Dissertation

ACYL CHAIN-DEPENDENT EFFECT OF LYSOPHOSPHATIDYLCHOLINE ON CYCLOOXYGENASE (COX)-2 EXPRESSION IN ENDOTHELIAL CELLS

submitted by

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DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used.

Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Graz, 25.10.2012 Signature

Lada Brkić

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SUMMARY

Endothelial lipase (EL) is a serum phospholipase produced mainly by vascular endothelial cells. EL generates substantial amounts of saturated palmitoyl lysophosphatidyl-choline (16:0 LPC) and unsaturated oleoyl (18:1 LPC), linoleyl (18:2 LPC) and arachidonoyl lysophosphatidyl-choline (20:4 LPC) by cleaving its major substrate high-density lipoprotein (HDL) phosphatidylcholine. Because EL is active on the surface of vascular endothelial cells and its expression is upregulated by inflammation, EL-derived LPCs generated in close proximity of vascular endothelium might be important modulators of endothelial function. While 16:0 LPC is an established activator of various signaling cascades and modulator of expression of various genes including cyclooxygenase (COX)-2, almost nothing is known about signaling and gene-expression properties of unsaturated LPCs. Therefore, the aim of the present study was to examine the signaling properties of unsaturated LPCs in comparison to 16:0 LPC with an emphasis on intracellular Ca^{2+} homeostasis, activation of MAP kinases and nuclear transcription factors as well as modulation of COX-2 expression.

Quantitative PCR experiment showed that 16:0, 18:1, 20:4, but not 18:2 LPC raised COX-2 mRNA expression in human umbilical vein endothelial cell-derived cell line EA.hy 926, with profoundly different potencies and kinetics. Similarly, Western blot analysis revealed profound differences in the capacity and kinetics of the tested LPCs to upregulate COX-2 protein. Interestingly, COX-e protein was increased by 18:2 but not with 18:1 LPC. Experiments performed in the presence of pharmacological inhibitors of various signalling pathways revealed the involvement of intracellular Ca^{2+} and p38 MAPK in LPC-elicited COX-2 expression. All tested LPCs were capable of increasing intracellular Ca^{2+} concentration, however with different potencies as found by fluorescence spectrometry in Fura-2/AM loaded EA.hy 926 cells. The LPC-elicited increase in $[Ca^{2+}]_i$ was dependent on phospholipase C (PLC) and inositol-triphosphate receptor (IP_3R). Furthermore 16:0. 18:1 and 20:4 LPC induced p38 MAPK activation with markedly different kinetics, as found by monitoring p38 MAPK phosphorylation by Western blot. The involvement and markedly different relative contribution of selected transcription factors, including cyclic AMP responsive element binding protein (CREB), c-Jun and nuclear factor kappa B (NF-κB), in LPC-induced COX-2 upregulation was found upon knock-down of those transcription factors by siRNA approach.

Collectively, the obtained data strongly argue that the tested LPCs exhibit remarkably different, acyl chain-related potencies and kinetics of COX-2 induction in human endothelial cells, dependent on intracellular Ca^{2+} and p38 MAPK, as well as

nuclear factors NF-κB, c-Jun and CREB. Considering their high plasma levels together with their simultaneous action on endothelial COX-2 in vivo, the tested LPCs emerged as potent and important regulators of vascular (patho)biology.

ZUSAMMENFASSUNG

Die Endothellipase (EL) repräsentiert eine an der Oberfläche von vaskulären Endothelzellen gebildete Phospholipase. Durch die Spaltung ihres Hauptsubstrates „high densitiy lipoprotein“ (HDL) generiert EL substantielle Mengen sowohl an gesättigtem Palmitoyl-Lysophosphatidylcholin (16:0 LPC) als auch an ungesättigten LPCs wie Oleoyl-(18:1 LPC), Linoleoyl-(18:2 LPC) und Arachidonoyl-LPC (20:4 LPC). Bedingt durch die Entzündungs-mediierte Induktion der EL-Expression könnten an der vaskulären Oberfläche generierte LPCs wichtige Modulatoren der endothelialen Funktion darstellen. Während 16:0 LPC als etablierter Aktivator mehrerer Signalkaskaden und als Modulator der Genexpression z.B. der Cyclooxygenase-(COX)-2 gilt, sind diesbezügliche Eigenschaften von ungesättigten LPCs noch weitestgehend unbekannt. Deshalb war es Ziel dieser Studie, die Eigenschaften von ungesättigten LPCs in Signalkaskaden, mit dem Hauptaugenmerk auf intrazelluläre Ca^{2+}-Homeostase, Aktivation von MAP-Kinasen, nukleären Transkriptionsfaktoren als auch der Modulation der COX-2 Expression, zu untersuchen.

In quantitativen PCR-Experimenten in der HUVEC-basierten Endothelzell-linie EA.hy 926 zeigte sich eine Induktion von COX-2 mRNA durch 16:0, 18:1, 20:4 (aber nicht 18:2) LPC, jedoch mit deutlichen Unterschieden in Bezug auf Potenz und Kinetik. Westernblot Analysen ergaben ebenfalls deutliche Unterschiede in der Kapazität und Kinetik der getesteten LPC, die COX-2 Proteinexpression zu induzieren. Interessanterweise wurde COX-2 Protein zwar durch 18:2 LPC - jedoch nicht durch 18:1 LPC -induziert. Experimente mit pharmakologischen Inhibitoren diverser Signaltransduktionskaskaden indizierten eine Involvierung intrazellulären Ca^{2+} und der p38 MAPK in der LPC-induzierten COX-2 Expression. Fluoreszenzspekrometrisch detektiertes intrazelluläres Ca^{2+} $[Ca^{2+}]_i$ wurde in Fura-2/AM-beladenen EA.hy 926 Zellen durch alle getesteten LPCs in unterschiedlicher Stärke induziert. Der LPC-generierte Anstieg in $[Ca^{2+}]_i$ war hierbei abhängig von Phospholipase C (PLC) und Inositoltriphosphat-Rezeptor (IP_3R). In Westernblot-basierten Phosphorylationsanalysen induzierten sowohl 16:0 und 18:1 als auch 20:4 LPC die p38 MAPK Aktivierung, jedoch mit stark unterschiedlicher Kinetik. Die Involvierung und stark unterschiedliche relative Beteiligung ausgewählter Trasnkriptionsfaktoren (inkl. CREB, c-Jun and NF-κB) an der LPC-induzierten COX-2 Upregulation zeigte sich beim Silencing dieser Transkriptionsfaktoren mittels siRNA.

Zusammengefasst wiesen die getesteten LPCs in humanen Endothelzellen deutliche acylgruppenspezifische Unterschiede - sowohl in der Potenz als auch der Kinetik der Induktion von COX-2 - auf; jeweils in Abhängigkeit von intrazellulärem Ca2+, p38 MAPK und CREB. Betrachtet man den hohen Plasmaspiegel der LPCs zusammen mit deren simultaner Aktion auf endothelilale COX-2 in vivo, so stellen sich die getesteten LPCs als potente und wichtige Regulatoren der vaskulären (Patho)-Biologie dar.

ABBREVIATIONS

AA = arachidonic acid

AChE = acetylcholinesterase

AP-1 = activator protein-1

BAEC = bovine arterial endothelial cells

cAMP = cyclid 3', 5'- adenosine monophosphate

CBP = CREB binding protein

C/EBP = CCAAT enhancer-binding protein

COX = cyclooxygenase

CRAC channel = Ca^{2+} release-activated Ca^{2+} channel

CRE = cyclic AMP-response element

CREB = cyclic AMP-response element - binding protein

DAG = Diacylglycerol

DMEM = Dulbecco's modified Eagle medium

EC = endothelial cells

EIA = enzyme immunoassay

EL = endothelial lipase

ER = endoplasmatic reticulum

ERK = extracellular signal-regulated kinase

FBS = fetal bovine serum

FFA = free fatty acid

GTP = guanosine triphosphate

GPCR = G protein coupled receptor

GPR4 = G protein coupled receptor 4

HAEC = human aortic endothelial cells

HCAEC = human coronary artery endothelial cells

HDL = high density lipoprotein

HL = hepatic lipase

HUVEC = human umbilical vein endothelial cells

IκB = inhibitor protein kappa B

IL = interleukin

IP_3 = inositol triphosphate

IP_3R = inositol triphosphate receptor

JNK/SAPK = c-jun amino (N)-terminal or stress-activated protein kinase

LCAT = lecithin:cholesterol acyltransferase

LDL = low density lipoprotein

LPA = lysophosphatidic acid

LPL = lipoprotein lipase

LPC = lysophosphatidylcholine

MAPK = mitogen - activated protein kinases

MAPKK = MAPK kinase

MAPKKK = MAPKK kinase

MIP-2 = macrophage inflammatory protein 2

MPK = MAPK phosphatise

MSK = mitogen- and stress-activated protein kinase

NAC = N-acetylcysteine

NFAT = nuclear factor of activated T cells

NF-IL6 = nuclear factor IL6

NF-κB = nuclear factor kappa B

OGR1 = ovarian cancer GPCR 1

p90 RSK = p90 ribosomal S6 kinase

PGH_2 = prostaglandin H_2

PI_3 kinase = phosphatidyl Inositol 3-OH kinase

PIP_2 = phosphatidylinositol-4,5-bisphosphate

PC = phosphatidylcholine

PG = prostaglandin

PGI_2 = prostacyclin

PLA2 = phospholipase A2

PLC = phospholipase C

PKA = protein kinase A

S1P = sphingosine-1-phosphate

SOCE –store-operated Ca^{2+} entry

SPC = sphingosylphosphorylcholine

STAT3 = signal transducer and activator of transcription 3

STIM1 = stromal interaction molecule 1

TDAG8 = T cell associated gene 8

TG = triglyceride

TNF-α = tumor necrosis factor alpha

TXA_2 = thromboxane A_2

1. Introduction

1.1 Lysophosphatidylcholine

Lysophosphatidylcholine (LPC, 1-palmitoyl-sn-glycero-3-phosphocholine) is a bioactive phospholipid. It belongs to a group of bioactive glycerol- or sphingosine- based lysophospholipids, including sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA). They are generated from membrane phospholipids as a part of normal physiological activities or disease processes. LPC consists of a glycerol backbone that serves as a link between a fatty acid chain and a phosphorylcholine.

LPCs are formed primarily as a product of plasma membrane and lipoprotein-phosphatidylcholine (PC) hydrolysis by phospholipase A2 (PLA2) enzymes, generating LPC concomitantly with arachidonic acid (Sato *et al.*, 2008), but are also created by the action of lecithin:cholesterol acyltransferase (LCAT) in high density lipoprotein (HDL) that transfers fatty acids on the sn-2 position of phosphatidylcholine to the free cholesterol in plasma, thus forming cholesterol esters and LPCs (Prokazova *et al.*, 1998; Rousset *et al.*, 2009), as well as by the oxidation of low density lipoprotein (LDL) (Parthasarathy *et al.*, 1985). Additional sources of LPCs are hepatic lipase (HL) (Santamarina-Fojo *et al.*, 2004) and endothelial lipase (EL).

Endothelial lipase (EL) is a member of triglyceride (TG) lipase family together with hepatic lipase and lipoprotein lipase (LPL), and it is localised on the surface of vascular endothelial cells (Hirata *et al.*, 1999; Jaye *et al.*, 1999). EL is a phospholipase and it cleaves HDL-phosphatidylcholine (HDL-PC) liberating free fatty acids (FFA) and LPCs (McCoy *et al.*, 2002; Strauss *et al.*, 2003).

Previously, we demonstrated that, in addition to the well characterised, and mostly used in the research 16:0 (palmitoyl) LPC, EL generates substantial amounts of unsaturated LPCs: 18:1 (oleoyl), 18:2 (linoleyl), and 20:4 (arachidonoyl) LPC (Gauster *et al.*, 2005). The EL-generated LPCs are also among the most abundant ones in human plasma (Ojala *et al.*, 2007).

LPCs are normal constituents of blood plasma, vascular tissue and lipoproteins. Total LPC concentration in the human plasma is high, around 150 μM (Rabini *et al.*, 1994; Subbaiah *et al.*, 1985), but it's levels are greatly enhanced in hyperlipidemic

patients reaching milimolar levels (Chen *et al.*, 1997). LPC is accumulated in pathological tissues like ischemic myocardium, atherosclerotic aortas, and other inflammatory lesions of blood vessels (Katz *et al.*, 1981; Sobel *et al.*, 1978). LPC is also a major phospholipid component (40-50%) of oxidized LDL and is an atherogenic factor of oxidized LDL (McIntyre *et al.*, 1999). In plasma, LPC is bound to albumin and other carrier serum proteins and lipoproteins (Croset *et al.*, 2000; Ojala *et al.*, 2006). Binding of LPC to albumin abolishes most of its cellular effects (Kim *et al.*, 2007). Free LPC may transiently exist when, due to the excessive lipolysis, the concentrations of FFA and LPC locally exceed the binding capacity of the carrier proteins (Croset *et al.*, 2000). The free LPC is rapidly delivered to cells where it can be either reacylated to give PC (Stoll *et al.*, 1992), of deacyleted to give FFA and choline (Croset *et al.*, 2000).

1.2 Vascular endothelium

The vascular endothelium is a monolayer of cells located between the blood vessel lumen and the vascular smooth muscle cells. The endothelium is now recognized as being far more physiologically and therapeutically important than what its name would imply. It is now recognised as a very versatile multifunctional organ controlling various physiological processes including the modulation of vascular tone, blood coagulation, cell-to-cell adhesion, vessel repair, cellular proliferation and angiogenesis (Tran *et al.*, 2006).

All the cells receive the signals from their surrounding and respond to them. Cellular activities and actions of cells inside multicellular organisms are governed by a complex communication system called cell signaling. Coordination between cells is achieved with the help of multiple signaling molecules that can be secreted or displayed on the surface of one cells and also be bound on the receptors displayed on the surface of other cells. By biding to the specific receptors, a whole cascade of intracellular events is set into motion, and they regulate all aspects of cellular behaviour including metabolism, movement, proliferation, survival and differentiation. Briefly, cell signaling network is operating through three mayor steps: 1) signal reception (signal is detected by a receptor protein located on the cell surface of inside of the cell); 2) signal transduction (it is often occurring as a sequence of changes in a series of different molecules/proteins); and 3) cell response (the transduced signal triggers a specific cellular response). Thus, gene expression occurs as the final result of complex signaling networks that communicate

environmental changes to the nucleus of the cell. It is important to note that the same signaling system can lead to very different responses in different cells or different organisms (Alberts *et al.*, 2002).

1.3 Biological effects of LPC

16:0 LPC is already well connected to a number of cellular responses, and the numerous signaling cascades triggered by 16:0 LPC have been characterised.

It has been demonstrated that LPC induces multiple functional alterations of the vasculature that are potentially involved in atherosclerosis. LPC acts on several types of cells involved in atherosclerosis. For example, LPC increases the cytoplasmic free Ca^{2+} concentration in endothelial cells (Watanabe *et al.*, 2006; Yokoyama *et al.*, 2002), leukocytes, macrophages, and monocytes (Lee *et al.*, 2004; Ojala *et al.*, 2007; Okajima *et al.*, 1998; Yun *et al.*, 2004). Additionally, LPC can upregulate the expression of adhesion molecules (Kume *et al.*, 1992; Murohara *et al.*, 1996; Zhu *et al.*, 1997; Zou *et al.*, 2007), the production of cytokines (Murugesan *et al.*, 2003; Riederer *et al.*, 2011), the secretion of O_2^- (Kugiyama *et al.*, 1999), and DNA-binding activity of NF-κB (Sugiyama *et al.*, 1998) in endothelial cells.

Free LPC can be chemotactic for monocytes and lymphocytes (Quinn *et al.*, 1988), inhibit EC migration (essential for re-establishing arterial integrity after vascular injury) (Chaudhuri *et al.*, 2003), increase endothelial permeability (Huang *et al.*, 2005), and promote expression of cyclooxygenase-2 (Rikitake *et al.*, 2001; Zembowicz *et al.*, 1995).

1.4 Cyclooxygenases and prostanoids

Cyclooxygenases (COX) catalyse the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2) in the committed step of prostanoid biosynthesis (Rouzer et al., 2009; Smith et al., 2000). Two isoforms of the enzyme, the COX-1 that is constitutively expressed and COX-2 that is thought to be inducible (Davidge, 2001), have been shown to be expressed in mammalian tissues. However, increasing reports indicate that vascular endothelial cells constitutively express COX-2 isoform (Inoue *et al.*, 2002; McAdam *et al.*, 1999). In endothelial cells, the enzymes are positioned in the luminal monolayer of

the endoplasmic reticulum and the inner membrane of the nuclear envelope (Otto et al., 1994; Spencer et al., 1998).

COX-2 catalyses two reactions: a cyclooxygenase and a peroxidase reaction. COX-2 catalysis begins with the generation of arachidonoyl radical from AA, and the addition of two O_2 molecules with concomitant rearrangements to form PGG_2 (Schneider et al., 2007). By peroxidase reaction PGG_2 is reduced to an unstable endoperoxide intermediate, PGH_2, which serves as substrate for cell-specific isomerases and specific PG synthases to generate PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane (TX) A_2 (Smith, 1992; Ueno *et al.*, 2005). COX-2 is a homodimer, composed of monomers with identical sequences. Each monomer has a distinct cyclooxygenase and peroxidase active site. The monomers are tightly associated and their dissociation can occur only upon denaturation (Dong *et al.*, 2011).

Prostaglandins PGD_2, PGE_2, $PGF2_\alpha$, PGI_2 and TXA_2 interact with specific G-protein-coupled receptors (GPCRs) classified in five subtypes DP, EP, FP, IP, and TP receptors according to their preferential affinity to the five prostanoids, respectively (Tsuboi *et al.*, 2002). Prostanoid receptors can bind and be activated by not only the respective prostaglandins, but the others too (Gluais *et al.*, 2005), and some other biologically active compounds like isoprostanes (Janssen, 2002) and hydroxyeicosatetraenoic acid (Feletou *et al.*, 2010), with various range of potency.

