Acyl chain-dependent effect of lysophosphatidylcholine on endothelial prostacyclin production[S]

Monika Riederer,* Pauli J. Ojala,† Andelko Hrzenjak,§ Wolfgang F. Graier,* Roland Malli,* Michaela Tritscher,* Martin Hermansson, Bernhard Watzer,†† Horst Schweer,†† Gernot Desoye,§§ Akos Heinemann,*** and Sasa Frank[1],***

Institute of Molecular Biology and Biochemistry,* Center of Molecular Medicine, Medical University of Graz, Graz, Austria; Finnish Red Cross Blood Service,† Helsinki, Finland; Division of Pulmonology,§ Department of Internal Medicine, Medical University of Graz, Graz, Austria; Department of Biochemistry,** Institute of Biomedicine, University of Helsinki, Helsinki, Finland; Department of Pediatrics,†† Philipps University of Marburg, Marburg, Germany; and Clinic of Obstetrics and Gynecology;§§ and Institute of Experimental and Clinical Pharmacology,*** Center of Molecular Medicine, Medical University of Graz, Graz, Austria

Abstract **Previously we identified palmitoyl-lysophosphatidylcholine (16:0 LPC), linoleoyl-LPC (18:2 LPC), arachidonoyl-LPC (20:4 LPC), and oleoyl-LPC (18:1 LPC) as the most prominent LPC species generated by the action of endothelial lipase (EL) on high-density lipoprotein. In the present study, the impact of those LPC on prostacyclin (PGI_2) production was examined in vitro in primary human aortic endothelial cells (HAEC) and in vivo in mice. Although 18:2 LPC was inactive, 16:0, 18:1, and 20:4 LPC induced PGI_2 production in HAEC by 1.4-, 3-, and 8.3-fold, respectively. LPC-elicited 6-keto PGF1α formation depended on both cyclooxygenase (COX)-1 and COX-2 and on the activity of cytosolic phospholipase type IVA (cPLA2). The LPC-induced, cPLA2-dependent ^{14}C-arachidonic acid (AA) release was increased 4.5-fold with 16:0, 2-fold with 18:1, and 2.7-fold with 20:4 LPC, respectively, and related to the ability of LPC to increase cytosolic Ca^{2+} concentration. In vivo, LPC increased 6-keto $PGF_{1\alpha}$ concentration in mouse plasma with a similar order of potency as found in HAEC. Our results indicate that the tested LPC species are capable of eliciting production of PGI_2, whereby the efficacy and the relative contribution of underlying mechanisms are strongly related to acyl-chain length and degree of saturation.**—Riederer, M., P. J. Ojala, A. Hrzenjak, W. F. Graier, R. Malli, M. Tritscher, M. Hermansson, B. Watzer, H. Schweer, G. Desoye, A. Heinemann, and S. Frank. **Acyl chain-dependent effect of lysophosphatidylcholine on endothelial prostacyclin production.** ***J. Lipid Res.*** **2010.** 51: **2957–2966.**

Supplementary key words cyclooxygenase • prostanoids • calcium • phospholipase A2 • endothelial cell • arachidonic acid

Lysophosphatidylcholine (LPC) is a bioactive phospholipid generated primarily by the action of phospholipase A2 (PLA2) enzymes on plasma membrane- and lipoprotein-phosphatidylcholine (PC) containing saturated fatty acid (FA) at the sn-1 and mostly unsaturated FA at the sn-2 position (1–3). LPC can also be produced as a by-product by lecithin cholesterol acyltransferase (LCAT) in high-density lipoprotein (HDL) (4) as well as from oxidation of low-density lipoprotein (LDL) (5) and by endothelial lipase (EL).

The physiological concentrations of LPC in body fluids is high, around 150 μM (6, 7), with even millimolar levels in hyperlipidemic subjects (8). LPC in plasma is distributed between albumin or some other carrier serum proteins (9, 10) and lipoproteins (11), with the likely existence of minute amounts of free LPC. This free LPC may transiently arise during an excessive lipolysis when the concentrations of FA and LPC locally exceed the binding capacity of albumin. Under such conditions, LPC partitions transiently to lipoproteins, from where it is rapidly delivered to cells. After uptake into cells, free LPC may be reacylated to yield PC (12) or deacylated to provide FA and choline (10). Despite abundant evidence for the capacity of the free LPC to increase cytosolic Ca^{2+} (13–16) and activate numerous signaling pathways (17, 18) resulting in an enhanced expression of inflammatory molecules (19, 20)

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; EL, endothelial lipase; HAEC, human aortic endothelial cell; IP_3, inositol-3 phosphate; LPC, lysophosphatidylcholine; 16:0 LPC, palmitoyl-lysophosphatidylcholine; 18:1 LPC, oleoyl-LPC; 18:2 LPC, linoleoyl-LPC; 20:4 LPC, arachidonoyl-LPC; PGI_2, prostacyclin; PGIS, prostaglandin I_2 synthase; cPLA2, cytosolic phospholipase A2; PLC, phospholipase C.

[1] To whom correspondence should be addressed.
e-mail: sasa.frank@medunigraz.at

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

and prostanoids (EC) (14, 21, 22), the role of the putative LPC receptors is still a matter of debate.

It is largely accepted that LPC may act via a subset of G protein-coupled receptors (GPCR), including GPR4, G2A (G2 accumulation), OGR1 (ovarian cancer G protein-coupled receptor 1), and TDAG8 (T cell death-associated gene 8), that are sensitive to both LPC and protons (23, 24). Of these receptors, GPR4 shows the widest tissue distribution and is abundantly expressed in vascular endothelial cells where its expression is enhanced by inflammation (25). In contrast, G2A, whose cell surface expression and stabilization are enhanced by LPC (26) but whose proton-induced actions are antagonized by LPC (27), is mainly expressed in lymphoid tissue, lymphocytes, and macrophages (24). Although the initial papers were retracted due to failure to reproduce the binding of LPC to GPR4 and G2A, respectively, several studies have shown that LPC stimulates a variety of cellular activities dependent on GPR4 (25, 28, 29). However, some studies that failed to observe the LPC actions in GPR4-expressing cells provided evidence for the capacity of GPR4 to sense extracellular protons, resulting in G-protein-mediated intracellular signaling and cAMP accumulation (30-32).

Prostanoid biosynthesis involves oxidation and subsequent isomerization of unesterified arachidonic acid (AA). The initial step of this metabolic pathway is the stimulus-induced release of AA from membrane phospholipids by phospholipase A2 (PLA2) enzymes, principally Ca^{2+}-dependent cytosolic PLA2IVA (cPLA2), followed by conversion to prostaglandin H_2 (PGH_2) by either cyclooxygenase (COX)-1 or COX-2, both constitutively expressed by vascular endothelial cells (EC) (33–35). PGH_2 is then converted to various prostanoids by the respective terminal prostanoid synthases (36, 37).

