Research Article

Unsaturated fatty acids liberated from VLDL cause apoptosis in endothelial cells

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Certain unsaturated fatty acids (UFAs), cleaved from lipoproteins, are known to activate the serine/threonine protein phosphatase type 2C (PP2C) α- and β-isoforms. To investigate the role of UFAs in apoptosis of endothelial cells, we cocultured human umbilical vein endothelial cells (HUVECs) with THP-1 monocytes. Phorbol-12-myristic-13-acetate (PMA)-treated THP-1 monocytes differentiated into macrophages and synthesized lipoprotein lipase (LPL), the major enzyme for hydrolysis of triglycerides. We demonstrated that LPL from THP-1 macrophages released UFAs from VLDL, which were capable of inducing apoptosis in HUVECs. Physiological concentrations of VLDL did not cause apoptosis in HUVECs, whereas the combination of VLDL with LPL-rich cell medium of THP-1 macrophages did. THP-1 macrophages and HUVECs in cocultivation did not interfere with each other. However, addition of VLDL to this coculture caused apoptosis in HUVECs. Furthermore, inhibition of LPL by adding orlistat to the culture medium and down-regulation of LPL by small interfering RNA (siRNA) reduced the extent of apoptosis of HUVECs. In conclusion, our results show that the amounts of UFAs liberated from lipoproteins are high enough to induce apoptosis in endothelial cells. This underlines the proatherogenic role of UFAs in hyperlipoproteinemias.

Keywords: Apoptosis / Atherosclerosis / Fatty acids / Human umbilical vein endothelial cells / Protein phosphatase type 2C

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1 Introduction

The "Response to Injury Hypothesis" is a generally approved theory for the pathological development of atherosclerosis. Apoptosis of endothelial cells leads to alterations in permeability, and a loss of adhesive proteins and growth factors in the vascular arterial wall [1, 2]. For instance, apoptosis of endothelial cells has been reported in human coronary atherosclerotic arteries [3]. Special chemo-attractants and regulatory molecules are released by the damaged endothelium and lead to the adhesion of monocytes, which migrate into the vessel wall and mature to macrophages. The differentiation of macrophages into so-called foam cells by the accumulation of lipids is believed to be a critical event in the pathological progression of atherosclerosis [4].

Reversible phosphorylation of proteins represents one of the most important regulatory mechanisms in cell signaling. This process is mediated by protein kinases and phosphatases [5]. The highly conserved Mg^{2+}-dependent PP2C is a member of one of four major groups of serine/threonine phosphatases in eukaryotes [6] and exists in various isoforms. PP2Cα and β plus other phosphatases are able to dephosphorylate Bad, a member of the Bcl-2-family [7]. Phosphorylated Bad is crucial for cell viability, whereas its dephosphorylated form is able to bind to the antiapoptotic Bcl-2, thereby displacing proapoptotic Bax. This latter protein forms pores within the mitochondrial membrane to release Cytochrome *C* and finally to cause cell death [8, 9].

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Abbreviations: HSPGs, heparan sulphate-proteoglycans; **HUVECs**, human umbilical vein endothelial cells; **LPL**, lipoprotein lipase; **PMA**, phorbol-12-myristic-13-acetate; **PP2C**, protein phosphatase type 2C; **RNAi**, RNA interference; **RT**, room temperature; **siRNA**, small interfering RNA; **TBS-T**, Tris buffered saline with Tween; **UFAs**, unsaturated fatty acids

In previous work we demonstrated that certain UFAs like oleic acid activated PP2Cα and PP2Cβ 10 – 15-fold and were able to induce apoptosis [10, 11]. The PP2C activating UFAs had to be *cis*-configurated and long enough (>15 C-atoms). Furthermore, we showed that isolated UFAs from lipoproteins, liberated by LPL *in vitro*, induced apoptosis in endothelial cells [12]. By means of RNAi technology we could demonstrate a causal relationship between activation of PP2C and induction of apoptosis in HUVECs [13].

The vascular endothelium is constantly exposed to lipoproteins. LPL catalyses the hydrolysis of triacylglycerol components of circulating VLDL and chylomicrons, providing nonesterified fatty acids and 2-monoacylglycerol for muscle- and adipose tissue [14]. The physiological site of LPL action is the luminal surface of vessel endothelial cells to which the enzyme is annexed *via* highly charged, membrane bound heparan sulphate-proteoglycans (HSPGs) [15, 16]. Interestingly, LPL is synthesized in adipose, cardiac, skeletal muscle cells, and macrophages, but not in endothelial cells [15, 16]. After synthesis, LPL is translocated through the endothelium to HSPGs [15]. The active form of LPL exists as a noncovalent dimer [15]. Apolipoprotein C2, a component of VLDL and chylomicrons acts as a cofactor and activator of LPL [17]. Since LPL plays a central role in lipid metabolism, it is involved in several diseases, *e.g.*, obesity [18], diabetes [19], and atherosclerosis [20, 21].

LPL plays different roles in the development of atherosclerosis. Capillary LPL is capable to clear the blood from lipoproteins. This clearance drives the blood lipoprotein profile into a positive, nonatherogenic direction by increasing the HDL fraction [22]. In contrast, LPL within the artery walls seems to be proatherogenic and is mainly synthesized by macrophages [23 – 26]. This is supported by clinical studies: diabetics express more macrophage LPL, probably one reason why these patients show a higher atherosclerosis incidence [27, 28].

