Lysoplasmenylcholine increases neutrophil adherence to human coronary artery endothelial cells

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White MC, Rastogi P, McHowat J. Lysoplasmenylcholine increases neutrophil adherence to human coronary artery endothelial cells. Am J Physiol Cell Physiol 293: C1467–C1471, 2007. First published August 29, 2007; doi:10.1152/ajpcell.00290.2007.—We demonstrated previously that thrombin stimulation of human coronary artery endothelial cells (HCAEC) results in release of choline lysophospholipids [lysophosphatidylcholine (lysoPtdCho) and lysoplasmenylcholine (lysoPlsCho)]. These amphiphilic metabolites have been implicated in arrhythmogenesis following the onset of myocardial ischemia, but studies examining their direct effects on the vasculature remain limited. We and others have shown that thrombin and lysoPtdCho can increase cell surface adhesion molecules and adherence of circulating inflammatory cells to the endothelium. This study supports our hypothesis that these changes may be mediated, at least in part, by lysoPlsCho, thus implicating this metabolite as an inflammatory mediator in the coronary vasculature and a modulator of the progression of atherosclerosis. Apical stimulation of HCAEC with thrombin resulted in the production and release of choline lysophospholipids from the apical surface of the HCAEC monolayer. Basolateral stimulation had no effect on choline lysophospholipid production or release from either the apical or basolateral surface of the HCAEC monolayer. Incubation of HCAEC with lysoPlsCho or lysoPtdCho resulted in significantly increased HCAEC surface expression of P-selectin and E-selectin. Furthermore, lysoPtdCho increased cell surface expression of P-selectin, E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 with a time course similar to that of thrombin stimulation. Increased presence of cell surface adhesion molecules may contribute to the significant increase in adherence of neutrophils to either thrombin- or lysoPlsCho-stimulated HCAEC. These results demonstrate that the presence of thrombin at sites of vascular injury in the coronary circulation, resulting in increased choline lysophospholipid release from the HCAEC apical surface, has the potential to propagate vascular inflammation by upregulation of adhesion molecules and recruitment of circulating inflammatory cells to the endothelium.

endothelium; arrhythmogenesis; inflammation; lysophospholipids

LYSOPHOSPHATIDYLCHOLINE (lysoPtdCho) concentrations are increased in venular and lymphatic effluents from ischemic myocardium (1, 16) prior to evidence of irreversible cell damage (15). Additionally, increased net production of lysoPtdCho has been observed in patients with mild coronary atherosclerosis (9). We previously demonstrated (18) that thrombin stimulation of human coronary artery endothelial cells (HCAEC) increases calcium-independent phospholipase A2 (iPLA2) activity, resulting in the release of choline lysophospholipids [lysoPtdCho and lysoplasmenylcholine (lysoPlsCho)] from the HCAEC monolayer, suggesting an endothelial source of these metabolites. In normoxic myocytes, both lysoPtdCho and lysoPtdCho have been shown to produce action potential derangements (10). Thus the release of choline lysophospholipids from HCAEC may result in alterations in the electrophysiological properties of cardiac myocytes, particularly in ischemic areas downstream of an evolving thrombus.

There are limited studies in the literature concerning the cardiovascular effects of lysoPlsCho. Interestingly, lysoPlsCho alters the electrophysiological properties of normoxic myocytes at significantly lower concentrations than those previously described for structurally similar compounds, suggesting that there may be a direct interaction between this metabolite and several integral membrane proteins (10). LysoPtdCho also activates myocardial cAMP-dependent protein kinase (19), suggesting a role in signal transduction analogous to that of diacylglycerol activation of protein kinase C.

