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# Superoxide Generated by Lysophosphatidylcholine Induces Endothelial Nitric Oxide Synthase Downregulation in Human Endothelial Cells

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**Key Words**

Superoxide • eNOS • Endothelial cell • Lysophosphatidylcholine • Vascular dysfunction

**Abstract**

We examined the mechanism through which lysophosphatidylcholine (LPC) induces endothelial nitric oxide (eNOS) downregulation. Human umbilical vein endothelial cells (HUVECs) were treated with LPC (50-150 μM) for 0.5-2 h or the reactive oxygen species (ROS) donors, xanthine/xanthine oxidase (X/XO), 1,4-hydroquinone (HQ) or tert-butylhydroperoxide (TBHP) for 2 h. Protein levels of eNOS, superoxide dismutase1 (SOD1), catalase, and phospho-extracellular signal regulated kinase 1/2 (pERK 1/2) were assessed using immunoblotting. LPC treatment reduced SOD1 levels but increased catalase levels. The superoxide donors X/XO and HQ showed similar effects. The hydroperoxide donor TBHP increased SOD1 levels but did not change catalase levels. LPC concentration- and time-dependently decreased eNOS levels, but this effect was blocked by antioxidants and SOD and potentiated by the SOD1 inhibitor, ammonium tetrathiomolybdate. LPC and X/XO inhibited ERK1/2 phosphorylation, whereas TBHP stimulated phosphorylation. Taken together, these data indicate that LPC induces superoxide overload in HUVECs via SOD1 inhibition and downregulates phospho-ERK1/2 and eNOS levels.



## Introduction

Endothelial dysfunction refers to the perturbation of anticoagulant, anti-inflammatory properties, and impaired vascular reorganization against several pathophysiological conditions [1]. Endothelial dysfunction is the primary step in the initiation and development of atherosclerosis and promotes the ingress of lipoproteins from plasma into the intima [2, 3]. The endothelium, which is the lining between tissues and the blood, responds to external factors such as shear stress and blood-borne signals to maintain homeostasis. One diffusible regulator, nitric oxide (NO), an endothelium-derived relaxing factor, is beneficial under conditions of vessel impairment or platelet and leukocyte adhesion [4]. It is produced by NO synthase (NOS) as a by-product of the biochemical conversion of the amino acid L-arginine to L-citrulline. Since NO functions as quencher of reactive oxygen species (ROS), endothelial NO synthase (eNOS) could help reduce oxidative status caused by risk factors such as hypercholesterolemia.

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ROS are pleiotropic factors that play important roles in a wide variety of cellular events, but excess production causes cell damage through lipid peroxidation and the disruption of structural proteins, enzymes, or DNA. Low-density lipoprotein (LDL) and its oxidative modification (ox-LDL) cause extreme oxidative stress in endothelial cells, which affects cell signaling, differentiation and apoptosis [5, 6]. Lysophosphatidylcholine (LPC) is an atherogenic phospholipid generated during LDL oxidation [7]. LPC leads to ROS-induced endothelial dysfunction via impairing endothelium-dependent vasorelaxation caused by loss of NO [8, 9]. Although LPC modulates NO production by upregulating eNOS in endothelial cells [10], the regulatory mechanism is unclear.

In the present study, we investigated the mechanism by which LPC-induced oxidative injury impacts eNOS regulation in human endothelial cells. Our results suggest that the loss of superoxide dismutase1 (SOD1) caused by LPC increases the production of intracellular superoxide and suppresses phospho-extracellular signal-regulated protein kinase 1/2 (pERK1/2) to downregulate eNOS.

## Materials and Methods

*Materials*

N-acetyl-L-cysteine (NAC), Tempol, Tiron, 1,4-hydroquinone (HQ), tert-butyl hydroperoxide (TBHP), ammonium tetrathiomolybdate (TM), SOD, catalase, dihydroethidine (DHE), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-α-lysophosphatidyl choline (LPC) from egg yolk were purchased from Sigma (St. Louis, MO); 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR), xanthine and xanthine oxidase (X/XO) were from Calbiochem. Mouse anti-eNOS monoclonal antibody was obtained from BD Bioscience (Rockville, MD). Rabbit anti-superoxide dismutase 1, rabbit anti-catalase, goat anti-extracellular signal-regulated protein kinase (ERK), and mouse anti-phospho-ERK (pERK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

