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Lysophosphatidic acid causes endothelial dysfunction in porcine coronary arteries and human coronary artery endothelial cells

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Abstract

Aim—The objective of this study was to determine the effects of lysophosphatidic acid (LPA) on endothelial functions and molecular alternations in both porcine coronary arteries and human coronary artery endothelial cells (HCAECs).

Methods and results—The vessel rings and HCAECs were treated with clinically relevant concentrations of LPA for different times. Vasomotor reactivity was studied with a myograph tension system. LPA (10 and 50 μ M) treatment for the vessel rings significantly reduced endothelium-dependent vasorelaxation in response to bradykinin (10^{-5} M) by 32% and 49%, respectively, compared with the control ($P<0.05$). LPA decreased endothelial nitric oxide synthase (eNOS) mRNA and immunoreactivity levels in the vessel rings. In HCAECs, LPA reduced eNOS mRNA, phospho-eNOS and total eNOS protein levels. In addition, superoxide anion levels in LPA-treated vessel rings and HCAECs were significantly increased by lucigenin-enhanced chemiluminescence assay and dihydroethidium staining, respectively. Mitochondrial membrane potential and ATP content in LPA-treated HCAECs were substantially decreased. The mRNA levels of reactive oxygen species generating enzymes NOX4 and p40^{phox} were increased, while endogenous antioxidant enzyme superoxide dismutase 1 was decreased in response to LPA treatment in HCAECs. Furthermore, exogenous antioxidant molecule selenomethionine (SeMet) effectively reversed these LPA-induced effects in both porcine coronary arteries and HCAECs.

Conclusions—LPA causes endothelial dysfunction by a mechanism associated with decreased eNOS expression and increased oxidative stress in porcine coronary arteries and HCAECs.

Keywords

lysophosphatidic acid; endothelial cells; vasorelaxation; eNOS; superoxide anion; selenomethionine; endothelial dysfunction

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Conflict of interest

None declared.

1. Introduction

Endothelial cell dysfunction and oxidation of low-density lipoproteins (LDLs) are processes known to be key initiators of atherogenesis. Whereas numerous exogenous toxins have been implicated in contributing to plaque formation, a better understanding of molecular signaling and the imbalance of homeostatic mechanisms have led to the discovery of endogenous mediators capable of influencing such pathologic processes. One such molecule is the biologically active lipid mediator lysophosphatidic acid (LPA). LPA is generated by a number of cell types including platelets [1], macrophages [2], epithelial cells [3], cancer cells [4], neural cells [5], and fibroblasts [6]. It has a role in the regulation of a variety of cellular processes such as migration, proliferation, neurogenesis, vascular development, wound healing, apoptosis regulation, and immunity [4,7]. Several studies implicate that LPA may act as a pathologic mediator of atherogenesis including its incorporation into human atherosclerotic lesions [8,9], its generation upon oxidation of LDLs [10], and its effect on endothelial cells [9,11] and platelets [12], and its role in neointimal formation [13].

LPA is formed by activated platelets and mild oxidation of LDLs [14]. In activated platelets, phospholipase C- and phospholipase D-mediated processes lead to the formation of phosphatidic acid, which is subsequently degraded by phospholipase A1 or phospholipase A2 into 1-acyl-LPA or 2-acyl LPA, respectively. Platelet derived LPA accounts for only a fraction of serum LPA. Upon mild oxidation of LDL particles, LPA levels are increased and it is exposed and enhances binding to LPA receptors leading to activation of platelets and endothelial cells [8]. LPA binds to specific G-coupled receptors on plasma membrane to mediate its downstream effects on cellular function [15].

Endothelial cells are critical in regulating vascular tone and permeability, and play a crucial role in coagulation and thrombosis [16]. Endothelial dysfunction has important consequences on vascular structure, neointimal hyperplasia and atherosclerosis. LPA affects endothelial functions by several mechanisms [17,18]. Mild oxidation of LDL containing LPA has been implicated as an early step in endothelial dysfunction [7]. LPA has been shown to alter endothelial permeability through mechanisms that involve regulation of the actin cytoskeleton and the extracellular matrix [19–21] as well as disruption of endothelial tight junctions [19,22]. LPA is able to induce inflammatory response by upregulating the expression of leukocyte chemoattractants including IL-8, MCP-1 and pentraxin-3 as well as several adhesion molecules in human endothelial cells [23–26]. LPA can induce the expression of autotoxin, which may impair endothelial functions [27,28]. In human umbilical vein endothelial cells, LPA treatment results in shedding of the lectin-like domain of the thrombomodulin, an early marker of endothelial damage [29]. There are five cell-surface LPA receptors (LPA1-LPA5), which may mediate biological activities of LPA in endothelial cells [17].