Endothelial lipase is a phospholipase localized on the surface of vascular endothelial cells (38, 39). We demonstrated previously that EL, by cleaving HDL-PC, generates substantial amounts of LPC 16:0, 18:1, 18:2, and 20:4, respectively (40). As studies investigating the impact of LPC on prostanoid production have so far used 16:0 LPC exclusively (1, 14, 21, 22), nothing is known about the impact of the length and degree of saturation of the LPC-acyl chain on prostanoid production. Therefore, the aim of the present study was to assess the impact of those LPC, found to be abundantly generated by EL, on PGI_2 production in vitro in HAEC and in vivo in mice.

MATERIALS AND METHODS

LPC

All LPC was purchased from Avanti Polar Lipids, and additional preparations of 18:2 and 20:4 were generated as described elsewhere (41). LPC was dissolved and stored at −20°C in chloroform/methanol under argon atmosphere. For experiments, required amounts of LPC were dried under a stream of nitrogen or argon and redissolved in PBS (pH 7.4) for cell culture experiments or in pyrogen-free saline for in vivo experiments.

Cell culture

Human primary aortic endothelial cells (HAEC) were obtained from Lonza and maintained in endothelial cell growth medium [EGM-MV Bullet Kit = EBM medium + growth supplements + FCS (Lonza)] supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were cultured in gelatin coated dishes at 37°C in a 5% CO_2 humidified atmosphere and were used for experiments from passage 5 to 10. Cells were seeded (75000/well) in 12-well plates 48 h before exposure to LPC.

LPC treatment of HAEC

Initial time- and concentration-dependent experiments revealed that in a serum-free medium in the absence of BSA, all tested LPC at concentrations up to 10 µM were not toxic to HAEC (for incubations up to 8 h), as determined by monitoring the release of lactate dehydrogenase (LDH) using the cytotoxicity detection kit (LDH) (Roche, Mannheim, Germany).

At 48 h after plating, cells were washed with PBS and incubated with EBM medium without supplements and serum for 3 h. This medium is referred to as "serum-free medium" throughout the article. Thereafter, medium was removed and replaced with fresh serum-free medium supplemented with different concentrations of LPC alone or in some experiments along with AA or A23187. In some experiments, as indicated in the respective figure legends, LPC was applied in complex with BSA at a molar ratio of 1:1 and 5:1, respectively. Medium was collected in prechilled tubes at different time points following exposure to LPC, spun to remove cells, and used for lactate dehydrogenase (LDH) assay and measurement of prostanoids. Cells were washed with PBS and lysed in respective buffers for isolation of RNA or proteins.

Pharmacological inhibitors

During final 30 min of incubation with serum-free medium prior to exposure to LPC as well as during exposure to LPC, HAEC were treated with respective pharmacological inhibitors (all from Calbiochem) or vehicle (DMSO). Specific COX-1 inhibitor SC-560 (100 nM), COX-2 inhibitor NS-398 (20 µM), and cPLA2 inhibitor (1 µM) [(N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl] acrylamide, HCl)] were applied.

Quantitative real-time PCR

RNA of cell extracts was isolated using the peqGOLD Total RNA Kit (Peqlab-biotechnology, Erlangen, Germany) according to the manufacturer's protocol, including on column DNase digestion. Then 1.5 µg of RNA were reverse transcribed using the Archive cDNA Kit (Applied Biosystems, Foster City, CA) and 0.7 U of an RNase Inhibitor (Qiagen, Hilden, Germany). 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 QantiFast SYBR green RT-PCR kit (Qiagen) and validated QuantiTect Primer Assays (Qiagen) according to the manufacturer's instructions for Light Cycler 480 instruments (Roche Diagnostics). In brief, after the initial heat activation step at 95°C for 5 min, cycling conditions consisted of 40 cycles of denaturation at 95°C for 10 s and combined annealing and extension at 60°C for 30 s. The PCR efficiency of the target and housekeeping genes was determined by cDNA dilution series prepared from an untreated sample, and results were accordingly efficiency-corrected with the LightCycler Relative Quantification software (Roche Diagnostics, Basel, Switzerland). mRNA levels of COX-1 and COX-2 (Primer Assays QT00210280 and QT00040586) were normalized to human β-2-microglobulin (Primer Assay QT01665006) and expressed as

relative ratio (ΔΔ Ct). All samples were assayed in duplicate, and the average value was used for quantification.

Prostanoid profiling by GC-MS/MS

The spectrum of prostanoids produced by HAEC under basal conditions was determined by gas chromatography-tandem mass spectrometry (GC-MS/MS) as described previously (42). In brief, 10 µl deuterated internal standard mixture (about 1 ng of each PG) was added to 0.2–1 ml of each sample. After acidification with formic acid (5%, v/v) to pH 2.6, a solution of 0.1 g methoxyamine hydrochloride in 1 ml sodium acetate (1.5 M, pH 5.0) was added. The prostanoid derivates were extracted with ethyl acetate/hexane (7:3, v/v). After evaporation, the residues were esterified with a reaction mixture of acetone (80 µl), diisopropylamine (7 µl), and pentafluorobenzyl bromide (6 µl) for 10 min at 40°C. Dried samples were then applied to TLC and developed in ethyl acetate/hexane (9:1, v/v). The target zones were scraped off (Rf = 0.03–0.39) and extracted with ethyl acetate. The extracts were then evaporated, derivatized twice with 10 µl BSTFA at 60°C, and analyzed using GC-MS/MS.

6-keto $PGF_{1\alpha}$ measurements by EIA

6-keto $PGF_{1\alpha}$ was measured in cell culture media and 5-fold diluted mouse plasma by a correlate-EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol. Protein content of cell culture wells was initially determined to be equal for all treatments.

