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Lysophosphatidylcholine induces inflammatory activation of human coronary artery smooth muscle cells

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Abstract

Lysophosphatidylcholine (LPC) is the major bioactive lipid component of oxidized LDL, thought to be responsible for many of the inflammatory effects of oxidized LDL described in both inflammatory and endothelial cells. Inflammation-induced transformation of vascular smooth muscle cells from a contractile phenotype to a proliferative/secretory phenotype is a hallmark of the vascular remodeling that is characteristic of atherogenesis; however, the role of LPC in this process has not been fully described. The present study tested the hypothesis that LPC is an inflammatory stimulus in coronary artery smooth muscle cells (CASMCs). In cultured human CASMCs, LPC stimulated time- and concentration-dependent release of arachidonic acid that was sensitive to phospholipase A_2 and C inhibition. LPC stimulated the release of arachidonic acid metabolites leukotriene-B_4 and 6-keto-prostaglandin $F_{1\alpha}$, within the same time course. LPC was also found to stimulate basic fibroblast growth factor release as well as stimulating the release of the cytokines GM-CSF, IL-6, and IL-8. Optimal stimulation of these signals was obtained via palmitic acid-substituted LPC species. Stimulation of arachidonic acid, inflammatory cytokines and growth factor release, implies that LPC might play a multifactorial role in the progression of atherosclerosis, by affecting inflammatory processes.

Key words: Human CASMCs, LPC, signal transduction

Introduction

Lysophosphatidylcholine (LPC), generated from phospholipase A_2-dependent hydrolysis of phosphatidylcholine is a major bioactive lipid component of oxidized LDL [1, 2]. It has been proposed that LPC is responsible for many of the cellular effects of oxidized LDL described *in vitro* [3] including monocyte chemotaxis, gene transcription and proinflammatory cytokine secretion [4]. In vascular cells, LPC stimulates vascular smooth muscle cell migration and

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proliferation [5–7]; however, the pro-inflammatory effects of LPC have been most widely described in endothelial cells [8–12]. These include induction of adhesion molecule and growth factor expression, as well as increased secretion of arachidonic acid (AA) [8, 9, 11].

Transformation of vascular smooth muscle cells from a contractile phenotype to a proliferative/secretory phenotype is a hallmark of atherogenesis [13]. Vascular wall remodeling, manifest as increased proliferation as well as increased apoptosis, in combination with an increase in the synthesis and release of inflammatory cytokines is an important step in the generation and maintenance of the atherosclerotic plaque [14]. However, despite the growing body of literature supporting a role for LPC as a pro-inflammatory component of oxidized LDL, characterization of LPC as a stimulant of vascular smooth muscle inflammation and the signaling pathways potentially involved has not been described.

The present study was designed to test the hypothesis that LPC represents an inflammatory stimulus for coronary artery smooth muscle cells. To accomplish this, it was decided to examine (a) cytokine synthesis and the release of AA and its metabolites, in cultured human coronary artery smooth muscle cells during manipulation of the phosphatidylcholine system.

Methods

Reagents

Human coronary artery smooth muscle cells (CASMCs) were purchased from Clonetics Corp (San Diego, CA). LPC (1-acyl-2-hydroxy-sn-glycero-3-phosphorylcholine; acyl chain isoforms of LPC studied were myristoyl, palmitoyl and stearoyl), sphingosylphosphorylcholine (SPC), lysophosphatidic acid (LPA) and lysolecithin (egg) were purchased from Sigma Chemical company (St. Louis, MO). Arachidonyl trifluoromethyl ketone ($AACOCF_3$), U-73122 and U-73343 were purchased from Biomol Inc. (Plymouth Meeting, PA). [^{3}H]AA (210 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Enzyme immunoassay (EIA) kits for LTB_4, 6-keto-$PGF_{1\alpha}$ and basic FGF were purchased from R&D Systems (Minneapolis, MN).

Lysophosphatidylcholine (LPC) solutions (10 mM stock) were made by dissolving in chloroform-methanol 2:1 (v/v), evaporated under N_2 and the lysophospholipids were resuspended in HEPES buffer (pH 7.4).

Cell culture

Human CASMCs were cultured by using SmGM2 kit medium (smooth muscle growth medium BioWhittaker) supplemented with 5% fetal bovine serum. Cells were positive for smooth muscle cell markers like α-actin and negative for von Wilebrand factor (vWF). Cells (between passages 3 and 7) were seeded in 24-well culture plates at 20,000–25,000 cells/well (500 ul media/well) 48 h before the respective treatment and serum-starved 18 h before treatment.

