Lysophosphatidylcholine Increases Vascular Superoxide Anion Production via Protein Kinase C Activation

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Abstract We tested the hypothesis that lysophosphatidylcholine (lyso-PC) could activate protein kinase C in intact vascular segments and sought to examine some of the physiological consequences of this activation. In segments of rabbit aorta, the patterns of protein phosphorylation determined by two-dimensional electrophoresis stimulated by lyso-PC and 12-O-tetradecanoylphorbol 13-acetate (TPA) were similar. Activation of protein kinase C can stimulate superoxide anion (O$_2^-$) production in other tissues, and we found that lyso-PC-treated rabbit aortas produced twofold more O$_2^-$ than control vessels. Calphostin C, a potent and specific inhibitor of protein kinase C, attenuated O$_2^-$ production in lyso-PC-treated vessels but had no effect in control vessels. The effect of lyso-PC on O$_2^-$ production was mimicked by TPA. In separate bioassay studies, release of the endothelium-derived vascular relaxing factor (EDRF) quantified by the response of detector vessels was markedly impaired after exposure of donor rabbit aortic segments to lyso-PC. After incubation with calphostin C, EDRF release in response to acetylcholine from lyso-PC-treated donor vessels was restored significantly. Thus, lyso-PC can activate protein kinase C in intact vessels, leading to an increase in O$_2^-$ production. Activation of protein kinase C by lyso-PC may also play a role in altering the release of EDRF in response to acetylcholine. Increased O$_2^-$ production in response to lyso-PC may have important consequences in the atherogenic process. (Arterioscler Thromb. 1994;14:1007-1013.)

Key Words • endothelium-dependent vascular relaxation • lysophosphatidylcholine • protein phosphorylation • protein kinase C • rabbit aorta • superoxide anion

Several lines of evidence have indicated that oxidatively modified low-density lipoprotein (LDL) plays a central role in atherogenesis.1-4 Lysophosphatidylcholine (lyso-PC) accumulates in oxidized LDL,5,6 is abundant in atherosclerotic lesions,7 and inhibits endothelium-dependent vascular relaxation.6,8 Lyso-PC is also a selective chemoattractant for mononuclear leukocytes9 and induces mononuclear leukocyte adhesion molecule mRNA in endothelial cells.10 Furthermore, lyso-PC inhibits agonist-stimulated calcium signaling in cultured endothelial cells.11,12

In in vitro experiments lyso-PC, like other phospholipids, has been shown to activate protein kinase C.13 This important signaling mechanism for lyso-PC has not been demonstrated either in intact cells or in intact vascular segments but could be responsible for many of the effects of oxidized LDL and lyso-PC previously reported. The protein kinase C family of enzymes represents an important mediator of cell signaling.14,15 Activation of protein kinase C increases expression of c-fos and c-jun and modulates c-fos and c-jun binding to the API transcriptional regulatory site. These effects have recently been reviewed.16 Activation of protein kinase C may also modulate vascular constriction.17 One important consequence of protein kinase C activation is the generation of O$_2^-$ . This effect of protein kinase C activation was first demonstrated in neutrophils and macrophages18 but has recently been shown to occur in endothelial cells.19,20 O$_2^-$ generated in response to protein kinase C activation could enhance vascular constriction both via direct mechanisms and via inactivation of nitric oxide. O$_2^-$ production may serve to further oxidize LDL21 and thus promote the atherosclerotic process.

On the basis of these considerations, we performed this study to achieve the following goals: (1) to determine whether lyso-PC could activate protein kinase C in intact vascular segments; (2) to determine whether lyso-PC increases O$_2^-$ production by vascular segments in a protein kinase C-dependent fashion; and (3) to examine the role of protein kinase C in altering endothelium-dependent vascular relaxation after exposure to lyso-PC.