^{14}C-Arachidonic acid and ^{14}C-6-keto PGF1α release

Cells were labeled with ^{14}C-AA (4 µM, spec. activity 58 mCi/mmol) in complete medium for 20 h. Unbound ^{14}C-AA was removed by excessive washing in PBS supplemented with 1% BSA. After incubation in serum-free medium for 3 h, and facultative preincubation with 1 µM cPLA2 inhibitor for 30 min, cells were exposed to 50 µM LPC + 3.7 µM BSA (± cPLA2 inhibitor) for 20 min. Cell media were then collected, spun to remove cells, and immediately frozen at −70°C until extraction. Cells were washed with PBS and lysed in 0.3 M NaOH/0.1% SDS. Aliquots of the cell lysates were mixed with scintillation cocktail, and the radioactivity was determined on a β-counter (Beckman). Acidified media were extracted with 2 vol of hexane/isopropanol (3:2, v/v), evaporated in the SpeedVac, and redissolved in chloroform, followed by TLC using ethylacetate/isooctane/water/acetic acid (11:5:10:2, v/v) as a mobile phase. The signals corresponding to ^{14}C-AA and ^{14}C-6-keto PGF1α were visualized upon exposure of the TLC plates to a tritium screen (GE Healthcare) on the STORM imager. Quantification was performed by densitometric volume report analysis or by liquid scintillation counting of cut out TLC spots corresponding to the comigrating AA- and 6-keto PGF1α-standard [visualized by spraying with primulin (0.01%, w/v) in acetone/water (60:40, v/v) and subsequent UV detection]. The amounts of ^{14}C-AA and ^{14}C-6-keto PGF1α released into medium were normalized to total cellular radioactivity measured by scintillation counting of cell lysates.

Western blotting

For quantification of COX-2 protein, three 12-well plates of HAEC treated with LPC or PBS were washed with PBS and lysed in 50 µl of loading buffer [20% (w/v) glycerol, 5% (w/v) SDS, 0.15% (w/v) bromophenol blue, 63 mmol/l Tris-HCl (pH 6.8), and 5% (v/v) β-mercaptoethanol] and boiled for 10 min. Then 40 µl of the lysate were subjected to each lane and analyzed by SDS-PAGE (10% gel) and subsequent immunoblotting using goat anti-COX-2 (Santa Cruz Biotechnology, M19, sc-1747, 1:100) and the HRP-labeled rabbit anti-goat IgG (Dako) as a secondary antibody. Protein signals were detected by ECL assay, and the intensity was normalized to actin (Pierce, ms Anti-Actin IgM: 1:4000) using densitometry.

Measurements of $[Ca^{2+}]_i$

HAEC were seeded in 6-well plates. At 48 h after plating, cells were serum starved for 2 h and then loaded with 2 µM fura-2/AM for 60 min. After washing, cells were trypsinized and resuspended either in calcium buffer (138 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM Hepes, 10 mM Glucose, pH 7.4) or EGTA buffer (0.1 mM EGTA, 138 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM Hepes, 10 mM Glucose, pH 7.4). The ratio of fura-2 fluorescence intensity at the two excitation wavelengths (340/380 ratio) was monitored spectrophotometrically in a stirring cuvette before and after the injection of 10 µM LPC. In some experiments, cells were resuspended in EGTA buffer followed by a preincubation with either 100 µM 2-APB for 2 min or with 2 µM U73122 for 5 min before the injection of LPC.

Experiments in mice

Following a 6 h fasting period, 10–12-weeks-old male C57Bl/6J mice (4–7 per group) were treated with 20 mg/kg LPC in 0.9% NaCl (or NaCl alone) via tail vein injection. After 20 h, blood was collected from the retro-orbital plexus into tubes containing indomethacin and EDTA, centrifuged immediately, and stored at −70°C for measurements of 6-keto $PGF_{1\alpha}$. For tail-vein injection and bleeding, mice were anesthetized with Isoflurane (Pharmacia and Upjohn SA, Guyancourt, France). Animal experiments were conducted in conformity with the Public Health Service Policy on Human Care and Use of Laboratory Animals and were approved by the Austrian Ministry of Science and Research according to the Regulations for Animal Experimentation.

Statistical analysis

Cell culture experiments were performed at least three times and values are expressed as mean plus SEM. Statistical significance was determined by the Student's unpaired *t*-test (two-tailed) with application of Welch´s correction, where required. Group differences were considered significant for $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

RESULTS

LPC induces PGI_2 production in HAEC

As determined by GC-MS/MS analysis, HAEC secrete 6-keto $PGF_{1\alpha}$ (a stable degradation product of PGI_2), thromboxane B_2 (TxB_2; a stable degradation product of TxA_2), $PGF_{2\alpha}$, and PGE_2 (**Fig. 1A**).

To examine the impact of LPC on endothelial PGI_2 production, HAEC were incubated with 10 µM LPC followed by EIA-based quantification of 6-keto $PGF_{1\alpha}$. As shown in Fig. 1B, 16:0 LPC increased slightly (1.4-fold) and not statistically significantly the formation of 6-keto $PGF_{1\alpha}$ relative to the PBS control. The increases in 6-keto $PGF_{1\alpha}$ elicited by 18:1 LPC and 20:4 LPC were much more pronounced at 3- and 8.3-fold, respectively. 18:2 LPC did not have any impact on 6-keto $PGF_{1\alpha}$ production (supplementary Fig. I) and was not further studied.

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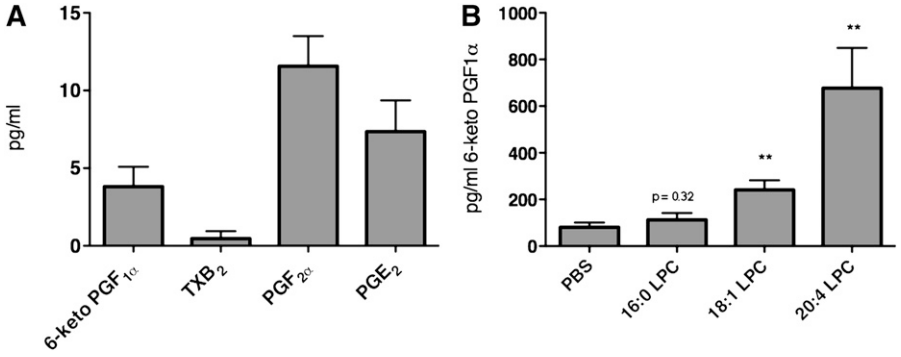

Fig. 1. Basal production of prostanoids and LPC-elicited 6-keto $PGF_{1\alpha}$ production in HAEC. HAEC were treated with 10 μM LPC (B) or solvent (PBS) (A) in serum-free medium for 5 h. Cell culture supernatants were subjected to prostanoid profiling by GC-MS/MS (A) or EIA for quantification of 6-keto $PGF_{1\alpha}$ (B). Results are means ± SEM of two (A) and five (B) experiments performed in duplicate. HAEC, human aortic endothelial cell; LPC, lysophosphatidylcholine.