In the present study we investigated whether LPL synthesized by THP-1 macrophages could release UFAs from lipoprotein-triglycerides in a concentration high enough to cause apoptosis in HUVECs.

2 Materials and methods

2.1 Materials, reagents, and equipment

HUVECs and endothelial cell growth medium containing 2% heat-inactivated fetal bovine serum, 0.1 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, 1 ng/mL basic fibroblast growth factor, and antibiotics were purchased from Promocell (Heidelberg, Germany). THP-1 monocytes were a gift from the Institute for Arteriosclerosis Research (Muenster, Germany). THP-1 cells were cultured in THP-1 monocyte medium containing RPMI-1640, 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM/L-glutamine, 100 mM sodium pyruvate.

Optimem, antibiotics, oligofectamine, MMLV reverse transcriptase, RNase inhibitor, and oligo-(dT)-primer were purchased from Invitrogen (Karlsruhe, Germany). Bovine LPL, LPL from *Pseudomonas ssp.*, BSA, phorbol-12-myristic-13-acetate (PMA), 2-mercaptoethanol, Nile blue, and Hoechst 33258 were received from Sigma – Aldrich (Taufkirchen, Germany). Orlistat was extracted from Xenical™ capsules (see Section 2.3). Bicinchoninic-acid-kit (BCA-Kit for measurement of protein concentration) was purchased from KMF (Sankt Augustin-Buisdorf, Germany). Analysis of apoptotic cells was accomplished with the fluorescent microscope Axiovert 100 Zeiss (Jena, Germany). RNeasy™ total RNA isolation kit and primers were obtained from Qiagen (Hilden, Germany). Mouse monoclonal antibodies against LPL were from J. D. Brunzell (University of Washington, Seattle). Enhanced chemoluminescence (ECL) Western blotting detection reagents and secondary antibody mouse IgG, peroxidase linked, from donkey, were from GE Healthcare (Munich, Germany).

VLDL was isolated from fresh-pooled human plasma drawn from healthy individuals by sequential preparative ultracentrifugation as previously described [12].

2.2 Cell culture

HUVECs were cultured in endothelial cell growth medium. Cells were used at passages 3 – 6 and morphology was controlled prior to treatment. The cells were seeded at a density of 3×10^5 cells *per* 19.6 cm^2 dishes (or 3×10^4 cells *per* 1.9 cm^2 well) and cultured for 24 h. THP-1 monocytes were cultured in suspension in THP-1 monocyte medium at a density of 5×10^5 cells/mL. For differentiation to macrophages, monocytes received 100 ng/mL PMA and 50 μM 2-mercaptoethanol and became adherent [29]. Experiments were performed in optimem with adherent macrophages (10^6 cells/19.6 cm^2 dishes). Both cell types were cultured at 37°C in an incubator supplemented with 5% CO_2 and 95% air.

For coculture experiments of HUVECs together with THP-1 cells, both cultures were initially grown separately. HUVECs (3×10^5) were seeded in 19.6 cm^2 dishes with endothelial cell growth medium. THP-1 cells (10^6) were seeded in 500 μL optimem containing 100 ng/mL PMA and 50 μM 2-mercaptoethanol in lids of 35 mm dishes. After 6 h of treatment with PMA adherent cells were washed with prewarmed (37°C) optimem to remove remaining phorbol ester and mercaptoethanol. The removal of PMA after 6 h showed no measurable influence on LPL expression in differentiated THP-1 macrophages. HUVECs were washed in the same way as THP-1 macrophages to remove serum proteins. For coculture, the lids with adherent THP-1 macrophages were then put down under into the 19.6 cm dishes containing HUVECs. The gap between both cell monolayers was 0.5 mm. The total volume of cell medium in coculture dishes was 1.5 mL.

2.3 Treatment of HUVECs with VLDL, LPL, and orlistat

HUVECs were treated for 16 – 30 h with 100 mg/dL VLDL in the presence or absence of *Pseudomonas* LPL (250 U), bovine LPL (0.625 U), or LPL released from THP-1 macrophages. LPL was inhibited by adding orlistat to the culture medium. Orlistat was extracted from Xenical capsules (120 mg *per* capsule) in 1 mL DMSO. Insoluble products were removed by centrifugation (14 000 × *g* for 5 min). The supernatant yielded a solution of orlistat, which was aliquoted and stored at −80°C until use. The final concentration of DMSO in the culture medium did not exceed 0.2%.

2.4 Knock-down of LPL in differentiated THP-1 cells with small interfering RNA (siRNA)

To carry out RNA interference (RNAi), THP-1 monocytes were transfected with chemically synthesized siRNA oligonucleotides directed against mRNA of LPL. The siRNA was designed as 21mers according to the published guidelines, and had dTdT 3′-overhangs [30]. For knock-down of LPL in THP-1 cells, we used 5′-GAA CCA GAC TCC AAT GTC A-3′ corresponding to the mRNA target. According to the manufacturer's protocol, oligofectamine and siRNA were separately diluted in optimem, then mixed and incubated for 30 min at room temperature (RT). Transfection of THP-1 cells with siRNA and oligofectamine occurred in parallel with PMA. For longer transfection times and before adding PMA, THP-1 monocytes were centrifuged, washed and suspended in optimem (2×10^6 cells/mL) and incubated with 10 µL oligofectamine and 150 pmol of siRNA *per* mL optimem for 10 h in a water bath at 37°C with repeated gentle rocking. Thereafter, PMA was added at the same concentration as previously described to induce the differentiation of THP-1 cells to macrophages. To investigate the effect of the LPL-knock-down in THP-1 macrophages on the VLDL-induced apoptosis in HUVECs, the HUVECs were cocultured with siRNA-treated THP-1 macrophages and incubated with 50 mg/dL VLDL for 18 h.

