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Hydrolysis products generated by lipoprotein lipase and endothelial lipase influence macrophage cell signalling pathways

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Abstract

Macrophages express lipoprotein lipase (LPL) and endothelial lipase (EL) within atherosclerotic plaques; however, little is known about how lipoprotein hydrolysis products generated by these lipases might affect macrophage cell signalling pathways. We hypothesized that hydrolysis products affect macrophage cell signalling pathways associated with atherosclerosis. To test our hypothesis, we incubated differentiated THP-1 macrophages with products from total lipoprotein hydrolysis by recombinant LPL or EL. Using antibody arrays, we found that the phosphorylation of 6 receptor tyrosine kinases and 3 signalling nodes – most associated with atherosclerotic processes – was increased by LPL derived hydrolysis products. EL derived hydrolysis products only increased the phosphorylation of tropomyosin-related kinase A, which is also implicated in playing a role in atherosclerosis. Using electrospray ionization-mass spectrometry, we identified the species of triacylglycerols and phosphatidylcholines that were hydrolysed by LPL and EL, and we identified the fatty acids liberated by gas chromatography-mass spectrometry. To determine if the total liberated fatty acids influenced signalling pathways, we incubated differentiated THP-1 macrophages with a mixture of the fatty acids that matched the concentrations of liberated fatty

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acids from total lipoproteins by LPL, and we subjected cell lysates to antibody array analyses. The analyses showed that only the phosphorylation of Akt was significantly increased in response to fatty acid treatment. Overall, our study shows that macrophages display potentially pro-atherogenic signalling responses following acute treatments with LPL and EL lipoprotein hydrolysis products, suggesting that elevated LPL and EL in lesions promotes the progression of atherosclerosis through several signalling mechanisms.

Keywords

lipoproteins; lipoprotein lipase; endothelial lipase; antibody array; cell signalling; atherosclerosis; mass spectrometry; triacylglycerol; phosphatidylcholine; free fatty acid

Introduction

Lipoprotein lipase (LPL) and endothelial lipase (EL) are members of an extracellular family of lipases that hydrolyse the triacylglycerols (TAG) and phospholipids (PL) associated with lipoproteins in the bloodstream [1-3]. While LPL and EL can both hydrolyse TAG and PL, LPL preferentially hydrolyses TAG whereas EL preferentially hydrolyses PL [4]. LPL and EL are associated with cell surfaces through binding to proteoglycans [5-8]; in addition, LPL is associated to cell surfaces through binding to glycerophosphatidylinositol high-density lipoprotein binding protein 1 [9]. The cell surface anchored lipases can capture lipoproteins independent of hydrolytic activity [10-12], thus bringing lipoproteins and lipid hydrolysis products in close proximity to various cell surface molecules associated with lipoprotein metabolism. Both LPL and EL are expressed in macrophages [13, 14], and the levels of these lipases are elevated in atherosclerotic lesions from animal models and humans [13, 15-17]. Macrophage LPL was shown to enhance inflammation [18] and lesion formation [19-21] *in vivo*. The relationship of macrophage EL with atherosclerosis is not known, but its suppression in cultured macrophages reduces proinflammatory cytokine secretion [22].

Hydrolysis products, generated from the hydrolysis of lipoprotein lipids by macrophage LPL or EL, may contribute to the progression of atherosclerosis by modulating cell signalling pathways. For example, human aortic endothelial cells incubated with interferon-γ, which can cause increased inflammatory chemokine expression and enhance leukocyte recruitment, exhibited an increased phosphorylation of the intracellular signalling molecules signal transducer and activator of transcription factor 1 (Stat1) and Janus kinase 2 [23]. The phosphorylation of these signalling molecules was synergistically increased when cells were also incubated in the presence of LPL [23]. Also, in the presence of EL and high-density lipoproteins, human aortic endothelial cells displayed an increased expression of the proinflammatory cytokine interleukin-8 possibly through a pathway associated with nuclear factor-κB [24]. Thus, we hypothesized that hydrolysis products from total plasma lipoproteins after hydrolysis by LPL and EL will modulate cell signalling pathways associated with atherosclerosis in macrophages. To test this hypothesis, we used antibody arrays to assess the effects of hydrolysis products generated by LPL and EL on receptor

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tyrosine kinases (RTK) and signalling nodes in the THP-1 human monocyte-derived macrophage cell line.

Materials and Methods

Cell culture

HEK293 cells (ATCC, Manassas, VA, USA) were cultured and transiently transfected using Lipofectamine™ (Invitrogen, Burlington, ON, Canada) in 100 mm dishes at 90% confluency with no DNA or 5.85 μg of an empty pcDNA3 mammalian expression vector (mock), or with 5.85 μg of pcDNA3 containing the cDNA for either human LPL [GenBank:NM_000237] or human EL [GenBank:NM_006033] as previously described [8]. At 24 h after the transfection, media were removed and replaced with serum-free DMEM media (HyClone, South Logan, UT, USA) containing 10 U/ml heparin (Organon, Toronto, ON, Canada). At 47.5 h after the transfection, an additional 10 U/ml heparin was added to the media in each plate. At 48 h after the transfection, media were collected and centrifuged at 1,200 rpm for 10 min to remove any cell debris. The supernatant was divided into aliquots and stored at −80°C.

THP-1 cells (ATCC) were incubated at 37°C with 5% CO_2 and cultured in RPMI-1640 containing 10% fetal bovine serum and 1% antibiotic/antimycotic (A/A) – (all from HyClone). To differentiate THP-1 cells, 9.65×10^5 cells/well in 6-well plates were cultured for 48 h in RPMI containing 10% fetal bovine serum, 1% A/A, and 100 nM phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO, USA). After 48 h, cells were washed three times with RPMI containing 0.2% w/v fatty acid free bovine serum albumin (FAF-BSA) (Sigma), then cultured for 24 h with RPMI containing 0.2% w/v FAF-BSA, 1% A/A, and 100 nM PMA. After 24 h, cells were washed three times with RPMI as above, then cultured for 1 h with RPMI containing 0.2% w/v FAF-BSA, 1% A/A, and 25 μg/ml of the lipase inhibitor tetrahydrolipstatin (THL) (Sigma) to inhibit endogenous lipases; this concentration was previously shown to inhibit LPL and EL activities *in vitro* and *in vivo* by at least 95% [7, 25, 26]. Cells were subsequently used for incubations with lipoprotein hydrolysis products.