Mostly studied prostaglandins are PGI_2 and TXA_2 because of their involvement in cardiovascular diseases, and the synthesis of both PGI_2 and TXA_2 are increased in patients with atherosclerosis (Belton *et al.*, 2000; FitzGerald *et al.*, 1984).

PGI_2 is a major PG produced by vascular endothelial cells. Prostacyclin synthase is highly expressed in endothelial cells (Tang *et al.*, 2008), but also in other types of cells as well (Wu *et al.*, 2005). It acts as a potent vasorelaxant (Fleisher *et al.*, 1982), a potent platelet inhibitor preventing their aggregation and adhesion to the endothelial cell surface, and an inhibitor of leukocyte adhesion (Thiemermann, 1991). Because of these actions, PGI_2 is thought to play a protective role in atherothrombosis.

On the contrary, TXA_2 is a potent inducer of vasoconstriction, and platelet activation, aggregation and adhesion. In addition to that, it is involved in allergies, modulation of acquired immunity, atherogenesis, neovascularization and metastasis of cancer cells (Nakahata, 2008). Its action is very local, as it is metabolised very fast to TXB_2 non-enzymatically. The relative concentrations of these two prostaglandins in the microenvironment are taught to be critical for vascular homeostasis because of the

opposing nature of their action.

Of other prostaglandins, PGD_2 is a major PG in immune cells and the central nervous system. Additionally, it can be expressed in endothelial cells, where PGD synthases can be upregulated by the increase in fluid shear stress (Taba *et al.*, 2000). However, PGD_2 has the main role in the regulation of sleep and in allergic responses (Smyth *et al.*, 2009).

PGE_2 is the most abundant PG in the human body involved in multiple physiological effects (Legler *et al.*, 2010). These diverse effects of PGE_2 can be attributed to the existence of four receptor subtypes (EP1-4) which are coupled to different signalling pathways (Hata *et al.*, 2004). It is an autocrine mediator which increases the risk of cardiovascular thromboembolic events with a potential role in plaque instability (Cipollone *et al.*, 2001).

$PGF_{2\alpha}$ is a prostaglandin required in the female reproductive system and in kidneys. It is produced in the vascular wall where it acts as a potent vasoconstrictor and may be involved in cardiac hypertrophy (Hata *et al.*, 2004; Smyth *et al.*, 2009).

The expression of COX-2 can be induced by growth factors and cytokines in many different cell types (Bartlett *et al.*, 1999; Lee *et al.*, 1992). It has been shown that COX-2 contributes significantly for PGI_2 synthesis in endothelial cells (Caughey *et al.*, 2001). We also additionally demonstrated the impact of multiple LPC species on PGI_2 production in endothelial cells (Riederer *et al.*, 2010). The effect of 16:0 LPC on COX-2 expression in human (Zembowicz *et al.*, 1995) and bovine (Rikitake *et al.*, 2001) endothelial cells was also previously demonstrated.

1.5 MAPKs

MAPKs are Ser/Thr kinases that convert of extracellular stimuli (e.g. growth factors and cytokines) into a wide range of cellular responses, by regulating the transcription factors, thus determining the exact patterns of gene expression. MAPK pathways regulate multiple cell functions like gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (Pearson *et al.*, 2001). Within the conventional MAPKs, four distinct subgroups have been well characterised: 1) extracellular signal-regulated kinase 1/2 (ERK 1/2), 2) c-Jun amino (N)-terminal or stress-activated protein kinase (JNK/SAPK), 3) p38 group of protein kinases, and 4)

ERK5 (Cargnello *et al.*, 2011).

Each subgroup of conventional MAPKs is composed of a set of three sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). In this "MAPK cascade", a series of phosphorylation events occure in which one kinase phosphorylates another. In response to extracellular stimuli, MAPKKKs are activated either through phosphorylation or through interaction with small GTP-binding protein of the Ras/Rho family. MAPKKK activation leads to activation through phosphorylation of MAPKK, which finally results in activation of MAPK through dual phosphorylation on Thr and Tyr residues located in a conserved Thr-X-Tyr motif within the activation loop of the kinase domain subdomain VIII (Robbins *et al.*, 1993). Once activated, MAPKs phosphorylate their respective substrates on Ser or Thr, followed by a Pro residue. Because of this characteristic, they can be considered Pro-directed kinases that have a very limited specificity for their consensus phosphorylation motifs.

p38 group of MAPK comprises of four p38 isoforms: ubiquitously expressed p38α and p38β, and of p38γ and p38δ that have more restricted expression patterns and specialized functions. Most of the published literature on p38 MAPK refers to the p38α as it generally more highly expressed than p38β.

p38 isoforms are activated by various environmental stresses and inflammatory cytokines, including UV light, heat, osmotic shock, hypoxia, ischemia, interleukin (IL) -1 and tumour necrosis factor alpha (TNF-α) (Cuadrado *et al.*, 2010). Most of the stimuli that activate p38 MAPK also stimulate JNK, as many MAPKKKs are shared between p38 and JNK cascades (Cargnello *et al.*, 2011). Additionally, p38 isoforms can extend the MAPK kinase cascade by phosphorylating and activating MAPK-activated protein kinases (MAPKAPKs), including MSK, MNK1, MK2/3, and MK5. Depending on the exact cell type and extracellular stimulus, p38 MAPK signaling cascade regulates numerous cellular functions, including inflammation, differentiation, development, cell cycle, and apoptosis (Zarubin *et al.*, 2005). Under physiological conditions, the activation of p38 is transient due to the dephosphorylation by specific phosphatases belonging to the MAPK phosphatase (MKP) family, and the level of p38 MAPK does not change throughout the course of stimulation.

COX-2 expression is modulated via members of MAPK cascade by multiple stimuli (Norata *et al.*, 2004; Said *et al.*, 2002). Once activated, the MAPKs can modulate the activity of several transcription factors such as NF-κB, CREB, AP-1, NFAT and C/EBPβ, which are involved in COX-2 expression (Callegari *et al.*, 2006; Iniguez *et al.*,

1999; Rikitake *et al.*, 2001; Said *et al.*, 2002). p38 MAPK reportedly impact the expression of COX-2 (Guan *et al.*, 1998b; Ridley *et al.*, 1997).

LPC is shown to activate p38 MAPK in a variety of cell types resulting in different physiological responses. It activates p38 in monocytic THP-1 cells, and is involved in its stimulated chemotaxis (Jing *et al.*, 2000). LPC induces IL-8 expression through p38 activation in endothelial cells (Riederer *et al.*, 2011). LPC increases neutrophil bactericidal activity through p38 activation (Hong *et al.*, 2010). In vascular smooth muscle cells it induces COX-2 expression (Yamakawa *et al.*, 2008), and it also induces apoptosis in endothelial cells (Takahashi *et al.*, 2002) through p38-dependent mechanism.

1.6 Singaling molecules and events implicated in regulation of COX-2 expression

COX-2 levels are tightly controlled and its gene regulation is dependent on gene transcription and post-transcriptional events (Harper *et al.*, 2008). Human COX-2 5'-flanking promoter region contains canonical TATA box and putative binding sites for transcription factors including cyclic AMP-response element (CRE)-binding protein (CREB), activator protein-1 (AP-1), nuclear factor (NF)-κB, NF-IL6 / CCAAT enhancer-binding protein (C/EBP), signal transducer and activator of transcription (STAT3) and SP1 and multiple other cis-activating regulator elements such as E-box transcriptional elements (Kosaka *et al.*, 1994). They all govern COX-2 gene transcription in a cell type- and stimulus-specific manner (Inoue *et al.*, 1994; Inoue *et al.*, 2000; Inoue *et al.*, 1995; Subbaramaiah *et al.*, 2002). COX-2 gene is also post transcriptionally regulated through its 3'-untranslated region (3'-UTR) that contains 17 copies of the AUUUA motif, which promotes the mRNA degradation (Kang *et al.*, 2007; Mbonye *et al.*, 2009).

One of the most extensively investigated transcription factor for the regulation of COX-2 expression is the eukaryotic transcription factor NF-κB. NF-κB belongs to the Rel family of transcription factors. The family consists of p65 (RelA), c-Rel, RelB, p50／p105 (NF-κB1), and p52／p100 (NF-κB2). In the resting state, they exist as homo- or heterodimers bound to the related inhibitory IκB family of proteins, which regulate the NF-κB activation. NF-κB proteins share a common domain responsible for dimerization, interaction with IκB and DNA binding. Under basal conditions, NF-κB is retained in the

cytosol and its nuclear translocation sequences are masked through the interaction with IκB. A proinflammatory signal results in a rapid phosphorylation of IκB and subsequent IκB ubiquitination and degradation by the proteasome (Siebenlist *et al.*, 1994). Degradation of IκB frees NF-κB that translocates to the nucleus and forms transcriptionally active complexes that direct transcription of immunity and inflammation-associated genes in a wide array of cell types, including COX-2 (Ghosh *et al.*, 2012; Inoue *et al.*, 1999). The classical pathway of NF-κB activation is triggered by proinflammatory cytokines, Toll like receptors and antigen receptors, but also some intracellular signals like reactive oxidative species. NF-κB has also been implicated in the response to 16:0 LPC in EC (Riederer *et al.*, 2011), a pathway previously shown to be dependent on PTK (Zhu *et al.*, 1997) and PKC ((Sugiyama *et al.*, 1998). Two NF-κB binding sites exist within COX-2 promoter: (-223/-214) and (-448/-439) (Appleby *et al.*, 1994; Kosaka *et al.*, 1994).

CREB is a 43 kDa basic leucine zipper nuclear transcription factor. It belongs to the CREB/ cyclic AMP-dependent transcription factor (ATF) family. Its activity is regulated by phosphorylation of Ser133 residue (Gonzalez *et al.*, 1989) . Cyclic 3',5'-adenosine monophosphate (cAMP) is generated upon binding of the ligand to its respective receptor coupled with a GTP-binding protein, which activates adenylyl cyclase that generates cAMP. Subsequently, cAMP activates protein kinase A (PKA). cAMP binds to the regulatory subunit of PKA and releases it's catalytic subunit (Cobb *et al.*, 1987) which subsequently modulates the function of CREB and of many other nuclear factors by phosphorylation (Lalli *et al.*, 1994). Except by cAMP, CREB has been reported to be regulated by kinases downstream from p38 like MAPK (p38/MAPKAPK-2) (Tan *et al.*, 1996) or ERK (ERK/p90 ribosomal S6 kinase (p90 RSK) (Xing *et al.*, 1996). CREB has a great biological importance in many cellular processes, demonstrated by enormous functional diversity of CREB-regulated genes and the encoded proteins. So far, more than 300 stimuli which can induce CREB phosphorylation have been described in the literature (Johannessen *et al.*, 2004a). CREB can form homodimers or heterodimers with other members of the ATF family, like ATF-1 and CREM, but the heterodimerization decreases its stability and binding affinity to the CRE element located in promoter regions of genes (Johannessen *et al.*, 2004b). COX-2 promoter region contains CRE site, and previously it has been shown that CRE sequence on the COX-2 promoter (-59/-53 bp) is bound by the transcription factor CREB in response to cytokines or PMA (Schroer *et al.*, 2002) and CREB has been shown to regulate COX-2 expression in ECs (Rikitake *et al.*, 2001).

AP-1 is another transcription factor involved in regulating the COX-2 expression. AP-1 is a dimer composed of a heterogeneous set of proteins including the members of Jun, Fos and ATF families of proteins. C-Jun can dimerize with diverse proteins of Jun, Fos and ATF families. C-Fos does not form homodimers but only heterodimers with Jun and ATF proteins (Chinenov *et al.*, 2001). Jun is constitutively expressed and activated by phosphorylation, whereas Fos is activated by regulation of its own protein expression (Karin *et al.*, 1997). c-fos/c-jun heterodimers possess stronger AP-1 binding activity than c-jun/c-jun homodimers, and are thus more biologically active (Angel *et al.*, 1991). Jun and Fos can both be activated by upstream kinases such as JNK, ERK and p38 MAPK (Ozanne *et al.*, 2007). JNK enhances the transcriptional activity of AP-1 by phosphorylating c-Jun on Ser63 and 73 of its transactivation domain. AP-1 complex is activated as a response to a wide range of stimuli, including growth factors, chemokines and extracellular matrix (Ozanne *et al.*, 2007), and is essential for the transcriptional control of numerous genes (Korenaga *et al.*, 1997; Shyy *et al.*, 1995).

AP-1 binding to CRE region has been previously reported to regulate COX-2 promoter activity (Chinenov *et al.*, 2001). However, the exact regulatory element that binds AP-1 is still controversial. AP-1 can bind to the CRE site and this binding is not influenced by mutation of the CRE site (Schroer *et al.*, 2002). It is also possible that it bind to the sequence inside of NFAT binding site.

1.7 Calcium homeostasis in endothelial cells

Endothelial cells (ECs) respond to various extracellular stimuli, like hormonal and chemical transmitters as well as to changes in physical parameters, with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$). Changes in $[Ca^{2+}]_i$ regulate to various extents many endothelial functions, like proliferation, apoptosis, prostaglandin synthesis, and NO synthesis. Ca^{2+} signaling controls many processes in cells because it is very versatile in terms of speed, amplitude and spatial-temporal patterning. Dysfunction of endothelium has been implicated in many cardiovascular diseases. Signaling pathways resulting in the increase of $[Ca^{2+}]_i$ will be shortly described in the following paragraphs. Changes in endothelial $[Ca^{2+}]_i$ depend on two main Ca^{2+} sources: release of Ca^{2+} from intracellular Ca^{2+} stores (endoplasmic reticulum, ER) and activity of Ca^{2+}-permeable ion channels in the plasma membrane.

Compounds like bradikynin, serotonin or acetylcholine act on receptors that are coupled to a guanosine nucleotide-binding protein (G-protein) and activate it, which subsequently activates phospholipase (PL) C-β1 which produces and releases to the cytosol inositol triphosphate (IP_3) and diacylglycerol (DAG) by hydrolysing phosphatidylinositol-4,5-bisphosphate (PIP_2) (Berridge, 1993; Tran *et al.*, 2000).

Other compounds, like certain growth factors, bind to the receptors linked to the tyrosine kinase leading to the activation of PLC-γ1. In addition to performing the same action of PLC-β1, it activates phosphatidyl inositol 3-OH (PI_3) kinase leading to regulation of Ras and activation of MAPKs (Berridge, 1993; Morris *et al.*, 1999; Tran *et al.*, 2000).

Once formed, IP_3 binds to its receptor (IP_3R) located on the surface of ER (Yamada *et al.*, 1994) leading to activation of IP_3R Ca^{2+} release channels and the depletion of internal Ca^{2+} stores which result in transient increase in $[Ca^{2+}]_i$ (Berridge *et al.*, 1984) often organized into characteristic spatial (elementary events (puffs) and waves) and temporal patterns (Ca^{2+} oscillations) (Berridge, 2009; Yao *et al.*, 1995). Thus, IP_3 is a Ca^{2+}-mobilizing second messenger which releases Ca^{2+} from intracellular Ca^{2+} stores, thus playing a central role in many of the Ca^{2+} releasing signaling systems. IP_3Rs are regulated by both IP_3 and Ca^{2+}. High $[Ca^{2+}]_i$ is inhibitory for IP_3 channel activity. When the endothelial cells are stimulated with IP_3 - generating agonist, the Ca^{2+} signal is typically biphasic consisting of an initial fast elevation of the cytosolic Ca^{2+} concentration which is a result of IP_3 - dependent Ca^{2+} release from ER, followed by a sustained Ca^{2+} entry (from the extracellular milieu) phase. The exact duration of Ca^{2+} intracellular increase and the shape of the signal vary considerably between cell types and the type of stimulation.

The major Ca^{2+} entry pathway in endothelial cells is store-operated Ca^{2+} entry (SOCE), a pathway activated by the reduction of intracellular Ca^{2+} stores. When the intracellular Ca^{2+} stores of a cell are reduced due to the cellular activity, the cell must refill these stores to maintain Ca^{2+} homeostasis and effective cellular function. Following the action of IP_3 on its receptor, and the subsequent release of Ca^{2+} from the internal store, the emptying of ER sends the signal to the Ca^{2+} release-activated Ca^{2+} (CRAC) channel named Orai1 that is responsible for maintaining the Ca^{2+} entry current (Parekh *et al.*, 2005). The coupling between store emptying and the activation of Orai1 is achieved through a stromal interaction molecule 1 (STIM1) on the surface of ER that functions as

the sensor of store emptying (Lewis, 2007; Roos *et al.*, 2005), and their interaction enables robust Ca^{2+} influx (Park *et al.*, 2009).

Subsequently, the interaction of STIM1 with the SOCE pore forming subunit Orai1 (Vig et al., 2006; Park et al., 2009; Yuan et al., 2009) enables robust Ca^{2+} influx. ER is a dynamic organelle that performs two major functions: synthesis and folding of proteins, and as a signaling organelle, it controls multiple cellular processes such as entry and release of Ca2+, sterol biosynthesis, apoptosis and the release of arachidonic acid (AA). It is the main Ca^{2+} store in endothelial cells having approximately 75% of the total intracellular Ca^{2+} reserve (Wood *et al.*, 1998) that is released either through IP_3Rs or through ryanodine receptors. The other important intracellular Ca^{2+} store is mitochondria (Tran *et al.*, 2006), accounting for the remaining 25% of the Ca^{2+} cellular reserve. Because of this, the functions of ER and mitochondria are intimately connected. These two organelles cooperate to generate Ca^{2+} signals within a highly interconnected network. The mitochondria assist with the recovery phase by rapidly sequestering Ca^{2+} and later returning it to the ER. It is important to note that mitochondria themselves are also excitable and capable of generating and conveying electrical and Ca^{2+} signals (Ichas *et al.*, 1997).