In addition, LPA's role in platelet function and activation results in initiation of thrombosis [8,9]. Activation of the above pathways leads to the formation of atherogenic plaque and development of atherosclerosis. Endothelial nitric oxide synthase (eNOS) actively participates in regulation of endothelial cell function, including modulating endothelium-dependent vasorelaxation and regulating endothelial gene expression and interactions with inflammatory cells and platelets [30]. Altered regulation of eNOS and subsequent development of endothelial dysfunction has been a subject of multiple studies into pathogenesis of atherosclerosis. Among a number of molecular pathways identified, increases in reactive oxygen species (ROS) including superoxide anion are involved in many conditions leading to atherosclerosis [31].

Despite multiple studies describing the role of LPA in the pathogenesis of atherosclerosis, its involvement in endothelium-dependent vasomotor reactivity has not been studied previously. In the present study, we tested the hypothesis that LPA may lead to endothelial dysfunction by downregulating eNOS expression via oxidative stress. Particularly, we studied the effects of LPA on endothelium-dependent vasorelaxation in porcine coronary artery rings, examined the effect of LPA on eNOS mRNA and protein levels, and described the mechanisms of increased ROS production with LPA treatment in both porcine coronary arteries and human coronary artery endothelial cells (HCAECs). This study may provide important novel mechanisms of LPA-induced endothelial dysfunction.

2. Materials and methods

2.1. Chemicals and reagents

Acyl-lysophosphatidic acid (LPA) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). The Tri-reagent kit, thromboxane A₂ analogue U46619 (9, 11-Dideoxy-11 α , 9 α -eposymethanoprostaglandin F₂ α), bradykinin, sodium nitroprusside (SNP), tyrosin/EDTA, dimethyl sulfoxide (DMSO), seleno-L-methionine (SeMet), and phosphate-buffered saline (PBS) solution were obtained from Sigma Aldrich Chemical Co. (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was obtained from Life Technologies, Inc. (Grand Island, NY). Antibiotic-antimycotic solution was obtained from Mediatech Inc. (Herndon, VA). Dihydroethidium (DHE) and 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) were obtained from Molecular Probes (Eugene, OR). The FITC-conjugated anti-mouse IgG and avidin-biotin complex kit were obtained from Vector labs (Burlingame, CA). iScript cDNA Synthesis Kit, iQ SYBR Green SuperMix Kit, and the protein assay kit were obtained from Bio-Rad laboratories (Hercules, CA). The polymerase chain reaction (PCR) primers were synthesized by Sigma Genosys (Woodlands, TX). The MitoScreen kit was obtained from BD Biosciences (San Jose, CA). The ATPLite kit was obtained from PerkinElmer (Wellesley, MA). HCAECs and endothelial growth medium-2 (EGM-2) were obtained from Cambrex BioWhittaker Inc. (Walkersville, MD). Anti-eNOS monoclonal antibody and anti- β -actin antibody were obtained from PharMingen Corp (San Diego, CA). Anti-phospho-eNOS (ser1177) antibody was obtained from Cell Signaling Technology Inc (Danvers, MA).

2.2. Porcine coronary artery cultures

Fresh porcine hearts were obtained from immediately sacrificed 6–8 months old farm-raised pigs at a local slaughterhouse, and coronary arteries were perfused with ice-cold PBS. Right coronary arteries were carefully dissected free of the perivascular loose connective tissue and cut into uniform 5 mm-rings. Rings were incubated in DMEM at 37°C and 5% CO₂ for 24 hours after they were divided into different groups including control, treatment with different concentrations of LPA (2, 10 or 50 μ M), and additional treatment with SeMet (20 μ M). After incubation under the above treatment conditions, rings were suspended in the Organ-bath chambers (Danish MyoTechnology Organ Bath 700 MO, Aarhus, Denmark) in 6 mL of oxygenated Krebs's solution at 37°C. Myograph studies were performed as previously described from our laboratory [32,33]. Briefly, rings were gradually subjected to predetermined optimal tension of 30 millinewtons (mN) and allowed to equilibrate for 30 minutes with frequent replacement of Krebs's solution. After equilibration, vessel contraction was achieved with thromboxane A₂ analogue, U 46619 (final concentration 3×10^{-8} M) for 60–90 minutes. Once maximal contraction was obtained, endothelium-dependent vasorelaxation was achieved with addition of increasing concentrations of bradykinin (1×10^{-11} to 1×10^{-5} M) every 3 minutes. SNP (final concentration 1×10^{-8} M) was added, and endothelium-independent vasorelaxation was recorded. Maximal contraction and percent relaxation (endothelium-dependent and endothelium-independent) were measured