Methods

Activation of Protein Kinase C by Lyso-PC in Intact Vascular Segments

An initial experiment was performed to determine whether lyso-PC could activate protein kinase C in intact vascular segments in a manner similar to that observed in tissue homogenates.13 Rabbits were killed by an overdose injection of sodium pentobarbital, and the aorta was removed. The thoracic aorta was then cut open longitudinally, sliced into 0.5-mm$^3$ cubes with a McIlwain chopper (Drumkinn Instruments Inc.), and suspended in 4 mL of phosphate-free RPMI cell culture medium. Aliquots (1 mL) of the suspended tissue were labeled with $^{32}$P (1 mCi/mL) for 2 hours at 37°C in the incubator, followed by an additional 15 minutes in the absence or presence of 0.2 μmol/L 12-O-tetradecanoylphorbol 13-acetate (TPA) or 10 μmol/L lyso-PC. The tissue samples were recovered by brief centrifugation, washed once with 2 mL of...
O$_2^-$ Production in Response to Lyso-PC

The above experiment demonstrated that protein kinase C in rabbit aorta was indeed activated by low concentrations of lyso-PC. To determine whether this activation of protein kinase C was associated with increases in vascular O$_2^-$ production, we employed a lucigenin assay similar to that used in prior studies. On the day of the study, rabbits were killed by an overdose injection of pentobarbital. The descending thoracic aorta was isolated and prepared, with care taken not to damage the endothelium. Five-millimeter ring segments of thoracic aorta dissected free of adventitia were incubated with Krebs-HEPES buffer maintained at 37°C for 30 minutes and then were gently transferred to glass scintillation vials containing 0.25 mmol/L lucigenin with or without 10 μmol/L lyso-PC and other additions (final volume of 2 mL). Chemiluminescence of lucigenin (bis-N-methylacridinum nitrate) was detected with a scintillation counter (LS 7000, Beckman Instruments, Inc) in out-of-coincidence mode with a single active photomultiplier tube as described previously. Counts were obtained at 2-minute intervals at room temperature. Vials containing all components with the exception of aortic rings were counted, and these blank values were subtracted from the chemiluminescence signals obtained from the aortic rings.

Assay for Protein Kinase C Activity

We studied the inhibitory capacity of calphostin C on protein kinase C in our assay system. Rabbit aortas were homogenized in 3 volumes of ice-cold extraction solution containing 50 mmol/L Tris/HCl (pH 7.5), 50 mmol/L 2-mercaptoethanol, and 5 mmol/L EGTA. The homogenate was centrifuged at 20,000g for 15 minutes at 4°C to obtain the soluble fraction, which was used as the source of protein kinase C. The enzyme activity was assayed as described previously.

Briefly, the standard reaction mixtures (0.2 mL) contained 5 μmol Tris/HCl (pH 7.5), 2 μmol MgCl₂, 2 μg sonicated phosphatidylserine, 40 μg histone H₁, either 0.8 μmol EGTA or 0.04 μmol CaCl₂, 1 mmol (γ⁻³²P)ATP (containing 1×10⁶ cpm), and appropriate amounts (2.5 and 5 μg protein) of the aortic soluble fraction. The reaction, started with the addition of the radioactive ATP, was carried out for 5 minutes at 30°C. The phosphotransferase activity that was stimulated by phosphatidylserine and Ca²⁺ was taken as that of the conventional group of protein kinase C. The enzyme activity was linear with respect to the incubation time and the enzyme amount under the assay conditions.

Detection of Endothelium-Derived Relaxing Factor Release by a Cascade Bioassay Preparation

We also performed experiments to determine whether activation of protein kinase C played a role in the alteration of endothelium-derived relaxing factor (EDRF) release by lyso-PC.

Preparation of Donor Segment

Rabbits were killed by an overdose injection of pentobarbital, and the thoracic aortas were dissected free. These segments were then cannulated with stainless steel cannulas, with care taken not to damage the endothelium. The aortas were then mounted in an organ bath containing Krebs buffer of the following millimolar composition: NaCl 118.3, KC1 4.69, CaCl₂ 1.57, MgSO₄ 1.20,KH₂PO₄ 1.03, NaHCO₃ 25.0, and glucose 11.1; pH 7.40. The solution was aerated continuously with 95% O₂/5% CO₂ and maintained at 37°C. The donor vessels were perfused in the direction of their normal flow with the same solution from a separate reservoir at 4 mL/min by a roller pump.

Preparation of Detector Segment

Segments 2 mm wide from the proximal left circumflex coronary arteries of mongrel dogs were used as detector rings. These were denuded of endothelium by gentle rubbing of the intimal surface with the closed tips of hemostatic forceps.