*Mammalian Cell Cultures*

Human umbilical vein endothelial cells (HUVECs, CRL-1730) were purchased from the American Type Culture Collection (Manassas, VA) and cultured as a monolayer in Medium 199 (M199, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 μg/ml endothelial cell growth supplement, or Eagle basal medium 2 (EBM2, Clonetics, Walkersville, MD) supplemented with 2% FBS, 0.1% vascular endothelial growth factor, 0.1% ascorbic acid, 0.1% gentamycin sulfate amphotericin-B, 0.04% hydrocortisone, and 0.1% heparin. All cells were maintained at 37 °C in humidified conditions under 5% $CO_2$. Media were changed twice weekly, and cultures were split at 1:5 weekly. For experiments, HUVECs were plated in 60 mm or 100 mm plates and equilibrated with 0.3% serum containing media for 16 h. The medium was then removed and replaced with fresh medium, and the cells were maintained for the time periods indicated.

*Measurement of Intracellular ROS*

ROS in HUVECs were determined using DHE or DCFH-DA as described previously [11]. Cells were seeded at $2\times10^4$ cells per well in 96-well plates with EBM2 media one day before the experiment. Cells were pre-treated with various agents in EBM2 media for 30 min followed by treatmemt with LPC in EBM2 media for 120 min. Cells were then loaded with DCFH-DA in Hank's balanced salt solution for 20 min in the dark, washed twice with phosphate-buffered saline (PBS) to eliminate contamination of diffused ROS, and treated with vehicle alone or the indicated compounds for 30 min and then stimulated with LPC for 2 h. Fluorescence was directly read at an emission wavelength of 538 nm after excitation at 485 nm. Cell numbers were normalized to detect maximum fluorescence increases in wells.

*Western Blotting*

HUVECs were pre-treated with various agents in 10% FBS containing media for 30 min followed by stimulation with LPC for 120 min. After drug incubation, cells were washed once in ice-cold PBS and lysed in protein extraction buffer containing a protease inhibitor cocktail. Protein concentrations in the supernatant were determined by the Bradford protein assay. For Western blot analysis, 30 μg of protein was subjected to SDS-PAGE gels (7.5 - 12%), and proteins were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 h with TBST (10 mM Tris-HCl, 150 mM NaCl, and 1 v/v% Tween 20, pH 7.6) containing 5% bovine serum albumin at room temperature. The blots were incubated for 3 h with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Bands were visualized by chemiluminescence. Data collection and processing were performed using a luminescent image analyzer LAS-3000 and IMAGE GAUGE software (Fuji Film, Japan).

*RT-PCR*

The expression of eNOS mRNA was analyzed by the RNeasy mini kit (Qiagen, GmbH) for RNA isolation and OneStep RT-PCR kit (Qiagen, GmbH) for RT-PCR. For blocking DNA contamination in isolated RNA, DNaseI was treated before the final washing step. RNA (1 μg) was subjected to RT-PCR for 28 cycles. Primers for human eNOS were 5′-ACC CTC ACC GCT ACA ACA TC-3′ (sense) and 5′-GCT CAT TCT CCA GGT GCT TC-3′ (antisense). eNOS mRNA expression is normalized to the house-keeping gene human, GAPDH [5-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-TTG ATT TTG GAG GGA TCT CG-3′(antisense)].

