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Lysophosphatidylcholines activate PPARδ and protect human skeletal muscle cells from lipotoxicity

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ABSTRACT

Metabolomics studies of human plasma demonstrate a correlation of lower plasma lysophosphatidylcholines (LPC) concentrations with insulin resistance, obesity, and inflammation. This relationship is not unraveled on a molecular level. Here we investigated the effects of the abundant LPC(16:0) and LPC(18:1) on human skeletal muscle cells differentiated to myotubes. Transcriptome analysis of human myotubes treated with 10 μM LPC for 24 h revealed enrichment of up-regulated peroxisome proliferator-activated receptor (PPAR) target transcripts, including *ANGPTL4*, *PDK4*, *PLIN2*, and *CPT1A*. The increase in both *PDK4* and *ANGPTL4* RNA expression was abolished in the presence of either PPARδ antagonist GSK0660 or GSK3787. The induction of *PDK4* by LPCs was blocked with siRNA against *PPARD*. The activation of PPARδ transcriptional activity by LPC was shown as PPARδ-dependent luciferase reporter gene expression and enhanced DNA binding of the PPARδ/RXR dimer. On a functional level, further results show that the LPC-mediated activation of PPARδ can reduce fatty acid-induced inflammation and ER stress in human skeletal muscle cells. The protective effect of LPC was prevented in the presence of the PPARδ antagonist GSK0660. Taking together, LPCs can activate PPARδ, which is consistent with the association of high plasma LPC levels and PPARδ-dependent anti-diabetic and anti-inflammatory effects.

1. Introduction

The detection of biomarkers in human plasma in order to early diagnose the prediabetic state or to predict the onset of type 2 diabetes became an important tool in diabetes research, where early diagnosis plays a pivotal role to prevent the manifestation of the disease or to delay secondary complications. Among other candidates [1], lysophosphatidylcholines (LPC) gained particular attention as potential biomarkers for identifying people at risk to develop diabetes or to even predict diabetes at early stages. Notably, lower plasma concentrations of long-chain acyl LPC species, in particular LPC(16:0), LPC(16:1), LPC(18:2), and LPC(20:0) were associated with insulin resistance, obesity, type 2 diabetes [2–5], and with an increased risk of developing type 2 diabetes [6,7].

LPC belong to the class of glycerophospholipids. They represent the major lysophospholipids in plasma with a concentration ranging from 100 to 300 μM [8–10]. The most abundant LPC in human plasma is LPC(16:0) representing approximately 50% of total plasma LPC followed by LPC(18:0) and LPC(18:1) [9,11]. Major parts of LPC are bound by serum proteins such as serum albumin and α1-acid glycoprotein that serve as vehicles [12]. A minor pool of LPC is carried by lipoproteins such as LDL, VLDL, and HDL [8,10].

Relevant amounts of plasma LPC are formed from membranes and lipoproteins by classes of A_1 and A_2 phospholipases (PLA_1 and PLA_2) using phosphatidylcholines (PC) as substrates [13]. Other enzymes contributing to plasma LPC formation are endothelial lipase and lecithin-cholesterol acyltransferase [14]. Several PLA_2 isoforms act intracellularly and hepatic secretion of LPC has been shown to be an important source of plasma LPC in rodents [15]. The acylation of LPC to PC by LPC acyltransferases (LPCAT) also regulates intrahepatic and plasma LPC levels [16].

The mechanisms leading to the change in circulating LPC levels in the prediabetic state are not resolved, but may involve inflammatory processes in the liver [17,18]. The association of high plasma LPC levels with low concentrations of inflammatory markers CRP, TNFα, and

Abbreviations: ANGPTL4, angiopoietin-like 4; ATF3, activating transcription factor 3; CK, creatine kinase; CPT1A, carnitine palmitoyltransferase IA; CXCL3, chemokine (C-X-C motif) ligand 3; ER stress, endoplasmic reticulum stress; αGPC, α-glycerophosphorylcholine; IL6, interleukin 6; $iPLA_2$, calcium independent phospholipase A2; LDH, lactate dehydrogenase; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; PC, phosphatidylcholine; PDK4, pyruvate dehydrogenase kinase 4; PLA_2, phospholipase A_2; PLIN2, perilipin 2; PPAR, peroxisome proliferator-activated receptor; RXRα, retinoid X receptor α; XBP1, X-box binding protein 1.

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MCP1 can also point to a potential anti-inflammatory effect of LPC [19]. LPC can act as signaling lipids with a wide range of cellular effects. Foremost among these are pathways involved in the regulation of immunologic and inflammatory events [20–22]. Moreover, LPC administration lowers blood glucose levels in normal and diabetic mice and stimulates glucose uptake in adipocytes [23]. On the other hand, exogenous LPCs have been reported to inhibit insulin signaling in skeletal muscle cells [24] and to induce lipoapoptosis in hepatocytes [25].

To summarize, the relation of reduced LPC plasma levels, the increased production of inflammatory cytokines and the development of type 2 diabetes is not unraveled on a molecular level. We hypothesize that a reduced LPC plasma concentration in the pre-diabetic state can contribute to the over-activation of inflammatory processes and fortify the development of type 2 diabetes. To this end, we investigated the effect of two abundant LPC species, LPC(16:0) and LPC(18:1) on human skeletal muscle cells. Both LPC species are highly abundant in plasma and their plasma concentrations are correlated positively with insulin sensitivity and negatively with circulating CRP levels [17]. Alterations in skeletal muscle metabolism and function may have great impact on whole body glucose tolerance and insulin sensitivity [26]. Based on whole genome expression analysis of human skeletal muscle cells, we identified LPC as activators of the transcription factor peroxisome proliferator-activated receptor (PPAR)δ and studied potential metabolic and anti-inflammatory consequences.

2. Material and methods

2.1. Materials

Palmitic acid, oleic acid, WY14643, albumin solution from bovine serum Fraction V 10% in DPBS, L-α-glycerophosphorylcholine (αGPC) from soybean, 1,2-dioleyl-sn-glycero-3-phosphocholine (PC(18:1/18:1)), 1,2-dipalmitoyl-glycero-3-phosphocholine (PC(16:0/16:0)), 1-*O*-palmityl-2-*O*-methyl-rac-glycero-3-phosphocholine (PC(O-16:0/O-1:0), 1-palmitoyl-sn-glycero-3-phosphocholine (LPC(16:0)), 1-oleoyl-sn-glycero-3-phosphocholine (LPC(18:1)), and monoclonal mouse antibody against MyHC (slow) clone NOQ7.5.4D were obtained from Sigma-Aldrich (Taufkirchen, Germany). Purity of LPC is given as ≥99% (TLC) by the supplier. GW501516 was purchased from Santa Cruz (Dallas, USA). GSK0660 and GSK3787 were from TOCRIS (Bristol, UK). Bromoenol lactone and FKGK11 were from Cayman Chemicals (Ann Arbor, Michigan, USA). FBS was supplied by Biochrom (Berlin, Germany). Polyclonal rabbit antibodies against phospho-ACC (ser79), monoclonal rabbit antbodies against AMPK α1, and Immobilion Western Chemiluminescence HRP Substrate Luminol reagent were obtained from Merck Millipore (Darmstadt, Germany). Monoclonal rabbit antibodies against phospho-AMPKα (thr172) and polyclonal rabbit phospho-Akt (ser473) were purchased from Cell Signaling Technologies (Cambridge, UK). Monoclonal mouse antibodies against Akt (clone 55) were from BD bioscience (San Jose, California, USA). Media and reagents for cell culture were from LONZA (Basel, Switzerland) if not stated otherwise.

2.2. Cell culture

Primary skeletal muscle cells were obtained from percutaneous needle biopsies of the lateral portion of quadriceps femoris (vastus lateralis) muscle of non-diabetic subjects. The donors gave informed written consent to the study. The Ethical Committee of the Tübingen University Medical Department had approved the protocol. Cells were grown in a 1:1 mixture of α-MEM and Ham's F-12 supplemented with 20% FBS, 1% chicken embryo extract (Seralab, Haywards Heath, UK), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, and 0.5 g/mL amphotericin B (Sigma-Aldrich, Taufkirchen, Germany) till 70–80% confluency. Cells were fused for 5–8 days to myotubes in α-MEM containing 2% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.5 g/mL amphotericin B. Experiments were performed in EMEM containing 2% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.5 g/mL amphotericin B.

LPC and PC were dissolved in ethanol, αGPC was dissolved in water, and GW501516, WY14643, GSK0660, and GSK3787 in DMSO. Palmitate was dissolved in ethanol and complexed at a concentration of 6 mM with 10% BSA (fatty acid-free) in PBS for 24 h at 37 °C under mild agitation (molar ratio palmitate/BSA 4:1, end concentration of BSA 0.4%).

2.3. Lactate dehydrogenase and creatine kinase measurements

Cell supernatant was centrifuged for 4 min with 13,000 rpm at 4 °C. Enzymatic activities from cell supernatants were analyzed with the ADVIA 1800 clinical chemical analyzer (Siemens Healthcare Diagnostics, Fernwald, Germany).