Analyses of lipase expression

Proteins in conditioned media samples from transfected cells were denatured and separated on 10% SDS-PAGE gels, then transferred to nitrocellulose membranes as previously described [27]. Nitrocellulose membranes were subjected to immunoblot analyses for LPL using a 1:1,000 of an anti-human LPL polyclonal antibody (#sc-32885, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and for EL using a 1:500 of an anti-human EL polyclonal antibody (#NB400-111, Novus Biologicals, Littleton, CO, USA). Detection was by chemiluminescence using a 1:1,000 dilution of a horseradish peroxidase-conjugated anti-rabbit IgG (#SA1-200, Pierce Biotechnology, Rockford, IL, USA) and ECL™ Prime (GE Healthcare, Baie d'Ufre, QC, Canada), according to manufacturer's instructions. Total RNA from transfected cells was extracted using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) and subjected to real-time PCR analyses for human LPL, EL, and β-actin using commercially available primers (Integrated DNA Technologies, Toronto, ON, Canada) and

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iQ™ SYBR-Green (Bio-Rad, Mississauga, ON, Canada), according to manufacturer's instructions.

Lipoprotein hydrolysis products and THP-1 cell incubations

Total lipoproteins (*d*<1.21 g/ml) were isolated from freshly isolated pooled human plasma prepared from fasted normolipidemic subjects by KBr density gradient ultracentrifugation as previously described [28]. Isolated lipoproteins were stored at 4°C under $N_{2(g)}$ and used within a week of isolation. The PL concentration of the total lipoproteins was measured using a commercial kit (Wako Diagnostics, Richmond, VA, USA). Lipoprotein hydrolysis assays were carried out as similarly described [29]. Briefly, total lipoproteins (from a diluted stock of 3.9 mM PL) were gently mixed with an equal volume of phosphate-buffered saline (PBS) or heparinized media without or with recombinant lipases from transfected HEK293 cells (as prepared above), plus FAF-BSA to a final concentration of 0.2% w/v. The mixture was incubated for 4 h at 37°C. Total free fatty acids (FFA) generated by the hydrolysis of lipoproteins were measured using a commercial kit (Wako Diagnostics). In addition, lipids were extracted from 10 μl of lipoprotein-media mixtures, in the presence of 0.5 μg triheptadecenoin (Nu-Chek Prep, Elysian, MN, USA), 5 μg diarachidoylphosphocholine (Avanti Polar Lipids, Alabaster, AL, USA), and 1 μg arachidic acid (Nu-Chek Prep) as internal standards, using the Bligh-Dyer method [30]; individual species of TAG and phosphatidylcholine (PtdCho) were quantified using direct injection tandem electrospray ionization mass spectrometry (ESI-MS) methods as previously described [31-33]. FFA were derivitized into pentafluorobenzyl esters and quantified by selective ion monitoring using gas chromatography-mass spectrometry (GC-MS) as previously described [34]. Following 1 h incubations with THL (described above), differentiated THP-1 cells were incubated for 30 min with RPMI containing 1% A/A, 25 μg/ml THL, and hydrolysis products at 0.9 mM (based on pre-hydrolysis PL). Following incubation with hydrolysis products, cells were washed three times with ice-cold phosphate buffered saline then lysed on ice for 5 min with 1× Cell Lysis Buffer (#9803, Cell Signaling Technology, Danvers, MA, USA). The lysed cell mixtures were centrifuged at 14,000 × *g* for 10 min at 4°C. The protein content of the supernatant was measured using a bicinchoninic acid assay [35]. The supernatant was divided into aliquots and stored at −80°C.

THP-1 cell incubations with fatty acids

Myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6) were purchased from Nu-Chek Prep and dissolved at 10 mg/ml in high performance liquid chromatography grade methanol and stored at −20°C under $N_{2(g)}$ until needed. To prepare 1 ml of media with fatty acids for cell culture, 37.3 nmol 14:0, 553.0 nmol 16:0, 47.7 nmol 16:1, 91.3 nmol 18:0, 486.1 nmol 18:1, 140.8 nmol 18:2, 1.7 nmol 20:4, and 0.8 nmol 22:6 were removed from stock solutions and methanol was evaporated under $N_{2(g)}$ at 35°C; fatty acids were resuspended with 10 μl dimethylsulfoxide. The fatty acid-dimethylsulfoxide mixture or 10 μl dimethylsulfoxide (as a vehicle control) was added at a rate of 1 μl/min to RMPI containing 0.2% w/v FAFBSA, 1% A/A, and 25 μg/ml THL while continuously vortexing. Control media or FFA containing media was added to differentiated THP-1 cells that were pre-treated with THL, as described above. Following a 30 min incubation with

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control or fatty acids containing media, cells were washed, lysed, and cell protein was quantified, as described above. Cell lysates were divided into aliquots and stored at −80°C.

Antibody arrays

Antibody arrays were carried out using the PathScan RTK Signaling Antibody Array Kit (#7982, Cell Signaling Technology). Each cell extract was diluted to 250 μg/ml and applied to its own multiplexed array. Chemiluminescence from the arrays were captured with film or digitally using an ImageQuant LAS 4000 system (GE Healthcare), and the intensity for each signal in the array was quantified by scanning densitometry using Image J software [36]; the intensity from the negative control within each array was subtracted from all signals, and all data from each array were normalized to the internal positive control within each array.

Statistical analyses

Where indicated, data were analyzed using either an unpaired t-test (t-test) or a one-way analysis of variance (ANOVA) with a 95% confidence interval, followed by a comparison of groups using a Bonferroni Test.

Results

We used conditioned media containing LPL, EL, or no lipase to hydrolyse total lipoproteins isolated from fasted human plasma, and we incubated THP-1 macrophages with the hydrolysis products to determine how LPL and EL might influence macrophage cell signalling pathways. Triplicate experiments showed that conditioned media from HEK293 cells expressing LPL generated significantly greater quantities of FFA during the hydrolysis of 2.0 mM (by PL) total lipoproteins over 4 h at 37°C versus media from cells expressing EL (Fig. 1C). Despite this difference in hydrolysis, the levels of lipase transcripts were not significantly different between LPL- and EL-transfected cells from these experiments (Fig. 1B). Furthermore, these transcripts were shown to be actively translated as shown using immunoblots (Fig 1A). Conditioned media from cells expressing LPL or EL liberated significantly more FFA than media from mock transfected cells (Fig. 1C). Cells that were mock transfected with no DNA or transfected with empty vector displayed no difference in lipoprotein hydrolysis (Supplementary Fig. 1).