and compared among control and treatment groups. All animal studies were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals and that the animal protocol was reviewed and approved by the Animal Care and Use Committee of Baylor College of Medicine.

2.3. Cell culture

HCAECs at passage 6 to 8 were grown in EGM-2 supplemented with 10% FBS at 37°C and 5% CO₂ to 80–90% confluence. Cells were incubated for 24 hours in EGM-2 supplemented with 2% FCS at 37°C and 5% CO₂ under the following treatment conditions: control, 2 μM LPA, 10 μM LPA, 50 μM LPA, 20 μM SeMet, and 10 μM LPA plus 20 μM SeMet. The cells were harvested for molecular analyses.

2.4. Detection of superoxide anion

Superoxide anion levels in endothelial cells of the porcine coronary artery rings were detected using the lucigenin-enhanced chemiluminescence method with Sairius Luminometer and FB12 software (Berthold Detection System GmbH, Pforzheim, Germany). Treated rings were briefly rinsed in a modified Krebs N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid) (HEPES) buffer solution and cut open longitudinally into approximately 5×5 mm pieces [32,33]. Assay tubes (12×75 mm) were filled with 500 μL of the HEPES solution and 25 μL of lucigenin and the ring pieces were placed endothelium-side down at the bottom. Time-based readings of the luminometer were recorded. Data were obtained in relative light units per second (RLU/sec) for each sample and were averaged between 5 and 10 minutes. Values of blank tubes with HEPES and lucigenin only were subtracted from the values obtained from the tubes containing the ring samples. The area of each vessel segment was measured using calipers and used to normalize the data for each sample. Final data were represented as mean ± SEM (RLU/sec/mm²). For HCAECs, superoxide anion levels were detected by DHE staining and flow cytometry analysis. HCAECs were harvested with 0.02% Trypsin/EDTA and adjusted to 1 × 10⁶ cells per FACS tube. Superoxide anion (O₂⁻) staining was performed by adding DHE (3 μM) and incubating at 37°C for 30 minutes. Final samples were collected in 500 μL of staining buffer and stored at 4°C. Samples were analyzed using FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) within 24 hours of preparation. A minimum of 10,000 events were analyzed per experiment.

2.5. Real-time polymerase chain reaction (PCR)

Total RNA from HCAECs and porcine artery endothelial cells was isolated using the Tri-Reagent kit (Sigma) following the manufacturer's directions. RNA was resuspended in 20 μL of RNase-free water and the concentration was determined by absorbance at 260 nm wavelength using a spectrophotometer. cDNA was generated by reverse transcriptase (RT) from mRNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. Thermal cycler conditions used for reverse transcription were as follows: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Real-time PCR was performed using the iQ SYBR Green SuperMix Kit (Bio-Rad Laboratories, Hercules, CA). A master mixture was used to reduce variability in primer and reagent concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control to account for variations in mRNA loading. One μg total RNA was used for each reaction. Primers for human eNOS, NOX4, p40^{phox}, superoxide dismutase 1 (SOD1), and GAPDH [32,33] were designed by Beacon Designer (Bio-Rad Laboratories, Hercules, CA), and synthesized by Sigma Genosys (Woodlands, TX). Real-time PCR was performed in an iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Thermal cycler conditions used for real-time PCR were as follows: 3 minutes at 95°C, 40 repeats of 20 seconds at 95°C, and 1 minute at

60°C. Controls were performed with no RT (mRNA sample only) or no mRNA (water only) to demonstrate the specificity of the primers and the lack of DNA contamination in samples. Relative mRNA levels of eNOS, NOX4, p40^{phox}, and SOD1 were presented as $2^{[Ct(GAPDH)-Ct(\text{gene of interest})]}$, where Ct is the threshold cycle as described above. Minimum of 3 samples using the same treatment conditions were used for each analysis.