2.4. RNA interference

Control siRNA against luciferase 5′-CGUACGCGGAAUACUUCGA-3′ was purchased from eurofins MWG operon (Eberberg, Germany). siGENOME SMART pool siRNA against *PPARD*, and *LPCAT* was purchased from Thermo Scientific (Karlsruhe, Germany). Differentiated human myotubes were transfected in 6-wells with 20 nM siRNA in fusion media using ViromerBlue reagent obtained from Lipocalyx (Halle, Germany). Transfection was performed according to the manufacturer's protocol. 24 h afterwards medium was changed and the cells were stimulated as indicated.

2.5. Luciferase assay

LexA-PPARδ-LBD and LexA-BS plasmids were kind gifts from Prof. Dr. Rolf Müller, Philipps-University Marburg, Germany [27]. Human primary myoblasts were seeded in 12-wells. At 80% confluency the myoblasts were transfected using GeneCellin (BioCellChallenge SAS, Toulon, France) with 0.1 μg/mL LexA-PPARδ-LBD plasmid DNA or pcDNA3.1 empty vector together with 0.9 μg/mL LexA-BS plasmid DNA per well in fusion medium according to the manual. After 24 h medium was changed and the cells were subjected to stimulation. 24 h after stimulation, cells were lysed using Glo Lysis buffer (Promega, Mannheim, Germany) and luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany).

2.6. In vitro time-resolved fluorescence resonance energy transfer assay (TR-FRET)

Ligand binding was determined by TR-FRET in vitro [28] using the LanthaScreen PPARbeta/delta competitive binding assay (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Assays were carried out in 20 mM Tris pH 7.9, 100 mM KCl, 0.01% (v/v) Triton X-100, 5 mM dithiothreitol, and 1 g/L bovine serum albumin at room temperature and incubated for 30 min. GW501516 was dissolved in DMSO, αGPC was dissolved in water, and other substances were dissolved in ethanol at 10 mM stock concentration each. Measurements were performed on a VICTOR3 V Multilabel Counter (WALLAC 1420; Perkin Elmer Life and Analytical Sciences, Waltham, MA) with instrument settings as described in the manufacturer's instructions for LanthaScreen assays.

2.7. Gel shift assay

Oligonucleotides containing a consensus binding site for PPARδ target genes with type II response [29] 5′-GATAAGTAGGGGAAAGGTCA-3′ (TIB MOLBIOL, Berlin, Germany) including a 5′-overhang were annealed at 95 °C for 2 min and subjected to a fill-in reaction with [α-^{32}P]dATP (Hartmann Analytics, Braunschweig, Germany) and Klenow enzyme (New England Biolabs, Frankfurt am Main, Germany). After enzyme deactivation with 24 mM EDTA pH 8.0 and 20 min at 75 °C, oligo-DNA

was cleaned up by QIAquick Nucleotide removal kit (Qiagen, Hilden, Germany). Recombinant proteins PPARδ and RXRα were purchased from Cayman Chemicals (Ann Arbor, USA) and Active Motif (La Hulpe, Belgium), respectively. 37.5 ng of each protein were incubated for 30 min on ice in 5 ng/μL polydIdC, 10 mM Tris-HCl pH 8, 150 mM KCl, 0.05% (v/v) NP-40, 0.2 g/L BSA, 11.25% glycerol (ROTH, Karlsruhe, Germany) (modified from [30]). Ligands dissolved in ethanol or DMSO were added together with 20,000 cpm end-labeled oligonucleotide and the samples were incubated for further 30 min at room temperature. The samples were run on a 6% non-denaturating polyacrylamide gel in a buffer containing 25 mM Tris-HCl, pH 8.0, 190 mM glycine, and 1 mM EDTA. Gels were analyzed by phospho-imaging using FujiFilm Fluorescent Image Analyzer FLA-3000 (FujiFilm, Düsseldorf, Germany).

2.8. RT-PCR analysis

Cells were homogenized by QIAshredder (Qiagen, Hilden, Germany). Total RNA was extracted by RNeasy Mini-Kit (Qiagen). Reverse transcription of 1 μg of total RNA was performed in a volume of 20 μL using random hexamer primers with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. cDNA was amplified by QuantiFast SYBR

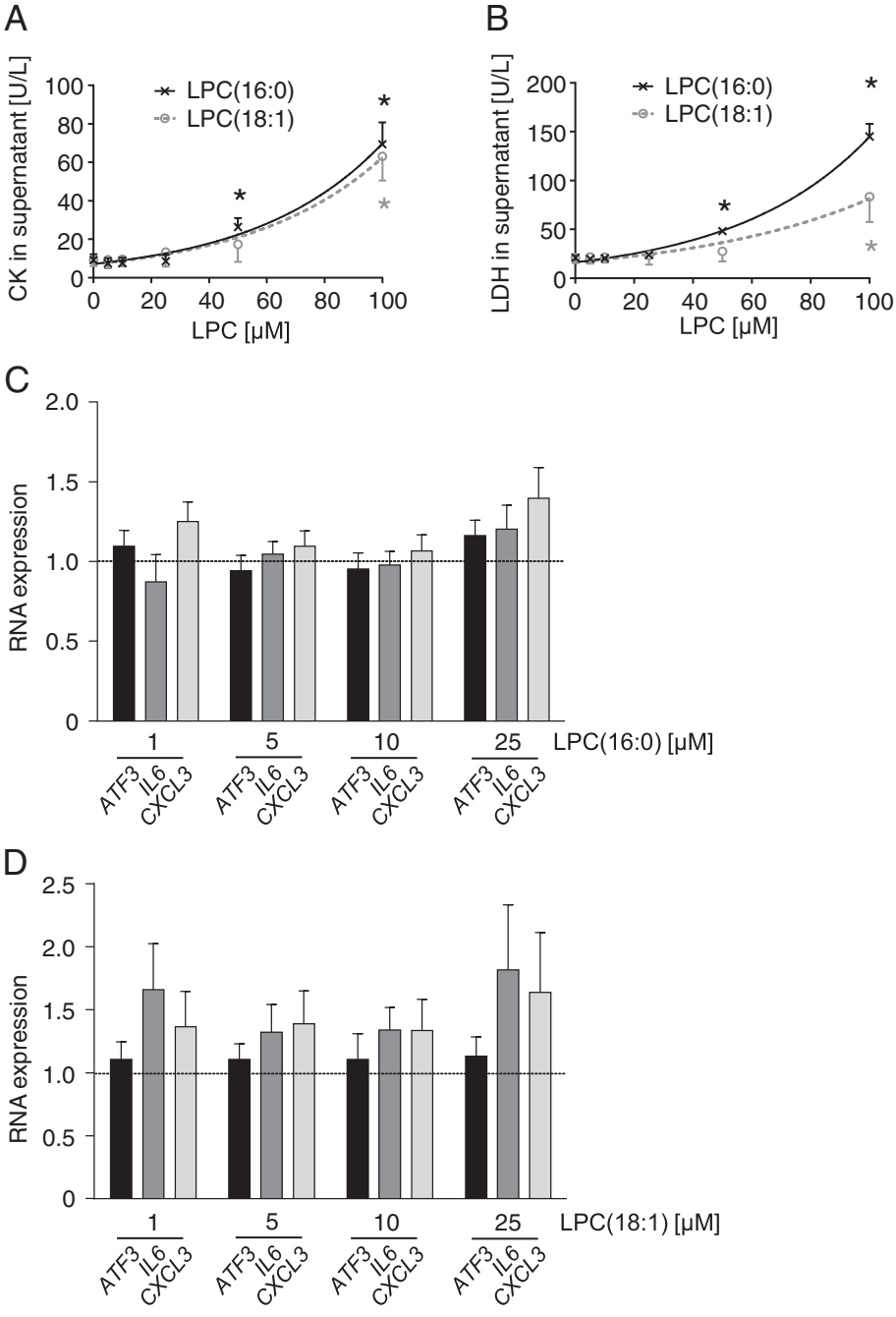

Fig. 1. Concentrations of LPCs up to 25 μM did not induce cell lysis or RNA expression of inflammatory cytokines. A,B: Human primary myotubes were stimulated with the indicated concentrations of LPC(16:0) and LPC(18:1) for 24 h. Release of myocellular proteins creatine kinase (CK) (A) and lactate dehydrogenase (LDH) (B) as markers of cell lysis was assessed in the supernatant by determination of CK and LDH enzyme activities and shown as [U/L]. Data points were fitted with an exponential model for LPC(16:0) (black solid line) and LPC(18:1) (grey dashed line) and are displayed as means ± SEM; * $p < 0.05$ vs. control cells without LPC treatment ($n = 6$). C,D: Induction of *IL6*, *ATF3*, and *CXCL3* gene expression was measured by qPCR after stimulation with the indicated concentrations of LPC(16:0) (C) or LPC(18:1) (D) for 24 h. Data are shown as fold change compared to cells without LPC treatment (set as 1 and indicated as dashed line) and are displayed as mean ± SEM ($n = 8$).

Green Master Mix (Qiagen) and detected by Roche LightCycler 480. Primers for *ACTB* (QT01680476), *ANGPTL4* (QT00003661), *ATF3* (QT00000273), *CXCL3* (QT00015442), *IL6* (QT00083720), *LPCAT1* (QT00057421), *LPCAT2* (QT00069419), *LPCAT3* (QT00094871), *PDK4* (QT00003325), and *PPARD* (QT00078064) were obtained from Qiagen. RNA expression levels were normalized to *ACTB* RNA levels.

