Lysophosphatidylcholines - Physiological Functions in Skeletal Muscle Cells

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2016

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	16.12.2016
Dekan:	Prof. Dr. rer. nat. Wolfgang Rosenstiel
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Contents

List of figures V

List of tables VII

Abbreviations X

List of publications XV

Abstract XVII

Zusammenfassung XIX

1 Introduction 1

1.1 The lipid class of glycerophospholipids 1

1.2 Chemical properties of lysophosphatidylcholine 4

1.3 LPC biosynthesis in humans 7

1.3.1 Kennedy pathway and other biosynthetic routes of PC 7

1.3.2 Phospholipase A_1 10

1.3.3 Phospholipase A_2 11

1.3.4 Etherlipid degradation 14

1.4 LPC converting processes 15

1.4.1 LPCAT 15

1.4.2 Phospholipase B, C, and D 16

1.4.3 LPCs in the blood 18

1.4.4 LPCs in lipoproteins 21

1.4.5 Dietary uptake of LPCs 21

1.5 Regulation of plasma LPC concentration . . . 22
1.6 LPCs as biomarkers . . . 23
1.7 Physiological functions of LPCs . . . 27
1.7.1 LPC receptors . . . 27
1.7.2 Effects of LPCs on insulin-glucose axis . . . 28
1.7.3 LPC in inflammation and atherosclerosis . . . 29
1.7.4 LPC and brain function . . . 30
1.7.5 Effects of LPC on liver . . . 31
1.7.6 Primary skeletal myotubes as model cell system . . . 32
1.7.7 Effects of LPC on skeletal muscle and own previous work (diploma thesis) . . . 33
1.8 Aims of the thesis . . . 36

2 Material and Methods 38
2.1 Materials . . . 38
2.1.1 Chemicals and consumables . . . 38
2.1.2 Software and devices . . . 45
2.2 Methods . . . 47
2.2.1 Cell culture . . . 47
2.2.2 Handling and storage of lipids . . . 49
2.2.3 Quantitative PCR . . . 50
2.2.4 Microarray analysis . . . 53
2.2.5 Thin layer chromatography . . . 54
2.2.6 RNA interference . . . 55
2.2.7 Luciferase reporter assay . . . 56
2.2.8 Transformation and isolation of plasmid DNA . . . 58
2.2.9 Agarose gel electrophoresis of DNA . . . 59
2.2.10 EMSA . . . 60
2.2.11 Palmitate oxidation . . . 63
2.2.12 Glucose uptake . . . 63
2.2.13 SDS-polyacrylamide gel electrophoresis and Western blotting . . 65

2.2.14 XBP1 splicing assay . . . 69
2.2.15 *In vitro* time-resolved fluorescence resonance energy transfer assay . . . 70
2.3 Statistics . . . 71
2.4 Nomenclature . . . 71

3 Results **72**
3.1 Characterization of LPC effects . . . 72
3.2 *PDK4* and *ANGPTL4* induction caused by LPCs is PPARδ dependent . 75
3.3 LPCs are PPARδ ligands . . . 79
3.4 LPCs protect against palmitate-mediated lipotoxicity . . . 85
3.5 Effects of interferences in the LPC metabolism on palmitate-mediated lipotoxicity . . . 92
3.6 Other signaling pathways might contribute to LPC effects and PPARδ activation . . . 96
3.7 Ether LPC analogue PC(O-16:0/O-1:0) is another PPARδ ligand . . . 98

4 Discussion **100**
4.1 *PDK4* and *ANGPTL4* induction by LPCs is PPARδ mediated . . . 100
4.2 LPCs are PPARδ ligands . . . 102
4.3 Cellular uptake of LPCs . . . 104
4.4 Effect of LPC metabolites on PPARδ . . . 105
4.5 Other signaling pathways might contribute to LPC effects . . . 107
4.6 Consequences of PPARδ activation by LPCs . . . 109
4.6.1 LPCs protect against lipotoxicity . . . 109
4.6.2 Mechanisms of the anti-inflammatory effects of LPCs . . . 112
4.6.3 Effects of LPCs on glucose uptake . . . 118
4.6.4 Targeting intracellular LPC formation during lipotoxicity . . . 118
4.6.5 Effects of LPCs on insulin resistance . . . 120
4.7 *In vivo* relevance of free LPC concentration . . . 121
4.8 Outlook: Pharmacological aspects of LPCs . . . 123

Bibliography **126**

5 Acknowledgements **172**

List of figures

1.1 Schematic view of glycerophospholipids 1
1.2 Overview of selected subclasses of phosphatidylcholines 3
1.3 Chemical structure of LPCs 4
1.4 Disruption of plasma membranes by LPCs 5
1.5 Biochemical pathways of glycerophospholipids 8
1.6 Cleavage sites of phospholipases on PC 10
1.7 Effects of LPCs on CK and LDH release 34
1.8 Stress marker responses to different concentrations of LPCs 35
1.9 Kinetics analysis of the *PDK4* and *ANGPTL4* response to LPC treatment 36

2.1 Gene map of plasmid LexA_PPARd-LBD fusion 57
2.2 Gene map of plasmid 7LTATA-I_Luc 57

3.1 Microarray analysis of mRNA after LPC treatment 73
3.2 *PDK4* and *ANGPTL4* induction by LPC(16:0) and LPC(18:1) 73
3.3 Uptake and metabolic fate of LPC(16:0) in HMT 74
3.4 *PDK4* and *ANGPTL4* are PPARδ target genes in HMT 75
3.5 Effects of PPARδ antagonists/inverse agonists on *PDK4/ANGPTL4* mRNA levels 76
3.6 Effects of siRNA against *PPARD* on *PDK4* and *ANGPTL4* mRNA levels 78
3.7 LPC treatment caused the activation of the PPARδ LBD in luciferase reporter assays 79
3.8 LPCs increased the DNA binding activity of PPARδ/RXRα complex . . . 80
3.9 Effect of different compounds on *PDK4* and *ANGPTL4* mRNA levels . . 81
3.10 TF-FRET PPARδ competitive binding assay with different compounds . 83

3.11 Effect of LPCs on palmitate oxidation 85
3.12 Effect of LPCs on glucose uptake of L6 skeletal muscle cells 86
3.13 Effect of LPCs on the phosphorylation of AMPK 87
3.14 Protective effects of LPC on lipotoxicity 89
3.15 Effect of LPCs on insulin resistance in HMT 90
3.16 Inhibition of the protective effect of LPCs by GSK0660 91
3.17 Effect of *LPCAT1/2/3* knockdown on palmitate-mediated lipotoxicity . . 93
3.18 Effects of different PLA_2 inhibitors on palmitate-mediated *IL6* induction . 94
3.19 Effects of silencing of *PLA2G6A* and *PLA2G6B* on palmitate-mediated lipotoxicity 95
3.20 Effects of forskolin on *PDK4* and *ANGPTL4* RNA over time 96
3.21 Effects of forskolin and H-89 treatment on *PDK4* and *ANGPTL4* RNA . 97
3.22 Effects of ionomycin and BAPTA-AM treatment on *PDK4* and *ANGPTL4* mRNA 98
3.23 PC(O-16:0/O-1:0) increased the DNA binding activity of the PPARδ/RXRα complex 99

4.1 Signaling pathways of LPCs 110
4.2 Signaling pathways of lipotoxicity 122

List of tables

1.1 Critical micellar concentration (CMC) of selected compounds 6
1.2 Official types of the human PLA_2s 12
1.3 Enzymes with LPC acyltransferase activity 16
1.4 List of enzymes that can convert LPCs 17
1.5 Plasma/serum concentration of different LPC species 19
1.6 LPCs as biomarkers of impaired glucose tolerance and T2DM 25
1.7 Association of LPCs with parameters of obesity, CVD, and inflammation 26

2.4 List of chemicals 38
2.7 List of consumables and other equipment 40
2.1 List of lipids/stimulants/recombinant proteins used for this work 42
2.2 List of radiochemicals used for this work 43
2.3 List of cell culture reagents 43
2.5 List of organic solvents 44
2.6 List of commercial kits 44
2.8 List of devices 45
2.9 List of software. 46
2.10 Collagenase solution 48
2.11 HMT growth medium (GM) 48
2.12 HMT differentiation medium (DM) 48
2.13 HMT stimulation medium (SM) 48
2.14 L6 growth medium (L6 GM) 49
2.15 L6 differentiation medium (L6 DM) 49
2.16 Solvents used in experiment of figure 3.9 on page 81 50

2.17 Reverse transcription reaction mix 51
2.18 cDNA synthesis PCR program for multiple reactions with random hexamer primers. 51
2.19 QuantiFast Two-Step RT-PCR for QuantiTect® Primer Assays. 52
2.20 Primer list of Qiagen QuantiTect® Primer Assays for SYBR® Green-based quantitative RT-PCR. 52
2.21 List of Thermo Scientific human siGENOME SMARTpool siRNA 56
2.22 List of eurofins mwg operon siRNA 56
2.23 DNA tracker dye 59
2.24 50X TAE buffer 59
2.25 10X DNA annealing buffer 60
2.26 Annealing reaction 60
2.27 Fill-in reaction 61
2.28 4X DNA binding buffer 61
2.29 DNA binding reaction 61
2.30 EMSA acrylamide gel 62
2.31 10X EMSA running buffer 62
2.32 HBS (HEPES buffered saline) 64
2.33 lysis buffer 64
2.34 5X sample buffer 65
2.35 10X phosphatase inhibitor 65
2.36 7.5 % acrylamide running gel 66
2.37 5.0 % acrylamide running gel 66
2.38 3.7 % acrylamide stacking gel 66
2.39 10X running buffer 66
2.40 10X blotting buffer 66
2.41 List of IgG antibodies used in this work for Western blotting 68
2.42 10X NET-G buffer 69
2.43 ECL solution 69
2.44 Stripping buffer 69

2.45 XBP1 PCR reaction mix 70
2.46 XBP1 amplification PCR reaction 70
2.47 FRET assay buffer 71

3.1 Fitting parameters of the dose response curves 84
3.2 EC_{50} of selected synthetic and endogenous PPAR ligands 84

Abbreviations

2DOG ..... 2-deoxy glucose
αGPC α-glycerol-3-phosphocholine
AC ..... acyl carnitine
ACC ..... acetyl-CoA carboxylase
ADCY ..... adenylate cyclase
ADP ..... adenosine diphosphate
AGMO ..... alkylglycerol monooxygenase
AGPAT ..... 1-acylglycero-3-phosphate acyltransferase
AKT ..... AKT serine/threonine kinase aka protein kinase B
ALB ..... serum albumin
ALP ..... alkyl phospholipid
AMP ..... adenosine monophosphate
AMPK ..... adenosine monophosphate-activated protein kinase
ANGPTL4 .... angiopoietin-like 4
APS ..... ammonium persulfate
ASPG ..... asparaginase
ATF ..... activating transcription factor
ATP ..... adenosine triphosphate
AU ..... arbitrary units
AYTL ..... acyltransferase-like
BEL ..... bromoenol lactone
BMI ..... body mass index
bp ..... basepairs
BSA ..... bovine serum albumin
CAMK ..... Ca^{2+}/calmodulin-dependent protein kinase
CAMKK ..... Ca^{2+}/calmodulin-dependent protein kinase kinase
cAMP ..... cyclic adenosine monophosphate
CCT ..... cytidine triphosphate:phosphocholine cytidylyltransferase
CD36 ..... cluster of differentiation 36
CDP ..... cytidine diphosphate
CDP-DG ..... cytidine diphosphate diacylglycerol
CEBPB ..... CCAAT/enhancer-binding protein β
CEPT ..... choline/ethanolamine phosphotransferase
CHK ..... choline kinase
CHPT ..... CDP-choline phosphotransferase
CI ..... confidential interval
CK ..... creatine kinase
CL ..... cardiolipin
CMC ..... critical micellar concentration

CoA ..... coenzyme A
con ..... control
cPA ..... cyclic phosphatidic acid
$cPLA_2$ ..... cytosolic phospholipase A_2
CPT1 ..... carnitine palmitoyl transferase 1
CRP ..... C-reactive protein
CVD ..... cardiovascular diseases
CXCL3 ..... chemokine (C-X-C motif) ligand 3
dATP ..... deoxyadenosine triphosphate
dCTP ..... deoxycytidine triphosphate
DG ..... diacylglycerol
DGAT ..... diacylglycerol *O*-acyltransferase
dGTP ..... eoxyguanosine triphosphate
DM ..... differentiation mediu,
DMSO ..... dimethyl sulfoxide
dNTP ..... deoxy nucleotides
DPBS ..... Dulbecco's phosphate-buffered saline
DTT ..... dithiothreitol
dTTP ..... deoxythymidine triphosphate
EC ..... endothelial cells
EC_{50} ..... half maximal effective concentration
ECL ..... enhanced chemiluminescence
EDTA ..... ethylenediaminetetraacetic acid
EGTA ..... ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid
EMSA ..... electrophoretic mobility shift assay
ENPP ..... ectonucleotide pyrophosphatase/phosphodiesterase
ER ..... endoplasmic reticulum
ESI ..... electrospray ionization
FA ..... fatty acid
FABP ..... fatty acid binding protein
FBS ..... fetal bovine serum
FIA ..... flow injection analysis
FRET ..... fluorescence energy transfer
G3P glycerol-3-phosphate
GC ..... gas chromatography
GDE ..... glycerophosphodiester phosphodiesterase
GK ..... glycerol kinase
GLC ..... gas-liquid chromatography
GM ..... growth medium
GPAT ..... glycerol-3-phosphate acyltransferase
GPCR ..... G-protein coupled receptor
GPL ..... glycerophospholipids
GPR119 ..... G-protein coupled receptor 119
GPR132 ..... G-protein coupled receptor 132 aka G2 accumulation
GSK ..... glycogen synthase kinase

HBS ..... HEPES buffered saline
HDL ..... high-density lipoproteins
HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HILIC ..... hydrophilic interaction liquid chromatography
HMT ..... human myotubes
HOMA ..... homeostatic model assessment
HPLC ..... high performance liquid chromatography
HRMS ..... high resolution mass spectrometry
IDL ..... intermediate-density lipoproteins
IGT ..... impaired glucose tolerance
IKK ..... IκB kinase
IL ..... interleukin
INSR ..... insulin receptor
$iPLA_2$ ..... calcium-independent phospholipase A_2
IRAK4 ..... interleukin-1 receptor-associated kinase 4
IRE1 ..... inositol-requiring enzyme 1
IRS ..... insulin receptor substrate
JNK ..... c-Jun N-terminal kinases
LB ..... Lysogeny broth
LBD ..... ligand binding domain
LC ..... liquid chromatography
LCAT ..... lecithin-cholesterol acyltransferase
LDH ..... lactate dehydrogenase
LDL ..... low-density lipoproteins
LIPC ..... lipase C aka hepatic lipase
LIPG ..... lipase G aka endothelial lipase
LKB1 ..... liver kinase B1
LPA ..... lysophosphatidic acid
LPC ..... lysophosphatidylcholine
LPCAT ..... lysophosphatidylcholine acyltransferase
LPE ..... lysophosphatidylethanolamine
LPL ..... lipoproteinlipase
LTQ ..... Linear Trap Quadropole
lysoPAF ..... lyso platelet activating factor
MAM ..... mitochondria-associated membranes
MBOAT ..... membrane bound O-acyl transferase
MFSD2A ..... major facilitator superfamily domain containing 2A
MG ..... monoacyl glycerol
MGLL ..... monoglyceride lipase
MS ..... mass spectrometry
MS/MS ..... tandem mass spectrometry
mTORC2 ..... mammalian target of rapamycin complex 2
MYH ..... myosin heavy chain
n.d. ..... not detected
NAFLD ..... non-alcoholic fatty liver disease

NASH ..... non-alcolic steatohepatitis
NCBI ..... National Center for Biotechnology Information
NET-G ..... NaCl-EDTA-TRIS-gelatine buffer
NFκB ..... nuclear factor κ-light-chain-enhancer of activated B cells
NP-40 ..... Nonidet P40
NRE ..... neuropathy target esterase related esterase
ORM ..... orosomucoid aka α 1-acid glycoprotein
oxLDL ..... oxidized low-density lipoproteins
PA ..... phosphatidate
PAF ..... platelet activating factor
PAFAH ..... platelet activating factor acetylhydrolase
PBS ..... phosphate buffered saline
PC ..... phosphatidylcholine
PCYT ..... CTP:phosphocholine cytidylyltransfersase
PDH ..... pyruvate dehydrogenase complex
PDK4 ..... pyruvate dehydrogenase kinase 4
PDPK1 ..... phosphoinositide-dependent kinase 1
PE ..... phosphatidylethanolamine
PEMT ..... phosphatidylethanolamine N-methyltransferase
PERK ..... PKR-like endoplasmic reticulum kinase
PG ..... phosphatidylglycerol
PI ..... phosphatidylinositol
PIK3 ..... phosphatidylinositol-4,5-bisphosphate-3-kinase
PIP_3 ..... phosphatidylinositol-3,4,5-trisphosphate
PISD ..... phosphatidylserine decarboxylase
PKA ..... protein kinase A
PKC ..... protein kinase C
PL ..... phospholipase
PLA_1 ..... phospholipase A_1
PLA_2 ..... phospholipase A_2
PLB ..... phospholipase B
PLC ..... phospholipase C
PLD ..... phospholipase D
PLPP ..... phosphatidate phosphatase
PNPLA ..... patatin-like phospholipase domain containing
PON1 ..... serum paraoxonase/arylesterase 1
PPAR ..... peroxisome proliferator-activated receptor
PPRE ..... peroxisome proliferator-activated receptor responsive element
PRDX6 ..... peroxiredoxin 6
PS ..... phosphatidylserine
PTDSS ..... phosphatidylserine synthase
PTX ..... pertussis toxin
Q-MS ..... Quadrupole-Orbitrap mass spectrometry
qPCR ..... quantitative polymerase chain reaction
qTOF ..... Quadrupole-time-of-flight

RXRα ..... retinoid X receptor α
SDS ..... sodium dodecyl sulfate
SEM ..... standard error mean
SGMS ..... sphingomyelin synthase
SLC ..... solute carrier family
SM ..... stimulation medium
SMPD ..... sphingomyelinase
sn ..... stereospecific numbering
$sPLA_2$ ..... secreted phospholipase A_2
T2DM ..... diabetes mellitus type II
TAE ..... TRIS-acetate-EDTA
TBC1D1 ..... TBC1 domain family member 1
TBK1 ..... TANK-binding kinase 1
TEMED ..... tetramethylethylenediamine
TLC ..... thin layer chromatography
TLR ..... toll-like receptor
TMEM86B .... transmembrane protein 86B
TNFα ..... tumor necrosis factor α
TR-FRET ..... time-resolved fluorescence energy transfer
TRIS ..... tris(hydroxymethyl)aminomethane
TZD ..... thiazolidinedione
U ..... enzyme unit
UPLC ..... ultra-performance liquid chromatography
UPR ..... unfolded protein response
V/V ..... volume/volume
VASP ..... vasodilator-stimulated phosphoprotein
VLDL ..... very-low-density lipoproteins
W/V ..... weight/volume
XBP1 ..... X-box protein 1

List of publications

Parts of this thesis has been already published in:

Peer reviewed journal papers

Klingler C, Zhao X, Adhikary T, Li J, Xu G, Häring HU, Schleicher E, Lehmann R, Weigert C 2016. ”Lysophosphatidylcholine activate peroxisome proliferator-activated receptor δ and protect human skeletal muscle cells from lipotoxicity.” *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, Sep 30;1861(12 Pt A):1980-1992

Diploma thesis

Klingler C 2012. ”Lysophosphatidylcholine - Regulators of Muscle Cell Metabolism and Apoptosis.” Eberhard Karls University Tübingen. Supervisor: Prof. Dr. Cora Weigert

Poster presentations

”Lysophosphatidylcholines activate PPARδ and protect skeletal muscle cells from lipotoxicity.” American Diabetes Association’s 76th Scientific Sessions in New Orleans, Louisiana (USA) (2016)

”Lysophosphatidylcholines as regulators of gene expression in human skeletal muscle.” 50th European Association for the Study of Diabetes in Vienna (Austria) (2014)

”Stable isotope-assisted non-targeted lipidomics analysis identifies lysophosphatidylcholine as potential mediators of lipotoxicity.” Insulin receptor congress in Barcelona

(Spain) XII International Symposium on Insulin Receptors and Insulin Action hosted by Committee of the International Symposium on Insulin Receptor and Insulin Action) (2013)

Oral presentations

”Lysophosphatidylcholine als Regulatoren der Genexpression humaner Skelettmuskelzellen.” German Diabetes Congress in Berlin (Germany) hosted by German Diabetes Society (2014)

”Lysophosphatidylcholines are mediators of lipotoxicity in human skeletal muscle cells.” German Diabetes Congress in Leipzig (Germany) hosted by German Diabetes Society (2013)

Abstract

Lysophosphatidylcholines (LPC) gained particular attention in metabolomics studies as potential biomarkers for metabolic diseases like obesity, insulin resistance, and type 2 diabetes mellitus. LPC plasma levels negatively correlate with insulin resistance, inflammatory parameters, and body mass index. Aim of this thesis was to investigate the physiological role of LPCs as potential regulators of metabolism and inflammation using skeletal muscle cells as model system. Human primary myotubes (HMT) were treated with 10 µM LPC(16:0) or LPC(18:1) for 24 h which were proven not to induce cytolysis. Microarray analysis and qPCR from total RNA were performed to study LPC effects. LPC treated HMT displayed activation of peroxisome proliferator-activated receptor (PPAR) target genes including *PDK4*, *ANGPTL4*, *PLIN2*, and *CPT1A*. The induction of *PDK4* and *ANGPTL4* was sensitive to PPARδ antagonists GSK0660 and GSK3787. The increase of *PDK4* was reduced by siRNA against *PPARD*. Luciferase assays showed activation of the ligand binding domain of PPARδ by LPCs. EMSAs demonstrated that LPCs can enhance the DNA binding activity of the PPARδ/RXRα complex. TR-FRET-based *in vitro* competitive binding assays displayed dose-dependent agonist displacement from the PPARδ ligand binding domain by both LPCs and by an ether analogue of LPCs proving that LPCs are direct ligands of PPARδ. The LPC analogue exhibited a robust induction of *PDK4*/*ANGPTL4* confirming the importance of the LPC structure. Both LPCs caused the phosphorylation of AMPK. LPC(16:0) marginally increased basal glucose uptake in L6 cells and palmitate oxidation in HMT. Both LPCs could reduce ER stress and inflammation caused by palmitate. The anti-inflammatory effects of LPCs were reduced by GSK0660 treatment. In conclusion, LPCs can activate PPARδ as direct ligands and AMPK by a yet unknown mechanism in skeletal muscle cells. As a result, LPCs pro-

tect against lipotoxicity caused by palmitate. This leads to the assumption that increasing plasma LPC levels can be beneficial via activation of PPARδ resulting in anti-inflammatory and anti-diabetic effects. It is proposed that LPC levels are of pharmacological relevance.

Zusammenfassung

Lysophosphatidylcholine (LPC) erhalten zunehmende Aufmerksamkeit in Metabolomics Studien, die zeigen, dass diese Lipide als potentielle Biomarker für Adipositas, Insulinresistenz und Typ 2 Diabetes mellitus fungieren können. Dabei sind Plasmaspiegel der LPCs negativ mit Insulinresistenz, Inflammation und Body Mass Index assoziiert. Ziel dieser Dissertation war es, die physiologische Funktion der LPCs anhand von Skelettmuskelzellen als Modellsystem zu erforschen. Dabei wurden humane Myotuben (HMT) für 24 h mit 10 µM LPC(16:0) oder LPC(18:1) behandelt, was nachweislich keine Zelllyse induzierte. Microarray Analysen und qPCR wurden durchgeführt um LPC Effekte zu ermitteln. Mit LPCs behandelte HMT zeigen eine Aktivierung von Peroxisome proliferator-activated receptor (PPAR) Zielgenen wie *PDK4*, *ANGPTL4*, *PLIN2* und *CPT1A*. Die Induktion von *PDK4* und *ANGPTL4* konnte mit den PPARδ Antagonisten GSK0660 und GSK3787 gehemmt werden. Zudem reduzierte siRNA gegen PPARδ die Induktion von *PDK4*. Luciferase Reportergenassays zeigten die Aktivierung der Ligandenbindungsdomäne von PPARδ durch LPCs. Eine Verstärkung der PPARδ/RXRα DNA Bindung wurde in EMSAs nachgewiesen. In TR-FRET basierten Kompetitionsassays erzielten LPCs und ein Ether-Analogon der LPCs eine dosisabhängige Verdrängung eines PPAR Agonisten aus der Ligandenbindungsdomäne von PPARδ. Das LPC-Analogon rief zudem eine ausgeprägte Induktion von *PDK4* und *ANGPTL4* hervor und bestätigt damit die Bedeutsamkeit der LPC Struktur. Beide LPCs lösten die Phosphorylierung von AMPK aus. LPC(16:0) konnte die basale Glukoseaufnahme in L6 Zellen, sowie die Palmitatoxidation in HMT in begrenztem Ausmaß erhöhen. Beide untersuchten LPCs verminderten ER-Stress und Inflammation, ausgelöst durch Palmitat Behandlung. Die anti-inflammatorischen Effekte waren zudem GSK0660-sensitiv. Diese Arbeit

zeigt auf, dass LPCs PPARδ als direkte Liganden aktivieren und AMPK durch noch unbekannte Mechanismen in HMT stimulieren. Als Konsequenz schützen LPCs vor Lipotoxizität, hervorgerufen durch Palmitat. Das führt zu der Annahme, dass erhöhte Plasma-LPC Spiegel positiv einzustufen sind, da durch sie PPARδ aktiviert wird, was anti-inflammatorische und anti-diabetische Effekte erzeugt. LPC Plasmaspiegel könnten daher von pharmakologischer Relevanz sein.

1 Introduction

1.1 The lipid class of glycerophospholipids

Lysophosphatidylcholines (LPCs, formerly known as lysolecithin) are a subgroup of lipids that belong to the family of glycerophospholipids (GPL) (Schmitz and Ruebsaamen, 2010). GPLs are composed of a glycerol backbone with different substituents at position *sn-1* (stereospecific numbering) and *sn-2*, and a phosphate group at position *sn-3*, as schematically depicted in figure 1.1.

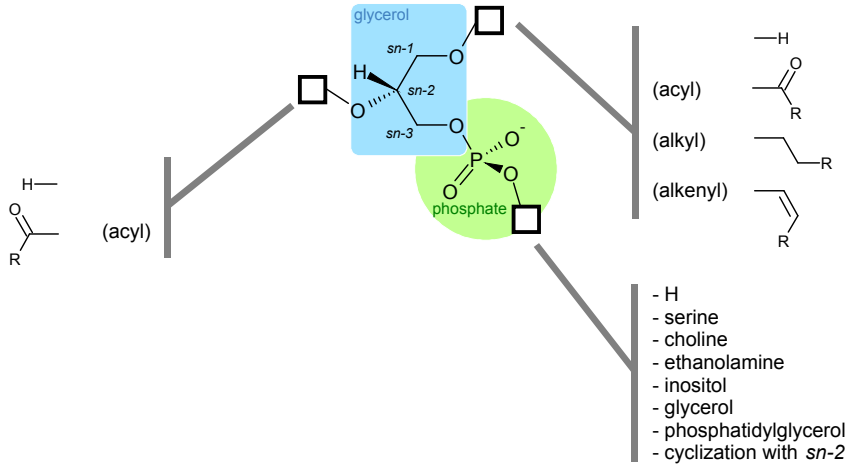


Figure 1.1: Schematic view of glycerophospholipids. The glycerol part is highlighted in blue, phosphate in green. The stereospecific numbering (sn) and possible substituents (boxes) at different position of the glycerophosphate backbone are given. The positions *sn-1/sn-2* contain either none (only hydroxyl groups), acyl, alkyl, or alkenyl groups. The phosphate can be linked to various substituents. R describes alkyl/alkenyl groups.

The nature of the substituents vary depending on the position. Radyl groups (alkyl/alkenyl or acyl groups) are located at position *sn-1* of the glycerol backbone while position *sn-2* typically contains acyl chains in mammals (Magnusson and Haraldsson, 2011; Nagan and Zoeller, 2001; Yamashita et al., 1997). A heterogeneous

spectrum of compounds are attached to the phosphate located at position *sn-3* (polar head group) which includes choline in the GPL class of phosphatidylcholines (PC aka lecithin), ethanolamine in phosphatidylethanolamines (PE), inositol in phosphatidylinositols (PI), serine in phosphatidylserines (PS), glycerol in phosphatidylglycerols (PG), phosphatidylglycerol in cardiolipins (CL), and no compound in phosphatidates (PA), to name a few examples (Yamashita et al., 1997). The nomenclature of these lipids according to LIPID MAPS (Fahy et al., 2005, 2009) and part of their diversity is shown exemplarily for several PCs at figure 1.2 on the following page.

Generally, the members of the GPL class are abbreviated according to the polar headgroup. The appearances, position, and linkage of the two radyl chains on the backbone are described in brackets: e.g. PC(*sn-1*/*sn-2*). The type of linkage of the radyl group to glycerol is added as prefixes in front of the radyl description: O for ether (termed plasmanylcholines in case of PC), P for 1*Z*-alkenyl ethers (termed plasmenylcholines in case of PC), and no descriptors are attached for ester. Examples are depicted in figure 1.2 on the next page.

The lyso forms of GPLs contain only one radyl group which changes the nomenclature to LPC(*sn-1* or *sn-2*) in case of the lysolipids of PC. A special class of PCs are platelet activating factors (PAF) with acetyl groups at the *sn-2* position and alkyl/alkenyl groups at *sn-1*, which are also depicted in figure 1.2 on the following page as acetyl form of PC. LPCs with alkyl or alkenyl chains instead of acyl chains are often abbreviated as lysoPAFs.

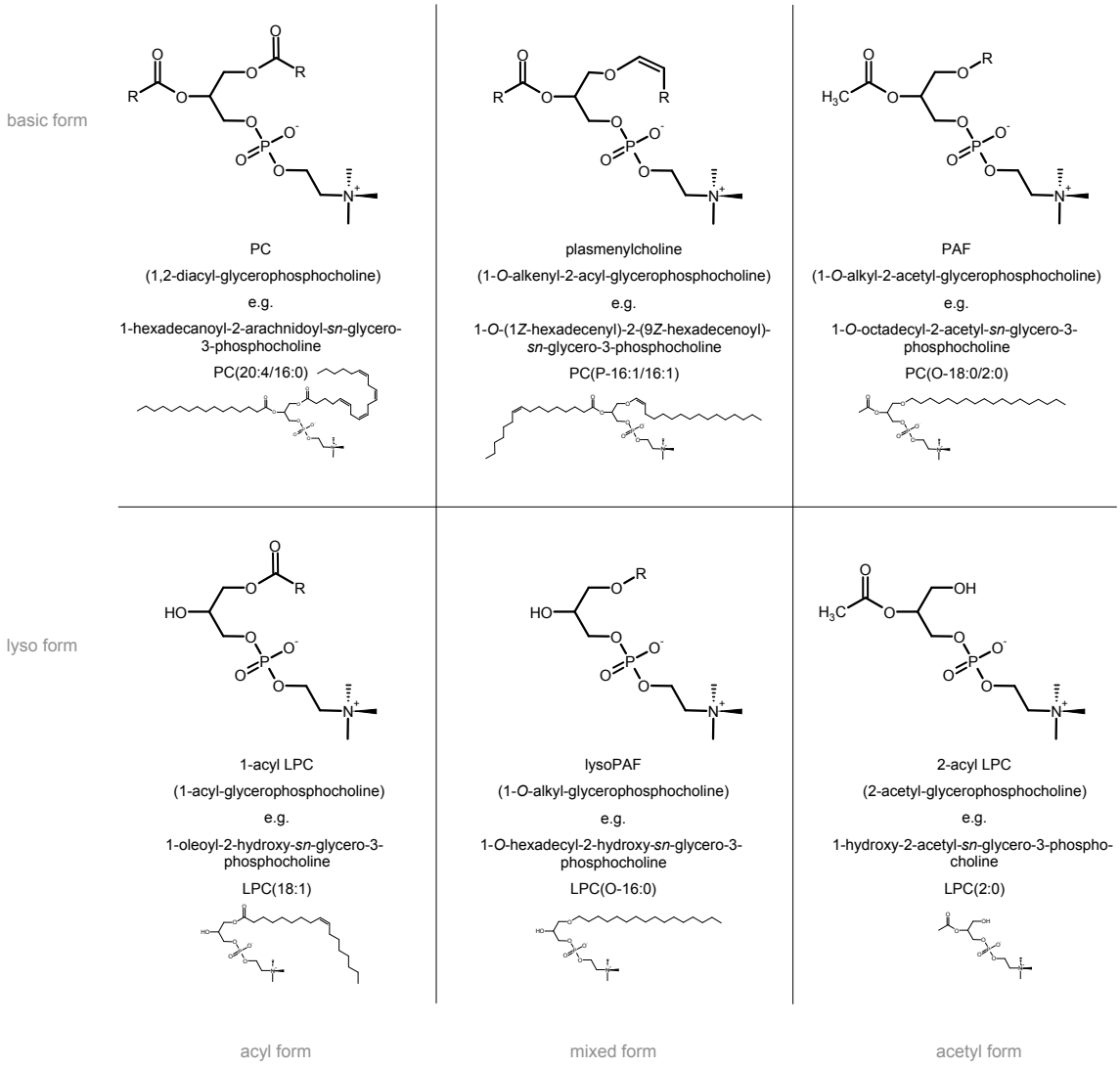


Figure 1.2: Overview of selected subclasses of phosphatidylcholines. This overview of different PC subclasses includes the corresponding structures and nomenclature examples. General chemical structures of the subclasses of PCs are depicted atop of the general category name or trivial name. Below, there is the systematic chemical expression of the subclass in brackets and one example of the subclass with the respective abbreviation according to the nomenclature used in this work and the corresponding chemical structure. The different PC subclasses are grouped in rows based on the basic forms and one possible example of a lyso form. The grouping in columns is in accordance with the linkage and type of the radyl chains. PCs with only acyl chains are depicted in the column acyl form. Mixed forms include both acyl and alkyl substituents. The special subclass of acetylated PCs are shown in the column acetyl form. Abbreviations: LPC (lysophosphatidylcholine), PAF (platelet activating factor), PC (phosphatidylcholine), R = alkyl or alkenyl groups.

1.2 Chemical properties of lysophosphatidylcholine

LPCs are monoradylglycerophospholipids composed of only a single radyl group leaving one vicinal located free hydroxyl group at either *sn-1* or *sn-2* position (requirement of the lyso-prefix in the term). The unoccupied hydroxyl group enables a quick migration of acyl chains from *sn-1* to *sn-2* and *vice versa* (Pluckthun and Dennis, 1982). At physiological conditions, pH 7.0 and 37 °C, 20 % of the C18:2 groups at *sn-2* of LPC(18:2) can migrate to the *sn-1* position within 2 min (Croset et al., 2000). At equilibrium and under these conditions the acyl chain at position *sn-1* is preferred (Croset et al., 2000).

LPCs have a special steric shape. The three methyl groups of choline are bulky while the radyl part sterically resembles a hydrophobic pole. In summary, these features create a cone-like shape as depicted in figure 1.3. Furthermore, LPCs are zwitterions under physiological conditions. This results from the pK_A of the phosphate group which is 0.8 in phosphatidylcholine (Moncelli et al., 1994). The phosphate group therefore carries a negative charge while choline has a permanent positive charge. LPCs are also highly hygroscopic due to the choline part (Beyer et al., 2004). In summary, the shape and amphiphatic (hydrophobic and hydrophilic) nature of LPCs renders them water-soluble detergents (Small, 1968; Henriksen et al., 2010; Helenius and Simons, 1975).

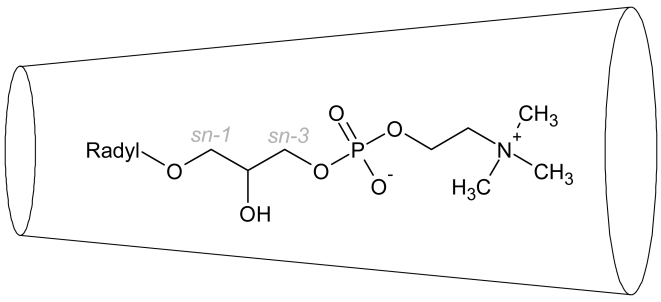


Figure 1.3: Chemical structure of LPCs. Chemical structure and stereo specific numbering of LPCs are depicted together with their steric shape of a cone-like form. Abbreviations: sn (stereospecific numbering)

LPCs are able to reversibly integrate into lipid bilayers with a half-time of 50 - 500 ms (Elamrani and Blume, 1982), and they are able to disrupt the integrity of physiological membranes by their shape, causing membrane curvature stress and pore

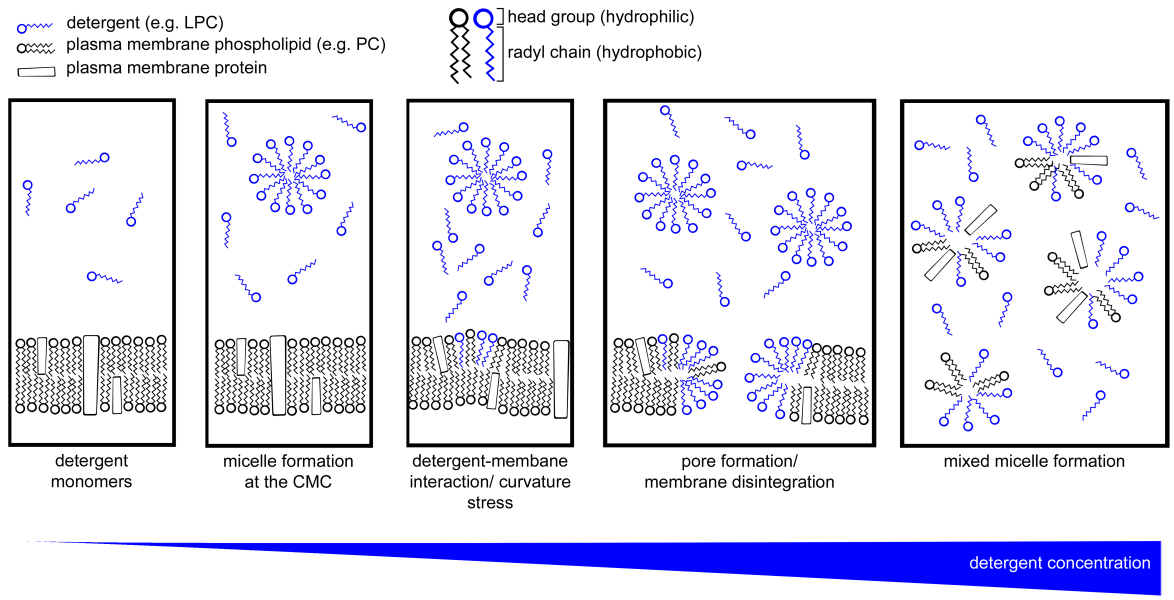


Figure 1.4: Disruption of plasma membranes integrity by LPCs acting as detergents. At the critical micellar concentrations (CMC) of LPCs, or detergents in general, the compound monomers aggregate to micelles. With increasing concentrations, the detergents start to integrate into plasma membranes causing curvature stress. At saturation with detergent molecules, the membrane starts to disintegrate into mixed micelles containing detergents, lipids, and membrane proteins (Arouri and Mouritsen, 2013; Heerklotz, 2008; Weltzien, 1979).

formation (Yoo and Cui, 2009; Weltzien, 1979; Heerklotz, 2008). This process is depicted in figure 1.4. At low concentrations detergent monomers in aqueous solutions form monolayers between the water and the air barrier or float freely in the solution. When the concentrations of detergents reach the critical micellar concentration (CMC), aggregates termed micelles start to form. In aqueous solution which contains plasma membranes, detergents are able to interact with the membranes. In a first step, detergent molecules associate with membranes until saturation of the membrane. In this step the membrane experiences curvature stress. If the concentration of detergents is high enough, mixed micelles containing detergents and plasma membrane components are formed by disintegration of the plasma membrane. At a certain level of detergents only this mixed micelles exist (Helenius and Simons, 1975; Heerklotz, 2008). The whole process depends on the membrane composition, which influences the affinity of the detergent for the host membrane and on the shape and length of

Table 1.1: Critical micellar concentration (CMC) of selected compounds. Abbreviations: acyl carnitine (AC), coenzyme A (CoA), lysophosphatidylethanolamine (LPE).

compound	CMC	temperature	reference
Triton X-100	0.37 mM	22 °C	Busto et al., 2007
LPC(12:0)	0.64-0.7 mM	25 °C	Stafford et al., 1989; Henriksen et al., 2010
	0.47 mM	37 °C	Matsuzaki et al., 1988
LPE(12:0)	0.33 mM	25 °C	Stafford et al., 1989
dodecylphosphocholine	1.1 mM	25 °C	Stafford et al., 1989
LPC(14:0)	0.045-0.07 mM	25 °C	Stafford et al., 1989; Henriksen et al., 2010
	0.048 mM	37 °C	Matsuzaki et al., 1988
LPC(16:0)	0.004-0.007 mM	25 °C	Stafford et al., 1989; Henriksen et al., 2010
	0.0029 mM	37 °C	Matsuzaki et al., 1988
AC(16:0)	0.015 mM	22 °C	Requero et al., 1993
C(16:0) CoA	0.035 mM	22 °C	Requero et al., 1993
LPC(18:0)	0.00025 mM	37 °C	Matsuzaki et al., 1988
PC(O-18:0/O-1:0)	0.035 mM	22 °C	Busto et al., 2007
LPC(18:1)	0.002 mM	37 °C	Matsuzaki et al., 1988

the detergent determining the extent of disturbance within the membrane (Henriksen et al., 2010; Heerklotz and Seelig, 2000; Heerklotz, 2008).

The CMCs of different LPCs and other compounds are compared in table 1.1. The table also shows the CMC of Triton-X 100 (Helenius and Simons, 1975), a commonly used nonionic detergent and the synthetic PC(O-18:0/O-1:0) also known as edelfosine (Munder et al., 1979). Acyl carnitines (AC) are intracellular fatty acid (FA) carriers and are structurally related to LPCs (Goni et al., 1996). The CMC is often used as predictor for the strength of a detergent but should be used with caution and only as rough estimation (Heerklotz, 2008; Henriksen et al., 2010). The hemolytic potency of LPCs on erythrocyte membranes at 37 °C decreases as follows LPC(18:0)>LPC(16:0)>LPC(14:0)>LPC(18:1)>LPC(12:0), while the CMCs at 37 °C increase in the following order: LPC(18:0)<LPC(18:1)<LPC(16:0)<LPC(14:0)<LPC(12:0) (Matsuzaki et al., 1988). The cytolytic properties of the different LPC species should be considered when the physiological functions of LPCs are studied (Weltzien, 1979).

Once integrated into membranes, LPCs can also perform a transbilayer movement called flip-flop. The flip-flop from the outer to the inner leaflet of plasma membranes has a half-time of 11 h for LPC(16:0) and 4 h for LPC(18:1) in human erythrocytes and is strongly dependent on the lipid composition of the plasma membrane and the acyl chain characteristics of the moving lysolipid (Bergmann et al., 1984).

1.3 LPC biosynthesis in humans

1.3.1 Kennedy pathway and other biosynthetic routes of PC

The biosynthesis and further metabolism of LPCs are shown in figure 1.5 on the following page. The *de novo* synthesis of LPCs is called *Kennedy pathway* (Kennedy and Weiss, 1956). This pathway starts with glycero 3-phosphate (G3P), a product of glycolysis, gluconeogenesis, or lipolysis. G3P can also originate from the diet in form of glycerol which is phosphorylated to G3P by glycerol kinase (GK). G3P is acylated at the position *sn-1* by glycerol-3-phosphate acyltransferase (GPAT) to lysophosphatidate (LPA). LPA is further acylated by 1-acylglycerol-3-phosphate acyltransferases (AGPAT) to phosphatidate (PA) (Yamashita et al., 2014).

PA is at the crossroad of two pathways. PA can either be metabolized to cytidine diphosphate diacylglycerols (CDP-DG) or diacylglycerol (DG) (Horvath and Daum, 2013). From CDP-DG, phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipins (CL) can be formed.

DG is acylated to triacylglycerides (TG) or is directly converted to PC by either choline phosphotransferase (CHPT) or CDP-choline/ethanolamine phosphotransferase (CEPT). Both enzymes require CDP-choline as co-substrate (Horvath and Daum, 2013).

Choline is phosphorylated by choline kinase (CHK) and converted to CDP-choline via CTP:phosphocholine cytidylyltransferase (PCYT) (Cornell and Ridgway, 2015). This reaction is supposed to be the rate limiting step in PC synthesis in the endoplasmatic reticulum (ER) (Cornell and Ridgway, 2015).