Our antibody array study showed that a 30 min incubation of THP-1 macrophages with total lipoprotein hydrolysis products liberated by LPL or EL significantly modulated signalling pathways (Fig. 2). Across independent experiments, the hydrolysis products generated by LPL significantly increased the phosphorylation of the RTKs anaplastic lymphoma kinase (ALK), macrophage-colony stimulating factor receptor (M-CSFR), platelet derived growth factor receptor (PDGFR), tropomyosin-related kinase (Trk) A, TrkB, and vascular endothelial growth factor receptor 2 (VEGFR2); hydrolysis products generated by LPL also significantly increased the phosphorylation of the signalling nodes Akt – both threonine-308 and serine 473, c-Abl, and Stat1. On the other hand, the hydrolysis products generated by EL significantly increased the phosphorylation of only TrkA. The phosphorylation of 8 signalling nodes and 22 RTKs was not significantly affected in THP-1 macrophages by LPL and EL hydrolysis products (Supplementary Table 1).

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We carried out shotgun lipidomic analyses of our hydrolysis reactions to provide some details of the TAG and PtdCho species that were hydrolysed, as well as GC-MS analyses to provide some details of the FFA liberated by LPL and EL (Fig. 3). LPL significantly reduced the levels of 12 of the 17 TAG species assessed versus the TAG levels from non-lipase media incubations, whereas EL modestly but significantly reduced the levels of the 50:3 and 54:3 species of TAG (Fig. 3A). Seven PtdCho species were commonly and significantly hydrolysed by LPL and EL, but an additional species (18:0-22:6) was significantly reduced only by LPL (Fig. 3B). Stearic acid (18:0) was a common hydrolysis product significantly liberated by LPL and EL, with palmitic acid (16:0) also being significantly liberated by LPL but approaching significant liberation (p=0.07) by EL; six additional FFA were significantly observed after only the hydrolysis of lipoproteins by LPL (Fig. 3C). These data further highlight the specificities of LPL and, such that FFA derived from LPL hydrolysis appears to be predominantly derived from TAG pools while FFA derived from EL hydrolysis appears to be selectively derived from PtdCho hydrolysis. No appreciable changes were observed to species of TAG and PtdCho between hydrolysis reactions in the presence of either mock-transfected media or PBS, demonstrating that no significant lipase activity is released by HEK293 cells (Supplementary Fig. 2A and 2B). The lack of hydrolysis of total lipoprotein TAG and PtdCho in the presence of either mock-transfected media or PBS suggest that the source for the observed very modest increases of select species of FFA in mock-transfected media (Supplementary Fig. 2C) is derived from the HEK293 cells.

Because the hydrolysis of total lipoproteins by LPL lead to products that appeared to have a more profound effect on the phosphorylation of RTKs and signalling nodes than EL, we tested the effects on the phosphorylation of RTKs and signalling nodes by a mixture of purified FFA species that matched the concentrations from the total lipoprotein hydrolysis by LPL that were applied to THP-1 macrophages in Fig. 2. In comparison to what we observed for total lipoprotein hydrolysis products by LPL, following a 30 min incubation of either a vehicle control or the FFA mixture with THP-1 macrophages, no phosphorylation of M-CSFR, PDGFR, Stat1, TrkA, TrkB, and VEGFR2 was observed by antibody array analyses. We also observed no differences in the phosphorylation of ALK and c-Abl between the vehicle control and FFA treated cells (data not shown). However, as shown in Fig. 4, we saw a significant increase in the phosphorylation of Akt in the FFA treated macrophages versus control macrophages. Thus, while other lipoprotein components and hydrolysis products generated by LPL may influence signalling molecules within THP-1 macrophages, the mixture of FFA liberated from total lipoproteins by LPL is responsible for influencing the phosphorylation of Akt.

Discussion

We show for the first time using antibody arrays that the hydrolysis of total lipoproteins by LPL or EL – two cell surface associated lipases with opposing substrate specificities – can raise the levels of phosphorylated signalling molecules in THP-1 macrophages. The hydrolysis of total lipoproteins was investigated in our studies over the assessment of individually isolated lipoproteins in order to better mimic the *in vivo* pool of lipoproteins that may be seen by macrophages. Since the human pool of lipoproteins is enriched in

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apolipoprotein (apo) B lipoproteins that are preferentially hydrolysed by LPL [37], we expected and observed that unlike EL, LPL would more effectively hydrolyse total lipoproteins and lead to a maximal number of cell signalling pathways modulated. Hepatic lipase is a lipase family member that is closely associated with LPL and EL but exhibits no distinct preference for either TAG or PL (reviewed in [37]); although not tested in the present study but of future interest, it may elicit an intermediate effect on cell signalling pathways.

The hydrolysis mixture generated from the hydrolysis of total lipoproteins by LPL had their most profound effect on the phosphorylation of PDGFR by 4-fold within THP-1 macrophages in our study. PDGFR was shown to be expressed in monocyte-derived macrophages, and the stimulation of its expression leads to a 2-fold increase in cell migration in response to platelet derived growth factor (PDGF) [38]. Interestingly, the expression of LPL in human monocyte-derived macrophages can be raised by PDGF through a protein kinase C mediated process [39], and the expression of PDGF in mesangial cells was previously shown to be driven in part by the presence of LPL and very low-density lipoprotein [40]. These previous observations, combined with our observation that LPL hydrolysis products increase PDGFR phosphorylation, suggest that a pathogenic paracrine system might exist in atherosclerotic lesion development that warrants future study, whereby LPL raises phosphorylated PDGFR and the expression of PDGF, and in turn PDGF increases LPL expression.