To examine the potential role of protein kinase C as a source of O$_2^-$ in lyso-PC-treated vessels, vessels from five additional rabbits were exposed to calphostin C (10 μmol/L), a potent inhibitor of protein kinase C. Chemiluminescence was measured 15 minutes after exposure to lucigenin as described above. To assess endothelial O$_2^-$ production, endothelium was removed by gentle rubbing of the vessel's intimal surface with the closed tips of hemostatic forceps.

To determine whether calphostin C could directly scavenge O$_2^-$, we examined its effect on the lucigenin-mediated chemiluminescence produced by pyrogallol. Lucigenin-mediated chemiluminescence produced by 10 μmol/L pyrogallol was assayed in the presence or absence of 50 mmol/L calphostin C. Chemiluminescence was measured 15 minutes after exposure to lucigenin as described above. We also studied the effects of the phorbol ester TPA on vascular O$_2^-$ production. Vessels from three additional rabbits were exposed to TPA (0.2 μmol/L), and chemiluminescence was measured 15 minutes after exposure to lucigenin and TPA.

Protocols

Additional rabbits (n=16) were randomly assigned to three groups. Six donor vessels were selected to be incubated with 10 μmol/L (5 μg/mL) lyso-PC for 30 minutes in aerated Krebs buffer maintained at 37°C. Then these aortas were mounted in an organ bath and perfused with buffer for 15 to 20 minutes to wash out lyso-PC. Five aortas were incubated with 50 mmol/L calphostin C and 10 μmol/L lyso-PC for 30 minutes and then mounted and washed as mentioned above. Incubation with calphostin C was performed under fluorescent light. Five aortas were used as controls. Donor vessels were not exposed to lyso-PC or calphostin C at all. Calphostin C was dissolved in dimethyl sulfoxide (DMSO) and added into aerated Krebs buffer. The final concentration of DMSO in Krebs buffer was 0.001%. To prevent the synthesis of vascular prostaglandins, we performed all experiments in the presence of 1 mmol/L indomethacin. Infusions of increasing concentration of the receptor-mediated agonist acetylcholine or non-receptor-mediated agonist A23187 were injected proximal to...
the donor vessel, and the effluent was allowed to superfuse the detector vessel that had been preconstricted with prostaglandin F\(_2\alpha\) (PGF\(_{2\alpha}\), 1 to 3 \mu mol/L). After a stable contraction plateau of detector vessels was reached, all donor vessels were exposed cumulatively to acetylcholine (0.1 to 10 \mu mol/L). After being washed out, donor and detector vessels were allowed to equilibrate for 30 minutes, and donor vessels were then exposed to A23187 (0.1 to 10 \mu mol/L). The amount of relaxation of detector vessels was calculated as a percent decrease in the active tension generated to PGF\(_{2\alpha}\).

**Materials**

Acetylcholine chloride, calcium ionophore (A23187), histone (lysine-rich) H1 subfraction, indomethacin, lucigenin, lysophosphatidylcholine (lyso-PC), 1,4-piperazinediethanesulfonic acid, phosphatidylserine (lysine-rich) H1 subfraction, indomethacin, lucigenin, pyrogallol, and TPA were obtained from Sigma Chemical Co. Calphostin C was obtained from Kamiya Bio-medical Co. [\(^{32}\)P]orthophosphate and [y-\(^{32}\)P]ATP were obtained from ICN Radiochemicals. RPMI cell culture medium was obtained from GIBCO BRL. Lucigenin and pyrogallol were dissolved in 0.1 M sodium phosphate buffer (pH 7.8) and dissolved in dimethyl sulfoxide and added into aerated Krebs buffer or Krebs-HEPES buffer (pH 7.4). Calphostin C was obtained from GIBCO BRL. Lucigenin and pyrogallol were dissolved in 0.1 M sodium phosphate buffer (pH 7.8) and dissolved in dimethyl sulfoxide and added into aerated Krebs buffer or Krebs-HEPES buffer.

**Statistical Analysis**

Measurements obtained under identical conditions from two ring segments were averaged for each animal. The number of experiments refers to the number of animals. Comparisons of \(\text{O}_2^\cdot\) production between control and lyso-PC-treated vessels were performed by unpaired \(t\) test or, when appropriate, paired \(t\) test. Percent relaxations caused by acetylcholine in the calphostin C-treated vessels versus untreated vessels were compared by ANOVA. The accepted level of significance was \(P<.05\). Data are expressed as mean±SEM.