2.5 Staining with Hoechst 33258 and Nile blue

HUVECs were washed with PBS (pH 7.4), fixed for 30 min with paraformaldehyde (4%) and then incubated for 30 min with the DNA fluorochrome Hoechst 33258 (10 µg/mL) in methanol at RT in the dark. After washing with PBS, the nuclear morphology of HUVECs was analyzed under a fluorescent microscope at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Cells showing shrunken or fragmented nuclei or chromatin condensation were counted as apoptotic cells.

Nile blue was used to reveal the uptake of fatty acids. After fixation with paraformaldehyde for 30 min, HUVECs were washed with PBS, stained with Nile blue solution (10 µg/mL) for 2 h and analyzed under a confocal laser scanning microscope at wavelengths of 488/525 nm.

2.6 Western blotting

LPL in cell lysates and medium was analyzed on 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Blots were treated for 1 h at RT with 5% skim milk powder in Tris buffered saline with Tween (TBS-T; 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20), incubated overnight at 4°C in TBS-T containing 5% BSA and primary antibody (anti-LPL 1:1000 – 1:500), followed by incubation in 5% skim milk powder in TBS-T for 1 h with peroxidase-conjugated antimouse IgG (1:2500). Blots were developed with ECL reagent. For detection of LPL we used the monoclonal anti-LPL antibody 5D2 (MAb 5D2).

2.7 Protein determination

The concentration of proteins was determined by BCA or Lowry assay using BSA as a standard.

2.8 RT-PCR

Total cellular RNA was prepared using the RNeasy total RNA isolation kit according to the manufacturer's instructions. For RT-PCR analysis, each isolated RNA sample (1 µg) was transcribed by MMLV reverse transcriptase in the presence of RNase inhibitor and oligo-(dT)-primer at 37°C for 60 min. The annealing temperature was 60°C and the total number of cycles was 25 for LPL and β-actin, respectively. These conditions were in the exponential phase of amplification and, therefore, provided a direct correlation between the amount of product and the RNA template abundance in the samples. The following primers were used for PCR amplification: LPL (5′-GGC TCT GCT TGA GTT GTA GA-3′ and 5′-TGT TCT GTA GAT TCG CCC AG-3′), β-actin (5′-ATT TGG CAC CAC ACT TTC TAC-3′ and 5′-TCA CGC ACG ATT TCC CTC TCA G-3′).

2.9 Statistical methods

All values are given as means ± SD. One way analysis of variance (ANOVA) followed by Scheffé's test was applied.

3 Results

3.1 Expression of LPL

The physiological site of LPL action is at the luminal site of the endothelium [15]. LPL mRNA was not detectable in HUVECs and native THP-1 monocytes (Fig. 1). However, LPL mRNA became visible in THP-1 cells after treatment with PMA (Fig. 1) what was in line with previous findings [31]. The expression of LPL in THP-1 cells after addition

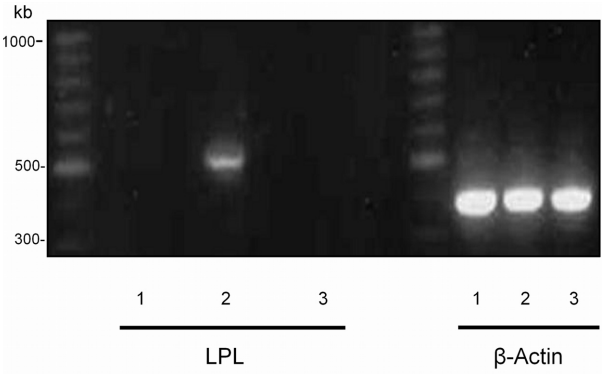

Figure 1. Expression of LPL mRNA. LPL mRNA was present in THP-1 cells treated with PMA (2), but not in HUVECs (1) and not in THP-1 cells without PMA treatment (3).

of PMA was transient and reached a maximum after 9 – 12 h (Fig. 2A). A large amount of LPL protein was detectable in the cell medium of differentiated THP-1 cells (Fig. 2B). This is in line with a report showing that LPL activity in the medium of differentiated THP-1 cells achieves the maximum 12 h after addition of PMA [31].

3.2 Induction of apoptosis in HUVECs cocultured with THP-1 macrophages

In previous work we demonstrated that LPL from *Pseudomonas ssp*. hydrolyzed lipoproteins to liberate UFAs which induced apoptosis in HUVECs [12]. Here we added bovine LPL, which has a high homology to human LPL, with or without VLDL to HUVECs. Physiological concentrations of VLDL added to the culture medium did not influence the viability of HUVECs. However, apoptosis was induced when LPL was added (Fig. 3A).

Next, we set out to analyze whether LPL from macrophages would hydrolyze VLDL and induce apoptosis to the same extent as bovine and *pseudomonas* LPL. Differentiated THP-1 cells released LPL into the culture medium 12 h after addition of PMA (Fig. 2). HUVECs were treated with this THP-1 medium in the absence or presence of VLDL. Again, native VLDL did not harm the cells whereas VLDL in LPL-rich THP-1 medium caused apoptosis of HUVECs (Fig. 3B). The uptake of lipids into cells was analyzed using the dye Nile blue. VLDL-treated cells showed an increased lipid uptake but no apoptotic morphology. UFAs liberated from VLDL by LPL of macrophages caused apoptosis in endothelial cells (Fig. 4).

