Lysophosphatidylcholine Potentiates Phenylephrine Responses in Rat Mesenteric Arterial Bed through Modulation of Thromboxane A₂

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ABSTRACT

Lysophosphatidylcholine (LPC) plays important physiological and pathophysiological roles in the cardiovascular system. Despite this, there is little information about its effects on vasoreactivity of resistance vessels. The present study was designed to characterize the effects of LPC in the isolated perfused rat mesenteric arterial bed (MAB) and to investigate the underlying mechanisms of the changes it produced. Perfusion with 10 μM LPC for 40 min did not significantly affect basal perfusion pressure or reactivity of MAB to the α₁-adrenoceptor agonist phenylephrine (PE) but almost completely abolished the maximal endothelium-dependent relaxation to acetylcholine (Ach), reducing it from 93 ± 5 to 7 ± 4% (p < 0.001). After washout of LPC for 60 min, the vasodilator response to Ach partially recovered, whereas the vasoconstrictor response to PE was markedly enhanced, the pD₂ value increasing from 7.50 ± 0.04 to 8.13 ± 0.15 and maximum response to 199 ± 24% of control (p < 0.001). Pretreatment with either indomethacin, a non-selective inhibitor of cyclooxygenase, or SQ-29548 ([1S-[1α,2α(Z),3α,4α]-7-3-[[phenylamino]carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid), a selective thromboxane receptor antagonist, completely prevented the potentiation of the PE response after washout of LPC. In untreated MABs, only the highest concentration of PE produced a significant increase in thromboxane A₂ (TXA₂) production (assessed by enzyme-immunoassay of thromboxane B₂ levels). This was prevented by perfusion with LPC but was significantly increased after LPC washout. The basal release of TXA₂ was not modified by LPC. These results demonstrate that LPC exerts both immediate and residual effects on the reactivity of the rat MAB and that these effects are at least partially due to modification of PE-induced TXA₂ production.

Lysophosphatidylcholine (1-acyl-sn-glycero-3-phosphocholine, LPC) is an important lysophospholipid that is generated as a breakdown product of phosphatidylcholine by phospholipase A₂. Present in cell membranes and oxidized lipoproteins, it plays important physiological and pathophysiologica roles in both humans and animals. For instance, LPC is responsible for the delivery of fatty acids and choline to tissues (Xu, 2002). On the other hand, elevated levels of LPC have been linked to the cardiovascular complications associ-
to its role in modulating vascular contractility, it is generally accepted that LPC is the major pathological component of oxidized low-density lipoprotein in promoting atherosclerosis (Takahara et al., 1997; Sonoki et al., 2003).

Several lines of evidence also suggest a vasoprotective role for LPC. In rabbits, LPC produces potent endothelium-dependent vascular smooth muscle relaxation, both in vivo and in vitro (Menon and Bing, 1991; Wolf et al., 1991). In umbilical vein endothelial cells, LPC has been shown to enhance endothelial expression of endothelial nitric-oxide synthase and cyclooxygenase-2 (COX-2), leading to increased synthesis of nitric oxide (NO) and prostacyclin (PGI2) (Zembowicz et al., 1995a,b). NO and PGI2 act synergistically to block monoamine oxidase (MAO) of LPC in regulating vascular resistance has not been completely elucidated, as the majority of previous studies have used either large blood vessels or isolated cells. The mesenteric arterial bed (MAB) is an important effector organ regulating blood pressure, with small structural and functional changes eliciting significant alterations in peripheral resistance (Caveney et al., 1998). The objective of the present study was to determine the vasoreactive effects of LPC in the isolated perfused MAB and to investigate the underlying mechanisms of the changes it produced.

Materials and Methods

Animals. The investigation conforms to the guidelines of the Canadian Council on Animal Care. Male Wistar rats were obtained from the Animal Care Center, University of British Columbia (Vancouver, BC, Canada). Animals were housed under a 12-h light/12-h dark regime and given free access to standard rat chow (PMI Feeds, Richmond, VA) and tap water.