2.9. Microarray analysis

Extracted RNA was further processed and analyzed on an Affymetrix human Genome U219 Genechip by the Microarray Genechip Facility Tübingen Service. The facility provided scaled, normalized, annotated and quality controlled data sets. (GEO Accession number GSE77337).

2.10. XBP1 splicing assay

For the *XBP1* amplification 0.1 μg total cDNA was subjected to PCR using the following primer pair: 5′-AAACAGAGTAGCAGCTCAGACTGC-3′ and 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′ (TIB MOLBIOL) amplifying the unspliced 474 bp and the spliced 448 bp fragment of *XBP1*. The PCR was performed at 94 °C for 4 min, followed by 35 cycles with 94 °C for 10 s, 66 °C for 30 s, and 72 °C for 30 s with a final step at 72 °C for 10 min. The two PCR products were differentiated by restriction enzyme digestion with *PST*I (Roche) for 1 h at 37 °C, since only the unspliced PCR product contains a *PST*I site resulting in two smaller fragments after digestion. The mixture containing the unspliced and cut *XBP1* fragments as well as the spliced and uncut *XBP1s* fragment was run on a 2% agarose gel (PeqLAB/VWR Erlangen, Germany). DNA bands were stained by GelRed (Biotium, Hayward, CA, USA).

2.11. Palmitate oxidation assay

[9,10-^{3}H]-palmitic acid was obtained from Perkin Elmer (Waltham, Massachusetts, USA). After pretreatment with 1 μM GW501516 or 10 μM LPC for 24 h, human myotubes were exposed to [9,10-^{3}H]-palmitic acid (33 μM palmitic acid with 0.5 μCi/mL) in EMEM for 4 h. The supernatant was transferred to OASIS HLB extraction cartridge 1 cm^3 30 mg (Waters, Milford, MA, USA) and assayed for tritiated water using liquid scintillation analyzer (TRI-CARB 2000TR, Perkin Elmer).

2.12. Western blotting

Cells were lysed with buffer containing (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, 100 mM NaF, 10.5 mM sodium pyrophosphate, 1 mM β-glycero phosphate disodium salt hydrate, 1 mM sodium orthovanadate). Protein were separated by sodium dodecyl sulfate (SDS) 5% polyacrylamide gel electrophoresis and transferred on nitrocellulose membranes via semi-dry blotting. Membranes were blocked in washing buffer consisting of 0.15 M NaCl, 5 mM EDTA, 50 mM Tris base pH 7.4, 0.05% (v/v) Triton X-100, 0.25% (w/v) gelatine. For probing, membranes were incubated over night with antibodies diluted 1:1000 in washing buffer at 4 °C under mild agitation. Secondary antibodies coupled to horse radish peroxidase were diluted 1:1000 in washing buffer and were applied to the membranes for 1 h at ambient temperature after washing with washing buffer. After another washing step, the peroxidase coupled antibodies were visualized by enhanced chemiluminescence solution and detected and digitalized via BioRad ChemiDoc Touch imaging system.

2.13. Statistical analysis

Data were calculated as means ± SEM, and groups of data were compared using Student's *t*-test. Statistical significance was considered as $p < 0.05$.

Table 1
LPC-regulated transcripts.

Gene symbol	LPC(16:0)	LPC(18:1)
ANGPTL4	2.32	4.72*
PDK4	2.14*	3.38
PLIN2	1.42	2.12*
UBE2C	1.33	1.69*
HJURP	1.36	1.68*
RGS5	1.25*	1.67
BIRC5	1.42	1.63*
KIF20A	1.30	1.63*
CPT1A	1.31*	1.59*
HMMR	1.17	1.57*
CENPF	1.21	1.56*
DEPDC1	1.30	1.54*
CCNB2	1.27	1.53*
CCNA2	1.19	1.53*
SKA1	1.36	1.51*

Transcripts with gene symbol showing a fold change >1.5 (* $p < 0.05$) in human myotubes treated with 10 μM LPC(16:0) or LPC(18:1) for 24 h as assessed by whole genome expression analysis (n = 4).

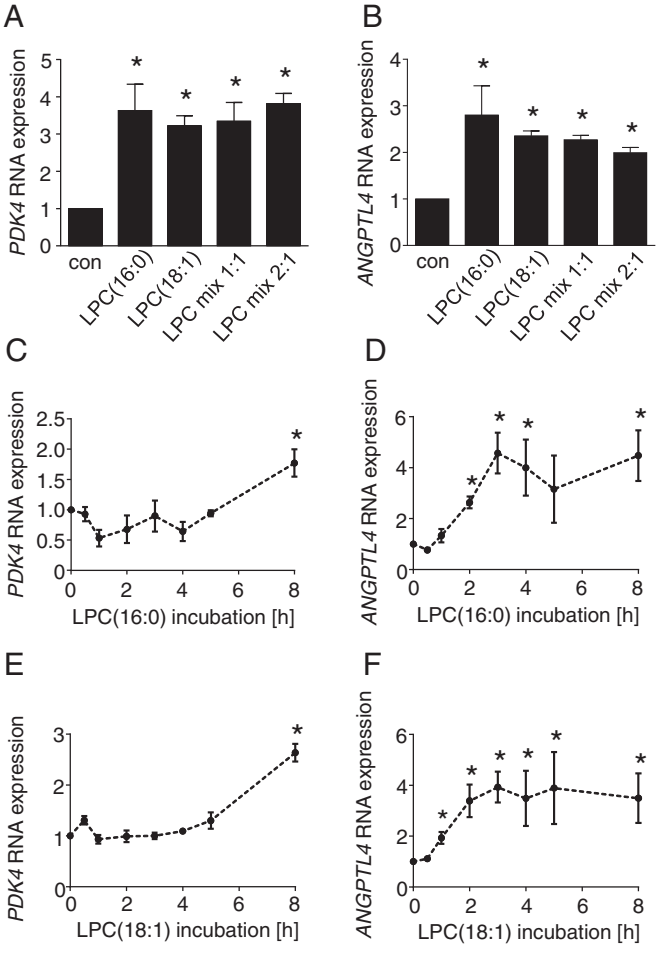

Fig. 2. LPC stimulate *PDK4* and *ANGPTL4* gene expression. A,B: Human primary myotubes were treated for 24 h with 10 μM of LPC(16:0), LPC(18:1), and 10 μM of mixtures of both LPC species with a LPC(16:0) to LPC(18:1) ratio of 1:1 or 2:1. C–F: Human primary myotubes were stimulated with either LPC(16:0) (C,D) or LPC(18:1) (E,F) for different time points. RNA levels of *PDK4* (C,E) and *ANGPTL4* (D,F) were assessed by qPCR and were shown as fold change compared to untreated cells. Data are mean ± SEM; * $p < 0.05$ vs. control cells without LPC treatment (n = 4).

3. Results

3.1. Concentrations of LPCs up to 25 μM did not induce cell lysis or RNA expression of inflammatory cytokines

First, the concentrations of LPC(16:0) and LPC(18:1) that can induce cytotoxicity and expression of inflammatory cytokines in human myotubes were studied. Human myotubes were incubated with increasing concentrations of LPC(16:0) and LPC(18:1). Below 50 μM cell lysis determined by the release of the intracellular enzymes creatine kinase and lactate dehydrogenase was not detected (Fig. 1A,B), and concentrations up to 25 μM of LPC(16:0) and LPC(18:1) showed no significant up-regulation of the expression of cellular stress genes activating transcription factor 3 (*ATF3*), interleukin 6 (*IL6*) and chemokine (C-X-C motif) ligand 3 (*CXCL3*) (Fig. 1C,D). Accordingly, 10 μM of LPC were used for further studies.