VLDL added at a final concentration of 100 mg/dL to the medium of cocultured HUVECs and THP-1 macrophages induced cell death in HUVECs exclusively. Apoptosis appeared in HUVECs already 16 h after treatment with VLDL (Fig. 3C).

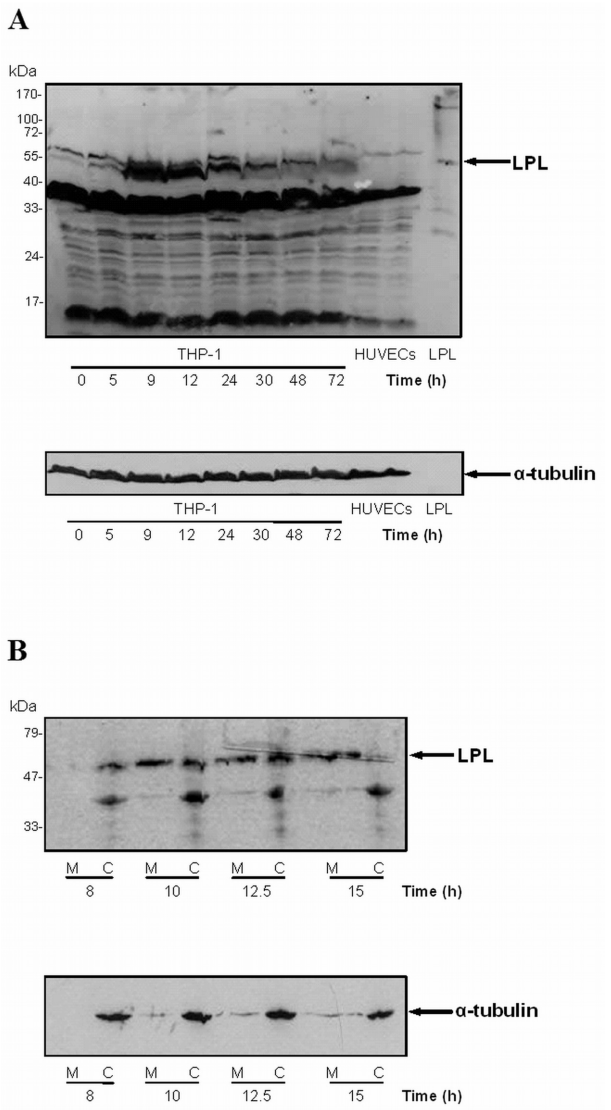

Figure 2. Expression of LPL protein in THP-1 macrophages. (A) Time-dependence of the expression of LPL protein in THP-1 cells after addition of PMA. Western blot analysis for detection of LPL in differentiated THP-1 cells and HUVECs (40 μg *per* lane) was performed on 12.5% SDS polyacrylamide gels after transfer to a nitrocellulose membrane. The membranes were immunoblotted with the monoclonal anti-LPL antibody (MAB 5D2, 1:1000). The expression of LPL (arrow, 55 kDa) was transient and maximal 9 – 12 h after addition of PMA. (B) Presence of LPL in the cell culture medium of differentiated THP-1 macrophages. Western blot analysis of THP-1 medium (M, 20 μL *per* lane) and cell lysate (C, 20 μg *per* lane) revealed a high concentration of LPL. LPL in the cell medium (M) was visible at a later stage (10 h) compared to cell lysate (8 h).

3.3 Inhibition and knock-down of LPL

We used orlistat, a specific lipase inhibitor [32], to confirm the role of LPL in causing apoptosis of HUVECs. Simultaneous treatment of HUVECs with orlistat, VLDL, and