2.6. eNOS immunohistochemistry staining in porcine coronary arteries

Treated porcine coronary artery rings were fixed in 10% neutral buffered formalin overnight and then transferred to 70% alcohol. The specimens were dehydrated using sequentially increasing concentrations of ethanol followed by xylene and were then embedded in paraffin. Five-micrometer cross sections were cut and stained with hematoxylin and eosin, and then subjected to immunohistochemistry staining for eNOS using the avidin-biotin complex immunoperoxidase procedure (LSAB kit, Dako Co., Carpinteria, CA). Sections were counterstained and viewed on an Olympus BX41 microscope (Olympus USA, Melville, NY). Images were captured with an attached SPOT-RT digital camera and software (Diagnostic Instruments, Sterling Heights, MI).

2.7. Western blot analysis for eNOS expression

HCAECs were treated with LPA (10 μ M), antioxidant SeMet (20 μ M) or LPA and SeMet for 24 hours. Total protein was isolated from HCAECs using cell lysis buffer. Total protein concentration was quantified using BCA Protein Assay Reagent system. Ten or 20 μ g of total proteins were mixed with SDS sample buffer, heated at 100°C for 5 minutes and loaded onto 10% SDS-PAGE (Lonza Rockland, Rockland, MD). Proteins were resolved electrophoretically at 120V for 90 minutes, and transferred to nitrocellulose membrane at 80V for 90 minutes at 4°C. Nitrocellulose membrane (Bio-Rad) was blocked with 10% milk in Tris buffered saline (TBS) with 0.05% Tween-20 for 30 minutes at room temperature, and incubated with a specified primary antibody (anti-eNOS antibody, anti-Page phospho-eNOS antibody or anti- β -actin antibody) in 5% milk in TBS+0.05% Tween overnight at 4°C. The membrane was washed 3 times with TBS-0.05% Tween for 5 minutes, and incubated with secondary antibody (1:10000 goat-anti-mouse HRP IgG) in 5% milk in TBS-0.05% Tween for 45 minutes at room temperature. After 5 additional washes with TBS-0.05% Tween, the membrane was incubated with an ECL-detection kit for 5 minutes, and exposed on BioMax Film.

2.8. Mitochondrial potential ($\Delta\psi$ m) detection in HCAECs

Loss of $\Delta\psi$ m was assessed using flow cytometry analysis of cells stained with 5,5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1, MitoScreen kit, BD Biosciences, San Diego, CA). Mitochondria with a normal $\Delta\psi$ m are able to concentrate JC-1 into aggregates producing a red fluorescence, but with a de-energized or depolarized $\Delta\psi$ m, JC-1 forms monomers producing a green fluorescence. HCAECs (5×10^5 cells) were incubated with 10 μ g/mL of JC-1 for 12 minutes at 37°C and analyzed by flow cytometry using the FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). The analyzer threshold was adjusted on the FSC channel to exclude most of the subcellular debris. Photomultiplier settings were adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered at 390 nm) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered at 340 nm). Data analyses were performed with Paint-a-Gate Pro Software (Becton Dickinson, Franklin Lakes, NJ). A minimum of 20,000 events were analyzed per experiment.

2.9. Measurement of the ATP content

ATP levels in HCAECs were measured with an ATPLite kit per manufacture's instructions. The ATPLite assay system (Perkin-Elmer, Waltham, MA) is based on the production of light caused by the reaction of ATP, D-luciferin, and luciferase. The emitted light is proportional to the ATP concentration. HCAECs were cultured and treated as previously described on 6 well plates for 24 hours. The lysis and substrate solutions were added to each well. The luminescence was measured by the TopCount Microplate Scintillation and Luminescence Counter (Perkin-Elmer, Waltham, MA).