Although lysoPtdCho has been implicated in arrhythmogenesis following the onset of myocardial ischemia, to date there have been no studies demonstrating the effect of this compound on the coronary vasculature after release from the endothelium. We have demonstrated (18) that thrombin- and tryptase-stimulated HCAEC increase platelet-activating factor (PAF) production and cell surface expression of P-selectin, both processes that can contribute to increased neutrophil adherence and transmigration, illustrating a role for these proteases in inflammation. In this study, we explore the hypothesis that our previously observed effects may be mediated, at least in part, by lysoPtdCho, implicating this metabolite as an inflammatory mediator in the coronary vasculature and a modulator of the progression of atherosclerosis, in addition to its previously described role in arrhythmogenesis.

MATERIALS AND METHODS

Reagents. Primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Cell culture of HCAEC. HCAEC were obtained from Lonza (Walkersville, MD). Endothelial cells were grown in MCDB-131 medium with 5% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 200 μg/ml endothelial cell growth supplement, and 90 μg/ml heparin. Cells were allowed to grow to confluence, achieving a contact-inhibited monolayer of flattened, closely apposed endothelial cells in 4–5 days. After achieving confluence, cells were passaged in a 1:3 dilution and cells from passages 3–4 used for experiments.

HCAEC stimulation. LysoPtdCho, lysoPtdCho, or thrombin was diluted with medium (for assay of neutrophil adherence) or Hanks’ balanced salt solution (for assay of adhesion molecule surface expres-
Stimulant was added to the cell culture plate, and the plate was gently rotated to ensure thorough mixing and even distribution of stimulant across the HCAEC monolayer.

Choline lysophospholipid production. LysoPtdCho and lysoPtdCho were measured with a modification of a radiometric assay method developed previously (11). Lipids were extracted from HCAEC and the surrounding medium by the method of Bligh and Dyer (4), and lysophospholipids were separated from other phospholipids by HPLC. The purified lysoPtdCho and lysoPtdCho fractions were acetylated with [14C]acetic anhydride, using 0.33 M dimethylaminopyridine as a catalyst. The acetylated lysophospholipid was then separated by thin-layer chromatography, and radioactivity was quantified by liquid scintillation spectrometry. Standard curves were constructed, and lysoPtdCho and lysoPtdCho content were derived for all samples and normalized according to the protein content of HCAEC. l4C]lysoPtdCho was added as an internal standard to all samples to correct for the loss of sample that occurred during extraction, purification, and acetylation.

Cell surface expression of adhesion molecules. HCAEC, grown to confluence in 16-mm culture dishes, were incubated with stimulant in Hanks’ buffer for 5 min at 37°C in 95% O2-5% CO2. At the end of the incubations buffer was quickly removed, and cells were immediately fixed with 1% paraformaldehyde and incubated overnight at 4°C. Cells were then washed three times with phosphate-buffered saline (PBS) and then blocked with Tris-buffered saline-Tween supplemented with 0.8% BSA (wt/vol) and 0.5% fish gelatin (wt/vol) for 1 h at 24°C. Appropriate primary antibody (1:50) was used before treatment with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (1:5,000). Subsequently, each well was incubated in the dark with the 3,3',5,5'-tetramethylbenzidine liquid substrate system. Reactions were stopped by the addition of sulfuric acid, and color development was measured with a microtiter plate spectrophotometer at 450 nm.

Isolation of neutrophils from human peripheral blood. Adult peripheral blood was collected from volunteers in vials containing 3.8% sodium citrate to inhibit coagulation. The protocol was approved by the Institutional Review Board of Saint Louis University School of Medicine, and all volunteers gave informed consent. Equal volumes of whole blood were layered over Polymorphprep (Axis-Shield PoC, Medica, and all volunteers gave informed consent. Equal volumes of whole blood were layered over Polymorphprep (Axis-Shield PoC, Oslo, Norway) in a 50-ml conical tube. Tubes were spun at 500 g for 30 min at 20°C with no brake. The top band at the sample/medium interface consisting of mononuclear cells and the lower band of polymorphonuclear cells were removed to a clean 50-ml conical tube. An equal volume of 0.5 N Hanks’ buffer was added to the cells in the 50-ml tube. Normal Hanks’ buffer was added to bring the total volume to 50 ml. Tubes were spun at 400 g for 10 min at 4°C. The supernatant was discarded, and the cell pellet was resuspended with 3 ml of 0.2% NaCl and incubated for 3 min at room temperature. Three milliliters of cold 1.6% NaCl was added, and the solution was transferred to a 15-ml conical tube. Ice-cold normal Hanks’ buffer was added to bring the total volume to 15 ml. Cells were centrifuged at 175 g for 10 min at 4°C. The supernatant was removed, and cells were resuspended in 5 ml of ice-cold Hanks’ buffer. An aliquot was taken for cell count with a hemacytometer. Cells were centrifuged at 175 g for 10 min, and the supernatant was discarded. Neutrophils were resuspended in MEM + 10% FBS at 1 × 106 cells/ml. Pretreatment of neutrophils. Freshly isolated neutrophils were incubated with 10 μM CV3988 (Sigma), a PAF receptor antagonist, for 10 min before their use in the neutrophil adherence assay.