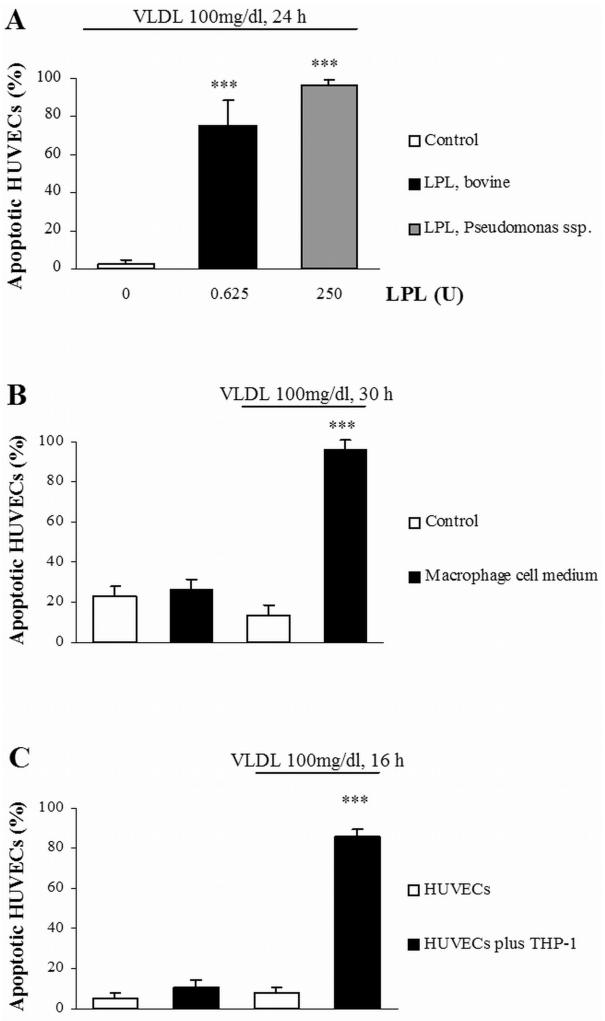

Figure 3. Apoptosis of HUVECs. (A) Induction of apoptosis in HUVECs treated with LPL and VLDL. Physiological VLDL concentrations did not cause cell death. In contrast, HUVECs incubated with VLDL combined with *Pseudomonas* LPL or bovine LPL were highly damaged. (B) Induction of apoptosis in HUVECs with LPL-rich THP-1 cell medium and VLDL. LPL-rich cell medium from differentiated THP-1 cells did not cause apoptosis. In contrast, addition of VLDL to this LPL-containing medium was able to cause cell death. (C) Induction of apoptosis in HUVECs cocultured with THP-1 macrophages and VLDL. UFAs from VLDL caused massive apoptosis in cocultured HUVECs. Values are given as means ± SD. *** $p < 0.001$, compared to controls.

bovine LPL significantly reduced the rate of apoptosis in a concentration-dependent manner. Orlistat inhibited apoptosis at a concentration as low as 4 μM (Fig. 5A). Endothelial cells treated with VLDL in LPL-rich THP-1 medium showed apoptotic morphology. Addition of orlistat to this system reduced the rate of apoptosis as mentioned before (Fig. 5B). To verify these results we used the RNAi-technique to knock-down LPL in THP-1 macrophages treated with PMA for 72 h. The amount of LPL mRNA was measured semiquantitatively by RT-PCR. We could demonstrate a down-regulation of LPL in THP-1 macrophages. It was not possible to show that this effect was transient because the cells did not tolerate the conditions of serum-free medium and transfection reagent for more than 60 h (data not shown).

As mentioned before, the expression of LPL in differentiated THP-1 cells was transient and reached a plateau 9–12 h after addition of PMA (Fig. 2A). During this time, we could demonstrate a high rate of apoptosis in HUVECs induced by LPL in the medium in combination with VLDL (Fig. 3B). Therefore, we tried to knock-down LPL in the time window between 12 and 24 h after addition of PMA. We used different preincubation times with siRNA (5–24 h) before adding PMA to the THP-1 cells and several concentrations of oligofectamine. The best down-regulation of LPL in the cell lysate (Fig. 6A) and culture medium was achieved by transfecting the cells at the same time point when PMA was added. HUVECs, cocultured with siRNA-treated THP-1 macrophages, were less damaged by UFAs released from VLDL by the reduced amount of LPL (Fig. 6B).

4 Discussion

The endothelium acts as a selectively permeable barrier between blood and tissues. The endothelial dysfunction, caused by apoptosis of endothelial cells, is assumed to be the critical initial step in atherogenesis [1, 2] and may compromise vasoregulation, promote infiltration of inflammatory cells and lipids into the intima, increase migration, and proliferation of smooth muscle cells as well as blood coagulation [4].

In previous studies we demonstrated that stimulation of PP2C by certain UFAs from lipoproteins, like oleic acid, correlated with the induction of apoptosis in endothelial cells [12]. We incubated HUVECs with oleic acid, oleic acid methylester, and eladaic acid and got a similar lipid loading of the cells. But only oleic acid which fulfills the prerequisites for activation of PP2C caused apoptosis. It has been shown recently by others that palmitate (500 μM) increases the saturated lipid content of the endoplasmatic reticulum (ER) of CHO-cells and of H9c2-cardiomyoblasts leading to an impairment of its structure and function [33]. In our hands, treatment of HUVECs with palmitate led to lipid loading of the cells but did not cause apoptosis (Krieglstein *et al*., submitted). However, we did not analyze the ER of the HUVECs wherefore we cannot say anything about changes of this organelle.

It is well known that the phosphorylation state of proapoptotic Bad, a member of Bcl-2 family, is crucial for cell viability [8, 9]. This protein can be dephosphorylated by PP2C α and β and other phosphatases [7] and is then able to induce apoptosis. Furthermore, we could show a causal

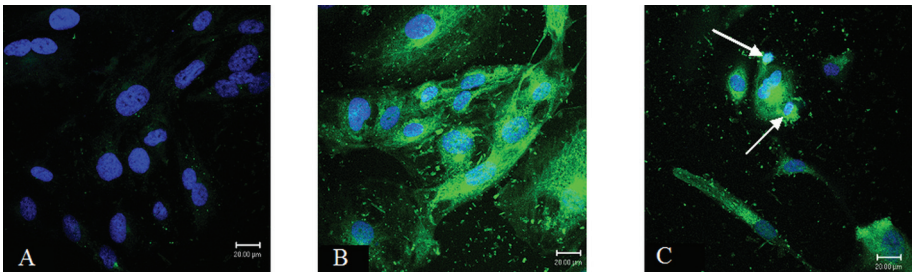

Figure 4. Lipid uptake and apoptosis of HUVECs after treatment with VLDL in THP-1 medium. Double staining of HUVECs with Nile blue and Hoechst 33258 shows the lipid uptake (green) and nuclei morphology (blue). (A) Control cells without addition of VLDL show no lipid uptake. (B) HUVECs treated with 100 mg/dL VLDL showed a high rate of lipid accumulation. (C) The same concentration of VLDL added to THP-1 cell medium was able to induce apoptosis in HUVECs, visible as fragmented nuclei (arrows).