3.2. LPC stimulate PDK4 and ANGPTL4 gene expression

To achieve a non-hypothesis driven overview of LPC-dependent cellular effects on human myotubes whole genome expression analysis was performed. After stimulation of myotubes with 10 μM LPC(16:0) or LPC(18:1) for 24 h, only 15 transcripts with a fold change >1.5 ($p < 0.05$) were detected, with all genes showing an up-regulation

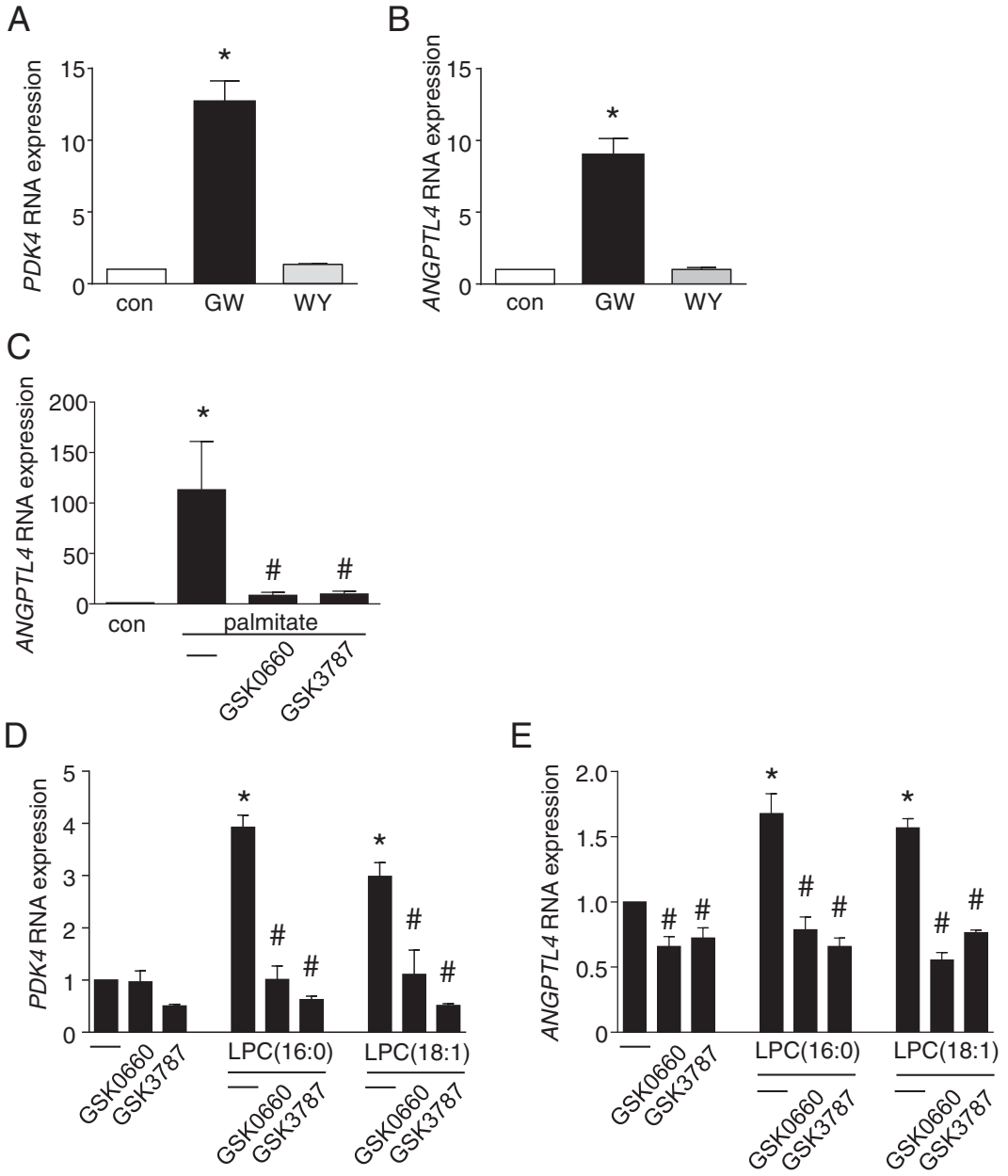

Fig. 3. LPC-dependent *PDK4* and *ANGPTL4* gene expression is mediated by PPARδ. A,B: Stimulation of primary human myotubes with PPARδ agonist GW501516 (1 μM) and PPARα agonist WY14643 (1 μM) for 24 h. RNA levels of *PDK4* (A) and *ANGPTL4* (B) were assessed by qPCR and were shown as fold change compared to untreated cells. Data are mean ± SEM; * $p < 0.05$ vs. untreated cells ($n = 4$). C: Cells were treated with palmitate (250 μM) for 24 h with or without PPARδ antagonist GSK3787 (10 μM) and GSK0660 (10 μM) pre-treatment for 2 h. *ANGPTL4* RNA levels were assessed by qPCR and shown as fold change compared to untreated cells. Data are mean ± SEM; # $p < 0.05$ vs palmitate, * $p < 0.05$ vs untreated cells ($n = 6$). D,E: Cells were treated with LPC(16:0) and LPC(18:1) for 24 h with or without PPARδ antagonist GSK3787 (10 μM) and GSK0660 (10 μM) pre-treatment. *PDK4* (D) and *ANGPTL4* (E) RNA levels were analyzed by qPCR and shown as fold change compared to untreated cells. Data are mean ± SEM; # $p < 0.05$ vs. LPC-treated cells, * $p < 0.05$ vs. untreated cells ($n = 4$). F-H: Cells were transfected with siRNA targeting PPARD and treated with LPC(16:0) (10 μM) and LPC(18:1) (10 μM) for 24 h. RNA levels of *PPARD* (F), *PDK4* (G), and *ANGPTL4* (H) were analyzed by qPCR and shown as fold change compared to control transfected cells w/o LPC. Data are mean ± SEM; # $p < 0.05$ vs. control transfected cells with LPC, * $p < 0.05$ vs. control transfected cells w/o LPC ($n = 4$).

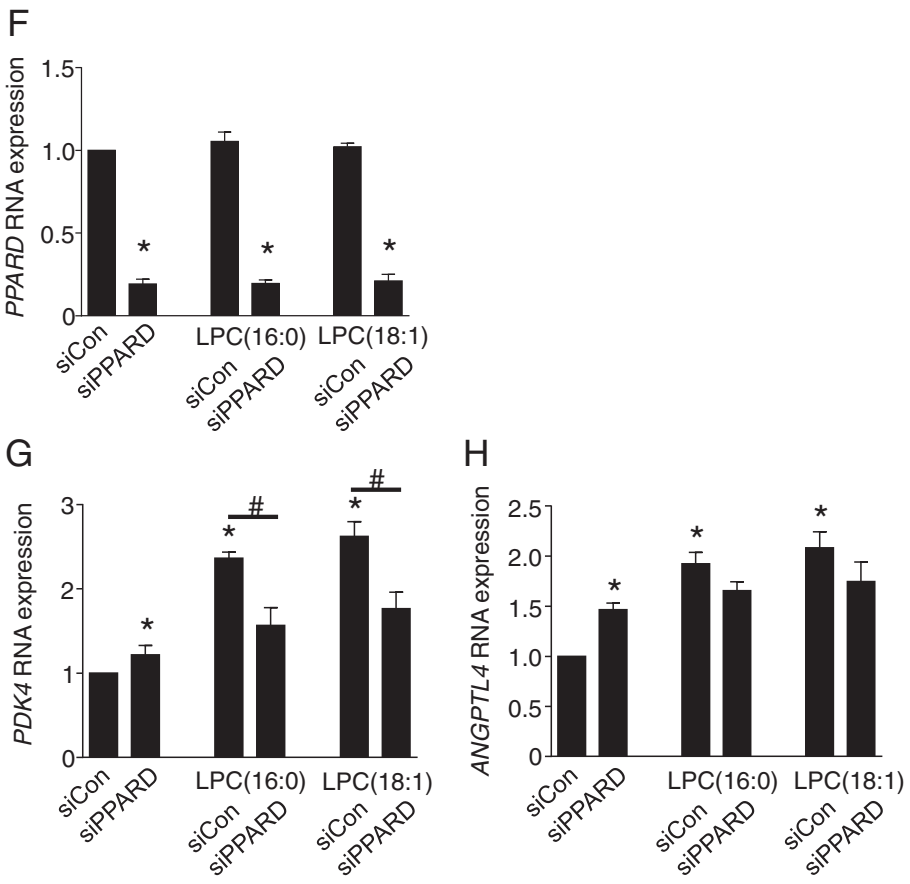

Fig. 3 (*continued*).

(Table 1). Genes with the highest fold change, angiopoietin-like 4 (*ANGPTL4*), pyruvate dehydrogenase kinase 4 (*PDK4*), perilipin 2 (*PLIN2*), and carnitine palmitoyltransferase (*CPT1*) are known to be regulated by the nuclear receptor and transcription factor PPARδ in skeletal muscle [31,32]. We focused on the regulation of *ANGPTL4* and *PDK4* gene expression. The increased expression of these genes after incubation with 10 μM LPC(16:0) or LPC(18:1) for 24 h was validated by qRT-PCR (Fig. 2A,B). The 10 μM mixes of LPC(16:0) and LPC(18:1) at a ratio of 1:1 and 2:1 were similar effective as the single compounds (Fig. 2A,B). The upregulation of *ANGPTL4* gene expression by LPC(16:0) and LPC(18:1) was observed after 2 h of stimulation, while significant effects on *PDK4* expression were found after 8 h of stimulation (Fig. 2C–F).

3.3. LPC-dependent PDK4 and ANGPTL4 gene expression is mediated by PPARδ

PPARδ binds to PPRE sequences and is activated upon ligand binding. Fatty acids and fatty acid-derived lipids have been shown to activate PPARδ-dependent gene activation in human skeletal muscle [31]. The synthetic PPARδ agonist GW501516, but not the PPARα agonist WY14643, increased the expression of *PDK4* and *ANGPTL4*, confirming the importance of PPARδ in the regulation of these genes in human myotubes (Fig. 3A,B). Both PPARδ antagonists GSK0660 and GSK3787 blocked the upregulation of *ANGPTL4* RNA levels by the known PPARδ activator palmitate completely (Fig. 3C). Similarly, both substances blocked the upregulation of *PDK4* and *ANGPTL4* by LPC(16:0) and LPC(18:1) (Fig. 3D,E). Moreover, knock-down of *PPARD* achieved by transfection of si-RNA oligonucleotides (Fig. 3F) reduced the LPC-mediated upregulation of *PDK4* gene expression (Fig. 3G). In the absence of a stimulatory ligand of PPARδ, the knock-down of *PPARD* resulted in increased expression of *PDK4* and *ANGPTL4* (Fig. 3G,H) as reported previously [29]. This corepressor activity of PPARδ described for certain genes including *ANGPTL4* may be the explanation for a mixed effect of the knock-down of *PPARD*. It can reduce the repressor activity of PPARδ and on the other hand it reduces the stimulatory effect of the agonist LPC (Fig. 3H). Together, the data show that stimulation of human myotubes with LPC activate PPARδ-dependent gene expression.