One of the functions of Ca^{2+} in cells is to activate transcription factors residing either in the cytoplasm (NF-AT, NF-κB) or in the nucleus (CREB) that can activate numerous target genes. The increase in Ca^{2+} is one of the signals that trigger proteolysis of IκB subunit leading to activation and translocation of NF-κB subunit to the nucleus. In the nucleus, CREB is phosphorylated by CAMKII and CAMKIV. Additionally, its co-activator CREB-binding protein (CBP) is also stimulated by Ca^{2+}.

LPC has been shown to increase $[Ca^{2+}]_i$ in endothelial cells resulting in calpain activation (Chaudhuri *et al.*, 2003), and in prostacyclin production, a research done by our group (Riederer *et al.*, 2010). Here we demonstrated the influence of multiple LPC species on the increase of $[Ca^{2+}]_i$, which was to our knowledge partially shown so far only in human neutrophils (Ojala *et al.*, 2007) and in HL-60 leukaemia cells (Okajima *et al.*, 1998). Additionally, in endothelial cells, the increase $[Ca^{2+}]_i$ was shown to lead to ERK activation (Watanabe *et al.*, 2006) , or to be suppressed by statins (Yokoyama *et al.*, 2002).

1.8 LPC receptors

There is evidence linking LPC-activated signals to possible orphan G protein-coupled receptors (GPCRs) GPR4 and G2A (Xu, 2002).

GPCRs are a superfamily of receptors that exert their activity by activating heterotrimeric guanine nucleotide binding proteins (G proteins), hence the name (Kristiansen, 2004). Binding of the ligand to the respective receptor promotes the release of free Gα and Gβγ subunits, which then initiate the intracellular signal transduction. GPCRs preferentially couple to G proteins grouped to four classes: $G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Simon *et al.*, 1991). Once activated, GPCRs promote a broad range of intracellular responses resulting in the modulation of many aspects of human physiology, growth, development and disease control (Pierce *et al.*, 2002).

GPR4 and G2A, together with T cell death associated gene 8 (TDAG8) and ovarian cancer GPCR 1 (OGR1) belong to OGR1 subfamily of GPCRs. This subfamily recognizes lysolipid molecules as ligands. Upon binding to their respective receptor, these lysolipid ligands activate various second messenger pathways like intracellular Ca^{2+}, inositol phosphate and cAMP.

In addition, this subfamily of GPCRs has been suggested to serve also as proton-sensing receptors activated by acidification of extracellular pH (Im, 2005; Meyer zu Heringdorf *et al.*, 2007; Tobo *et al.*, 2007). It was also shown that LPC serves as a ligand for GPR119, resulting in insulin secretion (Soga *et al.*, 2005). However, some actions of LPC seem to be independent of GPCR activation (Im *et al.*, 2006; Yun *et al.*, 2004).

GPR4 potentially serves as a receptor for sphingosylphosphorylcholine (SPC) and LPC. Whether this receptor serves as a receptor for LPC is still not clear because after the initial report showing ligand-receptor specificity, the same report has been withdrawn 4 years later (Zhu *et al.*, 2001).

GPR4 is widely expressed in the body, which suggests a broad biological importance (Zhu *et al.*, 2001). GPR4 is also highly expressed in many types of endothelial cells, whereas other members of the same family of receptors are expressed only weakly in the same types of cells (Kim *et al.*, 2005). Still, its physiological role is mostly unidentified. Both SPC and LPC are produced under normal and pathological conditions, which highlight its potential importance. It has been shown that inflammatory stress increases GPR4 expression in microvascular endothelial cells (Lum *et al.*, 2003), and upregulation of adhesion molecules by LPC in endothelial cells showed to be dependent

on GPR4 (Zou *et al.*, 2007). Even though some of the effects of LPC on GPR4 have been disputed, it was proven for SPC to have an angiogeneic effect in vivo that depends on GPR4 (Kim *et al.*, 2005).

1.9 Objective

Little is known about signal transduction promoted by EL-generated LPC species. In our present study, we aimed to elucidate the signaling cascade of different LPC species leading to upregulation of COX-2 in endothelial cells.

Since studies addressing the impact of LPC on endothelial COX-2 induction used exclusively 16:0 LPC (Rikitake *et al.*, 2001; Zembowicz *et al.*, 1995), nothing is known about the impact of length and degree of saturation of LPC- acyl chain on COX-2 expression in vascular endothelial cells, the main source of EL. In our previous study using human aortic endothelial cells (HAEC), LPC 16:0, 18:1, 18:2 and 20:4 only slightly increased COX-2 mRNA without affecting COX-2 protein expression (Riederer *et al.*, 2010). Therefore, the aim of present study was to compare the impact of the "standard" 16:0 LPC with that of unsaturated LPCs, on COX-2 expression in endothelial cell line EA.hy 926 and the underlying mechanisms.

To address this objective, we asked the following questions: (1) Do LPCs induce COX-2 mRNA and protein expression in endothelial cell line EA.hy926? (2) If so, which intracellular signaling mechanisms are responsible for this induction?

2. Materials and methods

2.1 Materials

2.1.1 LPC

Individual LPCs (16:0-, 18:1-, 18:2- and 20:4-LPC) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). LPCs were dissolved in chloroform/methanol solution under argon atmosphere and stored at -20 ºC. For cell culture experiments, required amount of LPCs were dried / evaporated under a stream of nitrogen or argon in glass tubes and resuspended in PBS (pH 7.4) to give a final concentration of 3 mM. The aliquots were vortexed at room temperature for 1 min (2x) to give a clear dispersion. The final concentration was achieved by dissolving required amount of LPCs/PBS solution in the cell culture medium.

2.1.2 Antibodies

Antibodies against COX-2 (M-19) (sc-1747) and β-actin (C4) (sc-47778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific antibody recognizing p38 dual-phosphorylated on Thr180 and Thr182 (#9211) and antibodies against total p38 (#9212), total c-Jun (#9165), total CREB (#9197) and total NF-κB p65 (#3987) were obtained from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated

2.1.3 Inhibitors

BAPTA/AM (Ca^{2+} chelator) was obtained from Calbiochem (San Diego, CA, USA). U-73122 (Phospholipase C (PLC) inhibitor), aminoethyl diphenylborinate (2-APB) (IP3 receptor inhibitor), SB203580 (p38 MAPK inhibitor), cycloheximide (inhibitor of protein synthesis), Actinomycin D (inhibitor of transcription), pertussis toxin (PTX) ($G\alpha_{i/o}$ inhibitor) and cholera toxin (CTX) ($G\alpha_s$) were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2 Methods

2.2.1 Cell culture

Human endothelial cell line EA.hy 926 is a hybridoma cell line formed by the fusion of human umbilical vein endothelial cells (HUVECs) with the human lung carcinoma cell line A549 (Edgell *et al.*, 1983). EA.hy 926 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% foetal bovine serum (FBS) (PAA) and 1% HAT Media Supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine). Cell culture medium was supplemented with penicillin G sodium sulphate (100 units/ml), streptomycin sulphate (100 µg/ml), and amphotericin B (2.5 µg/ml) (in the future text, referred as the cell culture medium). Cells were cultured in humidified atmosphere of 5% CO_2/ 95% air at 37°C and were sub-cultured every 3 days using 0.025% trypsin/ 0.01% EDTA at a ratio 1:4.

Cells were seeded in 12- or 6-well plates 24 hours before the start of the experiment.

2.2.2 LPC treatment of EA.hy 926 cells

EA.hy 926 cells were seeded in 6- or 12-well plates (2.5 x 10^5 or 1.1 x 10^5, respectively) 24 h before the start of LPC treatment. Subsequently, cell culture medium was replaced with the fresh cell culture medium supplemented with 200 µM LPCs. After the respective incubation times, cells were washed twice with ice cold PBS and lysed in buffers for isolation of RNA or proteins.

With this incubation medium, no cytotoxicity was observed, measured by lactate dehydrogenase release, at LPC concentrations up to 200 µM.

2.2.3 Pharmacological inhibitors

EA.hy 926 cells were seeded in 12-well plates (1.1 x 10^5 cells). Twenty four hours after plating, cells were pre-treated with respective pharmacological inhibitors of vehicle (DMSO) for 30 minutes before the addition of fresh culture medium containing LPCs supplemented with vehicle or inhibitors, respectively. The pharmacological inhibitors were used in following concentrations: BAPTA/AM (10µM), SB203580 (5 µM), U73122 (2 µM), cycloheximide (100 µM), Actinomycin D (800 nM), PTX (20 and 100 ng/ml) and CTX (200 ng/ml).

2.2.4 Quantitative real-time PCR

EA.hy 926 cells were grown in 12-well plates. After the respective treatment, cells were washed twice with PBS and total cellular RNA was isolated using the peqGOLD Total RNA Kit (Peqlab-biotechnology, Erlangen, Germany) according to the manufacturer's protocol. RNA purity and quantity was measured with a Nanodrop spectrophotometer (PeqLab) and samples with an absorbance of ≥1.8 measured at 260/280 nm were used for reverse transcription. 0,8 µg of total cellular RNA was used for synthesis of cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 2,8 U of an RNase Inhibitor (Qiagen, Hilden, Germany) added. Real-time PCR analysis was performed essentially as described previously (Riederer *et al.*, 2010). RT-PCR analysis was performed in 384-well plates in a total volume of 4 µl containing 2 ng of original total RNA using the QuantiFast SYBR green RT-PCR kit (Qiagen) and validated QuantiTect Primer Assays (Qiagen) according to the manufacturer's instructions for Light Cycler 480 instrument (Roche Diagnostics). In brief, after the initial heat activation step at 95 ºC for 5 min, reaction was performed for 40 cycles with denaturation at 95 ºC for 10 s, and combined annealing and extension at 60 ºC for 30 s. The PCR efficiency of the target and housekeeping genes was determined by cDNA dilution series prepared from un untreated sample, and results were accordingly efficiency-corrected with the LightCycler Relative Quantification software (Roche Diagnostics, Basel, Switzerland). mRNA levels of COX-2 (Primer Assay QT00040586) were normalized to human β-2-microglobulin (Primer Assay QT00088935) and expressed as relative ratio (ΔΔ Ct). All samples were assayed in duplicate, and the average value was used for quantification.

Other primers used: Jun (Primer Assay QT00242956), RelA (Primer Assay QT02324308) and CREB (Primer Assay QT00092435).

2.2.5 Protein isolation and Western blotting

EA.hy 926 cells grown in 6- or 12-well plates, transfected or nontransfected, were stimulated with LPCs (200 µM, 10% FBS) or PBS for respective time, rinsed twice with ice-cold PBS and lysed in ice cold RIPA buffer [25 mM Tris-HCl (pH 7.6), 1% NP-40, 150 mM NaCl, 1% sodium odeoxyccholate, 0.1% SDS] (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich), and in some experiments phosphatase inhibitor PhosSTOP (Roche), at 4 °C for 5 min. The whole cell lysates were briefly sonicated (3x

10s, 60% of the strength) on ice followed by 10 min centrifugation at 14000 x g, 4ºC to remove all the cytoskeletal structures. Lysates were stored at -20 ºC until use. Protein concentration was determined with the BCA protein assay kit (Novagen), using BSA as standard, according to the manufacturer's instructions. Samples were mixed with the 6x sample buffer, boiled for 10 minutes at 95 °C and equal amounts of cell lysate protein samples underwent gel electrophoresis using 10% SDS-polyacrylamide gels. Molecular weights of the detected proteins were estimated using Prestained Protein Ladder ~10-180kDa (Fermentas).

For detection of COX-2 protein, proteins were afterwards transferred to a nitrocellulose membrane (150 mA, 80 min) in a Bio-Rad Laboratories mini transblot apparatus. Membranes were cut and separated around protein size of 55 kDa. Membranes were blocked with 5% (w/v) non-fat powdered milk in TBS/T [25 mM Tris base (pH 7.4), 137 mM NaCl and 0.1% (v/v) Tween 20] for at least 1 h before probing with COX-2 (goat, 1:200) and β-actin (mouse, 1:1000) antibodies. Primary antibody incubation was performed 2% (w/v) milk on 4 °C overnight. Incubation with appropriate HPR-conjugated rabbit anti-goat (1:1500) and rabbit anti-mouse (1:2000) secondary antibodies (Dako) secondary antibodies incubation were performed in 2% (w/v) milk for 2 h at room temperature, all while slightly rocking. After primary and secondary antibody incubation, membranes were washed at least six times for 30 min using TBS/T.

For detection of NF-κB p65, CREB, c-Jun, p38 or phospho-p38 proteins were transferred to a PVDF membrane (150 mA, 60 min). Membranes were blocked with 3% (w/v) BSA in TBS/T for at least 1 h before probing with rabbit anti-human primary antibodies (all 1:1000) diluted in 3% (w/v) BSA on 4 °C overnight. Incubation with HPR-conjugated goat anti-rabbit (1:5000) secondary antibody (Thermo scientific) was performed in 2% (w/v) milk for 2 h at room temperature, all while slightly rocking. Again, after both primary and secondary antibody incubation, membranes were washed at least six times for 30 min with TBS/T.

Immunoreactive bands were visualised using a SuperSignal West Pico (Thermo Scientific) detection system. Densitometry analyses were carried out using ImageJ software.

2.2.6 siRNA transfection

Transfections were carried out using Nanofectin siRNA (PAA) according to the manufacturer's instructions. In all, 5.5 x 10^4 EA.hy 926 cells were plated in 12-well plates

24 h before the transfection with the relevant siRNA. 1 h before the transfection, 50-60% confluent cells were washed with pre-warmed PBS and incubated in DMEM without the addition of the serum and antibiotics. The transfection mixture was prepared by mixing 40 pmol (0,5 µg siRNA) of the respective siRNA with Nanofectin siRNA reagent in the ratio of 1:3 (µg of siRNA : µl of Nanofectin siRNA reagent) in medium without antibiotics and serum. After 20 minutes, the transfection complex was added drop-wise to the cells while slightly swirling. After 3 hours, the medium was exchanged with the fresh one containing antibiotics and serum. Cells were incubated at 37 ºC, 5% CO_2 for 48 h for target silencing. The siRNA library: RelA (SI003001672), c-Jun (SI00300580), CREB1 (SI00299894) and control (nonsilencing) siRNA (SI001027281) was from Qiagen.

2.2.7 Plasmid DNA transfection

Control pCXN2.1 and pCXN2.1+GPR4-hemagglutinin (HA) tagged plasmid constructs were kindly provided by Dr. Takao Shimizu, Faculty of Medicine, The University of Tokyo, Japan. In all, 8 x 10^4 EA.hy 926 cells were seeded in 12-well plates 24 h before the transfection with the relevant plasmids (GPR4 and the control plasmid) using Nanofectin (PAA) according to the manufacturer's instructions. In detail, the cell culture medium from 60% confluent cells was aspirated and replaced with 1 ml of serum and antibiotics free medium1 h before the transfection. In the first tube, 1 µg of plasmid DNA per well was mixed with 50 µl of the diluent provided with the Nanofectin kit, and in the second tube, 2 µl of Nanofectin reagent was dissolved in 50 µl of the diluent. The content of the second tube (Nanofectin) was added to the first tube (DNA) and mixed briefly by vortexing. The mixture was incubated on the room temperature for 20 min, and then added drop-wise to the cells and homogenized by gently swirling the plate. Three hours post transfection, the transfection medium was aspirated and replaced with fresh cell culture medium (containing serum and antibiotics). 36 h following transfection cells were treated with 16:0 LPC for 16 h. Total cell protein content was collected in RIPA buffer and Western blot for COX-2 was performed as described in section 2.2.5.

2.2.8 Measurements of intracellular Ca^{2+}

Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined using the fluorescent Ca^{2+} indicator fura-2-acetoxymethyl ester (Fura-2 /AM). EA.hy 926 cells were grown in 75 cm^2 flasks until confluent and were serum starved for 2 hours. Cells were harvested by trypsinization and loaded with 2 µM Fura-2 for 45 min at room temperature in the dark.

Then the extracellular Fura-2/AM was removed by centrifugation and for the measurements in the presence of Ca^{2+} the medium was exchanged with HEPES-buffer solution containing in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/NaOH, pH=7.4, whereas for the Ca^{2+} free measurements, the buffer composition was in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 0,1 EGTA,10 glucose and 10 HEPES/NaOH, pH=7.4. The Fura-2 fluorescence intensity was monitored spectrophotometrically every 0.1 sec in a stirred cuvette at alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm before and after the injection of 1, 3, 10 and 30 μM LPCs. In some experiments, cells were resuspended in EGTA containing buffer and preincubated with 2-APB (100 μM, 2 min) or U71322 (2 μM, 5 min) before the injection of LPCs. All the experiments were conducted at room temperature. Data were expressed as ratio values of Fura-2/AM fluorescence (340/380 nm).

2.2.9 Prostanoid measurement by competitive enzyme immunoassay

Measurements for 6-keto $PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2 were performed with Enzyme Immunoassay kits (EIA) (Cayman, Ann Arbor, MI) according to the manufacturer's protocol. In detail, 1.1 x 10^5 EA.hy 926 cells were seeded in 12-well plates 24 hours before the beginning of experiment. 90% confluent cells were exposed to 200 μM 16:0 LPC for 16 h in the cell culture medium, washed twice with PBS, and then incubated for 30 min with exogenous AA (10 μM) dissolved in medium without addition of the serum. 100 μl of medium from each well was collected and saved at -80 °C, and later was processed for prostanoid release according to the manufacturer instructions.

The test is based on the competition between prostanoid measured and a prostanoid-acetylcholinesterase (AChE), the prostanoid tracer. The EIA plates come precoated with mouse anti-rabbit IgG and blocked with a proprietary formulation of proteins. Wells were incubated with a tracer, an antiserum, and either a standard or a 10-fold diluted sample. Following 18 hour incubation on room temperature, the plates were washed to remove any unbound reagents, and Ellman's Reagent containing the substrate to AChE was added. After 1 hour of incubation, the resulting yellow colour was measured spectrophotometrically at 412 nm. Results were normalised to the protein content of cells collected from the same well (measured by BCA protein assay) and expressed as pg of prostanoid/μg of protein.