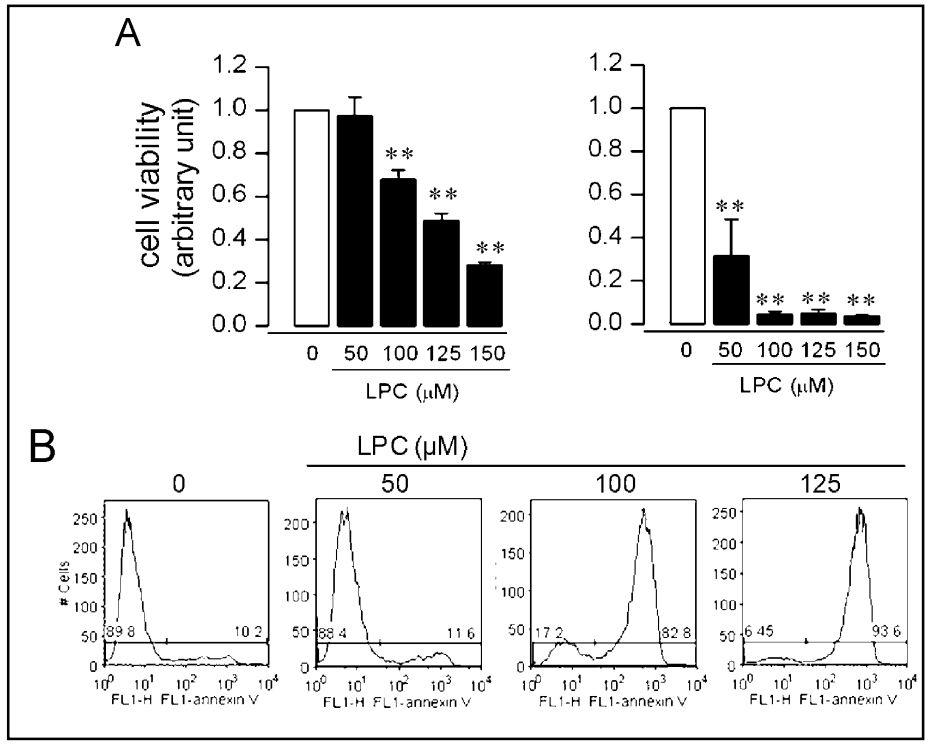


**Fig. 1.** HUVEC death induced by LPC. (A) Cell viability was determined by MTT assay after 2 h stimulation with LPC in M199 containing 10% (left panel) or 2% (right panel) serum. Data are shown as means ± S.E. from three independent experiments. Asterisks indicate significant differences from vehicle treated cells ($P < 0.01$). (B) After LPC treatment in M199 containing 10% serum for 2 h, cells were analyzed by flow cytometry. The numbers show the percentages of annexin V-positive cells. Data are representative of three experiments.

*Nuclear Extraction*

Cells were washed twice in ice-cold phosphate-buffered saline and suspended in ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin A, leupeptin, and aprotinin), and incubated on ice for 30 min. To prepare nuclear extracts, lysed cells were centrifuged at 8,000 rpm for 5 min at 4 °C, and the pellets were resuspended in a buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM β-glycerophosphate, 0.05 mM vanadate, and the protease inhibitor mixture. After extraction on ice for 30 min, samples were centrifuged at 14,000 rpm for 30 min at 4 °C. Supernatant, containing nuclear proteins, was transferred to a microcentrifuge tube, an aliquot was removed for protein determination, and samples were stored at -20 °C.

*Electrophoretic Mobility Shift Assay (EMSA)*

EMSAs were performed using a double-stranded oligonucleotide (5'-CCTGTGCTCCGGGAATTTCCCTGGCC-3') containing the NF-κB binding motif. For the binding reaction, nuclear protein extract (10 μg) was incubated in a total volume of 20 μl in binding buffer containing 10 mM HEPES, pH 7.5, 5% glycerol, 50 mM KCl, 1 mM dithiothreitol, 1 μg of poly(dI-dC), and radiolabeled (about 30,000 cpm) DNA for 30 min at room temperature. DNA-protein complexes were resolved in a pre-electrophoresed 6% nondenaturing polyacrylamide gel at 4 °C. Subsequently, the gel was dried under vacuum and exposed to film.

*Flow Cytometry*

HUVECs ($10^6$) were treated with LPC for 2 h. To evaluate apoptotic cell death, cells were stained with Annexin V-FITC (BD Bioscience, Rockville, MD) in Annexin-V staining buffer for 15 min at room temperature and counterstained with propidium iodide. Data were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

*MTT assay*

HUVEC cells were seeded onto 96-well plates ($2 \times 10^4$ cells/well) and stimulated with LPC in 2% or 10% FBS media for 120 min. MTT (0.1 mg) in phosphate-buffered saline was then added to each well, and the cells were incubated for an additional 4 h at 37 °C. After removing the medium, the cells were lysed with dimethyl sulfoxide to dissolve the formazan product. The absorbance of reduced MTT at 590 nm was measured with a plate reader.

*Statistical analysis*

Data are expressed as means ± S.E. Statistical analysis was performed using Student's t-test and ANOVA as appropriate. $P<0.05$ was considered statistically significant.