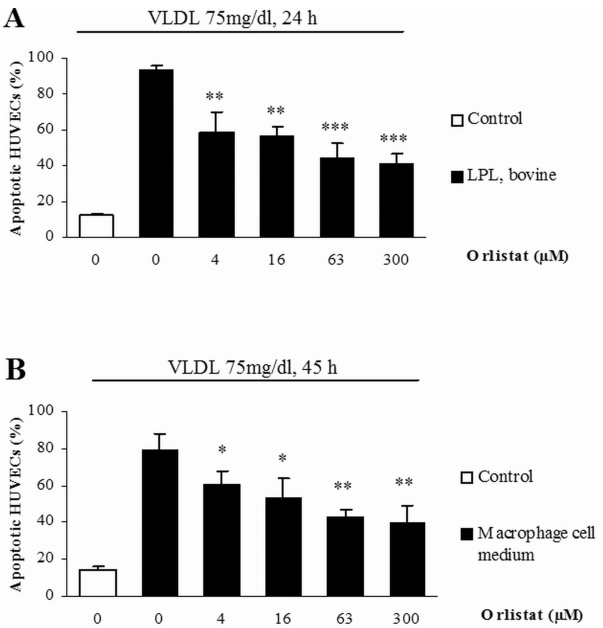

Figure 5. The specific lipase inhibitor orlistat was used to demonstrate the role of LPL. (A) HUVECs were treated with VLDL and bovine LPL to induce apoptosis. Simultaneous treatment with orlistat significantly reduced the number of apoptotic cells in a concentration-dependent manner. (B) The same reduction of apoptosis was observed in HUVECs incubated with LPL-rich THP-1 medium and VLDL. Values are given as means ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively, compared to control groups without orlistat.

relationship between PP2C activation by oleic acid and induction of apoptosis in endothelial cells *via* RNAi [13]. Finally, we were able to demonstrate that UFAs isolated from lipoproteins *in vitro* could activate PP2C and also caused apoptotic cell death in HUVECs [12]. On the other hand, we could significantly diminish apoptosis of HUVECs caused by oleic acid when PP2C was downregulated by the RNAi technique. The extent of apoptosis, however, was not completely abolished by PP2C knockdown suggesting that also other mechanisms of apoptotic damage might play a role [13].

The endothelium is exposed to fatty acids any time due to the fact that LPL, associated with HSPGs on the luminal site of the endothelial cells, hydrolyzes triglycerides from lipoproteins [15]. Endothelial LPL is synthesized in the arterial wall by smooth muscle cells, whereas LPL in atherosclerotic plaques is mainly produced by macrophages [23, 34, 35]. We addressed whether the concentration of UFAs *in vivo* is high enough to activate PP2C and to induce apoptosis in endothelial cells. The concentration of free, unbound fatty acids within the cells is hardly possible to measure. Therefore, our approach was to get indirect information on a sufficient concentration of UFAs when triglycerides from a physiological concentration of VLDL were hydrolyzed by LPL and the liberated UFAs induce apoptosis in HUVECs.

First, we attempted to characterize and compare the expression of LPL in HUVECs and THP-1 macrophages. We could determine LPL mRNA and protein only in differentiated THP-1 cells (Figs. 1 and 2A). These data are in line with published results by Auwerx *et al*. [31]. The expression of LPL in THP-1 macrophages was transient and reached a maximum already 9 – 12 h after addition of PMA. Furthermore, we measured a large amount of LPL in the cell medium of THP-1 macrophages (Fig. 2B) as published by Auwerx *et al*. [31].

We could demonstrate that LPL, added together with VLDL to the culture medium, induced apoptosis in HUVECs (Fig. 3A). However, a high amount of the enzyme was used in these previous experiments. Here, we set out to analyze whether LPL, expressed by THP-1 macrophages, is also able to hydrolyze triglycerides from lipoproteins thereby inducing apoptosis in endothelial cells when VLDL is added. Our results indicate that human macrophages adjacent to endothelial cells expressed LPL to cleave lipids and to cause apoptosis.

Exposure of HUVECs to intact VLDL in a physiological concentration range did not induce cell death. In contrast, VLDL added to LPL-rich medium from THP-1 macrophages caused apoptosis in endothelial cells (Fig. 3B). To imitate the physiological status, we cultivated HUVECs and THP-1 macrophages as separate monolayers in one culture