[^{3}H] AA release

Human CASMCs were loaded with [^{3}H]AA (0.5 μCi/well) in DMEM containing 0.01% fatty acid-free bovine serum albumin (BSA) for 18 h. To measure [^{3}H] AA release, cells were washed with DMEM and incubated with 0.5 ml/well DMEM + BSA (0.01% w/v) for 10 min at room temperature before treatment with stimuli. For inhibitor experiments, cells were pre-incubated with inhibitors (3 or 10 μM) for 10 min, and then treated with LPC for 20 min at 37 °C, after which time the incubation medium was collected and radioactivity determined using a scintillation counter.

Measurement of LTB_4, 6-keto-$PGF_{1\alpha}$ and basic FGF-2

Human CASMCs were seeded in 24-well plates (5.0×10^4 cells/well) in DMEM supplemented with 5% fetal calf serum and incubated for 48 h at 37 °C. The cells were washed twice in Hank's balanced salt solution and incubated with different concentrations (0.1–30 μM) of LPC ($C_{16:0}$) at 37 °C for 30 min. Culture medium (''conditioned'' medium) was collected and stored at −80 °C for measurements of LTB_4, 6-keto-$PGF_{1\alpha}$ and basic FGF-2.

LTB_4, 6-keto-$PGF_{1\alpha}$, and basic FGF-2 release was measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D systems, Minneapolis, MN). The sensitivity of the LTB_4 assay was approximately 5.6 pg/ml. Cross reactivity for LTC_4, LTD_4 and LTE_4 for this kit is <0.2%. The minimum detectable dose of 6-keto-$PGF_{1\alpha}$ assay is approximately 1.4 pg/ml. The basic FGF-2 assay kit does not cross-react with other FGFs. The minimum detectable dose of FGF basic is typically less than 3 pg/ml according to the supplier.

Cytokine measurement

Human CASMCs were grown to confluency in 24-well plates and incubated with serum-free medium for 24 h prior to experimentation. Cells were incubated with various concentrations of LPC ($C_{16:0}$) for an additional 24 h, and conditioned medium was collected and frozen. Cytokines in conditioned media from human CASMCs treated with

vehicle or increasing concentrations of lipids were measured by Luminex100^{TM} system (Molecular Devices, Sunnyvale, CA) using carboxylated polystyrene beads coupled covalently with capture Abs specific for individual cytokines (R&D). The beads coated with Abs for IL-1β, -2, -4, -5, -6, -8, -10 and -12; GM-CSF; IFN-γ and TNF-α were used. Assays were performed according to the manufacturer's instructions; samples were run in duplicate. Concentrations of individual samples were calculated and expressed as picogram (pg) of protein per ml.

Data analysis

Unless stated to the contrary all the measurements were performed in triplicate and the experiments were repeated two to three times. The data represent the mean ± SEM. The data were analyzed with LIGAND program and with nonlinear regression analysis of GraphPad InPlot program (GraphPad Software, Inc. San Diego, CA). Comparison between two values was assessed by unpaired Student *t*-test and also by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Effect of LPC on [^{3}H]AA release

At a concentration of 30 μM, egg lysolecithin, which is comprised of multiple forms of LPC, induced rapid, time-dependent release of AA in human CASMCs that reached a maximum of 547% above basal within 15 min and was sustained between ~500% over basal up to 60 min (Fig 1A). Based on these data, 15 min incubation was used for all subsequent experiments reported in the study.

As egg lysolecithin contains mainly saturated species of LPC, synthetic myristoyl ($C_{14:0}$), palmitoyl ($C_{16:0}$), stearoyl ($C_{18:0}$) and unsaturated LPC ($C_{18:1}$), were tested for their ability to stimulate AA release in human CASMCs. As shown in Fig. 1B, LPC containing palmitoyl and stearoyl chains induced higher release of [^{3}H]AA from human CASMCs compared to myristoyl or unsaturated LPC. Lysophosphatidic acid and SPC did not stimulate AA release from these cells. Based on these results, LPC containing palmitoyl ($C_{16:0}$) chain was used for further characterization.

LPC ($C_{16:0}$) stimulated AA release from human CASMCs in a concentration-dependent manner (Fig. 2), with a significant stimulation of 315% above basal observed at 10 μM, and a stimulation of 875% above basal at 30 μM. LPC at this concentration has been found to be nonlethal to vascular smooth muscle cells [15] and in the present study, LPC did not change the cell number and also confirmed the cell viability under the incubation conditions by the exclusion of trypan blue dye (data not shown). Hence, these conditions were used in subsequent experiments.