2.2.10 Luciferase assay

COX-2 luciferase reporter vector construct (nucleotide -1432/+57) was provided by Dr. Hiroyashu Inoue, Faculty of Human Life and Environment, Nara Women's University, Nara, Japan . 8 x 10^4 EA.hy 926 cells were seeded in 12-well plate 24 hours before the start of transfection. One hour before the start of the transfection, the cell culture medium was replaced with 500 µl of fresh culture medium. 70% confluent EA.hy 926 cells were co-transfected with COX-2 promoter luciferase construct and a pSV-β-galactosidase construct, in total 1.2 µg in the ratio 1:1 using FuGENE 6 transfection reagent (Promega) according to the manufacturer's instructions. In detail, FuGENE 6 transfection reagent was added to the cell culture medium (containing serum and antibiotics) and after mixing, it was incubated on room temperature for 5 min. Appropriate amount of DNA (1.2 µg) was added to FuGENE 6/medium to achieve the ratio of FuGENE 6 transfection reagent to DNA 3:1 (3 µl of FuGENE : 1 µg DNA). After 15 min incubation, the mixture was added to wells, containing exactly 1 ml of cell culture medium, and mixed gently. 6 hours after transfection, transfection medium was exchanged and replaced with medium containing 16:0 LPC (200 µM, 10% FBS) for 16 hours. Subsequently, cells were washed twice with prewarmed PBS, collected in 100 µl of the passive lysis buffer and centrifuged on 14000 g 10 min to remove the cell pellet.

First, β-galactosidase activity was assayed using β-galactosidase enzyme assay system (Promega) according to the manufacturer's instruction. In detail, 50 µl of the diluted cell lysate (1:4 dilution with passive lysis buffer) was mixed carefully by pipetting with 50 µl of Assay 2x buffer (containing the substrate for β-galactosidase o-nitrophenyl-β-D-galactopyranoside (OPNG) in a well of a 96-well plate. After 30 min of incubation at 37° C, during which a faint yellow colour has developed, reaction was stopped by adding 150 µl of sodium carbonate (1 M). The absorbance of the samples was read at 405 nm in the Victor 3 Multilabel Counter (PerkinElmer).

Subsequently, luciferase activity was assayed using Luciferase Assay System (Promega). In detail, 10 µl of cell lysate was added to 50 µl of luciferase assay reagent II placed in a well of a 96-well white luminometer plates, and immediately luminescence was measured for 10 s in the Victor 3 Multilabe Counter.

Results were expressed as the fold change of luciferase activity normalised to the β-galactosidase activity.

2.2.11 Statistical analysis

Experiments were performed at least three times and the data are represented as the mean ± standard deviation. To compare the differences between two groups, student´s unpaired t-test (two-tailed) was performed. To compare the differences between multiple groups, statistical analysis was performed using one-way Anova followed by Bonferroni's multiple comparison test. Statistically significant differences between groups are indicated by *P* values of < 0.05 (*), < 0.01 (**), or < 0.001 (***).

3. Results

3.1 LPC induce COX-2 mRNA in endothelial cells

Previously it was shown that 16:0 LPC increases COX-2 expression in a several cell lines (Zembowicz *et al.*, 1995),(Gwak *et al.*, 2006; Rikitake *et al.*, 2001). Since by the action of EL, many different LPC species are created (Gauster *et al.*, 2005), it is possible that all of them have an effect on COX-2 expression. To investigate the effect of 16:0, 18:1, 18:2 and 20:4 LPC on COX-2 gene expression, EA.hy926 cells were exposed to respective LPCs (200 μM in the presence of 10 % FBS) for up to 24 hours. The concentrations of LPC and FBS were similar as in previous studies (Riederer *et al.*, 2011; Zembowicz *et al.*, 1995). The cells were harvested for total RNA and analysed by quantitative real-time RT-PCR, using primers specific for COX-2. The quantitative analysis showed that when the cells were stimulated with 16:0 LPC, COX-2 mRNA, normalized to β2-microglobulin, increased already after 1 h of stimulation by 3.7-fold and had a sustained increase, and a maximal upregulation of 61- and 41-fold after 16 and 24 h, respectively. Under the same culture conditions, 18:1 LPC elicited only weak upregulation of COX-2 mRNA after 1 h of stimulation, which returned to the basal level in the later time points. 20:4 LPC showed a different pattern of COX-2 mRNA upregulation. It induced a significant COX-2 mRNA upregulation after 3 hours of stimulation (2.4-fold) with a further sustained increase after 5 h (3.6-fold) and 8 h (4.7-fold), followed by a decrease after 16 h (2.4-fold) and a maximal 13-fold upregulation after 24 hours of stimulation. On the contrary to other LPCs used, 18:2 LPC didn't induce COX-2 mRNA upregulation at any time point measured (Figure 1A).

To show that the concentration of 200 μM in the presence of 10% FBS is required to overcome scavenging of LPC by serum proteins, cells were incubated with increasing concentrations of 16:0 LPC ranging from 100 μM to 200 μM in the presence of 10% FBS (Figure 1B). COX-2 mRNA upregulation was dose dependent, with significant rise in COX-2 mRNA already at the concentration of 175 μM.

To address whether the upregulation of COX-2 mRNA levels induced by 16:0 and 20:4 LPC was the result of the *de novo* mRNA synthesis, EA.hy 926 cells were pretreated with the transcriptional inhibitor actinomycin D (800 ng/ml) for 30 min, and then incubated with 16:0 and 20:4 LPC (200 μM, 10 % serum) for 1 and 4 hours, respectively, in the presence of the inhibitor. The complete suppression of COX-2 mRNA upregulation

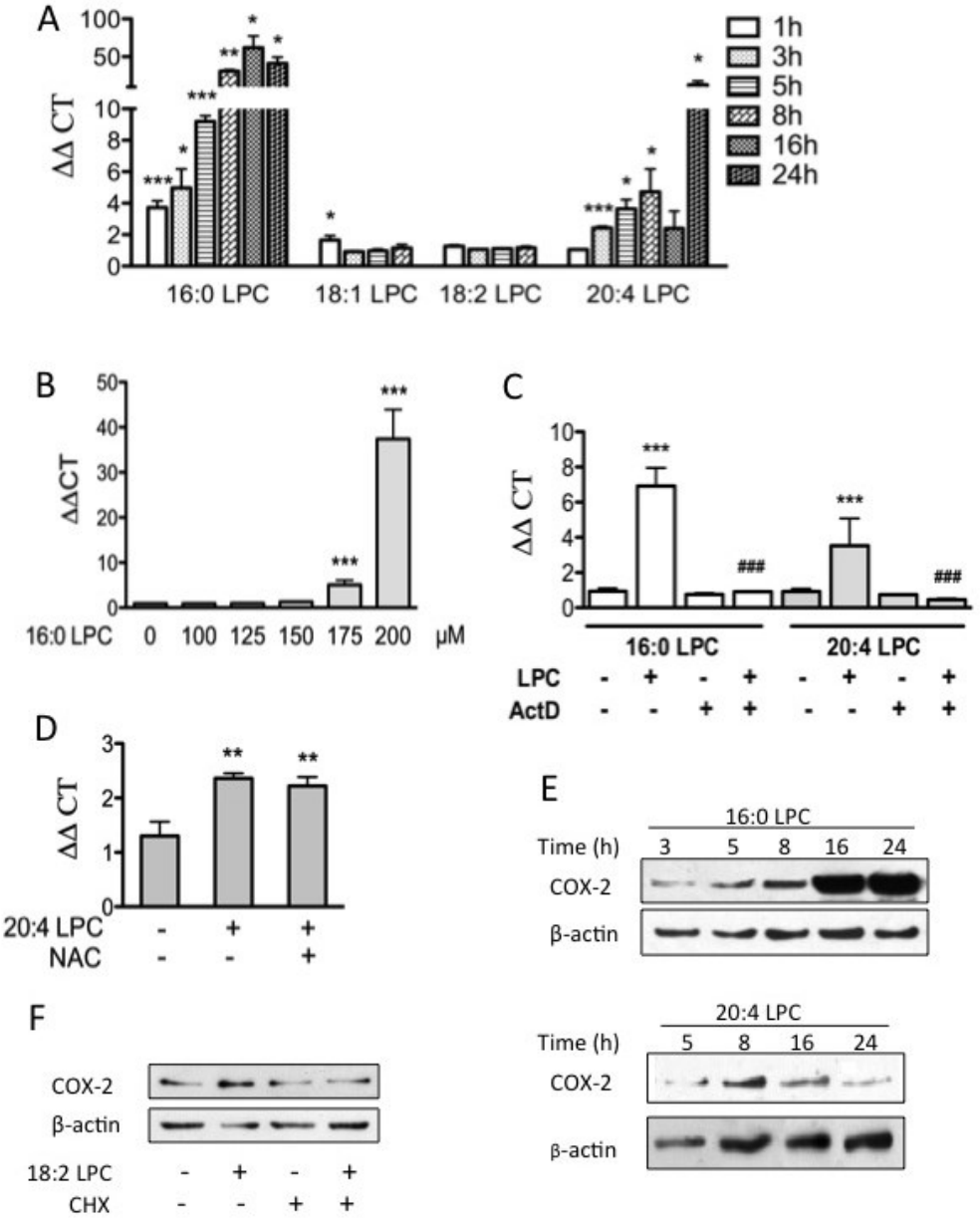

Figure 1. LPCs increase COX-2 m-RNA and protein expression. EA.hy 926 cells were incubated with 200 μM LPC or PBS in medium containing 10% FBS for the indicated time points, followed by the determination of (**A**) COX-2 mRNA by qRT-PCR and (**E**) Western blotting. In (B) cells were incubated with rising concentrations of 16:0 LPC for 16 h. In (**C**) cells were pre-incubated with actinomycin D (ActD) (800 nM) for 30 min, followed by incubation without or with LPC 16:0 and 20:4 for 1 or 4 hours, respectively in the presence or absence of ActD. In (**D**) cells were pre-incubated with N-acetyl cysteine (NAC) (1mM) for 30 min, followed by incubation with 20:4 LPC for 4 hours in the presence of NAC. Subsequently, relative COX-2 mRNA expression was determined by qRT-PCR as detailed in Materials and methods. In (F) cells were pre-incubated with cycloheximide (CHX) (100 μM) or vehicle, followed by incubation with or without 18:2 LPC in the presence or absence of CHX for 5 h. Subsequently, expression of COX-2 protein was analysed by Western blotting. Results shown in (A-D) are mean ± SD from three independent experiments performed in triplicates. Y axis indicates the fold induction of gene expression (corrected for β2M expression) and normalised to PBS, which is set to 1. Results in (E) and (F) are representative Western blots. *, **, *** indicate significant differences between LPC and PBS treated cells. $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ indicate significant differences between LPC treated cells in the presence or absence of the applied inhibitor.

indicates that 16:0 and 20:4 LPC-elicited upregulation of COX-2 mRNA is dependent on *de novo* RNA synthesis (Figure 1C).

To test whether the slow developing 20:4 LPC-induced response was the result of the 20:4 LPC oxidation occurring during the prolonged incubation period of 4 h, cells were co-incubated with antioxidant N-acetylcysteine (NAC). The incubation with NAC didn't prevent the 20:4 LPC-induced COX-2 upregulation (Figure 1D).

In line with the pronounced upregulation of COX-2 mRNA, both 16:0 and 20:4 LPC yielded elevation of cellular COX-2 protein content, however with different potency and kinetics (Figure 1E). Under similar culture conditions as described above, induction of COX-2 protein with 16:0 LPC was detectable from 5 h, reaching plateau after 16 h of stimulation with 200 μM LPC. The induction of COX-2 protein was the strongest with 16:0 LPC, but other LPCs tested also showed upregulation of COX-2 protein at certain time points: 20:4 LPC was able to induce COX-2 protein upregulation with maximal expression after 8 hours of incubation. 18:2 LPC, used in the same concentration, showed COX-2 protein upregulation after 5 hours of incubation, even though it showed to be incapable of inducing COX-2 mRNA, and this increase could be completely abolished with cycloheximide (Figure 1F). On the other hand, 18:1 LPC failed to promote COX-2 protein expression (data not shown). These results demonstrated acyl-chain dependent differences in the capacity and kinetics of LPC-mediated COX-2 induction at both mRNA and protein levels.

3.2 Effects of 16:0 LPC on prostanoid production in EA.hy 926 cells

Our findings showed that LPCs modulate the expression of COX-2 mRNA and protein in EA.hy 926 cells. In order to test the functionality of the newly synthesized COX-2 protein produced upon the exposure to 16:0 LPC, EA.hy 926 cells were incubated with 16:0 LPC (200 μM, 10% FBS) for 16 hours followed by 30 minutes of incubation with exogenous arachidonic acid (AA) (10 μM) in serum free medium.

In response to 16:0 LPC, EA.hy 926 cells were able to produce a wide array of prostanoids: 6-keto $PGF_{1\alpha}$ (a stable product of PGI_2 degradation), thromboxane B_2 (TxB_2, a stable product of TxA_2), PGE_2 and $PGF_{2\alpha}$ (Figure 2A-D).

As shown in Figure 2A, 16:0 LPC strongly increased the formation of 6-keto $PGF_{1\alpha}$ relative to PBS control. The increases in TxB_2 (Fig. 2B) and PGE_2 (Figure 2C) were

equally high, whereas the increase in $PGF_{2\alpha}$ was less pronounced (Figure 2D), but equally statistically significant.

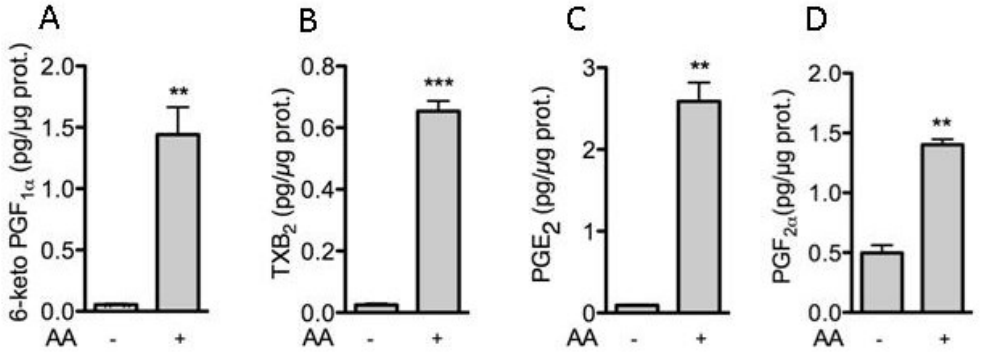

Figure 2. 16:0 LPC-elicited prostanoid production in EA.hy 926 cells. EA.hy 926 cells were treated with 16:0 LPC (200 µM) in medium containing 10% FBS for 16 h. Subsequently, cells were washed and incubated with 10 µM arachidonic acid in serum-free medium. Cell culture supernatants were subjected to EIA (described in Methods chapter 2.2.9) to measure the formation of (A) 6-keto $PGF_{1\alpha}$, (B) TxB_2, (C) PGE_2 and (D) $PGF_{2\alpha}$. Results are means ± SD of two experiment performed in duplicate. *,**, *** indicate significant differences when compared with values for medium treated cells.

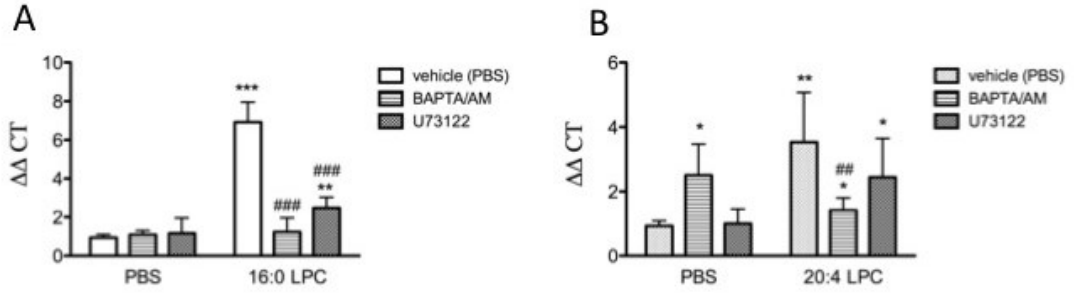

Figure 3. Intracellular Ca^{2+} is involved in LPC-elicited COX-2 upregulation. EA.hy 926 cells were pre-incubated with the intracellular Ca^{2+} chelator (BAPTA/AM) (10 µM) or PLC inhibitor (U71322) (2 µM) for 30 min before they were exposed to 200 µM LPC 16:0 and 20:4 with or without inhibitors in medium containing 10% FBS for 1 and 4 hours, respectively. Subsequently, COX-2 mRNA expression was determined by qRT-PCR. Results are mean ± SD of three independent experiments performed in triplicates. *, **. *** indicate significant differences between PBS and LPC treated cells. #, ##, ### indicate significant differences between LPC treated cells in the absence and presence of respective inhibitors.

3.3 The role of intracellular Ca^{2+} in LPC-elicited COX-2 mRNA upregulation

Since alterations in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ are implicated in activation of various signaling pathways (Knot *et al.*, 2005) and regulation of gene

expression (Dolmetsch *et al.*, 1997), we examined the influence of intracellular Ca^{2+} increase by incubating cells with Ca^{2+} chelator BAPTA/AM as well as PLC inhibitor U73122 on the COX-2 mRNA upregulation elicited by LPC 16:0 and 20:4. Intracellular Ca^{2+} buffering and inhibition of PLC abolished 16:0 LPC-elicited induction of COX-2 mRNA (Figure 3A). In contrast, 20:4 LPC-elicited induction of COX-2 mRNA was decreased by BAPTA/AM, but the effect of U73122 was weak and not statistically significant (Figure 2B). Surprisingly, BAPTA/AM increased COX-2 mRNA in vehicle treated cells after longer exposure time of 4 h (Figure 2B), whereas U73122 didn't exert a significant effect on the basal COX-2 mRNA expression at any time point measured. This data suggest that LPC-elicited COX-2 mRNA upregulation is dependent on the increase of intracellular Ca^{2+}, a pathway that involves PLC.