The hydrolysis mixture generated from the hydrolysis of total lipoproteins by LPL also increased the phosphorylation of additional signalling molecules in THP-1 macrophages (Fig. 2). The normophysiologic functions of ALK are unclear, however its activation is believed to negatively influence apoptosis [41]. The activation of c-Abl was previously shown to increase the migratory activity of both macrophages and neutrophils [42]. M-CSFR activation via colony stimulating factor-1 increases monocyte and macrophage recruitment, macrophage adhesion molecule expression, and atherosclerotic lesion formation [43]; M-CSFR activation also increases the production of pro-inflammatory cytokines [43, 44]. Stat1 is required to induce the apoptosis of macrophages in advanced atherosclerotic lesions [45]. The activation of VEGFR2 leads to increased cell migration and cell survival [46]. The heterozygous expression of TrkB in apoE-null mice was shown to reduce atherosclerotic lesion size versus the homozygous expression of TrkB [47]. The phosphorylation of TrkA was increased by the hydrolysis mixtures generated from the hydrolysis of total lipoproteins by both LPL and EL; a clear role for TrkA remains to be identified in macrophages, but it was previously reported that TrkA stimulates vascular smooth muscle cell proliferation and migration [48]. Taken together, the identified pathways that are modulated in THP-1 macrophages by the hydrolysis of total lipoproteins via LPL suggest that they might play a role in the early development of atherosclerosis.

Unlike our analyses of the effects on the phosphorylation of RTKs and signalling nodes by the hydrolysis mixture generated from the hydrolysis of total lipoproteins via LPL, no phosphorylation of M-CSFR, PDGFR, Stat1, TrkA, TrkB, and VEGFR2 was observed in THP-1 macrophages with either a mixture of purified FFA species (that matched the concentrations observed from the total lipoprotein hydrolysis by LPL) or a vehicle control.

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This suggests that protein or non-FFA lipid components associated with total lipoproteins might stimulate the phosphorylation of these molecules, and that the level of phosphorylation is augmented by LPL hydrolysis, possibly by enabling the hydrolysed lipoproteins to interact with the macrophages more effectively due to a reduced size that could reveal a cryptic ligand on the lipoprotein. Similarly, our data show that the phosphorylation of ALK and c-Abl is not affected by FFAs, thus suggesting they are modulated by protein or lipid components that are liberated from total lipoproteins following LPL hydrolysis. Future lipidomic and proteomic studies would be of importance toward identifying the molecules responsible for affecting these signalling molecules.

We show that the mixture of FFAs liberated from total lipoproteins by LPL specifically and uniquely induced the phosphorylation of Akt at both threonine-308 and serine-473. Akt is a member of the phosphoinositide 3-kinase pathway that is activated by membrane associated phosphatidylinositol (3,4,5)-triphosphate (PIP3) [49]. Akt functions to maintain cell growth and survival under normophysiologic conditions [49], however its role in atherosclerosis is unclear. A homozygous deficiency of Akt in apoE-null mice leads to severe atherosclerosis and plaque instability while reducing vascular smooth muscle cell migration and proliferation [50, 51]. However, apoE-null mice exhibit high expression levels of phosphorylated Akt and larger plaque sizes versus apoE-null mice with a homozygous deletion of the p110γ subunit of phosphoinositide 3-kinase, which prevented the phosphorylation of Akt [52]. We speculate that the FFAs liberated by LPL might influence the cell membrane to allow for the presence of more PIP3, or that one or more of the FFAs rapidly become incorporated into PIP3 to generate a ‘preferential’ molecular species of PIP3 that leads to Akt phosphorylation. Future work is necessary toward understanding the mechanisms behind how FFAs liberated by LPL stimulate Akt phosphorylation, and the subsequent roles of phosphorylated Akt in the early and late stages in foam cell formation and atherosclerosis.

While macrophage LPL is clearly associated with atherosclerosis [18-21] and our study identifies nine signalling molecules that may mechanistically tie LPL with atherosclerosis, we only observe an association of EL with TrkA, which does not have a clearly defined role in macrophages. Because we chose a 30 minute time point to assess changes to signalling molecules, we cannot exclude the possibility that EL, as well as LPL, might influence the phosphorylation of additional signalling molecules that could be observed during a time course using multiple antibody arrays. Studies addressing the role of EL in atherosclerosis are unclear. EL overexpression leads to molecular events that are both beneficial (such as raised selective uptake and cholesterol efflux) and adverse (such as increased monocyte adhesion, increased expression of pro-inflammatory cytokines, and decreased expression of anti-inflammatory cytokines) (reviewed in [37]). In addition, inhibition of the proprotein convertase inactivation of EL in mice lowers macrophage-to-feces reverse cholesterol transport [53]. Elevated plasma levels of EL in humans are positively associated with coronary artery calcification [54], and apoB-depleted sera from loss of function variants for EL in humans exhibit an increased cholesterol efflux capacity [55]. However, animal studies in EL-null mice have shown either no change in macrophage-to-feces reverse cholesterol transport [56] or a modest increase in reverse cholesterol transport [57]; furthermore, conflicting studies about lesion development in EL-null mice on an apoE-null background

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describe either reduced lesion development [58] or no effect [59]. Thus, the lack of a definitive role for EL in atherosclerosis may suggest that a link between TrkA and EL may not be associated with atherosclerosis, but rather another yet to be identified function.

In summary, we showed that the hydrolysis of total lipoproteins by LPL and EL influence cell signalling molecules in THP-1 macrophages, and that the FFAs liberated by LPL stimulate the phosphorylation of Akt. The mechanisms remain to be investigated; these might include direct associations of one or more hydrolysis products with signalling molecules, and regionalized alterations to cell membrane structure that may affect signalling cascades. Future studies toward identifying which hydrolysis products are responsible for modulating cell signalling, and how these products may acutely or chronically affect atherosclerosis, will contribute to defining how LPL and EL influence atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A/A	Antibiotic/antimycotic
ALK	Anaplastic lymphoma kinase
ANOVA	Analysis of variance
Apo	Apolipoprotein
EL	Endothelial lipase
ESI-MS	Electrospray ionization-mass spectrometry
FAF-BSA	Fatty acid free bovine serum albumin
FFA	Free fatty acid
GC-MS	Gas chromatography-mass spectrometry
LPL	Lipoprotein lipase
M-CSFR	Macrophage colony stimulating factor receptor
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor

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PDGFR	Platelet derived growth factor receptor
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PL	Phospholipid
PMA	Phorbol 12-myristate-13-acetate
PtdCho	Phosphatidylcholine
RTK	Receptor tyrosine kinase
Stat1	Signal transducer and activator of transcription factor 1
TAG	Triacylglycerol
THL	Tetrahydrolipstatin
Trk	Tropomyosin-related kinase
VEGFR2	Vascular endothelial growth factor receptor 2