To determine whether the increase in AA release is mediated via cytosolic PLA_2, LPC-mediated AA release was studied in these cells in the presence of arachidonoyl trifluoromethyl ketone ($AACOCF_3$, 1, 10 and 100 μM), a $cPLA_2$ inhibitor (Fig. 3A). At 10 and 100 μM inhibitor

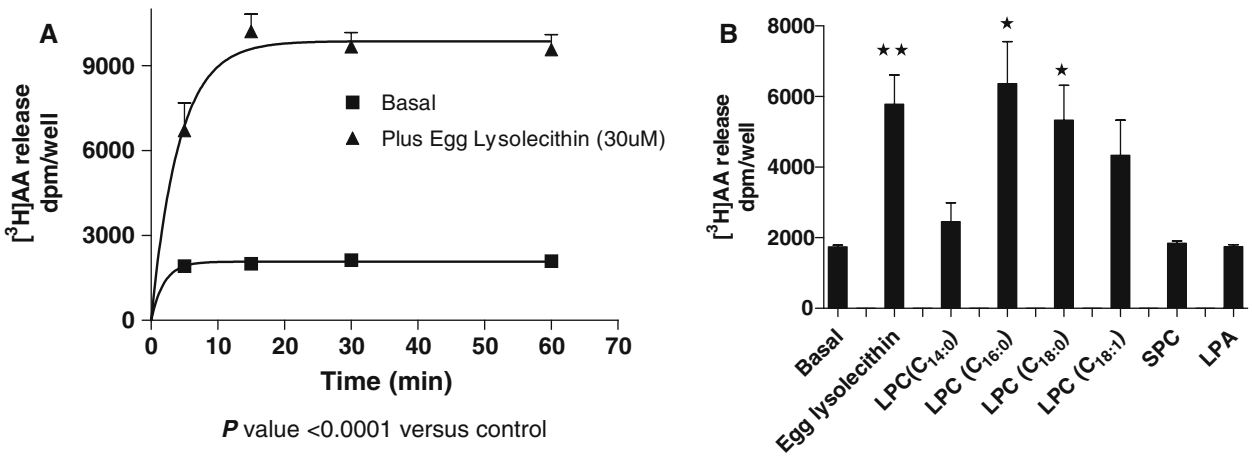

Fig. 1. Effect of LPC on AA release in human CASMCs. Cells were prelabeled with 1 μCi/ml of [^{3}H]AA in Dulbecco's medium containing 10% fetal calf serum for 16 h. Cells were washed three times with medium without serum prior to challenge. Panel A: Time course of egg lysolecithin induced [^{3}H]AA in human CASMCs. Cells were challenged with vehicle or 30 μM egg lysolecithin for the indicated times. Panel B: Prelabeled cells were challenged for 20 min with vehicle or 30 μM of the indicated LPC or other lipids. Egg lysolecithin; LPC ($C_{14:0}$), LPC ($C_{16:0}$), palmitoyl-LPC; LPC ($C_{18:0}$), stearoyl-LPC; LPC ($C_{18:1}$); SPC, sphingosylphosphorylcholine; LPA, lysophosphatidic acid. Arachidonate release into the medium was determined as described under "Methods." Basal release of [^{3}H]AA was 1745 ± 45 dpm well^{-1}. Values represent means ± standard error of the average from six separate experiments. $^{**}p = 0.007$, $^{*}p < 0.02$ vs. control.

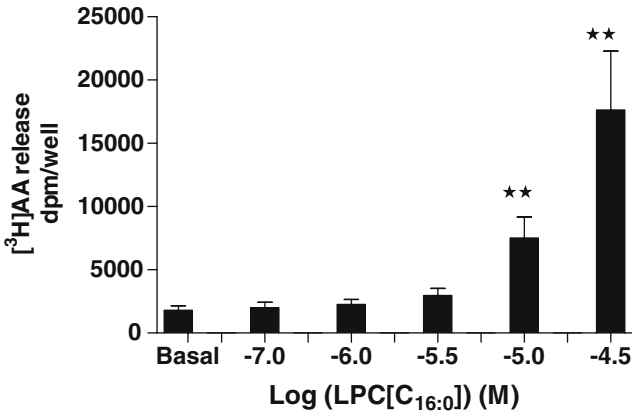

Fig. 2. LPC concentration-dependently stimulates arachidonate release in human CASMCs. Cells were prelabeled with [^{3}H]AA and challenged for 20 min with vehicle or with indicated concentrations of LPC ($C_{16:0}$). Arachidonate release into the medium was determined as described under "Methods". Basal release of [^{3}H]AA was 1808 ± 305 dpm well^{-1}.Values represent means ± standard error of the average from three separate experiments. $^{**}p$ = 0.007 vs. control.