Isolated, Perfused Rat Mesenteric Arterial Bed. Male Wistar rats (300–400 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and the MAB was isolated as described previously (He and MacLeod, 2002). In brief, the abdominal cavity was opened, and the superior mesenteric artery was cannulated through an incision at its confluence with the dorsal aorta. The whole MAB was then separated by cutting close to the intestinal border. The MAB was flushed with heparinized warm Krebs bicarbonate buffer with the following composition: 113 mM NaCl, 4.7 mM KCl, 11.5 mM glucose, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, and 25.0 mM NaHCO3. The pH of the buffer following saturation with a 95% O2, 5% CO2 gas mixture was 7.4. Subsequent to flushing, the MAB was transferred into a jacketed organ chamber and perfused through the cannula with Krebs buffer maintained at 37°C and gassed with 95% O2, 5% CO2. Perfusion pressure was kept constant at 3 ml/min using a peristaltic pump (Buchler Instruments, Buchler Fort Lee, NJ). The perfusate flowed out through the cut ends of the mesenteric arterial bed. Vascular responses were detected as changes in perfusion pressure, and this was continuously measured and recorded using a pressure transducer (PD23ID; Gould, Statham, CA) placed between the perfusate and the cannula and connected to a Grass polygraph (model 79D; Grass Instruments, Quincy, MA). The perfused MAB was allowed to stabilize for 40 min before four bolus injections of KCl (0.4 mmol). Perfusion pressure was allowed to return to baseline after each injection of KCl. After further equilibration for 40 min, dose–response curves (DRCs) to various agonists were performed.

Vasoconstrictor Responses. To determine the effects of LPC on vasoconstrictor responses of the MAB, bolus injections of phenylephrine (PE) (0.9–300 nmol) or KCl (50–800 μmol) were given. These first DRCs to PE or KCl served as controls. Once perfusion pressure returned to baseline, palmitoyl-LPC (0.1–10 μM) was added to the perfusion buffer. In preliminary experiments, none of these concentrations of LPC per se produced any observable change in contractility. In all of the subsequent experiments, 10 μM LPC was used. After 40 min of perfusion with LPC, the second PE/KCl DRC was constructed in the presence of LPC. The tissues were then switched back to normal Krebs buffer and perfused for 1 h followed by construction of a third PE/KCl DRC. The 60-min washout was designed to test whether the expected effect of LPC was reversible or not. Perfusion pressure was allowed to return to baseline between each PE/KCl injection. To test whether the vascular responses are affected by time, a control experiment consisting of three consecutive DRCs to PE and KCl were done without the addition of LPC.

To determine the potential contribution of NO and thromboxane A2 (TxA2) to the observed modulatory effects of LPC on PE responses, the LPC perfusion and washout procedures described above were performed in the presence of a NOS inhibitor, L-NMMA (Nω-monomethyl-L-arginine methyl ester; 300 μM), a COX inhibitor (indomethacin; 20 μM), or a TxA2 receptor antagonist, SQ-29548 [1S-[2S(Z),3a,4a]-7-[3-[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptanoic acid; 0.3 μM). All inhibitors were added to the perfusate after the first DRC and kept present throughout the second and third DRCs.

Vasodilatory Responses. To assess the effects of LPC on EDR, MABs were preconstricted with submaximal concentrations of PE (1–3 μM). Subsequently, increasing concentrations of acetylcholine (Ach; 3 nmol–0.3 μM) were administered until maximal relaxation was attained. MABs were then perfused for 40 min with 10 μM LPC, and the response to PE and Ach was repeated. A third Ach response was obtained after washout of LPC for 1 h. Control experiments consisted of three consecutive Ach-induced responses in the absence of LPC.

Effluent Collection. To measure the content of TxB2 in the MAB effluent, samples were collected for 2 min on six different occasions as follows: before the first application of PE, during the first PE-DRC at each PE dose, after the 40-min perfusion with LPC, during the second PE-DRC at each PE dose, after the 60-min washout of LPC, and during the third PE-DRC at each PE dose. Samples were stored at −70°C until assayed.

Enzyme Immunoassay of TxB2. The collected perfusates were extracted using Solid-Phase Extraction C-18 cartridges (Cayman Chemical, Ann Arbor, MI) after cartridge activation by 5 ml of methanol and 5 ml of ultrapure water. A 0.5-ml aliquot of each sample was acidified to pH 4.0 and loaded onto the cartridge. The cartridge was washed with 5 ml of ultrapure water followed by 5 ml of hexane. The TxB2 fraction was eluted with 5 ml of ethyl acetate containing 1% methanol. The eluate was dried under a stream of nitrogen and reconstituted with 0.5 ml of enzyme immunoassay buffer. The concentration of TxB2 in the eluate was determined by enzyme immunoassay, using a commercially available kit (Cayman Chemical).