**Results**

**Protein Phosphorylation in Aorta Slices**

The in situ phosphorylation of endogenous proteins in aortic segments indicated that several proteins were phosphorylated under basal conditions, notably proteins 1, 2, 3, and 4 (with an isoelectric point [pI] of 7.4) and proteins 5, 6, and 7 (with a pI of 6.5) (Fig 1A). In the presence of 0.2 \mu mol/L TPA, phosphorylation of proteins 5, 6, and 7 as well as proteins 8 and 9 (with a pI of 6.5) was enhanced, which was accompanied by a concomitant decrease in phosphorylation of proteins 1, 2, and 3 (Fig 1B). The effects of TPA were essentially mimicked by 10 \mu mol/L lyso-PC, although its effects were less pronounced than those of TPA (Fig 1C). It is unclear at present whether proteins 1, 2, 3, and 4 were related to proteins 8, 9, 6, and 7, respectively, of the same molecular masses. A change in the pI of 7.4 for the former group of proteins to that of 6.5 for the latter might reflect additional phosphorylation catalyzed by protein kinase C, which was activated by TPA and lyso-PC.

**\(\text{O}_2^\cdot\) Production in Control and Lyso-PC–Treated Vessels With or Without Endothelium**

The chemiluminescence produced by lucigenin increased with time and reached a plateau within 15 minutes (Fig 2).

\(\text{O}_2^\cdot\) production, estimated by measuring chemiluminescence 15 minutes after exposure to lucigenin, was 0.50±0.04 nmol/mg tissue (dry wt) per minute in control vessels and was approximately twofold higher in lyso-PC–treated vessels (0.97±0.14 nmol/mg tissue per minute, \(P<.05\) versus control vessels, Fig 3). Endothelial removal increased \(\text{O}_2^\cdot\) production in control vessels (0.70±0.09, \(n=5, P<.05\) versus denuded control vessels) while having no effect in lyso-PC–treated vessels (1.13±0.15, \(n=5, P=NS\) versus denuded lyso-PC–treated vessels, Fig 3).
Effects of Calphostin C on \(O_2^-\) Production in Control and Lyso-PC-Treated Vessels With Intact Endothelium

In lyso-PC-treated vessels, calphostin C markedly reduced \(O_2^-\) production to a value similar to that observed in control vessels (Fig 4A). Calphostin C was dissolved in DMSO, and the final concentration of DMSO in assay solution was 0.01%. In three experiments we found that this concentration of DMSO (vehicle) did not affect \(O_2^-\) production in lyso-PC-treated vessels. Importantly, calphostin C had no effect on \(O_2^-\) production in control vessels. The effect of calphostin C on the production of \(O_2^-\) was not related to direct scavenging of the \(O_2^-\), because calphostin C did not affect the lucigenin-mediated chemiluminescence produced by auto-oxidation of pyrogallol (Fig 4B).

Aortic Protein Kinase C Activity and Its Inhibition by Calphostin C

The activity of phosphatidylserine/Ca\(^{2+}\)-stimulated protein kinase C in the soluble fraction of the aorta was determined to be 496 pmol phosphate transferred per minute per milligram protein, which was inhibited 40% and 52% by 50 and 100 nmol/L calphostin C, respectively (n=4 for each). Protein kinase C activity was abolished by 5 \(\mu\)mol/L calphostin C.

Effects of Phorbol Ester TPA on \(O_2^-\) Production in Vessels With Intact Endothelium

The phorbol ester TPA (0.2 \(\mu\)mol/L) also markedly increased \(O_2^-\) production. Fifteen minutes after exposure to lucigenin, \(O_2^-\) production was 0.47±0.02 nmol/mg tissue (dry wt) per minute in control vessels and was approximately 2.5-fold higher in TPA-treated vessels (1.22±0.11 nmol/mg tissue per minute, \(P<.05\) versus control vessels, Fig 5).