## Results

*LPC-induced ROS involve in the downregulation of eNOS on HUVECs*

To examine which kinds of cell injury were induced by LPC treatment, we performed MTT assay and FACS analysis. LPC was concentration-dependently cytotoxic in 10% FBS media and concentration-independently in 2% FBS media in the MTT assay (Fig. 1A). Because the physiological concentration of LPC in body fluids is

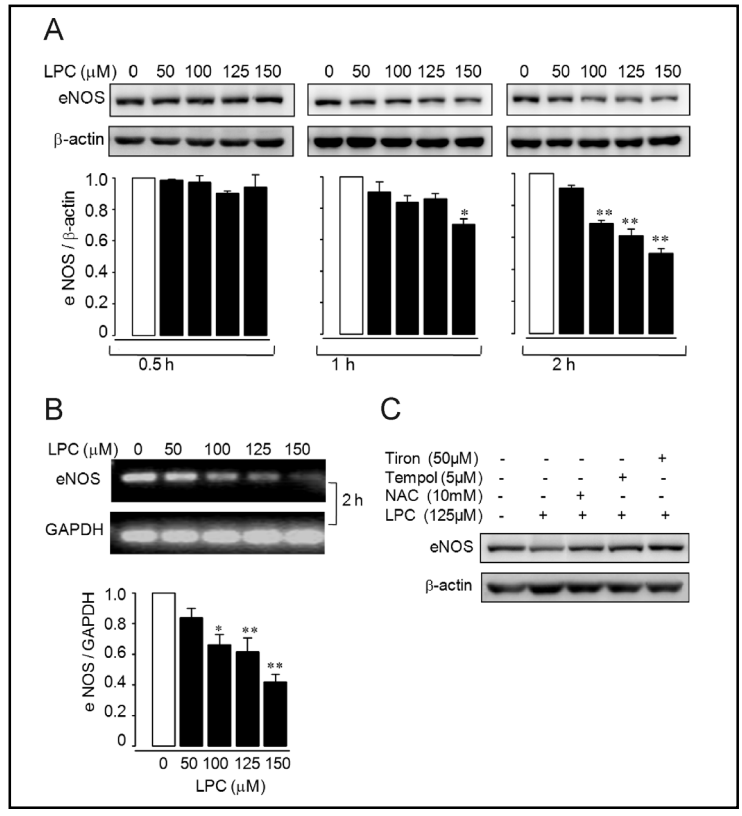


**Fig. 2.** Effects of LPC on eNOS. After stimulation with vehicle or LPC for 0.5, 1, or 2 h, expression of eNOS protein (A) or mRNA (B) was measured by western blotting or RT-PCR analysis. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ** $P < 0.01$, * $P < 0.05$ versus vehicle-treated cells. (C) HUVECs were treated with NAC, Tempol or Tiron for 0.5 h prior to stimulation with LPC for 2 h. Western blotting was performed with an anti-eNOS antibody. Results are representative of three independent experiments.