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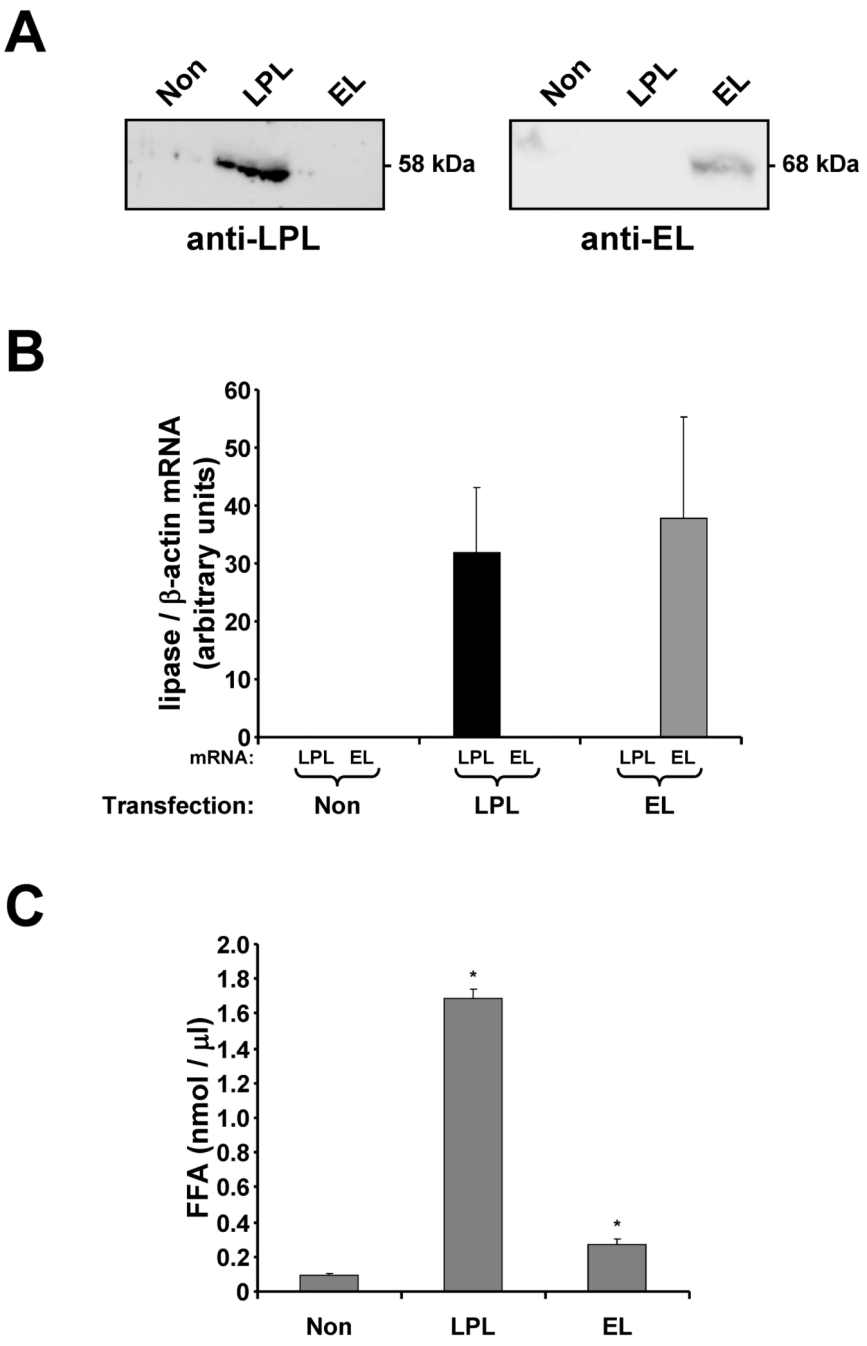

Fig. 1.

Expression of LPL and EL, and their hydrolysis of total lipoproteins. (A) Immunoblot analyses for LPL or EL in heparinized media from transfected HEK293 cells expressing no lipase (Non), LPL, or EL. Data are representative of three independent experiments. (B) Real-time PCR analyses of total RNA from transfected HEK293 cells expressing no lipase (Non), LPL, or EL to quantify LPL and EL expression. The average means of data ±SD from independent experiments (*n*=3) are shown. (C) Quantification of free fatty acids (FFA) released from the hydrolysis of total lipoproteins (*d*<1.21 g/ml, 2.0 mM by PL) over 4h at

37°C by heparinized media from transfected HEK293 cells expressing no lipase (Non), LPL, or EL. The average means of data ±SD from independent experiments (n=3) are shown. *p<0.01 versus Non (ANOVA).

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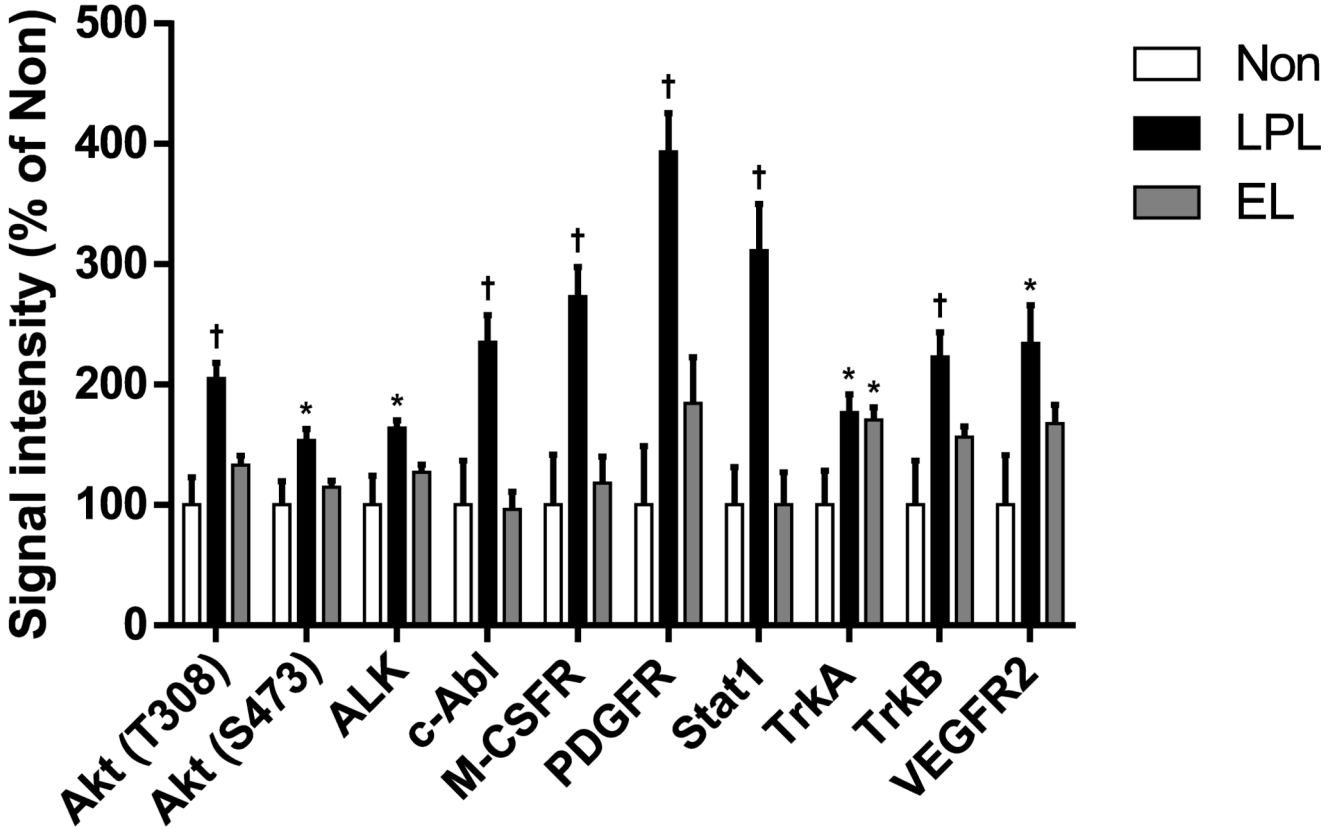

