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Alpha-Lipoic Acid Activates eNOS through Activation of PI_3-Kinase/Akt Signaling Pathway

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Abstract

Background—Lipoic acid (LA) exerts therapeutic effects on cardiovascular diseases. However, the mechanisms underlying these therapeutic effects remain elusive. Endothelial nitric oxide synthase (eNOS) plays a critical role in cardiovascular homeostasis. LA was shown to potently activate PI3-kinase/Akt pathway, and the latter is critical in the regulation of eNOS activity. In the present study, we test the hypothesis that LA improves endothelial function through PI_3-kinase/Akt-mediated eNOS activation.

Methods and Results—Western blot analysis showed that LA time- and dose-dependently induced phosphorylation of Akt and eNOS in human umbilical vein endothelial cells (HUVECs). Both PI_3-kinase and Akt inhibitors abolished LA-induced eNOS phosphorylation, indicating that LA induces eNOS phosphorylation through the PI_3-kinase/Akt pathway. This increase in eNOS phosphorylation was paralleled by an increase in NO release by HUVECs, supporting its relevance in eNOS activity regulation. Myograph analysis revealed that LA relaxed phenylephrine-induced contraction. Endothelium removal and NOS inhibition by L-NAME abolished this vasodilator action of LA, and Akt but not AMPK inhibition significantly reduced the vasodilator action of LA, indicating that it is mediated by PI_3-kinase/Akt pathway-dependent activation of eNOS. Consistent with *in vitro* results, intraperitoneal injection with LA significantly increased plasma nitrite and nitrate levels in C57Bl/6j mice.

Conclusions—LA activates eNOS through a PI3-kinase/Akt signaling pathway-dependent mechanism, offering a potential molecular basis for the therapeutic effects of LA on cardiovascular diseases.

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Keywords

Lipoic acid; eNOS; Akt; phosphorylation; endothelium

1. Introduction

α-Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring disulfide compound commonly found in a normal diet. LA is a necessary cofactor for the mitochondrial enzymes α-ketoglutarate and pyruvate dehydrogenase in mammals [1]. A number of studies have demonstrated that LA can exert therapeutic effects on cardiovascular diseases, such as diabetes mellitus [2], hypertension [3, 4] and atherosclerosis [5, 6]. However, the molecular and cellular mechanisms underlying these therapeutic effects are poorly understood.

LA is a potent biological antioxidant. In addition to directly scavenging for hydroxyl radicals, hypochlorous acid, and singlet oxygen [7], it also reduces oxidative stress through metal chelation, electrophilic mechanisms, and induction of heme-oxygenase-1 (HO-1) expression via the nuclear factor E2-related factor 2 (Nrf2) pathway [8]. Since there is compelling evidence that oxidative stress is crucial in the pathogenesis of cardiovascular diseases, it is believed that LA exerts its therapeutic effects at least partly via its antioxidant action [7, 9, 10]. However, this is somewhat challenged by the fact that other antioxidants do not have all these effects [8]. Furthermore, some effects of LA can be achieved with low micromolar levels of LA, suggesting that at least some of its therapeutic potential extends beyond the role of an antioxidant [8]. Notably, because administration of LA in a cell culture model increased GSH only after 24 hours [11], it is even postulated that LA may activate the Nrf2 pathway as a pro-oxidant and subsequently reduce oxidative stress [8].

The controlled regulation of nitric oxide (NO) synthesis by endothelial NO synthase (eNOS) in endothelial cells is essential for cardiovascular health [12]. Notably, several studies have demonstrated that LA increases eNOS activity [13–15], providing a potential mechanism that can explain its therapeutic effects on cardiovascular diseases. However, how LA activates eNOS remains unknown. A complex system has been identified to temporally and spatially regulate eNOS activity and subsequent NO production [12, 16, 17]. In addition to Ca^{2+}-dependent mechanisms, phosphorylation/de-phosphorylation plays an important role in regulating eNOS activity [12, 16, 17]. Upon stimulation, Akt/protein kinase B (PKB) phosphorylates eNOS at Ser-1177(1179) and possibly Ser-615(617), and consequently activates eNOS [18]. Recently, it has been shown that LA inhibits lipopolysaccharide (LPS)-induced NF-κB activation in THP-1 cells through activation of the PI_3 kinase/Akt signaling pathway [19]. Therefore, in the present study, we test the hypothesis that LA ultimately activates eNOS by initially activating the PI3 kinase/Akt pathway.

2. Materials and Methods

2.1. Animals

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.

