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Endothelial lipase (EL) and EL-generated lysophosphatidylcholines promote IL-8 expression in endothelial cells

Monika Riederer[a], Margarete Lechleitner[a], Andelko Hrzenjak[b], Harald Koefeler[c], Gernot Desoye[d], Akos Heinemann[e], Saša Frank[a,*]

[a] Institute of Molecular Biology and Biochemistry, Harrachgasse 21/III, Medical University Graz, 8010 Graz, Austria
[b] Division of Pulmonology, Department of Internal Medicine, Auenbruggerplatz 20, Medical University Graz, 8010 Graz, Austria
[c] Center for Medical Research, Stiftingtalstraße 24, Medical University Graz, 8010 Graz, Austria
[d] Department of Obstetrics and Gynecology, Auenbruggerplatz 14, Medical University Graz, 8010 Graz, Austria
[e] Institute of Experimental and Clinical Pharmacology, Universitätsplatz 4, Medical University Graz, 8010 Graz, Austria

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ABSTRACT

Objective: Previously we identified palmitoyl-lysophosphatidylcholine (LPC 16:0), as well as linoleoyl-, arachidonoyl- and oleoyl-LPC (LPC 18:2, 20:4 and 18:1) as the most prominent LPC species generated by the action of endothelial lipase (EL) on high-density lipoprotein (HDL). In the present study, the impact of EL and EL-generated LPC on interleukin-8 (IL-8) synthesis was examined *in vitro* in primary human aortic endothelial cells (HAEC) and in mice.

Methods and Results: Adenovirus-mediated overexpression of the catalytically active EL, but not its inactive mutant, increased endothelial synthesis of IL-8 mRNA and protein in a time- and HDL-concentration-dependent manner. While LPC 18:2 was inactive, LPC 16:0, 18:1 and 20:4 promoted IL-8 mRNA- and protein-synthesis, differing in potencies and kinetics. The effects of all tested LPC on IL-8 synthesis were completely abrogated by addition of BSA and chelation of intracellular Ca^{2+}. Underlying signaling pathways also included NFkB, p38-MAPK, ERK, PKC and PKA. In mice, adenovirus-mediated overexpression of EL caused an elevation in the plasma levels of MIP-2 (murine IL-8 analogue) accompanied by a markedly increased plasma LPC/PC ratio. Intravenously injected LPC also raised MIP-2 plasma concentration, however to a lesser extent than EL overexpression.

Conclusion: Our results indicate that EL and EL-generated LPC, except of LPC 18:2, promote endothelial IL-8 synthesis, with different efficacy and kinetics, related to acyl-chain length and degree of saturation. Accordingly, due to its capacity to modulate the availability of the pro-inflammatory and pro-adhesive chemokine IL-8, EL should be considered an important player in the development of atherosclerosis.

1. Introduction

EL is a member of the triglyceride (TG) lipase gene family localized on the surface of vascular endothelial cells [1,2]. By virtue of its phospholipase activity, EL cleaves HDL-phosphatidylcholine (HDL-PC) liberating free fatty acids (FFA) and lysophosphatidylcholine (LPC) [3,4]. Experiments in genetically modified mice, either over-expressing or lacking functional EL revealed an inverse relationship between plasma HDL cholesterol level and EL expression [5,6]. The capacity of EL to decrease HDL plasma concentrations together with its ability to promote monocyte adhesion to endothelial cells [7], suggest EL to be an atherogenic enzyme. However, the results obtained from atherosclerosis studies using EL knock out (ko) models were conflicting, with decreased aortic atherosclerosis in double EL-/apoE-ko mice [8] and no differences in a separate study where both EL-/apoE-ko and EL-/LDL receptor-ko mice were used [9].

In a previous study, we demonstrated that in addition to the well characterized 1-palmitoyl (16:0) LPC, EL generates substantial amounts of unsaturated LPC 18:1, 18:2 and 20:4, respectively [10]. Saturated 16:0 LPC represents the well characterized standard LPC, shown to induce various signaling pathways thereby promoting the production of inflammatory molecules, including IL-8, in human vascular endothelial cells [11].

The physiological concentrations of LPC in body fluids ranges between 100 and 170 μM [12] with even millimolar levels in hyper-

Abbreviations: LPC, lysophosphatidylcholine; EL, endothelial lipase; 16:0 LPC, palmitoyl-lysophosphatidylcholine; 18:2 LPC, linoleoyl-LPC; 20:4 LPC, arachidonoyl-LPC; 18:1 LPC, oleoyl-LPC; HAEC, human aortic endothelial cells; AA, arachidonic acid; NEFA, nonesterified fatty acids; FFA, free fatty acids; BSA, bovine serum albumin; PKA, protein kinase A; PKC, protein kinase C; NFκB, nuclear factor kappa B; 2-AG, 2-arachidonoyl-glycerol; p38-MAPK, p38-mitogen-activated protein kinase; ERK, extracellular regulated kinase; HDL, high-density lipoprotein; MIP-2, macrophage inflammatory protein 2-alpha; GFX, GF109203X.

* Corresponding author. Tel.: +43 316 380 4194; fax: +43 316 380 9615.
E-mail address: sasa.frank@medunigraz.at (S. Frank).