Neutrophil adherence assay. HCAEC were grown to confluence on a 12-well plate. Cells were washed twice with MEM + 10% FBS. Stimulant in 500 μl of Hanks’ buffer was added to HCAEC, and cells were stimulated for 10 min at 37°C. Five hundred microliters of neutrophils in suspension (5 × 106 cells) in MEM + 10% FBS was added to each of the wells and incubated for 10 min at room temperature. Medium and unbound neutrophils were removed and discarded. Plates were washed twice with prewarmed Dulbecco’s PBS. One milliliter of 0.2% Triton X-100 was added to each well to lyse adherent neutrophils and HCAEC. Cell lysates were scraped from the plate and transferred to an Eppendorf tube. A 500-μl aliquot of neutrophil suspension was added to 500 μl of 0.2% Triton X-100 and used as the theoretical maximal binding sample. Five hundred micro- liters of distilled H2O and 500 μl of 0.2% Triton X-100 was used as the reference blank. Samples, theoretical maximal binding sample, and blank were sonicated (500 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) for 10 s. To measure neutrophil peroxidase activity, 400 μl of cell lysate was transferred to a glass tube and 1 ml of PBS, 1.2 ml of Hanks buffer + BSA, 200 μl of 3,3'-dimethoxybenzidine, and 200 μl of 0.05% H2O2 were added. The cell lysate reaction mixture was incubated for 15 min at room temperature. Two hundred microliters of 1% sodium azide (Na3) was added to stop the reaction. The absorbance was then measured with a 4050 UV/visible spectrophotometer (Biochrom, Cambridge, UK) at 460 nm.

Lysis of neutrophils. LysoPtdCho. LysoPtdCho was prepared by alkaline hydrolysis of bovine heart choline glycerophospholipids as described previously (17). LysoPtdCho was isolated by column chromatography on a 2.5 × 60-cm column of silica with a stepwise gradient elution procedure (2). Palmityl lysoPtdCho was isolated by reverse-phase HPLC as described previously (5). Fast atom bombardment mass spectrometry (FAB-MS) analysis of the final product revealed <2 mol% alkyl ether choline lysophospholipid species.

Statistical analysis. Data were analyzed with Student’s t-test. P values of <0.05 were considered statistically significant; P values of <0.01 were considered highly statistically significant.

RESULTS

LysoPtdCho release from HCAEC on thrombin stimulation. Previously, we demonstrated the release of choline lysophospholipids from thrombin-stimulated endothelial cells. However, we had not determined whether choline lysophospholipid release occurred from apical, basolateral, or both sides of the endothelial cell monolayer. HCAEC were plated on Transwell inserts and grown for 7 days. Confluence of the monolayer on the Transwell insert was verified by monitoring the development of electrical resistance over time. Thrombin (0.05 IU/ml, 2 min) was added to the Transwell insert (apical surface of HCAEC), and choline lysophospholipid content was assessed in the medium in the upper (apical surface) and lower (basolateral surface) chambers, together with the content within the HCAEC monolayer (cell associated) (Fig. 1). Choline lysophospholipid content in the HCAEC monolayer and the amount released from the apical surface of the monolayer were significantly increased in response to apical thrombin stimulation (Fig. 1). However, no change in release of choline lysophospholipids from the basolateral surface of the monolayer was observed (Fig. 1). Electrical resistance across the HCAEC monolayer was not significantly decreased during the thrombin stimulation time, suggesting that monolayer integrity was not compromised by this concentration of thrombin (data not shown). When thrombin was added to the bottom chamber (basolateral surface of HCAEC), no significant change in choline lysophospholipid production was observed (Fig. 2). These data suggest that apical stimulation of HCAEC was required for choline lysophospholipid release and may indicate a sidedness for thrombin receptors. In addition, choline lysophospholipid release occurs primarily from the apical side of the monolayer.