Chemicals. l-Phenylephrine hydrochloride, 1-palmitoyl-sn-glycero-3-phosphocholine, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO). L-NMMA and SQ-29548 were purchased from BIOMOL International, L.P. (Plymouth Meeting, PA). Stock solutions of PE (0.01 M), LPC (0.01 M), and L-NMMA (0.1 M) were prepared in distilled water. Indomethacin and SQ-29548 were dissolved in 100% ethanol and prepared as stock solutions of 0.1 M and 0.1 mM, respectively. Solutions of PE and indomethacin were made fresh. LPC and all of the inhibitors were further diluted to the required concentration in the perfusate reservoir. The final ethanol concentrations (0.03–0.3%, v/v) were without effect on contractile responses.

Data Analysis. KCl-induced vasoconstrictor responses were expressed as the absolute increase in perfusion pressure. PE responses were expressed as a percentage of the maximal response of the first PE-DRC. The negative log of the PE concentration producing 50% of
maximum response (pD₂) was obtained by nonlinear regression analysis of individual DRCs using GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, CA).

All of the data are presented as mean ± S.E.M. Data were analyzed for significant differences using Number Cruncher Statistical Systems. Student’s unpaired t test was used for comparisons between two means. One-way ANOVA followed by Newman-Keuls test was used for comparison of more than two means. Two-way ANOVA, using the general linear model approach (repeated measures) followed by Newman-Keuls test, was used for comparisons between PE-DRCs and KCl-DRCs. P < 0.05 was considered statistically significant.

**Results**

**Effect of LPC on Basal Perfusion Pressure.** To examine the effect of LPC on basal perfusion pressure, isolated MABs were incubated with varying concentrations of LPC (0.1–10 μM). Perfusion pressure was measured during a 40 min perfusion with LPC or 1 h after washout. In Fig. 1 (left), the effects of 10 μM LPC on basal perfusion pressure are shown. LPC had no significant effect on perfusion pressure, either during incubation or following washout, suggesting that this lysophospholipid has no direct effect on the contractility of MAB. Basal MAB perfusion pressure in untreated control tissues remained unchanged throughout the experimental period (Fig. 1, right).

**KCl-Induced Contractile Responses following LPC.** Pressor responses of the MAB to bolus injections of KCl (50–800 μmol) were not significantly altered, either during perfusion with 10 μM LPC or after LPC washout (Fig. 2A). In untreated control tissues, KCl-DRCs remained constant over time (Fig. 2B).

**Effects of LPC on PE-Induced Contractile Responses.** Bolus injections of PE (0.9–300 nmol) produced a concentration-dependent increase in perfusion pressure that did not significantly change over time (Fig. 3A). The response to PE was unaffected by prior perfusion with LPC for either 40 (Fig. 3B) or 150 min (data not shown). Interestingly, after washout of LPC for 1 h, the PE response was markedly unchanged over time (Fig. 3B). The maximal response to PE increased to 199 ± 24% of control (P < 0.001) after LPC washout, whereas the PE pD₂ value increased from 7.50 ± 0.04 to 8.13 ± 0.15 (P < 0.001). Further increasing the dose of PE above 90 nmol resulted in a decline in the contractile response.

![Fig. 1](image.png)

**Fig. 1.** Basal perfusion pressure before, after 40-min perfusion with 10 μM LPC, and after washout of LPC for 60 min (n = 25, left). The effects of LPC at lower concentrations were not shown. The right panel depicts basal perfusion pressure in untreated control tissues (n = 7) at the same fixed time intervals. Data represent the mean ± S.E.M.

**Involvement of NO and TxA₂ in Mediating the Direct Effects of LPC Perfusion.** Before LPC perfusion, Ach (0.1–0.3 μmol) produced a maximal relaxation of 93 ± 5% of the response to PE (Fig. 4, left). The Ach response remained unchanged over time (Fig. 4, right). LPC perfusion almost completely abrogated the response to Ach, reducing the maximal relaxation to 7 ± 4% (Fig. 4, left), suggesting that LPC is diminishing the actions of both NO and EDHF. In a separate experiment, we tested the effects of a NOS inhibitor on PE responses. As expected, pretreatment with l-NMMA significantly enhanced the PE response (Fig. 5A), with both an increase in the maximal response and pD₂ value (control, 7.03 ± 0.45; l-NMMA, 7.49 ± 0.17; P < 0.001) being apparent. However, in the presence of LPC, the enhancement of the PE response by l-NMMA was prevented (Fig. 5A). These data suggest that, in addition to suppressing NO, LPC may be exerting other actions to limit vasoconstrictor release or responsiveness to PE.