concentration, LPC-mediated AA release was inhibited >90%. Incubation of human CASMCs with U-73122 (10 μM), a specific phospholipase C inhibitor, resulted in a significant inhibition (~80%) of LPC-mediated AA release (Fig. 3B). In contrast, however, pretreatment of these cells with U-73343 (an inactive isomer of U-73122) had no effect on LPC-mediated AA release (Fig. 3B).

Effect of LPC on LTB4, 6-keto-PGF$_{1\alpha}$, and FGF-2 synthesis

As shown in Fig. 4A, LPC significantly increased LTB_4 accumulation in human CASMCs with a threshold stimulus (124% above basal) occurring at 3 μM and the response at 30 μM LPC ($C_{16:0}$) was of 208% above basal. Egg lysolecithin, LPC ($C_{14:0}$) and SPC also stimulated LTB_4 accumulation [538%, 160% and 134% above basal at 30 μM of egg lysolecithin, LPC ($C_{14:0}$) and SPC, respectively] but LPA (up to 30 μM) failed to stimulate LTB_4 accumulation (Fig. 4B).

Measurement of 6-keto-$PGF_{1\alpha}$ was used to assess human CASMC cyclooxygenase-2 (COX-2) activity. To evaluate the level of 6-keto-$PGF_{1\alpha}$ in human CASMCs, the cells were exposed for 16 h to the indicated concentrations of LPC ($C_{16:0}$). The results showed that LPC induced 6-keto-$PGF_{1\alpha}$ levels in a concentration-dependent manner and the maximum increase observed was 909 ± 105% above basal (Fig. 5). The 6-keto-$PGF_{1\alpha}$ production was 878 ± 52 pg/well for control cells.

As shown in Fig. 6, LPC caused an increase in basic fibroblast growth factor-2 (bFGF-2) from human CASMCs. The effect was concentration dependent. Stimulation of FGF-2 was significant at 10 μM (1529% above basal).