3.4. LPC activate PPARδ

To provide evidence for a direct stimulatory effect of LPC on PPARδ-dependent gene activation, reporter gene assays were performed using a luciferase construct consisting of LexA binding sites upstream of a TATA box co-transfected with a plasmid harboring the PPARδ ligand binding domain fused to the LexA DNA binding domain. Stimulation of co-transfected human myoblasts with PPARδ agonist GW501516 revealed a strong transcriptional activation (Fig. 4A). In this system LPC(16:0) and LPC(18:1) also activated PPARδ transcriptional activity (Fig. 4A). Since certain lipids structurally related to LPC were identified as ligands of PPARδ [33], LPC may also act as ligand, leading to enhanced DNA binding of the PPARδ/RXR complex. Gelshift assays revealed enhanced formation of a PPARδ/RXRα/PPRE complex in the presence of LPC(16:0) and LPC(18:1) (Fig. 4B,C). Here, 0.5 and 1 μM LPC were most effective, and the induction of PPARδ binding activity is comparable with the agonist GW501516. LPC and related structures were also tested in their affinity to bind to PPARδ in a competitive in vitro ligand binding assay. In this assay, the ligand binding domain of PPARδ interacts with a fluorescent pan-PPAR ligand, which produces FRET. Displacement of the pan-PPAR ligand by unlabeled ligand results in a quantifiable attenuation of FRET. LPC(16:0), LPC(18:1) and the LPC ether analogue PC(O-16:0/O-1:0) showed a dose-dependent

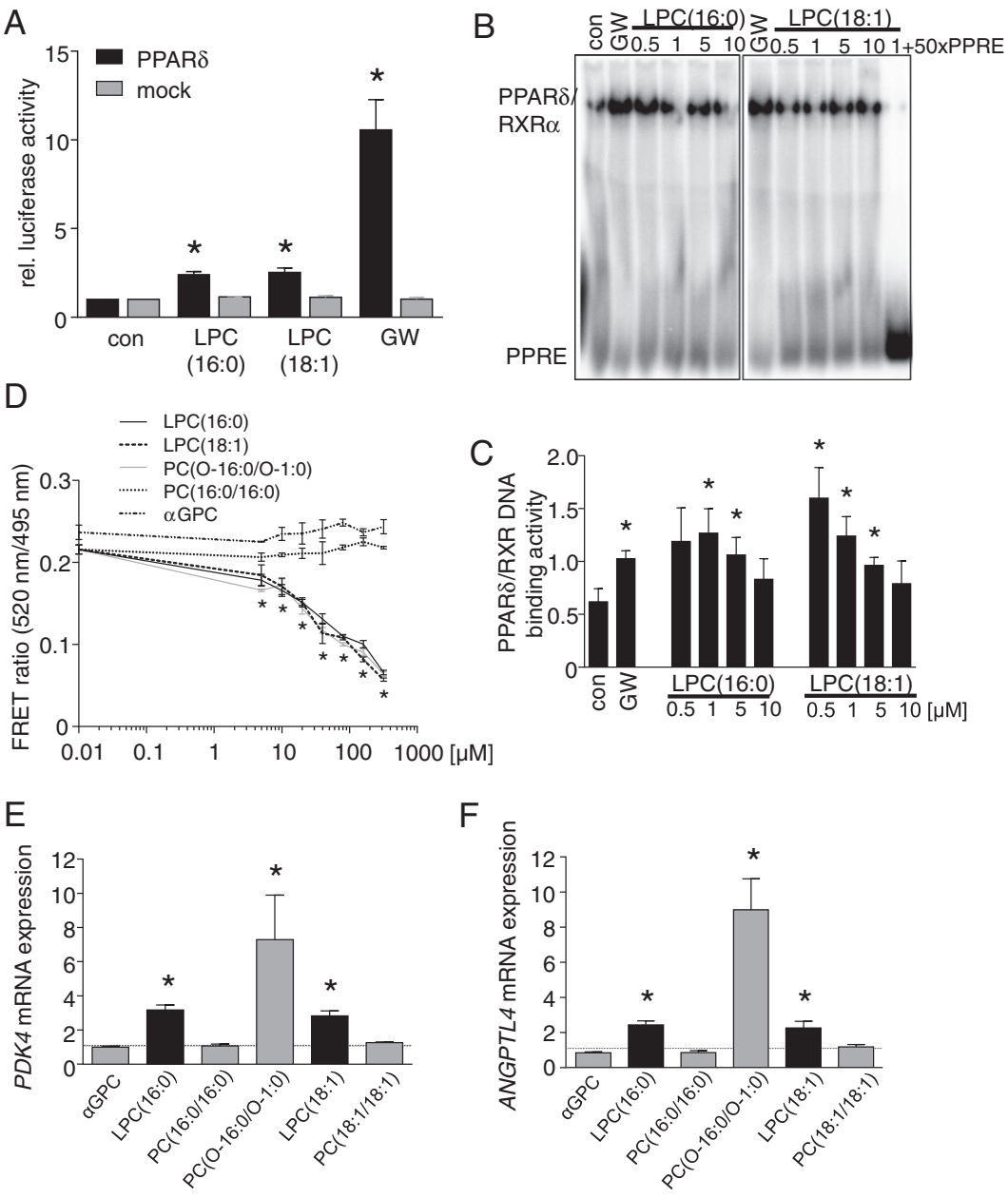

Fig. 4. LPC activate PPARδ. A: Activation of the PPARδ ligand binding domain was analyzed in a luciferase reporter gene assay co-transfecting LexA-BS with LexA-PPARδ-LBD (PPARδ) or pcDNA3.1 (mock). Effects of LPC(16:0) (10 μM), LPC(18:1) (10 μM), and GW501516 (1 μM) for 24 h were shown as fold change compared to transfected cells w/o treatment (con). Data are mean ± SEM; * $p < 0.05$ vs. con ($n = 4$). B,C: Gel shift assay was performed using recombinant hPPARδ and hRXRα protein and a consensus binding site for PPARδ. LPC(16:0) or LPC(18:1) at increasing concentrations from 0.5 to 10 μM or GW501516 were added. DNA binding activity was analyzed by phospho-imaging. Addition of a 50-fold molar excess of unlabeled PPRE (+50xPPRE) blocked the formation of the labeled protein/DNA complex completely. One representative gel image is shown (B). Densitometric quantification (C) was performed by the use of ImageJ image processing software. Data are mean ± SEM; * $p < 0.05$ vs. con ($n = 4–6$). D: TR-FRET displacement assay. The experiment was carried out with the indicated concentrations of putative ligands competing for displacement of Fluormone™ Pan-PPAR Green from the PPARbeta/delta LBD. Displacement results in diminished FRET from the Terbium-labeled anti-GST antibody to the fluorescein-labeled tracer ligand. The "0" μM data points correspond to the respective solvent controls. Data are mean ± SEM of three technical replicates for each data point. E,F: Human primary myotubes were stimulated with 10 μM of the indicated substances for 24 h. *PDK4* (E) and *ANGTPL4* (F) RNA levels were quantified by qPCR and shown as fold change compared to untreated cells. Dashed line indicates value of untreated cells set as 1. Data are mean ± SEM; * $p < 0.05$ vs. con ($n > 4$).

replacement of the pan-PPAR ligand in the μmolar range (Fig. 4D). PC(16:0/16:0) and α-glycerophosphorylcholine (αGPC) could not replace the pan-PPAR ligand (Fig. 4D). Notably, the ether analogue was a strong inducer of *PDK4* and *ANGPTL4* mRNA levels, whereas PC and αGPC did not increase the level of these transcripts (Fig. 4E,F).

3.5. Prevention of palmitate-induced lipotoxicity by LPC is PPARδ dependent

Next, the potential consequences of the LPC-dependent PPARδ activation were studied. A major metabolic consequence of PPARδ activation in skeletal muscle is enhanced fatty acid oxidation [34,35]. Stimulation of human myotubes with GW501516 (1.44 ± 0.20; $p = 0.021$, data not shown) and LPC (1.24 ± 0.12; $p = 0.039$ for LPC(16:0), data not shown) enhanced palmitate oxidation. Moreover, it has been reported that activation of PPARδ by GW501516 in rodent skeletal muscle leads to amelioration of fatty acid-induced expression of inflammatory cytokines and ER stress [36,37]. When the human myotubes were preincubated with GW501516, the palmitate-induced increase in *IL6*, *ATF3*, and *CXCL3* was clearly reduced (Fig. 5A–C). Comparable protection against palmitate-induced *IL6*, *ATF3*, and *CXCL3* gene expression was achieved using LPC(16:0) and LPC(18:1) (Fig. 5A–C).