Cell surface expression of adhesion molecules. Previous reports have shown that LysoPtdCho can increase endothelial cell surface expression of adhesion molecules (8, 13). To
determine whether lysoPlsCho and lysoPtdCho exert similar effects on HCAEC surface expression of selectins, we incubated confluent HCAEC monolayers with lysoPtdCho or lysoPlsCho (5 μM) for up to 60 min. LysoPlsCho and lysoPtdCho increased cell surface expression of P-selectin (Fig. 3) and E-selectin (Fig. 4) to a similar extent after 10 min of incubation. Increases in cell surface P- and E-selectin were also found to be concentration dependent (data not shown).

Stimulation of HCAEC confluent monolayers with thrombin (0.01 IU/ml) resulted in a time-dependent increase in cell surface expression of P-selectin, E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 (Fig. 5). An increase in cell surface expression of P-selectin was observed after 10 min that returned to unstimulated levels after 30 min. Maximal increases in E-selectin, ICAM-1, and VCAM-1 were observed after 1 h of incubation with thrombin (Fig. 5). Similar increases in cell surface expression were observed when HCAEC were incubated with 5 μM lysoPlsCho (Fig. 6).

LysoPlsCho stimulation increases adherence of neutrophils.

Previous studies by our laboratory (18) demonstrated significant increases in HCAEC surface expression of P-selectin and...
increased PAF production on thrombin stimulation, leading to significant increases in neutrophil adherence. LysoPlsCho-activated HCAEC exhibit increased cell surface expression of several molecules known to be involved in neutrophil adhesion and migration, potentially enhancing the binding of inflammatory cells bearing the corresponding ligands or receptors to the HCAEC layer. Therefore, we examined the effect of either thrombin or lysoPlsCho stimulation of HCAEC on the adherence of neutrophils to the monolayer. Thrombin stimulation (1 IU/ml, 10 min) led to a significant increase in neutrophil adherence (Fig. 7). Stimulation of HCAEC with lysoPlsCho (5 μM, 10 min) also led to a significant increase in neutrophil adherence (Fig. 7). Pretreatment of neutrophils with a PAF receptor antagonist significantly inhibited neutrophil adherence to both treated and untreated HCAEC (Fig. 7). To exclude the possibility that thrombin or lysoPlsCho activated neutrophil peroxidase activity, we incubated neutrophils for 10 min with each agent. Thrombin increased peroxidase activity by 8 ± 1% (n = 6) and lysoPlsCho decreased peroxidase activity by 4 ± 1% (n = 6) compared with untreated neutrophils. Additionally, thrombin or lysoPlsCho did not result in increased neutrophil adherence to the tissue culture surface in the absence of HCAEC, with <0.01% of added neutrophils sticking to the surface with thrombin or lysoPlsCho treatment. Together, these results demonstrate that, similar to thrombin-stimulated changes, lysoPlsCho-stimulated increases in neutrophil adherence are dependent, at least in part, on interaction with the neutrophil PAF receptor.