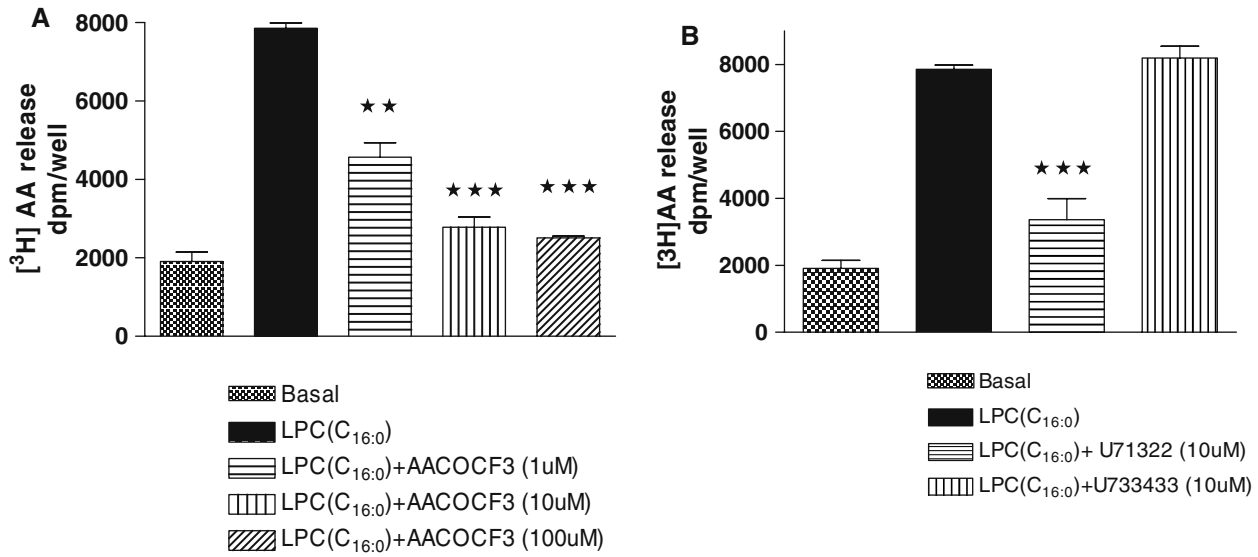

Fig. 3. Effect of phospholipase inhibition on LPC-stimulated [^{3}H]AA release in human CASMCs. Panel A. Cells were preloaded with [3H]AA and incubated with the indicated concentrations of the PLA_2 inhibitor $AACOCF_3$ or vehicle for 10 min prior to challenge with 10 μM LPC ($C_{16:0}$) for 20 min. The release of AA into the medium was then determined as described under "Methods". Results are expressed as mean ± SEM from three separate experiments. $^{**}p$= 0.001, $^{***}p < 0.0001$ vs. LPC ($C_{16:0}$). Panel B: Cells were preloaded with [3H]AA and incubated with the indicated concentrations of vehicle, the PLC inhibitor U73122, or the inactive isomer U73343 for 10 min prior to challenge with 10 uM LPC ($C_{16:0}$) for 20 min. AA release to the medium was determined as described under "Methods". Results are expressed as mean ± SEM from three separate experiments.$^{***}p < 0.0001$ vs. LPC ($C_{16:0}$).

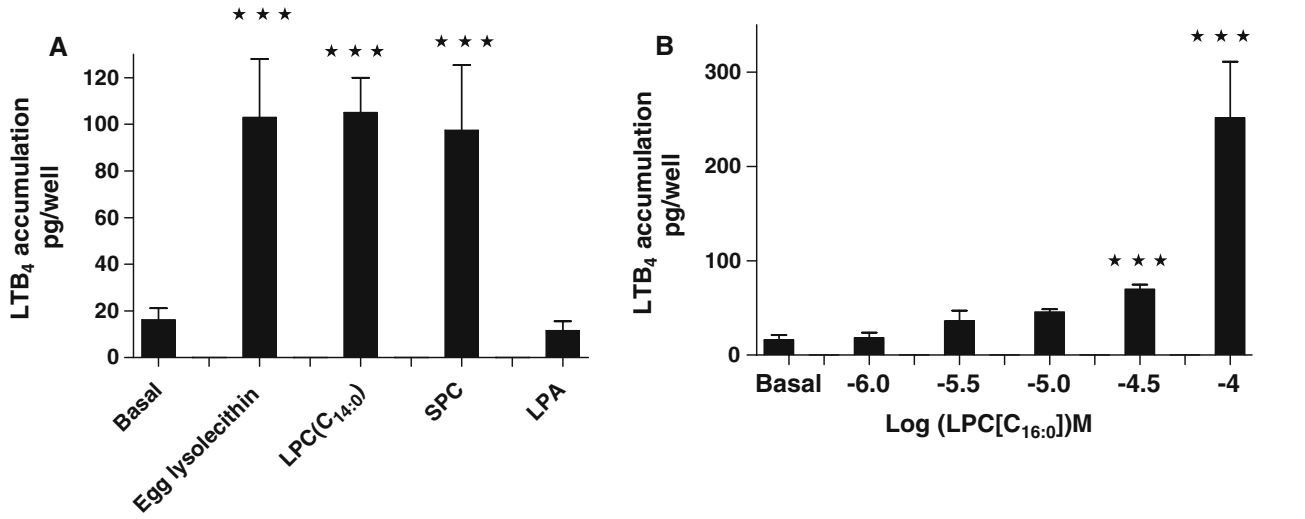

Fig. 4. Effect of lipids on LTB_4 levels in human CASMCs. Panel A: Cells in serum free cultured media were stimulated with 30 μM concentrations of egg lysolecithin, LPC (C14:0), LPC ($C_{16:0}$), SPC or LPA at 37 °C in an incubator for 16 h. LTB_4 levels were assayed in the supernatant by ELISA method. Results are expressed as mean ± SEM from three separate experiments. Basal LTB_4 level was 16.5 ± 5 pg/well. ***p = 0.001, ***$p\varpi$ = 0.0001 vs. basal. Panel B: Cells in serum free cultured media were stimulated with indicated concentrations of LPC ($C_{16:0}$) at 37 °C in an incubator for 16 h. LTB_4 levels were assayed in the supernatant by ELISA method. Results are expressed as mean ± SEM from seven separate experiments. Basal LTB_4 level was 16.5 ± 5 pg/well. ***p = 0.0002 vs. basal.

Effect of LPC on cytokine accumulation

To examine cytokine release, confluent human CASMCs were treated with LPC ($C_{16:0}$ 1–30 μM) for 12 h and the accumulation of eight cytokines/chemokines (IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, TNFα and GM-CSF) in the supernatant was measured using Luminex™ technology. Among the tested cytokines, IL-6, IL-8 and GM-CSF were found to be substantially increased in the LPC ($C_{16:0}$) treated cells (Fig. 7). There were no significant differences in cytokine production between vehicle and LPC ($C_{16:0}$) treated samples for IL-1β, IL-2, IL-3, IL-4, IL-10, IL-12, INF γ and TNFα.