2.10. Statistical analysis

Experimental data were presented as mean $\pm$ standard error of the mean. Statistical analysis was conducted by comparing results from the control and treatment groups using the two-tailed Student's t-test. Vasomotor reactivity, mRNA levels, superoxide anion and ATP content, and membrane potential obtained from multiple groups were compared by one way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Experimental results

3.1. LPA decreases endothelium-dependent vasorelaxation in porcine coronary artery rings

Endothelial dysfunction is thought to play an important role in progression of atherosclerosis. To investigate the effects of LPA on endothelial cell function, we performed vasomotor reactivity experiments using the myograph tension system. Treatment of porcine coronary artery rings with LPA did not affect maximal vessel contraction achieved with administration of thromboxane A2 analogue, U46619 (Fig. 1A). However, LPA treatment impaired endothelium-dependent vasorelaxation elicited by response to increasing concentrations of a vasodilator, bradykinin. The effects of LPA were concentration-dependent at most bradykinin concentrations used (Fig. 1B). For example, at 1×10^{-5} M bradykinin, the treatment with 2, 10 and 50 μ M LPA resulted in a decrease in endothelium-dependent relaxation by 4%, 32%, and 49%, respectively (Fig. 1C). LPA did not affect endothelium-independent vasorelaxation in porcine coronary artery rings (Fig. 1D).

3.2. LPA decreases eNOS expression in HCAECs and porcine coronary arteries

To investigate the role of LPA in eNOS expression in HCAECs and porcine coronary arteries, we performed real-time quantitative PCR, Western blot and immunohistochemistry. Treatment of HCAECs with increasing concentrations of LPA (2, 10 and 50 μ M) for 24 hours resulted in a concentration-dependent decrease in eNOS mRNA levels compared with untreated cells (Fig. 2A). For example, LPA at 10 μ M decreased eNOS mRNA levels by 30% over controls. This effect of LPA was observed at 6 hours, and remained persistent up to 48 hours of treatment (Fig. 2B). For eNOS protein analysis, HCAECs were treated with LPA (10 μ M) for 24 hours. Both phospho-eNOS (ser-1177) and total eNOS protein levels were determined by Western blot analysis. β -Actin was used as a house-keeping gene control. LPA treatment substantially reduced both phospho-eNOS and total eNOS levels in HCAECs (Fig. 2C). Immunohistochemistry staining showed a concentration-dependent decrease in eNOS protein levels in porcine coronary artery rings treated with increasing concentrations of LPA (Fig. 2D).

3.3. LPA induces superoxide anion production in HCAECs and porcine coronary artery rings

To elucidate potential mechanisms of LPA-induced decrease in endothelium-dependent vasorelaxation and in the eNOS expression, we sought to examine the effect of LPA on

superoxide anion production. Treatment of HCAECs with LPA resulted in a concentration-dependent increase in superoxide anion production as measured by DHE staining (Fig. 3A and B). Administration of 20 μ M SeMet to cells treated with 10 μ M LPA decreased superoxide anion production to the control level. Similarly, LPA increased superoxide anion production in a concentration-dependent manner in porcine coronary artery rings as measured by lucigenin-enhanced chemiluminescence assay (Fig. 3C), and this increase was abolished with co-treatment with 20 μ M SeMet.

3.4. LPA causes mitochondrial dysfunction in HCAECs

Mitochondria are the major source of ROS during mitochondrial respiration and ATP production. Mitochondrial membrane potential ($\Delta\psi$ m) provides measure of the function of the mitochondrial respiratory chain. Treatment of HCAECs with 10 μ M LPA for 24 hours resulted in a 35% decrease in $\Delta\psi$ m (Fig. 4A). Co-treatment of 10 μ M LPA with 20 μ M SeMet restored the $\Delta\psi$ m to the control level. Furthermore, 10 μ M LPA decreased ATP levels in HCAECs by 29%, and SeMet co-treatment rescued LPA-mediated decline in ATP levels in HCAECs (Fig. 4B).

3.5. LPA regulates ROS related enzymes

Real-time PCR measurement of mRNA levels of ROS generating enzymes, NOX4 and p40^{phox}, and an endogenous antioxidant enzyme SOD1 were analyzed in HCAECs treated with LPA. Treatment of HCAECs with LPA led to a concentration-dependent increase in mRNA levels of both NOX4 and p40^{phox} (Fig. 5A and B), while mRNA levels of SOD1 were decreased with increasing concentrations of LPA (Fig. 5C).