LPC-induced 6-keto $PGF_{1\alpha}$ production is mediated by COX

LPC upregulates COX-2 mRNA but not COX-2 protein. The ability of both specific COX-1 (SC-560; 100 nM) and COX-2 (NS-398; 20 μM) inhibitors to prevent the LPC-mediated increase of 6-keto $PGF_{1\alpha}$ clearly demonstrated the involvement of both COX-enzymes (**Fig. 2A**). To further characterize the involvement of COX enzymes COX-1 and COX-2, mRNA and protein abundance, respectively, were monitored in LPC-treated HAEC. COX-1 mRNA was unaltered by LPC (supplementary Fig. IIA). COX-2 mRNA was induced 3-fold by 16:0 LPC, 2-fold by 18:1 LPC, and 1.6-fold by 20:4 LPC, upon 1 h incubation with 10 μM of LPC under our standard conditions (i.e., in serum-free medium without addition of NEFA-free BSA) (Fig. 2B). The LPC failed to upregulate COX-2 mRNA when applied in combination with an equimolar amount of NEFA-free BSA (50 μM NEFA-free BSA + 50 μM LPC). When, based on the assumption that 1 mol of BSA binds 5 mol of LPC (43), the BSA concentration was adjusted to yield roughly 10 μM of free LPC (8.8 μM NEFA-free BSA + 50 μM LPC), the upregulation of COX-2 mRNA could be restored (Fig. 2B).

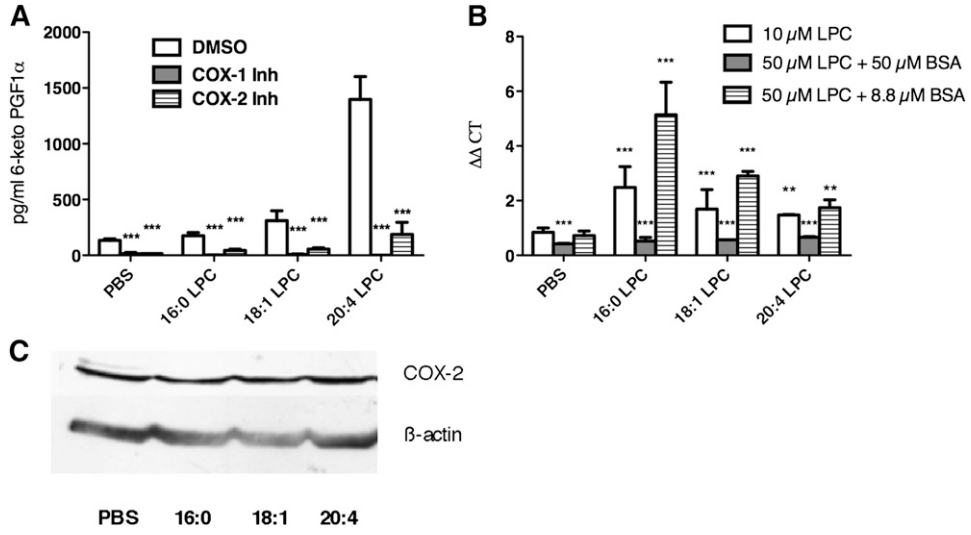

Fig. 2. Involvement of COX isoforms and the impact of LPC on COX-2 mRNA and COX-2 protein expression in HAEC. A: HAEC were preincubated with isotype-specific COX inhibitors (COX-1; SC-560), (COX-2; NS-398), or vehicle (DMSO) for 30 min before their exposure to 10 μM LPC in serum free medium for 5 h. 6-keto $PGF_{1\alpha}$ concentration in cell culture supernatants was determined by EIA. Results shown are mean ± SEM of one representative experiment (n = 3). B: For determination of COX mRNA levels, HAEC were incubated either with 10 μM LPC, 50 μM LPC + 50 μM BSA (equimolar) or 50 μM LPC + 8.8 μM BSA (~10 μM free LPC) for 1 h. Subsequently, RNA was isolated and the relative COX-2 mRNA abundance was determined by qRT-PCR as described in "Materials and Methods." Results shown are mean ± SEM of one representative experiment (n = 3). C: After 5 h of incubation with 10 μM LPC, HAEC COX-2 protein content was determined by 10% SDS-PAGE followed by immunoblotting using COX-2 and actin antibodies. Bands were visualized using appropriate secondary antibodies and an ECL substrate. A representative Western blot out of three is shown. COX, cyclooxygenase; HAEC, human aortic endothelial cell; LPC, lysophosphatidylcholine.

However, like COX-1 (supplementary Fig. IIB), COX-2 protein was not increased after a 5 h incubation with LPC as determined by Western blotting (Fig. 2C), leading us to the conclusion that COX protein upregulation could not account for the LPC-mediated increase of 6-keto $PGF_{1\alpha}$.

LPC promote cPLA2-dependent AA release in HAEC. To elucidate the underlying mechanism for LPC-elicited 6-keto $PGF_{1\alpha}$ production, we examined the role and contribution of cPLA2, the major PLA2 implicated in the generation of AA for PG synthesis. As shown in **Fig. 3A**, a specific cPLA2 inhibitor (see "Materials and Methods"; 1 μM) efficiently decreased the LPC-elicited 6-keto $PGF_{1\alpha}$ production in HAEC, strongly indicating a role for cPLA2. To further examine the impact of LPC on cPLA2, ^{14}C-AA release was determined in HAEC. As shown in Fig. 3B, the highest (4.5-fold) induction of ^{14}C-AA release was obtained with 16:0 LPC and was less pronounced (2- and 2.7-fold) with LPC 18:1 and 20:4, respectively. The effects of all LPC could be decreased by preincubation with the specific cPLA2-inhibitor (Fig. 3B), which also efficiently decreased A23187-induced ^{14}C-AA release and 6-keto $PGF_{1\alpha}$ production in HAEC (not shown). As cPLA2 activation is postulated to be dependent on a transient increase in cytosolic calcium concentration $[Ca^{2+}]_i$, we tested the ability of the LPC to modulate $[Ca^{2+}]_i$.

LPC increase $[Ca^{2+}]_i$. In contrast to a marked increase in $[Ca^{2+}]_i$ by 16:0 LPC, a less pronounced but sustained increase was evoked by LPC 18:1 and 20:4 in the presence of extracellular Ca^{2+} (**Fig. 4A**). In the absence of extracellular Ca^{2+} (EGTA), the increase in $[Ca^{2+}]_i$ triggered by 16:0 LPC was low and similar to that of 18:1 and 20:4, respectively (Fig. 4B). Furthermore, EGTA did not alter the magnitude of the initial $[Ca^{2+}]_i$ peak elicited by 18:1 and 20:4 LPC; however, it rendered the signal transient (Fig. 4B). Under EGTA-conditions, both U73122, a phospholipase C (PLC) inhibitor, and 2-APB, an inositol-3 phosphate (IP_3) receptor antagonist, completely blocked the increase in $[Ca^{2+}]_i$ elicited with all tested LPC (Fig. 4C and supplementary Fig. IIIA, B). From these results, we concluded that LPC-elicited increase in $[Ca^{2+}]_i$ is partially due to IP_3-triggered Ca^{2+} release from endoplasmatic reticulum (ER) and influx of extracellular Ca^{2+}. Additionally, these findings strongly suggest that the capacity of LPC to promote cPLA2-dependent AA release is related to its capacity to increase $[Ca^{2+}]_i$.