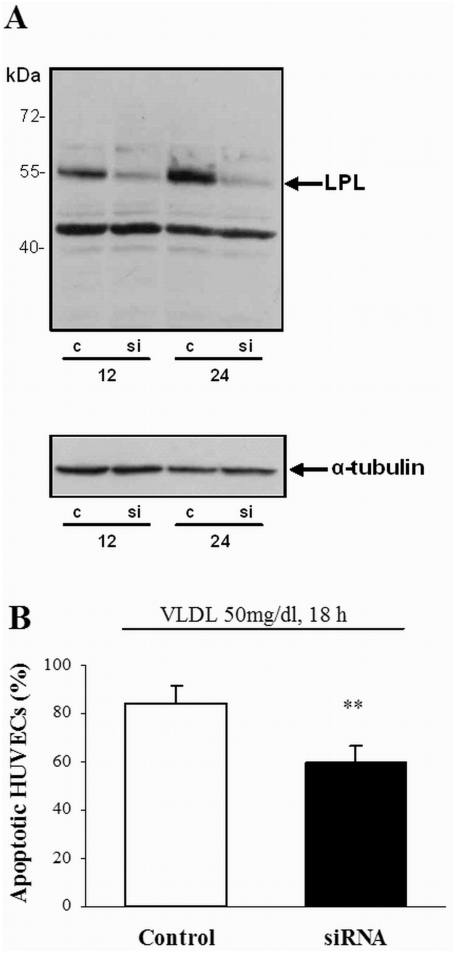

Figure 6. Knock-down of LPL with siRNA. (A) Expression of LPL in THP-1 cells after treatment with siRNA. Western blot analysis (40 μg cell lysate) for detection of LPL in differentiated THP-1 cells, treated with or without siRNA, were performed on 12.5% SDS polyacrylamide gels after transfer to a nitrocellulose membrane and immunoblotting with the monoclonal anti-LPL antibody (MAB 5D2, 1:500). LPL protein level in the cell lysate was down-regulated in differentiated THP-1 cells treated with siRNA (si) in comparison to control (c). The amount of LPL in the cell medium of these cells was also reduced. (B) Reduction of apoptosis in HUVECs cocultivated with siRNA treated THP-1 cells. UFAs from VLDL induced apoptosis in HUVECs which were cocultivated with THP-1 macrophages. The number of apoptotic HUVECs was significantly reduced in the siRNA-treated group in comparison to control. Values are given as means ± SD. ** $p < 0.01$ compared to controls.

dish. Then VLDL was added to this coculture-system and the UFAs, released from VLDL by macrophage LPL, were capable of inducing endothelial apoptosis (Fig. 3C). It is assumed that a similar procedure might occur *in vivo* and increased endothelial dysfunction could accelerate atherogenesis.

To verify the role of LPL in endothelial cell death we used orlistat as specific lipase inhibitor. As described above, VLDL and LPL-rich medium from THP-1 macrophages damaged HUVECs by apoptosis. Simultaneous treatment of these cultures with orlistat significantly reduced the number of apoptotic cells in a concentration-dependent manner (Fig. 5). This result underlines that LPL is responsible for the hydrolysis of VLDL triglycerides deliberating UFAs, which can trigger apoptosis in endothelial cells. Our next experiments focused on the knock-down of LPL in differentiated THP-1 cells with siRNA. Starting from the hypothesis that apoptosis of endothelial cells is caused by UFAs liberated from VLDL by LPL, HUVECs cocultured with THP-1 macrophages with a reduced expression of LPL should be less damaged compared to cocultures with normal LPL-expression. Therefore, expression of LPL in THP-1 macrophages was reduced by siRNA (Fig. 6A). Indeed, VLDL-treated HUVECs, cocultured with siRNA-treated THP-1 macrophages, showed a significantly reduced rate of apoptosis in comparison to control cultures (Fig. 6B). These results substantiate the responsibility of LPL from THP-1 macrophages to hydrolyze triglycerides from VLDL thereby inducing apoptosis in endothelial cells.

In conclusion, we could show that a physiological amount of LPL, produced by macrophages, was capable of liberating UFAs at a concentration high enough to cause apoptosis in endothelial cells. This apoptotic damage might contribute essentially to endothelial dysfunction and could represent a crucial initial step in atherogenesis. It also may provide further insight into the proatherogenic role of UFAs in the arterial wall.

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The authors have declared no conflict of interest.

5 References

[1] Rossig, L., Dimmeler, S., Zeiher, A. M., Apoptosis in the vascular wall and atherosclerosis. *Basic Res. Cardiol.* 2001, *96*, 11 – 22.

[2] Choy, J. C., Granville, D. J., Hunt, D. W., McManus, B. M., Endothelial cell apoptosis: Biochemical characteristics and potential implications for atherosclerosis. *J. Mol. Cell Cardiol.* 2001, *33*, 1673 – 1690.

[3] Tricot, O., Mallat, Z., Heymes, C., Belmin, J., Leseche, G., Tedgui, A., Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* 2000, *101*, 2450 – 2453.

[4] Lusis, A. J., Atherosclerosis. *Nature* 2000, *407*, 233 – 421.

[5] MacKeigan, J. P., Murphy, L. O., Blenis, J., Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat. Cell Biol.* 2005, *7*, 591 – 600.

[6] Cohen, P., Cohen, P. T., Protein phosphatases come of age. *J. Biol. Chem.* 1989, *264*, 21435 – 21438.

[7] Klumpp, S., Selke, D., Krieglstein, J., Protein phosphatase type 2C dephosphorylates BAD. *Neurochem. Int.* 2003, *42*, 555 – 560.

[8] Yang, E., Korsmeyer, S. J., Molecular thanatopsis: A discourse on the Bcl-2 family and cell death. *Blood* 1996, *88*, 386 – 401.

[9] Hsu, S. Y., Hsueh, A. J., Tissue-specific Bcl-2 protein partners in apoptosis: An ovarian paradigm. *Physiol. Rev.* 2000, *80*, 593 – 614.

[10] Klumpp, S., Thissen, M. C., Krieglstein, J., Protein phosphatases types 2Cα and 2Cβ in apoptosis. *Biochem. Soc. Trans.* 2006, *34*, 1370 – 1375.