3.6. Antioxidant SeMet reverses LPA-mediated endothelial dysfunction

Co-treatment of porcine coronary artery rings with 20 μ M SeMet and 10 μ M LPA reversed LPA-mediated attenuation of endothelium dependent vasorelaxation (Fig. 6A and B). SeMet alone had no significant effects on endothelium-dependent vasorelaxation. Decreased eNOS protein staining after LPA treatment was similarly abrogated with co-treatment of porcine coronary artery rings with 20 μ M SeMet (Fig. 6C). For HCAECs, treatment with 20 μ M SeMet in addition to 10 μ M LPA normalized eNOS mRNA levels to those seen in control cells in the quantitative real-time PCR experiments (Fig. 6D). SeMet (20 μ M) alone did not alter eNOS mRNA levels in HCAECs, while 10 μ M LPA significantly decreased eNOS mRNA levels as described above. Furthermore, antioxidant SeMet effectively blocked LPA-induced downregulation of phospho-eNOS and total eNOS protein levels (Fig. 2C). These data were consistent with those in Fig. 3 and Fig. 4 where co-treatment with SeMet reduced LPA-induced increase in superoxide anion production in both porcine coronary artery rings, and rescued mitochondrial membrane potential and the intracellular ATP levels to those seen in control samples. Furthermore, SeMet blocked LPA-induced increase in NOX4 and p40^{phox} mRNA levels (Fig. 5A and B) and decrease in SOD1 mRNA levels (Fig. 5C).

4. Discussion

LPA has been implicated in the pathogenesis of atherosclerosis and cardiovascular disease [22,23]. In the present study, we demonstrate that LPA may lead to endothelial dysfunction by decreasing eNOS levels and activity in porcine coronary arteries and HCAECs. Superoxide anion generation is in part responsible for the observed changes in eNOS levels and activity, and treatment with exogenous antioxidant, SeMet, attenuates the effects of LPA on endothelial function and eNOS regulation. These findings provide a novel mechanism of the deleterious effects of LPA on coronary vasculature.

Quantification of the LPA serum levels has been a subject of several different studies. A number of assays have been developed to measure serum LPA levels, with significant variability in obtained results [18]. Presence of several isoforms of LPA in human serum complicates these measurements. In addition, platelet poor plasma LPA levels are lower than serum LPA levels by approximately 5 fold [34]. Based on majority of currently available studies, serum LPA levels in low (1–5) μM range have been reported in healthy subjects [35]. Due to local production of LPA in atherosclerotic plaque (enhanced by activated platelets and oxidized LDLs), concentration of LPA in atherosclerotic plaque is increased 13 fold [36]. Therefore, 10 μM LPA used in the majority of currently presented experiments is a clinically relevant concentration for the effects of LPA on the endothelium.

Multiple prior studies support the importance of endothelial dysfunction in pathogenesis of atherosclerosis. Altered regulation and function of eNOS is a cornerstone of endothelial dysfunction. Treatment of bovine aortic endothelial cells with micromolar concentrations of LPA transiently increased eNOS activity, with return to baseline eNOS activity within minutes after treatment [37]. The increase in eNOS activity with LPA treatment is mediated by phosphorylation of eNOS via Akt/PI3K pathways. Sphingosine 1-phosphate (S1P) similarly increases eNOS activity in a transient fashion, via a process that requires phosphorylation of eNOS by Akt upon activation of the EDG-1 receptor [38]. eNOS activity levels returned to baseline within 2 hours of S1P treatment. The observed differences seen in eNOS activity with LPA treatment in the prior reports and in our current study are most likely due to activation of different molecular pathways upon longer exposure to LPA. Prior studies have not evaluated the role of prolonged (24 hours) treatment with LPA on eNOS in endothelial cell culture systems.

Involvement of LPA in atherogenesis has been implicated in multiple pathways in addition to its effects on endothelial cells. LPA plays an important role in platelet activity. In conjunction with other platelet activators, it increases platelet aggregation and adhesion, and platelet activation by mildly oxidized LDL and atherosclerotic plaque are thought to be mediated via LPA-dependent pathway [8,9]. LPA alters immune cell function [18] and recently has been shown to stimulate lipid accumulation in monocyte-derived cells, preventing monocyte reverse transmigration and contributing to atherogenesis [39]. Isolated smooth muscle cells (SMCs) proliferate and migrate following treatment with LPA [40].