Conversion of AA to 6-keto $PGF_{1\alpha}$ is not compromised by 16:0 LPC. The findings presented in Figs. 1 and 3 clearly showed that the capacity of 16:0 LPC to induce cPLA2-mediated AA release is unrelated to its capacity to elicit 6-keto $PGF_{1\alpha}$ production. Therefore, we examined whether 16:0 LPC compromises the conversion of AA to 6-keto $PGF_{1\alpha}$. As shown in **Fig. 5A**, the production of 6-keto $PGF_{1\alpha}$ elicited by exogenous AA was unaltered by 16:0 LPC. The conversion of AA released (from cellular phospholipids) upon A23187 treatment to 6-keto $PGF_{1\alpha}$ was even promoted by 16:0 LPC (Fig. 5A). Hence, the utilization of neither exogenous nor endogenous AA is compromised by 16:0 LPC.

Magnitude of 20:4 LPC-elicited 6-keto $PGF_{1\alpha}$ production is similar to that of AA. To explain the pronounced capability of 20:4 LPC to elicit 6-keto $PGF_{1\alpha}$ production despite its moderate capacity to promote AA release, we compared the magnitude of 6-keto $PGF_{1\alpha}$ induction elicited by 20:4 LPC with that of AA. As shown in Fig. 5B, cells incubated with 20:4 LPC (10 μM) secreted 6-keto $PGF_{1\alpha}$ with similar kinetics and efficiency as cells incubated with AA (10 μM), suggesting that 20:4 LPC might serve as a donor of AA for 6-keto $PGF_{1\alpha}$ production.

20:4 LPC is a weak inducer of endogenous 6-keto $PGF_{1\alpha}$ production. To examine the capacity of 20:4 LPC to

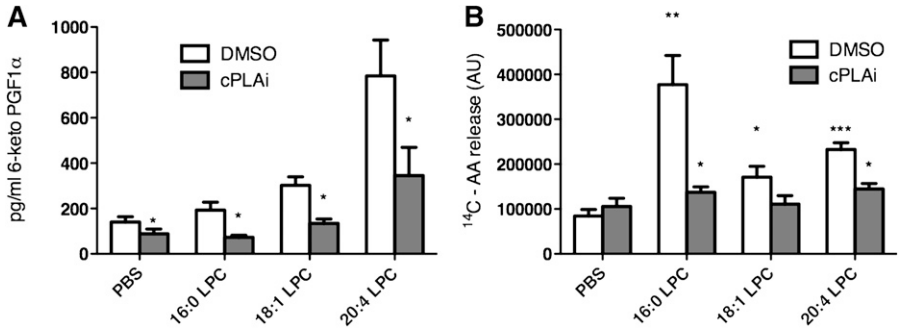

Fig. 3. Role of cPLA2 in LPC-elicited 6-keto $PGF_{1\alpha}$ production and impact of LPC on cPLA2-mediated AA release. A: HAEC were preincubated with a specific cPLA2 inhibitor or vehicle (DMSO) for 30 min before exposure to 10 μM LPC in serum-free medium for 5 h. 6-keto $PGF_{1\alpha}$ in cell culture supernatants was quantified by EIA. Results are mean ± SEM of seven separate experiments. B: HAEC were labeled with ^{14}C-AA for 20 h. After preincubation with cPLA2 inhibitor or vehicle (DMSO) for 30 min, cells were exposed to serum-free medium supplemented with 50 μM LPC + 3.7 μM BSA in the presence or absence of cPLA2 inhibitor for 20 min. Lipid extracts of cell media were separated by TLC and analyzed by densitometric quantification of ^{14}C-AA spots. The amount of ^{14}C-AA released into medium was normalized to total cellular radioactivity measured by scintillation counting of cell lysates (AU). Results are mean ± SEM of three experiments. AA, arachidonic acid; HAEC, human aortic endothelial cell; LPC, lysophosphatidylcholine; cPLA2, cytosolic phospholipase A2.

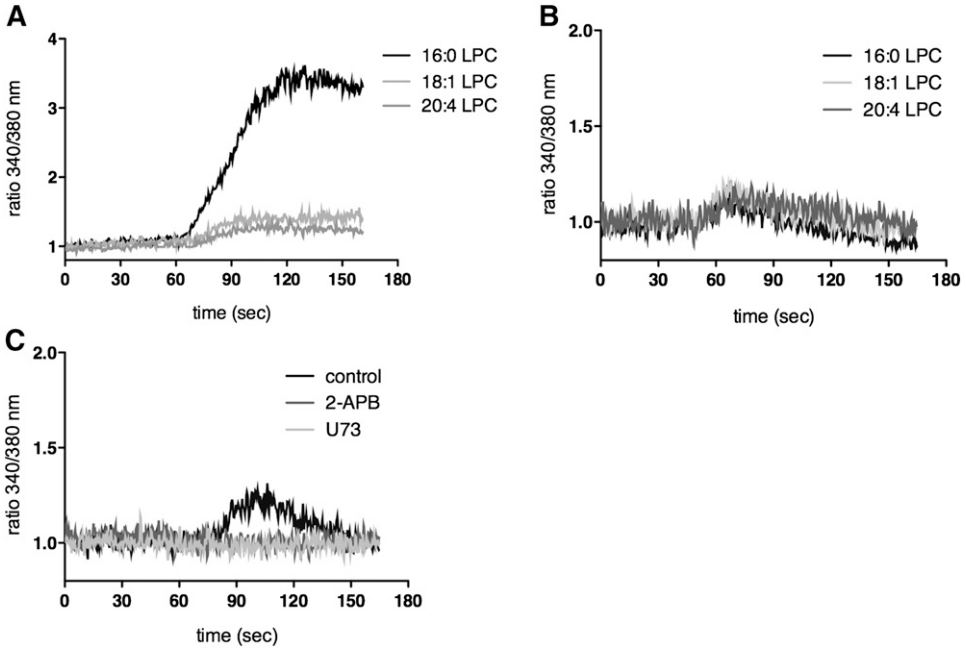

Fig. 4. Impact of LPC on cytosolic calcium concentration $[Ca^{2+}]_i$. Fura-2/AM-loaded HAEC were trypsinized and resuspended either in Ca^{2+}-containing buffer (A) or Ca^{2+}-free buffer containing EGTA (B and C). The ratio of fura-2 fluorescence intensity at the two excitation wavelengths (340/380 ratio) was monitored spectrophotometrically in a stirring cuvette during exposure to 10 μM LPC. In (C), cells were preincubated without or with 2-APB or U73122 (U73) for 2 and 5 min, respectively, before application of 18:1 LPC. Results are representative single traces out of three experiments performed in duplicate. HAEC, human aortic endothelial cell; LPC, lysophosphatidylcholine.