Cells were also protected against palmitate-induced ER stress as shown as restoration of unspliced *XBP1* (Fig. 5D,E). LPC(16:0), but not LPC(18:1), prevented also the palmitate-mediated reduction of insulin-stimulated phosphorylation of Akt (Fig. 5F). The role of PPARδ in the prevention of lipotoxic effects was tested with the PPARδ antagonist GSK0660. The presence of GSK0660 prevented the palmitate-induced activation of PPARδ as shown in Fig. 3C, and this inhibition of PPARδ activity further increased the palmitate-induced *IL6*, *CXCL3*, and *ATF3* gene expression (Fig. 5G–I). Moreover, the presence of GSK0660 attenuated the protective effect of LPC(16:0) and LPC(18:1) on the palmitate-induced expression of these genes (Fig. 5G–I). These data show that the LPC-mediated activation of PPARδ can reduce fatty acid-

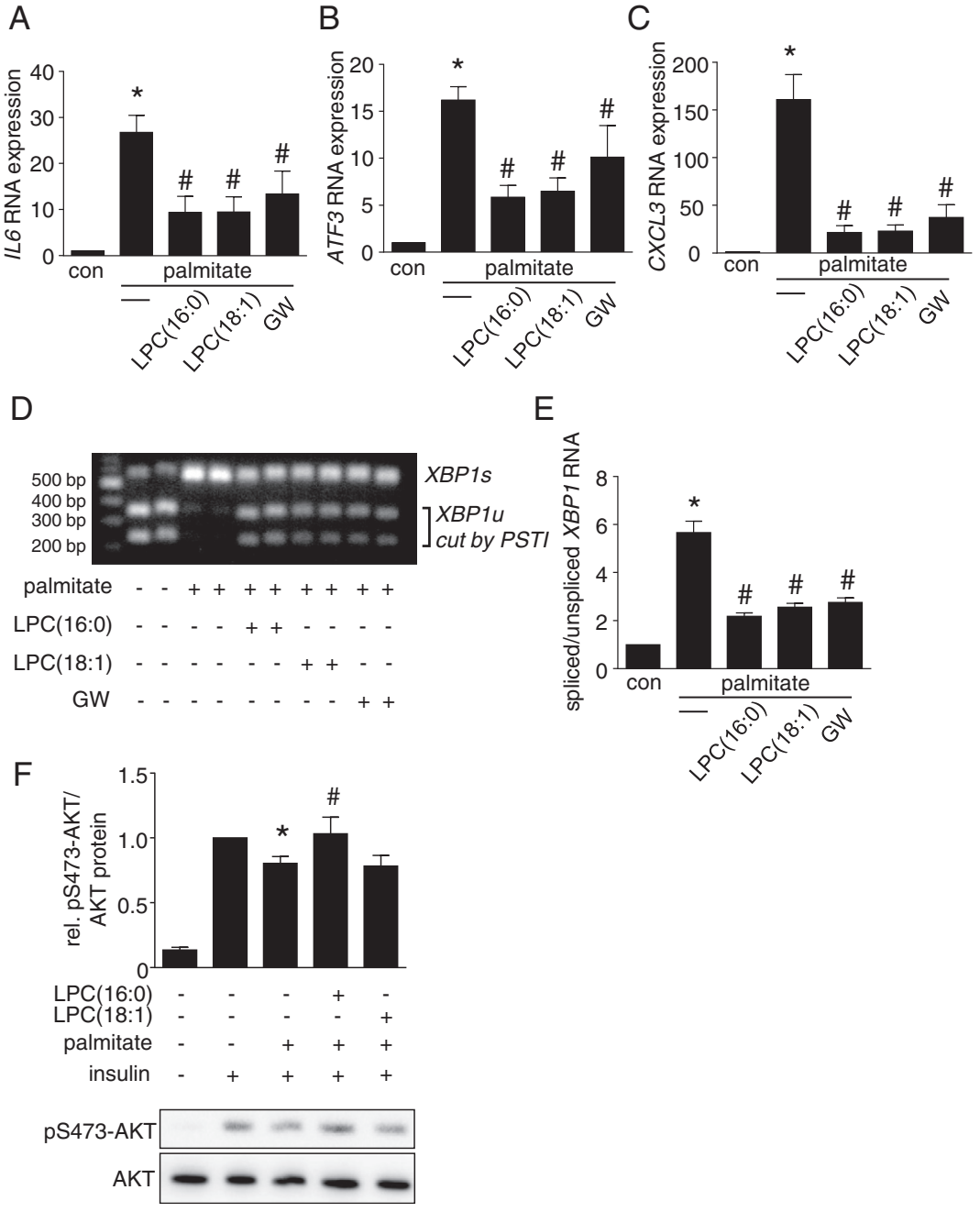

Fig. 5. Prevention of palmitate-induced lipotoxicity by LPC is PPARδ dependent. Pretreatment of human primary myotubes with LPC(16:0) (10 μM), LPC(18:1) (10 μM), or GW501516 (1 μM) for 24 h and subsequent palmitate (250 μM) stimulation for further 24 h. *IL6* (A), *ATF3* (B), and *CXCL3* (C) RNA levels were analyzed by qPCR and shown as fold change compared to untreated cells (con). Data are mean ± SEM; # $p < 0.05$ vs. palmitate, * $p < 0.05$ vs. con ($n = 6$). *XBP1* splicing was detected as the spliced and uncut (by *PSTI*) 448 bp PCR product (XBP1s) and the two smaller fragments of the cut, unspliced PCR product (XBP1u) (representative gel, D) and was quantified using ImageJ (E) and shown as fold change compared to untreated cells (con). Data are mean ± SEM; # $p < 0.05$ vs. palmitate, * $p < 0.05$ vs. con ($n = 4$). F: After treatment with LPC and palmitate, cells were serum starved for 3 h and stimulated with 100 nM insulin. Protein abundance of phospho-Akt (ser473), and total Akt was analyzed by Western blotting (representative blots are shown). Relative band intensities are shown as fold change compared with insulin-treated cells. Data are mean ± SEM; * $p < 0.05$ vs. insulin, # $p < 0.05$ vs. palmitate + insulin ($n = 8$). G–I: Human primary myotubes were subjected to GSK0660 (10 μM) pretreatment for 2 h followed by LPC(16:0) and LPC(18:1) treatment for 24 h and subsequent palmitate (250 μM) stimulation for 24 h. *IL6* (G), *ATF3* (H), *CXCL3* (I) RNA levels were quantified and shown related to palmitate-treated cells. Data are mean ± SEM; # $p < 0.05$ vs. palmitate, * $p < 0.05$ vs. cells w/o GSK0660 ($n = 6$). J–L: Human primary myotubes were treated with LPC(16:0) (10 μM) or LPC(18:1) (10 μM) for 1 h, 4 h, or 24 h. Protein abundance of phospho-AMPKα (thr172), AMPKα1, and phospho-ACC (ser79) was analyzed by Western blotting (representative blots are shown). Myosin heavy chain (slow) (MyHC) was used as loading control for ACC. Relative band intensities are shown as fold change compared with untreated cells. Data are mean ± SEM; * $p < 0.05$ vs. untreated cells ($n = 8$).