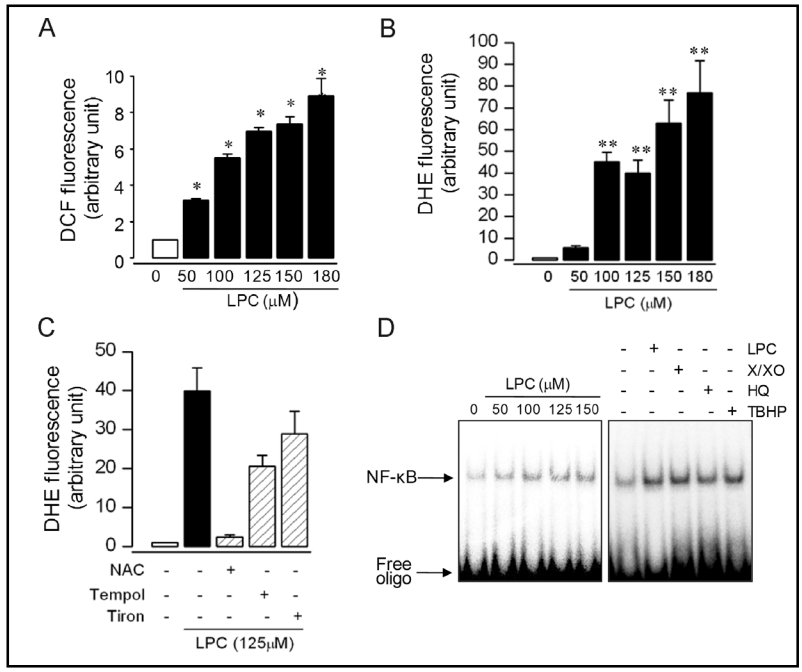


**Fig. 3.** ROS generation induced by LPC. HUVECs were treated with vehicle or LPC for 2 h, and the amounts of total ROS (A) or superoxide (B) were measured using DCF or DHE dye. (C) HUVECs were treated with vehicle or LPC (125 μM) for 2 h with or without pretreatment of NAC (10 mM), Tempol (5 μM) or Tiron (50 μM) for 30 min, and the amounts of superoxide were measured. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ** $P < 0.01$ versus vehicle-treated cells. (D) HUVECs were treated with vehicle, LPC (left panel), LPC (125 μM), X/XO (100 μM/100 mU), HQ (100 μM) or TBHP (100 μM) for 2 h (right panel). EMSA was performed in nuclear extracts incubated with $^{32}$P-end labeled oligonucleotide.

between 5 and 180 μM [12, 13] and low serum itself can damage cells, we used 10% FBS media for subsequent experiments. FACS results showed that LPC concentration-dependently induced apoptotic changes in HUVECs (Fig. 1B). LPC time-dependently decreased protein levels of eNOS (Fig. 2A) when treated for 1 h at 150 μM or 2 h at ≥100 μM. In addition, LPC decreased eNOS mRNA levels in RT-PCR analysis (Fig. 2B). The antioxidants, NAC, Tempol, and Tiron could block the effects of LPC (Fig. 2C), implying a ROS-mediated mechanism. Indeed, LPC increased levels of total ROS (Fig. 3A) as well as superoxide (Fig. 3B), consistent with previous reports [14], and these increases could be blocked with NAC, Tempol, or Tiron (Fig. 3C). LPC (50 – 150 μM) also in-

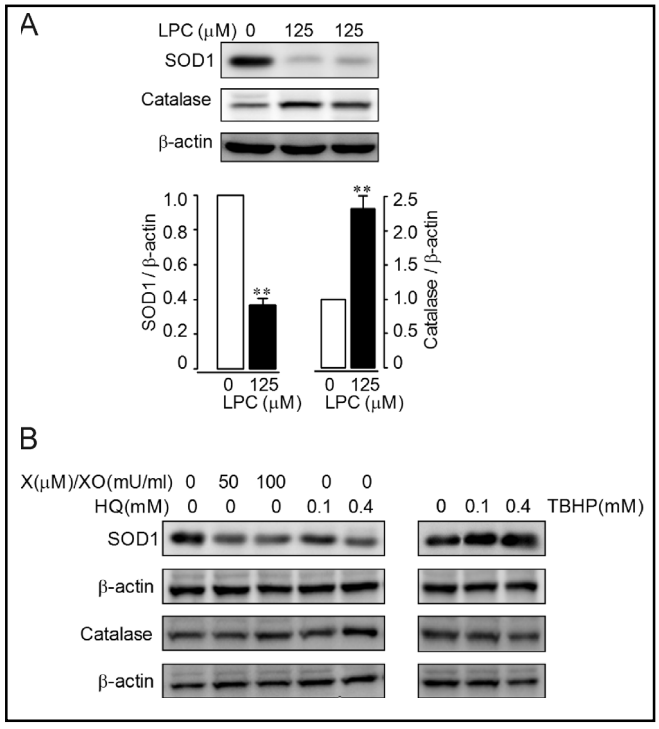


**Fig. 4.** Effects of LPC-induced ROS on antioxidant enzymes. HUVECs were treated with vehicle, LPC (125 μM) (A), X/XO, HQ or TBHP (B) for 2 h. Antibodies against SOD1 or catalase were used for blots. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ** $P < 0.01$ versus vehicle-treated cells.