Fig. 2.

Signalling molecules in THP-1 macrophages modulated by LPL and EL hydrolysis products. Total lipoproteins (*d*<1.21 g/ml, 2.0 mM by PL) were hydrolyzed for 4h at 37°C by recombinant LPL or EL, or non-lipase (Non) mock transfected media from HEK293 cells. Hydrolysis products (0.9 mM by pre-hydrolyzed PL) were added to THP-1 macrophages for 30 min. Cell lysates were assessed for phosphorylated signalling molecules using a commercial antibody array. Signal intensities normalized to an internal control within the array were collected by scanning densitometry, and expressed as a percent of Non. The average means of data ±SE from independent experiments (*n*=6) are shown. *$p<0.05$ versus Non, †$p<0.01$ versus Non (ANOVA).

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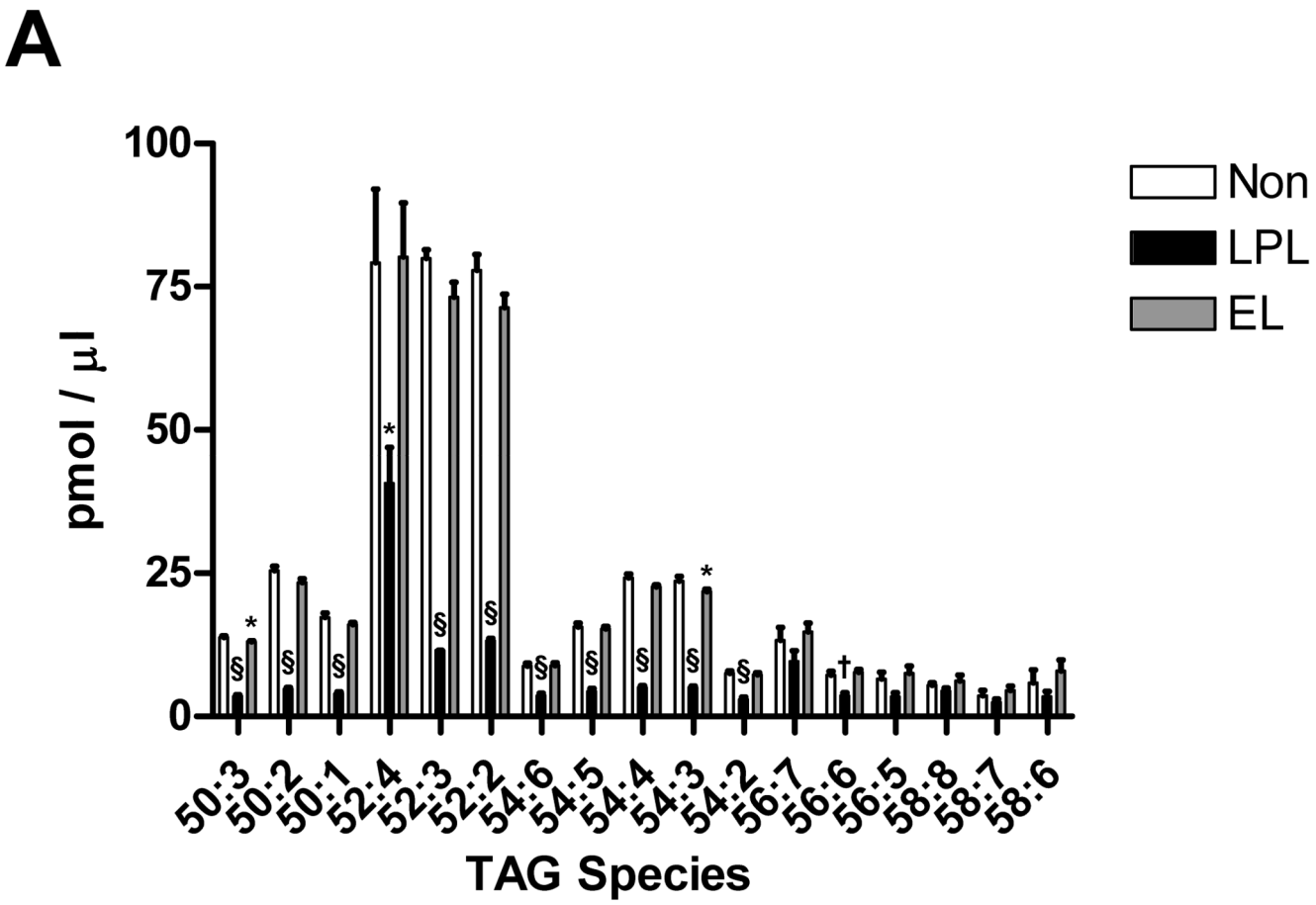