85–23, revised 1996), and approved by the Institutional Animal Care and Use Committees at the Ohio State University. C57Bl/6j (male, 8-week-old) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA).

2.2. Materials

LA (T5625), LY-294002 (L9908), and Dorsomorphin (P5499) were bought from Sigma (St. Louis, MO, USA). MK-2206 was obtained from Selleckchem (Houston, TX, USA). Dominant negative Akt over-expression plasmid was bought from Addgene (Cambridge, MA, USA).

2.2. Myograph Experiments

Briefly, C57Bl/6j mice were anesthetized with pentobarbital sodium, and the thoracic aorta was quickly removed and cleaned in physiological salt solution (PSS) containing (mM): NaCl, 130; $NaHCO_3$, 14.9; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$ 1.18; $CaCl_2 \cdot 2H_2O$, 1.56, EDTA, 0.026, glucose 5.5. The aorta was cut into 2-mm rings, and in some experiments, the endothelium was mechanically removed by gently rubbing the intimal surface with a stainless steel wire. The aortic rings were then mounted in a muscle bath containing PSS at 37°C and bubbled with 95% O_2-5% CO_2. Isometric force generation was recorded with a Multi Myograph System (Danish Myo Technology A/S, Aarhus N, Denmark). A resting tension of 4 mN was imposed on each ring, and the rings were allowed to equilibrate for 1 hour.

Arterial integrity was assessed first by stimulation of vessels with 80 mM KCl. Endothelium-integrity was assessed by measuring the dilatory response to ACh (10 μM) in PE-contracted vessels (1 μM). The failure of ACh to relax denuded aortic rings was considered as proof of endothelium disruption. To test the effect of LA on vascular tone, aortic rings were pre-contracted by PE (0.3 μM) that induced approximately 60% of maximal contraction. Vehicle or LA was then added in an accumulative manner. LA responses were also evaluated after a 10-minute incubation with vehicle or with the NO synthase inhibitor ω-nitro-L-arginine methyl ester (L-NAME 100 μM) and/or indomethacin (10 μM), an inhibitor of prostanoid synthesis.

2.3. Cell treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA, USA), and maintained in endothelial cell basic medium supplemented with 10% FBS (Promocell, Heidelberg, Germany). Before treatment with LA, cells were grown to 90% confluence in 60mm dishes, and starved in endothelial cell basic medium supplemented with 0.5% FBS overnight. After treatment with LA or vehicle, cells were immediately placed on ice, and washed twice with ice-cold PBS. All subsequent manipulations were then performed on ice. Cells were lysed with 100 μl of ice-cold RIPA buffer (Upstate, Jaffrey, NH, USA): 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM EGTA, 1 mM Na3VO4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin). Cells were scraped with a rubber policeman, rocked for 30 minutes at 4°C, and centrifuged at 14,000 g for 30 minutes at 4°C.

The supernatants were transferred to new tubes. Protein concentration in each sample was determined with BCA protein assay reagents (Pierce, Rockford, IL, USA).

2.4. Nitrite/Nitrate assay

The HUVECs were grown on 24-well plates (5×10^5 cells/well) and stimulated with LA. Following stimulation, the supernatants were harvested to measure NO release with a Total NO/Nitrite/Nitrate Assay (R&D System, Minneapolis, MN, USA). C57bl/6 mice were treated with LA and plasma samples were collected, filtered, and diluted 5-fold prior to a Total NO/Nitrite/Nitrate Assay (R&D System, Minneapolis, MN, USA). The measurement of the concentration of endogenous nitrite was performed according to the protocols provided by the manufacturer. The results were obtained from the optical density of each sample using a Model 680 Microplate Reader (BIO-RAD, Hercules, CA, USA) set at 540 nm. Nitrite and nitrate were measured with Parameter™ Total NO/Nitrite/Nitrate Assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Western blot analysis

20 μg/sample proteins were resolved with 8% SDS-PAGE and transferred to membrane. Immuno-staining was performed by standard techniques with primary antibodies as follows: anti-GAPDH, anti-AMPKα, anti-phospho-AMPKα, anti-Akt, and anti-phospho-Akt from Cell Signaling Technology (Boston, MA, USA); anti-eNOS and anti-phospho-eNOS (s1177) from Millipore (Billerica, MA, USA). Signals were detected by supersignal™ chemiluminescence (Pierce, Rockford, IL, USA) and analyzed by densitometry.