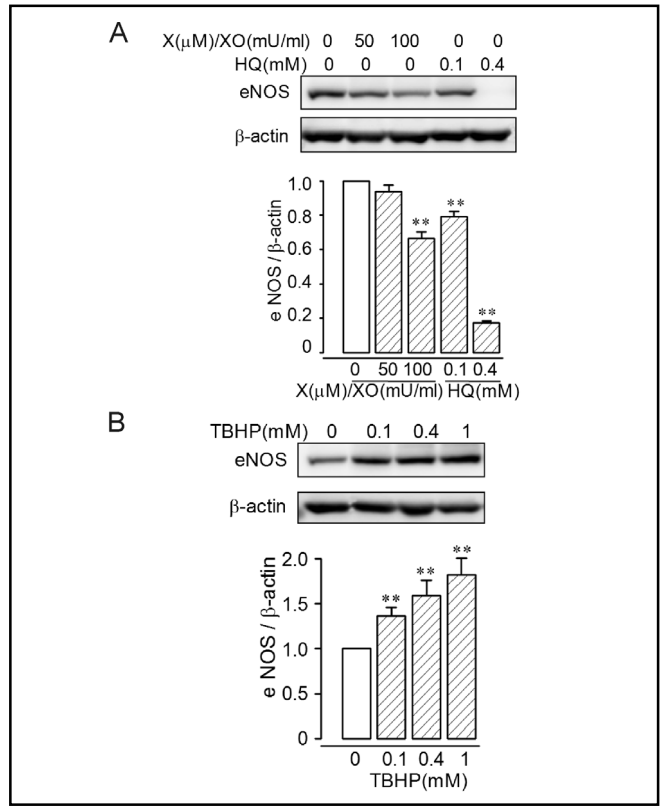


**Fig. 5.** Effects of ROS donors on eNOS regulation. HUVECs were treated with vehicle, X/XO, HQ (A), or TBHP (B) for 2 h. Western blot was performed using an anti-eNOS antibody. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ** $P < 0.01$ versus vehicle-treated cells.

creased levels of NF-κB, a redox-sensitive transcription factor (Fig. 3D). These results indicate that LPC-induced ROS regulate eNOS levels in human endothelial cells.

*LPC-induced ROS affect intracellular antioxidant enzymes*

Because intracellular oxidative stress is modulated by antioxidant enzymes such as SOD and catalase, we examined whether LPC affected the expression of these enzymes. LPC (125 μM) decreased protein levels of SOD1, which neutralizes superoxide radicals, and increased catalase, which detoxifies hydroperoxide (Fig. 4A). To differentiate the effects of superoxide radicals and hydroperoxide, we used an X/XO mixture or HQ as superoxide radical donors [15, 16] and TBHP as a hydroperoxide donor. X/XO and HQ decreased levels of intracellular SOD1, but TBHP increased them. The superoxide donors increased catalase levels as did LPC stimulation, but TBHP did not affect catalase expression. These results indicate that LPC changes antioxidant enzymes via superoxide.

*LPC leads to superoxide-mediated eNOS downregulation*

As for LPC, the superoxide donors, X/XO and HQ, diminished eNOS levels (Fig. 2 & 5), whereas TBHP increased them, suggesting that superoxide is a major effector in LPC-induced eNOS downregulation. To test the role of SOD1 in LPC activity, we used TM, a SOD1 inhibitor [17]. Whereas TM alone did not affect eNOS levels, preincubation of TM (100 μM) prior to LPC (100 μM) treatment potentiated the downregulation of eNOS (Fig. 6A). In contrast, exogenous SOD could block LPC downregulation of eNOS (Fig. 6B). These treatments also changed LPC-induced NF-κB activation (Fig. 6C), consistent with their effects on eNOS. Thus, superoxide generated by LPC promotes the downregulation of eNOS via SOD1 inhibition or direct superoxide donation.

*LPC leads to inactivation of ERK1/2*

Although pERK1/2 can modulate eNOS [18, 19], it is not known whether ROS affects ERK activation in endothelial cells. Thus our study was focused on the re-