3.4 LPCs induce rise in $[Ca^{2+}]_i$

To study the effect of LPCs on the increase of intracellular calcium, increasing concentrations (1, 3, 10 and 30 μM) of single LPCs (16:0, 18:1, 18:2 and 20:4 LPC) were applied to EA.hy 926 cells in the absence of foetal bovine serum (FBS) in a buffer with 2 mM calcium. In presence of extracellular Ca^{2+}, all tested LPCs induced slow, transient and concentration dependent increase in $[Ca^{2+}]_i$ in EA.hy 926 cells (Figure 4 A-D). Unlike other LPCs, 16:0 LPC elicited at 30 μM a pronounced and sustained Ca^{2+} response possibly due to the plasma membrane damage (Figure 4A).

The increase in cytosolic Ca^{2+} turned to be acyl chain-dependent. When the cells were incubated with 3 μM LPCs in the presence of extracellular Ca^{2+}, the increase in $[Ca^{2+}]_i$ induced with 16:0 LPC was the most pronounced, followed by 18:1, 18:2 and 20:4 LPC (Figure 5A).

In the absence of extracellular Ca^{2+} (EGTA containing buffer), 3 μM LPCs were still able to induce Ca^{2+} transients showing the same acyl-chain dependency, however, the magnitudes of the transients evoked by all LPCs were profoundly lower (Figure 5B).

In nominally calcium free environment, U73122 (Figure 5C) and 2-APB, an inositol-3 phosphate (IP_3) receptor antagonist (Figure 5D), both blocking Ca^{2+} depletion from endoplasmic reticulum (ER), completely blocked the increase of $[Ca^{2+}]_i$ elicited with all tested LPCs. From these results we could conclude that all LPCs, at the physiological concentration of 3 μM elicit an increase of $[Ca^{2+}]_i$ due to the Ca^{2+} release from intracellular stores via PLC/IP_3-mediated mechanism.

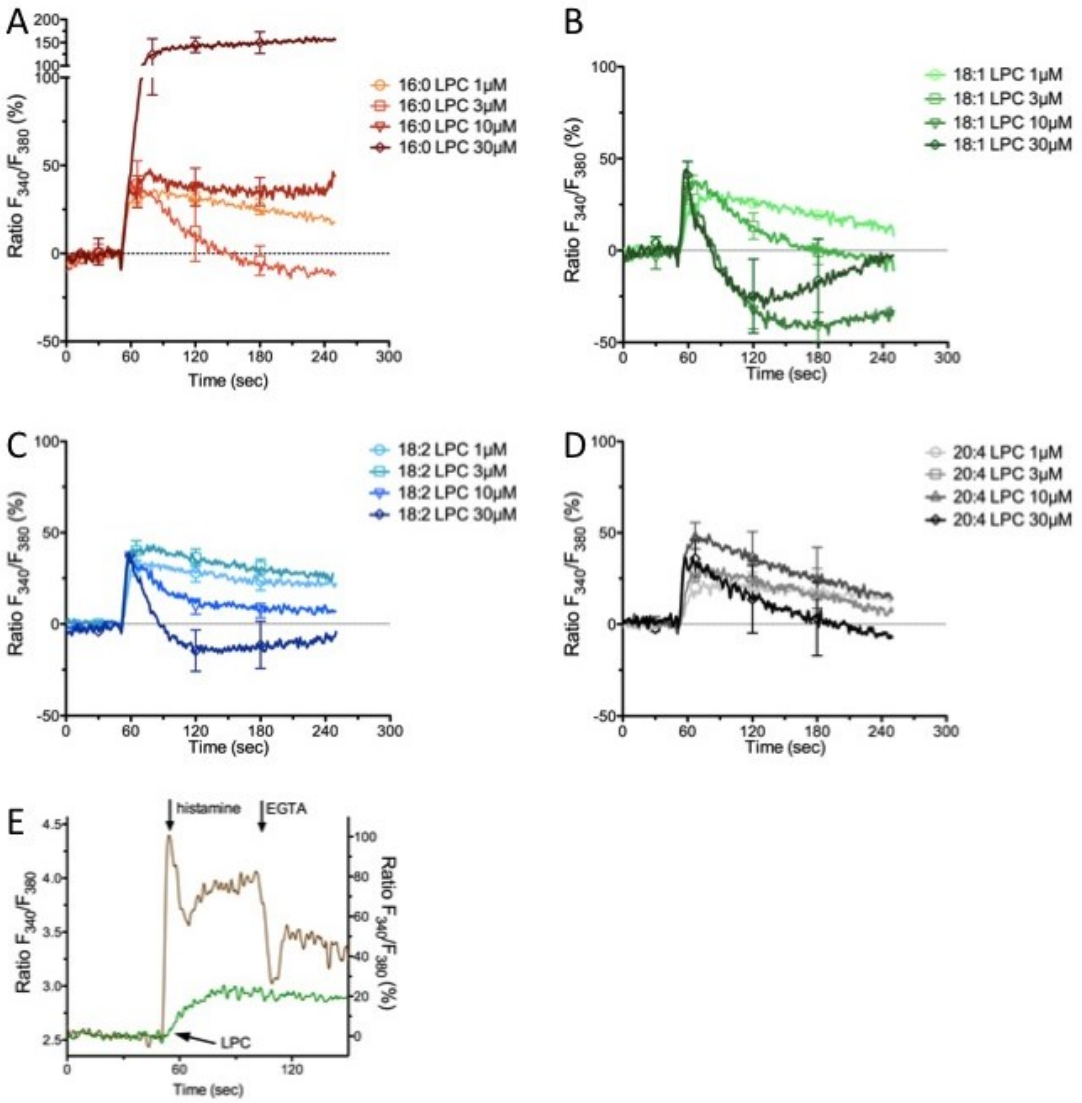

Figure 4. LPCs induce cytosolic Ca^{2+} increase in the presence of extracellular Ca^{2+} in EA.hy 926 cells. EA.hy 926 cells were loaded with fura-2/AM-loaded. Increasing concentrations (1, 3, 10 and 30 μM) of LPC 16:0 (A), 18:1 (B), 18:2 (C) and 20:4 (D) were applied at t=60 s. and the ratio of Fura-2/AM fluorescence intensity (340/380 ratio) was monitored spectrophotometrically in a stirring cuvette. Traces represent the change of Fura-2/AM fluorescence ratio normalized to the max. histamine response (100 μM) in the same cells (E). Results are means ± SE of two experiments performed in triplicate.

3.5 p38 MAPK mediates LPC-elicited COX-2 mRNA upregulation

Since p38 MAPK has been shown to mediate the induction of COX-2 mRNA triggered by various stimuli (Rikitake *et al.*, 2001), we investigated whether LPC 16:0 and 20:4 induced COX-2 expression through p38 MAPK activation. EA.hy 926 cells were pretreated with SB203580 (5 μM), a specific p38 MAPK inhibitor, for 30 min and then incubated with LPC 16:0 and 20:4 (200 μM) in the presence of SB203580, for 1 or 4

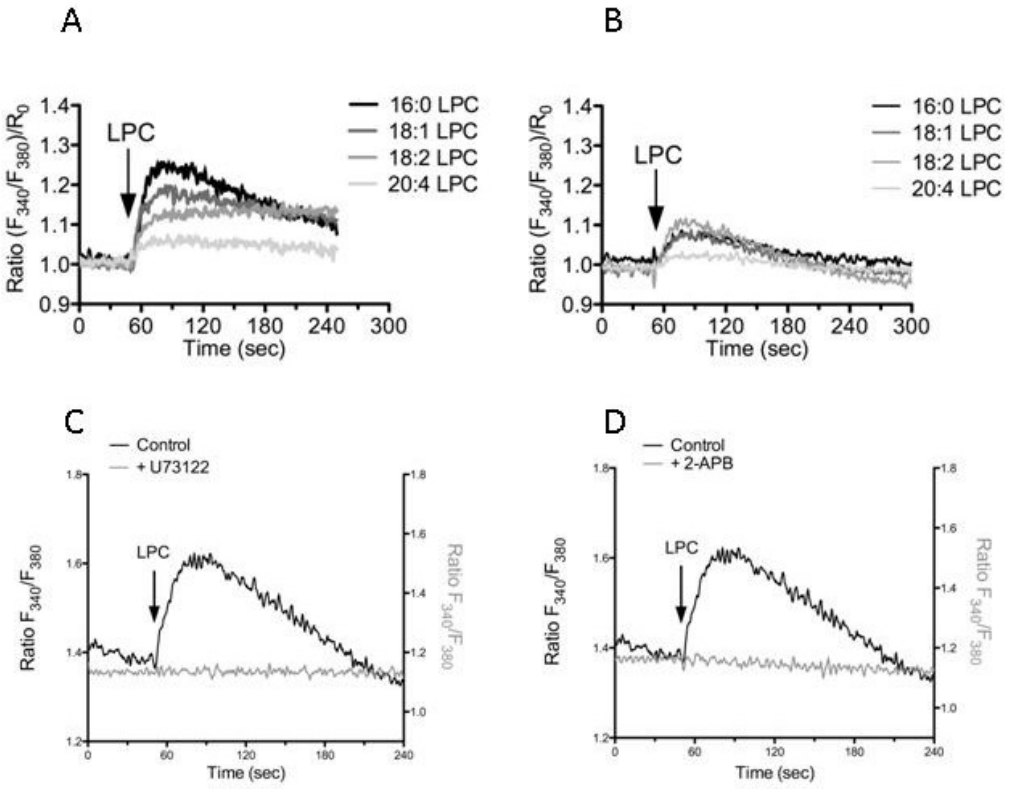

Figure 5. Impact of LPCs on cytosolic calcium concentration $[Ca^{2+}]_i$. Fura-2/AM loaded EA.hy 926 cells were trypsinized and resuspended either in Ca^{2+}-containing buffer (A) or a nominally Ca^{2+}-free buffer containing EGTA (B-D). The ratio of Fura-2/AM fluorescence intensity (340/380 ratio) was monitored spectrophotometrically in a stirring cuvette during exposure to 3 μM LPCs (A-D). In (C), cells were preincubated with U73122 (2μM, 5 min) and in (D) with 2-APB (100 μM, 2 min) before the addition of LPCs. Results in (A) and (B) are representative single traces out of four experiments performed in duplicates, and in (C) and (D) representative single traces showing the effect of inhibitors on the increase of $[Ca^{2+}]_i$ induced by 18-2-LPC (3μM) out of four experiments performed in duplicates.

hours respectively, and the RNA was collected. As shown in Fig. 6A and B, SB203580 markedly inhibited both 16:0 and 20:4 LPC-induced COX-2 expression. These data suggest that p38 MAPK is activated upon incubation with LPCs and that it may promote COX-2 upregulation induced by various LPCs.

To confirm that p38 MAPK is involved in signaling cascade induced with various LPCs, we examined the activation of p38 after incubation of endothelial cells with LPCs. As shown in figure 6C (upper panel), 16:0 LPC induced rapid and transient p38 MAPK phosphorylation which became evident after 20 min of exposure and reached a maximum after 30 min before returning to baseline levels by 2 hours. 18:1 LPC was also able to induce p38 MAPK phosphorylation with a similar pattern as 16:0 LPC but to a smaller extent (Figure 6C lower panel). In contrast to 16:0 and 18:1 LPC-induced p38 MAPK

phosphorylation, 20:4 LPC promoted a sustained, long lasting phosphorylation of p38 MAPK, with a maximum achieved only after 3 h of exposure and the signal lasting up to 8 h (Figure 6D upper panel). As expected, 18:2 LPC did not significantly modify p38 MAPK phosphorylation state at any time point measured (Figure 6D lower panel). In all cases the level of total p38 MAPK protein remained unmodified under the same experimental conditions. These data demonstrated that p38 MAPK is activated upon incubation with LPCs in acyl-chain-dependent manner.

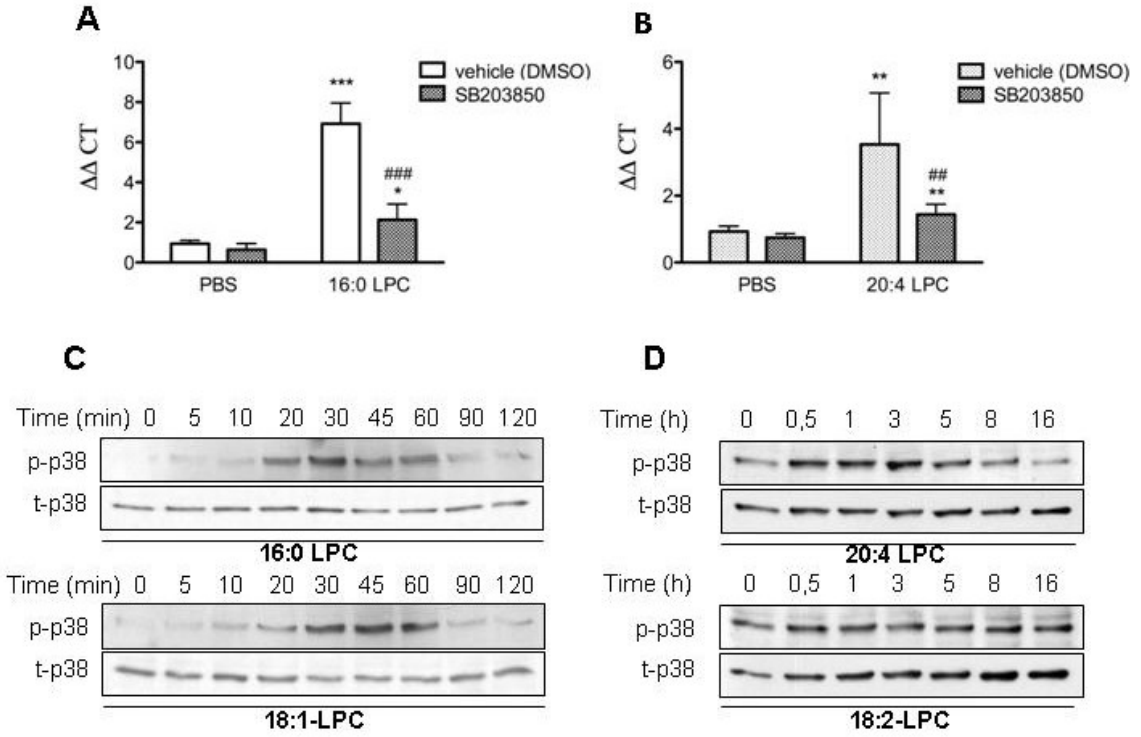

Figure 6. p38 MAPK is involved in LPC-elicited COX-2 mRNA upregulation. EA.hy 926 cells were pre-incubated with SB203580 (5μM), a p38 MAPK inhibitor, for 30 min before they were exposed to 200 μM LPC in the presence of the vehicle or inhibitor in medium containing 10% FBS for 1 h (16:0 LPC) (A) or 4 h (20:4 LPC) (B). Subsequently, relative COX-2 mRNA expression was determined with qRT-PCR. Results are mean ± SD of three independent experiments performed in triplicates. *, **, *** indicate significant differences between LPC and PBS treated cells; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ indicate significant differences between LPC treated cells in the presence of vehicle and the inhibitor. (C) and (D): EA.hy 926 cells were stimulated with (C) 16:0 and 18:1 LPC or (D) 18:2 and 20:4 LPC (all 200 μM in medium containing 10% FBS) for indicated periods of time. Phosphorylation of p38 was determined by Western blot analysis of phospho- (p) and total- (T) p38 MAPK (as described in section 2.2.5). The data are representatives from 3 independent experiments.

3.6 NF-κB, AP-1 and CREB mediate LPC-elicited COX-2 mRNA and protein upregulation

To demonstrate directly that 16:0 LPC upregulates COX-2 at the transcriptional level, we performed transient transfection experiments using full-length (-1432/+59) promoter-luciferase construct (a kind gift from-see methods) using EA.hy 926 cells. Upon incubation with 16:0 LPC, luciferase activity, normalised as described in the section 2.2.10, was 2 fold higher compared to vehicle treated cells proving that 16:0 LPC induces the activity of COX-2 promoter (Figure 7A).

COX-2 promoter activity is regulated by several transcription factors including NF-κB, NF-IL6, AP-1, CRE and NFAT (Iniguez *et al.*, 1999), (Callegari *et al.*, 2006), (Rikitake *et al.*, 2001). To understand the role of the selected transcription factors (NF-κB, CREB and c-Jun) and their involvement in COX-2 induction with LPC 16:0 and 20:4, experiments were performed in cells with silenced p65 subunit of NF-κB, CREB and c-Jun, a component of AP-1.

As shown in the figure 7B, the gene silencing procedure almost completely depleted p65 and CREB (99 and 98%, respectively) and markedly reduced c-Jun (50%), demonstrated by Western blot, as compared to the control siRNA-transfected cells.

In 16:0 LPC treated cells, after 16 h of incubation, the impact of p65 silencing on COX-2 protein induction was less pronounced compared to that of c-Jun and CREB silencing (Figure 7D, left panel). In contrast, the impact of p65 silencing had more profound attenuating effect on 20:4 LPC-, compared with 16:0 LPC-elicited COX-2 protein induction and was similar to the impact of CREB silencing (Figure 7D, right panel).

The impact of CREB silencing on COX-2 protein induction was similar for both LPC species tested (Figure 7D), i.e. CREB silencing efficiently attenuated LPC-induced COX-2 protein upregulation.

Those observations on the impact of silencing of various transcription factors on COX-2 protein levels were further confirmed at mRNA levels (Figure 7C).