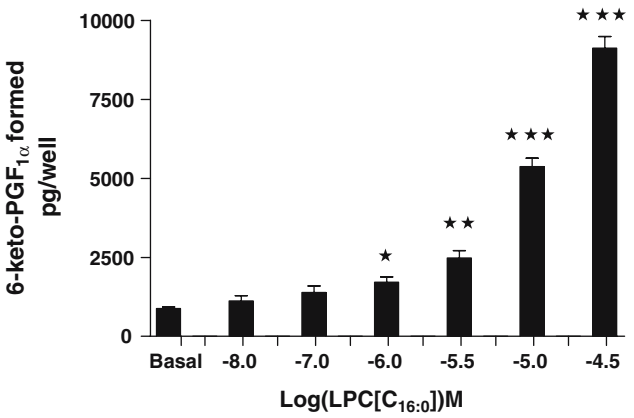

Fig. 5. LPC induced 6 keto $PGF_{1\alpha}$ release from human CASMCs. Human CASMCs cells were cultured in 24 well plates and at confluence the media was replaced by serum free medium. Cells were then treated with vehicle or at indicated concentrations of LPC ($C_{16:0}$) at 37 °C in an incubator for 16 h. 6-keto$PGF_{1\alpha}$ was measured in the conditioned media by ELISA. Basal 6-keto $PGF_{1\alpha}$ was 878 ± 52 pg/well. *p = 0.01 **p = 0.002 ***p < 0.0001 vs. basal.

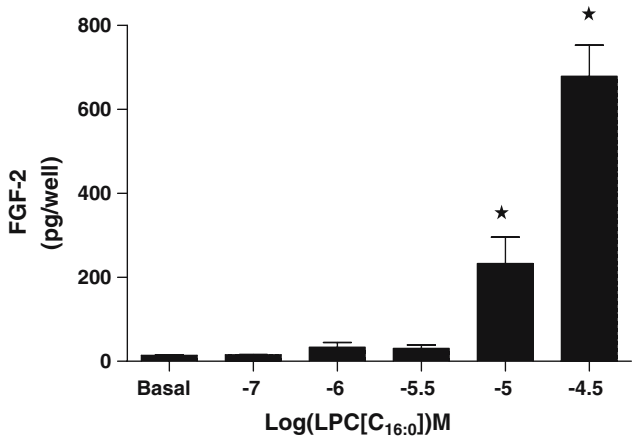

Fig. 6. LPC-mediated basic FGF-2 accumulation in human CASMCs: Human CASMCs cells were cultured in 24 well plate at 37 °C in a 5%CO2 incubator. The indicated concentrations of LPC ($C_{16:0}$) or vehicle were added to the quiescent cells for 2 h. The content of FGF-2 in the conditioned media was measured by an ELISA. Data are averages of duplicates; the experiment shown is representative results from four separate experiments. Basal FGF-2 level was 14.3 ± 1.4 pg/ml. *p < 0.02 vs. basal.