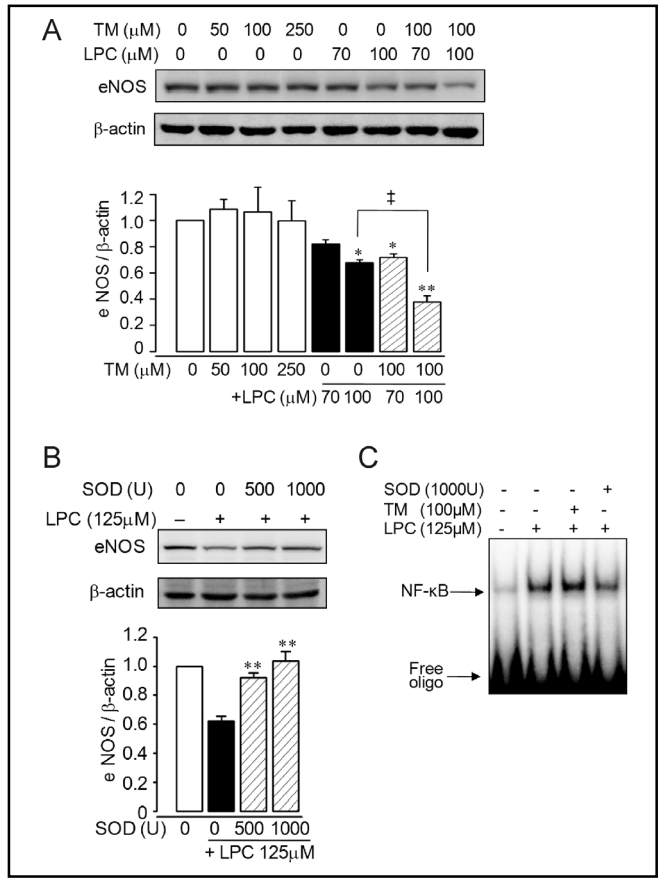


**Fig. 6.** The role of SOD1 on LPC-induced eNOS regulation. (A) HUVECs were treated with vehicle or LPC (70 or 100 μM) for 2 h with or without pretreatment of TM (100 μM) for 30 min. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ‡ indicates significant difference between LPC (100 μM) and TM (100 μM)-pretreated LPC (100 μM) at $P < 0.05$ by ANOVA. *, $P < 0.05$ or **, $P < 0.01$ versus vehicle-treated cells. (B) HUVECs were pretreated with SOD for 30 min and then stimulated with LPC (125 μM) for 2 h. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments. ** $P < 0.01$ versus LPC (125 μM)-stimulated cells. (C) HUVECs were treated with vehicle or LPC (125 μM) for 2 h with or without pretreatment of SOD (1000 U) or TM (100 μM) for 30 min. EMSA was performed in nuclear extracts incubated with $^{32}$P-end labeled oligonucleotide. Results are representative of three independent experiments.

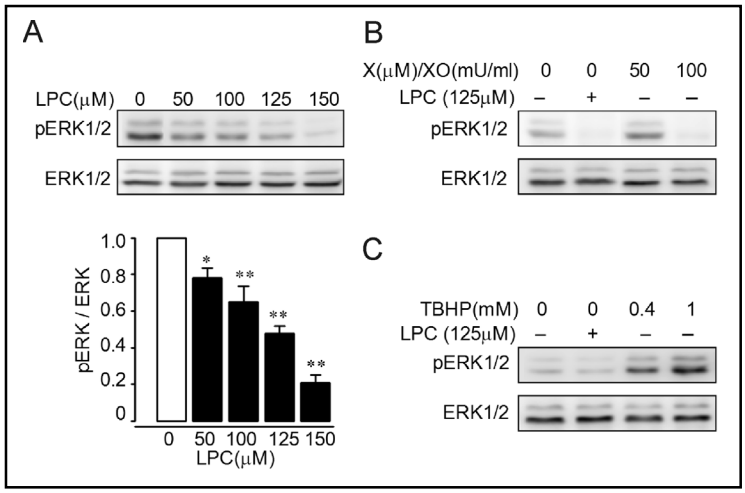


**Fig. 7.** Effects of LPC-induced superoxide on pERK1/2. HUVECs were stimulated with vehicle or LPC for 2 h. Western blot was performed using anti-pERK antibody. Results are representative of three independent experiments. The data shown are means ± S.E. of three independent experiments ** $P < 0.01$, * $P < 0.05$ versus vehicle-treated cells. (A) HUVECs were treated with X/XO (B), TBHP (C), or LPC 125μM (B, C) for 2 h. Western blot was performed using an anti-pERK antibody. Results are representative of three independent experiments.

lationship between LPC-induced ROS and ERK1/2 activation in HUVECs. LPC concentration-dependently inhibited ERK1/2 activation (Fig. 7A). To confirm whether LPC effect on pERK1/2 was caused by ROS, we tested the effect of X/XO or TBHP on pERK1/2. X/XO (100 mU/ml) inhibited ERK1/2 as did LPC (Fig. 7B), suggesting that LPC evoke superoxide-mediated ERK1/2 suppression. In contrast, TBHP activated ERK1/2 (Fig. 7C). These results indicate that LPC reduces ERK1/2-mediated eNOS expression by inducing superoxide.