2.6. Statistics

All data are expressed as mean ± SEM. Statistical comparisons of dose-response curves were performed with two-way repeated-measures analysis of variance (ANOVA) using GraphPad Prism version 4.0b (Graphpad Software Inc., La Jolla, CA, USA)]. Otherwise, statistical comparisons were performed with one-way ANOVA or student's t-test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. LA induces eNOS and Akt phosphorylations in HUVECs

Figures 1A and B show that LA time-dependently increased Akt and eNOS phosphorylations in HUVECs. These phosphorylations were increased as early as 5 minutes after treatment with LA, and peaked at 15 minutes. Figures 1C and D depict LA concentration-dependently increased Akt and eNOS phosphorylations. The half maximal effect concentrations (EC50s) were 14.8 (95% confident interval: 3.4–64.1) and 16.1 (95% confident interval: 4.1–64.2) μM, respectively.

3.2. LA-induced eNOS phosphorylation is mediated by PI_3-kinase/Akt pathway

Since it has been well established that PI_3-kinase/Akt pathway regulates eNOS activity through phosphorylation at the pro-active site (Ser1177), a PI_3-kinase inhibitor LY-294002 was used to test whether LA-induced eNOS phosphorylation is mediated by PI_3-kinase/Akt pathway. Consistent with a previous report [19], Figures 2A and B show that LY-294002

blocked LA-induced Akt phosphorylation at Ser473, and simultaneously abolished LA-induced eNOS phosphorylation at Ser1177, supporting that PI_3-kinase/Akt pathway mediates LA-induced eNOS phosphorylation. MK-2206 is a highly selective inhibitor of Akt. No inhibitory activities against 250 other protein kinases was observed [20]. To confirm that LA-induced eNOS phosphorylation is mediated by Akt, HUVECs were treated with LA in the presence of MK-2206. Figures 2C an D show that treatment with MK-2206 abolished LA-induced phosphorylation of Akt and eNOS. Furthermore, over-expression of dominant negative Akt [Akt(DN)] significantly reduced LA-induced eNOS phosphorylation (Figures 2E and F), whereas an AMPK inhibitor, dorsomorphin, reduced LA-induced phosphorylation of AMPKα but not eNOS (Figures 2G and H), strongly supporting that LA-induced eNOS phosphorylation at Ser1177 is mediated by Akt.

3.3. LA induces NO release in HUVECs

eNOS exerts its biological function through production of NO. To assess the effect of LA on eNOS function, we next examined if LA increases NO release in HUVECs through measurement of nitrate and nitrite, two metabolites of NO. Figure 3A–D show that LA time- and dose-dependently increased nitrite and nitrate in the supernatants. The calculated EC50s were comparable to those of Akt and eNOS phosphorylations (95% confident interval: 2.6–59.1 and 4.7–26.1 μM, nitrite and nitrate respectively). Consistent with its effect on eNOS phosphorylation (Figure 2A and B), LY-294002 treatment abolished LA-induced increases in nitrite and nitrate (Figure 3E and F), indicating that LA activates eNOS through the PI_3-kinase/Akt signaling pathway.

3.4. LA relaxes vasoconstriction through eNOS-dependent mechanism

NO generated by eNOS is critical in regulation of vascular tone, and the latter is implicated in the pathogenesis of cardiovascular diseases. To assess if the effect of LA on eNOS is pharmacologically relevant, we analyzed the effect of LA on vascular tone. Figure 4A and B reveal that LA relaxed phenylephrine-induced contraction of mouse aortic segments. The EC50 (95% confident interval: 5.1 – 39.5 μM) was comparable to that of its effect on eNOS phosphorylation, suggesting that LA may regulate vascular tone through activation of eNOS. To verify if LA relaxes vasoconstriction via activation of eNOS, we mechanically removed endothelium from mouse aortic segments. Figure 4C shows that removal of endothelium completely abolished the effect of LA on vasoconstriction. NO (a product of NOS), PGI_2 (a product of COX), and endothelium-derived hyperpolarization factor (EDHF, produced in a COX-dependent manner) are three main endothelium-derived vasodilators. Figure 4D demonstrates that L-NAME (a NOS inhibitor) but not indomethacin (a COX inhibitor) abolished the vasodilator action of LA, strongly suggesting that the vasodilator effect of LA is NOS-dependent.