**DISCUSSION**

In previous studies, we have demonstrated an increase in endothelial cell choline lysophospholipid production following thrombin stimulation. In this study, we grew HCAEC on Transwell inserts, applied thrombin to the apical or basolateral surface of the monolayer, and measured choline lysophospholipid release from the apical and basolateral surfaces, as well as that which remained cell associated (Figs. 1 and 2). After apical stimulation with thrombin, ~60% of lysoPlsCho was released from the apical surface and ~40% remained cell associated (Fig. 1). Additionally, ~60% of lysoPtdCho remained cell associated, with only 40% being released from the apical surface of the HCAEC monolayer. No increase in choline lysophospholipid release from the basolateral surface was observed, suggesting that lysoPlsCho and lysoPtdCho are released exclusively into the circulation and not directly into the subendothelial space. These data suggest that in order to play an arrhythmogenic role in the myocardium, choline lysophospholipids must cross the endothelium after being released from the apical surface. Previous data from our laboratory and others have demonstrated that choline lysophospholipids increase the permeability of the endothelial cell monolayer, possibly facilitating its passage to the underlying interstitium. The mechanisms whereby lysoPtdCho impairs endothelial cell barrier function have been found to be associated with activation of RhoA and formation of actin stress fibers, suggesting contraction of adjacent endothelial cells (3, 6, 7, 14). The first study to examine the effects of lysoPtdCho on HCAEC permeability demonstrated that this amphiphilic compound at concentrations between 7.5 and 30 μM increased the permeability of HCAEC grown on Transwell inserts (20). The authors of that study proposed that the increase in HCAEC...
may be a result of downregulation of tight junction proteins by lysoPtdCho. Release of choline lysophospholipids from HCAEC, passage of these metabolites across the endothelial cell monolayer, and their ability to cause alterations in the electrophysiologic properties of cardiac myocytes provide a possible link between thrombin generation at sites of vascular injury and cardiac myocyte dysfunction as a result of increased HCAEC choline lysophospholipid generation and release.

Additionally, the production of choline lysophospholipids by thrombin-stimulated HCAEC is dependent on the presence of thrombin on the apical surface of the monolayer. No significant release was observed when thrombin was present at the basolateral surface of the HCAEC monolayer. The concentration and time of thrombin incubation used in these experiments did not alter the electrical resistance measured across the HCAEC monolayer or cause visible cell retraction, suggesting that the HCAEC monolayer was intact. When the thrombin concentration was increased to 1 IU/ml, gaps appeared in the HCAEC monolayer and choline lysophospholipid release from the HCAEC apical surface was observed (data not shown). We hypothesize that HCAEC thrombin receptors demonstrate “sideness,” with all or a majority of the receptors present on the HCAEC apical surface. We are currently conducting electron microscopic studies to explore this possibility.

LysoPtdCho has been implicated in several studies as pro-inflammatory and atherogenic. In the vascular endothelium, lysoPtdCho increases the cell surface expression of several adhesion molecules and increases neutrophil adherence (8, 13, 21). In a previous study, we demonstrated (12) that thrombin stimulation of HCAEC results in activation of an iPLA2 that is selective for arachidonoyl-phospholipids. As shown in Fig. 1, the release of lysoPtdCho is over twofold that of lysoPtdCho after thrombin stimulation. Similar to the changes observed with lysoPtdCho stimulation, lysoPtdCho results in increases in HCAEC surface expression of P-selectin, E-selectin, VCAM-1, and ICAM-1 (Fig. 6). Although the increases in cell surface expression of adhesion molecules may result in increased neutrophil adherence, our data suggest that the interaction between PAF expression on the endothelial cell surface and the PAF receptor on neutrophils is necessary (Fig. 7). Pretreatment of neutrophils with a PAF receptor antagonist significantly reduced the percentage of adherent neutrophils, demonstrating the importance of the PAF-PAF receptor interaction in the adherence of these cells.

These results demonstrate that the presence of thrombin at sites of vascular injury in the coronary circulation, resulting in increased lysoPtdCho and lysoPtdCho release from the apical surface, has the potential not only to initiate arrhythmias but to induce changes within the endothelium leading to increased neutrophil adherence and vascular inflammation.

**REFERENCES**