While c-Jun silencing efficiently attenuated COX-2 mRNA (Figure 7C, middle panel) and protein expression (Figure 7D, left panel) elicited by 16:0 LPC, the data obtained for 20:4 LPC were controversial and not conclusive as in some sets of experiments c-Jun silencing decreased COX-2 mRNA (Figure 8A, left panel) and

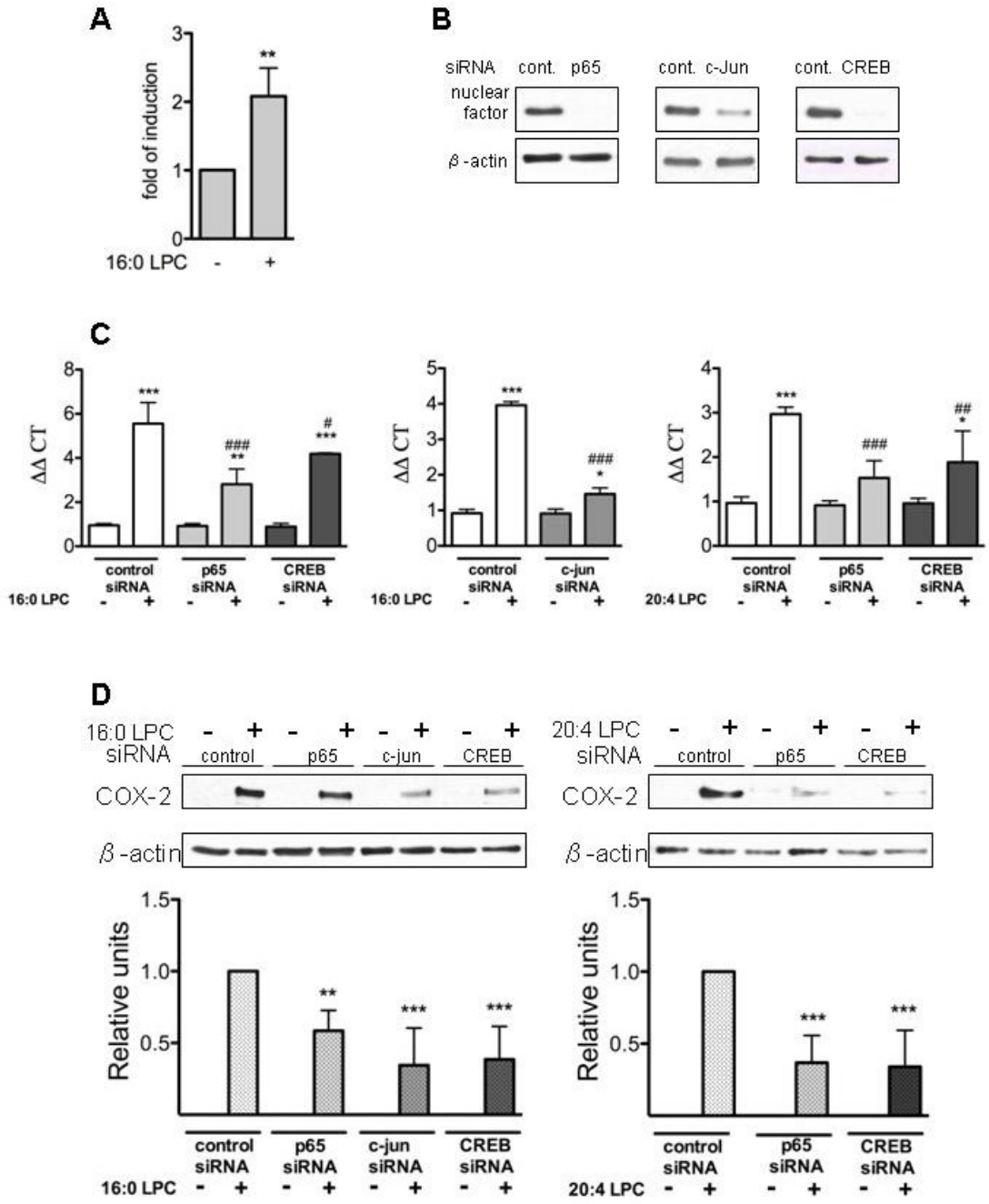

Figure 7. 16:0 LPC induces COX-2 promoter activity and NF-κB, c-Jun and CREB are involved in LPC-elicited COX-2 mRNA and protein upregulation. (A) Whole length COX-2 promoter (-1432/+59) luciferase construct was transfected into EA.hy 926 cells using FuGENE 6. 6 h post transfection cells were incubated with 16:0 LPC (200 μM, 10% FBS) for 16 h. The results are mean ± SD of 2 experiments performed in triplicate. The luciferase luciferase activity was normalized to β-gal activity and presented as a fold increase against the value obtained from vehicle treated cells. **P□0.01 versus control. 48 h after transfection with p65, c-Jun, CREB or scrambled siRNA (control), EA.hy 926 cells were treated with 16:0- or 20:4-LPC for 16 and 8 h, respectively. Subsequently, protein levels of (B) p65, c-Jun and CREB as well as of COX-2 mRNA (C) and protein (D) were analysed by quantitative real-time PCR and Western blot, respectively. In (C) mean ± SD of three experiments performed in duplicates are presented. *, ** and *** indicate significant differences between PBS and LPC treated cells. #, ##, ### indicate significant differences between LPC treated cells transfected with control vs. specific siRNA. In (D) representative images are presented. COX-2 densitometric values are normalized to β-actin

and expressed as fold-induction, setting the value of cells transfected with control siRNA and treated with LPCs as 1. Bars represent the mean ± SD of three experiments performed in triplicates. *, **, *** indicate significant difference between LPC treated cells transfected with control vs. specific siRNA.

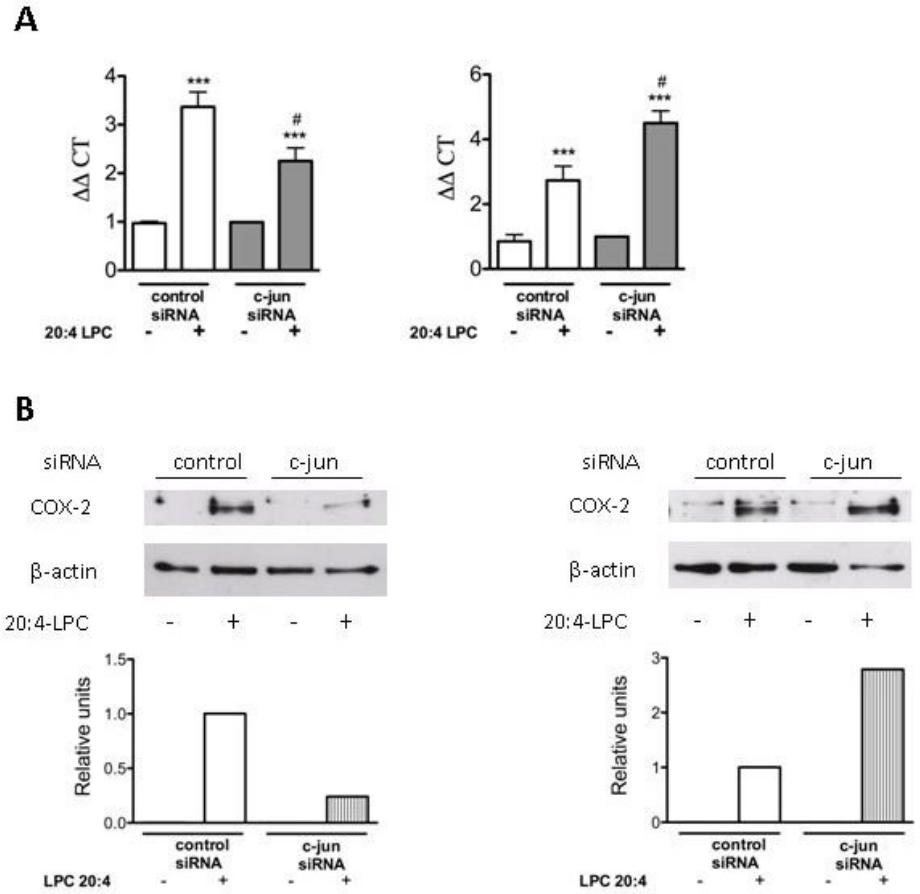

Figure 8. Impact of c-Jun on 20:4 LPC-elicited COX-2 mRNA and protein upregulation. 48 h after transfection with c-Jun scrambled siRNA (control), EA.hy 926 cells were treated with 20:4-LPC for 8 h. Subsequently, COX-2 mRNA (A) and protein (B) were analysed by quantitative real-time PCR and Western blot, respectively. In (A) mean ± SD of three experiments performed in duplicates are presented. *, ** and *** indicate significant differences between PBS and LPC treated cells. #, ##, ### indicate significant differences between LPC treated cells transfected with control vs. c-jun siRNA. In (B) representative images are presented. COX-2 densitometric values are normalized to β-actin and expressed as fold-induction, setting the value of cells transfected with control siRNA and treated with LPCs as 1.

protein (Figure 8B, left panel), but in other sets of experiments it led to increased COX-2 mRNA (Figure 8A, right panel) and protein (Figure 8B, right panel).

These results suggest that the two LPCs partially differ in utilization of signalling pathways for COX-2 upregulation.

3.7 GPCRs are possibly not involved in LPC-induced COX-2 induction

To determine whether LPCs act over GPCRs and which class of Gα subunit is involved in signal transduction upon exposure to LPCs, EA.hy 926 cells were preincubated with pertussis toxin (PTX), a known inhibitor of the $G\alpha_{i/o}$ subunits of GPCRs (New *et al.*, 2007), for 16 h, or with cholera toxin (CTX), a known inhibitor of the $G\alpha_s$ subunit of GPCRs (New *et al.*, 2007) for 30 min prior the exposure to 16:0 or 20:4 LPC.

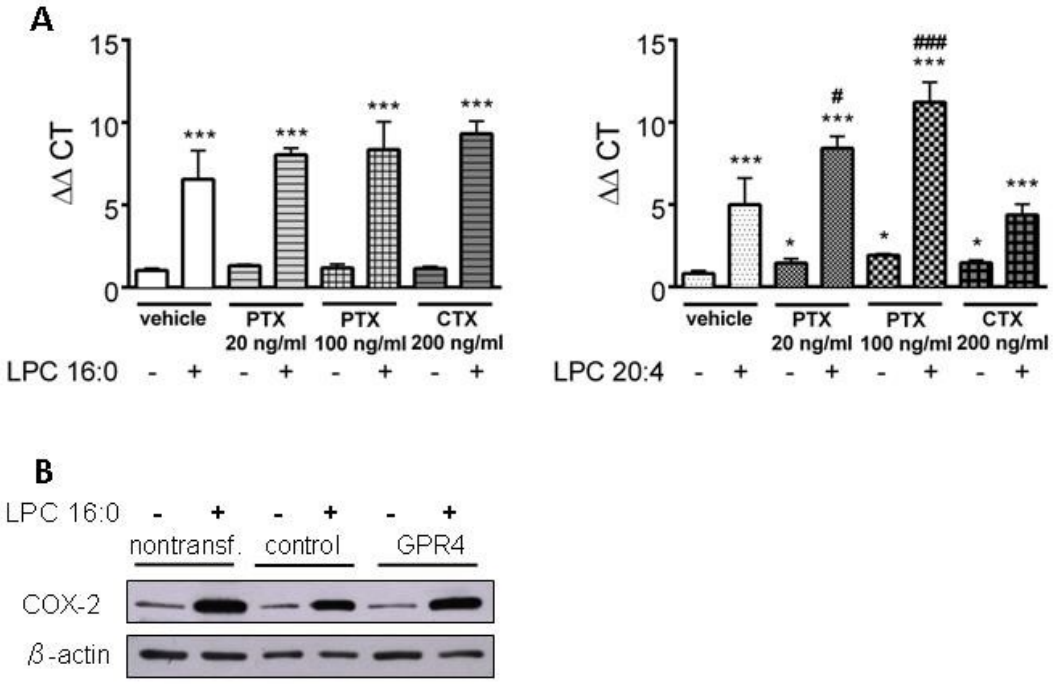

Figure 9. Impact of pertussis toxin (PTX), cholera toxin (CTX) and GPR4 on LPC-induced COX-2 mRNA and protein upregulation. (A) PTX, an inhibitor of $G\alpha_{i/o}$ subunits of GPCRs, and CTX, an inhibitor of the $G\alpha_s$ subunit of GPCRs were preincubated with EA.hy 926 cells for 16 h or 30 min, respectively, and 16:0 LPC (left panel) and 20:4 LPC (right panel) (200 μM, 10% FBS) were added to the cells for 1 or 4 h, respectively. COX-2 mRNA was measured with quantitative real-time PCR. The results are mean ± SD of two experiments performed in triplicate. *, ** and *** indicate significant differences between PBS and LPC treated cells. #, ##, ### indicate significant differences between LPC treated cells treated with inhibitors vs. vehicle treated cells. (B) EA.hy 926 cells were transfected with pCXN2.1+GPR4-HA or the control plasmid using Nanofectin. 36 h post transfection, cells were treated with 16:0 LPC (200 μM, 10% FBS) for 16 h. Representative picture of three experiments done in duplicates is presented.

PTX and CTX failed to affect the upregulation of COX-2 mRNA by both tested LPCs. PTX didn't influence the LPC 16:0-elicited COX-2 mRNA induction, whereas it potentiated the stimulatory effect of LPC 20:4 on COX-2 mRNA induction; however, it

also led to the constitutive increase of COX-2 basal levels in control cells at both concentrations tested.

Similarly, CTX also failed to affect the LCP 16:0-induced COX-2 mRNA upregulation, whereas it potentiated the stimulatory effect of 20:4-elicited COX-2 mRNA induction, and it led to the constitutive COX-2 upregulation in control cells.

In conclusion, incubation with PTX and CTX failed to exert inhibitory effect on COX-2 mRNA upregulation elicited by both tested LPCs.

Some of the previous studies have shown that some of the LPC-induced cellular responses are mediated through GPR4 (Zou *et al.*, 2007). EA.hy 926 cells were transfected with pCXN2.1+GPR4-HA plasmid or the control plasmid as described in detail in the chapter 2.2.7. 36 h later cells were treated with 16:0 LPC, and COX-2 protein expression was monitored. As shown in figure 9B, incubation of EA.hy 926 cells with 16:0 LPC resulted in the upregulation of COX-2 protein expression. This expression wasn't further upregulated upon transfection of EA.hy 926 with GPR4, suggesting that LPC-induced COX-2 upregulation is not mediated via putative LPC receptor GPR4.

4. Discussion

In this doctoral thesis, we investigated the signaling pathway downstream of 16:0, 18:1, 18:2 and 20:4 LPC leading to the stimulated expression of COX-2 in human endothelial cell line EA.hy 926. Previously, we identified LPC 16:0, 18:1, 18:2 and 20:4 as major LPC species generated by the action of EL on HDL (Gauster *et al.*, 2005). These LPC species are also prevalent in plasma and tissues (Ojala *et al.*, 2007), and they proved to be capable of promoting endothelial prostacyclin and IL-8 production in acyl-chain-dependent manner (Riederer *et al.*, 2011; Riederer *et al.*, 2010).

The LPC concentration of 200 μM in the presence of 10% FBS, used in this thesis, was not toxic to cells (Huang *et al.*, 2005) and was within the (patho)physiological range of 150 mM-1.7 mM (Chen *et al.*, 1997; Ojala *et al.*, 2007; Rabini *et al.*, 1994). LPCs are normally present in the plasma bound to albumin and other carrier proteins, and the concentration of monomeric LPCs in the circulation still remains to be determined. Albumin has been shown to contain one to three high-affinity binding sites and multiple low-affinity binding sites for LPC (Kim *et al.*, 2007). It has already been shown that the presence of albumin greatly influence the bioactivity of LPC (Huang *et al.*, 2005). Hence, an appropriate ratio of LPC to FBS, imitating situations where the amount of LPC locally exceeds the binding properties of albumin and other lipid binding proteins and is available to bind to the possible cell surface receptors, is required to yield the free LPC capable of evoking cellular responses (Riederer *et al.*, 2011; Riederer *et al.*, 2010). The relationship between LPC and FBS in the present study was similar as in previous studies, where cells were exposed to 100 μM LPC in the presence of 5% FBS (Riederer *et al.*, 2011; Zembowicz *et al.*, 1995).

Even though it was previously observed that method of LPC preparation can drastically affect the functional properties of LPCs (Lin *et al.*, 2005; Silliman *et al.*, 2003), for intracellular Ca^{2+} measurements, cells were exposed to LPCs dissolved in serum free aqueous solution, as this experimental setup is necessary for this type of measurements.

Throughout this study, we used an endothelial cell line EA.hy 926 derived from HUVECs, which has been shown previously to be a good model for endothelial cells not only regarding its morphology, expression of endothelial markers and response to

physiological agonists (Edgell *et al.*, 1990; Edgell *et al.*, 1983), but also in terms of prostacyclin production (Suggs *et al.*, 1986).

In contrast to our previous study performed in human aortic endothelial cells (HAEC) (Riederer *et al.*, 2010), where LPC elicited only upregulation of COX-2 at mRNA but not protein level, in the present study performed in EA.hy 926 cell line, LPC yielded increases in both COX-2 mRNA and protein content. This is in line with previous findings (Rikitake *et al.*, 2001; Zembowicz *et al.*, 1995) describing the impact of 16:0 LPC on COX-2 expression in endothelial cells where 16:0 LPC increased COX-2 both mRNA and protein. The discrepancy between the present and previous study (Riederer *et al.*, 2010) might be explained by the different experimental conditions: 200 μM LPC in the presence of 10% FBS in the present study versus 10 μM LPC in the absence of serum in the previous study. Additionally, the fact that EA.hy 926 cells originate from HUVEC where both COX-2 mRNA and protein were upregulated upon exposure to LPC 16:0 (Zembowicz *et al.*, 1995), might explain the observed better responsiveness of EA.hy 926 cells than HAEC in terms of COX-2 upregulation.