3.5. The vasodilator action of LA is mediated by Akt

Given that LA activated PI_3-kinase/Akt and subsequently induced eNOS phosphorylation at a pro-active site in HUVECs (Figure 1), MK-2206 was used to determine whether LA relaxes vasoconstriction through PI_3-kinase/Akt pathway. Figure 5 shows that pre-treatment with MK-2206 almost abolished the vasodilator action of LA. In contrast, inhibition of

AMPK did not significantly affect the vasodilator action of LA, consistent with studies on cultured HUVECs.

3.6. LA induces NO release in living mice

To confirm the pharmacological relevance of eNOS activation by LA, we assessed its effect on NO production in living animals. Figure 6A and B reveal that intraperitoneal injection with LA rapidly increased nitrite and nitrate in plasma. A dose-dependent increase in plasma nitrite and nitrate was also observed (Figure 6C and D), indicating that LA also activates eNOS in living animals.

4. Discussion

LA exerts therapeutic effects on cardiovascular diseases. However, the mechanism underlying these therapeutic effects remains elusive. In the present study, we demonstrate that: 1) LA induced eNOS phosphorylation in a PI_3-kinase/Akt-dependent manner and subsequently increased eNOS activity in HUVECs; 2) the activation of eNOS by LA resulted in relaxation of phenylephrine-constricted mouse aortic segments; 3) administration of LA increased NO production in living animals. Given the well-established protective role of eNOS in the pathophysiology of cardiovascular diseases, our results thus provide a molecular mechanism for the therapeutic effects of LA on cardiovascular diseases.

Endothelial dysfunction characterized by a decrease in endothelium-mediated vasodilation plays an important role in the pathogenesis of various cardiovascular diseases that LA exerts therapeutic effects on, such as hypertension, diabetes, and atherosclerosis [2–6, 20]. Studies have shown that LA may modulate endothelial function through multiple mechanisms. For example, LA inhibits oxidized low density lipoprotein-induced apoptosis in cultured HUVECs [21]. Dietary supplementation with LA significantly improves endothelium-dependent vasodilation in diabetic patients [22] and animal models [15, 23]. In the present study, our data demonstrate that LA directly induces vasodilation. To our knowledge, this is the first study showing that LA is a vasodilator. It is somehow consistent with previous studies showing that LA significantly increases NO synthesis in human aortic endothelial cells [24]. In addition to NO, endothelial-derived hyperpolarizing factors (EDHF) and prostaglandin also mediate endothelium-dependent vasodilation in some contexts [25].

A number of studies have shown that eNOS is constitutively expressed in endothelium and is the primary source of NO at physiological conditions [12, 16, 17]. Its dysfunction is a critical component of pathogenesis of cardiovascular diseases [26]. Post-translational modifications such as phosphorylation/de-phosphorylation play a key role in the regulation of eNOS activity [12, 16, 17]. In the present study, our data show that LA time- and dose-dependently increased eNOS phosphorylation at Ser1177, the most extensively studied pro-active phosphorylation site of eNOS [18, 27]. Consistently, LA increases NO release by HUVECs (Figure 2) and nitrite/nitrate production in living animals (Figure 4). Our results are consistent with several studies showing that LA increases eNOS activity [13–15], suggesting that this action of LA may be pharmacologically important. In addition to phosphorylation/de-phosphorylation, other post-translational modifications such as protein-protein interaction and the bioavailability of coenzyme tetrahydrobiopterin (BH4) also play

a role in regulation of eNOS activity [12, 16, 17]. However, given our data that inhibition of PI_3-kinase almost completely abolished LA-induced NO production (Figure 2E and F), those post-translational modifications may only play a trivial role in LA-induced eNOS activation.

Consistent with its critical role in diverse responses to stimuli that promote eNOS [18, 27], eNOS Ser1177 can be phosphorylated by several kinases, including Akt, protein kinase a (PKA), AMP-activated protein kinase (AMPK), protein kinase g (PKG), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) [28]. Our results show that LA time- and dose-dependently increases Akt activation in HUVECs, suggesting that Akt may mediate LA-induced eNOS phosphorylation. This is consistent with previous studies showing that LA activates Akt in monocytes and consequently inhibits inflammatory responses [19]. The almost complete inhibition of LA-induced eNOS phosphorylation by PI_3-kinase inhibitor (Figure 1E and F) strongly suggests that this phosphorylation is primarily mediated by the PI_3-kinase/Akt signaling pathway. Together with the result showing that inhibition of PI_3-kinase abolished LA-induced NO production (Figure 2E and F), our data indicate that LA activates eNOS through the PI_3-kinase/Akt signaling pathway.