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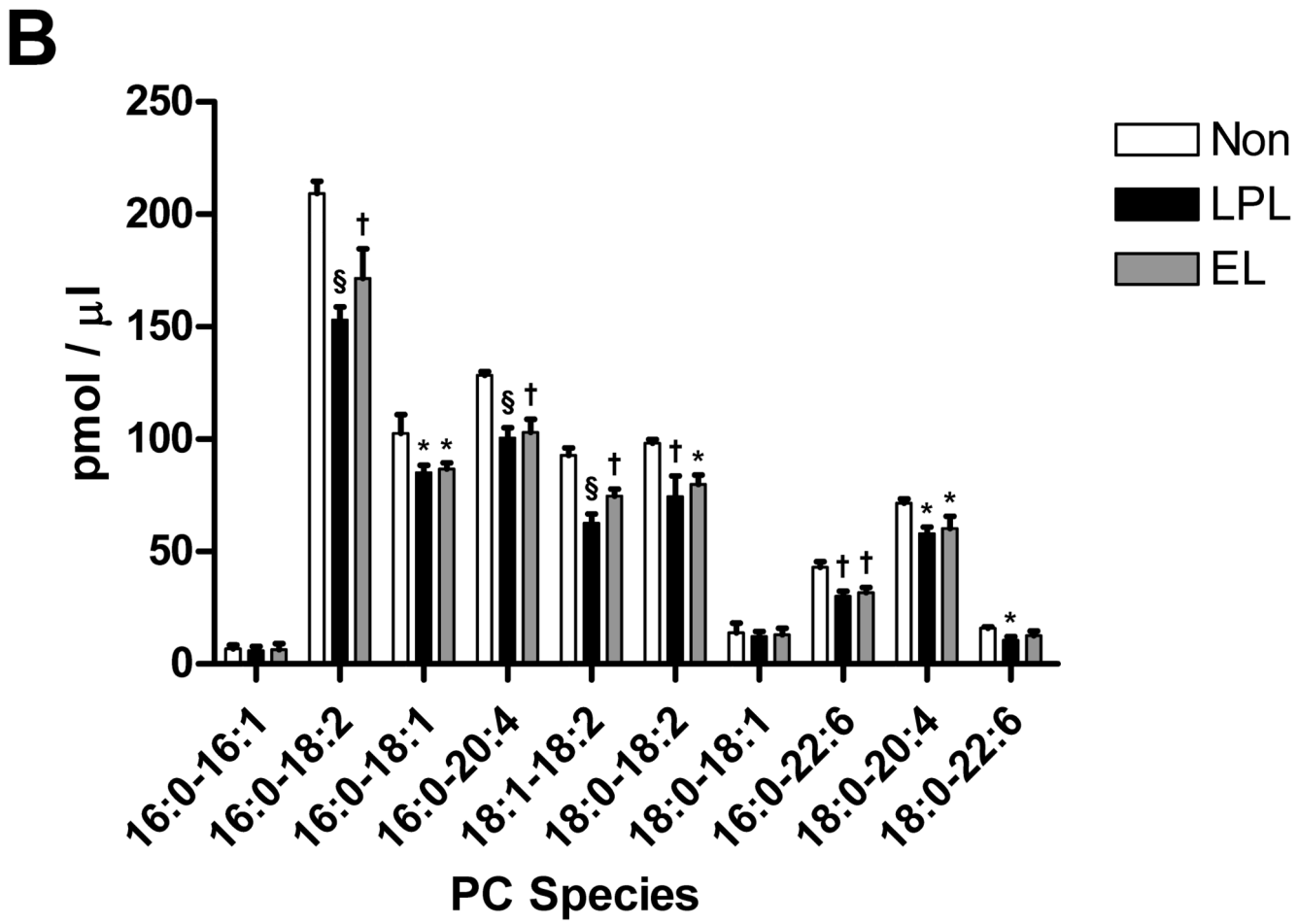

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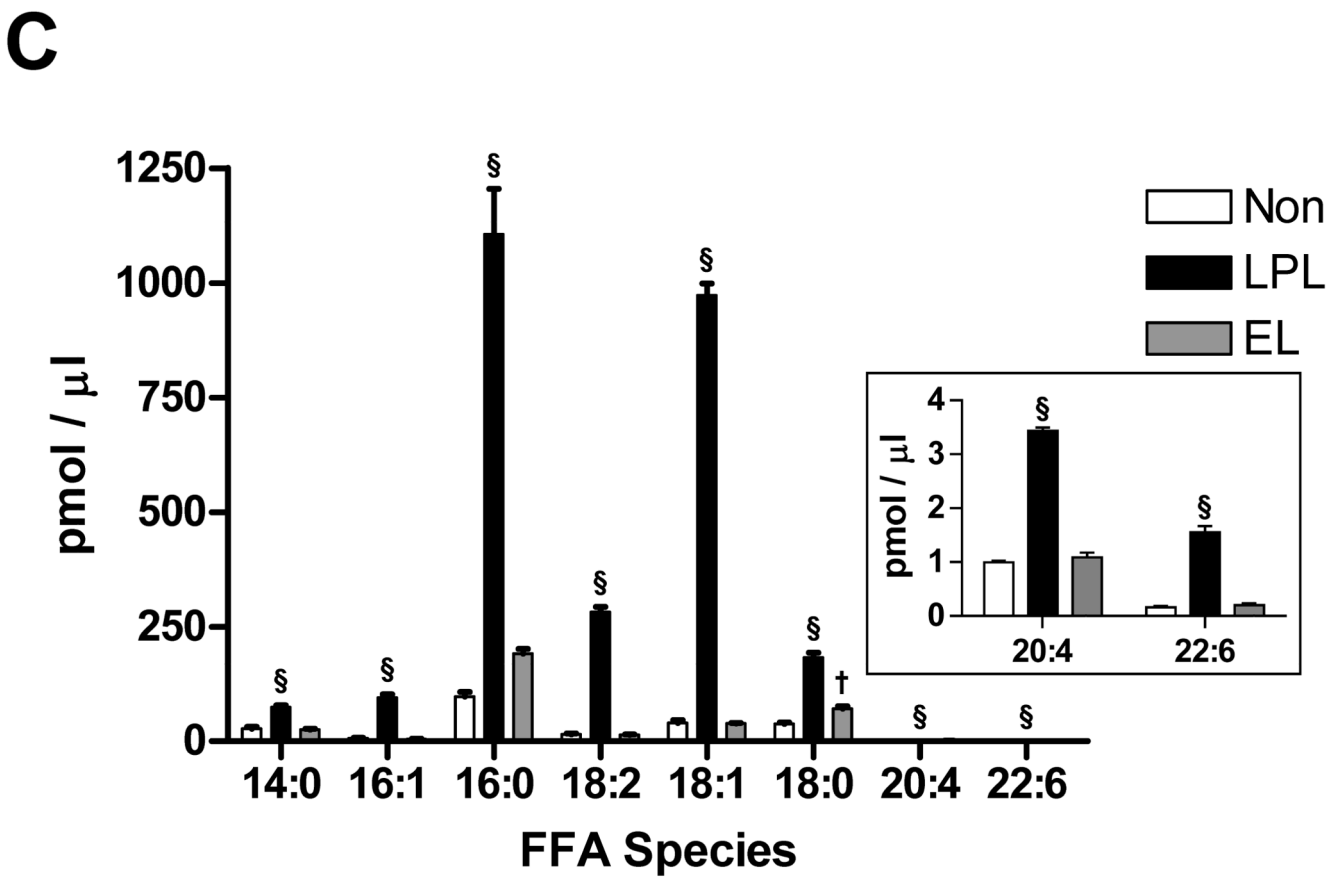