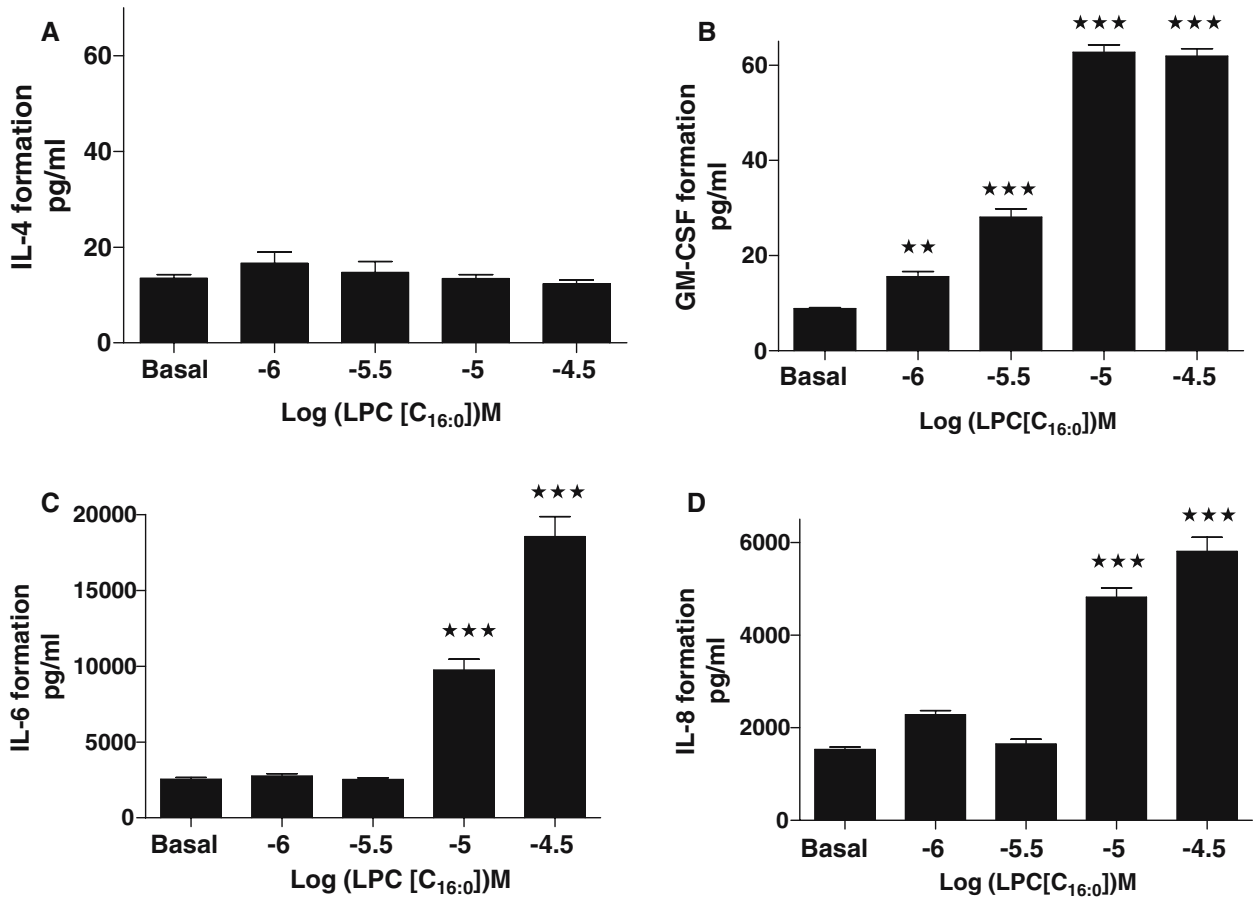

Fig. 7. Effects of LPC ($C_{16:0}$) on GM-CSF, IL-6 and IL-8 accumulation in human CASMCs. Cytokine assay by Luminex: LPC ($C_{16:0}$)-stimulated human CASMCs samples were assayed for cytokine levels through Luminex™. The data represent average values from three independent determinations done in duplicate. Basal GM-CSF, IL-6 and IL-8 levels were 8.9 ± 1.9, 2535 ± 125 and 1530 ± 55 pg/ml respectively. $^{**}p = 0.03$ and $^{***}p < 0.0001$ vs. control.

Discussion

The present data demonstrate the potential role of LPC to influence inflammatory activation of human CASMCs. LPC is the principal lysophospholipid formed during LDL oxidation [16] and is thought to play a major role in the pro-inflammatory effects of Ox-LDL, a key player in the initiation and progression of atherosclerosis. Despite reports of a wide range of effects induced by LPC, the specific inflammatory effects and potential signaling pathways involved in these phenomena have not been clearly defined.

LPC-induced AA release in human CASMCs in a time- and concentration-dependent manner, with LPC species containing long and saturated acyl chain causing a more pronounced effect than shorter acyl chain-containing isoforms. LPC-mediated AA release has been reported in H9c2 cells, a cell line derived from embryonic rat heart, and in bovine endothelial cells [11, 17], but this effect has not been described for human smooth muscle cells. AA release in these cells could occur either through a direct action of LPC on PLA_2 or indirectly by stimulation of PLC, which produces diacylglycerol and monoacylglycerol lipases that can be converted to AA [18]. Indeed, LPC-mediated AA release was inhibited by both $cPLA_2$-specific ($AACOCF_3$) and PLC-specific (U73122) inhibitors suggesting that LPC-mediated AA release could be mediated both by $cPLA_2$ and PLC activation in these cells.