[11] Zhu, Y., Schwarz, S., Ahlemeyer, B., Grzeschik, S. *et al*., Oleic acid causes apoptosis and dephosphorylates Bad. *Neurochem. Int.* 2005, *46*, 127 – 135.

[12] Hufnagel, B., Dworak, M., Soufi, M., Mester, Z. *et al.*, Unsaturated fatty acids isolated from human lipoproteins activate protein phosphatase type 2Cβ and induce apoptosis in endothelial cells. *Atherosclerosis* 2005, *180*, 245 – 254.

[13] Schwarz, S., Hufnagel, B., Dworak, M., Klumpp, S., Krieglstein, J., Protein phosphatase type 2Cα and 2Cβ are involved in fatty acid-induced apoptosis of neuronal and endothelial cells. *Apoptosis* 2006, *11*, 1111 – 1119.

[14] Cryer, A., Tissue lipoprotein lipase activity and its action in lipoprotein metabolism. *Int. J. Biochem.* 1981, *13*, 525 – 541.

[15] Braun, J. E., Severson, D. L., Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem. J.* 1992, *287*, 337 – 347.

[16] Braun, J. E., Severson, D. L., Tissue-specific regulation of lipoprotein lipase. *CMAJ* 1992, *147*, 1192.

[17] Salinelli, S., Lo, J. Y., Mims, M. P., Zsigmond, E. *et al.*, Structure-function relationship of lipoprotein lipase-mediated enhancement of very low density lipoprotein binding and catabolism by the low density lipoprotein receptor. Functional importance of a properly folded surface loop covering the catalytic center. *J. Biol. Chem.* 1996, *271*, 21906 – 21913.

[18] Pykalisto, O. J., Smith, P. H., Brunzell, J. D., Determinants of human adipose tissue lipoprotein lipase. Effect of diabetes and obesity on basal- and diet-induced activity. *J. Clin. Invest.* 1975, *56*, 1108 – 1117.

[19] Pulinilkunnil, T., Rodrigues, B., Cardiac lipoprotein lipase: Metabolic basis for diabetic heart disease. *Cardiovasc. Res.* 2006, *69*, 329 – 340.

[20] Mead, J. R., Ramji, D. P., The pivotal role of lipoprotein lipase in atherosclerosis. *Cardiovasc. Res.* 2002, *55*, 261 – 269.

[21] Mead, J. R., Cryer, A., Ramji, D. P., Lipoprotein lipase, a key role in atherosclerosis? *FEBS Lett.* 1999, *462*, 1 – 6.

[22] Yagyu, H., Ishibashi, S., Chen, Z., Osuga, J. *et al.*, Overexpressed lipoprotein lipase protects against atherosclerosis in apolipoprotein E knockout mice. *J. Lipid Res.* 1999, *40*, 1677 – 1685.

[23] O'Brien, K. D., Gordon, D., Deeb, S., Ferguson, M., Chait, A., Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J. Clin. Invest.* 1992, *89*, 1544 – 1550.

[24] Merkel, M., Kako, Y., Radner, H., Cho, I. S., *et al.*, Catalytically inactive lipoprotein lipase expression in muscle of transgenic mice increases very low density lipoprotein uptake: Direct evidence that lipoprotein lipase bridging occurs in vivo. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 13841 – 13846.

[25] Zimmermann, R., Panzenbock, U., Wintersperger, A., Levak-Frank, S., *et al.*, Lipoprotein lipase mediates the uptake of glycated LDL in fibroblasts, endothelial cells, and macrophages. *Diabetes* 2001, *50*, 1643 – 1653.

[26] Mamputu, J. C., Desfaits, A. C., Renier, G., Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells. *J. Lipid Res.* 1997, *38*, 1722 – 1729.

[27] Sartippour, M. R., Renier, G., Upregulation of macrophage lipoprotein lipase in patients with type 2 diabetes: Role of peripheral factors. *Diabetes* 2000, *49*, 597 – 602.

[28] Beauchamp, M. C., Michaud, S. E., Li, L., Sartippour, M. R., Renier, G., Advanced glycation end products potentiate the stimulatory effect of glucose on macrophage lipoprotein lipase expression. *J. Lipid Res.* 2004, *45*, 1749 – 1757.

[29] Dory, L., Post-transcriptional regulation of apolipoprotein E expression in mouse macrophages by phorbol ester. *Biochem. J.* 1993, *292*, 105 – 111.

[30] Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., *et al.*, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, *411*, 494 – 498.

[31] Auwerx, J. H., Deeb, S., Brunzell, J. D., Peng, R., Chait, A., Transcriptional activation of the lipoprotein lipase and apolipoprotein E genes accompanies differentiation in some human macrophage-like cell lines. *Biochemistry* 1988, *27*, 2651 – 2655.

[32] Lookene, A., Skottova, N., Olivecrona, G., Interactions of lipoprotein lipase with the active-site inhibitor tetrahydrolipstatin (Orlistat). *Eur. J. Biochem.* 1994, *222*, 395 – 403.

[33] Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., *et al.*, Disruption of endoplasmatic reticulum structure and integrity in lipotoxic cell death. *J. Lipid Res.* 2002, *47*, 2726 – 2737.

[34] Goldberg, I. J., Lipoprotein lipase and lipolysis: Central roles in lipoprotein metabolism and atherogenesis. *J. Lipid Res.* 1996, *37*, 693 – 707.

[35] Zilversmit, D. B., Atherogenesis: A postprandial phenomenon. *Circulation* 1979, *60*, 473 – 485.