Fig. 3.

Triacylglycerol, phosphatidylcholine, and free fatty acid species levels following total lipoprotein hydrolysis by heparinized media from LPL, EL, and no-lipase expressing HEK293 cells. Total lipoproteins (*d*<1.21 g/ml, 2.0 mM by PL) were hydrolyzed for 4h at 37°C by recombinant LPL or EL, or non-lipase (Non) mock transfected media from HEK293 cells. Lipids were extracted from independent experiments (*n*=3) and subjected to mass spectrometric analyses. The average means of data ±SD are shown. (A) Triacylglycerols (TAG) were quantified with triheptadecenoin as an internal control by measuring $[M+Na]^+$ using ESI-MS between *m/z* 800-950. (B) Sodiated phosphatidylcholines (PC) were quantified with diarachidoylphosphocholine as an internal control by assessing the neutral loss of choline (*m/z* 59.1) in positive ion mode using tandem ESI-MS. (C) Pentafluorobenzyl-derivitized free fatty acids (FFA) were quantified with arachidic acid as an internal control by selective ion monitoring using GC-MS; *inset*: 20:4 and 22:6 FFA. *p<0.05 versus Non, $^{\dagger}p$<0.01 versus Non, $^{\S}p$<0.001 versus Non (ANOVA).

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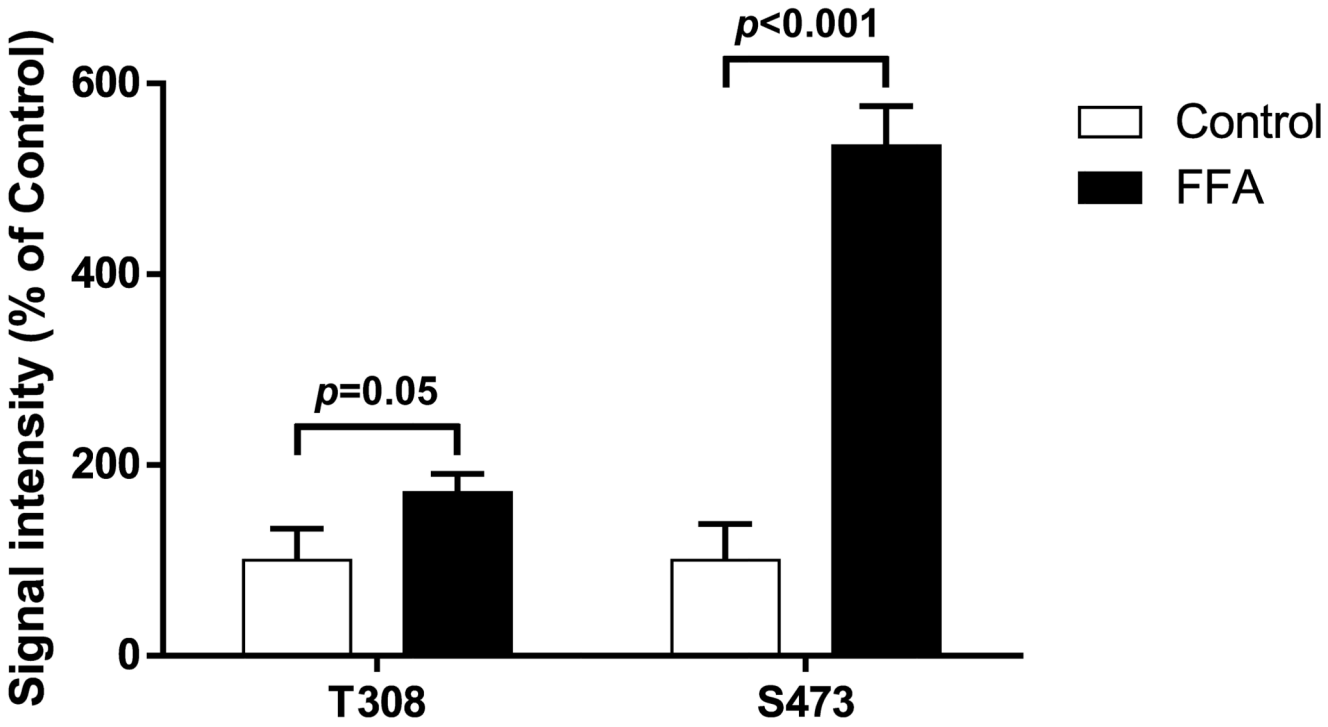

Fig. 4.

Modulation of Akt by the free fatty acids liberated by LPL. Vehicle control (Control) media and free fatty acid (FFA) containing media were prepared in RPMI with 0.2% w/v BSA as described under Materials and Methods. Media were added to THP-1 macrophages for 30 min. Cell lysates were assessed for phosphorylated signalling molecules using a commercial antibody array; data for Akt are shown. Signal intensities normalized to an internal control within the array were collected by scanning densitometry, and expressed as a percent of Control. The average means of data ±SE from independent experiments (n=4) are shown. Statistical analysis was performed using a t-test.