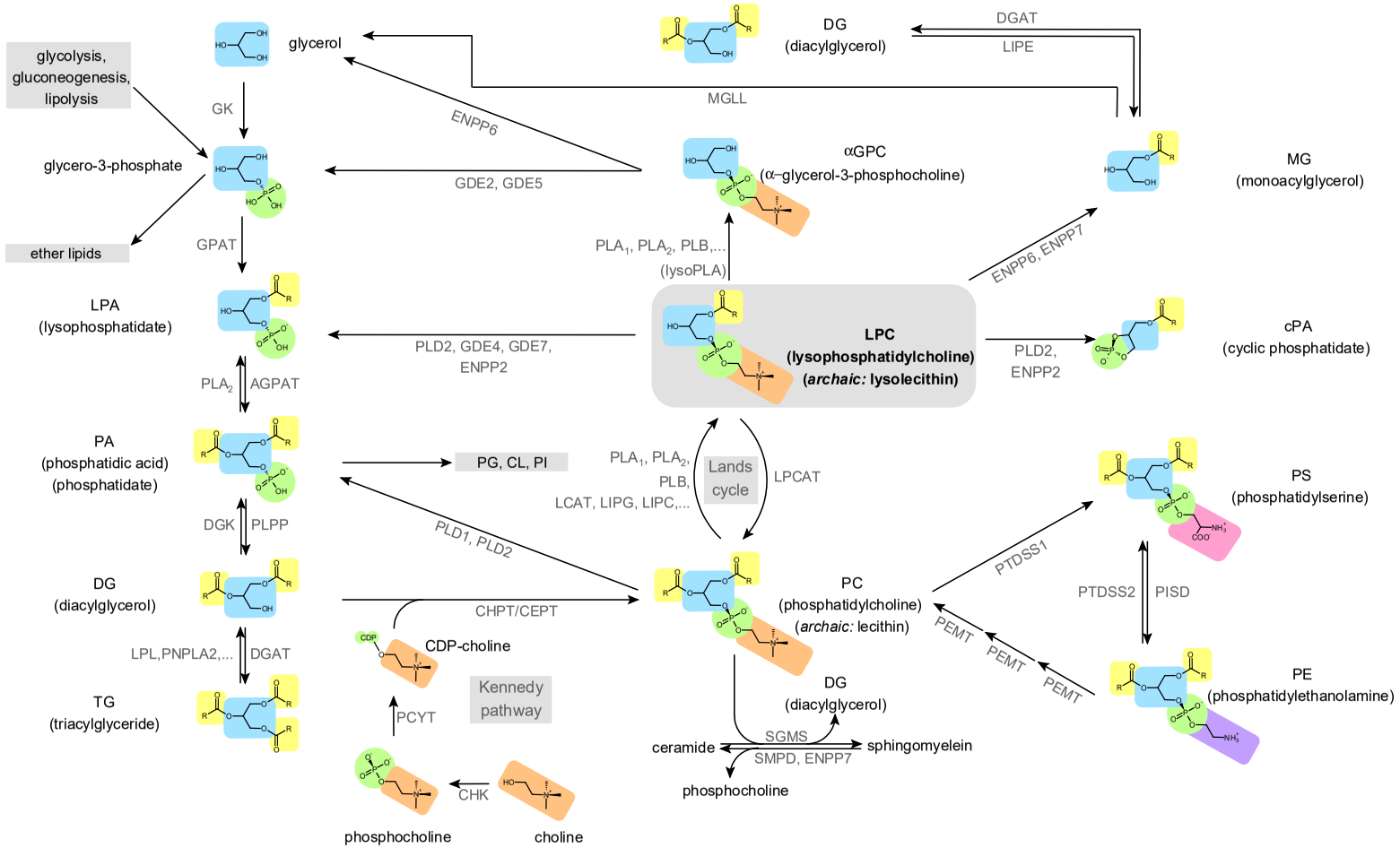


Figure 1.5: Biochemical pathways of glycerophospholipids. The biochemical pathways of glycerophospholipids focusing on PC/LPC metabolism in humans based on pathways from (Horvath and Daum, 2013; Kent, 1995; Cornell and Ridgway, 2015; Schmitz and Ruebsaamen, 2010; Yamashita et al., 1997; Stefan et al., 2005; Corda et al., 2014; Hannun and Obeid, 2008; Yuan et al., 2016) and other references mentioned in the text (adapted from (Klingler, 2012)). Lipid moieties are highlighted in yellow, glycerol backbone in blue, phosphate in green, choline in orange, serine in red, ethanolamine in violet. Abbreviations: AGPAT (1-acylglycerol-3-phosphate *O*-acyltransferase), CEPT (choline/ethanolamine phosphotransferase), CHK (choline kinase), CL (cardiolipin), CHPT (choline phosphotransferase), DGK (diacylglycerol kinase), DGAT (diacylglycerol *O*-acyltransferase), LIPG (endothelial lipase), ENPP (ectonucleotide pyrophosphate/phosphodiesterase), GDE (glycerosphosphodiester phosphodiesterase), GK (glycerol kinase), GPAT (glycerol-3-phosphate acyltransferase), LIPC (hepatic lipase), LCAT (lecithin-cholesterol acyltransferase), LPCAT (LPC acyl transferase), LPL (lipoprotein lipase), lysoPLA (lysophospholipase), MGLL (monoglyceride lipase), PCYT (CTP:phosphocholine cytidylyltransfersase), PEMT (phosphatidylethanolamine *N*-methyltransferase), PI (phosphatidylinositol), PISD (phosphatidylserine decarboxylase), PG (phosphatidylglycerol), PLA (phospholipase A), PLB (phospholipase B), PLD (phospholipase D), PLPP (phosphatidate phosphatase), PNPLA (patatin like phospholipase domain containing), PTDSS (phosphatidylserine synthase), SGMS (sphingomyelin synthase), SMPD (sphingomyelinase).

PC can undergo a headgroup exchange for the interconversion of PC, PS and PE (Kent, 1995). In mammals, PC together with serine can be metabolized by PS synthase I (PTDSS1) to choline and PS at the mitochondria-associated membranes (MAM), which are special loci where ER and mitochondria closely interact. PS can be decarboxylated by PS decarboxylase (PISD) to phosphatidylethanolamine (PE) in the mitochondrial outer membrane. PE can be re-converted to PS by PS synthase II (PTDSS2) at the MAM (reviewed in (Vance and Tasseva, 2013)). PE can also be methylated by phosphatidylethanolamine N-methyltransferases (PEMT) requiring S-adenosyl methionine as co-substrate to form PC. This triple methylation takes place only in liver (Vance et al., 1997) and accounts for 30 % of the total hepatic PC synthesis (DeLong et al., 1999; Vance, 2014; Reo et al., 2002). The rest of the PC production is provided by the *de novo* synthesis from DG and choline via the *Kennedy pathway* (Kent, 1995).

The ether forms of PC are formed in a completely different pathway starting from dihydroxyacetone phosphate, a product of triose-phosphate isomerase using G3P as substrate (not shown) (Watschinger and Werner, 2013b).

The acyl chains of PC are subjected to a constant remodeling in order to fulfill its physiological functions as major plasma membranes and lipoprotein component (Yao and Vance, 1988; van Meer et al., 2008), signaling lipid transporter (Thomas et al., 1984; Zhou and Nilsson, 2001), and lung surfactant (Holm et al., 1996). The remodeling and maturation pathway or *Lands cycle* (Lands, 1958), is a sequence of deacylation of PC by phospholipases A (PLA) forming LPC and one FA, followed by the reacylation to PC by LPC acyltransferases (LPCAT) using acyl coenzyme A (CoA) as substrate. The FA formed by PLAs are ATP-dependently activated to acyl CoA for re-entering the metabolic pathways of lipids (Yamashita et al., 2014). It is estimated that both, the *de novo* synthesis and the *Lands cycle*, contribute almost equally to the PC pool of many cell types (Zhao et al., 2008).

1.3.2 Phospholipase A_1

The family of phospholipases (PL) is divided into the subclasses PLA_1, PLA_2, PLB, PLC, and PLD. Each family has its own cleavage pattern which is depicted in figure 1.6.

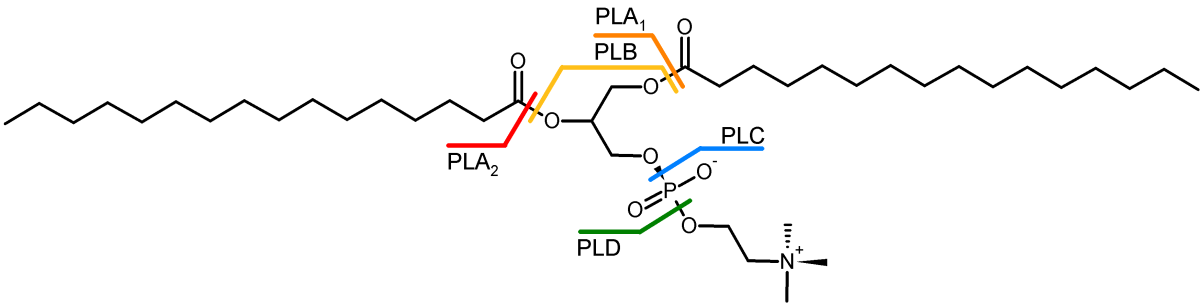


Figure 1.6: Cleavage sites of phospholipases. This figure depicts the cleavage site of PLA_1, PLA_2, PLB, PLC, and PLD on PC.

PLA_1 cleave the *sn-1* position of phospholipid forming *sn-2* radyl lysolipids. This subclass of phospholipases is small with only a few members (Richmond and Smith, 2011). Endothelial lipase (aka lipase G, LIPG), hepatic lipase (aka lipase C, LIPC), and lecithin cholesterol acyltransferase (LCAT) possess also PLA_1 activity and contribute to the LPC production in the circulation (Duong et al., 2003; Riederer et al., 2012; Subbaiah et al., 1992; Shamburek et al., 1996).

LIPG is expressed in liver, kidney, lung, placenta, ovary, thyroid gland, testis, and macrophages (Bartels et al., 2007; Jaye et al., 1999; Hirata et al., 1999), hydrolyzes preferentially PE and PC from high-density lipoproteins (HDL) (Jaye et al., 1999; Duong et al., 2003; Gauster et al., 2005; Riederer et al., 2012) and has only low lipase activity toward TGs (McCoy et al., 2002; Jaye et al., 1999; Hirata et al., 1999). Additionally, LIPG also has PLA_2 and lysophospholipase activity (Gauster et al., 2005). Lysophospholipase describes the ability to hydrolyze the remaining acyl chain of lysolipids. In case of LPC the product is a FA and α-glycero-3-phosphocholine (αGPC).

LIPC hydrolyzes TG and to a minor extend PC (Duong et al., 2003). LIPC is expressed in liver where it is located at the hepatic sinusoids and binds to HDL, very-low-density lipoproteins (VLDL), and low-density lipoproteins (LDL) (Santamarina-Fojo

et al., 2004). The catalytic activity is regulated by the composition of the lipoproteins. Sphingomyelin inhibits its general activity and influences its substrate specificity, a common feature of many PC hydrolyzing enzymes in the circulation including LIPG and LCAT (Yang and Subbaiah, 2015; Yang et al., 2014; Subbaiah and Liu, 1993).

LCAT does not belong to the family of PLA_1 (Richmond and Smith, 2011) but shows PLA_1 activity (Subbaiah and Liu, 1996) and contributes to the LPC pool of the circulation (Subbaiah et al., 1994; Shamburek et al., 1996). LCAT is secreted from the liver and is associated with the lipoproteins HDL, where it catalyzes the transfer of one acyl chain from PC to cholesterol, forming cholesterol acyl esters and LPCs (Ossoli et al., 2016). Concerning the head-group, LCAT favors PC and PE (Pownall et al., 1985; Grove and Pownall, 1991; Christiaens et al., 2000; Shamburek et al., 1996). LCAT can also use PAF and oxidized PC as substrates (Liu and Subbaiah, 1994; Goyal et al., 1997).

LCAT produces predominantly saturated LPCs, while LIPC and LIPG form unsaturated LPCs (Yang and Subbaiah, 2015; Glomset, 1968; Shamburek et al., 1996).

1.3.3 Phospholipase A_2

PLA_2 are specialized to hydrolyze the *sn-2* position of phospholipids releasing a lysolipid and one free FA. The superfamily of PLA_2 has numerous members. The classification of PLA_2 is shown in table 1.2 on the following page (Dennis, 1994; Schaloske and Dennis, 2006). The PLA_2 are divided in groups that are consecutively numbered: G1 - G16. The numbering has been established 1994 for snake venom PLA_2 and has been expanded ever since (Dennis, 1994). Some groups are summarized in types according to special requirements of the catalytic activity, their localization in the cell or other features. Their subgroups are assigned to an alphabetic character (Dennis et al., 2011; Schaloske and Dennis, 2006). For a more functional classification of the groups, PLA_2 are divided into the following types: secreted PLA_2s ($sPLA_2$), cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$), platelet-activating factor acetylhydrolases (PAFAH), lysosomal PLA_2 ($LPLA_2$), and adipocyte-specific PLA_2 ($AdPLA_2$) (Dennis et al., 2011; Schaloske and Dennis, 2006).

Table 1.2: Official types of the human PLA_2s. Official types of the human PLA_2 class and other enzymes with PLA_2 activity (Dennis et al., 2011; Ghosh et al., 2006; Murakami et al., 2015; Ramanadham et al., 2015; Fisher, 2011; Fisher et al., 2016; Rozenberg et al., 2003; Gauster et al., 2005; Pownall et al., 1985; Subbaiah and Liu, 1996; Maury et al., 2002). The six types of PLA_2 (secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), platelet activating factor acetylhydrolase (PAFAH), lysosomal PLA_2 ($LPLA_2$), and adipocyte specific PLA_2 ($AdPLA_2$)) are listed with their groups (symbols: PLA2G1 - PLA2G16) and subgroups (A-F). Also other enzymes with PLA_2 activity are given. Enzymes marked with * also have PLA_1 activity. Official symbols are italicized. Abbreviations: LCAT (lecithin-cholesterol acyltransferase), LIPG (lipase G aka endothelial lipase), Lp-PLA_2 (lipoprotein-associated PLA_2), PLA (phospholipase A), PLB (phospholipase B), PNPLA (patatin like phospholipase domain containing), PON1 (serum paraoxonase/arylesterase 1), PRDX6 (peroxiredoxin 6).

$sPLA_2$	$cPLA_2$	$iPLA_2$
PLA2G1B	*PLA2G4A* 1/2 aka $cPLA_2\alpha$*	*PLA2G6A* aka PNPLA9, $iPLA_2\beta$*
PLA2G2A	*PLA2G4B* aka $cPLA_2\beta$*	PLA2G6B aka *PNPLA8*, $iPLA_2\gamma$*
PLA2G2C	*PLA2G4C* aka $cPLA_2\gamma$*	PLA2G6C aka *PNPLA6*, $iPLA_2\delta$*
PLA2G2D	*PLA2G4D* aka $cPLA_2\delta$*	PLA2G6D aka *PNPLA3*, $iPLA_2\varepsilon$
PLA2G2E	*PLA2G4E* aka $cPLA_2\varepsilon$*	PLA2G6E aka *PNPLA2*, $iPLA_2\zeta$
PLA2G2F	*PLA2G4F* aka $cPLA_2\zeta$*	PLA2G6F aka *PNPLA4*, $iPLA_2\eta$
PLA2G3		
PLA2G5	PAFAH	$LPLA_2$
PLA2G10	*PLA2G7* aka Lp-PLA_2	*PLA2G15* aka $LPLA_2$*
PLA2G12A	PLA2G7B aka *PAFAH2*	
PLA2G12B	PLA2G8A aka *PAFAH1B*	adipocyte-specific PLA_2
		PLA2G16 aka AdPLA*
others		
*PLB1**		
PRDX6		
*LIPG**		
*LCAT**		
PON1		

The type of $sPLA_2$ consists of eleven groups that are reviewed elsewhere (Murakami et al., 2015). They all have a catalytic dyad of histidin and aspartate which require Ca^{2+} for their catalytic activity (Murakami et al., 2015). This group of phospholipases have low selectivity towards the *sn-1* or the *sn-2* position and low selectivity for the saturation degree of the acyl chains but show a general preference for PG and PC (Singer et al., 2002). $sPLA_2$s potentially also contribute to the plasma pool of LPCs (Gesquiere et al., 2002; Singh and Subbaiah, 2007). PLA2G1B (aka pancreatic PLA_2) is the best studied $sPLA_2$. It is secreted by the acinar cells of the pancreas into the intestinal lumen and takes part in the digestion and absorption of phospholipids and

TG, and also affects the plasma LPC levels after a meal (Huggins et al., 2002; Labonte et al., 2006).

The type of $cPLA_2$ only has one group known as group G4 which consists of six subgroups (PLA2G4A - F). All of them have a catalytic dyad of serine and aspartate that requires a conserved arginine residue in the active center. In addition to their PLA_2 activity, they all exhibit PLA_1 and lysophospholipase activity (Ghomashchi et al., 2010). Subgroup C and E are expressed in skeletal muscle (Pickard et al., 1999; Underwood et al., 1998; Ohto et al., 2005). Except for subgroup C, all $cPLA_2$ has a C2 domain that binds Ca^{2+} ions and therefore respond to intracellular Ca^{2+} rises which leads to membrane translocation of the phospholipases and enables the binding to their substrates (Dennis et al., 2011; Xu et al., 1998; Perisic et al., 1998; Dessen et al., 1999).

$iPLA_2$ consists of six subgroups PLA2G6A - F (Ramanadham et al., 2015). This type of PLA_2 also have the catalytic dyad of serine and aspartate but lacks the Ca^{2+}-dependence. The subgroup PLA2G6A (aka patatin-like phospholipase domain containing 9 (PNPLA9), $iPLA_2\beta$) was the first member discovered in this group and also has PLA_1 (Tang et al., 1997), lysophospholipase (Lio and Dennis, 1998), transacylase (Lio and Dennis, 1998), and acyl-CoA thioesterase activity (Jenkins et al., 2006; Carper et al., 2008). The transacylase activity describes the transfer of an acyl residue from PC or LPC onto another LPC (Lio and Dennis, 1998). The thioesterase activity - the hydrolysis of acyl CoA into FA and HS-CoA - supports FA oxidation in skeletal muscle by providing free HS-CoA (Carper et al., 2008). The enzyme has several splice variants from which two (PLA2G6A1 and PLAG6A2) are known to be active and at least one is expressed in skeletal muscle (Ma et al., 1999; Larsson et al., 1998; Carper et al., 2008). The predominant location of this $iPLA_2$ is the cytosol but it was also found in nucleus, ER, golgi, and mitochondria (Ramanadham et al., 2015). In latter compartment, the phospholipase is involved in CL remodeling (Malhotra et al., 2009; Hsu et al., 2013; Ye et al., 2016).

PLA2G6B (aka PNPLA8, $iPLA_2\gamma$) is expressed in many tissues including skeletal muscle (Tanaka et al., 2000; Mancuso et al., 2000). The phospholipase is localized

in the mitochondria, peroxisomes (Kinsey et al., 2007; Mancuso et al., 2007), and ER (Kinsey et al., 2005). The lipase also shows PLA_1, lysophospholipase, and transacylase activity (Yan et al., 2005; Dennis et al., 2011). PLA2G6B is also involved in CL remodeling (Mancuso et al., 2007; Ye et al., 2016). Knockout of PLAG6B in mice causes protection against diet induced insulin resistance and obesity (Song et al., 2010). This is supposed to be a consequence of an altered CL content which compromises the mitochondrial FA oxidation (Mancuso et al., 2010; Song et al., 2010; Yoda et al., 2010).

1.3.4 Etherlipid degradation

The alkyl-acyl (plasmanylcholine) and alkenyl-acyl (plasmenylcholine) forms of PC are degraded in two steps. The acyl chain is hydrolyzed by PAF acetylhydrolases (PAFAH). PAFAH is a family of intracellular and extracellular PLA_2 of the group PLA2G7 and PLA2G8, catalyzing the hydrolysis of the *sn-2* position of PAF and other plasmalogens creating lysoPAF or lysoplasmanalogens in general (McIntyre et al., 2009). There is also an enzyme that specifically cleaves acetyl residues at the *sn-2* position of PAFs called 1-*O*-alkyl-2-acetylglycerophosphocholine esterase (Blank et al., 1981; Watschinger and Werner, 2013b).

The ether linked alkyl and alkenyl chains are more stable. Lysoplasmenylcholines are cleaved into fatty aldehyde and G3P by lysoplasmalogenases (aka transmembrane protein 86B, TMEM86B). TMEM86B is expressed in liver, brain, and intestine (Wu et al., 2011; Jurkowitz et al., 1999). Lysoplasmanylcholines with saturated alkyl chains are individually degraded by a tetrahydrobiopterin-dependent monooxygenase named alkylglycerol monooxygenase (AGMO) forming fatty aldehyde and G3P (Watschinger and Werner, 2013a). AGMO is expressed in rat brain, liver, lung, intestine, and other tissue types except heart (Snyder et al., 1973; Tietz et al., 1964; Werner et al., 2007; Watschinger et al., 2010, 2012).

In 1979 a new pharmacological LPC analogue was generated that is resistant to many degradation pathways by substituting the *sn-1* acyl chain by an alkyl chain and placing an *O*-methyl group at position *sn-2* (Munder et al., 1979). These alkyl phos-

pholipid (ALP) are not cleavable by PLAs, TMEM86B, and AGMO (Munder et al., 1979; Kotting et al., 1987; Watschinger and Werner, 2013b). The first ALP was 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine or PC(O-18:0/O-1:0) (aka edelfosine, ET-18-OCH_3). Later, it was shown that ALPs cause apoptosis in cancer cells (Mollinedo et al., 1997). Their mechanism of action is still unclear but it is mainly membrane-related (Kuerschner et al., 2012; Gajate et al., 2012; Jaffres et al., 2016; Castro et al., 2013; Carrasco et al., 2010).

1.4 LPC converting processes

1.4.1 LPCAT

The half-life of LPC in the circulation is about 6 - 11 min in rats (Stein and Stein, 1966) with comparable results in squirrel monkeys (Portman et al., 1970). LPCs are taken up by different organs but predominately by liver (Stein and Stein, 1966; Shamburek et al., 1996; Portman et al., 1970). The fast elimination of LPCs from the circulation by uptake into organs is one mechanism to protect the body against the detergent effects of LPCs (Stein and Stein, 1966). One fate of LPC in organs is the re-acylation to PC by LPCATs as part of the *Lands cycle*.

Until today, five enzymes with the ability of acylating LPCs are known: LPCAT1-4 (Shindou and Shimizu, 2009; Shindou et al., 2009) and peroxiredoxin 6 (PRDX6) (Fisher et al., 2016). LPCATs have different expression patterns and localizations in the cell (Shindou and Shimizu, 2009). LPCAT1+2 are targeted to mitochondria and lipid droplets, while LPCAT3+4 are found in the ER (Moessinger et al., 2011; Shindou and Shimizu, 2009). Specific data of each LPCAT are displayed in table 1.3 on the next page. LPCAT1 (gene name: LPCAT1) (Harayama et al., 2008) and LPCAT2 (Shindou et al., 2007) are able to transfer acetyl groups onto lysoPAF and common acyl LPCs which creates PAF and acyl-PAF (PC with one acyl and an acetyl group), respectively. The cytosolic enzyme PRDX6 also have PLA activity together with its LPCAT activity (Fisher, 2011; Fisher et al., 2016).

Table 1.3: Enzymes with LPC acyltransferase activity. Enzymes with LPC acyltransferase (LPCAT) activity and their tissue distributions and substrate specificity are listed in this table. Abbreviations: AGPAT (acylglycerophosphate acyltransferases), AYTL (acyltransferase-like), LysoPAFAT (lysoPAF acyltransferase), MBOAT (membrane bound *O*-acyl transferase), PRDX6 (peroxiredoxin 6). Official symbols are italicized.

LPCAT	tissue distribution	acyl CoA substrate	reference
LPCAT1 aka AYTL2, AGPAT9	lung, spleen, brain, heart, skeletal muscle	saturated acyl CoA	Nakanishi et al., 2006; Harayama et al., 2014, 2008
LPCAT2 aka AYTL1, AGPAT11, LysoPAFAT	immune cells, skin, spleen, colon, brain, heart, stomach	C(20:4)	Shindou et al., 2007; Harayama et al., 2008, 2014
LPCAT3 aka MBOAT5	liver, adipocytes, pancreas, erythrocytes, lung, testis	C(18:2), C(20:4), C(22:6)	Kazachkov et al., 2008; Zhao et al., 2008; Gijon et al., 2008; Hishikawa et al., 2008
LPCAT4 aka MBOAT7	epididymis, brain, testis and ovary	C(16:1), C(18:1), C(18:2), C(10:4)	Harayama et al., 2014; Gijon et al., 2008; Hishikawa et al., 2008; Shindou et al., 2013
PRDX6	lung, liver, brain, heart	C(16:0)	Manevich and Fisher, 2005; Fisher et al., 2016

1.4.2 Phospholipase B, C, and D

Enzymes with phospholipases B (PLB) activity which often can act as lysophospholipase, are able to remove acyl chains of LPCs and other phospholipids (Richmond and Smith, 2011). Phospholipase C (PLC) and phospholipase D (PLD) are responsible for the removal of the head-groups. A list of enzymes that have at least one of the mentioned activities on LPCs are given in table 1.4 on the following page.

By definition, the family of PLBs are able to cleave either *sn-1* or *sn-2* localized acyl chains (Richmond and Smith, 2011). Three PLB members have been discovered so far: PLB1 - 3. Only PLB1 is described in humans (Maury et al., 2002).

As mentioned earlier, several PLA_2s show robust lysophospholipase activity especially the subgroup of PLA2G4 (Ghomashchi et al., 2010), as well as some $iPLA_2$ mem-

Table 1.4: List of enzymes that can convert LPCs. This is a list of enzymes that can convert LPCs into αGPC (PLB/lysophospholipase activity), MG + phosphocholine (PLC activity), LPA + choline (PLD activity), or cPA + choline (special PLD activity, marked with *). Abbreviations: ASPG (asparaginase, aka 60 kDa Lysophospholipase (LPP60)), cPA (cyclic phosphatidate), ENPP (ectonucleotide pyrophosphatase/phosphodiesterase family member), Gα (heterotrimeric G-protein Gα subunit), GDE (glycerophosphodiester phosphodiesterase), αGPC (α-glycero-3-phosphocholine), LPA (lysophosphatidate), lysoPLA (lysophospholipase), MG (monoacyl glycerol), NRE (neuropathy target esterase related esterase), PLB (phospholipase B), PLC (phospholipase C), PLD (phospholipase D), PNPLA (Patatin-like phospholipase domain containing).

activity	enzyme	reference
PLB/lysoPLA	PLB1	Maury et al., 2002
	PLA2G4 (aka $cPLA_2$)	Ghomashchi et al., 2010
	PNPLA9 (aka $iPLA_2\beta$, PLA2G6A)	Lio and Dennis, 1998
	PNPLA8 (aka $iPLA_2\gamma$, PLA2G6B)	Yan et al., 2005
	PNPLA6 (aka $iPLA_2\delta$, PLA2G6C)	van Tienhoven et al., 2002
	ASPG (aka LPP60)	Menniti et al., 2010
	PNPLA7 (aka NRE)	Kienesberger et al., 2008
	LIPG	Gauster et al., 2005
PLC	ENPP6	Sakagami et al., 2005
	ENPP7 (aka alkaline sphingomyelinase)	Duan et al., 2003
PLD	ENPP2 (aka autotaxin)*	Tokumura et al., 2002
	PLD1	Selvy et al., 2011
	PLD2*	Tsukahara et al., 2010
	$G\alpha_q$ and $G\beta_1$	Aoyama et al., 2011
	GDE4	Ohshima et al., 2015
	GDE7	Ohshima et al., 2015

bers like PLA2G6A - C (Yan et al., 2005; Lio and Dennis, 1998; Tang et al., 1997; van Tienhoven et al., 2002; Dennis et al., 2011) . Beyond the class of PLA_2s, also 60 kDa lysophospholipase (LPP60 aka asparaginase, official symbol: ASPG) (Menniti et al., 2010), neuropathy target esterase-related esterase (NRE, official symbol: PNPLA7) (Kienesberger et al., 2008), and LIPG (Gauster et al., 2005) exhibit lysophospholipase activity.

The final product of the lysophospholipase activity on LPC - αGPC - is an osmolyte (Nakanishi et al., 1990) which is degraded by ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) 6 (Morita et al., 2016) to glycerol and phosphocholine and by glycerophosphocholine phosphodiesterases (GDE) 2 and 5 (Corda et al., 2014) to choline and G3P. Phosphocholine is hydrolyzed in the circulation by still unknown enzymes to choline which can be taken up by the liver and other tissues and can be re-used to form PC (Morita et al., 2016; Gallazzini et al., 2008).

ENPP6 and ENPP7 have PLC activity and cleave LPCs into monoacyl glycerol (MG) and phosphocholine (Sakagami et al., 2005; Duan et al., 2003).

ENPP2 (aka autotaxin) has PLD activity and is able to cleave LPC into lysophosphatidate (LPA) and choline, and PC into DG and choline (Umezu-Goto et al., 2002; Tokumura et al., 2002). ENPP2 prefers saturated LPCs and PCs that are lipoprotein associated (Tokumura et al., 1999, 2002). Both, PLD1 (Hammond et al., 1995) and PLD2 (Colley et al., 1997) produce PA in the expense of PC and are able to cleave LPCs (Colley et al., 1997; Hammond et al., 1995; Tsukahara et al., 2010; Selvy et al., 2011). Other enzymes with PLD activity are GDE4, GDE7, and the two heterotrimeric G-protein subunits $G\alpha_q$ and $G\beta_1$ (Ohshima et al., 2015; Aoyama et al., 2011).

Cyclic phosphatidate (cPA) (Tsukahara et al., 2010) is a product of ENPP2 (Tsuda et al., 2006) and PLD2 (Tsukahara et al., 2010) with LPCs as substrates. cPA is bound by serum albumin (ALB) in the circulation (Kobayashi et al., 1999). cPA(16:0) has a serum concentration of $\approx$9 nM and cPA(18:1) $\approx$16 nM (Shan et al., 2008).

1.4.3 LPCs in the blood

LPCs can be found in many body fluids including breast milk (Shoji et al., 2006), tear fluid (Brown et al., 2016), cerebro-spinal fluid (Illingworth and Glover, 1971), seminal fluid (Glander et al., 2002), urine (Bouatra et al., 2013), and blood. The plasma/serum concentration of LPCs in healthy humans range from approximately 100 to 300 µM (Barber et al., 2012; Croset et al., 2000; Heimerl et al., 2014; Sutter et al., 2016; Ojala et al., 2007; Kishimoto et al., 2002; Rabini et al., 1994) comprising 8 - 20 % of total phospholipid content in plasma (Switzer and Eder, 1965; Nelson, 1967). It was shown that males have higher LPC levels than females (Weir et al., 2013; Gillett and Besterman, 1975). The plasma/serum LPC compositions of healthy subjects obtained from various studies are displayed in table 1.5 on the next page. This table highlights the relative abundance of different LPC species in serum/plasma. LPC(16:0) is the most abundant LPC species reported in each study, followed by LPC(18:2), LPC(18:0), and LPC(18:1).

Table 1.5: Plasma/serum concentration of different LPC species from healthy human subjects. The data are depicted with the statistical size of the study, age and BMI of the subjects, method used to analyze the sample and the sample type. Abbreviations: BMI (body mass index), ESI-MS/MS (electrospray ionization-tandem mass spectrometry), GLC (gas-liquid chromatography), LC-MS (liquid chromatography-mass spectrometry), MS (mass spectrometry), TLC (thin layer chromatography).

ref.	Barber et al., 2012	Croset et al., 2000	Heimerl et al., 2014	Wang et al., 2016b	Sutter et al., 2016	Ojala et al., 2007
N	11	8-10	23	123	14	5
BMI	23.3±0.4	unkn	22.6±2.1	unkn	24.5±3.48	unkn
age	51±2	unkn	38.2±13.8	18-81	56.4±7.5	unkn
method	ESI-MS/MS	GLC	ESI-MS/MS	ESI-MS/MS	LC-MS	LC-MS/MS
sample	fasting plasma	plasma	fasting plasma	fasting serum	plasma	plasma
units	[µM]	[µM]	[µM]	[µM]	[µM]	[% of tot. LPC]
LPC(14:0)	0.92±0.14					
LPC(15:0)	0.56±0.03					
LPC(16:0)	34.11±1.26	36.7±4.3	146±37	107.4±18.3	90.8 (77.4-121.1)	36.8±4.8
LPC(16:1)	2.57±0.61					2.6±1.0
LPC(18:0)	14.37±0.54	13.5±.2	56.5±14.9	18.2 (14.5-24.0)	30.7 (26.4-46.7)	11.9±1.7
LPC(18:1)	15.91±1.85	9.2±1.3	28.4±12.5	19.4 (16.4-22.1)	21.3 (15.4-28.5)	13.2±1.4
LPC(18:2)	21.57±1.40	19.5±2.0	34.5±12.5	55.0 (23.9-36.7)	32.5 (25.6-50.6)	20.3±3.1
LPC(20:0)	0.10±0.01	0.26±0.07			0.33 (0.05-0.06)	
LPC(20:1)	0.20±0.03					
LPC(20:2)	0.23±0.03					
LPC(20:3)	2.08±0.23	0.89±0.11				2.4±0.9
LPC(20:4)	5.45±0.39	4.0±0.06			3.4 (1.9-4.4)	7.6±1.1
LPC(20:5)	0.98±0.15	0.31±0.04				1.4±0.6
LPC(22:0)		0.26±0.88				
LPC(22:4)		0.14±0.03				
LPC(22:5)		0.08±0.26				1.0±0.5
LPC(22:6)	1.50±0.16					2.5±0.8
sum LPC	100.55±5.76	89.4±3.5	287±66		179.2 (156.8-245.2)	188±27 µM

In the circulation LPCs are mainly bound by serum proteins like ALB (Switzer and Eder, 1965; Ojala et al., 2006) and α1-acid glycoprotein (aka Orosomucoid, ORM) (Ojala et al., 2006). LPCs are the most abundant phospholipid species bound by ORM and ALB (Ojala et al., 2006). The binding of LPCs by serum proteins effectively prevents the rather high plasma concentration of LPCs to be cytolytic and also eliminates some of the physiological effects of LPCs (Klibansky and De Vries, 1963; Ojala et al., 2006; Mochizuki et al., 1982; Soga et al., 2005; Klingler, 2012).

ALB is the most abundant serum protein with a physiological range of 37 - 56 g/L (Lockitch et al., 1988; Hostmark et al., 2005; Reijnierse et al., 2015) (equals 560 - 848 µM) and accounts for around 50 - 60 % (Simard et al., 2006) of the total plasma proteins (Simard et al., 2006). ALB is expressed and secreted by the liver (Peters and Anfinsen, 1950). Human ALB has seven binding sites for FA of which three are high affinity sites and four low affinity sites which are presumably reserved for other ligands (Peters, 2008; Simard et al., 2006). The molar ratio of LPC bound by human ALB is around 3:1 (Ojala et al., 2006). The affinity of different lipids for ALB is as follows: FA>LPA>LPS>LPC>LPE>lysoPAF with lower affinity for unsaturated lipids (Ojala et al., 2006).

ORM is a acute phase protein, secreted by the liver with a plasma concentration of 0.5 g/L (equals 12 µM) with up to 4 g/L during inflammation (Ojala et al., 2006). While ALB mainly carries LPC(16:0), ORM complexes unsaturated LPCs like LPC(18:1), LPC(18:2), and LPC(20:4) (Ojala et al., 2006). ORM binds LPCs in a molar ratio of 1:1 and has higher affinity for LPCs than for FA (Ojala et al., 2006). The affinity increases as follows: LPC>LPS>LPE>FA>lysoPAF>LPA (Ojala et al., 2006). Additionally the more unsaturated and the longer the acyl chain the higher the affinity for ORM (Ojala et al., 2006).

In cells, LPCs can bind to lipid binding proteins termed fatty acid binding proteins (FABP). This protein family consists of ten members in humans (Smathers and Petersen, 2011): FABP1-9 and FABP12. FABP3 (aka heart/muscle-FABP (H-FABP)), FABP4 (aka adipocyte FABP), and FABP5 (aka epidermal FABP (E-FABP)) are known to be expressed in skeletal muscle (Smathers and Petersen, 2011). FABPs are able

to bind lipids and lipid-like compounds to facilitate their transport within the cell to different organelles for instance nucleus or ER (Lawrence et al., 2000; Tan et al., 2002; Smathers and Petersen, 2011). FABP1 (aka liver-FABP (L-FABP)) has been shown to transport LPCs (Burrier and Brecher, 1986). FABPs are also able to transfer FA or other ligands to their nuclear receptors like PPARs by direct interaction of both PPARs and FABPs (Tan et al., 2002; Wolfrum et al., 2001).

1.4.4 LPCs in lipoproteins

About 80 % of total LPCs in the plasma are found in the serum protein fraction, while the rest of it is mainly located in the lipoprotein fraction including HDL and LDL (Ojala et al., 2006; Croset et al., 2000; Dashti et al., 2011). The lipoproteins mostly enriched with LPCs are intermediate-density lipoproteins (IDL) and oxidized low-density lipoproteins (oxLDL) (Chen et al., 1997; Kugiyama et al., 1990; Dashti et al., 2011). In fact, during conversion of native LDL into oxLDL, the LPC content increases from about 1 - 5 % of total phospholipid mass to up to 40 - 50 % (Chen et al., 1997; Steinbrecher and Pritchard, 1989; Parthasarathy et al., 1985). The oxidation can occurs in PCs containing unsaturated acyl chains by the influence of reactive oxygen species (Panasenko et al., 1994; van den Berg et al., 1993). Oxidized PC can decay to LPCs spontaneously or via enzymes like LCAT or paraoxonase 1 (PON1) (Arnhold et al., 2001, 2002; Rosenblat et al., 2006; Rozenberg et al., 2003; Goyal et al., 1997). Saturated LPCs with a length of $\geq$C(16:0) are dominating in oxLDLs (Chen et al., 1997; Sasabe et al., 2014).

1.4.5 Dietary uptake of LPCs

Dietary PC is taken up during the passage through the jejunum in large part as LPCs (Ikeda et al., 1987), the cleavage product of PC by PLA2G1B (pancreatic PLA_2). LPCs formed by PLA2G1B are also supporting the absorption of cholesterol and TG from the gut (Richmond et al., 2001; Huggins et al., 2002).

LPCs absorbed into the mucosa are then processed into PC or hydrolyzed into products like αGPC (Ikeda et al., 1987; Parthasarathy et al., 1974). Only 15 % are not reacylated and travel as LPCs (Ikeda et al., 1987). The acylated PCs then find their way to the liver mainly via the portal vein (Ikeda et al., 1987).

1.5 Regulation of plasma LPC concentration

The LPC levels in the circulation are fueled by many sources. The aforementioned phospholipases LIPC (Shamburek et al., 1996), LIPG (Gauster et al., 2005; Riederer et al., 2012), LCAT (Shamburek et al., 1996; Schmid et al., 2003; Morimoto et al., 2010), and $sPLA_2$ (Boyanovsky and Webb, 2009) play a pivotal role. Substantial amounts of LPCs are also provided by the diet as PC or LPCs (Huggins et al., 2002; Labonte et al., 2006). During an oral lipid tolerance test the amount of saturated LPCs like LPC(16:0) and LPC(18:0) rise and decrease 300 min afterwards (Morris et al., 2015). Unsaturated LPCs like LPC(18:1), LPC(18:2), LPC(20:4) decrease first and rise again after 300 min (Morris et al., 2015). During an oral glucose tolerance test, LPC(16:0), LPC(16:1), LPC(18:1), LPC(18:2) rise all together significantly from time point 0 to 120 min, while LPC(18:0) peaked already after 90 min (Zhao et al., 2009). After a complex meal, LPCs decline until 45 min with a rise to baseline at 60 min and a subsequent second decline (Shrestha et al., 2015). Special diets can increase levels of certain LPCs. For instance, fish oil diet leads to increased levels of LPC(20:5) and LPC(22:6) (Abdolahi et al., 2014).

The liver is supposed to perform a substantial secretion of LPCs (Sekas et al., 1985; Krautbauer et al., 2016; Brindley, 1993). Notably, it has been shown that exercise can increase LPC levels in liver and muscle of mice (Hoene et al., 2016) while no changes are observed in exercising humans (Hansen et al., 2015). The secreted LPCs are mainly unsaturated (about 62 %) LPC(20:4)>LPC(18:2) or LPC(18:0) (in female rats) (Sekas et al., 1985; Baisted et al., 1988; Brindley, 1993). The LPC release is enhanced by the presence of acceptors like ALB and is not related to secreted lipases or lipoproteins. It is increased by a combination of dexamethasone and insulin, is

inhibited by cyclic adenosine monophosphate (cAMP) and glucagon, and is dependent on intracellular Ca^{2+} levels (Baisted et al., 1988; Brindley, 1993).

During atherosclerosis, oxLDL are formed, which are also a source of LPCs (Rosenblat et al., 2006; Rozenberg et al., 2003; Goyal et al., 1997; Arnhold et al., 2001, 2002).

LPC levels are actively reduced by enzymes with PLB/lysophospholipase, PLC and PLD activity from which ENPP2 is of relevance in the circulation (Pamuklar et al., 2009; Reeves et al., 2015), together with the clearing of LPCs by acylation via LPCATs in different organs but predominantly in liver (Stein and Stein, 1966; Shamburek et al., 1996; Portman et al., 1970).

A reduction of serum LPCs can be found during prolonged fasting of 36 h with significant decreases in LPC(18:0), LPC(18:1), and LPC(18:2) levels in humans (Rubio-Aliaga et al., 2011).

1.6 LPCs as biomarkers

In recent years metabolomics approaches gained importance as tool in the search of reliable clinical biomarkers and individual pathophysiological blood parameters in order to avoid invasive methods for the early detection of diseases (Guo et al., 2015). By higher sensitivity and reproducibility of mass spectrometry, LPCs reached the focus as powerful biomarkers (Xiao et al., 2000). The outcome of many diseases rely on an early diagnosis or prediction, amongst them also diabetes mellitus type II (T2DM) (Tabak et al., 2012). In general, studies investigating the association of systemic LPC levels with markers of glucose tolerance or insulin sensitivity point to an decrease in LPCs at each stage of the development of T2DM (table 1.6 on page 25) (Rhee et al., 2011; Ferrannini et al., 2013; Wang-Sattler et al., 2012; Gall et al., 2010; Zhao et al., 2010a; Pietilainen et al., 2007; Drogan et al., 2015; Barber et al., 2012; Floegel et al., 2013; Tonks et al., 2016; Wallace et al., 2014). These studies also include follow-up studies where the patients were characterized up to 12 years before the onset of T2DM (Rhee et al., 2011; Fernandez et al., 2013). This association is found for several LPC species, rendering them a risk marker for T2DM (Roberts et al., 2014).

Table 1.7 on page 26 shows the correlation of LPCs with different parameters like BMI, adipose tissues mass, and inflammatory markers like C-reactive protein (CRP). T2DM is associated with obesity (Boden and Shulman, 2002), cardiovascular diseases (CVD) (Pietilainen et al., 2007), and other morbidities, which are all summarized in the term metabolic syndrome (Laaksonen et al., 2002). The metabolic syndrome is often accompanied by a low-grade inflammatory response (Donath and Shoelson, 2011; Spranger et al., 2003; Hotamisligil, 2006). The table shows a negative association of LPCs with body mass index (BMI), CVD risk and the inflammatory marker CRP. This summary of data confirms the robustness of LPCs as biomarker not only for T2DM but also for other aspects of the metabolic syndrome.

Lower LPC levels are also observed in non-alcoholic fatty liver disease (NAFLD), a strong risk factor of T2DM (Oresic et al., 2013; Roden, 2006). LPC(16:0) can discriminate between insulin-resistant and insulin-sensitive NAFLD with higher levels of LPC(16:0) in insulin-sensitive NAFLD patients (Lehmann et al., 2013). In addition to that, saturated LPCs species are reduced in decompensated liver cirrhosis (Krautbauer et al., 2016).

LPCs are also important diagnostic parameters for other diseases. LPC(26:0) was found to be a robust biomarker for peroxisomal disorders like X-linked adrenoleukodystrophy and is analyzed in dried blood spot extracts from newborns (Haynes and De Jesus, 2016; Theda et al., 2014). LPCs are also biomarkers for various cancer types. LPC(20:4) and LPC(16:1) are blood parameters that can help to distinguish between different breast cancer subtypes with different prognoses and therapeutic responses (Fan et al., 2016). In addition to that, reduced LPC(18:0) levels are associated with a higher risk of breast, colorectal, and prostate cancer (Kuhn et al., 2016).