AA is an important signaling molecule, which modulates a variety of physiological processes via its metabolism by a number of different enzyme systems. AA is converted into leukotrienes by the lipoxygenase pathway and into prostanoids by the cyclooxygenase (COX) pathway [19, 20]. The former pathway was examined in the present study via measurement of LTB_4. LPC dose-dependently increased LTB_4 accumulation in culture media of human CASMCs conditioned media. To our knowledge, the present study is the first to describe the activation of LTB_4 release by LPC. A fatty acid chain length of $C_{16:0}$ at the *sn*-1 position and the

presence of phosphocholine as the head group were critical determinants for optimal stimulation. Structurally-related LPA failed to stimulate LTB_4 in these cells. The importance of LTB_4 in atherosclerosis is highlighted by reports of expression of high levels of all components involved in LTB_4 biosynthesis (5-lipoxygenase, 5-lipoxygenase activating protein and LTA_4 hydrolase) in human atherosclerotic lesions [21]. Furthermore, in mouse models of atherosclerosis, lesion development is blocked by antagonists of LTB_4 receptor [22] and by genetic deficiency of BLT1, the LTB_4 receptor [23]. As such blockade of LPC function might be an alternate mode of therapeutic intervention.

Previous reports have shown that LPC can induce gene expression of COX-2 in endothelial cells [24, 25]. In the present study, COX-2 activation was assessed by measuring the accumulation of COX metabolite, 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of prostacyclin (PGI_2). PGI_2 is considered the principal prostanoid of vascular tissue, and its synthesis has been attributed, in part, to the activity of COX-2. Thus, increase in 6-keto-$PGF_{1\alpha}$ production seen in LPC treated human CASMCs ($909 \pm 105\%$ above basal) appears to reflect the activity of COX-2. COX-2 is one of the two isoforms that catalyzes the formation of prostaglandins from arachidonic acid. COX-2 has been detected in macrophages, smooth muscle cells and endothelial cells in human atherosclerotic lesions. Several studies have also reported that COX-2 is involved in the destabilization of atherosclerotic plaques, leading to rupture and atherothrombotic syndromes. Induction of COX-2 expression leads to the extracellular secretion of prostanoids, which stimulate cell growth [26, 27].

LPC has been shown to stimulate expression of cytokines, chemokines, adhesion molecules and growthfactors in various cell systems [8, 9, 15, 28–32]. The observation from the current study that LPC stimulates IL-6, IL-8 and GM-CSF production in human coronary artery smooth muscle cells support the notion that this might be a potential mechanism mediating a detrimental role for LPC in atherosclerosis. This is supported by a wealth of information implicating inflammatory cytokines and inflammatory cell recruitment in the pathophysiology of atherosclerosis [33, 34].

Among the 8 cytokines measured, GM-CSF, IL-6 and IL-8 were induced by LPC treatment in human CASMCs. IL-6 secreted from vascular smooth muscle cells is an important contributor to inflammation, in part by inducing B-cell differentiation, T-cell activation, and synthesis of acute-phase proteins such as C-reactive protein (CRP) and fibrinogen in the liver [35, 36]. IL-6 also increases smooth muscle cell proliferation in a PDGF-dependent manner [37]. IL-8 is predominantly released by macrophages and endothelial cells and has multiple effects, including neutrophil chemotaxis, activation of 5-lypoxygenase, and the release of cell matrix resorbing gelatinase and elastase. Stimulation of IL-6 and IL-8 release by LPC in smooth muscle cells, as described in the current study, could represent a pleiotropic role for LPC in atherogenesis, by stimulating both inflammatory (e.g. chemotaxis, lipoxygenase activation) as well as remodelling pathways (e.g. proliferation, matrix protease activity) in multiple cell types in vascular wall.

The LPC stimulation of basic FGF-2 in human CASMC is similar to earlier results reported in rabbit and human aortic smooth muscle cells [6]. In a previous study, Chai *et al.* [6] reported that the proliferative effect of LPC in smooth muscle cells is due to autocrine actions of basic FGF-2 released in response to LPC stimulation. This raises an interesting potential role for LPC as a mediator of vascular wall remodeling in atherosclerosis, involving stimulation of both inflammation and cell proliferation.

The present study demonstrates that LPC, a major component of oxidized LDL, stimulates a variety of effects in human cultured coronary artery smooth muscle cells. These include stimulation of AA release and metabolism, synthesis of inflammatory LTB_4, release of inflammatory cytokines, and stimulation of proliferative signaling cascades. All of the evidence along with reported effects of LPC on endothelial, vascular smooth muscle cells and macrophages imply that LPC may play an active role in atherogenesis and the selective inhibition of the LPC could represent a novel strategy to inhibit the progression of atherosclerosis.

Acknowledgment

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