## Discussion

We found that LPC downregulates eNOS via a superoxide-mediated mechanism in human endothelial cells. LPC stimulates superoxide production by suppressing SOD and/or upregulating catalase expression to suppress ERK1/2 phosphorylation and eNOS expression. This is an important finding that differs from previous reports that LPC promotes total ROS generation and decreases nitric oxide diffusion [20, 21].

Our findings provide novel evidence that eNOS is downregulated by LPC-induced ROS. LPC-induced eNOS downregulation was ameliorated by exogenous SOD (Fig. 6B) and superoxide donors had similar effects as LPC on eNOS (Fig. 5A), suggesting that LPC-induced eNOS downregulation was mediated by superoxide. LPC suppressed SOD1 and increased catalase expression (Fig. 4), indicating LPC increases superoxide but not hydroperoxide. Inducers of oxidative stress in atherosclerotic disease like oxLDL, tumor necrosis factor-α, and thrombin also promote eNOS downregulation [22-24].

External stress and physiological stimuli can signal through the ERK cascades, the best known MAP kinase cascade [18]. LPC or superoxide donors inhibited ERK1/2 phosphorylation and eNOS expression, whereas THBP stimulated them (Fig. 2 & Fig. 5). Thus, inhibition of ERK1/2 may suppress eNOS expression, as suggested previously [25].

The effect of LPC on eNOS in endothelial cells is controversial. LPC induces a rapid, transient rise in intracellular $Ca^{2+}$ levels [26], a pathway that can affect eNOS activity or expression [27]. eNOS expression and function can be upregulated by hydroperoxide-dependent CaM Kinase II [28]. Conversely, LPC inhibits phosphorylation of eNOS-Ser1177 and modulates $Ca^{2+}$ signals through Akt phosphorylation or a PKCδ-independent pathway [29]. Thus, eNOS may be differentially regulated by LPC in different cell types.

SOD1 is an endogenous antioxidant, but the relationship between ROS and SOD1 and its influences on target molecules is unclear. SOD1 is essential to hydroperoxide-mediated oxidation and can regulate growth factors [30, 31].

ERK can regulate the endothelial cell cycle, proliferation, growth, and apoptosis [32] and regulates the expression of substrates such as eNOS [33]. LPC induces superoxide and inactivates ERK1/2 (Fig. 7). Liu et al [34] also suggested that NADPH oxidase, a prominent contributor of endothelial superoxide [35], inactivates ERK and activates P-p38, which LPC treatment also activated (data not shown).

NF-κB is sensitive to oxidative stress [36]. We confirmed that NF-κB was activated by LPC and both types of ROS in an ERK-independent manner (Fig. 3D), with exogenous SOD blocking this activation and TM potentiating it (Fig. 6C). Because NF-κB induces overexpression of the proapoptotic protein Fas ligand (FasL) in plaques of patients with carotid atherosclerosis [37], NF-κB may be involved in LPC-induce apoptosis in HUVECs.

eNOS and ROS are important in endothelial function in cardiovascular disease. Here, LPC induced superoxide levels and decreased SOD and eNOS, producing oxidative stress and causing cytotoxicity. Physiological LPC levels (5 to 180 μM) [12, 13] could induce similar toxicity. Lower levels of LPC (20 μM) can inhibit $Ser^{1177}$-eNOS phosphorylation but not protein levels [29, 38], indicating an alternative mechanism for LPC regulation: lower LPC (≤ 20 μM) decreases phosphorylation but higher levels (≥ 100 μM) induce eNOS downregulation.

In conclusion, our study highlights a mechanism for LPC-induced eNOS downregulation. Inhibition of eNOS, SOD1, and pERK mediated by LPC-induced superoxide suggests that superoxide can influence eNOS regulation. Pharmacological modulation of eNOS expression in the vascular endothelium may be key for the treatment of atherosclerosis and endothelial damage.

## Acknowledgements

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