TxA₂ is a major vasoconstrictor prostanoid implicated in regulation of vascular tone in response to α₁-adrenoceptor stimulation in the MAB. To investigate the involvement of TxA₂ in mediating PE responses, we measured levels of TxB₂, the stable metabolite of TxA₂. Basal release of TxB₂ was stable over time. In untreated MAB, only the highest dose of PE tested (300 nmol) produced an increase in TxB₂ production (Fig. 6). LPC perfusion for 40 min had no effect on basal levels of TxB₂ but suppressed its enhancement by PE.
Involvement of NO and TxA2 in Mediating the Indirect Effects (after Washout) of LPC.

Washout of LPC induced partial recovery of the Ach response (Fig. 4). Despite this fractional recovery, contractile responses to PE were augmented (Fig. 3B), suggesting that amplification of vasoconstrictor pathways, in addition to reduction of vasodilators such as NO, may be responsible for this effect. To investigate this possibility, after washout of LPC, a DRC to PE was performed in the presence of L-NMMA (Fig. 5B). Interestingly, even in the presence of the NOS inhibitor, the response to PE was shifted to the left after LPC washout (Fig. 5B).

(Fig. 6), suggesting that LPC prevents the ability of PE to increase TxA2 production.

Involvement of NO and TxA2 in Mediating the Indirect Effects (after Washout) of LPC. Washout of LPC induced partial recovery of the Ach response (Fig. 4). Despite this fractional recovery, contractile responses to PE were augmented (Fig. 3B), suggesting that amplification of vasoconstrictor pathways, in addition to reduction of vasodilators such as NO, may be responsible for this effect. To investigate this possibility, after washout of LPC, a DRC to PE was performed in the presence of L-NMMA (Fig. 5B). Interestingly, even in the presence of the NOS inhibitor, the response to PE was shifted to the left after LPC washout (Fig. 5B).
Fig. 6. TxB<sub>2</sub> release from MAB in response to PE before, after 40-min perfusion with 10 μM LPC, and after washout of LPC for 60 min (n = 8). Data represent the mean ± S.E.M. *, P < 0.05 versus all other PE doses in “before LPC” (one-way ANOVA followed by Newman-Keuls test). #, P < 0.05 versus all of the other groups at the same dose (two-way ANOVA followed by Newman-Keuls test).

0.05). These data suggest that the enhancement of PE response after LPC washout is mediated by mechanisms in addition to NO inhibition.

To further investigate mechanisms facilitating the enhanced PE response after LPC washout, TxB<sub>2</sub> levels in the perfusion medium were measured. Interestingly, TxB<sub>2</sub> levels only increased after LPC washout (Fig. 6), suggesting a role for this vasoconstrictor in the magnified response to PE. MABs were then pretreated with either a COX inhibitor or a TxA<sub>2</sub> receptor antagonist. Both indomethacin (pD<sub>2</sub> value after LPC washout, 8.24 ± 0.07; LPC washout plus indomethacin, 7.74 ± 0.25; P < 0.05) and SQ-29548 (pD<sub>2</sub> value after LPC washout, 8.24 ± 0.07; LPC washout plus SQ-29548, 7.73 ± 0.10; P < 0.05) completely prevented the enhancement of the PE DRC after LPC washout (Fig. 7). Neither indomethacin nor SQ-29548 significantly changed PE-induced vasoconstriction by themselves (data not shown).

**Discussion**

To our knowledge, the present study is the first to investigate the effects of LPC on the reactivity of a perfused resistance artery bed. As reported previously in conduit blood vessels, LPC perfusion of MAB caused impairment in Ach-induced maximum relaxation. The novel finding in the present study was the potentiation of PE-induced contractile responses after washout of LPC, an effect likely related to increased production of TxA<sub>2</sub>.

The balance between vasodilators (NO, EDHF, and PGL<sub>1</sub>) and vasoconstrictors (TxA<sub>2</sub> and endothelin-1) is pivotal in the regulation of vascular tone. Because most of these vasoactive factors originate from vascular endothelium, we examined the effects of LPC on endothelial function. Measurement of EDR supported the established view that LPC inhibits EDR, probably through inactivation of NO and EDHF (Cowen and Steffen, 1995; Froese et al., 1999; Rikitake et al., 2000). Impaired EDR is normally linked to potentiated vasoconstrictor responses, an observation we confirmed in preliminary studies, in which various methods were used to denude MAB of endothelial cells, including perfusion with air (Tatchum-Talom and Atkinson, 1997), distilled water, sodium deoxycholate (Cusma-Pelogia et al., 1993), and CHAPS (McCulloch and Randall, 1996). Mechanical denudation of endothelium, which was associated with loss of the relaxant response to Ach, caused an enhancement of PE responses (data not shown). However, the impaired Ach-induced EDR caused by LPC perfusion was not accompanied by an increased response to PE. Other studies using aortic rings from normal rats have also demonstrated similar effects of LPC, i.e., reduced EDR with no significant change in PE responses (Ceylan et al., 2004). These data suggest that, even in the presence of NO/EDHF inhibition by LPC, vasoconstrictor responsiveness or release may also be inhibited by LPC, thus preventing augmentation in responses to PE. This possibility is supported by the observation that LPC prevented amplification of PE responses in the presence of L-NMMA and reduced the PE-induced production of TxA<sub>2</sub>.