induce 6-keto $PGF_{1\alpha}$ production from endogenous cellular AA and to compare it with that of 16:0 LPC, we determined ^{14}C-6-keto $PGF_{1\alpha}$ secretion from ^{14}C-labeled HAEC exposed to respective LPC. As shown in Fig. 5C, the 20:4 LPC-elicited ^{14}C-6-keto $PGF_{1\alpha}$ production was markedly lower compared with 16:0 LPC, clearly showing the minor contribution of endogenous AA to the 20:4 LPC-elicited 6-keto $PGF_{1\alpha}$ production. This finding further corroborated the role of 20:4 LPC as a donor of AA.

LPC augment PGI_2 production in mice. Finally, we tested the ability of LPC to promote the production of 6-keto $PGF_{1\alpha}$ in vivo. For this purpose, LPC was injected into the tail vein of fasted C57Bl/6J mice. The dose of injected LPC was 20 mg/kg to achieve plasma concentrations of approximately 10 μM of free LPC, after saturation of LPC binding sites of albumin and other plasma proteins. In accordance with in vitro data, 16:0, 18:1, and 20:4 LPC induced 6-keto $PGF_{1\alpha}$ formation in vivo (**Fig. 6**), however, with a slightly different order of potency as found in HAEC.

DISCUSSION

Identification of LPC 16:0, 18:1, 18:2, and 20:4 as major LPC species generated by the action of EL on HDL (40) prompted us to examine their impact on endothelial prostanoid production with the focus on PGI_2. Whereas three studies provided evidence to support the role for 16:0 LPC as a modulator of endothelial prostanoid production (14, 21, 22), unsaturated LPC (18:1, 18:2, and 20:4) has not been studied so far. Therefore, we examined the capability of those unsaturated LPC species, along with established 16:0 LPC, to modulate PGI_2 production in HAEC and in mice.

As found by MS analysis of cell media, HAEC produce PGI_2, TXA_2, $PGF_{2\alpha}$, and PGE_2. Considering the fact that HAEC, like human coronary artery endothelial cells (HCAEC) (44), express PGI_2 synthase (PGIS), PGE_2 synthase (mPGES-1), and thromboxane synthase (TBXS) (not shown), it is conceivable that those prostanoids are products of enzymatic transformation of PGH_2. However, we cannot completely exclude the possibility that a portion of the secreted prostanoids is generated by spontaneous, nonenzymatic transformation of PGH_2 (45).

In the present study, we found remarkable acyl-chain-dependent differences in the capability of LPC species to augment endothelial production of PGI_2 measured as stable degradation product 6-keto $PGF_{1\alpha}$. In contrast to LPC 18:1 and 20:4, which were much more potent inducers of 6-keto $PGF_{1\alpha}$ than 16:0 LPC, LPC 18:2 was completely incapable of altering 6-keto $PGF_{1\alpha}$ production in HAEC. The observed 6-keto $PGF_{1\alpha}$ stimulation was sensitive to both COX-1- and COX-2-specific inhibitors, indicating that in HAEC exposed to LPC both COX enzymes are functionally coupled to PGIS (37). In line with a previous finding (21) describing the impact of 16:0 LPC on COX-2 expression in human umbilical vein endothelial cells (HUVEC), our LPC increased COX-2 mRNA without altering COX-1 mRNA. In contrast to our findings, Zembowicz et al. (21)

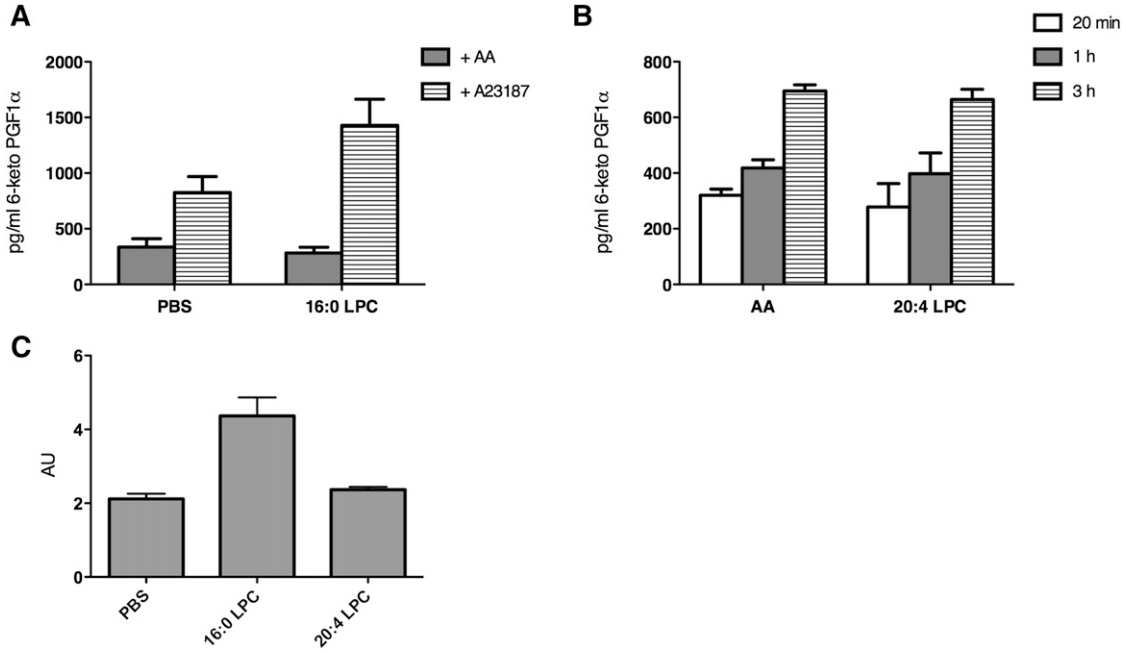

Fig. 5. Impact of LPC on exogenous and endogenous AA utilization for 6-keto PGF1α synthesis. A: HAEC were exposed to 10 μM 16:0 LPC or PBS in the presence of either 10 μM AA for 5 h or in the presence of 0.5 μM A23187 for 20 min. 6-keto $PGF_{1\alpha}$ in cell culture supernatants was quantified by EIA. Results are mean ± SEM of three separate experiments. B: HAEC were treated with either 10 μM AA or 10 μM 20:4 LPC and 6-keto $PGF_{1\alpha}$ in cell culture supernatants. was quantified by EIA at the indicated time points. Results are mean ± SEM of 3 separate experiments. C: ^{14}C-AA labeled HAEC were exposed to PBS or LPC for 5 h, followed by TLC separation of cell media lipids and densitometric quantification of ^{14}C-spots comigrating with 6-keto $PGF_{1\alpha}$. Results are mean ± SEM of three separate experiments. AA, arachidonic acid; HAEC, human aortic endothelial cell; LPC, lysophosphatidylcholine.