Effects of Calphostin C on \(O_2^-\) Production from Control and Lyso-PC-Treated Aorta With Endothelium Preincubated With or Without Calphostin C

![Fig 3. Bar graph showing \(O_2^-\) production from control with (+, n=5) or without (-, n=5) endothelium (endo) and lyso-PC (lyso-PC)-treated vessels with (+, n=6) or without (-, n=6) endothelium estimated by lucigenin chemiluminescence 15 minutes after exposure to lucigenin. Data are expressed as mean±SEM. *\(P<.05\) for control vessels with endothelium vs without endothelium (paired \(t\) test); †\(P<.05\) for control vs lyso-PC-treated aorta with endothelium (paired \(t\) test).](http://atvb.ahajournals.org/)

![Fig 5. Bar graph showing \(O_2^-\) production from control (n=3) and 0.2 \(\mu\)mol/L 12-O-tetradecanoylphorbol 13-acetate (TPA)-treated vessels (n=3) estimated by lucigenin chemiluminescence 15 minutes after exposure to lucigenin. Data are expressed as mean±SEM. *\(P<.05\) for control vessels vs TPA-treated vessels (paired \(t\) test).](http://atvb.ahajournals.org/)
Effects of Calphostin C on Endothelium-Derived Relaxing Factor Release in Lyso-PC-Treated Donor Vessels

Injection of acetylcholine and A23187 through donor vessels stimulates the release of EDRF, which, in these experiments, was quantified by relaxation of detector rings. As shown in Fig 6A, EDRF release in response to acetylcholine (0.1 to 1 μmol/L) was markedly impaired in lyso-PC–treated donor vessels compared with control vessels. Interestingly, in lyso-PC–treated vessels, calphostin C markedly restored EDRF release in response to acetylcholine (0.1 to 1 μmol/L). In contrast, as shown in Fig 6B, EDRF release in response to A23187 was impaired only at the concentration of 0.1 μmol/L in lyso-PC–treated donor vessels, and calphostin C did not affect EDRF release in response to A23187.

Discussion

The new findings of these studies are, first, that lyso-PC can activate protein kinase C in intact vessels, as evidenced by enhanced phosphorylation of certain proteins similarly phosphorylated in response to TPA. Lyso-PC also stimulated a substantial increase in O$_2^-$ production from isolated vessels. Because this was inhibited by calphostin C and mimicked by TPA, it is reasonable to assume that the increased vascular O$_2^-$ production following lyso-PC exposure was due to protein kinase C activation. Finally, we demonstrated that lyso-PC activation of protein kinase C is probably involved in alterations of EDRF release from isolated vascular segments.

Lyso-PC is a by-product of cholesterol esterification and is formed during oxidation of LDL.5,6 In cholesterol-fed animals, lyso-PC accumulates in the vessel wall,7,28 and lyso-PC has been found in atherosclerotic vascular lesions in humans.28 The levels of lyso-PC in human atherosclerotic lesions are similar to those used in the present experiments (10 μmol/L), although the intracellular concentration is not precisely known. It is known that lyso-PC may have nonspecific detergent-like cytotoxic properties in concentrations above those necessary to form micelles (>20 μmol/L).28 In preliminary studies, we found that exposure of vascular segments to 50 μmol/L lyso-PC for 30 minutes resulted in extensive endothelial cell disruption. Such an effect was not observed after exposure to 10 μmol/L lyso-PC.