Table 1.6: LPCs as biomarkers of IGT and T2DM. This table summarize associations of LPC species with parameters of insulin sensitivity like M-value (DeFronzo et al., 1979) or insulin resistance like HOMA-IR and HBA_{1c} (Singh and Saxena, 2010). Table includes data that are already reviewed in (Klein and Shearer, 2016). ↓ indicates negative association between the LPC species and the given parameter, while ↑ are positive associations. Abbreviations: CVD (cardiovascular disease), ESI (electrospray ionization), fast ins (fasting insulin), FIA (flow injection analysis), FP (fasting plasma), FS (fasting serum), HOMA (homeostatic model assessment), HPLC (high performance liquid chromatography), HRMS (high resolution mass spectrometry), IR (insulin resistance), IS (insulin sensitivity), LC-MS (liquid chromatography-mass spectrometry), LTQ (Linear Trap Quadropole), MS/MS (tandem mass spectrometry), P (plasma), qTOF (Quadrupole-time-of-flight), S (serum), UPLC (ultra-performance liquid chromatography).

LPC	IGT						T2DM												
15:0														↓					
O-16:0												↓							
16:0						↓	↓	↓		↓	↓		↓						
18:0						↓		↓		↓	↓			↓					
20:0														↓			↓		
18:1						↓				↑	↓								↓
20:1														↓			↓		
22:1																	↓		
18:2		↓	↓	↓	↓	↓			↓	↑	↓			↓	↓				
20:2														↓					
20:4														↓					
22:6	↓																		
total														↓		↑		↓	
ref	[1]	[2]	[2]	[3]	[4]	[5]	[6]	[8]	[2]	[4]	[4]	[9]	[10]	[11]	[12]	[13]	[14]	[7]	[15]
parameter	risk	M-value	M-value	M-value	IS vs IR	case	HBA_{1c}	M-value	M-value	M-value	IS vs IR	case	case	obese T2DM vs. Lean	case	case	IR vs IS	fast ins, HOMA-IR	HOMA-IR
sample	FP	P	P	FS	FP	FP		S	P	FP	FP	FS	FS	P	S	P	P	P	FP
method	LC-MS	UHPLC-MS/MS	UHPLC-MS/MS	LC-MS	UHPLC-ESI-MS/MS	UPLC-qTOF-MS	LTQ hybrid MS	UPLC-MS	UHPLC-MS/MS	UHPLC-ESI-MS/MS	UHPLC-ESI MS/MS	LC-HRMS	UPLC-MS	LC-ESI/MS	FIA-MS/MS	TLC	ESI-MS/MS	HPLC ESI-MS/MS	ESI-MS/MS
N(con)	189	1,115/ 1,811	779/ 2.249	866	211	39	216	24	1,115	211	211	30 total	300	11	2,282	10	23		
N(case)	189	146/ 642	123/ 151	238	82	12	211	24	47	188	188		300	9	800	24	21	86	39
study name	FHS	RISC/ BOTNIA	RISC/ BOTNIA	KORA	RISC		MDC	twin	RISC	RISC	RISC		EPIC		EPIC, KORA, TüF				MECHE

[1] Rhee et al., 2011, [2] Ferrannini et al., 2013, [3] Wang-Sattler et al., 2012, [4] Gall et al., 2010, [5] Zhao et al., 2010b, [6] Fernandez et al., 2013, [7] Nestel et al., 2014, [8] Pietilainen et al., 2007, [9] Garcia-Fontana et al., 2016, [10] Drogan et al., 2015, [11] Barber et al., 2012, [12] Floegel et al., 2013, [13] Rabini et al., 1994 [14] Tonks et al., 2016 [15] Wallace et al., 2014

Table 1.7: Association of LPCs with parameters of obesity, CVD, and inflammation. The data in this table depicts correlations of LPCs with different parameters of obesity and CVD like BMI, subcutaneous adipose tissue (scAT), and visceral adipose tissue (vAT) and the inflammatory marker CRP. ↓ indicates negative association between the LPC species and the given parameter, while ↑ are positive associations. Abbreviations: ESI (electrospray ionization), FP (fasting plasma), FS (fasting serum), HILIC (hydrophilic interaction liquid chromatography), HPLC (high performance liquid chromatography), LC (liquid chromatography), LTQ(Linear Trap Quadropole), MS/MS (tandem mass spectrometry), P (plasma), Q-MS (Quadrupole-Orbitrap mass spectrometry), S (serum), UPLC (ultra-performance liquid chromatography).

LPC	obesity											CVD risk		inflammation				
15:0		↓					↓			↓						↓		
16:0	↑		↑	↓	↓					↓		↓		↓				
17:0										↓								
18:0	↑	↓						↑		↓						↓		
20:0		↓					↓			↓						↓		
22:0										↓								
24.0										↓								
26:0										↓	↓							
18:1	↑	↓				↓	↓	↓		↓			↓		↓	↓	↓	↓
20:1		↓								↓	↓							
18:2	↑	↓				↓	↓	↓		↓			↓			↓	↓	↓
20:2										↓								
18:3							↓											
20:4	↑	↓			↓					↓		↓				↓		
22:1										↓								
22:6							↓			↓						↓		
total		↓							↓									
ref	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[11]	[9]	[9]	[5]	[6]	[4]	[6]	[7]	[8]	[10]
parameter	case	BMI	BMI	BMI	BMI	BMI	BMI	BMI	BMI	scAT	vAT	risk CVD	risk CVD	CRP	CRP	CRP	CRP	CRP
sample	S	P	FP	FS		S	FP	FS	P	P	P		S+P	FS	S	FP	FS	FP
method	UPLC-MS	LC-ESI/MS	LTQ hybrid MS	UPLC-MS	LTQ hybrid MS	UPLC Q-MS	ESI-MS/MS	HPLC HILIC ESI-MS/MS	LC ESI-MS/MS	ESI-MS/MS	ESI-MS/MS	LTQ hybrid MS	UPLC Q-MS	UPLC-MS	UPLC Q-MS	ESI-MS/MS	HPLC HILIC ESI-MS/MS	ESI-MS/MS
N(con)	24	11	42	300	216	970	23	123 total	1,076	23	23	216	2285	300	970	23	123 total	
N(case)	24	10	28	300	211		57			21	21	211	413	300		57		39
study name	twin			EPIC	MDC	PIVUS	OWRRS		SAFHS			MDC	Twin-Gene UL-SAM PIVUS	EPIC	PIVUS	OWRRS		MECHE

[1] Pietilainen et al., 2007, [2] Barber et al., 2012, [3] Graessler et al., 2009, [4] Drogan et al., 2015, [5] Fernandez et al., 2013, [6] Ganna et al., 2014, [7] Heimerl et al., 2014, [8] Wang et al., 2016b [9] Tonks et al., 2016 [10] Wallace et al., 2014 [11] Weir et al., 2013

1.7 Physiological functions of LPCs

1.7.1 LPC receptors

It has been proposed for years that LPCs bind to cell surface receptors thereby initiating intracellular signaling cascades. In 2001, two LPC receptors have been discovered: G-protein coupled receptor 132 (GPR132 aka G2 accumulation (G2A)) (Kabarowski et al., 2001) and G-protein coupled receptor 4 (GPR4) (Zhu et al., 2001). But the original publications demonstrating that LPCs bind to the two receptors have been retracted. The attempt to reproduce the experiments by other working groups failed for both GPR132 (Witte et al., 2005) and GPR4 (Bektas et al., 2003; Peter et al., 2008). Nevertheless, some actions of LPCs are demonstrated to be GPR132-dependent (Peter et al., 2008; Qin et al., 2014; Wang et al., 2005; Yang et al., 2005). The effects are not due to direct ligand binding but rather by influencing receptor localization and trafficking (Wang et al., 2005).

GPR132 is a G-protein coupled receptor (GPCR) and coupled to various small G-proteins likes G_q, G_{13} (Yang et al., 2005), G_i (Kabarowski et al., 2001), and G_s (Lin and Ye, 2003). GPCRs like GRP132 transduce their signal across the plasma membrane via these heterotrimeric small G-protein subunits (Syrovatkina et al., 2016). G_s is able to stimulate the intracellular adenylate cyclase (ADCY) to convert adenosine triphosphate (ATP) to cAMP, an important second messenger (Syrovatkina et al., 2016). G_i inhibits ADCY, G_q activate PLCs, and G_{13} stimulates members of the Rho family of G-proteins (Syrovatkina et al., 2016). GPR132 expression is restricted to immune cells like T-, B-lymphocytes, monocytes, and macrophages (Weng et al., 1998).

Four years later, G-protein coupled receptor 119 (GPR119) was discovered as another LPC receptor (Soga et al., 2005). LPCs are only weak and unspecific ligand for GRP119 (Lan et al., 2009; Overton et al., 2006; Hansen et al., 2011). The potency to activate GRP119 decreases as follows: LPC(18:1)<LPC(16:0)<LPC(18:0) (Soga et al., 2005). GRP119 is mainly expressed in pancreas but also in the gastrointestinal tract (Odori et al., 2013), skeletal muscle (Cornall et al., 2013), liver (Yang et al.,

2016), and testis (Soga et al., 2005). GPR119 is coupled to the G_α subunit type G_s (Overton et al., 2006; Chepurny et al., 2013; Moss et al., 2016).

To date, GPR132 and GPR119 are the only two known LPC-responsive receptors.

1.7.2 Effects of LPCs on insulin-glucose axis

As reviewed in section 1.6 on page 23, plasma LPC levels correlate negatively with insulin resistance and T2DM. In addition to that, LPCs are known to influence insulin secretion. In perfused rat pancreas LPC(18:1) enhances insulin secretion dose-dependently in the presence of 16.5 mM glucose (Soga et al., 2005). This finding was confirmed with NIT-1 cells (5 µM LPC(18:1)) (Soga et al., 2005), INS-1 cells (86 µM LPC(18:2)) (Ferrannini et al., 2013), and MIN6c4 cells (10 µM LPC(18:1)) (Ning et al., 2008). The effects are GRP119-dependent and are mediated by increasing intracellular cAMP and Ca^{2+} levels, two essential signaling events for insulin secretion (Soga et al., 2005; Ning et al., 2008). However, in RINm5f cells which do not express GRP119, LPC(18:1) also slightly increase the insulin secretion, pointing to a still unknown GPR119-independent mechanism (Ning et al., 2008).

Intravenous (Yea et al., 2009) or intraperitoneal (Takahashi et al., 2014) injection of LPC(16:0) leads to the reduction of blood glucose levels in mice (Takahashi et al., 2014; Yea et al., 2009). An enhanced glucose uptake caused by LPC(16:0) has been described in the 3T3-L1 adipocyte cell line (Takahashi et al., 2014; Yea et al., 2009). These findings are in contrast to findings showing that postprandial LPCs and intraperitoneal injection of LPCs can inhibit glucose uptake of various tissues (Labonte et al., 2006). In rat skeletal muscle cell line L6 it was demonstrated that LPCs inhibit insulin-dependent glucose uptake (Han et al., 2011).

It was also shown that LPCs can cause insulin resistance. A LPC concentration of 25 µM for 5 min causes the inhibition of insulin-dependent phosphorylation of AKT serine/threonine kinase (AKT aka protein kinase B, v-akt murine thymoma viral oncogene homolog) at Ser473 in rat vascular smooth muscle cells (Motley et al., 2002) and already 10 µM for 3 h in L6 cells (Han et al., 2011). In L6 cells it was demonstrated that LPC are responsible for palmitate-mediated insulin resistance (Han et al.,

2011). Inhibition of PLA_2 activity resulting in lower LPC production which ameliorates the reduction in insulin-dependent glucose uptake caused by palmitate treatment (Han et al., 2011).

To summarize, the effects of LPCs on insulin-glucose axis are mechanistically not well understood and controversial data are reported.

1.7.3 LPC in inflammation and atherosclerosis

It is known that LPCs play a pivotal role in inflammatory and atherogenic pathways. It has been reported that LPC(16:0) have pro-inflammatory effects when injected intracutanously leading to edema, erythema, and mixed cellular infiltrates containing T-lymphocytes, neutrophils as well as monocytes in humans (Ryborg et al., 2000). It is known that LPCs are chemoattractants for T-lymphocytes (Ryborg et al., 1994; McMurray et al., 1993) and monocytes (Rong et al., 2002; Quinn et al., 1988). LPC also promote the transition of differentiated monocytes to dendritic cells (Coutant et al., 2002).

LPCs are part of the so called find-me signal to attract phagocytes to apoptotic cells (Lauber et al., 2003) via GPR132 (Peter et al., 2008). Intravenous injection of LPC(16:0) or LPC(18:0) causes an increase in the cytokines interleukin (IL)6 and IL5 in serum (Bach et al., 2010). LPCs also help to maintain the pro-inflammatory M1 phenotype of macrophages and promote the release of pro-inflammatory cytokines like IL6 and tumor necrosis factor α (TNFα, official symbol: TNF) (Ngwenya and Yamamoto, 1986; Qin et al., 2014). In fact, as natural adjuvants they have the potential to be used in vaccines to boost the immune response (Perrin-Cocon et al., 2006; Bach et al., 2010).

Atherosclerosis and other metabolic diseases are often associated with an inflammatory reaction (Hotamisligil, 2006). It was reported that LPCs produced in oxLDL are able to modulate the immune response (Steinberg, 1997; Coutant et al., 2004, 2002) and influence endothelial cells (EC) as sites of atherosclerotic lessions (Di Pietro et al., 2016). LPC stimulate the expression of adhesion molecules for monocytes on EC, a pro-atherogenic phenotype of these cells (Kume et al., 1992). Furthermore, LPC

induce mitochondrial ROS production in EC via Ca^{2+} entry into cytosol and mitochondria (Li et al., 2016). This also contributes to the pro-atherogenic phenotype of EC (Li et al., 2016). Especially LPC(16:0) stimulate coronary artery smooth muscle cells to secrete IL6, IL8, growth factors, and arachidonic acid leading to the transition of the smooth muscle cells to a proliferative/secretory phenotype which can contributes to atherosclerosis (Aiyar et al., 2007).

Despite of being pro-atherogenic and pro-inflammatory, LPCs also display anti-inflammatory effects. LPC(22:6) inhibits the production of lipopolysaccharide-induced TNFα in RAW 264.7 cells (mouse monocyte cell line) (Hung et al., 2011) and in MM6 cells (human monocyte cell line) (Jackson et al., 2008). It was shown that the protective effects in MM6 cells are in part GPR132-dependent (Jackson et al., 2008). Serum LPC levels of septic patients are reduced (Cho et al., 2012; Park et al., 2014; Drobnik et al., 2003; Ferrario et al., 2016). When administrated orally to mice, LPC(22:6) and LPC(20:4) can inhibit Zymosan-A induced peritonitis (Hung et al., 2009, 2011) as well as peritoneal sepsis and pneumonia caused by *Acinetobacter baumannii* (Smani et al., 2015). It was also reported that LPCs can enhance the immuno-repressive function of regulatory T lymphocytes GPR132-dependently (Hasegawa et al., 2011). LPC administration during sepsis can prevent and treat the disease in mice (Yan et al., 2004). Most effective LPCs are LPC(18:0) and LPC(18:1) (Yan et al., 2004). The protective effect of LPCs on sepsis is GPR132- (Yan et al., 2004) and adenosine monophosphate-activated protein kinase (AMPK)-dependent (Kim et al., 2015).

In summary, the effects of LPCs on inflammation are controversial, but LPCs surely can act as modulators of an immune response.

1.7.4 LPC and brain function

When labeled LPCs are injected intravenously into squirrel monkeys, 1 % of labeled LPCs are found in the brain within 20 min after injection (Illingworth and Portman, 1972). In the brain, about 70 % of LPCs are converted to PC while the rest is hydrolyzed to serve as choline source in the form of acetylcholine, betaine, and phosphocholine (Illingworth and Portman, 1972). Choline is a conditional essential compound

during pregnancy and lactation, and during development of fetuses and neonates (Blusztajn, 1998). LPCs are one source and transporter of choline as it was shown for hepatocytes (Brindley, 1993).

LPCs can be transported across the blood-brain barrier (Illingworth and Portman, 1972). Recently, major facilitator superfamily domain containing 2A (MFSD2A) has been identified as a LPC carrier expressed in the blood brain barrier (Nguyen et al., 2014; Quek et al., 2016), liver (induced by fasting), intestine, kidney, brown adipose tissue (Angers et al., 2008), and in the retinal pigment epithelium in embryonic eyes (Wong et al., 2016). MFSD2A is a sodium-dependent symporter for LPCs with a chain length of >C14 with highest affinity for unsaturated LPCs (Nguyen et al., 2014). This transporter does not transport FAs (Nguyen et al., 2014). The importance of LPCs in the brain are shown by MFSD2A defects causing severe microcephaly syndrome due to insufficient omega-3 fatty acid delivery into the brain and an increase in total plasma LPC levels which is especially pronounced in unsaturated LPCs (Alakbarzade et al., 2015; Guemez-Gamboa et al., 2015). LPCs are important carriers for essential FAs that are also targeted to brain where they support development and cognitive functions (Alakbarzade et al., 2015; Oresic et al., 2012).

1.7.5 Effects of LPC on liver

In the liver, LPC influence the hepatic metabolism by inhibiting hepatic cholesterol biosynthesis (Shin et al., 1999) and activating peroxisome proliferator activator receptor (PPAR) α (official symbol: PPARA) (Takahashi et al., 2014). The latter feature of LPCs also have effects on LPC levels itself. It was shown in mice that bezafibrate, a pan-PPAR agonist (Willson et al., 2000; Krey et al., 1997), lowers blood glucose and increases LPC(16:0) in plasma and liver (Takahashi et al., 2014). *In vitro*, bezafibrate and fenofibrate (PPARα agonist) both increase LPC(16:0) in the cell culture media of primary hepatocytes (Takahashi et al., 2014). Bezafibrate treatment of the cells causes an elevation of PLA2G7, a PPARα target gene which influences the hepatic secretion of LPC(16:0) (Takahashi et al., 2014).

Although, PPAR agonists are beneficial and increase LPC levels at the same time, the intravenous injection of 60 mg/kg LPC in mice causes an increase in aspartate transaminase and alanine transaminase - two liver damage markers - after 24 h and hepatitis after 3 d (Han et al., 2008). It was also reported that LPCs inhibit insulin-dependent glycogen synthesis and FA oxidation in liver (Labonte et al., 2006, 2010). In addition to that, it is demonstrated that LPCs are the major cause of lipotoxicity mediated by palmitate treatment in liver Chang cells (Han et al., 2008).

1.7.6 Primary skeletal myotubes as model cell system

Due to the described glucose lowering effects of LPCs, skeletal muscle is considered as interesting target for LPCs. Skeletal muscle is a major organ in the human body with about 20 - 25 kg on average in men (Morgan and Partridge, 2003) and determines the major resting energy expenditure (Zurlo et al., 1990). It is also the most important tissue for insulin-dependent glucose uptake and therefore contributes to peripheral insulin sensitivity (DeFronzo et al., 1985; Baron et al., 1988).

Skeletal muscle consists of bundles of multinucleated post-mitotic myofibers. The myofibers are the product of proliferation and fusion of mononucleated and fusiform satellite cells which can undergo proliferation as myoblasts (Hawke and Garry, 2001; Shi and Garry, 2006). Satellite cells reside between the sarcolemma and the basal membrane (Muir et al., 1965) in a quiescent state where they wait for being activated for proliferation and fusion (Pallafacchina et al., 2010). Their stem-cell like behavior (Gross and Morgan, 1999) is required for muscle repair (Schultz et al., 1986; Watt et al., 1987) and hypertrophy (Rosenblatt et al., 1994) of the muscle.

Mononucleated satellite cells can be isolated by enzymatic digestion of biopsy material (Yasin et al., 1977). After isolation, the cells can be cultured during which they become proliferative myoblasts. At a certain confluency the serum content of the medium is reduced in order to stimulate differentiation of myoblasts to myotubes (Henry et al., 1995; Blau and Webster, 1981).

Fully differentiated human myotubes (HMT) display a cellular model system that is very close to the *in vivo* situation (Meola, 1991). Human skeletal muscle expresses

three myosin heavy chain (MYH) types in different expression levels according to the muscle function and muscle phenotype (Smerdu et al., 1994; Caiozzo et al., 2003; Harridge et al., 1996): MyHC-β (MYH7), MyHC-2A (MYH2), and MyHX-2X (MYH1). Cultured HMT express all three markers after differentiation (Nikolic et al., 2012; Bonavaud et al., 2001; Gaster et al., 2001; Aas et al., 2013). HMT carry donor specific properties, e.g. individual PPARδ (official symbol: PPARD) responsiveness (Ordelheide et al., 2011), adaptability of FA oxidation on higher substrate supply (Ukropcova et al., 2005), and stearoyl CoA desaturase 1 expression levels (Peter et al., 2009), and are therefore a valuable model system.

1.7.7 Effects of LPC on skeletal muscle and own previous work (diploma thesis)

Not much is known about the function of LPCs in skeletal muscle. In mouse myoblasts, the LPC content decreases during differentiation and LPCs influence the fusion and differentiation of myotubes (Blondelle et al., 2015). LPC can inhibit the formation of myotubes, a process that strongly depends on plasma membrane interactions and their physical fusion (Reporter and Raveed, 1973; Leikina et al., 2013). The inhibition of membrane fusion processes by LPCs has been reproduced in artificial membrane systems (Yeagle et al., 1994), other biological membranes (Chernomordik et al., 1993), and during viral fusion processes (Ciechonska and Duncan, 2014). This agrees with findings showing that LPC can inhibit the disassembly of protein complexes that are important for vesicle fusion, which could prolong the fusion process (Shin et al., 2012).

During my diploma thesis, I have started investigating biological effects of LPCs on HMT. The concentration at which cytolytic effects occur were examined for LPC(16:0) and LPC(18:1) on HMT. The release of intracellular creatine kinase (CK) and lactate dehydrogenase (LDH) are suitable markers for cytoplasm leakage of different tissues including skeletal muscle and is routinely measured in clinical diagnostics. Here, the cell culture supernatants were analyzed for the amount of CK and LDH after LPC treatment for 24 h. According to the results (figure 1.7 on the next page), cytolytic

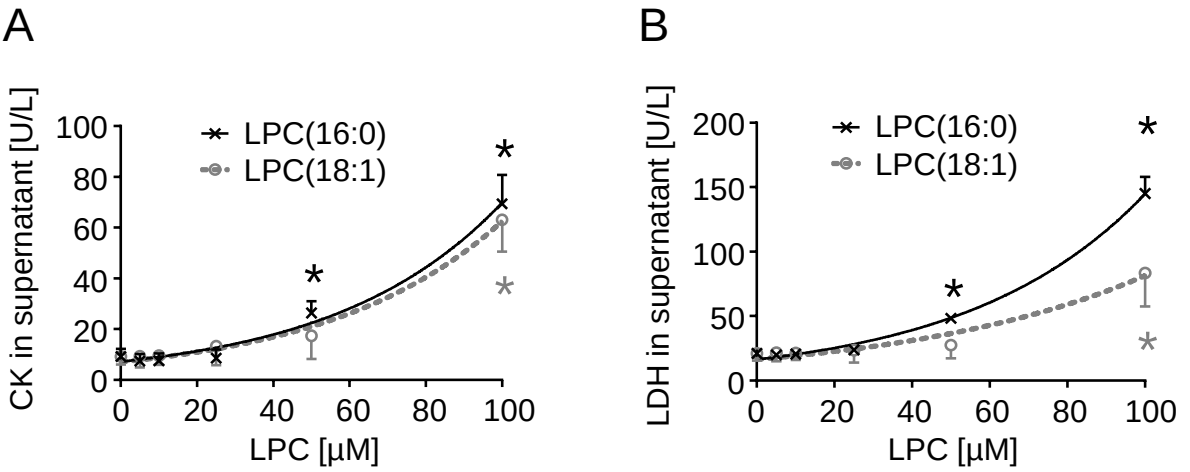


Figure 1.7: Effects of LPCs on CK and LDH release. HMT were stimulated with different concentrations of LPC(16:0) (black marks) or LPC(18:1) (gray marks) for 24 h. The release of (**A**) creatine kinase (CK) and (**B**) lactate dehydrogenase (LDH) were quantified in the cell culture supernatant. Data were fitted to an exponential model (LPC(16:0) black solid line, LPC(18:1) gray dashed line) according to equation $y=Y_0 \cdot e^{k \cdot x}$. Data are shown as mean ±SEM. * $p < 0.05$ vs solvent as control (n=6). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

effects increased exponentially with the applied LPC concentration. Thereby, the two LPCs tested in this assay behaved differently. LPC(16:0) at 50 µM or higher led to a significant release of LDH/CK, while for LPC(18:1) higher concentrations of 100 µM were needed for detectable cytolysis. Both assays, CK and LDH release, displayed similar results.

The non-cytolytic concentration range (< 50 µM) of the two LPCs was further examined. It is known that some lipids especially saturated fatty acids are capable of activating various stress pathways including inflammation and ER stress (Brookheart et al., 2009). To analyze potentially activated stress signaling cascades, the mRNA levels of three different transcripts were quantified: *activating transcription factor 3* (ATF3, official symbol: *ATF3*), *IL6* and *chemokine (C-X-C motif) ligand 3* (aka growth-regulated protein ɣ, official symbol: *CXCL3*). The increase of *ATF3* mRNA can be a result of ER stress (Schroder and Kaufman, 2005), while *IL6* and *CXCL3* are activated markers after nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB, official symbol: NFKB) stimulation (Weigert et al., 2004; Anisowicz et al., 1991). LPC concentrations of 1 to 25 µM were applied to differentiated HMT for 24 h. The RNA

levels of the three transcripts showed no significant response to the LPCs at the given concentration range (figure 1.8). For both LPCs the results were comparable.

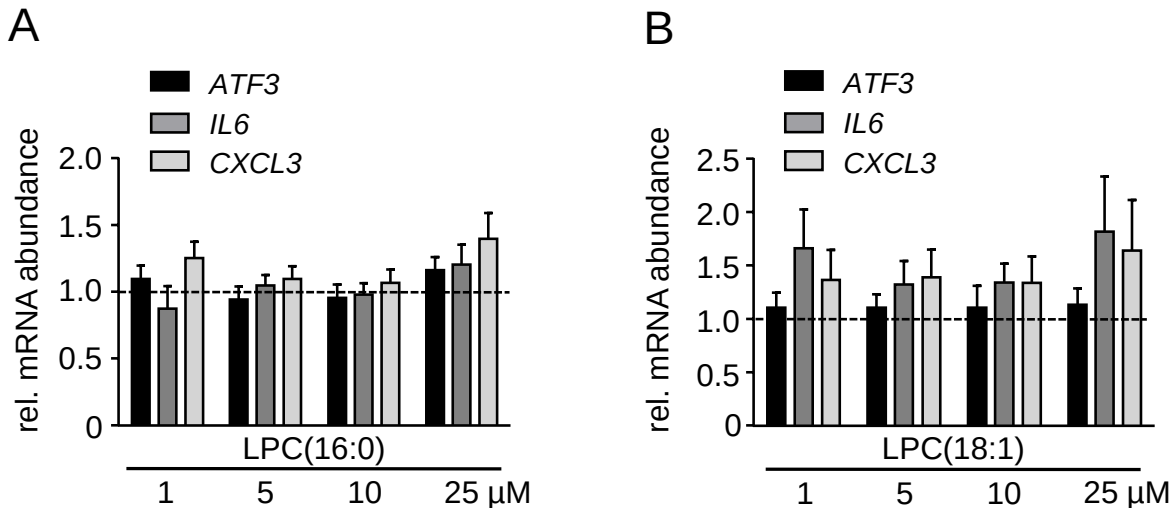


Figure 1.8: Stress marker responses to different concentrations of LPCs. HMT were stimulated with different concentrations of (**A**) LPC(16:0) or (**B**) LPC(18:1). Total RNA was extracted. mRNA levels of *ATF3* (black), *IL6* (dark gray), and *CXCL3* (light gray) were quantified by qPCR. Data are mean ±SEM related to solvent. Solvent control was set as 1 (dashed line) (n=8). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

For further experiments a suitable working concentration was necessary. This concentration should fulfill the requirement of being non-toxic for the given cellular model system HMT and should be in the physiological range of the plasma LPC concentrations. Based on the present results, a working concentration of 10 µM was used in further experiments in my diploma thesis and in this work.

It was demonstrated in that *pyruvate dehydrogenase kinase 4* (*PDK4*) and *angiopoietin-like 4* (*ANGPTL4*) were induced by LPC treatment (Klingler, 2012). This effect is potentially PPARδ mediated as studied in C2C12 cells (mouse skeletal muscle cell line). siRNA targeted against *Ppard* could block the induction of *Angptl4* mRNA by LPC(16:0) in C2C12 myotubes (data not shown).

The kinetics of the induction of *PDK4* (figure 1.9A,C on the following page) and *ANGPTL4* (figure 1.9B,D) evoked by LPC treatment were analyzed. In a time frame of 30 min to 8 h, both LPCs induced a similar response. *PDK4* increased after 8 h. *ANGPTL4* mRNA levels were significantly elevated after 2 h for LPC(16:0) and 1 h for LPC(18:1).

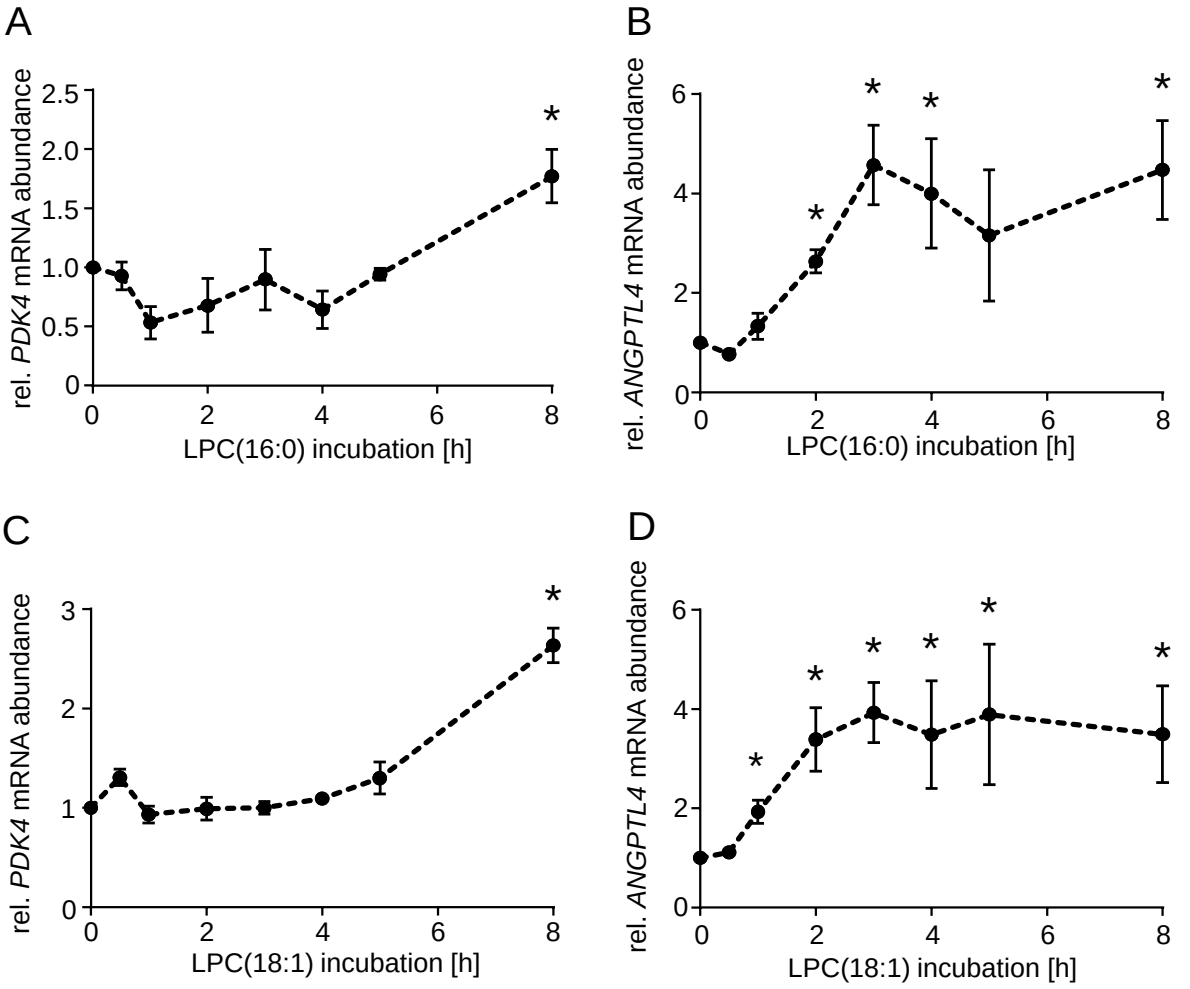


Figure 1.9: Kinetics analysis of the *PDK4* and *ANGPTL4* response to LPC treatment. HMT were treated with (**A+B**) LPC(16:0) or (**C+D**) LPC(18:1) for the indicated time points. Total RNA was isolated. mRNA of *PDK4* and *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * < 0.05 vs. solvent (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

1.8 Aims of the thesis

LPCs are important in many physiological processes including fatty acid transport, immunological reactions, and metabolism. LPCs may also be involved in pathophysiological conditions like T2DM. The plasma level and acyl composition of LPCs is dynamic and mirrors diseases and benign conditions. Hence, LPCs are widely studied biomarkers. The question if LPCs are actively influencing body metabolism has been

investigated insufficiently and findings are inconsistent. To this end, the metabolic effects of LPCs should be studied in more detail.

Aim of this PhD thesis is to explore the physiological functions of LPCs on HMT. Skeletal muscle is a major representative tissue for energy expenditure, insulin sensitivity, and whole body metabolism. HMT differentiated from myoblasts isolated from biopsy material are a convenient model system that sufficiently resembles the *in vivo* tissue. LPC(16:0) and LPC(18:1), which are highly abundant in the circulation and differ in their length and saturation, were chosen as representative LPC species.

The specific aims of this PhD thesis are:

1) Examination of the global effects of LPCs on RNA levels

2) Are the nuclear receptors PPARs activated by LPC treatment?

3) What are the mechanism of the PPARδ activation by LPCs?

4) Are LPCs mediators or protectors of lipotoxicity in HMT?

The methods applied are microarray analysis, quantitative PCR, electromobility shift assay, thin layer chromatography, luciferase reporter assays, glucose uptake assay, palmitate oxidation analysis, Western blotting, *XBP1* splicing assay, TR-FRET-based assays, and siRNA-based gene knockdown.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals and consumables

Table 2.4: List of chemicals.

chemicals	manufacturer
acetic acid (glacial) 100 %	Merck Millipore, Darmstadt (Germany)
ammonium persulfate (APS)	Sigma-Aldrich, Munich (Germany)
ampicillin	Sigma-Aldrich, Munich (Germany)
Bam HI	Roche, Mannheim (Germany)
Bio-Rad protein assay dye reagent concentrate	Bio-Rad, Hercules (US-CA)
collagenase B from *Clostridium histolyticum*	Roche, Mannheim (Germany)
boric acid	Sigma-Aldrich, Munich (Germany)
bovine serum albumin, lyophilized powder (BSA)	Sigma-Aldrich, Munich (Germany)
bovine serum albumin, 20 mg/mL	Roche, Mannheim (Germany)
bromphenol blue sodium salt	Sigma-Aldrich, Munich (Germany)
calcium chloride dihydrate	AppliChem, Darmstadt (Germany)
2',7'-dichlorofluorescein spray solution	Carl Roth, Karlsruhe (Germany)
dithiothreitol (DTT)	Sigma-Aldrich, Munich (Germany)
DNA polymerase I, large (Klenow) fragment	New England BioLabs, Ipswich, (US-MA)
dNTPs set	Eurogentec, Cologne (Germany)
EDTA disodium salt dihydrate	Sigma-Aldrich, Munich (Germany)
EDTA solution pH 8 0.5 M	Sigma-Aldrich, Munich (Germany)
EGTA	Carl Roth, Karlsruhe (Germany)
Glo lysis buffer 1X	Promega, Madison (US-WI)
glycerol	Carl Roth, Karlsruhe (Germany)

β-glycerophosphate disodium salt hydrate	Sigma-Aldrich, Munich (Germany)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Carl Roth, Karlsruhe (Germany)
HindIII	Roche, Mannheim (Germany)
hydrochloric acid	Merck Millipore, Darmstadt (Germany)
hydrogen peroxide, 30 % in aqueous solution	VWR, Darmstadt (Germany)
Immobilion Western chemiluminescence HRP substrate luminol reagent	Merck Millipore, Darmstadt (Germany)
4-iodophenol	Sigma-Aldrich, Munich (Germany)
LB agar	Sigma-Aldrich, Munich (Germany)
LB broth	Sigma-Aldrich, Munich (Germany)
luminol	Sigma-Aldrich, Munich (Germany)
magnesium chloride	Sigma-Aldrich, Munich (Germany)
magnesium sulfate solution 1 M	AppliChem, Darmstadt (Germany)
β-mercaptoethanol	Sigma-Aldrich, Munich (Germany)
nitrogen, liquified 4.8 EN ISO 14175-N1-N 99.998 % purity	Westfalen AG, Münster (Germany)
Nonidet® P40 (NP-40)	Affymetrix, Santa Clara (US-CA)
peqGOLD universal agarose	peqlab, Erlangen (Germany)
poly(deoxyinosinic-deoxycytidylic) acid sodium salt (poly(dI-dC))	Sigma-Aldrich, Munich (Germany)
potassium chloride	Merck Millipore, Darmstadt (Germany)
potassium hydroxide solution	AppliChem, Darmstadt (Germany)
Precision Plus protein dual color standards	Bio-Rad, Hercules (US-CA)
PSTI	Roche, Mannheim (Germany)
Quick-Load® 1 kbp DNA ladder	New England BioLabs, Ipswich, (US-MA)
Quick-Load® 100 bp DNA ladder	New England BioLabs, Ipswich, (US-MA)
Rothiphorese Gel 30 (37.5:1)	Carl Roth, Karlsruhe (Germany)
Rothiphorese Gel 40 (29:1)	Carl Roth, Karlsruhe (Germany)
scandicaine	Astra Zeneca, Wedel (Germany)
sodium chloride	VWR, Darmstadt (Germany)
sodium fluoride	Sigma-Aldrich, Munich (Germany)
sodium hydroxide	AppliChem, Darmstadt (Germany)
sodium lauryl sulphate/sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe (Germany)
sodium orthovanadate	Sigma-Aldrich, Munich (Germany)
sodium pyrophosphate tetrabasic decahydrate	Sigma-Aldrich, Munich (Germany)
spermidine	Sigma-Aldrich, Munich (Germany)

SuRE/Cut buffer B	Roche, Mannheim (Germany)
SuRE/Cut buffer H	Roche, Mannheim (Germany)
Taq DNA polymerase with standard Taq buffer	New England BioLabs, Ipswich, (US-MA)
tetramethylethylenediamine (TEMED)	Carl Roth, Karlsruhe (Germany)
TRIS EDTA buffered solution	Sigma-Aldrich, Munich (Germany)
Triton X-100	Sigma-Aldrich, Munich (Germany)
Trizma base	Sigma-Aldrich, Munich (Germany)
xylene cyanol FF	Sigma-Aldrich, Munich (Germany)

Table 2.7: List of consumables and other equipment.

consumables and other equipment	manufacturer
Amersham Protran 0.45 µm nitrocellulose blotting membrane	GE Healthcare Life science, Little Chalfont (UK)
Assistent® Neubauer counting chamber, 0.100 mm depth, 0.0025 mm^2	Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim v. d. Rhön (Germany)
BAS-IP MS 2025 imaging plate	Fujifilm, Düsseldorf (Germany)
Biosphere Filter tips 20 neutral	Sarstedt, Nümbrecht (Germany)
Cellstar tubes, 50 mL, PP, graduated, conical bottom with support skirt, sterile	Greiner bio-one, Frickenhausen (Germany)
Cell Strainer, 40 µm, blue	BD Bioscience, Heidelberg (Germany)
Combitips advanced 5 mL	Eppendorf, Hamburg (Germany)
CoolCell® cell freezing container	BioCision, San Rafael (US-CA)
costar 5 mL Stripette disposable serologial pipette	Corning, Wiesbaden (Germany)
costar 10 mL Stripette disposable serologial pipette	Corning, Wiesbaden (Germany)
costar 25 mL Stripette disposable serologial pipette	Corning, Wiesbaden (Germany)
costar 50 mL Stripette disposable serologial pipette	Corning, Wiesbaden (Germany)
Cryotubes 1.8 mL SI INT, foot, round	Thermo Fisher Scientific, Waltham (US-MA)
disposable cell scrapers, blade length 3.1 cm	Sarstedt, Nümbrecht (Germany)
ep Dualfilter T.I.P.S. 2-100 PCR clean	Eppendorf, Hamburg (Germany)
ep Dualfilter T.I.P.S. 2-200 PCR clean	Eppendorf, Hamburg (Germany)
Eppendorf 0.1-2.5 research plus pipette	Eppendorf, Hamburg (Germany)
Eppendorf 2-20 research plus pipette	Eppendorf, Hamburg (Germany)
Eppendorf 20 research pipette	Eppendorf, Hamburg (Germany)
Eppendorf 100 research pipette	Eppendorf, Hamburg (Germany)

Eppendorf 1000 research pipette	Eppendorf, Hamburg (Germany)
Falcon 50 mL Polypropylene Conical Tube	Corning, Wiesbaden (germany)
fine needle punch biopsy technique	Peter Pflugbeil GmbH, Zorneding (Germany)
LightCycler 480 Multiwell plate 96, white	Roche, Basel (Swiss)
LightCycler 480 sealing foil	Roche, Basel (Swiss)
Microplate PS, 96 well, F-bottom (Chimney well), white, Lumitrax, med. binding	Greiner bio-one, Kremsmünster (Austria)
Microplate PS, 96 well, F-bottom, med. binding	Greiner bio-one, Frickenhausen (Germany)
Microplate PS, 96 well, F-bottom, med. binding	Greiner bio-one, Frickenhausen (Germany)
Multiply-Pro cup 0.5 mL, PP	Sarstedt, Nümbrecht (Germany)
neoLab-caps 8 mm, with septum silicone white/PTEE red	neoLab, Leonberg (Germany)
neoLab-Wheaton Sample Vials ND8, 1,5 mL brown glass 32x11,6 mm	neoLab, Leonberg (Germany)
Nunc™ CryoTubes® cryogenic vial, 1.8 mL	Thermo Fisher Scientific, Waltham (US-MA)
Oasis HLB extraction cartridge 1 cc/30 mg	Waters, Eschborn (Germany)
Parafilm	Pechineay Plastic Packaging, Menasha (US-WI)
PerfectBlue™ Twin-Gel ExW S electrophoresis system	VWR, Darmstadt (Germany)
Safe-Lock tubes 0.5 mL	Eppendorf, Hamburg (Germany)
Safe-Lock tubes 1.5 mL	Eppendorf, Hamburg (Germany)
Safe-Lock tubes 2 mL	Eppendorf, Hamburg (Germany)
Safe-Lock tubes 5 mL	Eppendorf, Hamburg (Germany)
Steriflip-GV, 0.22 µm, PVDF, radio-sterilized	Merck Millipore, Darmstadt (Germany)
Tissue culture dishes 100 Ø100x20 mm	TPP, Trasadingen (Swiss)
Tissue culture dishes 150 147.8 cm^2	TPP, Trasadingen (Swiss)
Tissue culture testplate 6	TPP, Trasadingen (Swiss)
Tissue culture testplate 12	TPP, Trasadingen (Swiss)
TLC Silica Gel 60 (25 glass plates 20x20 cm)	Merck Millipore, Darmstadt (Germany)
tubes, 15 mL, PP, graduated, conical bottom sterile	Greiner bio-one, Frickenhausen (Germany)
Whatman gel blotting papers, grade GB005, 1.5 mm thick	Whatman, Maidstone (UK)

Table 2.1: List of lipids/stimulants/recombinant proteins used for this work.

lipids/stimulants/recombinant proteins	manufacturer
BAPTA-AM	Sigma-Aldrich, Munich (Germany)
bovine serum albumin, solution 10 % in DPBS (BSA) (FA-free)	Sigma-Aldrich, Munich (Germany)
cytochalasin B	Sigma-Aldrich, Munich (Germany)
1,2-dioleyl-*sn*-glycero-3-phosphocholine (PC(18:1/18:1))	Avanti Polar Lipids, Alabaster (US-AL)
1,2-dipalmitoyl-*sn*-glycero-3-phospho-choline (PC(16:0/16:0))	Avanti Polar Lipids, Alabaster (US-AL)
(+)-etomoxir sodium salt	Sigma-Aldrich, Munich (Germany)
forskolin	AppliChem, Darmstadt (Germany)
L-α-glycero-3-phosphocholine	Sigma-Aldrich, Munich (Germany)
GSK0660	TOCRIS, Bristol (UK)
GSK3787	TOCRIS, Bristol (UK)
GW501516	Santa Cruz, Dallas (US-TX)
H-89, dihydrochloride	Cell Signaling Technology, Leiden (The Netherlands)
insulin solution human	Sigma-Aldrich, Munich (Germany)
ionomycin calcium salt from *Streptomyces conglobatus*	Sigma-Aldrich, Munich (Germany)
lipopolysaccharides from Escherichia coli 0111:B4	Sigma-Aldrich, Munich (Germany)
oleic acid	Sigma-Aldrich, Munich (Germany)
1-oleoyl-*sn*-glycero-3-phosphocholine (LPC(18:1))	Sigma-Aldrich, Munich (Germany)
palmitic acid	Sigma-Aldrich, Munich (Germany)
1-palmitoyl-*sn*-glycero-2,3-cyclic phosphate ammonium salt (cPA(16:0))	Avanti Polar Lipids, Alabaster (US-AL)
1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC(16:0))	Sigma-Aldrich, Munich (Germany)
1-*O*-palmityl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (PC(O-16:0/O-1:0))	Sigma-Aldrich, Munich (Germany)
PPAR delta (human recombinant)	Cayman, Ann Arbor (US-MI)
recombinant RXR-α protein	Active Motif, La Hulpe (Belgium)
WY14643	Sigma-Aldrich, Munich (Germany)