In current study, we show that LPA induces endothelial dysfunction in cultured endothelial cells and isolated pig coronary artery rings through eNOS downregulation and oxidative stress, which may contribute to the vascular lesion formation. However, we did not perform *in vivo* study to confirm our current results. Although the molecular mechanisms of the effects of LPA on the vascular system are not fully understood, many *in vivo* studies have indicated that LPA may act as an atherogenic factor [41,42]. In an ApoE^{-/-} mouse model, single intraperitoneal injection of LPA markedly elicited sustained CXCL1-dependent arterial leukocyte adhesion *in vivo*, and intraperitoneal injection of LPA (2 nmol, twice weekly for 4 weeks) also promoted atherosclerotic plaque progression and macrophage accumulation in ApoE^{-/-} mice [43]. In an LDLR^{-/-} mouse model, LPA progressively accumulated in carotid artery plaques in these mice [44]; these mouse data are consistent with findings observed in human atherosclerotic tissues [8,9]. In addition, local administration of LPA stimulated carotid artery neointimal formation in rats [13], and induced vasoconstriction in the cerebral circulation of newborn pigs [45]. Intravenous injection of LPA elevated arterial blood pressure in rats [46] and neointimal hyperplasia in mice [47]. In future, these animal models could be used to study the effects of LPA on eNOS expression, vasomotor functions and oxidative stress, which are important mechanisms of the vascular lesion formation.

ROS play a crucial role in the pathogenesis of vascular inflammation and atherosclerosis. Several mechanisms have been proposed, including NO consumption and depletion [48]. In this study, we demonstrate that LPA treatment significantly increases production of ROS in porcine coronary arteries and in isolated HCAECs. Superoxide anion production was increased in porcine coronary artery rings exposed to LPA in a concentration-dependent manner, and our experiments from isolated HCAECs suggest impairment of mitochondrial respiratory chain function with LPA treatment. This study also addressed details of the molecular mechanisms responsible for increased ROS production, by demonstrating an upregulation in ROS generating enzymes p40^{phox} and NOX4 and decreasing mRNA levels of the endogenous intracellular antioxidant, SOD1. Little information has been published to date on the pathways involved in ROS upregulation upon LPA treatment in endothelial cells. LPA-mediated increases in ROS production have been reported in a number of other cell types, including in aortic smooth muscle cells. LPA mediated concentration-dependent increase in ROS production in these cells, a process that was inhibited by pre-treatment with either NADPH oxidase inhibitor, DPI, or with HMG-CoA reductase inhibitor, pitavastatin. Further studies to elucidate this mechanism in aortic smooth muscle cells demonstrated activation of the Rac-1 upon LPA treatment, a process that subsequently leads to increases in intracellular ROS levels [49]. Rac-1 mediated activation of NADPH oxidase and increased production of ROS upon LPA treatment was similarly demonstrated in fibroblasts [50] and macrophages [51]. SeMet, a power dietary antioxidant that acts as a free-radical scavenger and upon induction of several selenoproteins known to reduce oxidative stress [52], reversed LPA-mediated effects on eNOS in HCAECs. Particularly, treatment with 20 μ M SeMet, a concentration previously establish to exhibit antioxidant properties in endothelial cells [31], reversed LPA-induced decrease in eNOS mRNA and protein expression, reduced ROS production, restored mitochondrial function, and alleviated LPA-mediated impairment in endothelium-dependent vasorelaxation in porcine coronary artery rings.

In summary, our study demonstrates that LPA reduces eNOS mRNA and protein levels in HCAECs and in porcine coronary artery rings in a concentration-dependent manner, leading to impairment in the endothelium-dependent vasorelaxation. LPA treatment increases production of ROS, which mediate the observed changes in eNOS expression and function. Antioxidant treatment may have an important role in reversing LPA-induced endothelial dysfunction. Future studies will need to address detailed molecular mechanisms of LPA-mediated regulation of eNOS expression and vasomotor dysfunction.

Highlights

In the present study, we demonstrate that LPA may lead to endothelial dysfunction by decreasing eNOS levels and activity in porcine coronary arteries and HCAECs. Superoxide anion generation is in part responsible for the observed changes in eNOS levels and activity, and treatment with exogenous antioxidant, SeMet, attenuates the effects of LPA on endothelial function and eNOS regulation. These findings provide a novel mechanism of the deleterious effects of LPA on coronary vasculature.

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Fig. 1.