In the present study we found remarkable acyl chain-dependent differences in the capacity of LPCs to induce COX-2 expression. 16:0 LPC was the most potent inducer of COX-2 mRNA and protein expression which is in line with eliciting the most potent release of Ca^{2+} from intracellular stores. 18:2 and 20:4 LPC also showed to be capable of increasing COX-2 protein, and 18:1 and 20:4 LPC also demonstrated the capacity to increase COX-2 mRNA. LPC 20:4, despite capacity to induce COX-2 mRNA, however upon a prolonged incubation with cells, was the least potent inducer of intracellular Ca^{2+} increase. In our previous study, LPCs didn't show the ability to modulate COX-2 protein expression (Riederer *et al.*, 2010), but this might be due to lower responsiveness of HAEC to LPC in terms of COX-2 induction in comparison to EA.hy 926, a cell line derived from HUVECs.

The concentration of LPCs used in this study was 200 μM. When checking the concentration dependency of 16:0 LPC in the range from 100-200 μM, the 175 μM concentration of 16:0 LPC was also able to raise COX-2 mRNA levels upon 16 h-incubation period. That clearly points out the requirement for the free, non-protein bound LPC, as the concentrations of 150 μM or lower didn't induce the upregulation of COX-2 mRNA. In a previous study addressing the response of macrophages to LPC (Olofsson *et al.*, 2008), the importance of serum concentration relative to that of LPC was emphasized

as the authors noticed that LPC didn't induce macrophage inflammatory protein-2 (MIP-2) expression at 100 μM in the presence of 10% serum. This is also in accordance with several other studies demonstrating the attenuating imapct of albumin on the bioactivity of LPC (Huang *et al.*, 2005; Ojala *et al.*, 2007; Vuong *et al.*, 2003).

The COX-2 mRNA increase elicited by 20:4 LPC was slow developing. Since oxidized phospholipids, that could be generated during the prolonged incubation period, have been shown to induce some other cytokines involved in the process of atherosclerosis (Yeh *et al.*, 2004), we tested if the slow developing 20:4 LPC induced COX-2 mRNA increase could reflect the action of oxidized 20:4 LPC. The effect of 20:4 LPC could not be prevented with the addition of an antioxidant NAC which led us to the conclusion that oxidative modification of 20:4 LPC was not likely to be the reason for the observed kinetics of the COX-2 increase induced by 20:4 LPC.

Alternatively, the slow-developing increase of 20:4 LPC-induced COX-2 mRNA could also be explained by the action of slow-emerging metabolic conversion products of 20:4 LPC, like AA whose bioavailability may increase during prolonged incubation. AA can be formed by different cellular phospholipases including $cPLA_2$ that can also by its lysophospholipase activity accomplish the release of AA from 20:4 LPC (de Carvalho *et al.*, 1995). AA is capable of modulating COX-2 expression in endometrial stromal cells and in pancreatic cancer cells (Hughes-Fulford *et al.*, 2005; Zhao *et al.*, 2012) through a mechanism dependent upon downstream synthesis of PGE_2. It is conceivable that similar situation might occur in EA.hy 926 cells as 16:0 LPC, in addition to induction of various other prostanoids, induced the production of PGE_2 (Figure 3C).

Another striking finding of the present study was upregulation of COX-2 protein but not mRNA by 18:2 LPC. It is possible that some of the effects exerted with 18:2 LPC come from the 18:2 (linoleic) FFA itself. Several groups demonstrated the effect of linoleic acid on the expression of adhesion molecules in endothelial cells (Jung *et al.*, 2012; Sanadgol *et al.*, 2012). Additionally, it was demonstrated that linoleic acid affects the COX-2 expression induced by TNF-α (Matesanz *et al.*, 2012). Linoleic acid can also exhibit some other effects on endothelial cells - it was shown that linoleic acid can induce endothelial injury resulting in decreased barrier function and appearance of cytosolic lipid droplets (Ramasamy *et al.*, 1991).

Future experiments should reveal mechanisms underlying 18:2 LPC-elicited COX-2 protein upregulation unrelated to COX-2 mRNA.

What are the physiological implications of our data? In this research, we have only briefly addressed this issue. So far, the role of COX-2 in atherosclerosis is not completely known. COX-2 is overexpressed in human atherosclerotic lesions (Schonbeck *et al.*, 1999). It has been proposed that it can have both antiatherogenic and proatherogenic role, depending on the exact eicosanoids produced and the exact cells of the arterial wall where it is expressed (Linton *et al.*, 2002). Many physiological processes involved in atherosclerosis, including leukocyte-endothelial cell adhesion and vasorelaxation, are also regulated by eicosanoids.

In order to check the functionality of COX-2 upregulation, we examined the impact of 16:0 LPC on the production of wide array of different prostanoids, as the increased PG synthesis in EA.hy 926 cells after treatment with 16:0 LPC would reflects an increase in functional COX-2 protein.

Previous studies already established the impact of 16:0 LPC (Rikitake *et al.*, 2001; Zembowicz *et al.*, 1995) and unsaturated 18:1, 18:2 and 20:4 LPC (Riederer *et al.*, 2010) on endothelial prostanoid production with the focus on PGI_2 because it is the prostaglandin predominantly produced by endothelial cells (Thiemermann, 1991). It is believed that PGI_2 has a protective role in atherosclerosis (Thiemermann, 1991). Additionally, COX-2 is involved in PGI_2 biosynthesis in healthy humans (McAdam *et al.*, 1999). As found by Elisa analysis of the cell culture medium, EA.hy 926 cells showed to be capable of secreting both proconstricting and prorelaxation prostanoids.

EA.hy 926 exposed to LPC showed increased production of PGI_2 (measured by detection of its stable degradation product 6-keto $PGF_{1\alpha}$), PGE_2, $FGF_{2\alpha}$ and TxA_2 (measured by detection of its degradation product TxB_2). The production of PGI_2 showed to be the most upregulated, in comparison to other prostanoids measured. The observation that COX-2 induced by 16:0 LPC resulted in increased production of both antiatherogenic prostanoids like PGI_2, and proatherogenic prostanoids, like PGE_2, PGF_2 and TBX_2 indicates that COX-2 expression in the blood vessel wall could play both proatherogenic and antiatherogenic role. Thereby the relative abundance and activity of elicited prostanoids as well as the presence of corresponding receptors in endothelial as well as underlyimng smooth muscle cells would be crucial for the overall impact and vessel wall response.

Our results are in accordance with the previous research demonstrating that LPC increases PGI_2 release in EA.hy 926 cells (Olszanecki *et al.*, 2006). Our previous finding, showing that 16:0 LPC is not a potent inducer of PGI_2 synthesis (Riederer *et al.*, 2010), is

in contradiction with the present data. The reason for lower amount of PGI_2 produced by HAECs could be that this cell line didn't show responsiveness to 16:0 LPC in terms of induction of COX-2 protein, unlike EA.hy 926 cells. Additionally, COX-2 in the previous study was induced with 16:0 LPC for only 5 hours, a time interval which even in the present study was not sufficient for an efficient induction of COX-2 protein. Furthermore, in the present study, in contrast to the previous one (Riederer *et al.*, 2010) COX-2 induced with 16:0 LPC was supplied with exogenous substrate, AA.

Our finding of a high PGI_2 induction is also in contrary to the observations of Zembowicz et al. who demonstrated a weak induction of PGI_2 production in response to 16:0 LPC in spite of reported COX-2 mRNA and protein upregulation (Zembowicz *et al.*, 1995). This could again be explained with a different incubation time with 16:0 LPC and provision of exogenous AA.

Our previous study demonstrated that HAECs produce PGE_2 in the resting state (Riederer *et al.*, 2010), and other group showed that nonstimulated EA.hy 926 cells produce much more PGE_2 than PGI_2 (Olszanecki *et al.*, 2006). The same group failed to demonstrate the increase of PGE_2 production when the cells were given the substrate AA, which was stated for PGI_2. However, the increase of PGE_2 was demonstrated in curcumin treated human coronary artery endothelial cells (HCAECs) after supplementation of culture medium with AA, and this increase was blocked with a COX-2 inhibitor (Tan *et al.*, 2011).

It is known that endothelial cells express PGI_2 synthase, PGE_2 synthase and thromboxane synthase (Olszanecki *et al.*, 2006; Riederer *et al.*, 2010; Tan *et al.*, 2007), so those prostanoids are most likely produced from PGH_2 by enzymatic transformation. In our experiments, we cannot exclude the possibility that a small portion of the prostanoids are a product of nonenzymatic transformation of PGH_2 (Camacho *et al.*, 1998).

The colocalisation and functional coupling of $cPLA_2$ and COX-2 upon exposure to calcium-mobilizing agents were observed in EA.hy 926 cells (Grewal *et al.*, 2005). In our study it is not clear whether COX-2 induction after 16 h has physiological importance, since calcium-dependent phospholipase A_2 ($cPLA_2$)- mediated AA supply to COX-2 is most likely not active any more after that long exposure (16 h) to 16:0 LPC. In our experimental setup we bypassed this problem by giving exogenous AA, but several groups demonstrated that by giving AA, even resting cells can produce PGI_2 and PGE_2 (Olszanecki *et al.*, 2006; Tan *et al.*, 2011). As in the state of inflammation the levels of endothelial lipase, capable of enzymatically cleaving HDL-derived PC, are elevated thus

producing various LPCs, one could speculate that in such cases endothelial cells could constantly be exposed to a mixture of LPCs, including 20:4 LPC, which can by providing its own AA serve as a substrate for COX-2 (Riederer *et al.*, 2010). Accordingly, it could be speculated that the cells would constantly be provided with a COX-2 substrate. Furthermore, any event leading to an increase in endothelial $[Ca^{2+}]_i$ would activate the $cPLA_2$ and provoke the mobilization of arachidonic acid, and we have demonstrated that all the mayor LPC species are capable of inducing the mobilization of Ca^{2+} from intracellular stores.

However, it is important to mention that previously it was demostrated that upon the uptake to the cells, LPCs are converted to PC (Stoll *et al.*, 1992), and that most of the AA released by the action of $cPLA_2$ would be used for reacylation of LPCs, whereby only small amounts of free AA serve as the substrate for COX-2.

Further studies to clarify the exact potential role of prostaglandins produced by the LPC-induced COX-2 protein in the endothelium would be necessary.

The activation of COX-2 after LPC exposure seems to be calcium dependent, as the activation of COX-2 could also be prevented by preincubation with BAPTA, an internal calcium chelator.

Indeed, in the present study we found acyl chain-dependent differences in the capacity of LPCs to induce the increase in $[Ca^{2+}]_i$. Some reports have already demonstrated the increase in $[Ca^{2+}]_i$ induced by 16:0 LPC in endothelial cells, and some have shown the effect of different LPC species on human PMNs (Lee *et al.*, 2004; Ojala *et al.*, 2007; Silliman *et al.*, 2003). This study is the first one to show that multiple LPC species can trigger the release of Ca^{2+} from intracellular stores in endothelial cells.

16:0 LPC proved to be the most potent inducer of rise in $[Ca^{2+}]_i$, followed by 18:1, 18:2 and finally 20:4 LPC. In the presence of EGTA, the extracellular Ca^{2+} chelator, those LPCs were still able to induce the increase in $[Ca^{2+}]_i$ with the same potency order suggesting that at least part of the Ca^{2+} released comes from the intracellular stores.
As mentioned before, the most potent induction of COX-2 mRNA by 16:0 LPC might be explained by it's most pronounced capacity to increase $[Ca^{2+}]_i$. Indeed, both chelation of intracellular Ca^{2+} with BAPTA/AM and inhibition of PLC with U73122 markedly attenuated the effect of 16:0 LPC on COX-2 mRNA. Along these lines, a negligible effect of LPC 18:1 and a complete failure of LPC 18:2 to induce COX-2 mRNA might be due to

their weaker capacity to increase $[Ca^{2+}]_i$ (Riederer *et al.*, 2010). However, the 20:4 LPC-elicited induction of COX-2 mRNA, although considerably lower than that induced by 16:0 LPC, was pronounced and sustained, despite a weak capacity of 20:4 LPC to increase $[Ca^{2+}]_i$, suggesting the involvement of additional Ca^{2+}-independent mechanism(s).

PLC has been shown to be an important component of the signaling pathways in activation of p38 (Jing *et al.*, 2000). Even though the PLC inhibitor U73122 considerably blocked COX-2 expression induced by 16:0 LPC, it showed only a tendency of COX-2 downregulation when the cells were incubated with 20:4 LPC. This might be due to the limited bioavailability of U73122 because of its chemical reactivity with the components of cell culture medium i.e. 10% serum used in our experimental model (Wilsher *et al.*, 2007). The compound didn't display a significant effect on the basal COX-2 mRNA expression.

Because BAPTA/AM increased basal COX-2 mRNA and U73122 failed to decrease 20:4 LPC-induced COX-2 mRNA, the impact of intracellular Ca^{2+} on 20:4 LPC mediated upregulation of COX-2 mRNA could not be conclusively evaluated.

It is well known that COX-2 expression has been linked with activation of MAPK pathways and that the particular signaling pathway involved is dependent on the type of stimuli (Guan *et al.*, 1998a; LaPointe *et al.*, 1999). p38 MAPK plays an important role in the expression of proinflammatory molecules and the regulation of cellular responses during infection and has been widely investigated for an effect on COX-2 at translational and transcriptional levels (Chien *et al.*, 2006; Rikitake *et al.*, 2001).

Therefore, in the present study we investigated whether the inhibition of p38 MAPK affects the LPC-induced COX-2 mRNA expression. We showed that the p38 MAPK pathway is responsible for the induction of COX-2 by 16:0 and 20:4 LPC. This is in the accordance with the previous results demonstrating the involvement of p38 MAPK in 16:0 LPC-induced COX-2 upregulation in bovine arterial endothelial cells (BAECs) (Rikitake *et al.*, 2001).

In contrast to a rapid and transient activation of p38 MAPK induced by 16:0 LPC, which was similar as shown previously in endothelial cells (Murugesan *et al.*, 2003; Rikitake *et al.*, 2001; Yamakawa *et al.*, 2008) and other cell types (Jing *et al.*, 2000), 20:4 LPC elicited a sustained, long-lasting p38 MAPK activation. Additionally, here we showed for the first time that 18:1 LPC also induces activation of p38 MAPK in a pattern

similar to 16:0 LPC, even though 18:1 LPC didn't demonstrate potency in inducing COX-2 mRNA, and it didn't induce COX-2 protein at any time point measured. This could partially be explained by the lower potency in raising intracellular $[Ca^{2+}]_i$, a signaling mechanism that acts in addition to MAPK activation. We also showed that LPC 18:2 did not activate p38 MAPK at any time point measured, which wasn't surprising considering its failure to induce upregulation of COX-2 mRNA.

The sustained p38 activation elicited with 20:4 LPC might be responsible for a substantial COX-2 mRNA increase independent of, or in addition to rise in $[Ca^{2+}]_i$. We examined the connetion between LPC-induced activation of p38 MAPK and Ca^{2+} signalling. We found , in accordance with the previous study (Rikitake *et al.*, 2001) that blocking of Ca^{2+} signaling with BAPTA/AM could not prevent LPC-induced p38 phosphorylation (data not shown). This finding indicates that LPC by inducing both p38 MAPK and increase in intracellular Ca^{2+} triggers two independent signalling pathways which contribute to COX-2 upregulation.

Although COX-2 mRNA upregulation induced by 16:0 and 20:4 LPC was completely suppressed by actinomycin D, indicating that the increase in COX-2 mRNA levels occurs mainly at the transcriptional level, we cannot exclude that the observed increase was at least in part due to stabilization of COX-2 mRNA. This is likely, because both 16:0 and 20:4 LPC activate p38 MAPK, a potent promoter of COX-2 mRNA stability (Yamakawa *et al.*, 2008). p38 MAPK plays a housekeeping role in maintaining COX-2 mRNA stability via the recognition of the AUUUA motifs present in the 3' untranslated region of the COX-2 (Inoue et al. 2002). Previously, p38 MAPK has been implicated in the stabilization of COX-2 mRNA in some cell lines (Dean *et al.*, 1999; Norata *et al.*, 2004; Yamakawa *et al.*, 2008). Other research also implicate that p38 MAPK is activated by LPC and that it induces certain stimulating factors which then stabilize COX-2 mRNA (Yamakawa *et al.*, 2008).

To give additional proof that the increase of COX-2 mRNA happens mainly as a result of a new, LPC-elicited transcription, we performed luciferase assay using a luciferase construct containing COX-2 promoter region -1432 to +59. The exposure to 16:0 LPC of EA.hy 926 cells transfected with a that COX-2 promoter construct led to a significant increase in reporter gene activity. The increase in RLU together with qRT-PCR results confirmed activation of COX-2 promoter and *de novo* COX-2 mRNA synthesis upon exposure to LPC.

The relative contribution of underlying signaling pathways and induction of COX-2 protein are markedly different for 16:0 and 20:4 LPC independently of whether induction of transcription or stabilization of mRNA or both is responsible for COX-2 mRNA upregulation.

The transcriptional regulation of the COX-2 gene is complex and varies according to the cell type and the stimulus applied. Here we show that both 16:0 and 20:4 LPC, activate multiple transcription factors, most likely simultaneously, suggesting that LPCs can activate multiple signaling cascades. That concept is not a novel finding as the binding of more than one transcription factor to the promoter regions was already demonstrated.

The relative contribution of various promoter elements to COX-2 transcription so far has not been completely understood. The promoter region of the COX-2 gene contains multiple sequences that act as positive regulatory elements in various cells types (Smith *et al.*, 2000) and multiple potential cis-acting elements (Kosaka *et al.*, 1994). Up to the present time, the involvement of NF-κB, AP-1, NF-IL6 and CRE in COX-2 expression have been reported. In this study, 16:0 and 20:4 LPC increased the COX-2 mRNA and protein levels in a NF-κB, c-jun and CREB dependent way.