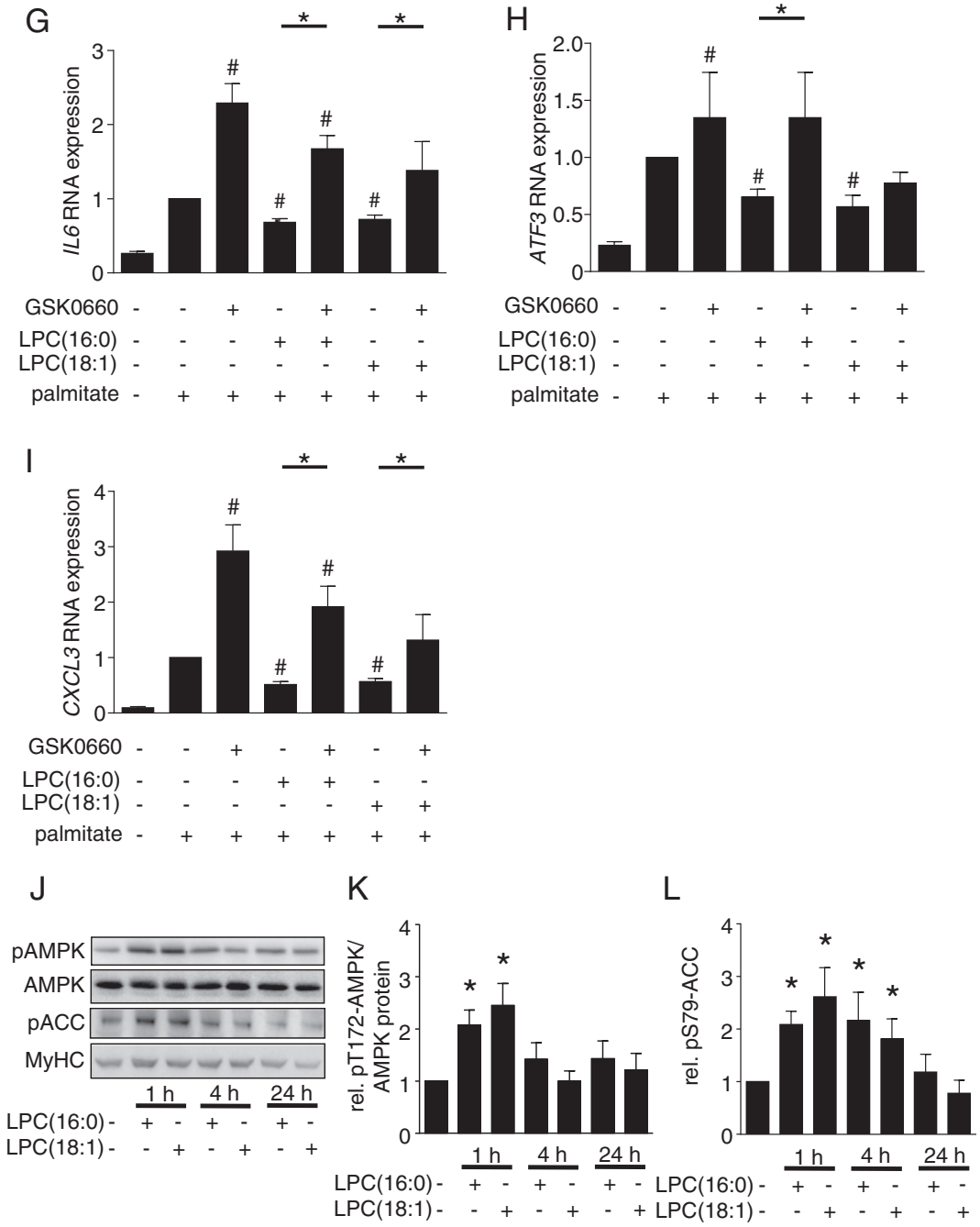

Fig. 5 (*continued*).

induced inflammation in skeletal muscle cells. The PPARδ-dependent activation of AMPK has been implicated in the prevention of lipotoxicity in myotubes by PPARδ agonists [36]. Incubation of human myotubes with LPC(16:0) and LPC(18:1) increased the phosphorylation of AMPK after 1 h of stimulation and the phosphorylation of the AMPK substrate ACC after 1 and 4 h of stimulation (Fig. 5J–L). These results suggest a contribution of AMPK activation in the protective effect of LPC on lipotoxicity.

3.6. Role of intracellular LPC production from palmitate in lipotoxicity

Other studies have linked the intracellular production of LPC from palmitate to palmitate-mediated lipoapoptosis [25,38]. Thus, the question arises whether this intracellular increase in LPC is involved in the palmitate-induced lipotoxicity to some extent, or whether the elevation of LPC can be even protective. To address this question, the intracellular production of LPC from palmitate was targeted by inhibition of calcium-independent phospholipase A_2 ($iPLA_2$), which was shown to be relevant for palmitate-induced lipotoxicity in hepatocytes [38]. Treatment of human myotubes with the $iPLA_2$ inhibitor FKGK11 or with bromoenol lactone using R- and S-enantiomeres with higher affinity to $iPLA_2$ PNPLA8 or PNPLA9, respectively [39], did not prevent the palmitate-induced increase in *IL6*, *ATF3*, and *CXCL3* expression (Fig. 6A–C). FKGK11 resulted even in a further increase in mRNA levels. These data suggest that blocking the LPC production from palmitate can lead to a further increase of palmitate-induced lipotoxicity. Next, the conversion of LPC to PC was targeted by siRNA-mediated knock-down of lysophosphatidylcholine acyltransferases (*LPCAT1–3*), which catalyze the acylation of LPC to PC. The knock-down of LPCAT has been shown to increase LPC and to reduce PC levels [16]. The expression of *LPCAT1*,

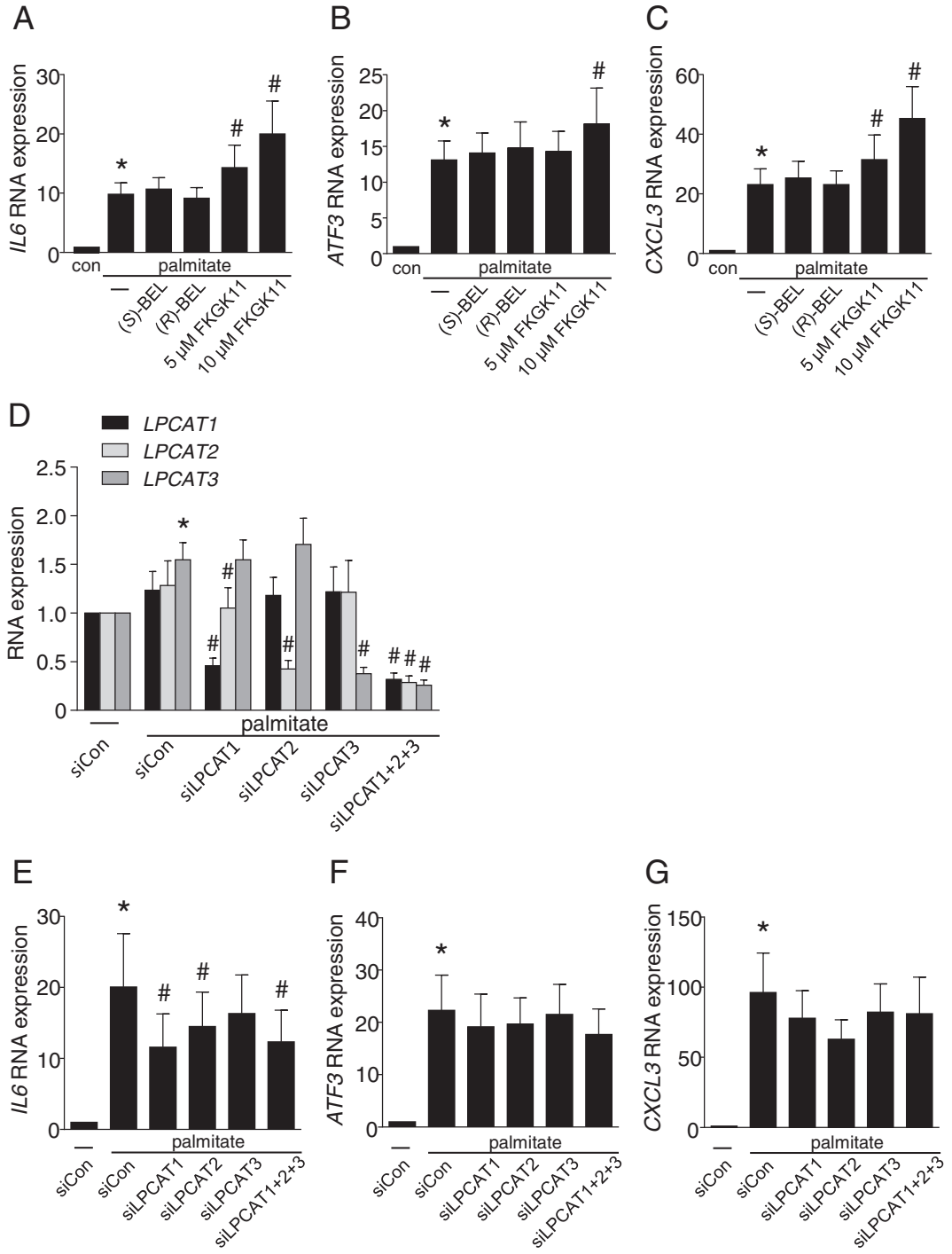

Fig. 6. Role of intracellular LPC production from palmitate in lipotoxicity. A–C: Human primary myotubes were treated with iPLA$_2$ inhibitors bromoenol lactone (BEL; S- and R-enantiomer, 10 μM each) or FKGK11 (10 μM) and palmitate (250 μM) for 24 h. D–G: Human primary myotubes were transfected with siRNA against *LPCAT1*, *LPCAT2*, and *LPCAT3* or control siRNA (siCon) for 24 h followed by stimulation with 250 μM palmitate for 24 h. RNA levels of *IL6* (A,E), *ATF3* (B,F), *CXCL3* (C,G), and *LPCAT1–3* (D) were analyzed by qPCR and shown as fold change compared to control transfected and untreated cells. Data are mean ± SEM; * $p < 0.05$ vs. (si)Con, # $p < 0.05$ vs.(si)Con + palmitate ($n = 4–6$).

2, and *3* was strongly reduced by either a single knock-down or knock-down of all three genes together (Fig. 6D). In particular the knock-down of *LPCAT1* expression and of all three targeted *LPCAT* ameliorated the palmitate-induced expression of *IL6* (Fig. 6E), while no significant effect on *ATF3* and *CXCL3* expression was detected (Fig. 6F,G). Thus, targeting LPC levels with iPLA2 inhibitors to reduce the production of LPC or with knock-down of *LPCAT* to prevent the further conversion of LPC to PC argue against a role of palmitate-derived LPC as mediators of lipotoxic effects. Parts of the data even suggest that the intracellular production of LPC from palmitate can be protective.