and Rikitake et al. (22) demonstrated an increase in COX-2 protein upon exposure of HUVEC and bovine aortic endothelial cells (BAEC) to 16:0 LPC. The lack of COX-2 protein upregulation in our experimental model might be due to a lower responsiveness of HAEC in contrast to HUVEC and BAEC to LPC in terms of COX-2 induction. Additionally or alternatively, the amount and the form in which LPC is applied, namely 100 μM LPC in the presence of 5% FCS (21) or 20 μM LPC (22) versus 10 μM LPC in serum-free medium in our experimental system, might explain the discrepancy between our findings and those of others. Our findings provide clear evidence that the basal expression of COX-2 is sufficient to accomplish LPC-elicited 6-keto $PGF_{1\alpha}$ production in HAEC. This is in line with the crucial role of basal endothelial COX-2 expression for the maintenance of vascular health, exemplified by cardiovascular events upon the inhibition of COX-2 by "coxibs" (46–48).

Strikingly, 16:0 LPC compared with 18:1 and 20:4 LPC was the least potent inducer of 6-keto $PGF_{1\alpha}$, whereas it was the most potent promoter of $[Ca^{2+}]_i$ increase and of cPLA2-mediated AA release. The profound activation of cPLA2 and concomitant AA release were also reported for 16:0 LPC in HUVEC; however, this study unfortunately did not address the impact of LPC on 6-keto $PGF_{1\alpha}$ production (14). From our findings, it appears that 16:0 LPC promotes AA release but reduces its full utilization toward the production of 6-keto $PGF_{1\alpha}$. As 16:0 LPC is efficiently converted to PC after uptake into cells (12), it is conceivable that substantial amounts of AA released by activated cPLA2 are used rather for reacylation of 16:0 LPC than as substrate for COX enzymes. Additionally, 16:0 LPC might interfere with functional coupling between cPLA2 and COX enzymes, which is a prerequisite for efficient supply of AA released by cPLA2 to COX enzymes (37), or alternatively, it might attenuate the activity of PGIS, resulting in reduced conversion of released AA to PGI_2. However, all these possibilities seem not to apply in our experimental

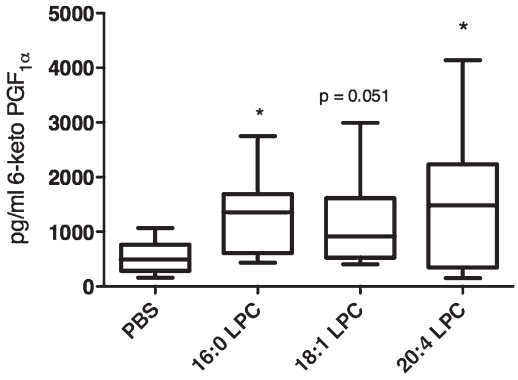

Fig. 6. Effect of LPC on 6-keto $PGF_{1\alpha}$ production in mouse plasma. Following a 6 h fasting period, 4–7 mice per group were injected via tail vein with either 20 mg/kg LPC in a total volume of 100 μl 0.9% NaCl or 0.9% NaCl alone. After 20 h, blood was collected from the retro-orbital plexus into tubes containing indomethacin and EDTA, followed by immediate centrifugation for the determination of 6-keto $PGF_{1\alpha}$ in plasma. Results represent two experiments, whiskers indicate the range from MIN to MAX. AA, arachidonic acid; LPC, lysophosphatidylcholine.

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system, as experiments addressing a possible inhibitory effect of 16:0 LPC (Fig. 5A) on the conversion of either exogenous or endogenous AA to 6-keto $PGF_{1\alpha}$ production failed to provide evidence for an inhibitory effect of 16:0 LPC. In HAEC, 16:0 LPC even augmented A23187-induced 6-keto $PGF_{1\alpha}$ production. A very weak 6-keto $PGF_{1\alpha}$ response to 16:0 LPC, despite COX-2 upregulation, was also found in HUVEC (21). Further experiments are required to clarify the failure of 16:0 LPC to induce a more efficient 6-keto $PGF_{1\alpha}$ production.

20:4 LPC was the most potent inducer of 6-keto $PGF_{1\alpha}$ formation, whereas it was in the same range as 18:1 LPC regarding induction of $[Ca^{2+}]_i$ and cPLA2-mediated AA release. Accordingly, the induction of cPLA2-mediated AA release could not explain the marked 8.3-fold increase in 6-keto $PGF_{1\alpha}$ elicited with 20:4 LPC. Because the rate of 6-keto $PGF_{1\alpha}$ production elicited by 20:4 LPC was similar to that of AA, it is likely that 20:4 LPC serves as a source of AA for COX enzymes. This, in our opinion, makes the major contribution to the marked increase in 20:4 LPC-elicited 6-keto $PGF_{1\alpha}$ production. Beside different cellular lysophospholipases, cPLA2 may by its lysophospholipase activity (49) accomplish the release of AA from 20:4 LPC. Indeed, 20:4 LPC-elicited 6-keto $PGF_{1\alpha}$ production was significantly decreased by cPLA2 inhibitor (Fig. 3A), which probably reflected the inhibition of both cPLA2 activities, the phospholipase activity involved in liberation of AA from cellular phospholipids and the lysophospholipase activity responsible for deacylation of 20:4 LPC. Importantly, the most potent inducer of 6-keto $PGF_{1\alpha}$ production among tested LPC, the 20:4 LPC, was a much weaker inducer of 6-keto $PGF_{1\alpha}$ production from endogenous AA than 16:0 LPC (Fig. 5C). These findings corroborate further the role of 20:4 LPC as a provider of exogenous AA. We are aware that our conclusions regarding the role of 20:4 LPC as a source of AA are based on indirect experimental approaches, but the lack of a commercially available 20:4 LPC labeled in the AA moiety precludes the possibility of directly demonstrating the conversion of 20:4 LPC-derived AA to labeled 6-keto $PGF_{1\alpha}$.