Table 2.2: List of radiochemicals used for this work.

radiochemicals	manufacturer
[α-^{32}P]dATP	Hartmann Analytics, Braunschweig (Germany)
2-[1,2-^{3}H(N)]-deoxy-D-glucose	Perkin Elmer, Waltham (US-MA)
Packard unquenched standards	Perkin Elmer, Waltham (US-MA)
[9,10-^{3}H]-palmitic acid	Hartmann Analytics, Braunschweig (Germany)
L-α-[palmitoyl-9,10-^{3}H]-lyso-phosphatidyl-choline	American Radiolabeled Chemicals, St Louis (US-MO)

Table 2.3: List of cell culture reagents.

cell culture reagents	manufacturer
amphothericin B solution	Sigma-Aldrich, Munich (Germany)
AlphaMEM Eagle w/o *L*-glutamine	LONZA, Cologne (Germany)
AlphaMEM with deoxyribonucleotides and ribonucleotides and UltraGlutamine 1	LONZA, Cologne (Germany)
carbondioxide, liquified C1 EN ISO 14175 99.8 % purity	Westfalen AG, Münster (Germany)
chicken embryo extract - lyophilized	Seralab, Haywards heath (UK)
Dulbecco's phosphate buffered saline (DPBS)	Sigma-Aldrich, Munich (Germany)
FBS Superior, Lot# 1149C	Merck Millipore, Darmstadt (Germany)
GeneCellin	BioCellChallenge, Toulon (France)
L-glutamine	LONZA, Cologne (Germany)
Ham's F12 medium with *L*-glutamine	LONZA, Cologne (Germany)
MEM Eagle with Earle's BSS, without L-glutamine	LONZA, Cologne (Germany)
OptiMEM	Thermo Fisher Scientific, Waltham (US-MA)
penicillin-streptomycin stock, 10K/10K	LONZA, Cologne (Germany)
5X siRNA buffer	GE Dharmacon, Lafayette (US-CO)
Trypan Blue 0.4 %, 0.85 % NaCl	LONZA, Cologne (Germany)
Trypsin EDTA 200mg/L EDTA, 170.000 U/L Trypsin	LONZA, Cologne (Germany)
Viromer®Blue	Lipocalyx, Halle (Saale) (Germany)

Table 2.5: List of organic solvents.

organic solvents	manufacturer
chloroform for analysis	Merck Millipore, Darmstadt (Germany)
dimethyl sulfoxide (DMSO)	Carl Roth, Karlsruhe (Germany)
ethanol 96% AnalaR NORMAPUR	VWR, Darmstadt (Germany)
formic acid	Merck Millipore, Darmstadt (Germany)
isopropanol	VWR, Darmstadt (Germany)
methanol	VWR, Darmstadt (Germany)
ULTIMA GOLD high flash-point LSC cocktail	Perkin Elmer, Waltham (US-MA)

Table 2.6: List of commercial kits.

kits	manufacturer
Dual-Glo Luciferase Assay System	Promega, Madison (US-WI)
GelRed Nucleic Acid Gel Stain	Biotium, Hayward (US-CA)
MinElute PCR Purification Kit	QIAGEN, Hilden (Germany)
QIAquick Nucleotide Removal Kit	QIAGEN, Hilden (Germany)
QIAGEN Plasmid Mini Kit	QIAGEN, Hilden (Germany)
QIAGEN Plasmid Maxi Kit	QIAGEN, Hilden (Germany)
QuantiFast SYBR Green PCR Kit	QIAGEN, Hilden (Germany)
RNeasy Mini Kit	QIAGEN, Hilden (Germany)
RNase-Free DNase Set	QIAGEN, Hilden (Germany)
ROCHE Transcriptor First Strand cDNA Synthesis Kit	Sigma-Aldrich, Munich (Germany)

2.1.2 Software and devices

Table 2.8: List of devices.

device	manufacturer
Axiovert 40 C inverted microscope	Carl Zeiss, Oberkochen (Germany)
Benchtop-Transilluminator ECX-20.C	LTF lLbortechnik GmbH & Co. KG, Wasserburg (Germany)
Biofuge fresco centrifuge	Heraeus, Hanau (Germany)
Biofuge pico centrifuge	Heraeus, Hanau (Germany)
Biofuge primo R	Heraeus, Hanau (Germany)
BioPhotometer	Eppendorf, Hamburg (Germany)
block thermostat BT 200	Kleinfeld Labortechnik, Gehrden (Germany)
Centrifuge 5415 R	Eppendorf, Hamburg (Germany)
Centrifuge 5810	Eppendorf, Hamburg (Germany)
ChemiDoc Touch imager	Bio-Rad, Hercules (US-CA)
CO_2 incubator	Binder, Tuttlingen (Germany)
FLA-3000 Fluorescent image analyzer	Fujifilm, Tokio (Japan)
Glomax Multi Detection System	Promega, Madison (US-WI)
Heraeus HERA cell incubator	Thermo scientific, Waltham (US-MA)
incubator hood TH 30	Edmund Bühler GmbH, Hechingen (Germany)
IP plate eraser	raytest, Straubenhardt (Germany)
LightCycler 480	Roche, Basel (Swiss)
liquid scintillation analyzer Tri-Carb 2900TR	Perkin Elmer, Waltham (US-MA)
Magnetic stirrer RCT basic	IKA Labortechnik, Staufen/Breisgau (Germany)
Mastercycler gradient	Eppendorf, Hamburg (Germany)
Microplate Reader Model 680	Bio-Rad, Hercules (US-CA)
PowerShot A710 IS digital camera	Cannon, Tokio (Japan)
reciprocating platform shaker Promax 2020	Heidolph Instruments, Schwabach (Germany)
Selphy CP910 compact photo printer	Cannon, Tokio (Japan)
Semidry-blotter, 24-200	GEWO Feinmechanik GmbH, Wörth/Hörlkofen (Germany)
Thermomixer comfort	Eppendorf, Hamburg (Germany)
universal shaker SM 30	Edmund Bühler GmbH, Hechingen (Germany)
VF2 vortexer	Janke & Kunkel, Staufen/Breisgau (Germany)
Vortex Genie 2	Scientific Industries Inc., Bohemia (US-NY)

Vortex VV3	VWR, Darmstadt (Germany)
WALLAC 1420, Victor3 V multilabel counter	Perkin Elmer, Waltham, (US-MA)
water bath serie MP	Julabo, Seelbach (Germany)
1003 water bath, 14 liters	GFL Gesellschaft für Labortechnik mbH, Burgwedel (Germany)
waving platform shaker Polymax 2040	Heidolph Instruments, Schwabach (Germany)

Table 2.9: List of software.

software	**version**
ACD/Labs ChemSketch	12.01
Adobe Photoshop Elements	6.0
Bio-RAD Image Lab Touch Software	5.2 build 14
GraphPad PRISM	6.05
IBIS Biosciences Bioedit	7.0.9
ImageJ (dev. by Rasband W. at the National Institutes of Health)	1.47
JabRef (dev. by JabRef Development Team; Morten OA., Nizar N. Batada, et al.)	3.1
Microsoft Excel 2010	
MiKTeX (dev. by Schenk C.)	2.9
R: A Language and Environment for Statistical Computing (dev. by R Core Team; Ihaka R., Gentleman R. et al.)	3.2.2
raytest Advanced image Data Analyzer for windows	V3.44.035
raytest Control Software for FujiFilm BAS and FLA scanners	V3.14
Roche LightCycler 480 Software	1.5
TeXnicCenter (dev. by The TeXnicCenter Team; Wiegand S., Weinkauf T. et al.)	2.02
Thomson Reuters Endnote	X7.4

2.2 Methods

2.2.1 Cell culture

Human satellite cells were isolated from biopsy material of non-diabetic patients. Biopsy material was gained by percutaneous needle-biopsy of the vastus lateralis portion of the musculus quadriceps femoris (Bergstrom, 1975; Krutzfeldt et al., 2000). The patients were informed and gave written consent to the biopsy. The protocol was approved by the ethical committee of the Tübingen University Medical Department.

Before the punctuation with a fine needle punch biopsy technique, a 2 % solution of the local anesthetic scandicaine was injected into the biopsy area. The biopsy material was transferred into ice cold Ham's F12 medium. The material was macerated in a sterile filtered and freshly prepared collagenase solution (table 2.10 on the next page) for 15 min at 37 °C and 1200 rpm agitation. The digested biopsy material was sedimented by short centrifugation on a table top centrifuge. To stop the collagenase digestion, 10 volumes of growth medium (GM) (table 2.11 on the following page) was added. The supernatant was stored at 37 °C and 5 % CO_2. The pellet was digested repeatedly to a total of three digestion cycles. The supernatants were united and filtered through a 40 µm cell strainer. The filtrate was centrifuged for 8 min at 2,580 g and ambient temperature. The pellet was resuspended in GM and dispensed on 147.8 cm^2 cell culture dishes. Pre-plating was performed for 3 h to enrich satellite cells (Richler and Yaffe, 1970). The supernatants containing the satellite cells were cultured to a confluency of approximately 80 % before trypsinization and cryopreservation in liquid nitrogen for long-term storage.

For trypsinization, myoblasts were washed with Dulbecco's phosphate-buffered saline (DPBS) and incubated with 850 U trypsin-EDTA solution per 15 cm^2 cell culture dish for 5 - 10 min at 37 °C and 5 % CO_2. Trypsinization was stopped by 10 mL GM. The cell suspension was centrifuged for 5 min at 900 g and ambient temperature. The pellet was resuspended in cryopreservation medium (fetal bovine serum (FBS) with 10 % DMSO), transferred into cryogenic vials, and frozen overnight at -80 °C by using a cell freezing container and long-term stored in liquid nitrogen.

Table 2.10: Collagenase solution

final conc.	substance
85 % (V/V)	Ham's F12
15 % (V/V)	PBS
1.5 % (W/V)	BSA
0.5 % (W/V)	collagenase B

Table 2.11: HMT growth medium (GM)

final conc.	substance
40 % (V/V)	alphaMEM
40 % (V/V)	Ham's F12
20 % (V/V)	FBS
100 U/mL	penicillin
100 U/mL	streptomycin
2 mM	L-glutamine
0.5 µg/mL	amphotericin B
1 % (V/V)	chicken embryo extract

After thawing, the cells were seeded in GM in cell culture dishes of different sizes according to the experiments. Medium was changed to fresh GM every 2 - 3 d until the cells reach a confluency of 70 - 80 %. Medium was changed to differentiation medium (DM) (table 2.12) with medium change every 2 - 3 d for a total of 5 - 8 d for differentiation to multinucleated HMT. HMT were used for experiments after medium change to stimulation medium (SM) (table 2.13) if not stated otherwise. In experiments that examined insulin signaling pathways (figure 3.15 on page 90), the cells were serum starved by incubation in DM without FBS for 3 h.

Table 2.12: HMT differentiation medium (DM)

final conc.	substance
base medium	alphaMEM
2 % (V/V)	FBS
100 U/mL	penicillin
100 U/mL	streptomycin
2 mM	L-glutamine
0.5 µg/mL	amphotericin B

Table 2.13: HMT stimulation medium (SM)

final conc.	substance
base medium	EMEM
2 % (V/V)	FBS
100 U/mL	penicillin
100 U/mL	streptomycin
2 mM	L-glutamine
0.5 µg/mL	amphotericin B

The rat skeletal muscle cell line $L6_{GLUT4myc}$ (provided by The Hospital of Sick Children, Toronto, Ontario (Canada)) is stably transfected with a myc epitope tagged rat Scl2A4 cDNA (Kishi et al., 1998). Cells were thawed and cultured in L6 GM (table 2.14 on the following page). The medium was changed every 2 - 3 days. Before the cells reach a confluency of 60 %, the cells were passaged and seeded for glucose uptake assays in 12-well dishes at $0.8X10^5$cells/well in L6 DM (table 2.15 on the next page).

For cell counting, the cells were trypsinized as described before. An aliquot of the cell suspension is diluted 1:10 with Trypan Blue solution. Trypan Blue solution visualizes living cells by the dye exclusion method. Cells with intact cell membrane are not stained, while dead cells with permeable plasma membrane are stained blue. Living cells in 10 µL of this stained suspension were counted in a Neubauer counting chamber and seeded in the desired cell amount on cell culture dishes. Medium was renewed every 2 - 3 days. After 7 d, the cells were stimulated and glucose uptake assays were performed (section 2.2.12 on page 63).

Table 2.14: L6 growth medium (L6 GM)

final conc.	substance
base medium	alphaMEM with deoxyribonucleotides and ribonucleotides
10 % (V/V)	FBS
100 U/mL	penicillin
100 U/mL	streptomycin

Table 2.15: L6 differentiation medium (L6 DM)

final conc.	substance
base medium	alphaMEM with deoxyribonucleotides and ribonucleotides
2 % (V/V)	FBS
100 U/mL	penicillin
100 U/mL	streptomycin

2.2.2 Handling and storage of lipids

LPC(16:0) and LPC(18:1) were purchased from Sigma Aldrich (Munich, Germany). The purity of LPC(16:0) was given as $\geq$98.5 % as ascertained by gas chromatography (GC), and $\geq$99 % by thin layer chromatography (TLC). A purity of $\geq$99 % was assessed by GC and TLC analysis for LPC(18:1). The compounds were purchased as lyophilized powder and were aliquoted into brown glass vials, overlaid with nitrogen gas, and long-term stored at -20 °C. Before experimental usage, LPCs were dissolved in 100 % ethanol at a stock concentration of 10 mM. LPC(18:1) readily dissolves in ethanol, while LPC(16:0) requires to be incubated in a 37 °C water bath for 5 min for solubilization. The stock solutions were used for experiments or short-term stored at -20 °C.

For the experiment in figure 3.9 on page 81, LPCs and the other compounds were incubated in SM at a concentration of 10 µM at 37 °C for 3 h in 50 mL tubes under

mild agitation of 150 rpm. Stock concentrations and final solvent concentrations in the medium are depicted in table 2.16. The medium containing the compounds was directly added on the cells for 24 h.

Table 2.16: Solvents, stock concentrations, and corresponding solvent concentrations of the compounds used in experiment of figure 3.9 on page 81.

compound	solvent	stock	final solvent conc.
LPC(16:0)	ethanol	10 mM	1 ‰ (V/V)
LPC(18:1)	ethanol	10 mM	1 ‰ (V/V)
cPA(16:0)	DMSO	2 mM	0.5 % (V/V)
PC(O-16:0/O-1:0)	ethanol	10 mM	1 ‰ (V/V)
αGPC	water	10 mM	1 ‰ (V/V)
PC(16:0/16:0)	ethanol	10 mM	1 ‰ (V/V)
PC(18:1/18:1)	ethanol	10 mM	1 ‰ (V/V)
palmitate	ethanol	10 mM	1 ‰ (V/V)
oleate	ethanol	10 mM	1 ‰ (V/V)

In the remaining experiments, the saturated FA palmitate was complexed to BSA in order to increase the solubility in aqueous media and for increasing the physiological availability (Pond et al., 1992; Fillerup et al., 1958; Brodersen et al., 1989). Palmitate was dissolved in 100 % ethanol at a concentration of 200 mM at ambient temperature by mixing. The stock solution was added to a commercial FA-free and sterile 10 % BSA (1.5 mM) solution at a concentration of 6 mM palmitate. After adding the ethanol-palmitate stock solution to BSA, a cloudy precipitates appeared, which dissolved after an incubation time of 24 h at 37 °C under 150 rpm agitation. Clear and complexed palmitate-BSA solution was used for experiments. BSA at a concentration of 63.3 µM was used as vehicle control.

2.2.3 Quantitative PCR

For assessing the mRNA levels of a transcripts of interest, quantitative polymerase chain reaction (qPCR) was performed. HMT were grown in 6-well plates. After stimulation, total RNA was isolated via QIAGEN RNease Kit. The cells were harvested by vigorous resuspension in lysis buffer provided of the kit in the cell culture dish and running through QIAshredder spin columns as method for disruption of the cells and

homogenization. RNA was isolated according to the manufacturer's protocol. The RNA isolation included a step with DNase digestion of the total nucleic acid fraction to remove genomic DNA contamination. The eluation from the columns was performed with 30 µL PCR grade water. The eluate was re-applied to the column for higher yields. After isolation the RNA content was assessed at 280 nm using a spectrophotometer. For qPCR, the RNA was reversely transcribed to cDNA with the help of ROCHE Transcriptor First strand cDNA synthesis kit. The reverse transcription was performed according to the manufacturer's manual for multiple reactions cDNA synthesis with random hexamer primers and 1 µg total RNA (table 2.17). The synthesis reaction was performed in a Eppendorf gradient PCR cycler (table 2.18).

Table 2.17: Reverse transcription reaction mix

final conc.	substance
0.5 U/µL	Transcriptor reverse transcriptase
1X	Transcriptor RT reaction buffer
1 U/µL	Protector RNase inhibitor
1 mM each	dNTP mix
60 µM	random hexamer primers
50 ng/µL	total RNA

Table 2.18: cDNA synthesis PCR program for multiple reactions with random hexamer primers.

step	target temp.	hold	cycles
random hexamer prime	25 °C	10 min	1X
cDNA synthesis	55 °C	30 min	1X
inactivation	85 °C	5 min	1X

The qPCR was performed with 2 µL of the reverse transcription product and the QuantiFast SYBR Green PCR kit according to the manual for Two-Step RT-PCR for QuantiTect Primer Assays with QIAGEN QuantiTect Primer Assays using a ROCHE LightCycler 480 (table 2.19 on the next page).

QIAGEN QuantiTect Primer Assays were provided as ready-to-use mix of a primer pair that are designed to be intron-spanning and therefore are useful to avoid the amplification of genomic DNA which would result in false positive values. Accuracy of the amplifications were controlled by melting curve analysis. The primer sets are listed in table 2.20 on the following page.

Table 2.19: QuantiFast Two-Step RT-PCR for QuantiTect® Primer Assays.

step	target temp.	hold	ramp rate	cycles
denaturation	95 °C	3 min	4.4 °C/s	1X
denaturation annealing/elongation	95 °C 60 °C	3 s 30 min	4.4 °C/s 2.2 °C/s	40X
melting	95 °C 62 °C 98 °C	10 s 5 s	4.4 °C/s 2.2 °C/s 0.11 °C/s	1X
cooling	40 °C	30 s	2.2 °C/s	1X

Table 2.20: Primer list of Qiagen QuantiTect® Primer Assays for SYBR® Green-based quantitative RT-PCR.

gene	assay name	detected transcript
ACTB	Hs_ACTB_2_SG	NM_001101, XM_006715764
ANGPTL4	Hs_ANGPTL4_1_SG	NM_016109, NM_139314, XM_005272484
ATF3	Hs_ATF3_1_SG	NM_001030287, NM_001040619, NM_001206484, NM_001206485, NM_001206486, NM_001206488, NM_001674, NM_004024, XM_005273146
CXCL3	Hs_CXCL3_1_SG	NM_002090
IL6	Hs_IL6_1_SG	NM_000600, XM_005249745
LPCAT1	Hs_LPCAT1_1_SG	NM_024830, XM_005248373
LPCAT2	Hs_LPCAT2_1_SG	NM_017839, XM_006721211
LPCAT3	Hs_LPCAT3_1_SG	NM_005768
PDK4	Hs_PDK4_1_SG	NM_002612
PLA2G6A	Hs_PLA2G6_1_SG	NM_001004426, NM_003560, XM_005261766
PLA2G6B	Hs_PNPLA8_1_SG	NM_001256007, NM_015723, XM_005250396
PPARA	Hs_PPARA_1_SG	NM_001001928, NM_001001929, NM_001001930, NM_005036, XM_005261653, XM_005261655, XM_005261656, XM_005261657, XM_006724269, XM_006724270
PPARD	Hs_PPARD_1_SG	NM_001171818, NM_001171819, NM_006238, NM_177435, XM_005249193, XM_005249194, XM_006715120, XM_006715121, XM_006715122, XM_006715123

The PCR reactions were run with internal standards of known amplificate concentration ranging from 5 ag/µL - 0.5 pg/µL DNA. This enabled an absolute quantification of the PCR product to determine the absolute mRNA levels of the transcripts.
For relative quantification the absolute values of the samples were related to mRNA levels of β-actin (gene name: *ACTB*) as reference transcript. The resulting values were further related to control conditions.

2.2.4 Microarray analysis

To gain a global and unbiased overview of the effects of LPCs, microarray analysis was performed. Microarray analysis is a high-throughput method to analyze different types of nucleic acid sample material on a gene chip on trancriptome level.

Total RNA was isolated from human differentiated myotubes by using QIAshredder and RNeasy Mini Kit from Qiagen (Hilden, Germany). Extracted mRNA was further processed and analyzed on an Affymetrix Human Genome U219 Genechip Array by the Microarray Genechip Facility Tübingen Service. This chip provides probes from more than 36,000 transcripts and variants annotated in the National Center for Biotechnology Information (NCBI) RefSeq v36 database (13 July 2009), UniGene database 219 (build date March 30, 2009), and full-length human mRNA's from GenBank (downloaded May 12, 2009). The facility provided scaled, normalized, annotated, and quality controlled data sets. The data was analyzed using Microsoft Excel software. Fold changes were calculated and subjected to Student's t-test analysis. Student's t-test was applied to obtain a rough overview of regulated transcripts by LPC treatment. Imaging of the fold changes as heatmap was performed by using R programming language and software environment for statistical computing. Array raw data were uploaded and published on the NCBI Gene Expression Omnibus server with the GEO accession number: GSE77337.

2.2.5 Thin layer chromatography

A method to visualize the lipid profile of a cell is thin layer chromatography (TLC). The cellular lipids were isolated by a solvent-based extraction method (Folch et al., 1957). The lipids of the extracts were separated by TLC.

LPC(16:0) labeled with tritium was used as tracer to examine uptake and metabolism of LPCs. To this end, HMT grown on 15 cm^2 cell culture dishes were stimulated with 56 nCi/mL (0.93 nM) L-α-[palmitoyl-9,10-^{3}H]-LPC together with 10 µM non-radioactive LPC(16:0) for different time points in 15 mL SM (Stoll et al., 1992). An aliquot of 1 mL cell culture supernatant was collected for each time point for quantification of the remaining radioactivity in the medium. The cells were washed once with cold PBS containing 4.16 g/L (63.13 µM) BSA (FA-free) (Stoll et al., 1992) and twice with cold PBS. The cells were scraped off the plate and pelleted with 16,000 g for 1 min at 4 °C. For homogenization and disruption of the cell membrane, a freeze/thaw cycle was applied and the cells were resuspended vigorously in 20 µL 0.9 % NaCl. An aliquot of 2 µL was counted for radioactivity to calculate the total LPC uptake in relation to the remaining radioactivity in the supernatant. 100 µL chloroform/methanol 2:1 (V/V) (Stoll et al., 1992; Folch et al., 1957) was added to the cell suspension and mixed vigorously for 2 min, followed by shaking for 20 min at ambient temperature and 1400 rpm. The phases separation was accelerated by centrifugation for 10 min at 500 g and 4 °C. Both the aqueous and organic phases were collected, combined, and the remaining debris were extracted once again with 100 µL chloroform/methanol 2:1 (V/V). Again, the two phases were collected and combined. The collected extracts were applied to a silica TLC plate. Previously, the plate was pre-conditioned by soaking with 1.2 % boric acid in ethanol/water 1:1 (V/V) sparing the origin line for a better separation of the lipids (Fine and Sprecher, 1982), followed by drying at 100 °C for 1 h. The plate was run in a chamber containing chloroform/methanol/formic acid/water 60:30:7:3 (V/V/V/V) (Penzo et al., 2004) for approximately 1.75 h till the mobile phase front migrated a distance of 16.5 cm. The dried plate was sprayed partially with 2',7'-dichlorofluorescein spray solution to visualize non-radioactive lipid standards (150 nmol LPC(16:0), PC(16:0/16:0), or palmitate) that were also run on the

TLC plates. 2',7'-dichlorofluorescein spray solution stained lipophilic compounds as bright yellow spots that additionally show fluorescence under UV light irradiation. According to the migration of the non-radioactive standards, LPC, PC, and FA fractions were scraped off the plate into scintillation vials containing 5 mL scintillation cocktail. Tritium was quantified in a 0 to 18.5 keV window with a count time of 5 min at 18 °C by the use of a liquid scintillation counter. Background was subtracted and counting efficiency was assessed by unquenched standards and amounted to 63 %.

2.2.6 RNA interference

A useful tool to reduce mRNA level of a transcript of interest in living cells is siRNA-based gene silencing. With this method, siRNA targeted to one or more transcripts of a gene are transfected into the cells. siRNAs are 19 - 22 bp RNA duplexes with a two nucleotide 3'-overhang (Leung and Whittaker, 2005). The duplexes are processed in the cytosol by the RNA-induced silencing complex (Leung and Whittaker, 2005) which degrades the target mRNA and therefore prevents the translation of the protein (Leung and Whittaker, 2005).

To enable the entry into the cell, siRNA was complexed to the transfection reagent Viromer®Blue, which is a zero-charged synthetic polymer. The reagent-siRNA complex can enter the cell via endocytosis by mimicking the influenza virus cell penetrating peptides. In the cell interior, the endocytotic vesicle fuses with the endosome leading to the acidification of the vesicle. The low pH changes the charge of the reagent-siRNA complex that enables the secretion of the complex from the endosome into the cytosol. The pH difference in the cytosol leads to the release of the siRNA which can be recognized by the RNA-induced silencing complex causing the degradation of the target mRNA.

For transfection, HMT were grown on 6-well plates. Medium was changed to fresh DM. HMT were transfected with 20 nM Thermo Scientific human siGENOME SMARTpool siRNA and Viromer®Blue according to the manual of Lipocalyx Viromer®Blue for 24 h at 37 °C and 5 % CO_2. Thermo Scientific human siGENOME SMARTpool siRNA is a set of four siRNA targeting a single gene of interest for an efficient gene silenc-

Table 2.21: List of Thermo Scientific human siGENOME SMARTpool siRNA.

target gene	target transcripts	order number
PPARD	NM_177435, NM_001171819, NM_001171819, NM_001171818, NM_006238, XM_005249194, XM_005249191, XM_005249191, XM_005249192, XM_005249193, NM_001171820	M-003435-02
PLA2G6A	NM_001004426, XR_244392, NM_003560, XM_005261765, XM_005261767, XM_005261766, XM_005261764, XR_244390, XR_244391, XM_005261771, XM_005261770, XM_005261772, NM_001199562, XM_005261769, XM_005261768	M-009085-04
PLA2G6B	NM_001256010, NM_001256011, NM_001256009, XM_005250396, XM_005250395, NM_001256008, NM_001256007, NM_015723	M-010284-01
LPCAT1	XM_005248373, XM_005248374, NM_024830, XM_005248375	M-010289-00
LPCAT2	NM_017839	M-010285-00
LPCAT3	NM_005768	M-010273-01

Table 2.22: List of eurofins mwg operon siRNA.

target gene	purity	siRNA sequence
LuciferaseGL2 (siCon)	siMax siRNA desalted 0.2	5'-CgUACgCggAAUACUUCgA-3

ing. The siRNA sets used in this work are listed in table 2.21. siRNA targeted against luciferase is used as a control (siCon) (table 2.22). The medium was changed to SM afterwards and the cells were subjected to stimulation with different compounds. Efficacy of the RNA interference was checked by qPCR.

2.2.7 Luciferase reporter assay

In order to detect direct activation of the PPARδ ligand binding domain (LBD), luciferase assays were performed. Human primary myotubes were co-transfected with two different plasmids (figure 2.1 on the next page and 2.2 on the following page) (Fauti et al., 2006; Rieck et al., 2008; Jerome and Muller, 1998) obtained as kind gift from Prof. Dr. Rolf Müller (University of Marburg (Germany)). Plasmid LexA_PPARd-

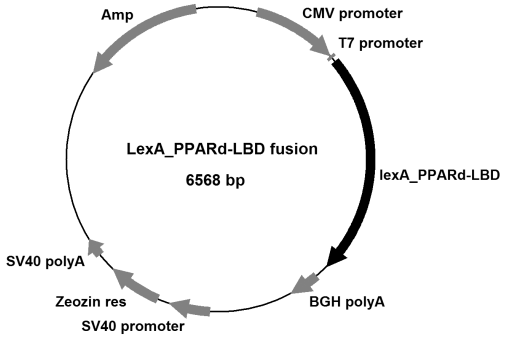


Figure 2.1: Gene map of plasmid LexA_PPARd-LBD fusion. The pcDNA3.1$_{zeo}$ vector contains a fusion protein of the LBD of mouse PPARδ and the bacterial transcription factor LexA with a Kozak sequence and nuclear translocation signal. Selection antibiotic resistance marker is ampicillin and zeocin.

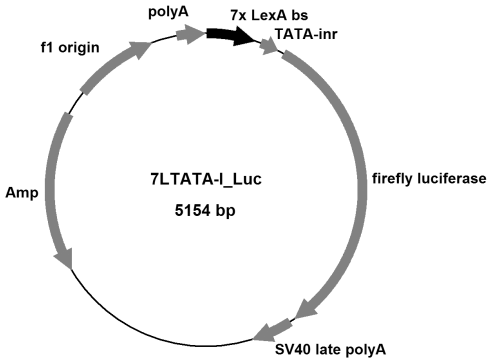


Figure 2.2: Gene map of plasmid 7LTATA-l_Luc. The pGL3 basic luciferase vector contains 7 repeats of the LexA binding site behind a TATA initiator and the firefly luciferase gene. Selection antibiotic resistance marker is ampicillin.

LBD fusion contains the LBD fragment of mouse Pparδ which is fused to the bacterial transcription factor LexA (Butala et al., 2009) together with a Kozak sequence (Kozak, 1987) and the nuclear translocation signal. The Kozak sequence is necessary for the initiation of the eukaryotic translation process (Kozak, 1987). The nuclear translocation signal guides the translated fusion protein to the nucleus.

The other plasmid contains the binding sites of LexA in front of a TATA initiator and a firefly luciferase gene. The constitutive binding of the LexA-PPARδ-LBD to the LexA binding sites is unproductive unless there is a ligand that activates the PPARδ LBD which then acts as an activation switch for the recruitment of the transcriptional protein complex of the cells (Dowell et al., 1997; Godowski et al., 1988). This leads to the transcription and subsequent expression of the firefly luciferase, which can be detected by luminescence quantification after offering the natural substrates luciferin, ATP, and oxygen (Ugarova, 1989). In a Mg^{2+}-dependent reaction AMP, pyrophosphate, and oxyluciferin are produced together with luminescence emission at 562 - 570 nm (Ugarova, 1989).

In order to transfect the two plasmids simultaneously, HMT were grown on 12-well plates. The medium was changed to fresh DM (1 mL per well). The plasmid

DNA was complexed with the polymer-based transfection reagent GeneCellin according to the manual. For an optimal result, 0.2 µg (0.2 µg/mL final concentration) LexA_PPARd-LBD or 0.2 µg (0.2 µg/mL final concentration) pCDNA3.1 as control and 0.8 µg (0.8 µg/mL final concentration) 7LTATA-I_Luc plasmid DNA were complexed with 4 µL GeneCellin in 100 µL OptiMEM for 15 min at ambient temperature and was added to one well. The medium was changed to SM for stimulation 24 h later.

After stimulation, the cells were rinsed with PBS and lysed with 50 µL 1X Glo Lysis buffer per well for 10 min at ambient temperature. 50 µL Promega Dual-Glo was added for 10 min at ambient temperature before luminometric detection of the luciferase signal by a luminometer. The luminescence protocol of the luminometer was adjusted to 0.5 s detection time. Technical triplicates were assessed per condition and biological replicate.

2.2.8 Transformation and isolation of plasmid DNA

To amplify the DNA plasmids LexA_PPARd-LBD fusion and 7LTATA-I_Luc in *E. coli*, 0.5 µg plasmid DNA was added to 100 µL competent DH5α genotype of competent *E. coli* (Thermo Fisher Scientific, Waltham (US-MA)) and mixed for 5 s, followed by incubation of 30 min on ice and 2 min at 42 °C in a water bath. 1 mL Luria broth (LB) medium was added for 30 min at 37 °C. The bacteria were pelleted for 2 min and 4000 g. The pellet was plated on a LB agar plate containing 0.1 mg/mL ampicillin. The colonies were grown over night at 37 °C and were picked for plasmid DNA isolation applying QIAGEN MiniPrep Kit according to the manufacturer's protocol. Control digests with restriction enzymes were performed for the isolated DNA. To this end, approximately 8 µg DNA was incubated with 5 U of each restriction enzymes HindIII and BamHI in 1X SuRE/cut buffer B and a total volume of 10 µL for 1 h at 37 °C. The products were separated by gel electrophoresis in a 1 % (W/V) agarose gel. HindIII cut site were located before either insert (7X LexA binding sites or lexA_PPARd-LBD) and BamHI downstream of the inserts. The gel was prepared as described in section 2.2.9 on the next page and run at 70 V for 3 h. The cut resulted in two expcected products of 4986 bp (vector) and 1582 bp (lexA_PPARd-LBD) in case of LexA_PPARd-LBD

fusion plasmid and four expected products 3022 bp, 151 bp (7X LexA binding sites), 30 bp and 1951 bp (luciferase) for the plasmid 7LTATA-I_Luc.

To propagate the colonies, 2 mL LB medium with 0.1 mg/mL ampicillin were inoculated with transformed bacteria for 9 h at 37 °C in a 15 mL reaction tube under 150 rpm agitation. The culture was added to 100 mL LB medium with 0.1 mg/mL ampicillin in a 1 L Erlenmeyer flask for 16 h at 37 °C and under 150 rpm agitation. The plasmid DNA was isolated form the culture by using QIAGEN MaxiPrep Kit according to the manual in order to increase the amount of plasmid DNA. The products were controlled by agarose gel electrophoresis.

2.2.9 Agarose gel electrophoresis of DNA

For the separation of DNA of various length, agarose gel electrophoresis was used. The DNA was previously stained by GelRed Nucleic Acid Gel Stain. To this end, the 10,000X stock of the stain was diluted 1:100 in DNA tracker dye (table 2.23) and was added to the sample at a final concentration of 16.7X.

Table 2.23: DNA tracker dye

final conc.	substance
0.1 % (V/V)	bromophenol blue sodium salt
0.1 % (V/V)	xylene cyanol FF
60 % (V/V)	glycerol
10 % (V/V)	TAE buffer

Table 2.24: 50X TAE buffer

final conc.	substance
25 mM	TRIS base
5,71 % (V/V)	glacial acetic acid
50 mM	EDTA

The agarose gel was prepared by adding agarose powder in 1X TAE buffer (table 2.24) and boiling in a microwave oven until the agarose was melted and completely dissolved. The gel was poured into the electrophoresis tray and cooled down. After sample loading, constant voltage of 50 - 70 V was applied for approximately 3 h in 1X TAE buffer. The negatively charged DNA travels in the agarose gel according to their length. DNA stained by the intercalating DNA dye GelRed can be detected by UV light illumination at 254 nm on a tabletop UV-transilluminator. The size of the DNA

fragments was estimated by a DNA ladder, which was run simultaneously together with the samples. The fluorescence image was digitalized by a digital camera and densitometrically analyzed by ImageJ software.

2.2.10 EMSA

Electromobility shift assay (EMSA aka gel shift assay) is a method to detect protein-DNA interactions. DNA fragments containing the binding site for the protein or transcription factor are incubated together with the protein of interest. In this work the transcription factors PPARδ and its essential co-receptor RXRα (Kliewer et al., 1992; Desvergne and Wahli, 1999), and the PPARδ binding site termed PPARδ responsive element (PPRE) were used. The PPRE was designed based on consensus PPREs of type II PPARδ target transcripts (Adhikary et al., 2011). The DNA fragments were 20 bp in length and are designed as two complementary DNA strands with an overhang of three bases at the 5'-ends. The overhang contains one thymidine for the labeling procedure with radioactively labeled dATP. The single stranded DNA (purchased from TIB MOLBIOL, Berlin (Germany)) were annealed at a concentration of 10 µM in 1X DNA annealing buffer (table 2.25) in a total reaction volume of 100 µL for 2 min at 90 °C (table 2.26) and cooled down slowly.

Table 2.25: 10X DNA annealing buffer

final conc.	substance
500 mM	TRIS base pH 8.2
100 mM	$MgCl_2$
0.1 mM	EDTA
50 mM	DTT
1 mM	spermidine

Table 2.26: Annealing reaction

final conc.	substance
10 µM	PPRE strand #1 5'-gATAAgTAggggAAAggTCA-3'
10 µM	PPRE strand #2 5'-gATTgACCTTTCCCCTACTT-3'
1X	annealing buffer

After annealing, the double stranded DNA oligos were subjected to the labeling process via 5'→3' fill-in reaction (table 2.27 on the following page) catalyzed by Klenow fragment which is the large subunit of the bacterial DNA polymerase I. [^{32}P]-dATP was added in place of unlabeled dATP for being attached by the polymerase on the oligos.

The reaction was performed in a total volume of 20 µL for 15 min at 25 °C. To stop the polymerase reaction, EDTA solution (pH 8.0) at a final concentration of 24 mM was added. The polymerase was heat inactivation for 20 min at 75 °C. The labeled DNA oligos were purified by applying QIAGEN nucleotide removal Kit according to the manufacturer's protocol and eluted with 30 µL water. The purified DNA oligos were used in the protein-DNA binding reaction (table 2.29). 20,000 cpm labeled oligo DNA were incubated together with the human recombinant PPARδ and RXRα proteins. For heterodimerization of the receptors, the two recombinant proteins were pre-incubated in 1X DNA binding buffer (table 2.28, adapted from (Mochizuki et al., 2001)), glycerol, and poly(dI-dC). Approximately 50X molar excess (≈0.83 µM) of unlabeled annealed oligo DNA was added to one sample to determine the specific DNA-protein band after gel electrophoresis.

Table 2.27: Fill-in reaction

final conc.	substance
5 µM	annealed oligo dsDNA
250 µM each	dGTP, dTTP, dCTP
1X	Klenow fragment buffer
5 mU/µL	DNA Polymerase I, Large (Klenow) Fragment
0.5 µCi/µL (0.16 µM)	[^{32}P]-dATP

Table 2.28: 4X DNA binding buffer

final conc.	substance
40 mM	TRIS base pH 8
600 mM	KCl
0.08 % (W/V) (12.1 µM)	BSA (FA-free)
0.2 % (V/V)	NP-40

Table 2.29: DNA binding reaction

final conc.	substance
500 ng/µL	Poly(dI-dC)
1X	DNA binding buffer
1.875 ng/µL	rec. hPPARδ
1.875 ng/µL	rec. hRXRα
11.25 % (V/V)	glycerol
≈1,000 cpm/µL	labeled oligo PPRE
5 % (V/V)	LPC, GW501516, PC(O-16:0/O-1:0), or solvent

The pre-incubation was performed on ice for 30 min. Subsequently, labeled oligo DNA together with different compounds or solvent were added for 30 min at ambient temperature. To detect the potency of the compounds to activate the binding of

the nuclear receptors to their consensus binding sequence, native gel-electrophoresis in a 6 % acrylamide gel (table 2.30) was performed. This procedure separates fast migrating free PPRE from slow migrating PPRE-PPAR/RXR complexes.

Table 2.30: EMSA acrylamide gel

final conc.	substance
1X	EMSA running buffer
16.5 % (V/V)	Rotiphorese Gel 40
1 ‰ (V/V)	TEMED
0.05 % (W/V)	APS

Table 2.31: 10X EMSA running buffer

final conc.	substance
250 mM	TRIS base pH 8
1.9 M	glycine
10 mM	EDTA

To remove substances that might interfere with the DNA-protein complex stability during the gel loading and to pre-cool the gel, a pre-run of the gel was carried out for 30 min at 4 °C and constant amperage of 30 mA in 1X EMSA running buffer (table 2.31). The gel was loaded with the incubated samples and run for 2.5 h at 4 °C and constant amperage of 30 mA. The gel was wrapped between plastic foils and exposed to a phospho-imager screen over night at ambient temperature to detect the radioactivity of the labeled DNA in the gel.

According to the manufacturer, the screen is a complex sensor plate consisting of a protective layer, a radio-sensitive layer, and a support layer. The sensor layer containing BaFBr grains of 5 µm in size. When the screen is exposed to radioactive material, a mirror image of the material is created on the screen (Takahashi et al., 1984; Yasuo et al., 1994). To read out the image on the screen, a monochromatic laser light of 633 nm was applied causing photostimulated luminescence at 400 nm which can be detected by a fluorescent image analyzer.

The digitalized pictures of the gels are processed by raytest Advanced image Data Analyzer software and densitometrically analyzed using ImageJ. The exposed plate was placed in an IP plate erase to fully remove the image from the phospho-imager screen after reading the image in the fluorescent image analyzer.

2.2.11 Palmitate oxidation

FA oxidation is an important energy supply pathway that takes place in the mitochondria. End products of the FA oxidation are CO_2 and H_2O. If one of the H atoms of the FA is labeled with tritium, the FA oxidation end product will be tritium labeled water, which can be detected by scintillation counting (Kler et al., 1992).

HMT were grown and differentiated in 6-well plates and stimulated for 24 h with different compounds. Medium was changed to 1 mL/well EMEM without supplements. The cells were stimulated with ^{3}H-palmitate at a total (^{1}H+^{3}H)-palmitate concentration of 33 µM and 0.5 µCi/mL ^{3}H-palmitate for 4 h at 37 °C and 5 % CO_2. Etomoxir at a concentration of 100 µM for 30 min was added to one well to determine non-mitochondrial FA oxidation. Etomoxir is an antagonist of carnitine palmitoyl transferase 1 (CPT1). Water produced during FA oxidation of palmitate can also be found in the supernatant of the cells. Therefore, the supernatants were collected and extracted by universal reverse-phase sorbent containing cartridges. The extraction cartridges were equilibrated with 1 mL methanol and 1 mL water before loading with 200 µL incubated cell culture supernatant. The loaded cartridges were washed with 800 µL water. The resulting eluates containing tritiated water were collected in scintillation vials filled with 5 mL scintillation cocktail. Lipophilic substances were retained in the sorbent material of the cartridges. Tritiated water in the eluate was quantified by a liquid scintillation counter at 18 °C with a count time of 5 min and a count region of 0 - 18.5 keV. Technical triplicates were assessed per condition and biological replicate.

2.2.12 Glucose uptake

Glucose uptake of skeletal muscle cells mainly relies on two glucose transporters: solute carrier family 2A1 (official symbol: SLC2A1 aka GLUT1) and solute carrier family 2A4 (official symbol: SLC2A4 aka GLUT4) (Klip and Paquet, 1990; Sarabia et al., 1992) from which SLC2A4 is able to translocate quickly to the plasma membrane in an insulin-dependent manner, while SLC2A1 is responsible for the basal glucose transport (Olson and Pessin, 1996).

To study the effects of different compounds on the insulin-dependent and independent glucose uptake, 2-deoxy glucose (2DOG) was used as tracer (Mitsumoto et al., 1991). 2DOG is taken up by the cell and is phosphorylated by hexokinases. The product 2-deoxy-glucose-6-phosphate cannot leave the cell anymore but also cannot be further metabolized and is therefore metabolically trapped in the cytosol (Hansen et al., 1994).

$L6_{GLUT4myc}$ cells (Kishi et al., 1998) were used as a model cell system to study basal and insulin-dependent glucose uptake. The cells were grown and differentiated to myotubes on 12-well dishes. After stimulation for 24 h with LPCs or GW501516 in SM, the cells were serum starved in alphaMEM with Deoxyribonucleotides and Ribonucleotides without any other supplements for 3 h to reduce effects of insulin and growth hormones contained in FBS. The cells were stimulated with or without 100 nM human insulin for 20 min. The cells were washed once with HEPES buffered saline (HBS) (table 2.32). 350 µL of glucose transport solution, containing 0.25 µCi/mL (31.25 pmol/mL) 2-[1,2-^{3}H(N)]-deoxy-*D*-glucose and 11.25 µM deoxy-*D*-glucose in HBS was added to the cells for 7 min at 37 °C and 5 % CO_2. 10 µM Cytochalasin B was added to control cells with or without insulin and with glucose transport solution in order to determine non-specific uptake. Cytochalasin B is an antagonist of SLC2A1 and SLC2A4 (Joost and Thorens, 2001). Afterwards, the cells were placed on ice and washed three times with cold 0.9 % NaCl. The cells were lysed in 350 µL lysis buffer (table 2.33) for 15 min at 4 °C. The lysate was added to 5 mL scintillation cocktail. Tritium was quantified by a liquid scintillation counter at 18 °C with a count time of

Table 2.32: HBS (HEPES buffered saline)

final conc.	substance
140 mM	NaCl
20 mM	HEPES
5 mM	KCl
2.5 mM	$MgSO_4$
2.5 mM	$CaCl_2 \cdot 2\,H_2O$

Table 2.33: lysis buffer

final conc.	substance
50 mM	HEPES pH 7.5
150 mM	NaCl
1.5 mM	$MgCl_2$
1 mM	EGTA
10 % (V/V)	glycerol
1 % (V/V)	Triton X-100
100 mM	NaF
10 mM	sodium pyrophosphate

5 min and a count region of 0 - 18.5 keV. Technical triplicates were assessed for each condition and biological replicate.

