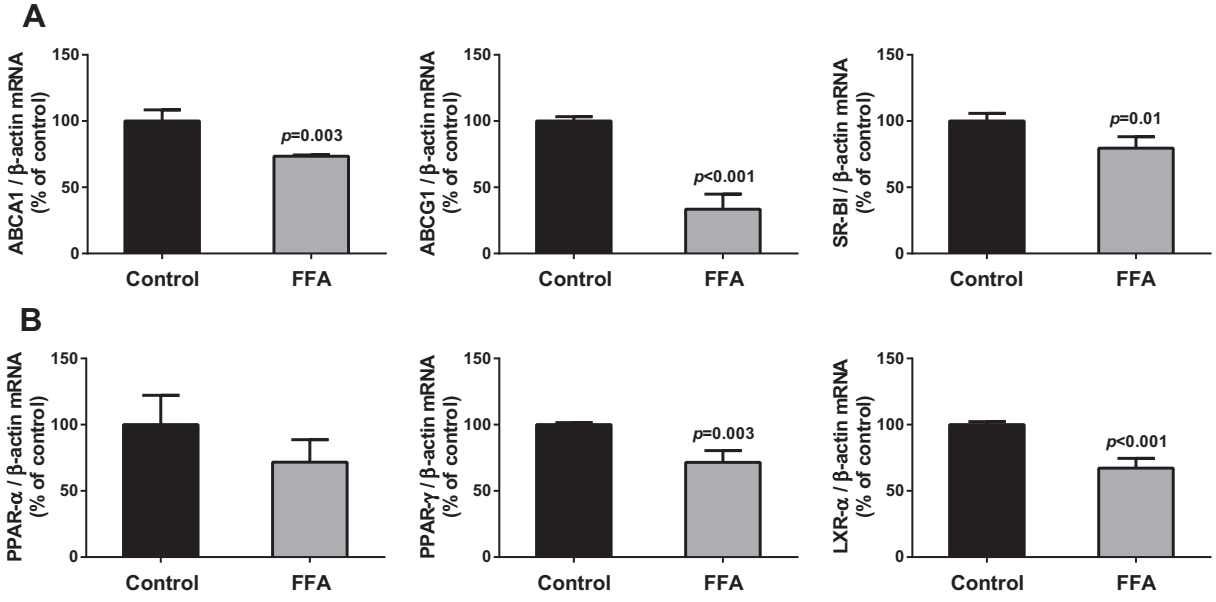

Fig. 2. Expression of cholesterol transporters and nuclear receptors in response to FFA mixture. A 0.68 mM FFA mixture representing the FFA ratios liberated by LPL was prepared as described under "Section 2". Vehicle control (control) or the FFA mixture (FFA) were added to THP-1 macrophages for 18 h and RNA were isolated for real-time PCR analyses. (A) Real-time PCR analyses of ABCA1, ABCG1, and SR-BI. (B) Real-time PCR analyses of PPAR-α, PPAR-γ, and LXR-α. All data are means ± SD from triplicate experiments.

Kawashima and Medh [36] recently reported that the lentiviral-mediated knockdown of LPL in THP-1 macrophages leads to a significant increase in ABCA1 mediated cholesterol efflux, while the addition of exogenous bovine LPL impaired cholesterol efflux. Our data support these findings and our study suggests a mechanism behind how LPL influences cholesterol efflux from macrophages: the hydrolysis of lipoproteins by LPL generates a FFA mixture that targets PPAR-γ and LXR-α to subsequently reduce the expression of ABCA1, ABCG1, and SR-BI, thus ultimately impairing cholesterol efflux. However, we cannot exclude the possibility that the FFAs liberated by LPL from lipoproteins might result in post-translational effects on cholesterol efflux. Wang et al. [37] previously showed that unsaturated FFAs can attenuate cholesterol efflux by increasing the intracellular degradation of ABCA1, thus it is possible that the sum of the FFA species liberated by LPL might also lead to similar events. While we are assured that the FFA component of total lipoprotein hydrolysis products generated by LPL ultimately results in impaired cholesterol efflux, we

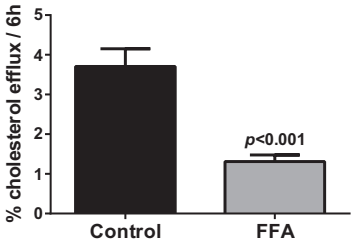

Fig. 3. Cholesterol efflux to apoA-I in response to FFA mixture. A 0.68 mM FFA mixture representing the FFA ratios liberated by LPL was prepared as described under "Section 2". [^{3}H] Cholesterol labelled THP-1 macrophages were treated with the vehicle control (control) or FFA mixture (FFA) for 18 h and apoA-I specific efflux over 6 h was determined. All data are means ± SD from six experiments.

also cannot exclude the possibility that non-FFA hydrolysis products, such as lysophospholipids, monoglycerides, and diglycerides, may play a role in affecting efflux.

In summary, we showed that the FFA component of the hydrolysis products generated from total lipoproteins from normolipidemic subjects by LPL impaired the efflux of cholesterol from THP-1 macrophages to apoA-I. Our *in vitro* results show in part how LPL could contribute to the promotion of atherosclerosis development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.040.

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