Unexpectedly, the response to PE was dramatically potentiated only after the removal of LPC. A time-dependent mechanism can be ruled out, as prolonged perfusion with LPC (150 min) did not produce any potentiation of PE responses. The enhancement of the PE response could be blocked by both indomethacin and SQ-29548, suggesting an important contribution of vasoconstrictor prostanoids, which was confirmed by the finding of potentiation of PE-induced TxA<sub>2</sub> production. At present, the mechanism for this rebound production of excessive TxA<sub>2</sub> is not known, nor is it clear whether this effect is global or localized to the MAB. The contribution of time in producing the potentiated contractile responses and TxA<sub>2</sub> production in response to PE can be ruled out, as these were stable over time in the absence of LPC. These effects were unlikely to be due solely to continued endothelial impairment, because Ach-induced relaxation had partially recovered. Furthermore, the enhancement of PE responses produced by mechanical removal of the endothelium could not be abolished by pretreatment with SQ-29548 (data not shown).

As a potent inducer of platelet aggregation, vasoconstriction, and bronchoconstriction, TxA<sub>2</sub> has been implicated in vascular pathogenesis (Dogne et al., 2004). Although the major source of TxA<sub>2</sub> is platelets, this vasoconstrictor can also be produced within the vascular walls by both endothelial (Ally and Horrobin, 1980) and smooth muscle cells (Shikokoshi et al., 2002). The mechanism of TxA<sub>2</sub> synthesis includes phospholipid hydrolysis by PLA<sub>2</sub>, release of arachi...
donic acid, and metabolism to TXA₂ by the COX-TXA₂ synthesis pathway. α₁-Receptor activation initiates phospholipid hydrolysis and release of TXA₂ (Terzic et al., 1993; Nishio et al., 1996; Ruan et al., 1998; Bolla et al., 2002; Parmentier et al., 2004). To our knowledge, an effect of LPC on α₁-adrenoceptor-mediated TXA₂ production in the vasculature has not previously been reported. However, our observation that LPC abrogated the increase in TXA₂ production produced by PE in the MAB is consistent with a previous study in platelets, in which an inhibitory effect of LPC on agonist-induced TXA₂ production by LPC, an interaction between these two agents has previously been suggested. For instance, LPC was found to enhance the ability of TXA₂ to increase vascular smooth muscle cell proliferation (Koba et al., 2000). Taken together, these findings strongly suggest that LPC is able to alter the biosynthesis of TXA₂, as well as its downstream signaling. Details of the mechanism of these modulatory effects need to be further investigated. Irrespective of the mechanism(s), the increased release of TXA₂ could be a major contributor toward hypertensive (Seeger et al., 1989), thrombotic, and ischemic diseases (Ally and Horrobin, 1980; Muller, 1991).

It is tempting to speculate that conditions that evoke an increase in LPC levels initially trigger compensatory mechanisms to neutralize its injurious effects. Thus, the suppression of PE-induced TXA₂ production could compensate for the endothelium dysfunction. Wu and co-workers (Zembowicz et al., 1995a,b) have also suggested that LPC is a double-faced molecule, which can produce both vasoprotective and proatherogenic mechanisms. An additional caveat is the suggestion that residual effects of LPC after washout of TXA₂ might have a membrane effect to lysophosphatic acid (Tokumura, 2004). Given this complex biology, investigation of the in vivo temporal effects of LPC under normal and pathological conditions would be beneficial.

In conclusion, our results for the first time demonstrate the effects of LPC in the rat resistance arterial bed, even after washout. These included potentiated PE responses and enhanced TXA₂ production. Thus, when evaluating the effects of LPC in the vasculature, both its immediate and residual effects need to be considered. Although unclear, the mechanism for this residual effect, especially the relationship between LPC and lysophosphatic acid, is currently being evaluated.

References


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