2.2.13 SDS-polyacrylamide gel electrophoresis and Western blotting

Western blotting is a method that can be used for the quantification of protein expression. HMT were cultured in tissue culture dishes of Ø100x20 mm. After stimulation the cells were lysed with 200 µL lysis buffer (table 2.33 on the previous page) including 1X phosphatase inhibitor (table 2.35). The lysate was scraped from the plate by using cell scrapers and incubated at 4 °C for 20 min on a rotation wheel followed by centrifugation at 4 °C for 5 min at maximum speed on a table top centrifuge. The supernatants were collected and stored at -80 °C. Aliquots were used for determination of the protein concentration by staining proteins with Coomassie Brilliant Blue G-250 (Bradford, 1976). For the staining, the samples were diluted 1:200 in 200 µL 1X Bio-Rad Bradford protein assay solution. The dye was incubated for 5 min at ambient temperature and analyzed by a Bio-Rad ELISA reader at 595 nm. For quantification of the protein amount via a standard curve, BSA standards were included at a concentration range of 0.05 - 0.5 µg/µL. The samples were adjusted to an equal protein amount per experiment ranging from 20 - 40 µg. Loading buffer (table 2.34) was added to the samples to a final concentration of 1X followed by boiling for 7 min at 95 °C.

Table 2.34: 5X sample buffer

final conc.	substance
60 mM	TRIS base pH 6.8
25 % (V/V)	glycerol
69.35 mM	SDS
5 % (V/V)	β-mercaptoethanol
0.1 % (V/V)	bromophenol blue sodium salt

Table 2.35: 10X phosphatase inhibitor

final conc.	substance
10 mM	NaF
5 mM	sodium pyro-phosphate tetrabasic decahydrate
10 mM	β-glycerophosphate disodium salt hydrate
10 mM	sodium orthovanadate

In order to separate the proteins of the lysates, SDS-polyacrylamide gel electrophoresis was performed. To this end, discontinuous acrylamide gels were pre-

pared. The discontinuous acrylamide gels used in this work were 1.5 mm thick. A gels consist of a stacking gel part and a running gel part. Two running gels of different acrylamide concentrations, 5.0 and 7.5 % (table 2.36 and 2.37), were used. 7.5 % gels resolve a protein size range of 30 - 100 kDa (AKT), while 5 % gels were used for larger proteins of 50 - 300 kDa (MYHC, ACC, AMPK). The boiled lysates were loaded on the stacking gel (table 2.38). The samples were run for 2 - 3 h at ambient temperature in 1X running buffer (table 2.39) in a gel running chamber.

Table 2.36: 7.5 % acrylamide running gel

final conc.	substance
0.375 M	TRIS base pH 8.8
0.5 % (W/V)	SDS
25 % (V/V)	Rotiphorese Gel 30
0.0675 % (W/V)	APS
0.165 % (V/V)	TEMED

Table 2.37: 5.0 % acrylamide running gel

final conc.	substance
0.375 M	TRIS base pH 8.8
0.5 % (W/V)	SDS
16.7 % (V/V)	Rotiphorese Gel 30
0.0675 % (W/V)	APS
0.165 % (V/V)	TEMED

Table 2.38: 3.7 % acrylamide stacking gel

final conc.	substance
0.115 mM	TRIS base pH 6.8
15.96 mM	SDS
12 % (V/V)	Rotiphorese Gel 30
0.1 % (W/V)	APS
0.33 % (V/V)	TEMED

Table 2.39: 10X running buffer

final conc.	substance
0.25 M	TRIS base
1.92 M	glycine
34.67 mM	SDS

Table 2.40: 10X blotting buffer

final conc.	substance
0.48 M	TRIS base
0.39 M	glycine
13.87 mM	SDS
20 % (V/V)	methanol

SDS in the running buffer, is an anionic detergent and binds to the hydrophobic parts of the denatured proteins which will lead to a negative net charge of the proteins regardless of their original net charge. The newly gained net charge enables a

homogenous electrophoretic mobility of the proteins in the gel that is only affected by their size. A stained protein size marker was also loaded on the gel for estimating the protein sizes. After the run, the acrylamide gel was transferred on a nitrocellulose membrane and wrapped between Whatman gel blotting papers. The blotting sandwich is soaked in 1X blotting buffer (table 2.40 on the preceding page). In a blotting chamber for semi-dry blotting, the proteins of the acrylamide gel are transferred onto the nitrocellulose membrane at constant amperage of 0.8 A/cm^2 of membrane for 3 h at ambient temperature. After blotting, the membranes were incubated in 1X NaCl-EDTA-TRIS-gelatine (NET-G) buffer (table 2.42 on page 69) for 1 h at ambient temperature on an orbital shaker. This prevents the unspecific binging of antibodies by blocking unspecific binding sites. IgG antibodies listed in table 2.41 on the following page were probed on the membranes over night at 4 °C under mild agitation. Afterwards, the membranes were washed three times with 1X NET-G buffer for 45 min in total to remove unbound antibodies.

Secondary antibodies against rabbit or mouse IgG were applied at a dilution of 1:1000 in 1X NET-G buffer for 45 min at ambient temperature, followed by washing of the membranes. The secondary antibody are coupled to horse radish peroxidase and can be detected by enhanced chemiluminescence (ECL) reaction. To this end, the membranes were soaked in ECL working solution (table 2.43 on page 69) for the detection of normal or in commercial ECL solution for weak antibody binding. The ECL solutions contain H_2O_2 and luminol, which are substrates of the peroxidase. The enzymatic reaction causes light emission of 428 nm which can be detected and digitalized by the ChemiDoc Touch chemiluminescence imaging system. The data were densitometrically analyzed by the software ImageJ.

For re-probing with antibody against total protein as loading control and reference, the membranes were incubated in stripping buffer (table 2.44 on page 69) for 30 min at 56 °C in a water bath to remove remaining antibodies. The membranes were washed and re-probed with antibodies as described before.

Table 2.41: List of IgG antibodies used in this work for Western blotting

antigen	clonality	source	dilution in NET-G	company	order number
anti-phospho-acetyl CoA carboxylase (Ser79)	polyclonal	rabbit	1:1000	upstate/Millipore, Darmstadt (Germany)	07-303
phospho-Akt (ser473)	polyclonal	rabbit	1:1000	Cell Signaling Technology, Cambridge (UK)	9271
phospho-Akt (thr308)	polyclonal	rabbit	1:1000	Cell Signaling Technology, Cambridge (UK)	9275
clone 55/PKBa/Akt (RUO)	monoclonal	mouse	1:500	BD biosciences, San Jose (US-CA)	610860
phospho-AMPKα (Thr172) (40H9)	monoclonal	rabbit	1:1000	Cell Signaling Technology, Cambridge (UK)	2535
anti-AMPK α1	polyclonal	rabbit	1:1000	upstate/Millipore, Darmstadt (Germany)	07-350
anti-myosin (skeletal, slow)	monoclonal	mouse	1:1000	Sigma-Aldrich, Munich (Germany)	M8421
goat anti-rabbit IgG-HRP	polyclonal	goat	1:1000	Santa Cruz Biotechnology, Inc., Dallas (US-TX)	sc-2004
goat anti-mouse IgG-HRP	polyclonal	goat	1:1000	Santa Cruz Biotechnology, Inc., Dallas (US-TX)	sc-2005

Table 2.42: 10X NET-G buffer

final conc.	substance
1,5 M	NaCl
50 mM	EDTA
500 mM	TRIS base pH 7.4
0.5 % (V/V)	Triton X-100
2.5 % (W/V)	gelatine

Table 2.43: ECL solution

solution A

final conc.	substance
4.41 mM	luminol
4.32 mM	4-iodophenol
0.1 M	TRIS base pH 9.35

solution B

final conc.	substance
0.0075 % (V/V)	H_2O_2
0.1 M	TRIS base pH 9.35

ECL working solution

final conc.	solution
50 % (V/V)	solution A
50 % (V/V)	solution B

Table 2.44: Stripping buffer

final conc.	substance
66 mM	TRIS base pH 6.8
0.5 % (V/V)	β-mercaptoethanol
2 % (W/V)	SDS

2.2.14 XBP1 splicing assay

X-box protein 1 (*XBP1*) mRNA splicing is a consequence of ER stress (Han and Kaufman, 2016) and can easily be quantified by agarose gel electrophoresis. Stimulated HMT were lysed for RNA isolation. The isolated RNA was reversely transcribed to cDNA. The cDNA of total *XBP1* was amplified via PCR reaction (tables 2.45 on the next page, 2.46 on the following page) (Calfon et al., 2002; Calligaris et al., 2009). Primers were purchased from TIB MOLBIOL, Berlin (Germany). To separate spliced from unspliced *XBP1*, the PCR product was cut by the restriction enzyme PST1. To this end, 10 µL of the PCR product in 1X SuRE/Cut buffer H was cut with 10 U/µL PST1 in a total volume of 25 µL for 1 h at 37 °C. The DNA products of the reaction were stained by GelRed and were separated via electrophoresis in a 2 % agarose gel. GelRed stained DNA was visualized via UV light radiation and quantified via

densitometric analysis by ImageJ. Relative splicing of *XBP1* was calculated as the ratio of the band intensity of uncut and spliced vs. cut and unspliced product.

Table 2.45: XBP1 PCR reaction mix

final conc.	substance
1X	Taq polymerase buffer
200 µM	dNTP mix
200 nM	hXBP1.3S 5'-AAACAgAgTAgCAgCTCAgACTgC-3'
200 nM	h/mXPB1.12AS 5'-TCCTTCTgggTAgACCTCTgggAg-3'
4 ng/µL (0.1 µg)	cDNA
5 % (V/V)	DMSO
25 mU/µL	Taq polymerase
25 µL	total volume

Table 2.46: XBP1 amplification PCR reaction

step	target temp.	hold	cycles
denaturation	94 °C	4 min	1X
denaturation	94 °C	10 s	
annealing	66 °C	30 s	35X
elongation	72 °C	30 s	
elongation	72 °C	10 min	1X

2.2.15 *In vitro* time-resolved fluorescence resonance energy transfer assay

Ligand binding was determined by time-resolved fluorescence energy transfer (TR-FRET)-based *in vitro* assays (Stafslien et al., 2007) using LanthaScreen PPAR-beta/delta competitive binding assay kit and coactivator assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Assays were performed by Dr. Till Adhikary (working group leader: Prof. Dr. Rolf Müller) at the Institute of Molecular Biology and Tumor Research of the University of Marburg (Germany).

The competitive binding assay was performed for 30 min at ambient temperature in FRET assay buffer (table 2.47 on the next page). GW501516 was dissolved in

DMSO, αGPC was dissolved in water, and LPCs were dissolved in ethanol at a 10 mM stock concentration each. Measurements were performed on a VICTOR3 V multilabel counter with instrument settings as described in the manufacturer's instructions for LanthaScreen assays.

Table 2.47: FRET assay buffer

final conc.	substance
20 mM	TRIS base pH 7.9
100 mM	KCl
0.01 % (V/V)	Triton X-100
5 mM	DTT
1 g/L	BSA

2.3 Statistics

Data depicted in this work are mean ±SEM (standard error mean), if not stated otherwise. In order to compare groups of data, Student's t-test was applied using Microsoft Excel software. P-values < 0.05 were considered as statistically significant.

2.4 Nomenclature

The symbols of proteins and genes used in this thesis are in accordance with Human Genome Gene Nomenclature Committee guidelines (Wain et al., 2002). In short, gene and protein symbols are equal, except that gene symbols are italicized while protein symbols are not. In this work, symbols commonly found in publications (e.g. PPARδ instead of the official symbol: PPARD) were used for a better understanding but the official symbols are also mentioned to avoid misleading.

3 Results

3.1 Characterization of LPC effects

To examine transcriptome wide effects caused by LPCs on HMT, global RNA analysis by Affymetrix microarray analysis was performed. HMT were stimulated with 10 µM LPC(16:0) or LPC(18:1) for 24 h. The isolated RNA was subjected to microarray analysis performed by the Microarray Genechip Facility Tübingen service. The data were processed according to the material and methods section. The quality controlled data were used to calculate fold change values.

The data showed a maximal fold change of 4.72 compared to solvent as control (figure 3.1 on the following page). The two most upregulated transcripts were *PDK4* and *ANGPTL4*. Together with *PLIN2* (Perilipin 2 aka Adipose differentiation-related protein) and *CPT1A* (carnitine palmitoyltransferase 1A (liver)) amongst the ten most up regulated genes, this pointed to an activation of the nuclear receptors family of PPAR (Adhikary et al., 2011).

To confirm the results of the microarray analysis, qPCR was performed under the same conditions (figure 3.2 on the next page). mRNA levels of *PDK4* and *ANGPTL4* were elevated by LPC treatment to a similar extend as shown by the microarray analysis. It was tested if the co-stimulation of the two LPCs have effects different than single LPC treatment. A LPC(16:0)/LPC(18:1) ratio of 1:1 was used as well as a ratio close to the physiological situation (table 1.5 on page 19) of 2:1. The mixtures showed similar results and were not different to stimulation with single LPCs.

In my diploma thesis, kinetics analysis of LPC treatment caused a cellular response on *ANGPTL4* mRNA levels already after 1 h (figure 1.9 on page 36). Hence, LPCs

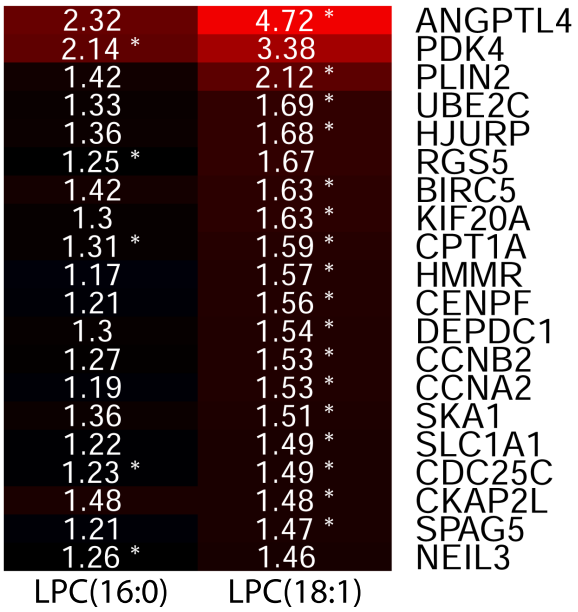


Figure 3.1: Microarray analysis of mRNA after LPC treatment. HMT were treated for 24 h with 10 µM LPC(16:0), LPC(18:1), or solvent. mRNA was subjected to microarray analysis. Raw data were uploaded in NCBI Gene Expression Omnibus homepage with the accession number GSE77337. The 20 of most upregulated transcripts were depicted in a heatmap. Fold changes related to solvent are given as mean in white font as well as a color code from blue=0, black=1 to red=5. Values were ordered for decreasing fold changes of LPC(18:1) treatment. * $p < 0.05$ vs. solvent (n=4). Adapted from (Klingler et al., 2016)

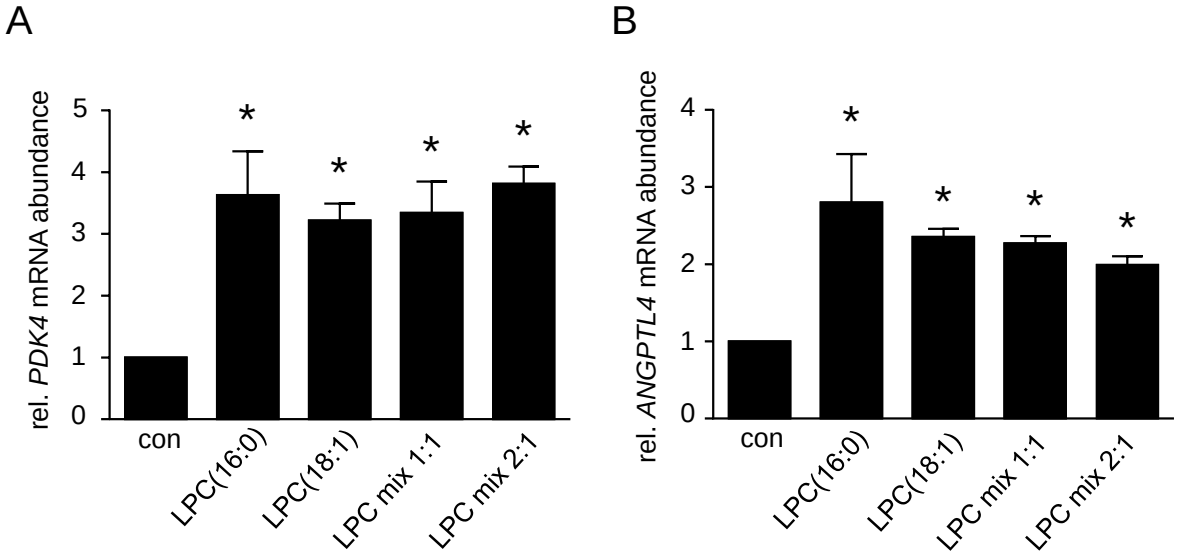


Figure 3.2: *PDK4* and *ANGPTL4* induction by LPC(16:0) and LPC(18:1). HMT were treated for 24 h with 10 µM LPC(16:0), 10 µM LPC(18:1), 1:1 and 2:1 mixtures of LPC(16:0)/LPC(18:1) (10 µM LPC in total), or solvent (con). Total RNA was isolated and the mRNA levels of (**A**) *PDK4* and (**B**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent (set as 1). * < 0.05 vs. solvent (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

presumably enter the cell within less than 1 h. To examine the uptake of LPCs into the cells, 10 µM LPC(16:0) were used together with 56 nCi/mL (0.93 nM) ^{3}H-labeled LPC(16:0) as a tracer. The uptake was analyzed at different time points of the treatment. The ratio of remaining ^{3}H-labeled LPC(16:0) in the supernatant and ^{3}H-labeled compounds in the cells were assessed as measurement for LPC uptake (figure 3.3A). A significant cellular uptake of ^{3}H-LPC(16:0) was detected 30 min after administration. 24 h after stimulation, 80 % of the ^{3}H-labeled LPC was taken up by the cells.

Furthermore, the fate of ^{3}H-LPC(16:0) was examined by TLC of lipid extracts (figure 3.3B). The radioactivity of the lipid fractions LPC, PC, and FA were quantified for different time points after ^{3}H-LPC(16:0) treatment. The incorporation of ^{3}H-LPC(16:0) into PC was significant after 30 min and increased over time. No significant changes were observed for LPC and FA fractions.

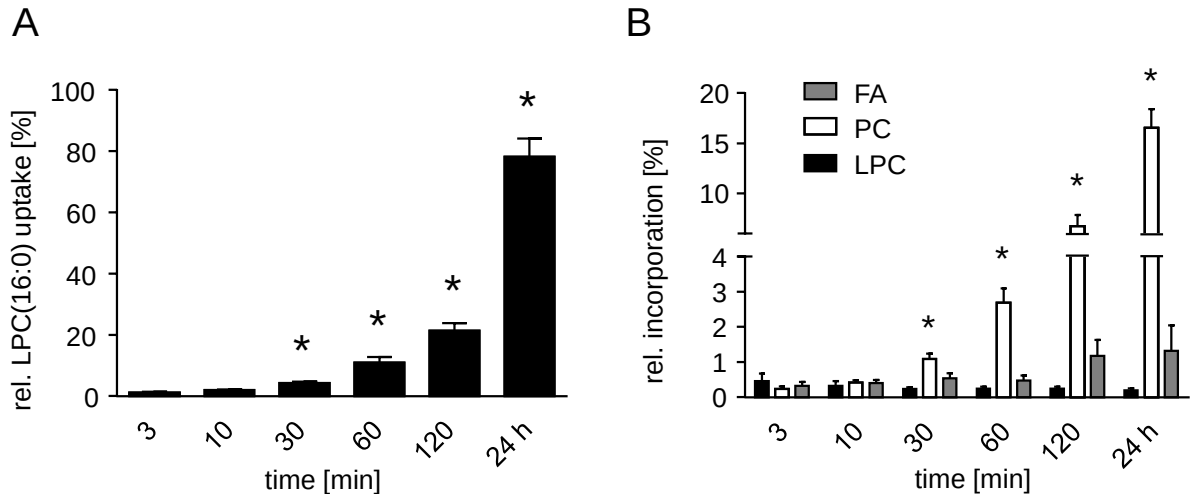


Figure 3.3: Uptake and metabolic fate of LPC(16:0) in HMT. HMT were treated with 56 nCi/mL (0.93 nM) ^{3}H-LPC(16:0) together with 10 µM LPC(16:0) for the indicated time points. (**A**) To verify LPC(16:0) uptake, the ratio of ^{3}H in the cells and ^{3}H in the supernatant was calculated. Data are depicted as mean ±SEM. * < 0.05 vs. cells stimulated for 3 min with ^{3}H-LPC(16:0) n=(4-5). (**B**) The lipids in the treated cells were extracted by chloroform/methanol 2:1 and separated by TLC. ^{3}H-incorporation into the different lipid fractions were quantified by liquid scintillation counting and are related to the amount of ^{3}H-LPC(16:0) applied to the cells. Data are mean ±SEM. * < 0.05 vs. stimulation time of 3 min (n=4 - 5).

3.2 *PDK4* and *ANGPTL4* induction caused by LPCs is PPARδ dependent

Microarray results pointed to a PPAR activation. The induction of the top two transcripts was confirmed via qPCR and was examined further to unravel the mechanism behind the induction. Three members of the PPAR family are known: PPARα (PPARA), PPARβ/δ (PPARD), and PPARɣ (official symbol: PPARG). According to the literature, *PDK4* and *ANGPTL4* inductions in skeletal muscle cells point to a PPARα or PPARδ activation (Staiger et al., 2009; Wu et al., 1999; Muoio et al., 2002; Adhikary et al., 2011). The synthetic agonist of PPARδ GW501516 and of PPARα WY14643 (aka Pirinixic acid) were tested in HMT for their efficacy to increase *PDK4* and *ANGPTL4* mRNA levels. The results indicated that PPARδ activation and not PPARα caused the induction of *PDK4* and *ANGPTL4* in HMT (figure 3.4). The PPARα activator WY14643 showed no significant induction of the two transcripts.

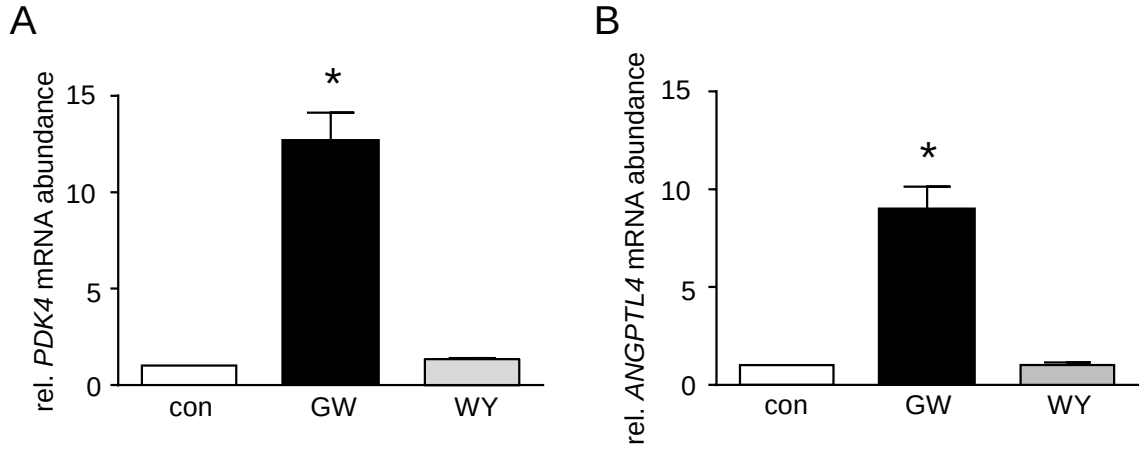


Figure 3.4: *PDK4* and *ANGPTL4* are PPARδ target genes in HMT. HMT were stimulated with 1 µM GW501516 (PPARδ agonist) or 1 µM WY14643 (PPARα agonist) for 24 h. Total RNA was isolated. mRNA levels of (**A**) *PDK4* and (**B**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * < 0.05 vs. solvent (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

To confirm a PPARδ participation in the LPC effects on *PDK4*/*ANGPTL4*, two antagonists of PPARδ, GSK3787 and GSK0660, were examined on the palmitate evoked induction of *ANGPTL4*. Palmitate is an endogenous PPARδ agonist (Xu et al.,

1999). GSK3787 is an irreversible antagonist of PPARδ (Shearer et al., 2010), while GSK0660 is an inverse PPARδ agonist (Shearer et al., 2008).

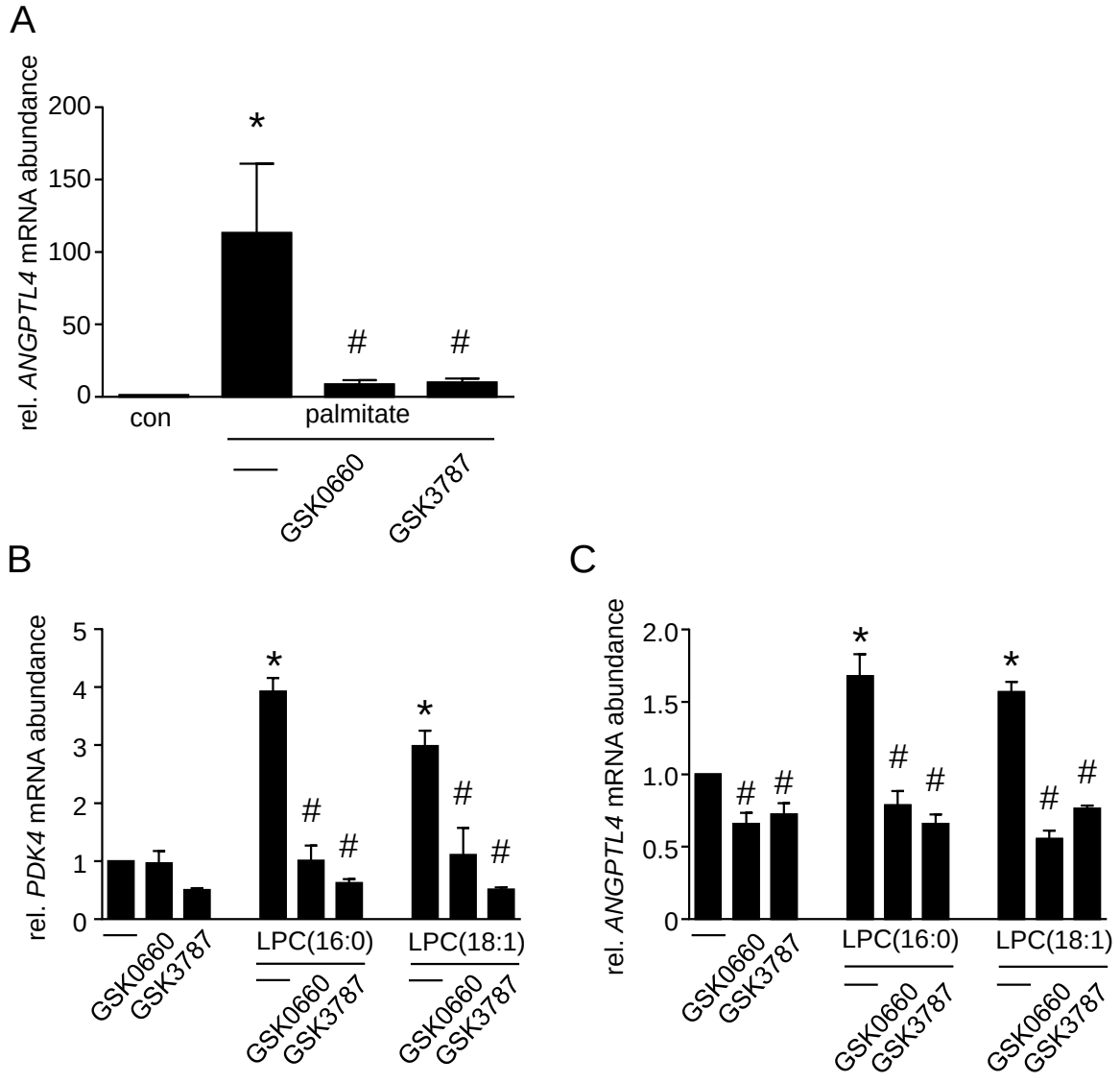


Figure 3.5: Effects of PPARδ antagonists/inverse agonists on *PDK4/ANGPTL4* mRNA levels. HMT were stimulated with either DMSO as solvent, 10 µM GSK3787 (PPARδ antagonist) or 10 µM GSK0660 (PPARδ inverse agonist) for 2 h. Subsequently, (**A**) 250 µM palmitate complexed to BSA or BSA alone as control were added for 24 h. *ANGPTL4* mRNA levels were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * $p < 0.05$ vs. cells treated with BSA, # $p < 0.05$ vs. cells treated with palmitate (n=6). (**B+C**) HMT were stimulated with 10 µM LPC(16:0) or LPC(18:1) for 24 h after GSK3787 or GSK0660 treatment. *PDK4* and *ANGPTL4* mRNA levels were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * $p < 0.05$ vs. cells treated with solvent, # $p < 0.05$ vs. cells treated with LPC w/o GSK3787/GSK0660 (n=6). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

The results showed that palmitate increased *ANGPTL4* mRNA levels and that this induction was inhibited by the compounds GSK3787 and GSK0660 (figure 3.5A). The

same effects were seen with LPC(16:0) and LPC(18:1) administration. The inductions of *PDK4* and *ANGPTL4* caused by LPC treatment were completely blocked by GSK3787 and GSK0660 pre-treatment (figure 3.5B,C). GSK3787 or GSK0660 itself had no effects on *PDK4* mRNA levels but reduced *ANGPTL4* mRNA abundance of approximately 25 % in the absence of LPCs.

It was tested if silencing of *PPARD* can influence the LPC-mediated induction of *PDK4* and *ANGPTL4*. HMT were transfected with siRNA targeted against *PPARD* prior to LPC treatment. The presence of siRNA against *PPARD* reduced the mRNA levels of *PPARD* by approximately 75 % (figure 3.6A). This reduction of *PPARD* mRNA resulted in a significant decline of LPC-mediated *PDK4* induction (figure 3.6B). The induction of *ANGPTL4* by LPCs was not affected by *siPPARD* (figure 3.6C). In the absence of LPCs, *siPPARD* caused a significant increase of both *PDK4* and *ANGPTL4* mRNA levels. This effect was most prominent for *ANGPTL4* with 50 % increased mRNA levels.

To confirm that LPCs activate the LBD of PPARδ, luciferase reporter assays were conducted. The plasmids for this assay were kindly provided by Prof. Dr. Rolf Müller from the University of Marburg (Germany) (Fauti et al., 2006; Rieck et al., 2008). The two plasmids (figure 2.1 and 2.2 on page 57) were co-transfected for 24 h followed by stimulation with LPC(16:0), LPC(18:1), solvent, and GW501516 as positive control for further 24 h. The plasmids LexA_PPARd-LBD fusion encodes for a fusion protein of the bacterial (*E. coli* (strain 12)) transcription repressor LexA and the PPARδ LBD behind a TATA box (figure 2.1 on page 57). The plasmid 7LTATA-I_Luc contains seven tandem repeats of the LexA binding site downstream of the firefly luciferase gene (figure 2.2 on page 57). In the presence of a PPARδ ligand the expressed fusion protein recruits co-activators and other transcriptional factors due to activation of the LBD of PPARδ (Dowell et al., 1997; Godowski et al., 1988). This recruitment of transcriptional machinery results in the expression of the firefly luciferase gene. That expression was verified via luminescence signal quantification.

Both LPCs caused the activation of the PPARδ LBD indicated by an increased luciferase activity. The increase of the luciferase activity by LPC(16:0) and LPC(18:1)

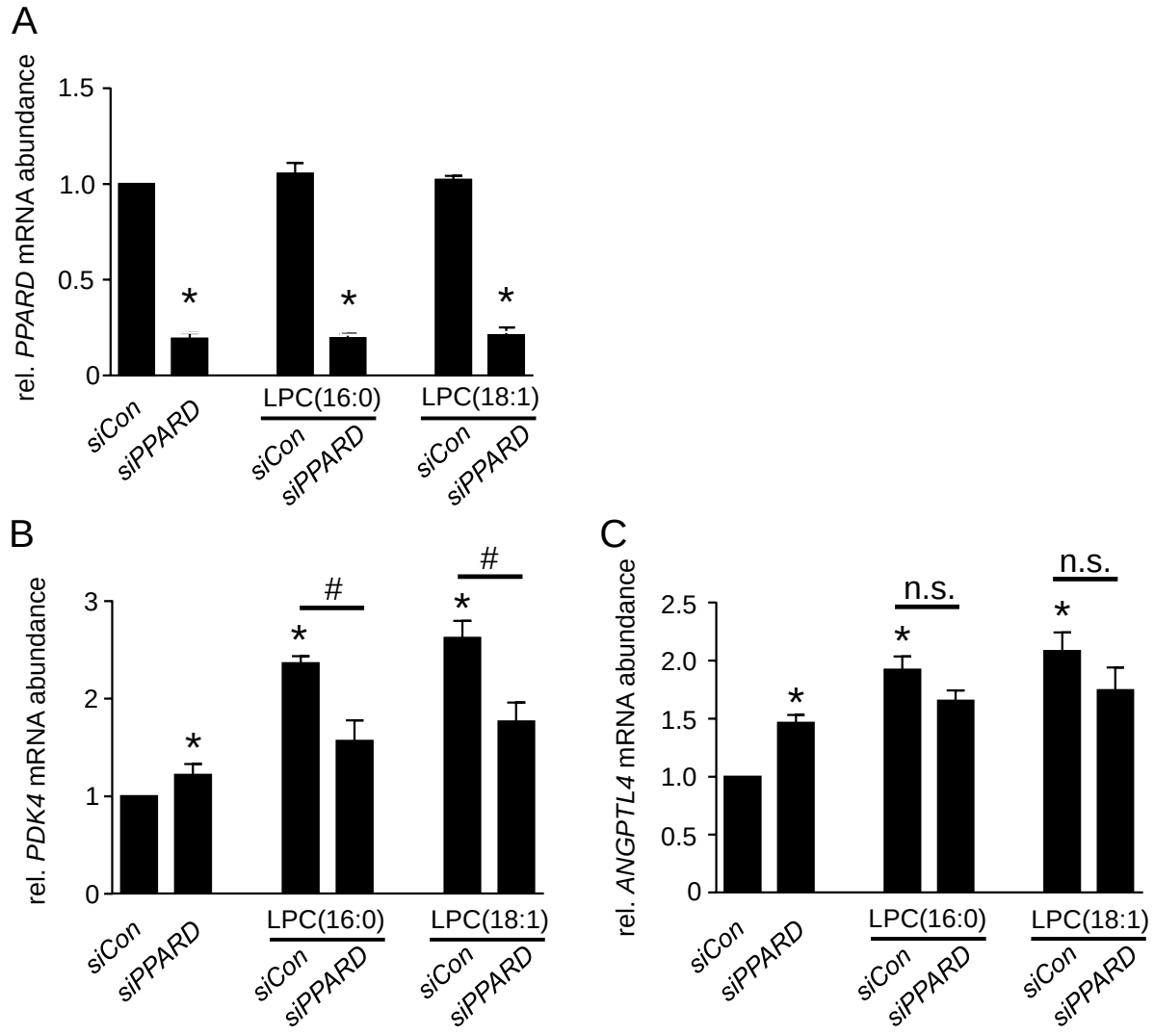


Figure 3.6: Effects of siRNA against *PPARD* on *PDK4* and *ANGPTL4* mRNA levels. HTM were transfected with siRNA against PPARD for 24 h followed by treatment with 10 µM LPC(16:0), 10 µM LPC(18:0), or solvent for 24 h. (**A**) *PPARD*, (**B**) *PDK4*, and (**C**) *ANGPTL4* mRNA levels were quantified by qPCR. Data are shown as mean ±SEM related to siCon transfected cells treated with solvent (set as 1). * $p < 0.05$ vs. cells transfected with siCon and treated with solvent, # $p < 0.05$ vs. cells transfected with *siPPARD* and treated with LPC (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

accounted for 2.4- and 2.5-fold vs. control, respectively, while the synthetic PPARδ agonist GW501516 had a 10-fold response vs. solvent control (figure 3.7 on the next page). Cells transfected with the empty vector pcDNA3.1 instead of the plasmid LexA_PPARd-LBD fusion did not show increased luciferase activity upon GW501516 or LPC treatment.

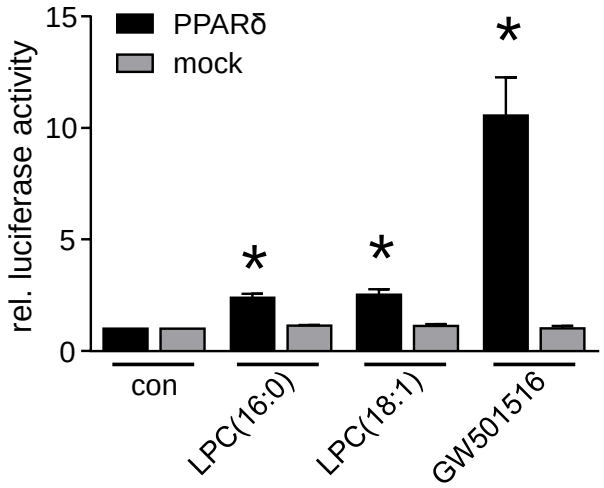


Figure 3.7: LPC treatment caused the activation of the PPARδ LBD in luciferase reporter assays. HMT were co-transfected with two plasmids for 24 h. One plasmid contained the fusion protein of PPARδ LBD with LexA and the other plasmid included LexA binding sites in front of a firefly luciferase gene. As mock control the former was replaced by an empty pcDNA3.1 plasmid. Afterwards, the cells were treated with 10 µM LPC(16:0), 10 µM LPC(18:1), 1 µM GW501516 for 24 h. The luciferase activity was quantified by luminescence reading. Data are depicted as mean ±SEM related to solvent as control (set as 1). * $p < 0.05$ vs. solvent as control (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

3.3 LPCs are PPARδ ligands

It was tested if LPCs can activate PPARδ directly as endogenous ligands. For this purpose, EMSAs were conducted with the recombinant human PPARδ and RXRα (retinoid X receptor α, official symbol: RXRA) proteins together with a consensus PPAR responsive element (PPRE) of PPARδ target transcripts with a type II response. Type II PPARδ target transcripts fulfill two criteria (Adhikary et al., 2011): (a) induction of the PPARδ target gene by the presence of siRNA targeted against PPARδ and (b) induction of the PPARδ target gene by PPARδ agonist GW501516. Both criteria are fulfilled by *PDK4* and *ANGPTL4* as showed by figure 3.4 on page 75 and figure 3.6 on the previous page.

The EMSAs showed that the two recombinant proteins caused a shift of the free PPRE probe in the acrylamide gel. The shifted complex of PPRE and PPAR/RXR appeared as weak band marked as ”PPARδ/RXRα” (figure 3.8A on the next page). This band became more prominent in the presence of the potent PPARδ agonist GW501516 and by different concentrations of LPC(16:0) and LPC(18:1) (figure 3.8A).

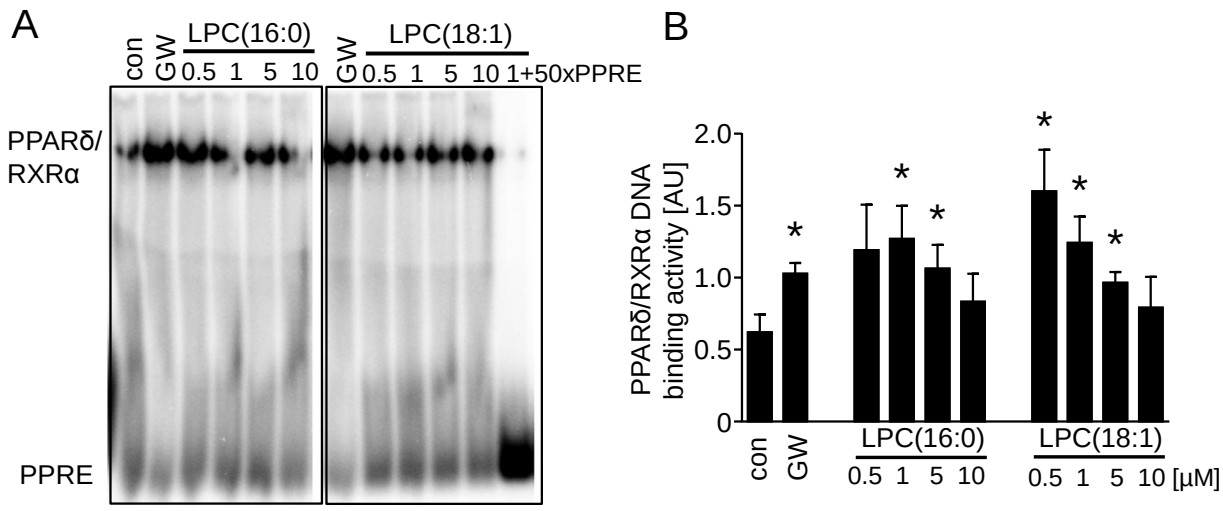


Figure 3.8: LPCs increased the DNA binding activity of PPARδ/RXRα complex. EMSA was performed with recombinant human PPARδ, and RXRα together with an oligo DNA sequence containing the consensus binding site for PPARδ named PPRE. The PPRE was radioactively end-labeled with [^{32}P]dATP. For the binding reaction, 1.875 ng/µL of each recombinant protein together with approx. 20,000 cpm radioactively end-labeled PPRE were used in the presence of 5 µM GW501516 or increasing concentrations of LPC(16:0) or LPC(18:1) or 50x molar excess of unlabeled PPRE. The radioactivity was visualized via phospho-imaging. (**A**) One representative gel is depicted. (**B**) Densitometrical evaluation was performed. Data are mean of values related to total PPARδ/RXRα band intensities per experiment ±SEM . * $p < 0.05$ vs. solvent (n=4 - 6). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

The band almost disappeared in the presence of 50X molar excess of non-radioactive PPRE indicating the specificity of the band. The strongest DNA binding activity was caused by LPCs was detected for 0.5 µM LPC(18:1) and 1 µM LPC(16:0). The effects of GW501516, 1 and 5 µM LPC(16:0), and 0.5 - 5 µM LPC(18:1) on the DNA binding activity of the proteins were statistically significant different from control samples. Increasing concentration of LPCs in the reaction mix reduced the DNA binding activity of the PPARδ/RXRα complex on the PPRE (figure 3.8B). This effect was observed for both LPCs.