The findings of the present study examining the acute effect of lyso-PC differ somewhat from those of our previous studies of cholesterol-fed rabbits.24 In the previous study, we found that segments of rabbit aorta from cholesterol-fed rabbits generated excess O$_2^-$. Endothelial removal or treatment with oxypurinol normalized O$_2^-$ production from vessels of hypercholesterolemic animals. These data were interpreted as indicating that the source of O$_2^-$ in hypercholesterolemic vessels was probably xanthine oxidase in the endothelium or cells closely associated with the endothelium. Our present findings indicate that hypercholesterolemia and the accumulation of lyso-PC within the vessel may increase O$_2^-$ production via separate pathways: lyso-PC via activation of protein kinase C and hypercholesterolemia via either activation or induction of xanthine oxidase. We considered the possibility that xanthine oxidase may be activated via a protein kinase C–mediated pathway and that the consequent increase in O$_2^-$ production was due to xanthine oxidase. In preliminary studies, however, oxypurinol did not affect lyso-PC–mediated O$_2^-$ production (n=2). One explanation for these findings is that our previous studies were performed using tissues from the early stage of hypercholesterolemia, only 4 to 6 weeks of cholesterol feeding, well before the development of intimal proliferation and, perhaps, before the accumulation of modified lipids. It is conceivable that oxidation of LDL and the accumulation of lysoprophospholipids within the intima might occur at a later stage of hypercholesterolemia. Another feature by which chronic hypercholesterolemia and acute exposure to lyso-PC seem to vary relates to the portion of the vessel that is stimulated to increase O$_2^-$ production. In hypercholesterolemic vessels, the source of O$_2^-$ was not the smooth muscle layer but rather the endothelial cell itself or perhaps monocyte-macrophages closely associated with the endothelium. In contrast, the source of O$_2^-$ in this setting is not the intima but predominantly the smooth muscle layer. Despite these considerations, it is clear that lyso-PC can accumulate in severely atherosclerotic regions, and it is likely that it may contribute to O$_2^-$ production via activation of protein kinase C.
protein kinase C leads to increases in $\text{O}_2^-$ production in other cell types, however, is not new. In neutrophils, $\text{O}_2^-$ production has been repeatedly stimulated by administration of tumor-promoting phorbol compounds known to activate protein kinase C. This has also been demonstrated in cultured endothelial cells. The production of $\text{O}_2^-$ by neutrophils is proportional to the activity of intracellular protein kinase C. Lyso-PC, in the presence of diacylglycerol and phosphatidylserine, can activate protein kinase C at physiological calcium concentrations. Conversely, lyso-PC, in the presence of calcium and phosphatidylserine, can potentiate the effect of diacylglycerol in activating protein kinase C. Protein phosphorylation and activation of an NADPH oxidase are probably involved in the ultimate pathway leading to $\text{O}_2^-$ production. NADPH oxidase is a major source of $\text{O}_2^-$ in several mammalian cell types.

$\text{O}_2^-$ production in vessels exposed to lyso-PC may predispose to vasoconstriction. It has been suggested that $\text{O}_2^-$ serves as an endothelium-derived vascular contracting factor. The mechanism whereby $\text{O}_2^-$ produces vasoconstriction is not known but could involve altering sarcoplasmic reticulum uptake of calcium. In addition, $\text{O}_2^-$ produced in response to lyso-PC could inactivate endothelium-derived nitric oxide and thus unmask the vasoconstrictor effects of numerous neurohumoral mediators of vascular tone. It is unlikely, however, that the only mechanism responsible for altered EDRF release after exposure to lyso-PC is $\text{O}_2^-$ degradation of the EDRF. Calphostin C completely prevented the increase in $\text{O}_2^-$ degradation of the EDRF. Calphostin C completely prevented the increase in $\text{O}_2^-$ production of vascular segments in response to lyso-PC and normalized EDRF release in response to acetycholine. Despite this, calphostin C failed to correct the altered release of EDRF in response to the calcium ionophore A23187 after exposure to lyso-PC. The mechanism responsible for this discrepancy remains unclear. Other mechanisms, such as alteration of calcium handling (independent of $\text{O}_2^-$-induced alterations), may be involved in this defect.

$\text{O}_2^-$ may participate in the oxidation of LDL. It is interesting to speculate that lyso-PC formed during oxidation of LDL increases production of $\text{O}_2^-$ in the vessel wall, which may further enhance LDL oxidation. This would probably result in a self-perpetuating cycle of LDL modification: release of lyso-PC, stimulation of $\text{O}_2^-$ production, and further LDL modification. Such a cycle might contribute to the rather marked acceleration of lesions and symptoms that may occur in certain clinical settings. $\text{O}_2^-$ also provides a source of other oxygen-centered radicals, such as $\text{H}_2\text{O}_2$ and $\cdot \text{OH}$, which may participate in lipid peroxidation and serve to damage cellular membranes. In addition, the $\text{O}_2^-$ may react with NO- (which is also produced in excess within the endothelium of hypercholesterolemic animals) to produce the highly injurious peroxynitrite radical. Oxygen-centered radicals have been implicated as promoters of vascular smooth muscle growth and thus may contribute to myointimal proliferation. Interestingly, the antioxidant $\alpha$-tocopherol inhibits LDL-induced proliferation and protein kinase C activity in vascular smooth muscle cells. It is interesting to speculate that this effect of $\alpha$-tocopherol may have been related to prevention of oxidation and lyso-PC activation. In these respects, the increase in $\text{O}_2^-$ production induced by lyso-PC may not only inactivate endothelium-derived nitric oxide but also serve as an early event in the atherosclerotic process.


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