4. Discussion

In this study, whole genome expression analysis revealed the increased expression of PPARδ target genes in LPC-treated human myotubes. The activation of PPARδ transcriptional activity by LPC was

shown as enhanced expression of target genes, PPARδ-dependent luciferase reporter gene expression, and enhanced DNA binding of the PPARδ/RXR dimer. A major functional consequence can be the contribution of LPC to the anti-inflammatory effect of PPARδ activation.

Several lines of evidence support that LPC can ameliorate fatty acid-induced cytokine production and ER stress. In human myotubes, treatment with LPC clearly reduces both palmitate-induced expression of *IL6*, *ATF3*, and *CXCL3* and palmitate-induced ER stress. LPC activate PPARδ, and PPARδ activation has not only been implicated in the protection from fatty acid-induced inflammation in skeletal muscle, but is an anti-inflammatory regulator in adipocytes [40], macrophages [41], and prevents leukocyte recruitment to the endothelium [42]. An important mechanism in this anti-inflammatory activity of PPARδ is the reduced expression and release of cytokines and chemoattractants from activated muscle cells, adipocytes, and endothelial cells. The anti-inflammatory properties of the LPC-PPARδ axis reported in this study are well in accordance with the correlation of higher plasma LPC levels and lower concentrations of inflammatory markers reported in prediabetic humans [9,17,19].

The investigation of the human plasma lipidome and its relationship to markers of inflammation can give insights into potential causalities, but it is not possible to differentiate whether the observed alterations in plasma LPC are cause or consequence of inflammatory processes. In a mouse model of non-alcoholic steatohepatitis, the expression of hepatic lysophosphatidylcholine acyltransferases is upregulated, resulting in a higher conversion of LPC to PC and reduced plasma levels of LPC [18]. Thus a condition of the metabolic syndrome which is associated with hepatic inflammation can contribute to the reduction of plasma LPC.

Our results even suggest a causal relationship of LPC and the protection from obesity-related expression of cytokines, which can result in lower concentrations of these inflammatory markers in plasma. LPC have been implicated in the modulation of inflammatory processes in various cells types both in an inhibitory [43,44] and a stimulatory manner [20,21,45]. Our results provide evidence for a protective effect of LPC on palmitate-induced expression of inflammatory cytokines and ER stress in human myotubes. Results obtained in primary hepatocytes and hepatoma cells point to an involvement of LPC as death mediators of palmitate treatment leading to lipoapoptosis [25,38]. To explain these opposite data it is important to consider that in higher concentrations LPC can be inserted into cellular membranes due to their amphiphilic chemical structure, which then can lead to cell lysis [46,47]. To avoid this lytic action we determined the concentration which is needed to induce cell lysis and used 10 μM LPC which is clearly below the lytic concentration of 50 μM or higher. This extracellular concentration of 10 μM of each LPC is sufficient to induce the activation of PPARδ-dependent gene expression and the observed prevention of lipotoxicity. In contrast, concentrations of LPC(16:0) above 10 μmol/L were used when toxic effects were reported [25,38]. A role for palmitate-derived LPC as mediators of lipotoxicity in human myotubes is also not supported by blocking the production of LPC from palmitate by the $iPLA_2$ inhibitors bromoenol lactone and FKGK11. The data obtained with FKGK11 and by targeting LPC metabolism by siRNA interference against *LPCAT1–3* even suggest that reducing the intracellular LPC levels can aggravate palmitate-induced cytokine expression. Notably, FKGK11 is more specific than bromoenol lactone in targeting $iPLA_2$, since bromoenol lactone also possesses inhibitory activity against phosphatidic acid phosphatase [48], triacylglycerol lipases, transacylases and thioesterases [49]. Although we did not measure the effect on intracellular LPC concentrations when targeting LPC production in palmitate-treated myotubes, using these different approaches support anti-inflammatory properties of LPC in human myotubes which are at least partially mediated by activation of PPARδ.

The cytolytic concentrations of LPC are clearly below total LPC plasma levels, but in plasma LPC are bound to proteins, mainly albumin and α1-glycoprotein, which prevents cell lysis and reduces other cellular effects of LPC [12]. Thus, the unbound, free fraction of LPC is critical for cellular effects. We are not aware of any data on the concentration of free LPC in plasma, but it was shown that 10-fold higher concentrations of LPC are necessary when albumin is present to activate transmembrane potentials [50]. The 10 μM LPC used in our experiments correspond to approximately 3–10% of total plasma LPC, which may reflect the fraction of free LPC. We hypothesize that with the lower LPC plasma levels reported in insulin resistance and obesity the fraction of free LPC is also decreased, leading to a reduced effect of LPC on PPARδ.

Lower plasma LPC levels are not only associated with increased concentrations of inflammatory cytokines in plasma, but also with insulin resistance. It could be speculated that a LPC-mediated PPARδ-dependent increase in lipid oxidation in skeletal muscle and the activation of AMPK can contribute to the maintenance of lipid homoeostasis and insulin sensitivity, but further mechanisms should be taken into account which could explain the protection from palmitate-induced reduction of insulin-stimulated Akt phosphorylation by LPC(16:0). Agonists of PPARα/δ had been developed as antidiabetic drugs and for treatment of the metabolic syndrome [51]. Notably, while we clearly showed that LPC can activate PPARδ, we cannot exclude that LPC may also be able to activate other PPAR transcription factors in muscle cells. For example, LPC(16:0) was shown to activate PPARα transcriptional activity and PPARα target gene expression in murine hepatocytes [52]. Thus LPC as endogenous PPARα/δ agonists might play a role in regulation of metabolic homoeostasis. Moreover, a glucose lowering effect of LPC has been reported in rodents [23]. LPC stimulate glucose uptake in adipocytes paralleled by increased abundance of GLUT4 at the plasma membrane and improve glucose uptake in insulin-resistant adipocytes [52]. To conclude, lower concentrations of circulating LPC may contribute to a reduced PPARδ activity and thereby to a deterioration of metabolic disturbances, increased inflammation and development of insulin resistance. In addition, LPC may prevent insulin resistance by PPARδ-independent mechanisms, since the effect of LPC(16:0) and LPC(18:1) on palmitate-mediated reduction in insulin-stimulated Akt phosphorylation is different, albeit both compounds activate PPARδ.

The activation of PPARδ requires the binding of a ligand. Our results provide evidence that LPC(16:0) and LPC(18:1) can act as direct PPARδ agonists, since they can replace the pan-PPAR ligand from the PPARδ ligand binding domain. The affinity of LPC to the ligand binding domain of PPARδ in the μmolar range is similar to that of other endogenous low affinity ligands such as arachidonic acid [27]. Thus direct binding of LPC to PPARδ can be involved in the activation of PPARδ-dependent gene expression, but other mechanisms may contribute as well. Once internalized into cells, LPC are rapidly reacylated to PC and metabolized by lysophospholipases providing αGPC and fatty acid as reported previously [53]. The exogenous addition of 10 μM αGPC and PC did not induce a PPARδ-dependent gene activation comparable to 10 μM LPC(16:0) or LPC(18:1), but an intracellular formation of LPC-derived PPARδ agonists like fatty acids can be involved in the activation of PPARδ. It has also been shown that the intracellularly formed PC(16:0/18:1) acts as agonist of a PPARα-dependent gene expression in hepatocytes, but it is described as a weak ligand for PPARδ [54]. On the other hand, the LPC ether analogue PC(O-16:0/O-1:0), which cannot be hydrolyzed to release palmitate or be converted to PC has a strong effect on *PDK4* and *ANGPTL4* mRNA abundance suggesting that the LPC-like structure itself can be responsible for the activation of PPARδ and that the intracellular conversion of LPC to other PPARδ agonists is not needed. It is also possible that other signaling mechanisms that are activated by extracellular LPC are involved in the observed gene expression pattern. The most prominent biological activities of extracellular LPC include the mobilization of intracellular $[Ca^{2+}]$ mediated via G protein coupled receptor activation [55,56]. However, treatment of human myotubes with the calcium ionophore ionomycin did not induce *ANGPTL4* and *PDK4* gene expression (data not shown),

which makes a role of increased [Ca^{2+}] in the up-regulation of *ANGPTL4* and *PDK4* less likely.

5. Conclusion

LPC(16:0) and LPC(18:1), which represent approximately 60% of total plasma LPC, can act as lipid signaling molecules, activate PPARδ-dependent gene expression and reduce fatty acid-induced inflammation in human myotubes. Thus, our data suggest that the reduced plasma LPC levels reported in obese and insulin resistant subjects can contribute to the development of the metabolic syndrome, while high LPC levels might be protective.

Author contributions

C. Klingler, H.U. Häring, E. Schleicher, R. Lehmann, and C·Weigert designed research; C. Klingler, X. Zhao, T. Adhikary, and J. Li performed research; C. Klingler, X. Zhao, R. Lehmann, and C. Weigert analyzed data; G. Xu and T. Adhikary contributed new analytical tools; C. Klingler, R. Lehmann, and C. Weigert wrote the paper.

Transparency document

The Transparency document associated with this article can be found, in online version.

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