In the present study, our data demonstrate that LA induces eNOS-mediated vasorelaxation through a PI_3-kinase/Akt pathway-dependent mechanism. In addition to our results, several other studies have shown that LA increases eNOS activity in both cultured cells and living animals [13–15, 22, 29, 30], suggesting that LA may exert its therapeutic effects on those cardiovascular diseases through targeting eNOS. Although in the majority of those studies it is postulated that eNOS activation by LA is subsequent to its antioxidant action, direct evidence of this is lacking. Notably, while more than 100 μM of LA is usually required for its antioxidant action [11, 29], our results show that a low micromolar concentration of LA is sufficient to activate eNOS. Since previous studies showed that at least some of therapeutic effects of LA can be achieved with low micromolar levels [8], these results suggest that activation of eNOS by LA may also be independent of its antioxidant action.

Acknowledgments

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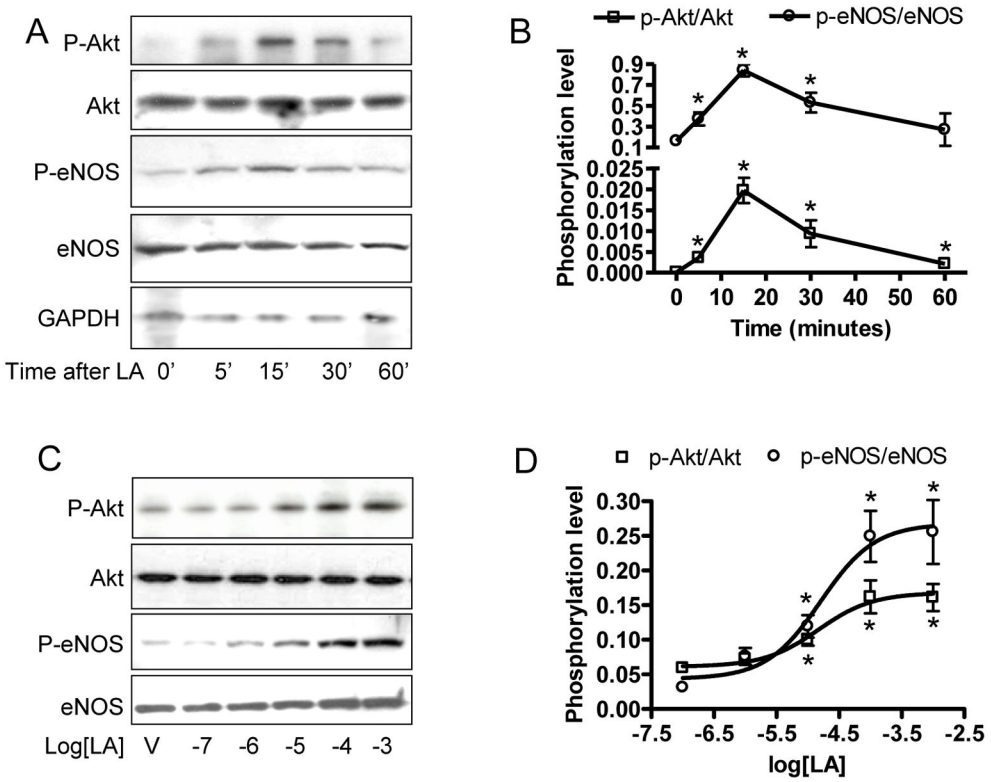

Figure 1.

LA induces Akt and eNOS phosphorylations. A and B, HUVECs were treated with LA (1 mM) for the indicated time, and the phosphorylation level of Akt and eNOS was analyzed by western blot. A representative picture (A) and the summary (B) of four independent experiments are presented.. *$p<0.05$ vs. 0; student's t-test with bonferroni correction. C and D, HUVECs were treated with the indicated concentration of LA for 15 minutes. The phosphorylation level of Akt and eNOS was analyzed by western blot. A representative picture (C) and the summary (D) of four independent experiments are presented. V, Vehicle. *$p<0.05$ vs. Vehicle; student's t-test with bonferroni correction.