To gain insights into which LPC metabolite is also capable of increasing *PDK4* and *ANGPTL4* mRNA levels and to compare LPC effects with their efficacy, a spectrum of compounds (figure 3.9A on the next page) was applied at equimolar concentrations for 24 h on HMT. The compounds were previously complexed for 3 h at 10 µM in SM containing 2 % FBS to provide comparable stimulation conditions among the compounds. Among the compounds (figure 3.9A) was the synthetic PC(O-16:0/O-1:0) that belongs

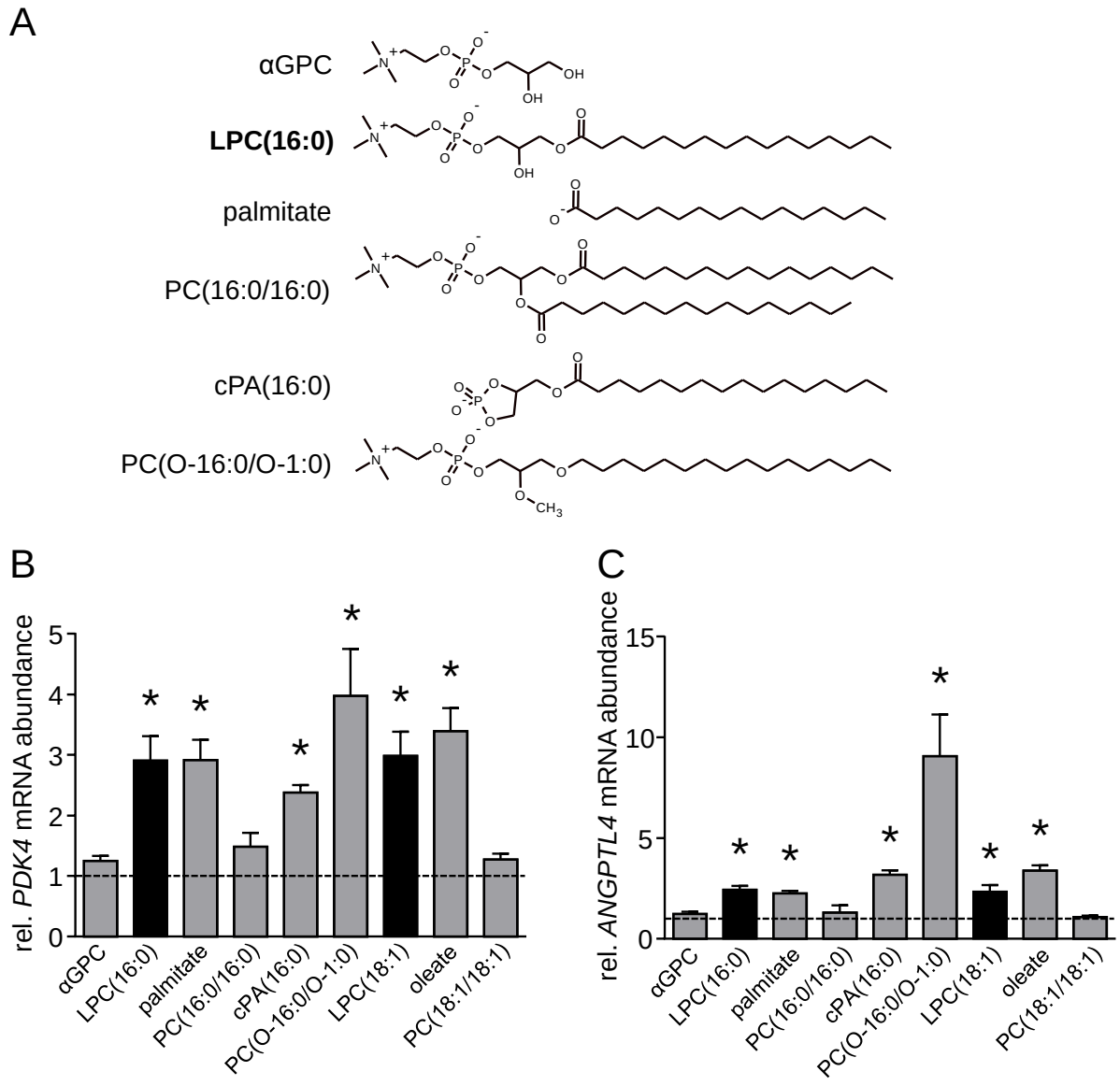


Figure 3.9: Effect of different compounds on *PDK4* and *ANGPTL4* mRNA levels. HMT were stimulated with (**A**) different compounds at 10 µM each for 24 h. RNA was isolated. The mRNA levels of (**B**) *PDK4* and (**C**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1 displayed as dashed line). * < 0.05 vs. solvent (n=6). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

to the class of ALPs and was used as compound that structurally resembles LPCs. This particular lipid is not hydrolyzable by phospholipases of the class A_1 and A_2.

The FA palmitate and oleate displayed similar efficacy as LPCs on the induction of *PDK4* and *ANGPTL4* (figure 3.9B,C). Also the cyclic compound cPA(16:0) displayed a robust induction of the two transcripts. Furthermore, PC(O-16:0/O-1:0) caused the strongest response among the set of compounds. The metabolites PC(16:0/16:0), PC(18:1/18:1), and αGPC demonstrated no effect on *PDK4* or *ANGPTL4* mRNA levels.

To confirm that LPCs are potential direct ligands of PPARδ, a LanthaScreen TR-FRET PPARδ competitive binding assay was performed in cooperation with Dr. Till Adhikary at the University of Marburg. This assay uses Fluormone Pan-PPAR Green™, a fluorescein-labeled pan-PPAR ligand that binds to the GST-tagged LBD of PPARδ. A terbium-labeled anti-GST antibody is added which generates a TR-FRET signal when the ligand is bound. Terbium acts as donor fluorophore for the acceptor fluorophore fluorescein. Fluorescein emits light at 520 nm via FRET when terbium is excited with a light source of 340 nm. The emission signal of terbium at 495 nm acts as reference. When the pan-PPAR ligand is competitively displaced by a non fluorescent ligand the TR-FRET signal at 520 nm declines. LPC(16:0), LPC(18:1), PC(O-16:0/O-1:0), PC(16:0/16:0), and αGPC as well as GW501516 were tested at different concentrations for their efficacy to replace the pan-PPAR ligand. The dose response curves were fitted to a three-parameter regression model according to equation 3.1 in order to calculated half maximal effective concentration (EC_{50}) of effective compounds.

$$y = \frac{\text{top-bottom}}{1+10^{\log(EC_{50}-x)\cdot \text{Hill slope}}} \tag{3.1}$$

This assay demonstrated that LPC(16:0), LPC(18:1), and PC(O-16:0/O-1:0) were equally potent in displacing the pan-PPAR ligand from the LBD of PPARδ (figure 3.10A on the next page). Their potency at 10 µM was moderate compared to 100 nM GW501516 (figure 3.10B). PC(16:0/16:0) and αGPC were not able to replace the ligand. EC_{50} values could only be estimated for LPC(16:0), LPC(18:1), and PC(O-16:0/O-1:0) (figure 3.10C - E), because the high concentration range was not

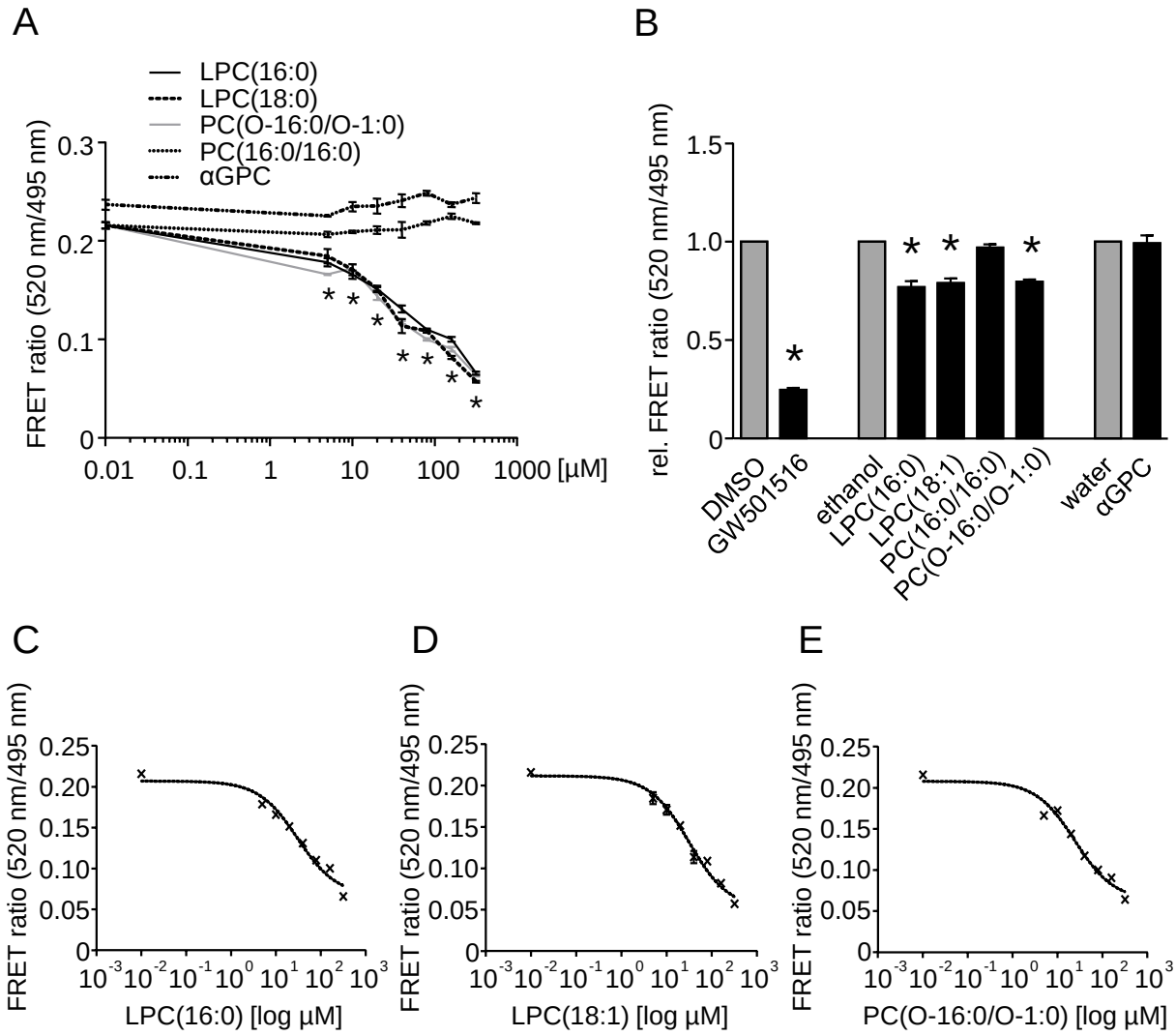


Figure 3.10: TF-FRET PPARδ competitive binding assay with different compounds. TR-FRET PPARδ competitive binding assay was performed with LPC(16:0), LPC(18:1), αGPC, PC(16:0/16:0), PC(O-16:0/O-1:0), GW501516, and the corresponding solvents. (**A**) The reduction of the fluorescence ratio of 520 nm/495 nm indicates replacement of the pan-PPAR ligand form the PPARδ LBD by the compounds at the indicated concentrations. Data are mean ±SEM. * $p < 0.05$ vs. solvent (n=3 technical replicates). Reprinted from (Klingler et al., 2016), with permission from Elsevier. (**B**) The values of 100 nm GW501516 and 10 µM LPC(16:0), LPC(18:1), PC(16:0/16:0), PC(O-16:0/O-1:0) are displayed as bar chart. Data are mean ±SEM. * $p < 0.05$ vs. corresponding solvent (n=3 technical replicates). (**C-E**) Dose response curves were created by plotting logarithmized concentrations against the FRET ratio of the single compounds (**C**) LPC(16:0), (**D**) LPC(18:1), (**E**) PC(O-16:0/O-1:0). The curves were fitted to equation 3.1 on the previous page. Fitting parameters are displayed in table 3.1 on the following page.

evaluated. The results (table 3.1) were similar for all three compounds under the given error means and 95 % confidential interval (CI) and amounted for approximately 30, 31 and 24 µM for LPC(16:0), LPC(18:1), and PC(O-16:0/O-1:0), respectively. The EC_{50} of the three compounds was in a similar range as the endogenous PPAR ligands palmitate and oleate (table 3.2).

Table 3.1: Fitting parameters of the dose response curves fitted to a three-parameter logistic model according to equation 3.1 on page 82 using GraphPad PRISM software. Abbreviations: CI (confidential interval), AU (arbitrary units).

parameter	LPC(16:0)	LPC(18:1)	PC(O-16:0/O-1:0)
bottom±SEM [AU]	0,067±0,006	0,051±0,006	0,063±0,006
top±SEM [AU]	0,207±0,005	0,212±0,005	0,208±0,005
EC_{50}±SEM [µM]	30,54±1,21	31,45±1,18	24,31±1,19
Hill slope±SEM [μM^{-1}]	0,140±0,007	0,160±0,007	0,145±0,006

95 % CI	LPC(16:0)	LPC(18:1)	PC(O-16:0/O-1:0)
bottom [AU]	0.054 to 0.08	0.038 to 0.064	0.051 to 0.075
top [AU]	0.197 to 0.217	0.201 to 0.222	0.198 to 0.219
EC_{50} [µM]	20.54 to 45.41	22.24 to 44.46	16.87 to 35.02
Hill slope [μM^{-1}]	0.126 to 0.154	0.147 to 0.174	0.132 to 0.158
R^2	0,957	0,967	0,961

Table 3.2: EC_{50} of selected synthetic and endogenous PPAR ligands. Abbreviations: n.d. (not detected)

ligand	EC_{50} [nM]			reference
	PPARδ	PPARα	PPARɣ	
GW501516	1	1100	850	Sznaidman et al., 2003
WY14643	>100,000	630	32,000	Willson et al., 2000
MBX-8025	2	too high	too high	Bays et al., 2011
GFT505	100-150	10-20	n.d.	Cariou et al., 2011
GSK0660	155	>10,000	>10,000	Shearer et al., 2008
GSK3787	6.6	too high	too high	Shearer et al., 2010
PC(O-16:0/O-1:0)	≈24,000	n.d.	n.d.	
LPC(16:0)	≈30,000	n.d.	n.d.	
palmitate	7,400	1,500	>30,000	Xu et al., 1999
LPC(18:1)	≈31,000	n.d.	n.d.	
oleate	5,300	600	4,100	Xu et al., 1999

3.4 LPCs protect against palmitate-mediated lipotoxicity

The physiological consequences of LPC treatment on skeletal muscle were studied. PPARδ has been shown to have effects on many metabolic pathways amongst them the enhancement of FA oxidation. LPCs as activators of PPARδ might also improve FA oxidation of HMT. Therefore, oxidation of ^{3}H-labeled palmitate was quantified by scintillation counting of tritiated water as an end product of FA oxidation. Tritiated water was isolated by column solid phase extraction of the cell culture supernatants.

The overall effects of LPC and GW501516 treatment for 24 h on palmitate oxidation were moderate. LPC(16:0) at 5 µM increased palmitate oxidation 1.24-fold. 10 µM LPC(16:0) were not effective. LPC(18:1) also had no detectable impact on palmitate oxidation in HMT. GW501516 significantly increased palmitate oxidation with a fold change of 1.44 vs. control (figure 3.11).

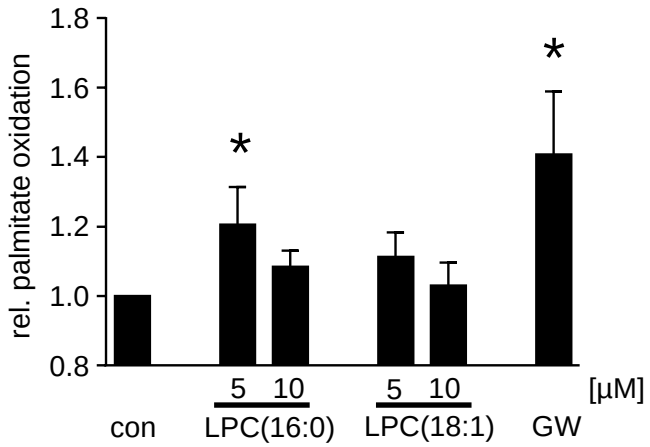


Figure 3.11: Effect of LPCs on palmitate oxidation. HMT were stimulated with 1 µM GW501516, 5 or 10 µM LPC(16:0), or 5 or 10 µM LPC(18:1) for 24 h. Subsequently, the cells were treated with 0.5 µCi/mL (33 µM total palmitic acid) [^{3}H] labeled palmitate for 4 h. The supernatant was applied to solid phase extraction columns to isolate tritiated water for scintillation counting as measurement for enhanced palmitate oxidation. Data are shown as mean ±SEM related to solvent. * $p < 0.05$ vs. solvent (n=7 - 8).

It was also tested if LPCs might affect glucose uptake as it was reported the literature. 20 µM LPCs for 3 h in L6 myotubes (Han et al., 2011) are supposed to decrease glucose uptake while enhanced glucose uptake was demonstrated with 1 µM

LPC(16:0) for 10 min in 3T3-L1 adipocytes (Yea et al., 2009). HMT are not suitable to study insulin-dependent glucose uptake since they have low SLC2A4 levels (Sarabia et al., 1992; Ciaraldi et al., 2005; Stuart et al., 2006). Therefore, $L6_{GLUT4myc}$ myotubes (rat skeletal muscle) were used which had a mean increase in glucose uptake of 2.2-fold upon 100 nM human insulin stimulation for 20 min (figure 3.12). $L6_{GLUT4myc}$ myotubes work are stably transfected with cDNA of rat homologue of SLC2A4 tagged with a myc epitope. The cells were treated with 10 µM LPCs for 24 h followed by serum starvation. The starved cells were treated with or without 100 nM human insulin for 20 min followed by administration of ^{3}H-labeled 2DOG as tracer for glucose uptake. ^{3}H-2DOG uptake into the cells was quantified as radioactivity of the cell lysates.

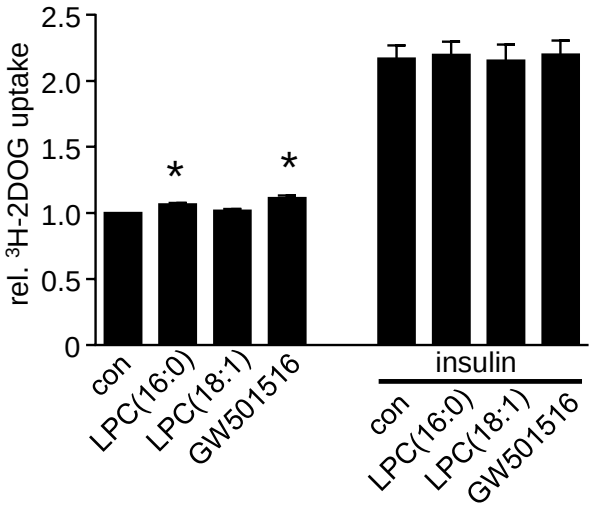


Figure 3.12: Effect of LPCs on glucose uptake of L6 skeletal muscle cells. $L6_{GLUT4myc}$ myotubes were stimulated with 10 µM LPC(16:0), 10 µM LPC(18:1), 1 µM GW501516, or solvent for 24 h. The cells were serum starved for 3 h and exposed to 0.25 µCi/mL [^{3}H]-labeled 2DOG (11.28 µM total 2DOG) for 7 min. The cells were washed and lysed. Lysate were analyzed for ^{3}H incorporation via scintillation counting as measurement for 2DOG uptake. Data are shown as mean ±SEM related to solvent without insulin as control (set as 1). * $p < 0.05$ vs. solvent as control (n=4).

LPC(16:0) and GW501516 but not LPC(18:1) marginally increased basal 2DOG uptake in $L6_{GLUT4myc}$ myotubes (figure 3.12). No significant effects of the compounds were observed when 100 nM insulin was present.

AMPK is a serine/threonine kinase and an important regulator of cell metabolism. AMPK activates glucose uptake and FA oxidation (Hardie et al., 2012). It was therefore examined if LPCs could trigger the activation of AMPK by phosphorylation at thr172

and the phosphorylation of its downstream target acetyl CoA carboxylase (ACC, official symbol: ACAC) at ser79. HMT were stimulated with LPC(16:0) and LPC(18:1) for different time points.

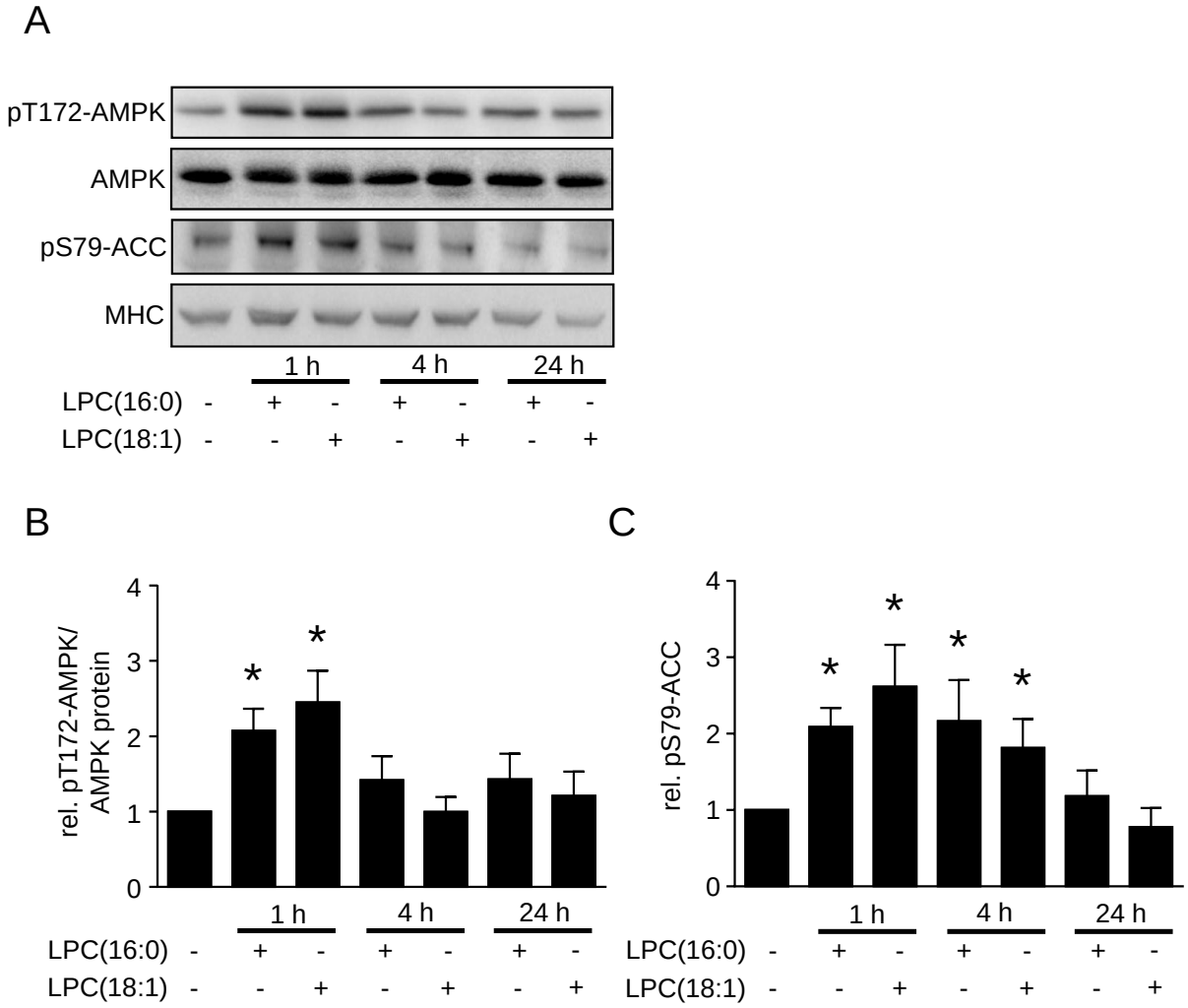


Figure 3.13: Effect of LPCs on the phosphorylation of AMPK. HMT were stimulated with 10 µM LPC(16:0), 10 µM LPC(18:1), or solvent for 10 min, 4 h, or 24 h. Afterwards, cells were lysed. The lysates were subjected to Western blotting and probed with anti-pAMPK thr172 and anti-pACC ser79 antibodies. Before re-probing with antibodies against anti-AMPK α1 and anti-MYH, the membrane were stripped off any antibodies. Representative blots are depicted (**A**). ECL signal of the secondary antibodies were detected, digitalized and quantified densitometrically. (**B**) The relative ratio of pAMPK thr172/AMPK protein and (**C**) values of pACC ser79 are given with MYH as loading control. Data are shown as mean ±SEM related to solvent as control (set as 1). * $p < 0.05$ vs. solvent as control (n=8). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

The data (figure 3.13) showed that LPC stimulation for 10 min caused the phosphorylation of thr172 on AMPK. On later time points the phosphorylation declined to

base line levels. The kinetics of the phosphorylation of ACC were similar compared to AMPK but persisted for 4 h. Both LPCs had comparable effects on the phosphorylation of both proteins.

As summarized in table 1.7 on page 26, LPCs negatively correlate with inflammatory markers like CRP. In addition, it is also known that PPARδ activation has anti-inflammatory effects (Wahli and Michalik, 2012; Vazquez-Carrera, 2016). Therefore, experiments were conducted to examine a potential anti-inflammatory role of LPCs during lipotoxicity. Lipotoxicity in HMT was provoked by stimulation with the saturated FA palmitate for 24 h. Lipotoxic results in the induction of cytokines like *IL6* and *CXCL3* (Peter et al., 2009) as well as the manifestation of ER stress with detectable splicing of *XBP1* mRNA (Yoshida et al., 2001; Calfon et al., 2002; Calligaris et al., 2009) and the induction of *ATF3* (Jiang et al., 2004). A potential protective role of LPCs was examined by LPC pre-incubation for 24 h prior to palmitate treatment of the cells.

With 250 µM palmitate, the cells had manifested lipotoxicity apparent by the induction of *IL6*, *ATF3*, and *CXCL3* (figure 3.14A - C on page 89) together with enhanced *XBP1* mRNA splicing (figure 3.14D,E). The induction of the transcripts and the *XBP1* splicing by palmitate were reduced by more than 50 % when the cells were pre-treated with LPCs or GW501516. LPCs and GW501516 had comparable efficacy in the protection against the lipotoxic effects caused by palmitate.

Palmitate treatment can also compromise insulin signaling as another part of lipotoxicicity (Belfort et al., 2005; Aas et al., 2005). Furthermore, LPCs negatively correlate with parameters of insulin resistance (table 1.7 on page 26). It was examined if LPCs could act in a protective manner on the palmitate-mediated impairment of insulin signaling. HMT were stimulated with LPC(16:0), LPC(18:1), or solvent for 24 h followed by palmitate stimulation for 24 h. To assess insulin-mediated AKT phosphorylation at ser473 and thr308 as markers for insulin sensitivity, the cells were serum starved for 3 h followed by stimulation with 100 nM human insulin for 10 min.

The results showed that the insulin-dependent phosphorylation of both sites on AKT was significantly abolished in the presence of palmitate (figure 3.15 on page 90). LPC(16:0) but not LPC(18:0) blocked the reduction of the phosphorylation of ser473

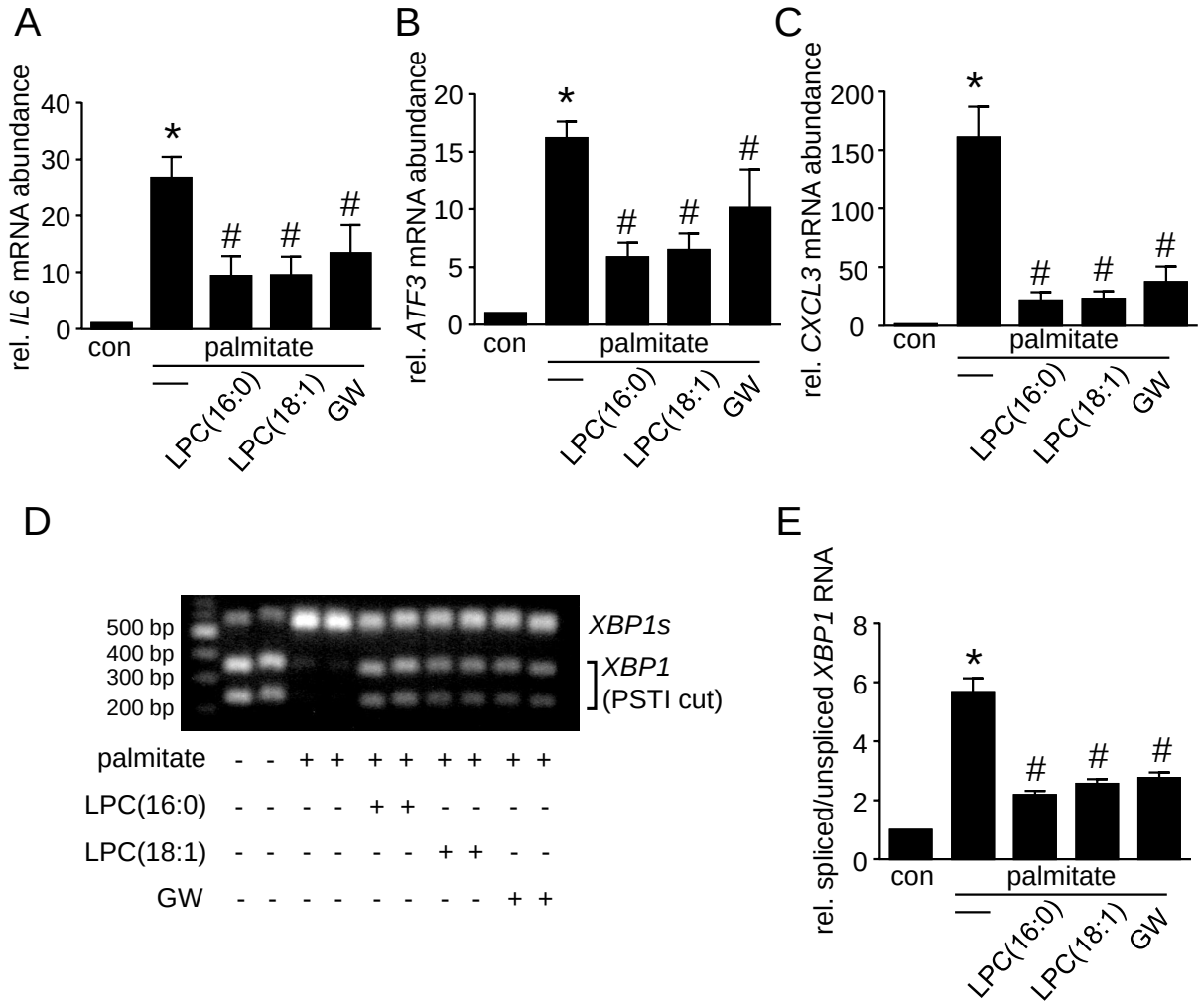


Figure 3.14: Protective effects of LPC on lipotoxicity. HMT were pre-treated with 10 µM LPC(16:0), 10 µM LPC(18:1), 1 µM GW501516 (abbreviated as GW) or solvent for 24 h, followed by administration of 250 µM palmitate complexed to BSA or BSA as control for 24 h. Total RNA was isolated. mRNA levels of (**A**) *IL6*, (**B**) *ATF3*, and (**C**) *CXCL3* were quantified by qPCR. *XBP1* RNA (transcript NM_005080.3) was amplified by PCR from isolated total RNA, cut with the restriction enzyme PST1 and quantified densitometrically after agarose gel electrophoresis. (**D**) A representative agarose gel is shown. (**E**) The ratio of spliced and PSTI resistant *XBP1s* (PCR product expected at 448 bp) to unspliced and PSTI cut *XBP1* (PCR products expected at 285 + 189 bp) was calculated as indicator of ER stress. Data are mean ±SEM. * $p < 0.05$ vs. BSA + solvent, # $p < 0.05$ vs. palmitate + solvent (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

by palmitate. The phosphorlyation of thr308 was only protected by LPC(18:1) and not by LPC(16:0).

To test if the protective effect of LPCs on lipotoxicity potentially was PPARδ-dependent, GSK0660 was pre-incubated for 2 h prior to LPC/GW501516 treatment

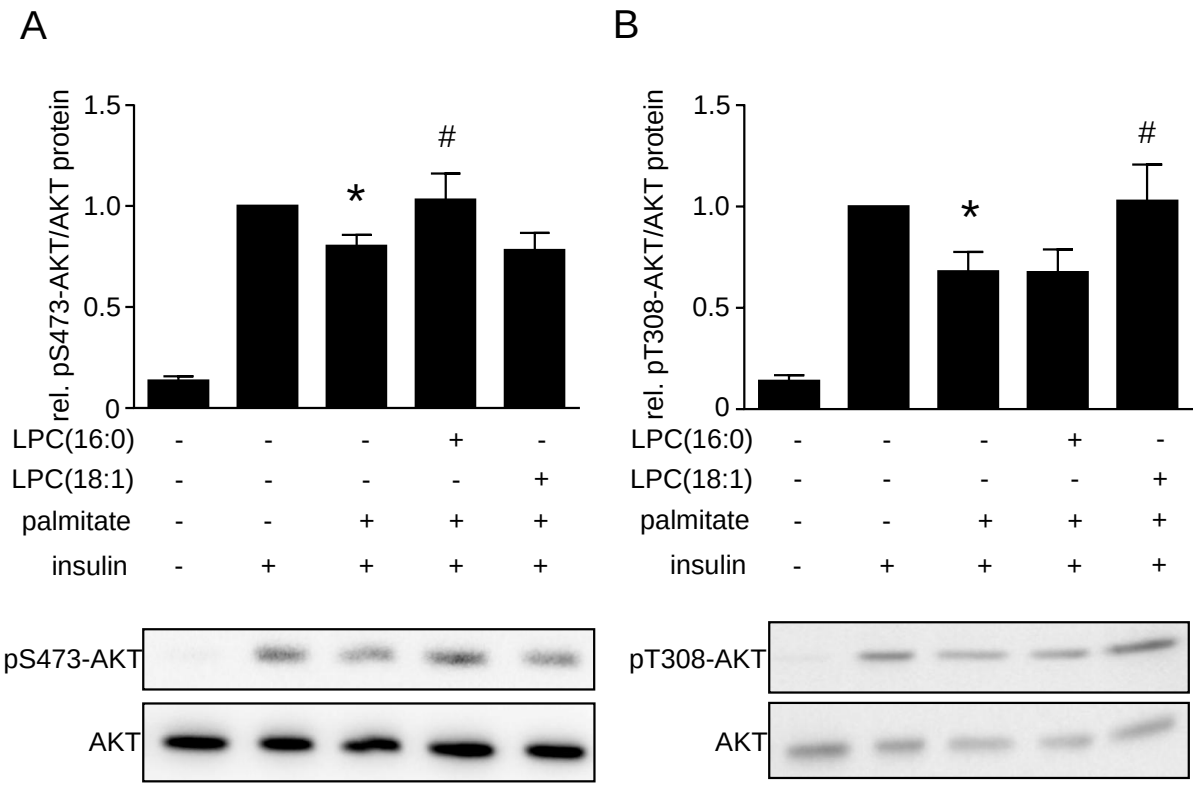


Figure 3.15: Effect of LPCs on insulin resistance in HMT. HMT were stimulated with 10 µM LPC(16:0), 10 µM LPC(18:0), or solvent for 24 h followed treatment with 250 µM palmitate or BSA as control for further 24 h. The cells were serum-starved for 3 h and stimulated with 100 nM human insulin for 10 min. Afterwards, the cells were lysed. The lysates were subjected to Western blotting and probed with (**A**) anti-pAKT ser473 and (**B**) anti-pAKT thr308 antibodies. Before re-probing with antibodies against anti-AKT protein, the membranes were stripped off any antibodies. ECL signal of the secondary antibodies was detected via chemiluminescence imaging system and quantified densitometrically. Data are shown as mean ±SEM related to solvent + palmitate + insulin treatment. * $p < 0.05$ vs. solvent + BSA + insulin stimulation, # $p < 0.05$ vs. solvent + palmitate + insulin stimulation (n=8).

for 24 h and the addition of palmitate/BSA for further 24 h. The mRNA levels of *IL6*, *ATF3*, and *CXCL3* were used as read-out.

The pre-incubation with the PPARδ reverse agonist GSK0660 increased the mRNA abundance of *IL6*, *ATF3*, and *CXCL3* in the presence of palmitate (figure 3.16 on the following page). GSK0660 treatment prevented the protective effect of both LPCs visible in all three marker transcripts in comparable manner except for LPC(18:1) on *ATF3* mRNA levels.

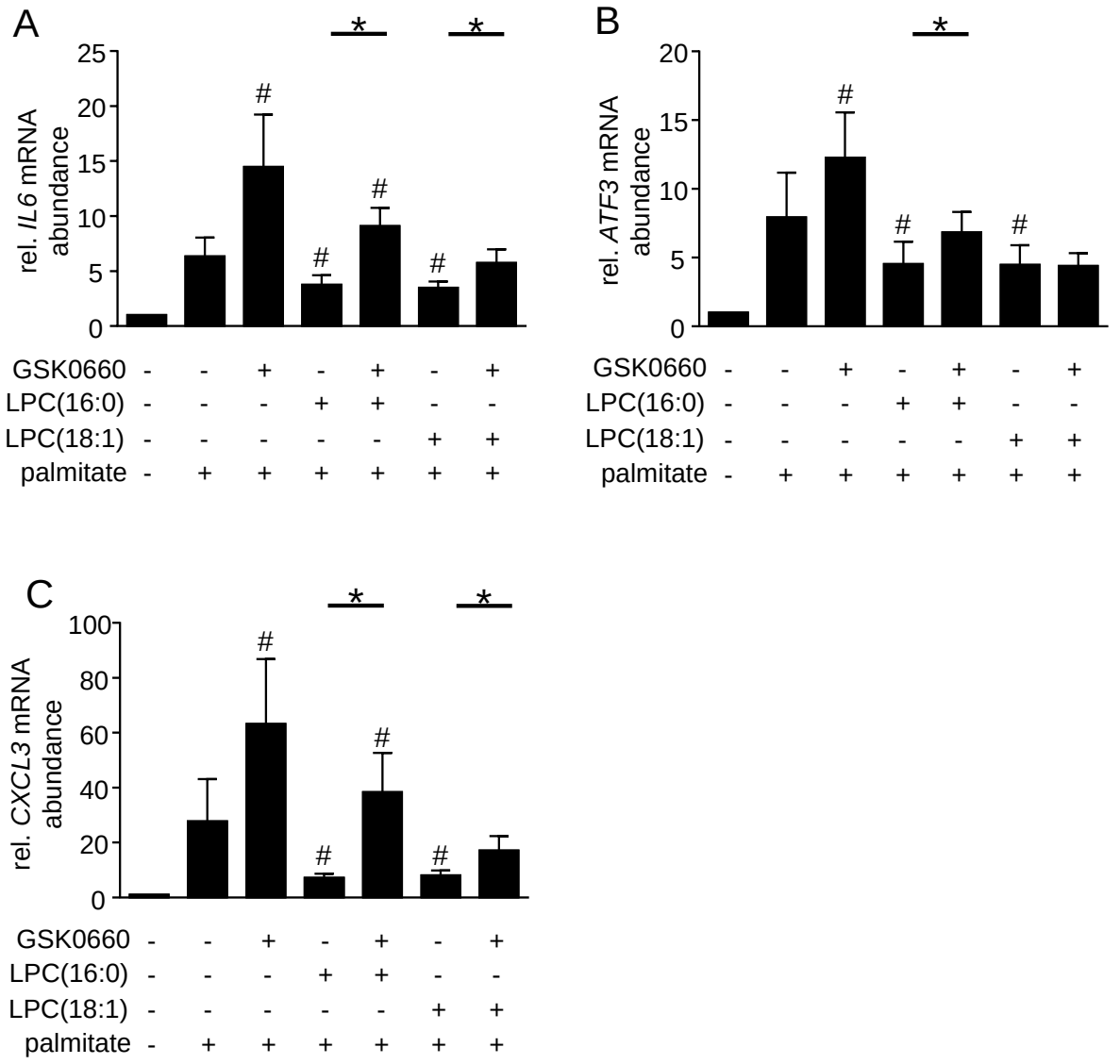


Figure 3.16: Inhibition of the protective effect of LPCs by GSK0660. HMT were stimulated with 10 µM GSK0660 or DMSO for 2 h followed by addition of 10 µM LPC(16:0), 10 µM LPC(18:1), or solvent. After 24 h, 250 µM palmitate complexed to BSA or BSA alone was added for further 24 h. Total RNA was isolated. mRNA levels of (**A**) *IL6*, (**B**) *ATF3*, and (**C**) *CXCL3* were quantified by qPCR. Data are shown as mean ±SEM related to BSA + solvent treatment without LPCs or GSK0660 (set as 1). * < 0.05, # p < 0.05 vs. palmitate treatment w/o LPCs and GSK0660 (n=7). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

3.5 Effects of interferences in the LPC metabolism on palmitate-mediated lipotoxicity

LPCs protected against lipotoxicity caused by palmitate. It was expected that also a reduced turn-over of LPCs to PCs might have similar effects as LPC administration on palmitate-mediated lipotoxicity. Therefore, LPCATs were chosen according to their mRNA levels determined by microarray analysis (data not shown), literature data about their tissue expression profile (table 1.3 on page 16), and their relevance in inflammatory reactions (Shindou et al., 2007) and other metabolic aspects (Zhao et al., 2008). LPCAT1+2+3 were silenced by siRNA.

The efficiency of the silencing was higher than 50 % for all three transcripts (figure 3.17 on the following page). Silencing of *LPCAT1* and *LPCAT2* and the combination of all three had comparable effects and moderately reduced *IL6* induction caused by palmitate treatment. Silencing of *LPCAT3* had no effects on *IL6* levels during palmitate challenge. However, this effect was not observed for *ATF3* and *CXCL3*. None of the siRNAs could affect the palmitate caused induction of these two stress markers.

It was also examined if a reduced formation of LPCs by inhibition of PLA_2s could intensify palmitate-mediated lipotoxicity. In this approach different PLA_2 inhibitors were administrated. Bromoenol lactone (BEL aka haloenol lactone suicide substrate) is a commonly used suicide inhibitor of $iPLA_2$ (Dennis et al., 2011; Jenkins et al., 2002; Balsinde et al., 2006; Hazen et al., 1991; Song et al., 2006; Han et al., 2011). BEL is a chiral compound. The *S* enantiomer inhibits preferentially PLA2G6A, while the *R* enantiomer blocks PLA2G6B (Jenkins et al., 2002). FKGK11 belongs to the class of polyfluoro ketones and inhibits $iPLA_2$ in general with high specificity (Baskakis et al., 2008). The inhibitors were co-stimulated with palmitate.

The enantiomers of BEL had no effect on the palmitate-mediated induction of *IL6*, *ATF3*, and *CXCL3* (figure 3.18 on page 94). As opposed to this, FKGK11 treatment dose-dependently fortified the effects on *IL6*, *ATF3*, and *CXCL3* mRNA abundance caused by palmitate. To further evaluate the results with the inhibitors BEL

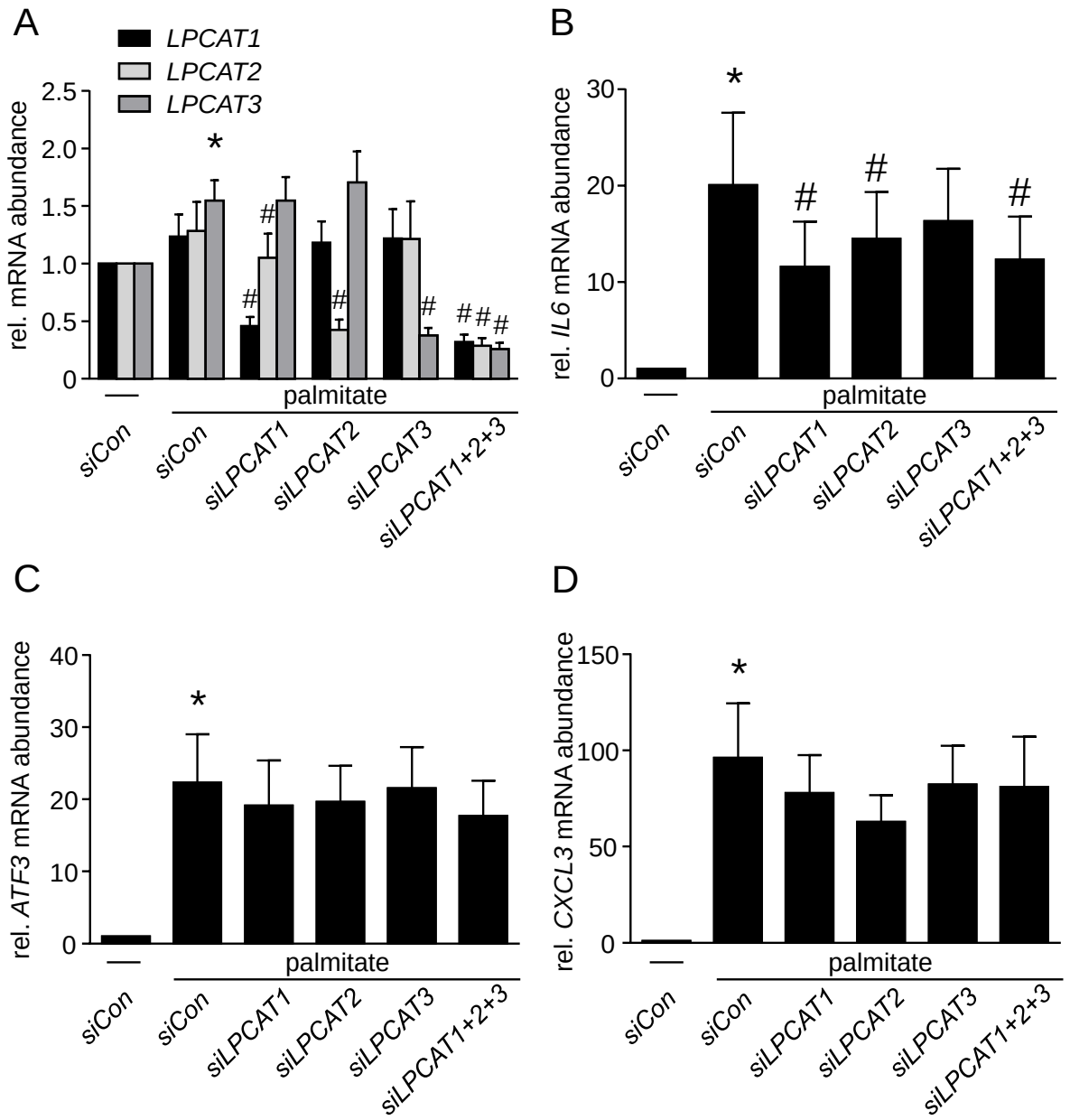


Figure 3.17: Effect of *LPCAT1/2/3* knockdown on palmitate-mediated lipotoxicity. HMT were transfected with siRNA targeted against *LPCAT1*, *LPCAT2*, *LPCAT3* and the combination of all for 24 h. 250 µM palmitate complexed to BSA or BSA alone as control was administrated for further 24 h. Total RNA was isolated. mRNA levels of (**A**) *LPCAT1* (black bars), *LPCAT2* (light gray bars), *LPCAT3* (dark gray bars), (**B**) *IL6*, (**C**) *ATF3*, and (**D**) *CXCL3* were quantified by qPCR. Data are shown as mean ±SEM related to siCon transfected cells with BSA treatment (set as 1). * < 0.05 vs. siCon transfected cells with BSA treatment, # p < 0.05 vs. siCon transfected cells with palmitate treatment (n=6). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

and FKGK11 and to exclude side effects of the inhibitors, siRNA targeted against *PLA2G6A* and *PLA2G6B* were transfected 24 h before palmitate treatment.