To confirm the role of NF-κB, c-Jun and CREB role in the LPC induced COX-2 expression, EA.hy 926 cells were transfected with siRNA specific for RelA (p65), c-Jun or CREB to specifically silence these proteins and then stimulated with 16:0 and 20:4 LPC.

Western blot and real time PCR analysis revealed that silencing of those transcription factors almost completely reduced their protein levels and had an impact on LPC-induced COX-2 expression, thus suggesting the role for NF-κB, c-Jun and CREB in COX-2 transcriptional activation.

NF-κB plays an important role in pathogenesis and development of atherosclerosis as many genes whose expression is increased in atherosclerotic plaques, like ICAM-1 (Poston *et al.*, 1992), VCAM-1 (O'Brien *et al.*, 1993), MCP-1 (Takeya *et al.*, 1993) or COX-2 (Schonbeck *et al.*, 1999), have NF-κB binding sites in their promoter region. NF-κB is one of the most extensively investigated transcription factors for the regulation of COX-2. It has been shown that NF-κB is a positive regulator of COX-2 expression in a wide array of cell types (Inoue *et al.*, 1999; Inoue *et al.*, 1995; Janicke *et al.*, 2003; Lee *et al.*, 2012; Nakao *et al.*, 2000; Yan *et al.*, 2002).

In many cell types, in basal state, NF-κB can be found in the cytosol in the inactive state bound to it's inhibitory protein, IκB. Following a plethora of different stimuli, it is activated by dissociation from IκB and translocated to the nucleus (Ghosh *et al.*, 2012). Previously, 16:0 LPC-induced activation of NF-κB was demonstrated in endothelial cells (Sugiyama *et al.*, 1998), resulting in ICAM-1 expression (Zhu *et al.*, 1997), or in the release of macrophage inflammatory protein-2 (MIP-2) in macrophages (De Plaen *et al.*, 2006; Olofsson *et al.*, 2008). Furthermore, the activation of NF-κB by 16:0 LPC appears to be dependent on LPC concentration. Thereby lower concentrations of LPC were found to be capable of promoting endothelial NF-κB activity, and in contrast higher concentrations of LPC attenuated NF-κB activity (Sugiyama *et al.*, 1998). Our data are in agreement with above mentioned results of previous studies, since siRNA-mediated silencing and in turn downregulation of RelA subunit of NF-κB significantly attenuated and almost abrogated both 16:0 LPC- and 20:4 LPC-induced COX-2 upregulation. Thus, our results demonstrate for the first time that NF-κB mediates, in addition to well established signalling and COX-2 upregulation elicited by 16:0 LPC, the signaling and COX-2 upregulation by 20:4 LPC as well. Since the impact of NF-κB silencing on COX-2 protein was even more pronounced for 20:4 LPC, one can conlude that in our experimental systmen primarily NF-κB (along with CREB) executes 20:4 LPC-induced COX-2 expression.

cAMP-responsive element-binding protein (CREB) is known as a regulator of COX-2 expression in several cell lines (Eliopoulos *et al.*, 2002; Rikitake *et al.*, 2001). CREB binds to CRE sequence within COX-2 promoter when after its activation, it binds to its transcription coactivator protein, CREB binding protein (CBP), or the related protein p300. Their interaction with p300/CBP-associated protein initiates the transcription by recruiting RNA polymerase II on the promoter site. Thus, CRE element in the COX-2 promoter serves as an anchor for p300 interaction with upstream transactivators and downstream transcription machinery (Schroer *et al.*, 2002). It is indispensible for the induction of COX-2 transcription mediated by a number of stimuli including nitric oxide, proteasome inhibitors, and lipopolysaccharide (LPS) (Chen *et al.*, 2005; Park *et al.*, 2005). Using siRNA transfection method to downregulate CREB, we demonstrated that CREB has an important role in 16:0 and 20:4 LPC-induced COX-2 upregulation in EA.hy 926 cells. This is consistent with the findings of Rikitake et al. and

Ueno et al., both showing the capacity of 16:0 LPC to activate CREB in BAECs (Rikitake *et al.*, 2001; Ueno *et al.*, 1999).

AP-1 is another transcription factor involved in regulating the COX-2 expression. Transcription factor AP-1 is composed of a heterogeneous set of proteins belonging to Jun, Fos and ATF families. AP-1 proteins are dimeric leucine zipper transcription factors that bind as homo- or heterodimers to AP-1 responsive elements (5'-TGA(G/C)TCA-3') on the promoters of many genes. Major inducers of AP-1 activity are pro-inflammatory cytokines, phorbol esters, and cAMP. The importance of the AP-1 transcription factor in the transcriptional control of numerous genes has already been demonstrated (Korenaga *et al.*, 1997; Shyy *et al.*, 1995), but the exact regulatory element within COX-2 promoter sequence that binds AP-1 is still controversial (Schroer *et al.*, 2002).

Whereas the knockdown of c-jun suppressed the 16:0 LPC-induced COX-2 upregulation, the effect of 20:4 LPC on COX-2 upregulation wasn't conclusive because in some experiments it suppressed, and in others it enhanced 20:4 LPC induced COX-2 upregulation. The reason for observed both COX-2 up- and down-regulation, both on mRNA and protein level, upon c-Jun silencing in 20:4 LPC- treated cells is not clear.

However, one can speculate that incomplete c-Jun depletion, together with slight inter-experimental differences in the efficiency of c-Jun silencing might impact the relative abundance of various AP-1 complexes in terms of homo- or heterodimerization of c-Jun with c-fos or ATF1/2 (Chinenov *et al.*, 2001). One simple way of explaining the different responses to the same signal could be that c-Jun is critical protein in the network and in these experiments it is differentially expressed. It is clear that the central protein in the network cannot interact with all of its partner proteins simultaneously and some of the interactions are mutually exclusive. That is, if an upstream central protein is expressed at lower concentration, then the affinities of competing binding partners could determine the signaling pathway taken leading to the different outcome. Thus in case of a prevalence of c-Jun/c-fos heterodimers, known to possess strong AP-1 binding activity (Yeh *et al.*, 2004), the c-Jun silencing would decrease 20:4 LPC-induced COX-2 upregulation. Conversely, due to a strong CRE binding activity of c-Jun/ATF (Hai *et al.*, 1991), and concomitantly diminished CREB mediated COX-2 upregulation (Fig. 4B), the c-jun silencing might lead to COX-2 upregulation.

Additionally, the observed COX-2 up- and down-regulation could be explained by the influence of LPC on c-jun expression. Indeed, Ueno et al. demonstrated the 16:0 LPC-dependent increase in c-jun gene expression via a mechanism involving 16:0 LPC-induced CREB phosphorylation which thereby activates the jun2 12-*O*-tetradecanoylphorbol 13-acetate response element (jun2TRE) site of the c-jun promoter (Ueno *et al.*, 1999). That appears to be the main molecular mechanism involved in LPC-induced c-jun gene expression in BAECs. It is possible that in our model, the same signaling cascade occurs, as we did demonstrate the importance of CREB in 20:4 LPC induced COX-2 expression. In that case, the discrepancy between the role of c-jun in 16:0 and 20:4 LPC-induced COX-2 upregulation could be explained by different time points at which the samples were collected, namely 16 and 8 h, respectively. At those time points COX-2 protein reached maximal expression upon stimulation with 16:0 and 20:4 LPC, respectively.

All these results support our data, where the major transcriptional factors (NF-κB, AP-1 and CREB) are essential in COX-2 gene regulation by LPC.We demonstrated that all three of them are involved in LPC-induced COX-2 upregulation. The combinatorial action of transcription factors in the induction of COX-2 expression was already demonstrated for IL-1β induced transcription of COX-2 in a human microvascular endothelial cell line. In that study, the involvement of activated protein-2 (AP2), NF-IL-6 and CRE in COX-2 expression was demonstrated (Kirtikara *et al.*, 2000). Others have demonstrated the activation of multiple transcription factors involved in COX-2 expression, namely c-Fos, c-Jun, CREB2, ATF-2 and USF-2, following PMA or TNF-α treatment (Schroer *et al.*, 2002). In bronchial airway epithelial cells COX-2 has been shown to require both NF-IL6 and CRE transcription factor binding sites for expression after treatment with LPS and phorbol ester (Inoue *et al.*, 1995), and in human chondrocytes both AP-1 and CRE binding proteins were required for COX-2 expression (Miller *et al.*, 1998).

We did not address the question to which cis-acting elements the tested transcription factors bind. Previously it was demonstrated that CRE in the COX-2 promoter is similar to TRE element, and in addition to CREB factors /ATF, it may also bind AP-1 transcription factors (Nomura *et al.*, 1993). AP-1, in addition to CRE site can

bind to NFAT binding site within COX-2 promoter (Schroer *et al.*, 2002). NF-κB can bind to 2 putative NF-κB elements within COX-2 promoter (Kosaka *et al.*, 1994).

It is well established that the amplitude and duration of Ca^{2+} signals control differential activation of signaling kinases and transcription factors (Dolmetsch *et al.*, 1997). Therefore, it is conceivable that LPC-induced Ca^{2+} signals, which were in particular different between 16:0 and 20:4 LPC, were at least in part responsible for differential relative contributions of the tested transcription factors, which in turn might have impacted the kinetics and magnitude of COX-2 induction.

In this research, we did not address the question whether p38 activation precedes the activation of nuclear factors. Most likely, that is the case, as the role of p38 MAPK is activation of transcription factor, but also of other kinases including the nuclear kinases called mitogen- and stress-activated protein kinase 1 (MSK1) and MSK2. One of the roles for MSK1 is activation by phosphorylation of RelA which is followed by increase in transcriptional activity of NF-κB (Vermeulen *et al.*, 2003). Additionally, the connection of p38 and NF-κB pathways was proven as the inhibion of the former reduces the activation of the latter in several cell types (Olson *et al.*, 2007; Ulivi *et al.*, 2008). Their most likely direct interaction has been demonstrated only in corneal cells (Rajaiya *et al.*, 2008).

Other possible substrates for MSK1 are transcription factors ATF-1 and CREB. Previously it was shown that MSK1-dependent CREB/ATF-1 activation also regulates the expression of COX-2 in macrophages (Eliopoulos *et al.*, 2002) and 16:0 LPC-stimulated induction of CREB activation in BAECs ultimately leading to the increase of COX-2 expression (Rikitake *et al.*, 2001).

Activation of AP-1 is also achieved by the upstream MAP kinases, mostly by JNK, but possibly also p38 and ERK (Zarubin *et al.*, 2005). As it can be activated by all the main MAPK pathways, AP-1 serves as the common integrator of these signaling pathways towards the expression of a specific target gene (Karin, 1995). Previously, it was shown that different isoforms of p38 differentially regulate the activation of AP-1 transcriptional activities (Pramanik *et al.*, 2003). Therefore, it is conceivable that differential patterns of p38 activation induced by 16:0 and 20:4 LPC account for contrasting role of c-jun in COX-2 upregulation elicited by these LPC.

LPC signaling is presumed to be mediated by a family of G protein coupled receptors and many endothelial cells including human umbilical vein endothelial cells express GPR4 (Kim *et al.*, 2005). GPR4 receptor is shown to be PTX sensitive suggesting a role for $G_{i/o}$. Gi/o might have a major role in the activation of NF-κB (Xu, 2002). However, incubation of EA.hy 926 with PTX didn't have an effect on LPC-mediated COX-2 upregulation, even though the knockdown of NF-κB resulted in reduced COX-2 upregulation. This might be due to a more pronounced contribution of PTX-sensitive pathways to LPC signaling in MCF10A cells than in EA.hy 926 cells (Zhu *et al.*, 2001).

Additionally, overexpression of GPR4 did not alter COX-2 expression elicited by LPC. It could be that the endogenous levels of GPR4 in EA.hy 926 cells are already sufficient for COX-2 induction, precluding further augementing effect of GPR4 overexpression The endogenous mRNA level of GPR4 in EA.hy 926 was similar to the basal level of COX-2 mRNA expression (data not shown). A possible additional experimental approach to address the role of GPR4 in LPC-mediated induction of COX-2 would be silencing of endogenous GPR4. Such an approach was applied in a study addressing the role of GPR4 in LPC-induced impairment of endothelial barrier function (Qiao *et al.*, 2006). However, silencing experiments were not pursued due to the lack of appropriate specific GPR4 antibody.

Collectively, these data strongly argue that the tested LPCs exhibited remarkably different, acyl chain-related potencies and kinetics of COX-2 induction in human endothelial cells, dependent on intracellular Ca^{2+}, p38 MAPK, NF-κB, c-Jun and CREB.

Considering their high plasma levels and their simultaneous action on vascular endothelium in vivo, the tested LPC might be important stimuli implicated in the maintenance of the basal endothelial COX-2 expression, as similar is shown for shear stress (Inoue *et al.*, 2002). Those studies showed that COX-2 is mainly involved in PGI_2 formation in blood vessels exposed to laminar sheer stress in the physiological range, which is a further confirmation that COX-2 rather than COX-1 is functionally coupled to PGI_2-synthase and represents accordingly the major source of endothelial PGI_2 (Ueno *et al.*, 2001). This is crucial for vascular health, taking into account an increased incidence of cardiovascular events in patients taking selective COX-2 inhibitors, so called "coxibs" (Ray *et al.*, 2002; Solomon *et al.*, 2006). That is most likely connected with the systemic selective COX-2 inhibition and concomitantly decreased PGI_2 in vasculature of healthy humans (McAdam *et al.*, 1999). On the other hand, strikingly increased plasma levels of

those LPC, as found in hyperlipidaemia (Chen *et al.*, 1997), might trigger overexpression of COX-2, leading to overproduction of vasoconstricting and proinflammatory prostanoids and in turn impaired endothelial and vascular function. The tested LPCs as the principal products of EL-mediated lipolysis might be produced locally at the sites where EL is upregulated by inflammatory cytokines, like in inflammed vascular wall or in fully developed atherosclerotic plaques (Ishida *et al.*, 2004).

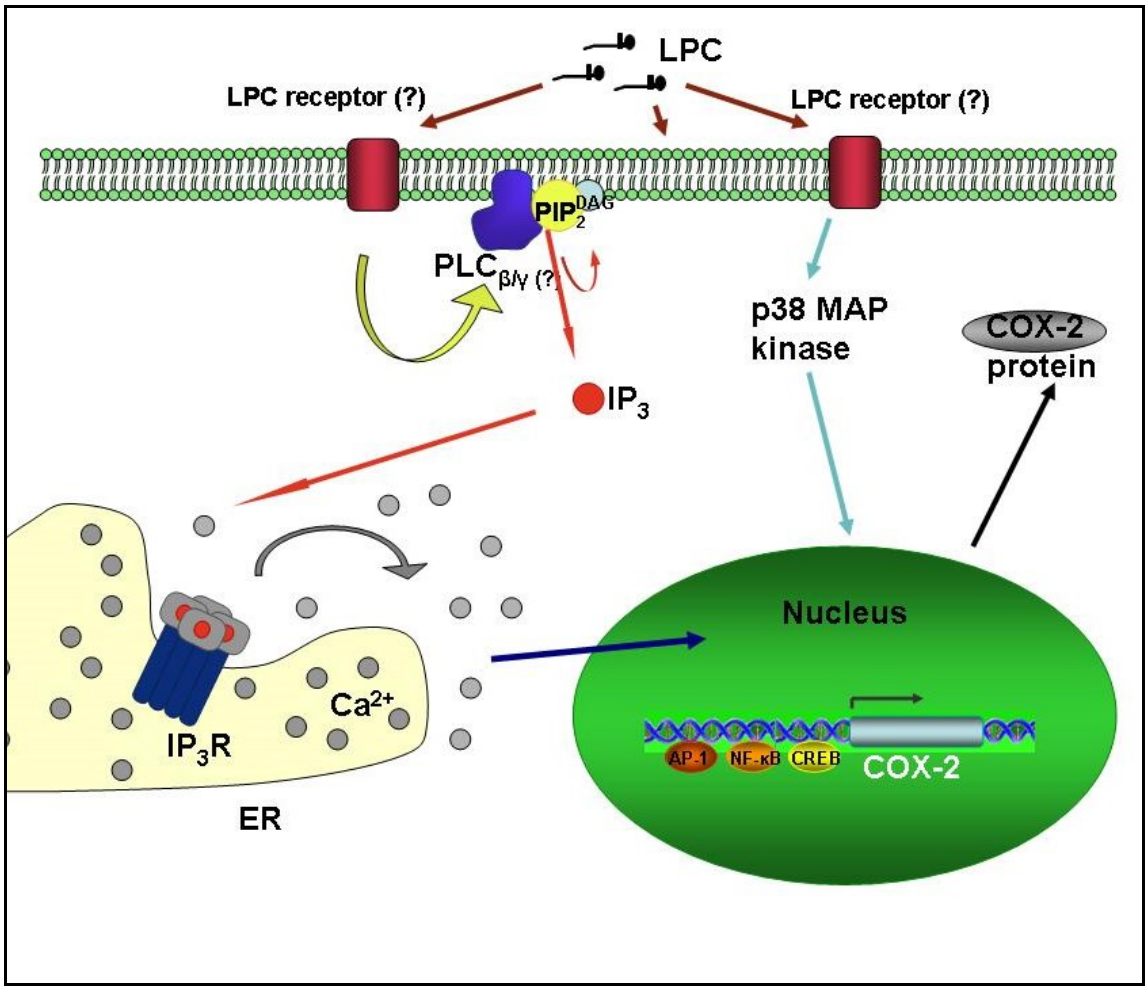

Figure 10. Mechanism of LPC-induced COX-2 upregulation in human endothelial cells

In summary, here we show that the tested LPC species, generated primarily by EL, HL and LCAT, and present in human plasma at high concentrations under physiological and at very high concentrations und pathophysiological conditions, differ markedly in their potency to modulate endothelial COX-2 expression. Thereby the magnitude and kinetics of LPC-elicited COX-2 upregulation as well as the involvement of underlying

signalling pathways and mechanisms are largely dependent on the LPC acyl-chain length and degree of unsaturation (Fig. 10).

5. Literature

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