Effect of LPA on vasomotor function in porcine coronary arteries. The vessel rings ($n=9$ for each group) were treated with specified amounts of LPA for 24 hours. **(A)** Vessel contraction in response to thromboxane A2 analog, 1×10^{-7} M U46199. **(B)** Endothelium-dependent vasorelaxation in response to increasing concentrations of bradykinin (10^{-9} to 10^{-5} M). Results are plotted as percent change from maximal pre-contraction. **(C)** Concentration-dependent response of LPA in endothelium-dependent vasorelaxation in response to 1×10^{-5} M bradykinin. Results plotted as percent change from pre-contraction. **(D)** Endothelium-independent vasorelaxation in response to 1×10^{-5} M sodium nitroprusside (SNP). Results plotted as percent change from maximal pre-contraction. * $P < 0.05$.

Fig. 2.

Effects of LPA on eNOS expression in HCAECs and porcine coronary arteries. **(A)** HCAECs were treated with different concentrations of LPA for 24 hours. eNOS and GAPDH mRNA levels were determined by quantitative real-time PCR. eNOS mRNA levels were normalized to GAPDH and plotted as $2^{[Ct(GAPDH)-Ct(eNOS)]}$, $n=3$. **(B)** HCAECs were treated with vehicle (control) or 10 μM LPA for specified times. mRNA levels were determined by quantitative real-time PCR. Results were normalized to control samples and plotted as percent of the control. $n=3$. **(C)** eNOS protein levels in HCAECs were analyzed by Western blot analysis. HCAECs were treated with LPA (10 μM), antioxidant SeMet (20 μM) or LPA and SeMet for 24 hours. Both phospho-eNOS (ser1177) and total eNOS levels were determined. β-Actin was used as a house-keeping gene control. **(D)** Representative slides showing decreased eNOS immunoreactivity in the endothelium of porcine coronary artery vessels treated with increasing concentrations of LPA compared with the control. Dark brown color represents positive staining of eNOS (arrows). Magnification: 400×. * $P < 0.05$.

Fig. 3.

Effects of LPA and SeMet on superoxide anion production in HCAECs and porcine coronary arteries. **(A)** HCAECs were treated with LPA and SeMet at specified concentrations for 24 hours, and intracellular O_2^- levels were determined by dihydroethidium (DHE) staining and flow cytometric analysis. Representative histograms are shown. **(B)** Data were plotted as percent DHE-positive cells, a quantification of O_2^- production ($n=3$). **(C)** Porcine coronary artery rings were treated with LPA and SeMet as specified for 24 hours, and O_2^- levels were quantified with the lucigenin-enhanced chemiluminescence assay. * $P < 0.05$.

Fig. 4. Effects of LPA and SeMet on mitochondrial membrane potential and ATP production in HCAECs. **(A)** HCAECs were treated with LPA and SeMet (20 μ M) for 24 hours. Mitochondrial membrane potential was determined by JC-1 staining and flow cytometric analysis. Representative histograms are shown. **(B)** Cellular ATP content was measured by ATPLite kit from HCAECs treated with LPA (0 to 50 μ M) and SeMet (20 μ M) for 24 hours (n=6). *P < 0.05.

Fig. 5.

Effects of LPA and SeMet on the expression of ROS related enzymes in HCAECs. HCAECs were treated with specified concentrations of LPA in the absence or presence of SeMet (20 μM) for 24 hours. The mRNA levels of NOX4 (A), p40^{phox} (B) and SOD1 (C) as well as GAPDH were determined by quantitative real-time PCR, and data were presented as $2^{[Ct(GAPDH)-Ct(gene\ of\ interest)]}$. *P < 0.05 compared with control samples as indicated by arrows.

Fig. 6. Effects of SeMet on LPA-induced endothelial dysfunction in porcine coronary arteries and HCAECs. Porcine coronary artery rings were treated with 10 μ M LPA and 20 μ M SeMet as specified for 24 hours prior to Myograph analyses (n=9). **(A)** Endothelium-dependent vasorelaxation in response to increasing concentrations of bradykinin (1×10^{-9} M to 1×10^{-5} M) in the presence or absence of 20 μ M SeMet. **(B)** Endothelium-dependent vasorelaxation elicited by application of 1×10^{-5} M bradykinin, in the presence or absence of co-treatment with 20 μ M SeMet. **(C)** Representative slides showing restoration of the decreased immunohistochemical staining for eNOS when porcine coronary arteries co-treated with 20 μ M SeMet in addition to 10 μ M LPA. Dark brown color represents positive staining of eNOS (arrows). **(D)** HCAECs were treated with specified concentrations of 10 μ M LPA and 20 μ M SeMet as indicated (n=5). eNOS mRNA levels were determined by quantitative RT-PCR. *P < 0.05.