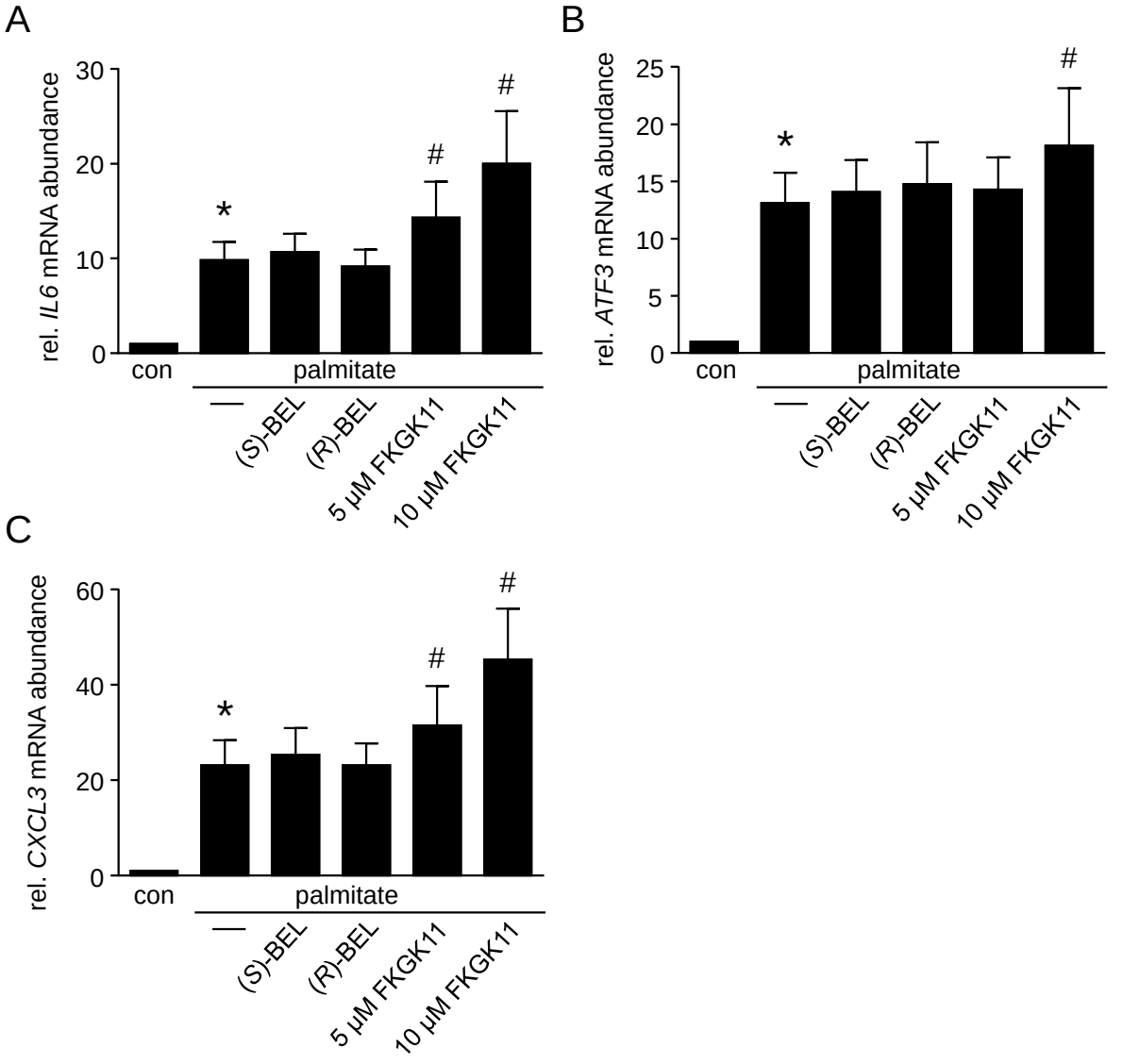


Figure 3.18: Effects of different PLA_2 inhibitors on palmitate-mediated *IL6* induction. HMT were co-stimulated with either 10 µM *(S)*-BEL, 10 µM *(R)*-BEL, 5 µM or 10 µM FKGK11 together with 250 µM palmitate complexed to BSA or BSA alone as control for 24 h. Total RNA was isolated and mRNA levels of (**A**) *IL6*, (**B**) *ATF3*, and (**C**) *CXCL3* were quantified by qPCR. Data are shown as mean ±SEM related to cells treated with solvent (set as 1). * < 0.05 vs. BSA treatment, # < 0.05 vs. palmitate without inhibitors (n=4). Reprinted from (Klingler et al., 2016), with permission from Elsevier.

Palmitate treatment significantly increased mRNA levels of *PLA2G6B* and reduced *PLA2G6A* (figure 3.19A on the following page). *siPLA2G6A* had comparable effects as palmitate treatment on *PLA2G6A* mRNA levels, although not statistically significant. In the presence of siRNA targeted against *PLA2G6B*, the increase of this par-

ticular PLA_2 caused by palmitate was reduced to control levels. On the other hand silencing of *PLA2G6B* increased *PLA2G6A* mRNA levels. The co-transfection of both *siPLA2G6A* and *siPLA2G6B* equalized both transcript levels to as comparable level as siCon treated cells without palmitate.

siPLA2G6A and *siPLA2G6B* but not co-transfection significantly fortified *IL6* induction caused by palmitate treatment (figure 3.19B). These effects were not observed for *ATF3* and *CXCL3* induction (figure 3.19C,D).

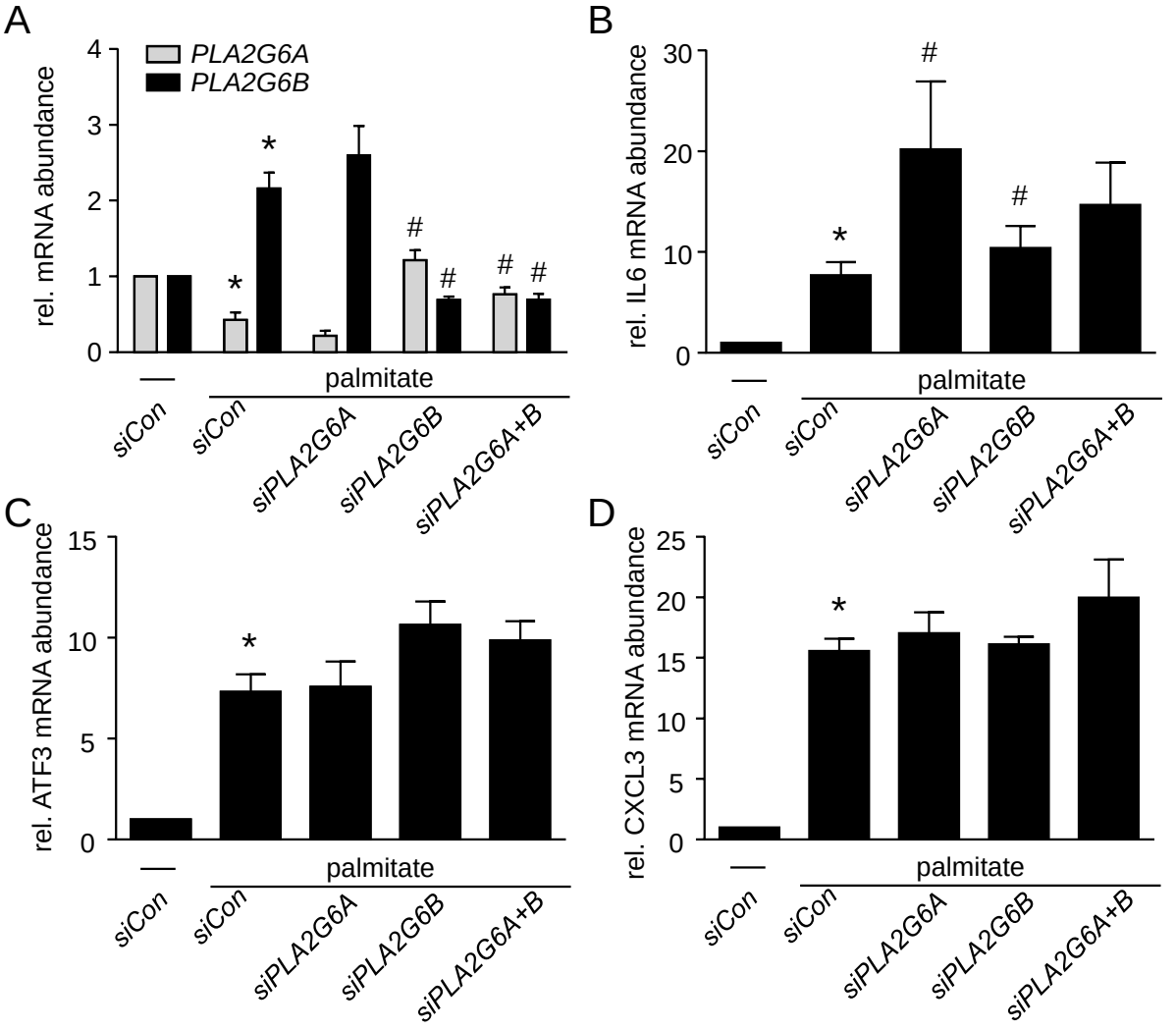


Figure 3.19: Effects of silencing of *PLA2G6A* and *PLA2G6B* on palmitate-mediated lipotoxicity. HMT were transfected with siRNA targeted against *PLA2G6A* and *PLA2G6B* for 24 h followed by administration of 250 µM palmitate complexed to BSA or BSA alone as control for 24 h. Total RNA was isolated. mRNA levels of (**A**) *PLA2G6A* (black bars), *PLA2G6B* (gray bars), (**B**) *IL6*, (**C**) *ATF3*, (**D**) *CXCL3* were quantified by qPCR. Data are shown as mean ±SEM related to siCon transfected cells with BSA treatment (set as 1). * < 0.05 vs. siCon transfected cells with BSA treatment, # p < 0.05 vs. siCon transfected cells with palmitate treatment (n=6).

3.6 Other signaling pathways might contribute to LPC effects and PPARδ activation

It is known that LPCs can transiently activate extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, official symbol: MAPK3 and MAPK1) and c-Jun N-terminal kinase 1 and 2 (JNK1 and JNK2, official symbol: MAPK8 and MAPK9) and inhibit protein kinase A (PKA, official symbol: PRKA) (Klingler, 2012; Han et al., 2011; Burkholder, 2009). It might be possible that these signaling events can contribute to an activation of PPARδ and its target genes via phosphorylation of the nuclear receptor in the absence of ligands (Burns and Vanden Heuvel, 2007). Forskolin activates the intracellular ADCY resulting in elevated cAMP levels. This triggers the activation of PKA. Forskolin was supplied to the cells for different time points to examine its effect on *PDK4* and *ANGPTL4* mRNA levels.

Forskolin stimulation led to an increase of *PDK4* mRNA abundance over time with first significant effects at 4 h (figure 3.20A). *ANGPTL4* mRNA levels declined starting at 4 h after stimulation (figure 3.20B).

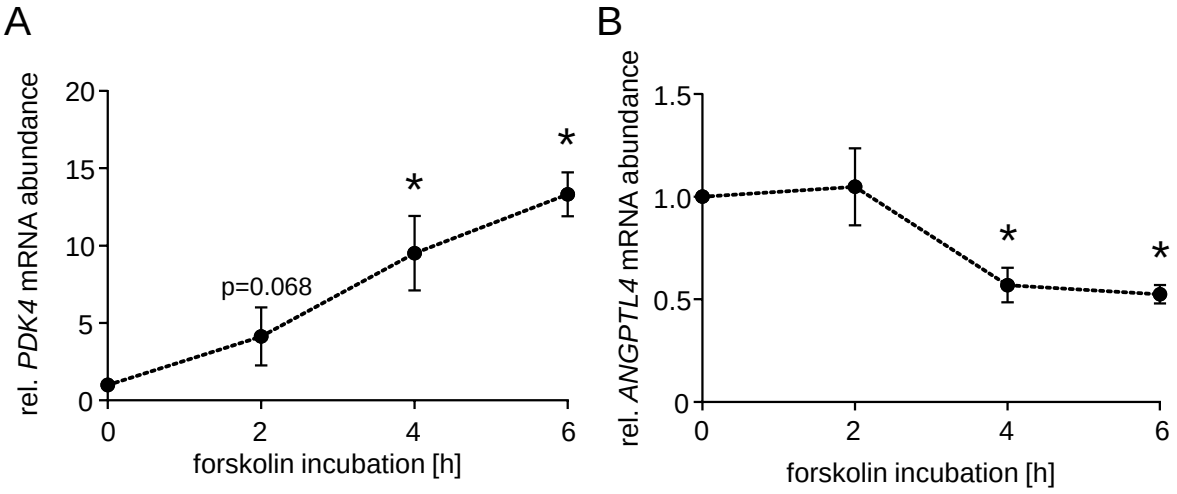


Figure 3.20: Effects of forskolin on *PDK4* and *ANGPTL4* RNA over time. HMT were stimulated with 20 µM forskolin for the indicated time periods. Total RNA was isolated. mRNA levels of (**A**) *PDK4* and (**B**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * $p < 0.05$ vs. solvent treatment (n=4).

It was examined if the PKA inhibitor H-89 could compensate for the effects of forskolin. H-89 and forskolin were administrated for 4 h. H-89 stimulation increased

PDK4 mRNA levels and reduced the effects of forskolin on *PDK4* mRNA abundance significantly. H-89 had no effect on *ANGPTL4* mRNA levels and also not in combination with forskolin (figure 3.21B).

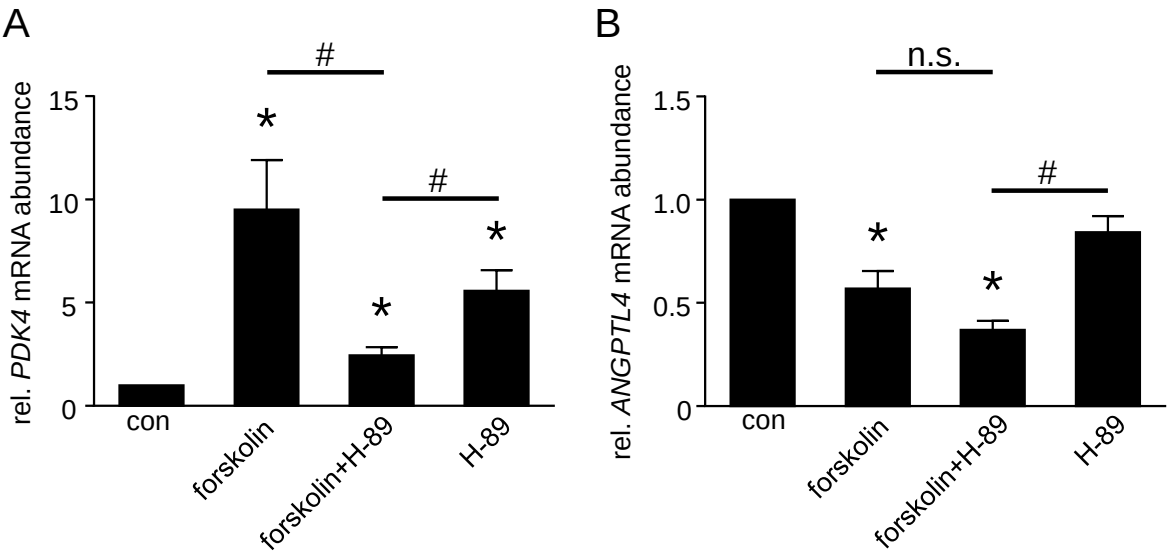


Figure 3.21: Effects of forskolin and H-89 treatment on *PDK4* and *ANGPTL4* mRNA. HMT were stimulated with 20 µM forskolin and/or 30 µM H-89 for 4 h. Total RNA was isolated. mRNA levels of (**A**) *PDK4* and (**B**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * $p < 0.05$ vs. solvent treatment, # $p < 0.05$ (n=4).

Putative LPC receptors are GPCRs that transduce their signal via various classes of G_α subunits including G_q (chapter 1.7.1 on page 27). G_q is able to increase intracellular Ca^{2+} levels. Apart from receptor-mediated alterations of intracellular Ca^{2+} levels, LPCs might also change membrane fluidity, ion channel activity, or influence the intracellular Ca^{2+} levels by other still unknown mechanisms (Jabr et al., 2000; Tanaka et al., 2011). It was investigated if alterations of intracellular Ca^{2+} levels could affect *PDK4* and *ANGPTL4* mRNA abundance. Ionomycin is an ionophore that increases cytosolic Ca^{2+} levels (Dedkova et al., 2000). BAPTA-AM is a strong chelator of Ca^{2+} ions that can cross the plasma membrane and inactivate cytosolic Ca^{2+} by complexation (Naraghi and Neher, 1997). Ionomycin had no effects on neither *PDK4* or *ANGPTL4* mRNA levels. BAPTA-AM increased *PDK4* mRNA levels with borderline significance (figure 3.22A on the next page). Ionomycin in combination with BAPTA-AM increased

PDK4 mRNA levels to a similar extend as BAPTA-AM alone. BAPTA-AM and in combination with ionomycin decreased *ANGPTL4* mRNA levels (figure 3.22B).

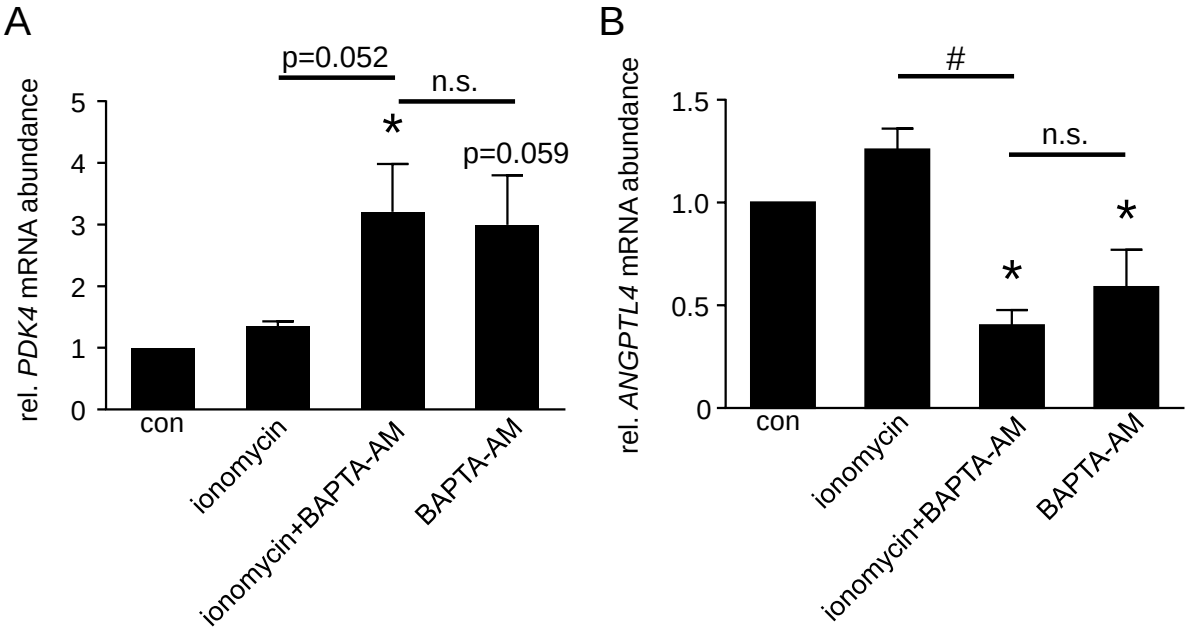


Figure 3.22: Effects of ionomycin and BAPTA-AM treatment on *PDK4* and *ANGPTL4* mRNA. HMT were stimulated with 1 µM ionomycin and/or 10 µM BAPTA-AM for 4 h. Total RNA was isolated. mRNA levels of (**A**) *PDK4* and (**B**) *ANGPTL4* were quantified by qPCR. Data are shown as mean ±SEM related to solvent treatment (set as 1). * $p < 0.05$ vs. solvent treatment, # $p < 0.05$ (n=4).

3.7 Ether LPC analogue PC(O-16:0/O-1:0) is another PPARδ ligand

In figure 3.9 on page 81 and figure 3.10 on page 83 it was shown that PC(O-16:0/O-1:0) is able to activate PPARδ and its target transcripts. PC(O-16:0/O-1:0) structurally resembles LPCs but is not metabolized in the cell. The effects of this compound might give a hint about the relevance of the chemical structure of LPCs. EMSAs were performed to confirm that PC(O-16:0/O-1:0) also can act as a PPARδ activator.

The results of the EMSAs demonstrated that PC(O-16:0/O-1:0) enhanced the DNA binding activity of the PPARδ/RXRα complex to its consensus binding se-

quence (figure 3.23). The DNA binding activity increased moderately with increasing PC(O-16:0/O-1:0) concentrations.

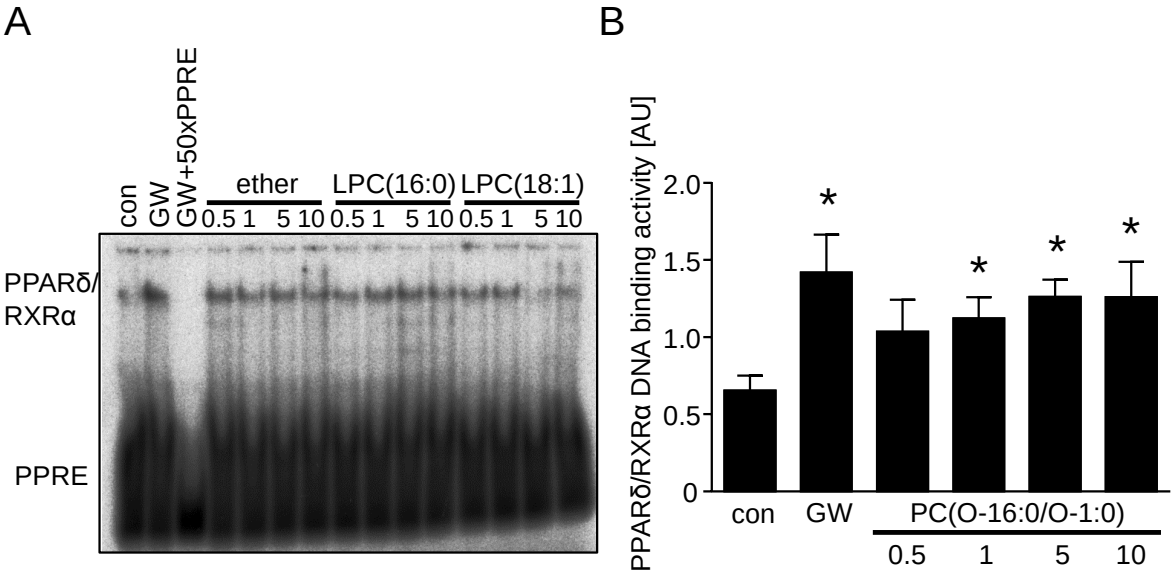


Figure 3.23: PC(O-16:0/O-1:0) increased the DNA binding activity of the PPARδ/RXRα complex. EMSA was performed with recombinant human PPARδ, RXRα and an oligo DNA sequence containing the consensus binding site for PPARδ named PPRE. The PPRE was radioactively end-labeled with [^{32}P]dATP. For the binding reaction, 1.875 ng/µL (37.5 ng) of each recombinant protein together with approx. 20,000 cpm radioactively end-labeled PPRE were used in the presence of 5 µM GW501516 or increasing concentrations of PC(O-16:0/O-1:0) (”ether”), LPC(16:0), and LPC(18:1). The radioactivity was visualized via phospho-imaging. (**A**) One representative gel is depicted. (**B**) Densitometrically evaluation was performed. Data are mean of values related to total radioactivity per replicate ±SEM. * $p < 0.05$ vs. solvent (n=4-6).

4 Discussion

4.1 *PDK4* and *ANGPTL4* induction by LPCs is PPARδ mediated

Previous data from my diploma work showed that LPC(16:0) and LPC(18:1) can cause the induction of *PDK4* and *ANGPTL4* mRNA in HMT (figure 1.9 on page 36). This was confirmed in this work by microarray experiments (figure 3.1 on page 73), where both LPCs increased the abundance of *PDK4* and *ANGPTL4*. *ANGPTL4* and *PDK4* are PPAR target genes (Staiger et al., 2009; Adhikary et al., 2011; Degenhardt et al., 2007). PPARs belong to the nuclear receptors superfamily (Mangelsdorf et al., 1995). The PPAR receptor family consists of three members: PPARα (official symbol: PPARA), PPARδ/β (official symbol: PPARD), and PPARɣ (official symbol: PPARG). PPARα is expressed in liver, kidney, heart, skeletal muscle, and adipose tissue (Issemann and Green, 1990; Loviscach et al., 2000; Su et al., 1998; Tontonoz et al., 1994; Escher et al., 2001). PPARδ is ubiquitously expressed except in testis and only low expression levels are found in liver (Braissant et al., 1996; Kliewer et al., 1994; Girroir et al., 2008; Escher et al., 2001). PPARɣ is predominantly expressed in adipose tissue (Tontonoz et al., 1994; Chawla et al., 1994; Vidal-Puig et al., 1997; Escher et al., 2001). In skeletal muscle all three members are expressed (Loviscach et al., 2000; Su et al., 1998). In HMT, the mRNA transcripts of all three PPARs were detected in microarray experiments (data not shown). Transcripts of *PPARD* and *PPARA* mRNAs were also quantified via qPCR and showed a comparable abundance (data not shown).

It is shown in HMT (Staiger et al., 2009) and by different *in vitro* approaches (Degenhardt et al., 2007), that *ANGPTL4* and *PDK4* are activated by PPARδ (Staiger et al., 2009; Adhikary et al., 2011; Degenhardt et al., 2007). In previous experiments of my diploma thesis, a participation of PPARδ in the LPC(16:0)-mediated induction of *Angptl4* was indicated by RNA interference experiments in mouse C2C12 myotubes (Klingler, 2012). Several findings in this thesis support that LPCs activate PPARδ. Beside *PDK4* and *ANGPTL4*, also other PPARδ target genes were found to be induced by both LPCs, including *CPT1A* and *PLIN2* (figure 3.1 on page 73) (Adhikary et al., 2011). Furthermore, the effects of LPCs on *PDK4*/*ANGPTL4* mRNA levels were mimicked by PPARδ agonist GW501516 but not by PPARα agonist WY14643 (figure 3.4 on page 75). *PDK4* and *ANGPTL4* induction caused by LPCs was blocked in the presence of the PPARδ antagonists GSK0660 and GSK3787 (figure 3.5 on page 76). siRNA targeted against *PPARD* reduced LPC-mediated *PDK4* induction (figure 3.6 on page 78). Activation of the LBD of PPARδ by LPCs was observed in luciferase reporter assays (figure 3.7 on page 79).

PPARs are nuclear receptors and are activated by ligand binding. But also ligand-independent modes of action that either lead to the induction or repression of PPAR target transcripts are known (Feige et al., 2006). siRNA experiments of this work pointed to the complexity of the impact of PPARδ on its target genes in the absence of PPARδ ligands. The presence of siRNA targeted against *PPARD* increased the mRNA levels of *ANGPTL4* and *PDK4* compared to the siCon transfected cells when no PPARδ ligands were administrated (figure 3.6 on page 78). This observation is also reported in the literature (Adhikary et al., 2011). It is postulated that PPARδ can act as repressor on some of its target genes in the absence of ligands (Santos et al., 2011). This repressive effect is apparently reduced in the presence of *siPPARD*.

Upon PPARδ antagonist treatment, mRNA levels of *ANGPTL4* were reduced in the absence of PPARδ activators (figure 3.5 on page 76). This is in contrast to the siRNA experiments and might be explained by the fact that antagonists *per se* do not necessarily lead to reduced PPARδ levels in contrast to *siPPARD*. In addition, ligand binding can increase the biological half-life of PPARs (Wadosky and Willis, 2012;

Genini and Catapano, 2007; Rieck et al., 2007). Therefore, silencing and antagonism of PPARδ had opposing effects on *ANGPTL4* mRNA levels. The discrepancy in the response of *PDK4* and *ANGPTL4* mRNA levels might be attributed to different amounts of PPREs located at their promoter regions. *ANGPTL4* has three PPREs while *PDK4* only has two PPREs in the regulatory region of their gene (Adhikary et al., 2011; Degenhardt et al., 2007).

The loss of the repressive effect of PPARδ by siPPARD in the absence of ligands might be the reason why the LPC-mediated induction of *ANGPTL4* is not significantly affected by *siPPARD* (figure 3.6 on page 78). Here, a mixed effect of a *siPPARD* but ligand-independent induction and a *siPPARD*-dependent reduction of ligand effects is detected.

4.2 LPCs are PPARδ ligands

The ligand-dependent activation of PPARs is the most prominent mechanism how the nuclear receptors act on their target genes. This multi-step process is still not fully understood. The heterodimerization with its co-receptor RXR is a requisite for this action of PPARs (Feige et al., 2005). According to current mechanistical models for the activation of PPARs by ligands, PPARs are moving along the DNA as heterodimers searching for their recognition sequence (Feige et al., 2006). Ligand binding of PPARδ enables the recruitment of a large protein complex consisting of co-regulators and transcriptional factors. This protein complex slows down the mobility of the PPAR/RXR complex while scanning the DNA and increases the affinity of that complex to its target sequence (Michalik et al., 2006; Carlberg, 2010; Feige et al., 2005; Dowell et al., 1997).

This work examined the effects of LPCs as putative PPARδ ligands. Concerning the ligand binding, PPAR members differ in size and shape of their binding pockets which affects their ligand spectrum. The endogenous ligands of PPARs are FA >C12 (Xu et al., 1999) and derivatives thereof including prostaglandins, eicosanoids, leukotrienes, and phospholipids as well as other lipids like retinoic acid (Ehrenborg

and Krook, 2009; Xu et al., 1999; Chakravarthy et al., 2009; Forman et al., 1997; Chakravarthy et al., 2009; McIntyre et al., 2003; Oishi-Tanaka and Glass, 2010; Shaw et al., 2003). Although some overlapping specificities are reported, it should be mentioned that not all ligands bind with similar affinity to each PPAR member, as demonstrated for FA (Forman et al., 1997; Xu et al., 1999) and other ligands. PC(16:0/18:1) binds specifically to PPARα (Chakravarthy et al., 2009) while retinoic acid is a ligand of PPARδ only (Shaw et al., 2003).

Because the ligand spectrum of PPARs is broad and includes different kinds of phospholipids, it was examined if LPCs are putative PPARδ ligands. TR-FRET based competition assay were used to verify if LPCs are ligands of PPARδ (figure 3.10 on page 83). Here the potency of LPCs to replace a pan-PPAR ligand from the LBD of PPARδ was examined. The efficacy of LPCs to compete for the LBD was moderate compared to GW501516, which is in accordance with cell-based studies of this work: 1 µM GW501516 increased *PDK4* mRNA levels 13-fold whereas 10 µM LPC increased *PDK4* mRNA levels 3-fold (figure 3.4 on page 75 and 3.9 on page 81). No competitive action was shown for PC(16:0/16:0) and αGPC which also failed to induce *PDK4* and *ANGPTL4* mRNA levels. From the competition assays, ED_{50} values of LPC(16:0) and LPC(18:1) were calculated. Both LPCs had similar values at approximately 30 µM. The ED_{50} values calculated here should be considered as rough approximation because for a precise calculation more TR-FRET values of higher LPC concentrations are needed (Sebaugh, 2011). Nevertheless, the values of LPC and the corresponding FA (ranging from 5 - 7 µM) (table 3.2 on page 84) are in an comparable range. This points to a possible relevance of LPCs as endogenous PPARδ ligands.

The functional activation of PPARδ by LPCs as ligands was assessed via EMSAs (figure 3.8 on page 80), which were adapted in a way that only the recombinant proteins PPARδ and RXRα as transcription factor components were used. This was necessary, because no significant specific binding to the PPRE using nuclear extracts could be detected (data not shown). In the presence of LPCs, the DNA binding activity of PPARδ/RXRα on the consensus PPRE was enhanced to a comparable extend as with GW501516. With higher concentrations of LPCs, the DNA binding activity was

increasingly abolished. The reason for that is not clear, but might be the result of an impairment of the protein structure integrity by the detergent-like action of LPCs. The lysolipid LPE exerts chaperone-like effects on denaturated citrate synthase and helps to refold the protein (Kern et al., 2001). Increasing concentrations of LPE increase the effectiveness of the refolding. In contrast to that, increasing LPC concentrations are decreasingly effective (Kern et al., 2001). Another possible explanation might be that LPCs interacted as cationic amphiphils with the PPRE DNA and compromised the interaction with the PPARδ/RXRα complex. Phospholipid formulations are commonly used as carriers for nucleic acid for their delivery into cells and therefore are capable of interacting with DNA (Paukku et al., 1997; MacDonald et al., 1999; Matulis et al., 2002; Farhood et al., 1995; Hui et al., 1996).

In an other approach to test the functional activation of PPARs, 100 µM LPC(16:0) and LPC(18:1) were able to significantly increase the recruitment of the C33 co-activator peptide in a TR-FRET based assay to the LBD of PPARδ with a fold change of 1.088±0.020 and 1.073±009 (±SEM, n=3 technical), respectively, compared to solvent control (data not shown). 100 nM GW501516 was strongest with a fold change of 1.455. To conclude, LPCs can activate the DNA binding activity of the PPARδ/RXRα complex as direct ligands with functional consequences on the activity of PPARδ.

4.3 Cellular uptake of LPCs

Since LPCs can act as PPAR ligands, their metabolization to other lipids is not necessary for the activation of PPARδ, but they must enter the cells. The interplay of internalization, transport, and kinetics of their metabolization was examined.

As already shown in the diploma thesis, the effects of LPCs on *ANGPTL4* mRNA abundance were apparent within less than 2 h of stimulation of HMT (figure 1.9 on page 36). This finding adds to the observation that a first significant increase of labeled LPC in HMT was apparent after 30 min after administration (figure 3.3A on page 74) which could be attributed to LPCs integrated to the membrane or internalized LPCs.

Notably, it was shown that radioactively labeled LPCs are taken up by aortic endothelial cells within a comparable short time frame (Stoll et al., 1992). This points to a quick internalization of LPCs that cannot be explained by a relative slow flip-flop rate as observed in other cell-systems with a half-time of several hours (Bergmann et al., 1984). According to that, a protein-assisted transport is more likely. The only known LPC transporter MFSD2A, has low expression levels in skeletal muscle (Guemez-Gamboa et al., 2015; Angers et al., 2008). However, robust mRNA levels are observed in HMT according to our microarray results. The expression levels of MFSD2A might be sufficient to facilitate the internalization of LPCs.

In summary, LPCs are internalized into the cell in a quick manner and most likely in a protein-assisted manner. Small changes of the LPC structure, like a difference in length of two carbon atoms or a double bond, did not have significant impact on the activation of PPARδ, hence, presumably also not on the kinetics of the internalization.

4.4 Effect of LPC metabolites on PPARδ

It was also evaluated if LPC metabolites can also cause the activation of PPARδ. This is an important consideration, since many of the metabolite of LPCs (figure 1.5 on page 8) are endogenous PPAR ligands, including FA (table 3.2 on page 84, figure 3.5A on page 76) (Forman et al., 1997; Xu et al., 1999), PC(16:0/18:1) (Chakravarthy et al., 2009), LPA (McIntyre et al., 2003; Zhang et al., 2004), and cPA (Oishi-Tanaka and Glass, 2010).

Furthermore, the results of the TLC experiments demonstrated a quick metabolism of the internalized LPCs to PC (figure 3.3B on page 74). No significant changes of the FA and LPC fraction were observed, while other LPC-derived metabolites were not covered by the TLC analysis.

Different LPC metabolites were screened for their ability to affect *ANGPTL4* and *PDK4* mRNA abundance (figure 3.9A on page 81). PC(16:0/16:0) and PC(18:1/18:1) did not increase PDK4 and ANGPTL4 mRNA levels when given extracellularly. This does not exclude their ability to activate PPARδ when they are formed intracellularly,

but they are reported as relevant ligands of PPARα only in the literature (Chakravarthy et al., 2009). Palmitate or oleate at equimolar concentrations showed similar responses on the induction of *PDK4* and *ANGPTL4* compared to LPCs (figure 3.9 on page 81). This was not surprising because FA are endogenous PPAR ligands (table 3.2 on page 84, figure 3.5A on page 76) and most likely have similar effects concerning the efficacy of the PPAR activation. The glycerol backbone of LPC, as represented in αGPC did not increase the mRNA levels of *PDK4* and *ANGPTL4*. αGPC might be too hydrophilic to activate PPARδ.

The LPC derivative cPA(16:0) was reported to be a PPARɣ antagonist (Oishi-Tanaka and Glass, 2010). In this work, this compound showed a robust induction of the two target genes. cPA which lacks the positively charged choline part (figure 3.9A on page 81) demonstrated the importance of a negative charged hydrophobic end opposed to the lipid part for the binding to the PPARδ LBD. This would presumably add other lysolipids like LPE, LPS and LPA to the list of ligands. In fact, LPA (not tested) was reported to be a PPARɣ agonist (McIntyre et al., 2003; Zhang et al., 2004).

The strongest effect on *PDK4* and *ANGPTL4* mRNA in this experiment was found for PC(O-16:0/O-1:0) treatment. PC(O-16:0/O-1:0) as an ether is chemically very resistant to hydrolysis under physiological conditions and structurally resembles LPC(16:0) (figure 3.9A on page 81). This compound can only be metabolized at the head-group position. PC(O-16:0/O-1:0) was able to increase *PDK4* and *ANGPTL4* mRNA levels to a higher extend than LPCs (figure 3.9 on page 81), increased the DNA binding activity of PPARδ/RXRα complex in EMSAs (figure 3.23 on page 99), and was able to replace the pan-PPAR agonist in TR-FRET based competition assays (figure 3.10 on page 83). PC(O-16:0/O-1:0) is therefore also an PPARδ ligand and emphasized the importance of the chemical structure of LPCs in the activation of PPARδ as ligand. And it supports the conclusion that LPCs without further metabolization to FA or PC can activate PPARδ target genes in HMT.

4.5 Other signaling pathways might contribute to LPC effects

The activity of PPARs is regulated by ligand binding and ligand-independent mechanisms. Latter mechanisms might contribute to the LPC-mediated effects on *PDK4* and *ANGPTL4*. Post-translational modifications like phosphorylation can affect PPARs by influencing the biological half-life of PPARs, affinity to ligands, heterodimerization with RXR, intracellular localization, or the affinity to the target DNA sequence (Feige et al., 2006). Kinases like AMPK, glycogen synthase kinase (GSK), ERK, PKA, and protein kinase C (PKC, official symbol: PRKC) are able to phosphorylate PPARα/ɣ at different sites (Burns and Vanden Heuvel, 2007). Not much is known about the regulation of PPARδ by phosphorylation. Some studies have demonstrated that PKA is able to influence PPARδ activity (Lazennec et al., 2000; Hansen et al., 2001; Krogsdam et al., 2002). The details of the interaction of PKA and PPARδ are not known and a direct phosphorylation has not been examined so far. Data obtained during my diploma thesis suggest that LPCs can inhibit PKA and the phosphorylation of its downstream target vasodilator-stimulated phosphoprotein (VASP) (Klingler, 2012). Therefore, it was examined if changes in intracellular cAMP levels as PKA regulating mechanism might affect PPARδ target genes expression. *PDK4* was induced by forskolin in a H-89-sensitive manner (figure 3.21 on page 97). In my diploma thesis, I had shown that pertussis toxin (PTX) could increase *PDK4* mRNA levels (Klingler, 2012). Similar to forskolin, PTX also increases intracellular cAMP levels by inhibiting the G_i type of heterotrimeric G-protein subunits that are coupled to GPCRs (Locht and Antoine, 1995; Carbonetti, 2010; Katada et al., 1983). In contrary, *ANGPTL4* was repressed by forskolin which could not be rescued by H-89 (figure 3.21 on page 97). The data suggests that, *PDK4* is regulated by intracellular cAMP levels, while *ANGPTL4* might be regulated in a complex fashion different from *PDK4*. According to that difference, a link between PPARδ and PKA is less likely.

It is known that LPCs are affecting the intracellular Ca^{2+} flux in endothelial cells, cardiac myocytes, immune cells like neutrophils and monocytes, and in skeletal muscle

cells by activating Ca^{2+} channels (Riederer et al., 2010; Ojala et al., 2007; Yokoyama et al., 2002; Wong et al., 1998; Frasch et al., 2007; Chaudhuri et al., 2003; Rolin et al., 2014; Wang and Hekimi, 2016; Khan et al., 2010; Boittin et al., 2010). The mechanisms for the activation of the channels by LPCs is not fully clarified but involves PLC activation in endothelial cells and neutrophils that causes the production of inositol-3 phosphate (IP_3) and subsequent Ca^{2+} channel activation (Yokoyama et al., 2002; Frasch et al., 2007). PLCs can be activated by GPCRs like GRP132 and GPR119, which are known to be activated by LPCs (Frasch et al., 2007; Ning et al., 2008), potentially via Gβ/ɣ subunit complex (Katz et al., 1992) and via different types of Gα subunits (Khan et al., 2010). Therefore, it was tested if the Ca^{2+} signaling might play a role in the induction of *PDK4* and *ANGPTL4*, since Ca^{2+} can activate several kinases among them are PKC and Ca^{2+}/calmodulin-dependent protein kinase (CAMKK). Furthermore, LPCs can activate PKCζ directly (Siddiqi and Mansbach, 2015) and other PKC isoforms by other mechanisms (Yea et al., 2009; Motley et al., 2002; Bassa et al., 1999; Sasaki et al., 1993; Oishi et al., 1988; Liao et al., 2013; Nishizuka, 1995). Such kinases could potentially affect PPARδ activity by phosphorylation (Burns and Vanden Heuvel, 2007).

BAPTA-AM used as an intracellular Ca^{2+}-depleting compound, increased *PDK4* mRNA abundance with borderline significance and reduced *ANGPTL4* mRNA levels. Ionomycin, that increases intracellular Ca^{2+} levels had no effects. Thus, only intracellular Ca^{2+} depletion but not increasing Ca^{2+} levels affects *PDK4* and *ANGPTL4* mRNA levels or BAPTA-AM has side effects and changes of intracellular Ca^{2+} are not of relevance in this context. In fact, chelation-independent effects of BAPTA-AM are described in the literature (Furuta et al., 2009). It is therefore assumed that changes of intracellular Ca^{2+} levels are not affecting *PDK4* and *ANGPTL4* mRNA abundance and PPARδ activity.