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lipidemic subjects [13]. LPC in plasma are distributed between albumin and other carrier serum proteins and lipoproteins [14,15]. Furthermore, free LPC may transiently exist due to an excessive lipolysis when the concentrations of FFA and LPC locally exceed the binding capacity of albumin and carrier proteins [15].

Interleukin-8 (IL-8) is a pro-inflammatory chemokine which acts as an important chemoattractant for neutrophils and monocytes, and has been implicated in the pathogenesis of atherosclerosis [16]. It is synthesized by various types of vascular cells, including endothelial cells, where inflammatory stimuli and bioactive lipids have been shown to augment its production [11,17].

Since studies investigating the impact of LPC on endothelial chemokine secretion used exclusively 16:0 LPC [11,18] nothing is known about the impact of length and degree of saturation of the LPC-acyl chain on IL-8 production in vascular endothelial cells, the main source of EL.

Therefore, the aim of the present study was to determine the effect of EL on the generation of IL-8 and to compare the impact of the "standard" 16:0 LPC with that of unsaturated LPC, abundantly generated by EL, on IL-8 production in primary human aortic endothelial cells (HAEC) and *in vivo* in mice.

2. Materials and methods

2.1. LPC

LPC 16:0, 18:1, 18:2 and 20:4 were purchased from Avanti Polar Lipids. LPC were dissolved and stored at −20 °C in chloroform/methanol under argon atmosphere. Required amounts of LPC were dried under a stream of nitrogen or argon and re-dissolved in PBS (pH 7.4) for cell culture experiments or in pyrogen-free saline (NaCl) for *in vivo* experiments.

2.2. 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 gelatine-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 HDL or LPC.

2.3. Isolation of human HDL

HDL (subclass 3, d = 1.125–1.21 g/ml) was prepared by sequential ultracentrifugation of plasma obtained from normolipidemic blood donors as described previously [19].

2.4. Adenovirus generation

Adenoviruses (Ad) encoding human EL (EL), catalytically inactive mutant EL (MUT) containing $Asp^{192} \rightarrow Asn$ substitution and bacterial β-galactosidase (LacZ) were prepared exactly as described previously [19].

2.5. Adenoviral infection of HAEC

HAEC (75,000/well) were seeded in 12-well plates. Forty-eight hours later, confluent cells were infected with EL-Ad, EL-MUT-Ad or LacZ-Ad at multiplicity of infection (MOI) of 50 in endothelial basal medium (EBM) without fetal calf serum (FCS). Following a 2 h-infection period, cells were grown in complete EBM for additional 24 h. Thereafter, cells were washed with PBS and incubated with EBM medium without supplements and without serum for 3 h. This medium is referred to as a serum-free medium throughout the text. Thereafter, medium was removed and replaced with fresh serum-free medium supplemented with indicated concentrations of HDL.

2.6. LPC treatment of HAEC

2.6.1. LPC cytotoxycity:

Initial time- and concentration-dependent experiments revealed that in a serum-free medium in the absence of bovine serum albumin (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 a cytotoxicity detection kit (LDH) (Roche, Mannheim, Germany).

2.6.2. Experiments under serum-free conditions:

48 h after plating, cells were washed with PBS, and incubated in serum-free medium for 3 h. Thereafter, medium was removed and replaced with fresh serum-free medium supplemented with different concentrations of LPC. In some experiments as indicated in the respective figure legends, LPC were applied along with BSA at a molar ratio of 1:1 and 5:1, respectively.

2.6.3. Experiments in the presence of serum:

Here, washing steps and preincubation steps with serum-free medium were omitted. Due to the presence of BSA and other binding proteins in serum, 100 μM LPC was applied for the incubation in serum-containing medium (5%) (to ascertain the presence of free LPC).

Medium was collected in pre-chilled tubes following exposure to HDL or LPC, centrifuged and used for lactate dehydrogenase (LDH) assay and IL-8 quantification. Cells were washed with PBS and lysed for RNA or protein isolation.

2.7. Pharmacological inhibitors

HAEC were pre-treated with respective pharmacological inhibitors or vehicle (DMSO) for 45 min in serum-containing medium before the addition of fresh FCS-medium containing LPC. Following inhibitors were applied: Bapta/AM (Ca^{2+} chelator; 10 μM), PDTC (NFκB inhibitor; 30 μM), SB203580 (p38-MAPK inhibitor; 5 μM), PD98059 (ERK inhibitor; 30 μM), GFX (PKC inhibitor; 1 μM), H-89 (PKA inhibitor; 10 μM), genistein/daizein (tyrosin kinase inhibitor/negative control; 100 μM), U-73122/U73343 (PLC inhibitor/negative control; 2 μM each). For treatment with pertussis toxin (PTX) cells were preincubated with 0.1 μg/ml PTX for 17 h. All inhibitors were from Sigma.

2.8. Quantitative real-time PCR (qRT-PCR)

Please see Supplemental Methods.

2.9. Western blotting

EL expression was examined exactly as described [19]. Please see supplemental Methods for details.

2.10. IL-8 and MIP-2 measurements by ELISA

IL-8 was measured in cell culture media by an ELISA Kit (eBioscience, San Diego, USA) according to the manufacturer's protocol. Protein content of cell culture wells was initially determined to be equal for all treatments.

MIP-2, a mouse IL-8 analogue, was measured in mouse plasma by an ELISA Kit according to manufacturers instructions (Komabiotech, Seoul, Korea).