Regarding 18:2 LPC, we speculate that its failure to promote 6-keto $PGF_{1\alpha}$ production might be due to a negligible impact on cPLA2-mediated AA release and inability to act as a substrate provider for COX enzymes like 20:4 LPC. Indeed, 18:2 FA is a poor substrate for the elongation/desaturation machinery in endothelial cells (50, 51).

A transient increase in $[Ca^{2+}]_i$ is required for the translocation of cPLA2 from cytosol to the cellular membrane surface (52). In line with this, we found that the extent of LPC-mediated AA release was fully related to the LPC-elicited increase in $[Ca^{2+}]_i$. All tested LPC generated a biphasic increase in $[Ca^{2+}]_i$ with a rapid, IP_3-triggered initial increase caused by the release of Ca^{2+} from intracellular stores, which was similar for all LPC, followed by sustained influx of extracellular Ca^{2+}, which is strongly induced by 16:0 LPC and only slightly by 18:1 and 20:4 LPC. 16:0 LPC has been shown to promote the transmembrane Ca^{2+} influx in endothelial cells by opening store-operated Ca^{2+} channels independent of ER-empting (53), as well as by promoting membrane hyperpolarization due to the opening of Ca^{2+}-activated K^+ channels (54). Future studies should clarify the difference in the capability of the tested LPC species to elicit an increase in $[Ca^{2+}]_i$ and to identify the routes of Ca^{2+} in HAEC.

To demonstrate the physiologic relevance of our findings, we injected LPC intravenously into mice. The amount of injected LPC was chosen to yield approximately 5–10 μM of free LPC, assuming scavenging by albumin and other carrier plasma proteins like α-1-acid glycoprotein (AGP) (9). Several studies have shown that the presence of albumin profoundly influences the bioactivity of LPC (41, 55–57), clearly pointing to the requirement for the free, nonprotein-bound LPC. In accordance with those findings was the inability of LPC to upregulate COX-2 mRNA when applied along with equimolar amounts of BSA and restoration of LPC-elicited COX-2 mRNA upregulation when BSA concentration was adjusted to yield approximately 10 μM of free LPC (Fig. 2B). All LPC species elicited 6-keto $PGF_{1\alpha}$ production in mice, clearly arguing for their role in PGI_2 production in vivo. The order of potency was slightly different compared with HAEC, with the less pronounced effect of 18:1 LPC compared with 16:0 and 20:4 LPC. This discrepancy might be due to differences in metabolic and pharmacokinetic properties of 18:1 LPC compared with 16:0 and 20:4, like half-life in plasma, clearance by the liver, or scavenging by albumin, resulting in lower bioavailability and consequently diminished ability to promote 6-keto $PGF_{1\alpha}$ production in vivo. Of note, preliminary monitoring of 6-keto $PGF_{1\alpha}$ in mice showed a pronounced dissipation in basal plasma levels in untreated mice, explaining the variance observed in experimental groups upon LPC treatment.

Under physiological conditions, plasma albumin scavenges LPC, thus blunting its action on the vascular endothelium. However, it is very likely that under inflammatory conditions when EL (58, 59) and other serum phospholipases (1, 2, 60) are upregulated, the excessive lipolysis in the plasma compartment raises the concentrations of FAs and LPC, leading to saturation of LPC binding sites on albumin and other plasma proteins and allowing the action of free LPC on vascular endothelium. The direct consequence of the action of LPC on vascular endothelium might be vasoprotective due to promotion of PGI_2 production. However, as the expression of PGE_2, TxA_2, and $PGF_{2\alpha}$ might also be altered by LPC in a similar manner as PGI_2, further experiments are required to examine the impact of LPC on those prostanoids with opposite impact on vascular function.

To our knowledge, this is the first study addressing the impact of the acyl-chain length and degree of saturation on the capacity of LPC to augment the formation of endothelial 6-keto $PGF_{1\alpha}$. Here we show that the magnitude of increase in LPC-elicited 6-keto $PGF_{1\alpha}$ and the relative contribution of underlying mechanisms, including increase in $[Ca^{2+}]_i$ and cPLA2 activation as well as provision of exogenous substrate, are strongly related to acyl-chain length and degree of saturation.

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ERRATA

The authors of "Acyl chain-dependent effect of lysophosphatidylcholine on endothelial prostacyclin production" (*J. Lipid Res.* 51: 2957–2966) have advised the journal that two authors were inadvertently omitted from the original list of authors.

The author list that initially appeared online and in print was as follows:

Monika Riederer, Pauli J. Ojala, Andelko Hrzenjak, Michaela Tritscher, Martin Hermansson, Bernhard Watzer, Horst Schweer, Gernot Desoye, Akos Heinemann, and Sasa Frank

The author list should have read:

Monika Riederer, Pauli J. Ojala, Andelko Hrzenjak, **Wolfgang F. Graier, Roland Malli**, Michaela Tritscher, Martin Hermansson, Bernhard Watzer, Horst Schweer, Gernot Desoye, Akos Heinemann, and Sasa Frank

Additionally, corrections were needed to the authors' affiliations.

The original affiliations appeared in print as follows:

Institute of Molecular Biology and Biochemistry,* Center of Molecular Medicine; Finnish Red Cross Blood Service;† Division of Pulmonology,§ Department of Internal Medicine; Department of Biochemistry,** Institute of Biomedicine, University of Helsinki, Helsinki, Finland; Department of Pediatrics,†† Philipps University of Marburg, Marburg, Germany; and Clinic of Obstetrics and Gynecology;§§ Institute of Experimental and Clinical Pharmacology,*** and Institute of Molecular Biology & Biochemistry,††† Center of Molecular Medicine, Medical University of Graz, Graz, Austria

The affiliations should have read:

Institute of Molecular Biology and Biochemistry,* Center of Molecular Medicine, Medical University of Graz, Graz, Austria; Finnish Red Cross Blood Service,† Helsinki, Finland; Division of Pulmonology,§ Department of Internal Medicine, Medical University of Graz, Graz, Austria; Department of Biochemistry,** Institute of Biomedicine, University of Helsinki, Helsinki, Finland; Department of Pediatrics,†† Philipps University of Marburg, Marburg, Germany; and Clinic of Obstetrics and Gynecology;§§ and Institute of Experimental and Clinical Pharmacology,*** Center of Molecular Medicine, Medical University of Graz, Graz, Austria

The incorrect author list and affiliations initially appeared online but have since been corrected in the article and the table of contents.

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