A LPC-mediated regulation of PPARδ by phosphorylation via PKA or Ca^{2+}-dependent kinases is unlikely according to these findings. Manipulations of intracellular cAMP levels therefore affected PPARδ target genes *PDK4* and *ANGPTL4* independent of PPARδ. It cannot be excluded that LPCs potentially alter intracellular

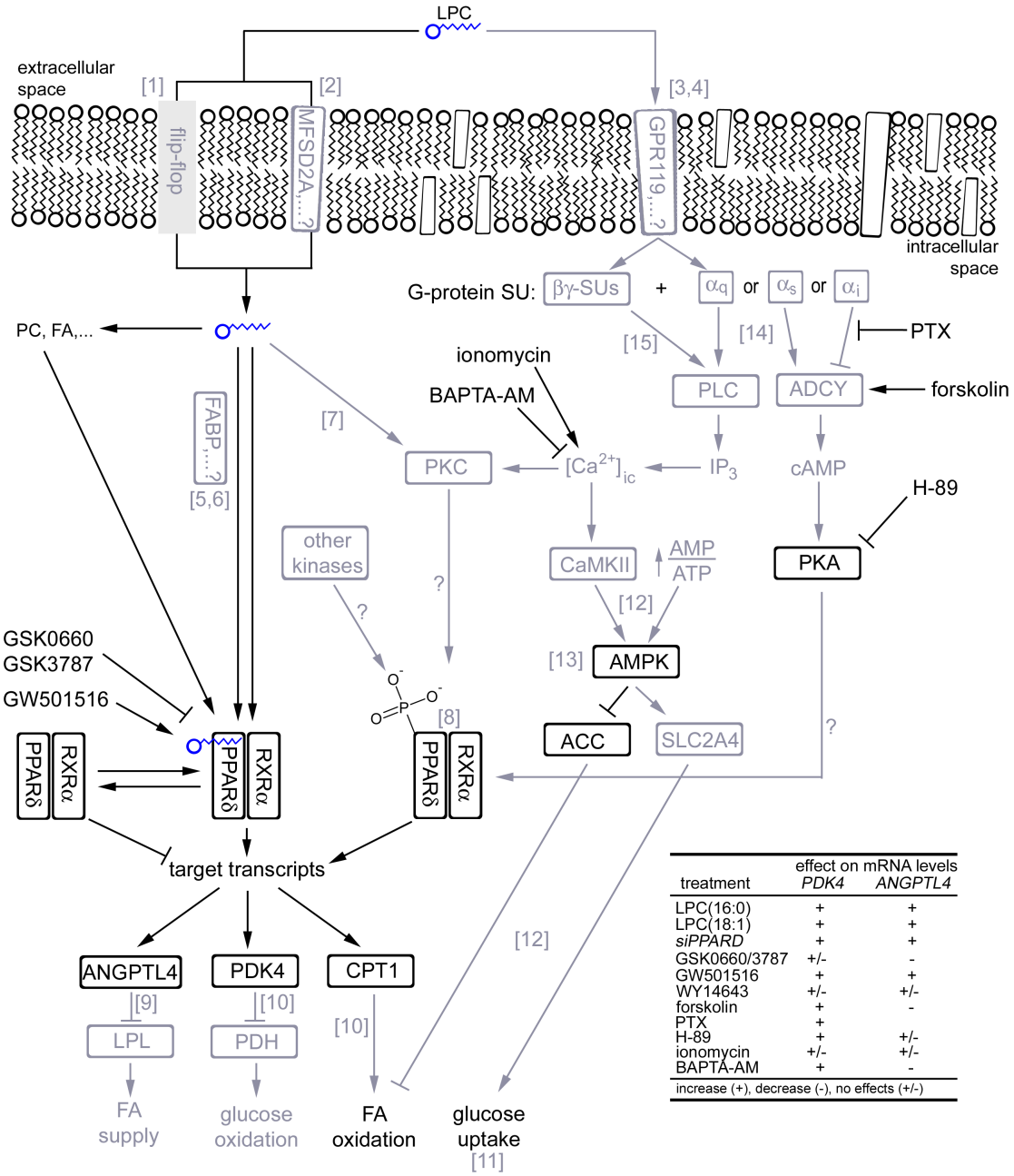
second messenger levels by mechanisms that involve receptors other than PPARs, for instance GPR119 (Cornall et al., 2013). That actions potentially can add to PPARδ effects and can lead to a mixed regulation of *PDK4*, *ANGPTL4* and other effects of LPCs on HMT reported in this thesis (summarized in figure 4.1 on the next page).

4.6 Consequences of PPARδ activation by LPCs

4.6.1 LPCs protect against lipotoxicity

PPARδ activation has been linked to anti-inflammatory effects (Wahli and Michalik, 2012; Vazquez-Carrera, 2016) during sepsis (Zingarelli et al., 2010) and on lipotoxicity caused by saturated FAs in skeletal muscle cells (Coll et al., 2010; Salvado et al., 2014; Wahli and Michalik, 2012).

Lipotoxicity describes a spectrum of pathophysiological events caused by ectopic lipid overload and maladaptation to this situation (Brookheart et al., 2009; Ritter et al., 2015) (summarized in figure 4.2 on page 122). An excessive supply of FA from the circulation leads to an intracellular lipid accumulation that exceeds the natural tissue capacity (Szendroedi and Roden, 2009). This results in the compensatory increase of putatively detrimental lipid metabolites. This scenario can evoke consequences like insulin resistance, chronic low-grade inflammation, ER stress, and apoptosis leading to organ dysfunction and metabolic diseases *in vivo* (Schaffer, 2016; Han and Kaufman, 2016; Erikci Ertunc and Hotamisligil, 2016; Peter et al., 2009). *In vitro*, prominent lipotoxic effects are predominantly induced by saturated FAs. They can enter a cell via protein-independent routes (flip-flop) and via the assistance of proteins like cluster of differentiation 36 (CD36) (Bonen et al., 2007) and are metabolized to different lipids, subsequently. The sole amount of lipids and/or the spectrum of different lipids can cause disturbances of the lipid composition of all membranes in the cell, especially of the ER membrane (Hou and Taubert, 2014). This might cause ER stress which can trigger a process called unfolded protein response (UPR) (Hou et al., 2014; Ariyama et al., 2010; Hou and Taubert, 2014; Wei et al., 2006). UPR can ultimately lead to apoptosis (Schroder and Kaufman, 2005; Unger and Orci, 2002).



treatment	effect on mRNA levels *PDK4*	*ANGPTL4*
LPC(16:0)	+	+
LPC(18:1)	+	+
siPPARD	+	+
GSK0660/3787	+/-	-
GW501516	+	+
WY14643	+/-	+/-
forskolin	+	-
PTX	+	
H-89	+	+/-
ionomycin	+/-	+/-
BAPTA-AM	+	-

increase (+), decrease (-), no effects (+/-)

Figure 4.1: Signaling pathways of LPCs. This chart summarized the findings of this work and (Klingler, 2012) concerning the signaling pathways of LPCs in HMT (black font and arrows). Findings of the literature are also included (gray font and arrows). LPCs can either be internalized by transporters like MFSD2A or via flip-flop, or can act via GPCRs like GRP119. Internalized LPCs activate PPARδ which leads to the transcription of genes like *PDK4*, *ANGPTL4*, and *CPT1*. Signaling via plasma membrane receptors trigger the activation of small G-protein α, β, and ɣ subunits and their individual downstream signaling cascades. Effects of selected treatments on *PDK4*/*ANGPTL4* mRNA abundance in HMT mentioned in this work are summarized in a table. Abbreviations: ADCY (adenylate cyclase), FABP (FA binding protein), JNK (c-Jun N-terminal kinase), ic (intracellular), IP3 (phosphatidyl inositol-3-phosphate), LPL (lipoprotein lipase), PDH (pyruvate dehydrogenase), PTX (pertussis toxin), SU (subunit). References: [1] Bergmann et al., 1984, [2] Nguyen et al., 2014, [3] Hansen et al., 2012, [4] Cornall et al., 2013, [5] Burrier and Brecher, 1986, [6] Armstrong et al., 2014, [7] Siddiqi and Mansbach, 2015, [8] Burns and Vanden Heuvel, 2007, [9] Yoshida et al., 2002, [10] Patel et al., 2014, [11] Yea et al., 2009, [12] Hardie et al., 2012, [13] Jiang et al., 2013, [14] Syrovatkina et al., 2016 [15] Katz et al., 1992.

However, also single lipid species were reported to be responsible for one or more aspects of lipotoxicity. The potential candidates are discussed controversially (Ritter et al., 2015). Among them are acyl CoAs (Li et al., 2010), ceramides (Watson et al., 2009; Frangioudakis et al., 2013; Summers, 2006), DGs (Macrae et al., 2013; Itani et al., 2002; Xu et al., 2015; Szendroedi et al., 2014), PAs (Zhang et al., 2015), and LPCs (Han et al., 2011, 2008; Kakisaka et al., 2012).

Saturated FA can also act on receptors on the cell surface. The most prominent receptors of long chain FA in this context are toll-like receptor (TLR) 2 and 4 (Pal et al., 2012; Hwang et al., 2016). Activated TLRs trigger several pathways which lead to the activation of NFκB via the interleukin-1 receptor-associated kinase 4 (IRAK4)-IκB kinase (IKK) axis (Leifer and Medvedev, 2016; Hwang et al., 2016) causing the induction of inflammatory cytokines like IL6 and CXCL3 (Weigert et al., 2004; Anisowicz et al., 1991). The activation of the kinase JNK by TLRs and the activation of especially PKCθ by various pathways cause the phosphorylation of insulin receptor substrates (IRS) which are downstream of the insulin receptor (IR, official symbol: INSR) signaling (Taniguchi et al., 2006; Boura-Halfon and Zick, 2009; Hwang et al., 2016; Ritter et al., 2015). These particular ser/thr phosphorylations of IRS proteins by these kinases lead to the pathophysiological downregulation of IRS proteins by different mechanisms. In consequence, all downstream events, like phosphorylation of AKT, are impaired resulting in insulin resistance of the cell (Boura-Halfon and Zick, 2009).

Treatment with the saturated FA palmitate is a common and efficient way to evoke lipotoxicity in cell culture systems (Newsom et al., 2015; Kausch et al., 2003; Montell et al., 2001). The resulting lipid profile has already been resolved in HMT and shows that amongst others, also LPCs are formed (Li et al., 2013). The question rises if LPCs could be a factor that contributes to lipotoxicity or if the LPC-mediated activation of PPARδ acts protective.

In this work, 250 µM palmitate was used to cause an increase of *ATF3*, *IL6*, and *CXCL3* mRNA abundance in HMT together with enhanced *XBP1* mRNA splicing as marker of ER stress as aspect of lipotoxicity. LPC pre-incubation ameliorated the

effects of palmitate (figure 3.14 on page 89) on the inflammatory markers *IL6* and *CXCL3* and decreased ER stress observed in decreased *ATF3* mRNA levels together with reduced *XBP1* mRNA splicing. Hereby, LPCs showed robust anti-inflammatory activity and are clearly not mediating lipotoxicity. In studies where LPCs are proposed as mediators of lipotoxicity (Han et al., 2008, 2011; Kakisaka et al., 2012) high amounts of LPCs were used. At such concentrations toxic effects that relate to unspecific and detergent-like actions of LPCs can be expected. The stimulation condition used in this thesis was tested for toxic and detergent-like effects, in order to avoid these effects. 10 µM of LPCs did not result in the release of intracellular LDH and CK (figure 1.7 on page 34) and did not cause the induction of *IL6*, *ATF3*, and *CXCL3*, pointing to a non-toxic LPC concentration (figure 1.8 on page 35). In one of those studies the acyl chains of the applied LPCs are not mentioned (Han et al., 2011). This makes it difficult to compare the results of such studies to this work or to the *in vivo* situation.

The findings here support the correlations listed in table 1.7 on page 26, where lower LPCs correlate with higher levels of inflammatory markers which provide a link to the *in vivo* situation. Studies of NASH in mice point to a upregulation in LPCATs by PPARα activation and inflammatory cytokines (Tanaka et al., 2012; Zhao et al., 2008; Li et al., 2015). This increase of LPCATs reduces plasma LPC levels (Tanaka et al., 2012). Fatty liver diseases like NASH and NAFLD are strongly associated with insulin resistance and the metabolic syndrome (Stefan et al., 2008). This gives rise to the hypothesis that during the metabolic syndrome, the main LPC source liver is compromised. This leads to reduced serum LPC levels which also prevents their protective effects and fortifies the low grade inflammation that is often seen during the metabolic syndrome (Hotamisligil, 2006).

4.6.2 Mechanisms of the anti-inflammatory effects of LPCs

Effects of PPARδ activation

Possible mechanisms of the protection against lipotoxicity by LPCs were examined. PPARδ activation is known to be protective against lipotoxicity (Cao et al., 2012; Sal-

vado et al., 2014; Coll et al., 2010; Yang and Yang, 2016). In fact, GW501516 protected the cells against palmitate-mediated ER stress and inflammation to a similar extend as LPCs. This indicates that PPARδ activation is sufficient to protect the cells (figure 3.14 on page 89). Other findings in this work are supporting this hypothesis by showing that PPARδ antagonism raised basal *IL6*, *ATF3*, and *CXCL3* mRNA levels after palmitate stimulation (figure 3.16 on page 91) and reversed the protective effects of both LPCs on *IL6* and *CXCL3*. Thus, PPARδ activation by LPCs or palmitate itself could act protective against palmitate-mediated lipotoxicity. The question rises how PPARδ causes this effect.

PPARs play an important role in the activation of metabolic pathways resulting in an enhanced substrate oxidation. Enhancement of palmitate oxidation is one important feature of PPARδ in skeletal muscle (Dressel et al., 2003; Tanaka et al., 2003). CPT1 is an important regulators of FA oxidation and its mRNA abundance was increased by LPC treatment (figure 3.1 on page 73). Furthermore, FA oxidation was reported to be one factor that causes protection against lipotoxicity (Henique et al., 2010).

GW501516 as positive control had also only minor effects on palmitate oxidation in HMT, showing that this cellular model system can increase its FA oxidation only to a small extend. The effects of LPCs on palmitate oxidation were also moderate (figure 3.11 on page 85). 5 µM LPC(16:0) had significant effects on palmitate oxidation. No significant effects were detected for LPC(18:1) treatment. Since only LPC(16:0) had significant effects but not LPC(18:1), enhanced FA oxidation might contribute but cannot be the only cause for the anti-lipotoxic effects of LPCs.

PPARs can exert actions that are not related to simple binding of the PPAR/RXR complex to their target DNA sequence. Such non-genomic mechanisms enable the crosstalk with other transcription factor pathways. Two main actions has been determined for this non-genomic actions: tethering and squelching (reviewed in (Feige et al., 2006)).

Tethering describes the ability of PPARs, upon ligand binding, to physically interact with other transcription factors and removing them from their target DNA sequence or preventing their interaction with the DNA. The second mechanism involves the inter-

action of PPARs with transcriptional co-regulators (Bugge and Mandrup, 2010). The recruitment of co-regulators to PPARs makes such co-regulators inaccessible for other transcription factors. This effect is called squelching.

Examples for the two mechanisms could explain some anti-inflammatory actions of PPARs. PPARα activation can cause the interaction of PPARα with subunits of the transcription factors NFκB and activator protein-1 which both thereafter are temporarily unable to bind to their target sequence on the *IL6* promoter (Delerive et al., 1999) preventing *IL6* induction. It should be noted that NFκB is also a transcription factor for *CXCL3* (Anisowicz et al., 1991) and that *CXCL3* might share a similar fate as *IL6*. PPARδ activation might act in a similar fashion on *IL6* and *CXCL3* as PPARα. This could explain why LPCs and GW501516 pre-treatment of HMT can decrease the palmitate caused induction of *IL6* and *CXCL3* (figure 3.14 on page 89).

PPARα activation causes the tethering of the transcription factor CCAAT/enhancer-binding protein β (aka C/EBPβ, official symbol: CEBPB) away from the promoter of ORM (Mouthiers et al., 2005). ORM, the important LPC carrier during inflammation (Ojala et al., 2006) is therefore reduced upon PPARα activation. This might increase the free LPC concentration and enhance their anti-inflammatory action during saturated FA overload in the serum, found during dyslipidemia as part of the metabolic syndrome (Laaksonen et al., 2002) until the total LPC concentration declines due to LPCAT induction in the liver as described earlier (Tanaka et al., 2012). It should be mentioned, that CEBPB is also an important transcription factor for CRP (Agrawal et al., 2001). PPARα activation might cause the downregulation of CRP by tethering of CEBPB and provides a possible explanation how increased LPC levels in the circulation are linked to the downregulation of the CRP gene (table 1.7 on page 26). All these effects have been found for PPARα. PPARδ could have similar effects. LPC(16:0) and LPC(18:1) not only activate PPARδ but also the PPARα LBD in luciferase reporter assays in HMT (data not shown) and it has been reported in the literature that LPCs activate PPARα in hepatocytes (Takahashi et al., 2014). According to that, LPCs would be able to cause tethering and squelching by either PPARα or

presumably by PPARδ as putative mechanisms for the reduction of *IL6*, *CXCL3* and other anti-lipotoxic effects reported in this work.

Target genes of PPARδ could contribute to the protective effect of LPCs, especially *in vivo*. ANGPTL4 is an inhibitor of the lipoprotein lipase (LPL aka LIPD) (Robciuc et al., 2012; Staiger et al., 2009). LPL is expressed in adipocytes and muscle cells and translocates to the endothelial cells of the blood vessels via transport proteins (Olivecrona, 2016). LPL is responsible for the hydrolysis of TG in lipoproteins like chylomicrons and VLDL. ANGPTL4 therefore indirectly reduces the uptake of FAs by reducing their production by LPL (Robciuc et al., 2012; Lichtenstein et al., 2007; Sukonina et al., 2006; Yoshida et al., 2002). This might prevent their accumulation in skeletal muscle cells and potential lipotoxic events.

PDK4 is an important regulator of glucose oxidation. The family of PDK consists four members: PDK1 - 4. PDKs phosphorylate the pyruvate dehydrogenase complex (PDH), thereby inhibiting this multi-enzyme complex which catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA (Patel et al., 2014). *Pdk4* knockout mice are protected against the deleterious effect of a diet enriched in saturated FA (Hwang et al., 2009). Therefore, PDK4 is presumably not a candidate that mediates the protection against lipotoxicity.

Effects of AMPK activation

This work describes an increase of FA oxidation by LPC(16:0) (figure 3.11 on page 85). AMPK and PPARδ synergistically enhance FA oxidation (Kramer et al., 2007). Thereby, AMPK inhibits the malonyl CoA producing enzyme ACCs and as a result fortifies FA oxidation (Hardie et al., 2012). It was reported that AMPK activation can protect against lipotoxicity in several cell types and systems (Cacicedo et al., 2011; Hong et al., 2014; Mayer and Belsham, 2010; Rodriguez-Calvo et al., 2015; Jung et al., 2015; Kramer et al., 2007; Salvado et al., 2014; Vazquez-Carrera, 2016; Henique et al., 2010) but the details of the mechanisms are still unclear. Recently, it was reported that LPC(18:0) is able to activate AMPK in macrophages (Quan et al.,

2016; Kim et al., 2015). AMPK activation could be another potential factor in the protection against lipotoxicity by LPCs.

AMPK consists of three subunits α, β, and ɣ from which α exists in two (α1 and α2; official symbols: PRKAA1 and PRKAA2), β in two (β1 and β2, official symbols: PRKAB1 and PRKAB2), and ɣ subunit in three isoforms (ɣ1 - 3, official symbols: PRKAG1 - 3) (Hardie, 2011). The α subunit harbors the phosphorylation site thr172, which when phosphorylated can increase the activity of AMPK by a factor of 100 (Suter et al., 2006). The kinases that are responsible for the phosphorylation of this site are liver kinase B1 (LKB1) (Hawley et al., 2003) and Ca^{2+}/calmodulin-activated protein kinase α and β (CamKIIα and CamKIIβ, official symbols: CAMK2A and CAMK2B) (Hawley et al., 2005; Woods et al., 2005; Hurley et al., 2005). LKB1 is a tumor suppressor gene and is activated by the formation of a protein complex with the pseudokinase Ste20-related adapters and the scaffold protein mouse protein-25 (Baas et al., 2003; Boudeau et al., 2003). Intracellular Ca^{2+} rise causes the phosphorylation and activation of CAMK2B. AMPK is also stimulated by the hunger signal AMP. AMP is able to allosterically activate AMPK, increasing its activity 10-fold and also enables the phosphorylation of AMPK by binding to the ɣ subunit (Hawley et al., 1995; Davies et al., 1995). Likewise, ADP is able to enhance the ability of AMPK to be activated by phosphorylation but lacks the ability to activate AMPK allosterically (Oakhill et al., 2011; Xiao et al., 2011). The AMP:ATP ratio indicates the energy status of a cell. In other words, AMPK is activated upon energy demand (Hardie, 2011; Hardie et al., 2012).

This work clearly shows, that both LPCs equally trigger the phosphorylation of AMPK and its downstream target ACC (figure 3.13 on page 87). The kinetics analysis revealed an activation that persists about 4 h and declines to baseline afterwards. The question rises, how LPCs activate AMPK. PPARδ activation could be one possible explanation. PPARδ causes the upregulation of uncoupling proteins (Chevillotte et al., 2001; Villarroya et al., 2007) that reduces ATP production and increases the AMP:ATP ratio (Kramer et al., 2007), an important signal for AMPK activation. The microarray results in this work (figure 3.1 on page 73) did not support an increase in

uncoupling proteins mRNA levels by LPC treatment. This is in accordance with the literature, showing that FA treatment of HMTs also does not increase uncoupling protein 3 (Staiger et al., 2009). This mechanism could be the case for strong PPARδ agonists like GW501516, but comparably weak endogenous agonists like FA and LPCs might require other additional metabolic events that contribute to the activation of AMPK via an increase of the AMP:ATP ratio. In fact, LPC(18:1) is able to activate GPR119 (Soga et al., 2005; Overton et al., 2006). GPR119 activation, recently, has been reported to activate AMPK by increasing the AMP:ATP ratio (Yang et al., 2016). Additionally, the activation of this receptor was linked to anti-diabetic properties in intestinal and pancreatic cells by increasing the release of incretins and insulin and anti-steatotic activity in hepatocytes by inhibiting lipogenesis via sterol regulatory element binding protein (Yang et al., 2016). This would endorse the protective properties of LPCs and shows that AMPK is an important factor for the protective effects of LPCs.

But also altered Ca^{2+} fluxes might contribute to the AMPK activation. Intracellular Ca^{2+} flux is indeed affected by LPCs, as reported by several studies in the literature (Riederer et al., 2010; Ojala et al., 2007; Yokoyama et al., 2002; Wong et al., 1998; Inoue et al., 1992; Frasch et al., 2007; Chaudhuri et al., 2003; Rolin et al., 2014; Wang and Hekimi, 2016).

Taking together, AMPK is presumably activated by LPCs via two pathways: increase in AMP:ATP ratio by binding to GPR119 or by increases of intracellular Ca^{2+} levels. AMPK activation could be an important factor in the protective effects of LPCs on lipotoxicity.

Another important feature of LPCs are their chemical properties, which affects membrane behavior and also receptor activity. It is reported that LPCs can affect TLR4 translocation and their exposition to ligands like FAs (Jackson et al., 2008). This could be another important feature of LPCs, how they influence inflammatory pathways (Fessler et al., 2009; Senn, 2006).

In summary, it is difficult to point to one single mechanistical pathway for the anti-inflammatory action of LPCs. It is more likely, that multiple effects of LPCs as signaling molecule and as cone-shaped amphiphil might play a role and act synergistically.

4.6.3 Effects of LPCs on glucose uptake

AMPK activation increases both glucose and FA uptake and oxidation (Sakamoto et al., 2005; Sakamoto and Holman, 2008; Chen et al., 2008; Pehmoller et al., 2009; Merrill et al., 1997) (Carling et al., 2003). AMPK is known to activate glucose uptake by an inhibitory phosphorylation of TBC1 domain family member 1 (TBC1D1) (Pehmoller et al., 2009; Chen et al., 2008). TBC1D1 inhibits SCL2A4 translocation (Cartee, 2015). This mechanism of the SCL2A4 translocation is independent of insulin but important for the contraction-induced glucose uptake in skeletal muscle via the Ca^{2+}-AMPK-TBC1D1 axis (Cartee, 2015).

In this work, LPC(16:0) treated $L6_{GLUT4myc}$ cells demonstrated a marginally increased basal glucose uptake in comparison to 100 nM insulin treated cells (figure 3.12 on page 86). LPC(18:1) had no effects in this experiment. This is in accordance with finding in adipocytes, demonstrating that LPC(16:0) but not LPC(18:1) increases 2DOG uptake already after less than 5 min in 3T3-L1 adipocyte (Yea et al., 2009) and 300 µM LPC(16:0) in insulin resistant adipocytes (Takahashi et al., 2014).

When insulin is present, the effects of LPC(16:0) on glucose uptake were not detectable. It was shown in HMT, that LPCs do not affect AKT phosphorylation upon insulin stimulation (data not shown). This could explain why there is no effect of LPCs on insulin-dependent glucose uptake.

Taking together, LPCs only marginally increase basal glucose uptake in $L6_{GLUT4myc}$ cells. Since AMPK is activated by LPC(16:0) and LPC(18:1) similarly, the mechanism for this observation is unclear.

4.6.4 Targeting intracellular LPC formation during lipotoxicity

It was reported that during palmitate stimulation, LPCs are rapidly increased in HMT (Li et al., 2013) and liver cells (Kakisaka et al., 2012). It was shown here that LPC stimulation can exerts protective effects. Thus, interference with the LPC metabolism during palmitate stimulation might also have an impact on lipotoxic events and could

give hints about the efficacy and role of the intracellularly formed LPC on the prevention of an overshooting lipotoxicity.

Silencing of LPCAT would presumably lead to an increase of intracellular LPCs and was expected to have similar effects as exogenous LPC treatment namely the protection against lipotoxicity. Indeed, silencing of LPCATs was protective against lipotoxicty in HMT (figure 3.17 on page 93). Inhibition of $iPLA_2$ should block LPC formation and fortify lipotoxicity. $iPLA_2$ were chosen as target for inhibition because in the literature two members PLA2G6A and PLA2G6B are responsible for palmitate-mediated increase of intracellular LPC content in L6 cells (Han et al., 2011). BEL is a suicide substrate for $iPLA_2$ (Hazen et al., 1991). It is commonly used in this context but also inhibits PLPP and many enzymes that have cysteine residues in the active center (Song et al., 2006; Balsinde and Dennis, 1996; Balboa et al., 1998; Daniels and Katzenellenbogen, 1986; Mitchell et al., 1998). BEL is a chiral compound. The enantiomers can discriminate between different $iPLA_2$ subclasses, especially between PLA2G6A and PLA2G6B. However, neither of the two enantiomers showed effects on lipotoxicity caused by palmitate (figure 3.18 on page 94). FKGK11 has less side effects than BEL and specifically inhibits $iPLA_2$ (Kokotos et al., 2010; Baskakis et al., 2008). At 10 µM, FKGK11 treatment significantly worsened *IL6*, *ATF3*, and *CXCL3* mRNA induction caused by palmitate. The more specific approach of silencing of *PLA2G6A+B* demonstrated that both lipases are in part mutually regulated (figure 3.19 on page 95). The mRNA abundances of both lipases were regulated by palmitate treatment and *PLA2G6B* mRNA was increased when *PLA2G6A* was silenced. This complex regulation caused difficulties in interpreting the effect on *IL6*, *ATF3* and *CXLC3* mRNA abundance. Similar to the finding during the LPCAT knockdown, also here only effects on *IL6*, but not on *ATF3* and *CXCL3* were observed. Nevertheless, silencing of either *PLA2G6A* or *PLA2G6B* significantly intensified *IL6* induction caused by palmitate treatment. It should be also pointed out, that the approaches of this section has not been tested for their effects on intracellular LPC levels.

To conclude, the metabolites that are responsible for lipotoxicity caused by saturated FA are still not completely resolved. This work shows by multiple approaches

that LPCs are not mediators of lipotoxicity in HMT, but LPC protect against lipotoxic events (summarized in figure 4.2 on page 122). Furthermore, intracellular formed LPCs might prevent an exaggerated lipotoxicity.

4.6.5 Effects of LPCs on insulin resistance

Several findings point to a potential insulin-sensitizing effect of LPCs. LPCs are biomarkers for IGT and T2DM (table 1.6 on page 25) and PPARδ activation can improve diseases associated with the metabolic syndrome, including insulin resistance (Odegaard et al., 2008; Oliver et al., 2001; Lee et al., 2006). It was shown in this thesis, that LPC(16:0) can increase basal glucose uptake in $L6_{GLUT4myc}$ cells although only marginally. Furthermore, LPCs acted in an anti-inflammatory way and protected against ER stress during lipotoxicity. It is likely that LPCs also prevent impairment of the insulin signaling by palmitate as an other aspect of lipotoxicity (Ritter et al., 2015).

To this end, it was investigated if cellular insulin resistance, observed as reduced insulin-mediated phosphorylation of AKT caused by palmitate, can be prevented by LPC treatment. This work showed that both LPCs differ in their potency concerning the protection of the insulin signaling pathway (figure 3.15 on page 90). LPC(16:0) but not LPC(18:1) could prevent palmitate caused reduction of insulin-dependent phosphorylation of AKT at ser473. For the site thr308 only LPC(18:1) was protective but not LPC(16:0). The discrepancy between the two LPCs could be due to the fact that both phosphorylation sites are targeted by different kinases.

AKT is a serine/threonine kinase that exists in three isoforms and all are partially activated upon phosphorylation on thr308 and completely activated by the additional phosphorylation of ser473 (Scheid and Woodgett, 2003). To enable the phosphorylation, AKT is recruited to the plasma membrane by binding to PI-3,4,5-trisphosphate (PIP_3) (Bellacosa et al., 1998). PIP_3 is a product of the PI-4,5-bisphosphate-3-kinase (PIK3), which is activated by growth hormone signaling cascades triggered for instance by insulin. PIP_3 also activates phosphoinositide-dependent kinase 1 (PDPK1) which thereafter phosphorylates AKT at thr308. Thr308 is also phosphorylated by inhibitor of kappa B kinase (IKK) (Guo et al., 2011). Ser473 is phosphorylated by

mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al., 2005; Frias et al., 2006), PDPK2 (Liao and Hung, 2010), CaMKK (Soderling, 1999), IKK (Guo et al., 2011), and TANK-binding kinase 1 (TBK1). This shows that many signaling pathways are between insulin and AKT which all are putatively affected by palmitate and LPC treatment. How the two tested LPC interfere individually with the pathways that lead to the phosphorylation of either ser473 or thr308 requires further detailed studies.

4.7 *In vivo* relevance of free LPC concentration

The question rises if the conditions used in this thesis sufficiently resemble the *in vivo* situation. *In vivo*, 100 - 300 µM LPCs (section 1.4.3 on page 18) are found in the blood together with approximately 600 µM (Simard et al., 2006) ALB (ALB:LPC = 1:0.5). The binding to ALB also reduces some of the physiological effects of LPCs (Klibansky and De Vries, 1963; Ojala et al., 2006; Mochizuki et al., 1982; Soga et al., 2005; Klingler, 2012). The presence of 68 µM ALB, the major LPC complexing protein, eliminated the induction of *PDK4*/*ANGPTL4* by 10 µM LPCs (ALB:LPC = 1:0.15), as shown in my diploma thesis (Klingler, 2012). At this ALB:LPC ratio, there were obviously no or too less biologically available LPCs. In the cell culture system used in this work 10 µM LPCs were incubated in medium (table 2.13 on page 48) containing $\approx$5 µM ALB resulting in an ALB:LPC ratio of 1:2.

In vivo, LPCs are bound by different serum proteins. Other compounds like FA, but also almost all lipophilic compounds in plasma compete for the binding. While it is assumed that under physiological conditions the free LPC concentration is small, but no data on the free concentration exist. To conclude, the free LPC levels are most likely only small but dynamic. These small LPC levels can have profound effects as illustrated by this work.

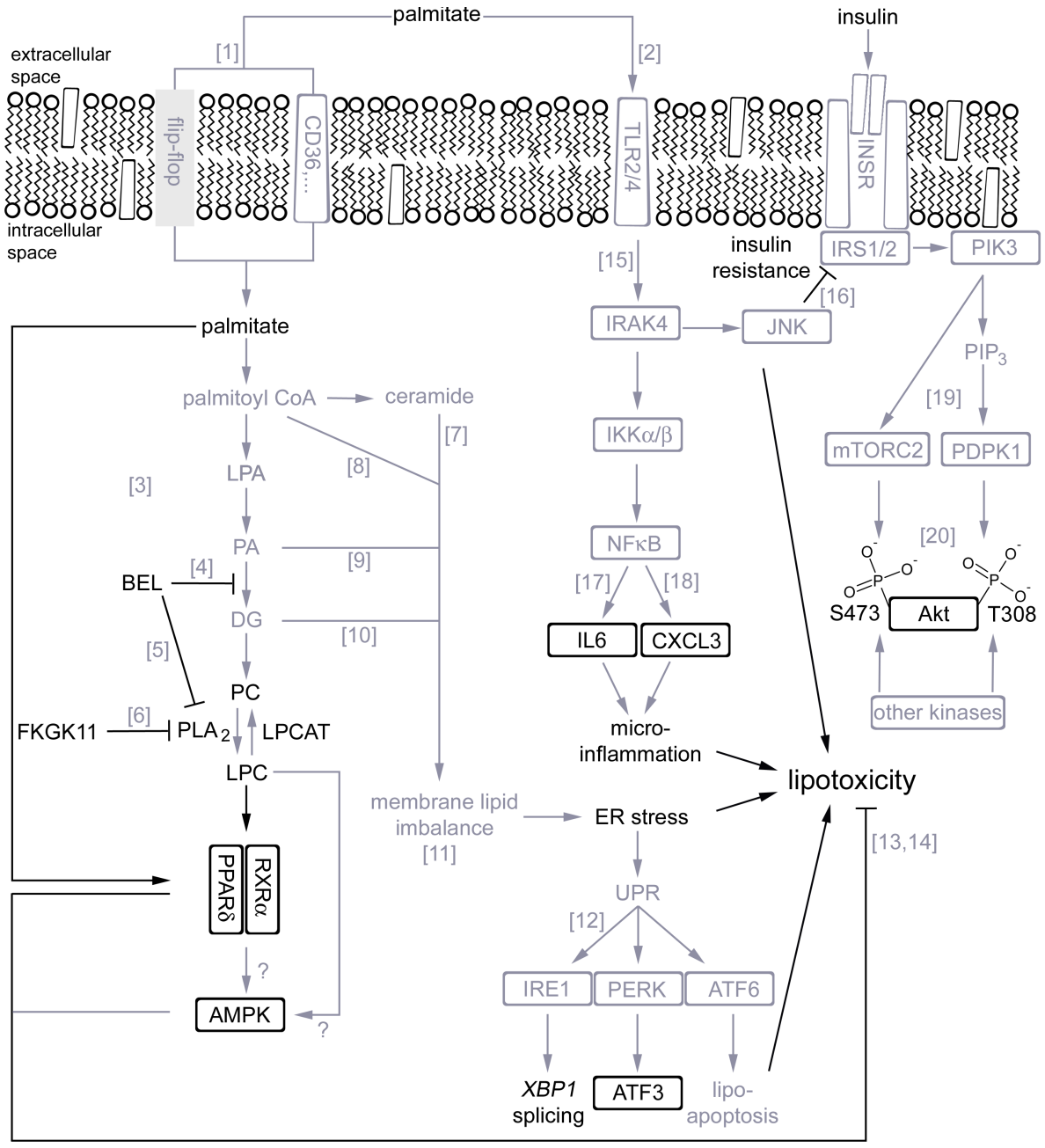


Figure 4.2: Signaling pathways of lipotoxicity. This chart summarized the findings of this work concerning the signaling pathways of lipotoxicity (black font/arrows). Findings of the literature are included (gray font/arrows). Palmitate causes lipotoxicity which includes ER stress, inflammation, insulin resistance, and apoptosis. This process is triggered by different pathways that involves the uptake and metabolization of palmitate as well as signaling events activated by the binding of palmitate to TLRs. Abbreviations: ATF (activating transcription factor), BEL (bromenol lactone), CD36 (cluster of differentiation 36), IKK (IκB kinase), INSR (insulin receptor), IRAK4 (interleukin-1 receptor-associated kinase 4), IRE1 (inositol-requiring enzyme 1), IRS (insulin receptor substrate), JNK (c-Jun N-terminal kinases), mTORC2 (mammalian target of rapamycin complex 2), NFκB (nuclear factor κ-light-chain-enhancer of activated B cells), PDPK1 (phosphoinositide-dependent kinase-1), PERK (PKR-like endoplasmic reticulum kinase), PIK3 (PI-4,5-bisphosphate 3-kinase), PIP_3 (PI-3,4,5-trisphosphate), TLR (toll-like receptor). References: [1] Bonen et al., 2007, [2] Pal et al., 2012, [3] Li et al., 2013, [4] Balsinde and Dennis, 1996, [5] Hazen et al., 1991, [6] Baskakis et al., 2008, [7] Summers, 2006, [8] Li et al., 2010, [9] Zhang et al., 2015, [10] Szendroedi et al., 2014, [11] Hou et al., 2014, [12] Han and Kaufman, 2016, [13] Kramer et al., 2007, [14] Salvado et al., 2014, [15] Hwang et al., 2016, [16] Boura-Halfon and Zick, 2009, [17] Weigert et al., 2004., [18] Anisowicz et al., 1991, [19] Taniguchi et al., 2006, [20] Scheid and Woodgett, 2003.

4.8 Outlook: Pharmacological aspects of LPCs

This thesis illustrates the beneficial effects of LPCs in the context of the metabolic syndrome. On a pharmacological point of view, this would suggest to increase the LPC concentration to a normal range. PPAR agonist and activators of the nuclear receptors RXR and retinoic acid receptor were shown to increase the LPC concentration in the circulation of mice (Takahashi et al., 2014; Weiss et al., 2011; Franko et al., 2016). According to that, LPCs could increase their own levels in the circulation by activating PPARs.

In this context, the direct substitution of LPCs when they are reduced could be of interest. But LPCs have a short half-live in the circulation due to quick uptake and degradation. Alkyl phospholipids could be a possible application form of LPCs with prolonged biological half-life (Arnold et al., 1978). Some alkyl phospholipid-related compounds are already in therapeutic use as it is the case for miltefosin (hexadecylphosphocholine) (Leonard et al., 2001; Fernandez et al., 2014), which recently has also been shown to activate AMPK in liver and therefore acting protective against hepatic steatosis (Fang et al., 2016). To overcome detergent-like effects, a lipid nanoparticle dosage form was successfully tested for miltefosine which reduced complications by oral and intravenous applications (da Gama Bitencourt et al., 2016).

The main action of LPCs is the activation of PPARs. PPAR activators, especially PPARα agonists (class of fibrates) and PPARɣ agonists (class of thiazolidinediones (TZD)s), are of great importance of today's treatment of the metabolic syndrome. Fibrates like fenofibrate are used to counteract dyslipidemia (Tenenbaum and Fisman, 2012). Pioglitazone and rosiglitazone are two TZDs that are under therapeutic use as insulin-sensitizers for patients with T2DM (Hauner, 2002). TZDs have several side effects, for instance increased risk of cardiac failure, bone fractures, and bladder cancer (Nissen and Wolski, 2007; Graham et al., 2010; Vallarino et al., 2013).

In order to intensify and combine the effects of fibrates and TZD and to reduce side effects, dual PPARα/ɣ agonist were developed. But the attempts failed due to unexpected side effect of different types (Cheang et al., 2015). Thereby, PPARδ came into focus.

PPARδ activation has been proven to be insulin-sensitizing in mouse models (Zhang et al., 2016; Lee et al., 2006). PPARδ knockout mice are glucose intolerant, have lower energy expenditure, and gain more weight during high fat diet compared to wild type littermates (Lee et al., 2003; Wang et al., 2003). Activation of PPARδ in adipose tissue can prevent diet-induced obesity (Wang et al., 2003). In fact, GW501516 was under investigation as a therapeutic (Sprecher et al., 2007; Riserus et al., 2008) but the trials were stopped because mice developed tumors during the treatment (Gupta et al., 2004; Pollock et al., 2010). According to that, dual agonists were discussed.

Currently, at least three PPARδ agonists are in clinical trials. The PPARδ agonist MBX8025 is in phase II (Bays et al., 2011; Choi et al., 2012), PPARδ agonist KD3010 in phase Ib (Dickey et al., 2016; Iwaisako et al., 2012), and the dual PPARα/δ agonist GFT505 (aka elafibranor) in phase III (Staels et al., 2013). MBX8025 is under investigation to treat dyslipidemia and insulin resistance (Hanf et al., 2014). KD3010 protects against liver fibrosis (Iwaisako et al., 2012) and the neurodegenerative Huntington's disease (Dickey et al., 2016). GFT505 counteracts NAFLD and non-alcolic steatohepatitis (NASH) (Staels et al., 2013; Ratziu et al., 2016), is insulin-sensitizing (Cariou and Staels, 2014; Cariou et al., 2013, 2011), and lacks the deleterious side effects of TZDs (Hanf et al., 2014).

From the results in this work, LPCs might be of clinical interest, not only as biomarkers but also as PPARδ and putatively also as dual PPARα/δ agonist. As endogenous ligands with rather moderate efficacy, they harbor the safety of less side effects than strong synthetic agonists. In the field of TZDs, it was shown that the weaker agonist pioglitazone has lower side effects than the stronger rosiglitazone (Colca et al., 2014; Winkelmayer et al., 2008). Strong PPARδ agonists increase PPARδ levels which might be the reason for unwanted side effects (Zhang et al., 2016).

LPCs also activate AMPK, which makes them an even more attractive pharmacological target. AMPK has been shown to be important during exercise. Mice lacking the β subunits of AMPK have reduced exercise capacity (O'Neill et al., 2011). The combination of AMPK and PPARδ activation can act as an exercise mimetic (Narkar et al., 2008; Manio et al., 2016; Wall et al., 2016). PPARδ activation not only counter-

acts T2DM and the metabolic syndrome by decreasing fasting insulin/glucose (Riserus et al., 2008; Lee et al., 2006), dyslipidemia (Sprecher et al., 2007; Riserus et al., 2008) including liver fat (Riserus et al., 2008), and body weight (Christodoulides et al., 2009; Wang et al., 2003) but also increases endurance capacity during exercise (Narkar et al., 2008). Accordingly, LPCs can intensify the effects of exercise by acting on PPARδ and AMPK, simultaneously.

Apart from PPAR/AMPK activation, LPCs were considered as future carriers for lipophilic drugs into the brain (Wang et al., 2016a), as immune modulators during immunizations (Bach et al., 2010; Perrin-Cocon et al., 2006), or as anti-inflammatory drugs against sepsis (Yan et al., 2004; Smani et al., 2015; Parra Millan et al., 2016). With that many possible applications of LPCs it is vital to find out what the exact mechanisms of their action are and to unravel the role of single LPC species.

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5 Acknowledgements

Am Ende meiner Doktorandenzeit möchte ich noch einigen Personen danken, die mich währenddessen begleitet und unterstützt haben. Großen Dank möchte ich vor allem meiner Doktormutter Prof. Dr. Cora Weigert aussprechen, die mich schon zu meiner Diplomandenzeit betreut und gefördert hat. Sie hatte mich immer ermutigt, geduldig unterstützt und mit kompetenten Vorschlägen beraten als Revisions anstanden, Experimente nicht funktioniert haben oder als der Wissenschaftleralltag düster war. Außerdem konnte ich dank ihr für ein viertel Jahr schottische Laborluft schnuppern. Meiner Co-Betreuerin Prof. Dr. Gabriele Dodt möchte ich dafür danken, dass Sie immer ein offenes Ohr für mich hatte und mich mit Ideen und Ratschlägen unterstützt hat. Prof. Dr. Rainer Lehmann möchte ich für die Kooperation mit Dalian danken. Prof. Dr. Erwin Schleicher hat mir mit seinem umfassenden Wissen über die Chemie und die Biochemie gezeigt, dass man vor allem die Chemie immer im Auge behalten sollte. Prof. Dr. Hans-Ulrich Häring danke ich für die Ideen und Vorschläge aus medizinischer Sicht und die Förderung durch Auslandsaufenthalte und Kongresse.

Natürlich wäre der Alltag im Labor sehr eintönig und bedeutend schwerer gewesen ohne Kolleginnen und Kollegen aus der Abteilung Med IV. Bei Heike Runge und Ann Kathrin Horlacher möchte ich für die schöne Zeit im Labor 537 bedanken. Sie haben mich bei allem unterstützt was Laboralltag und Experimente betrifft aber auch alles Andere, was sonst so über die Jahre hinweg passiert ist. Den Mit-Forschern aus Lab 580 und anderen Laboren möchte ich mich nicht nur für die Zeit im Labor sondern auch für die legendäre Zeit außerhalb danken. Dank geht an Dr. Miriam Hoene (für die Expertise), Dr. Christoph Hoffmann (für die Grill-Events), Dr. Sabine Neukamm (dass sie noch mit mir redet obwohl ich ihr Drehrad ausgeschaltet habe), Lisa Kappler

(für die Aufheiterungen, Split-Exkursionen und einfach nur so), Dr. Sebastian Hörber (für die Übernahme von Lab537), Dr. Marketa Kovarova (für die Gespräche unter Gleichgesinnten), Dr. Andrea Drescher (für die GRK1302-Zeit), Dr. Mika Scheler (für die gemeinsame Kongresszeiten), Carina Hermann (für die Reese's), Julian Geiger (für die Backkunst), Dr. Louise Fritsche (für die Betreuung als ich noch Praktikant war), Dr. Andras Frankó (für die Motivation) und an alle die ich noch in meinen Zeit in Tübingen kennengelernt habe für die schöne Zeit.

Und schließlich danke ich meiner Familie für alles was sie je für mich getan hat und dass sie immer für mich da war. Meinem Freund Dennis danke ich, weil er mich liebt auch wenn uns mein Naturwissenschaftler-Dasein des öfteren räumlich getrennt hat und er das Nachsehen hatte.