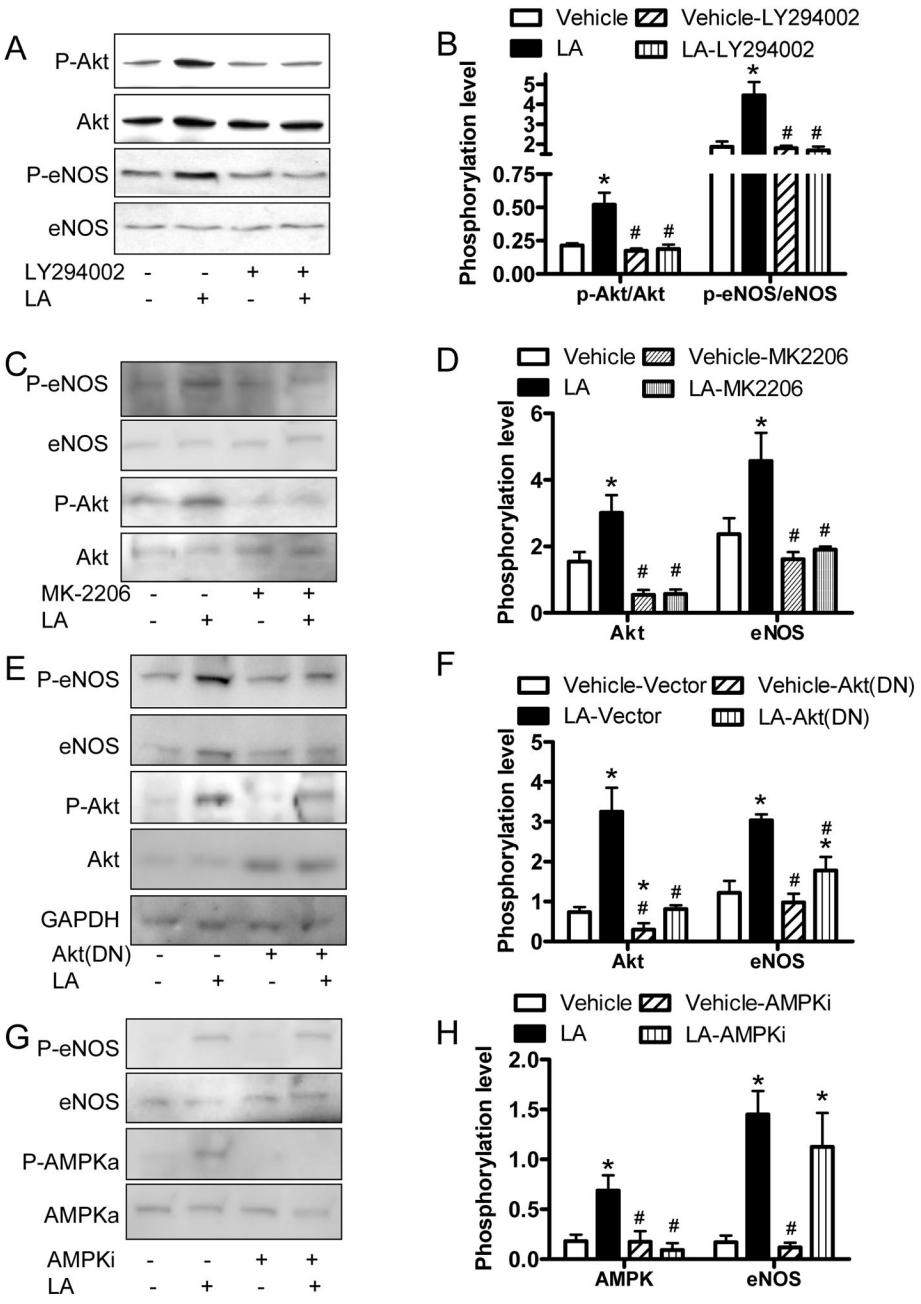

Figure 2.

LA-induced eNOS phosphorylation is mediated by PI_3-kinase/Akt pathway. A and B, HUVECs were pre-treated with vehicle or LY-294002 (20 μM) for 15 minutes and then treated with LA (1 mM) for 15 minutes. The phosphorylation level of Akt and eNOS was analyzed by western blotting. A representative picture (A) and the summary (B) of three independent experiments are presented. *p<0.05 vs. Vehicle; #p<0.05 vs. LA; student's t-test with bonferroni correction. C and D, HUVECs were pre-treated with vehicle or MK-2206 (10 μM) for 15 minutes and then treated with LA (1 mM) for 15 minutes. The phosphorylation level of Akt and eNOS was analyzed by western blotting. A representative

picture (C) and the summary (D) of three independent experiments are presented. *$p<0.05$ vs. Vehicle; $^{\#}p<0.05$ vs. LA; one way ANOVA. E and F, HUVECs were subjected to transfection of vector or dominant negative Akt (Addgene, Cambridge, MA, USA) with Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). After 48 hours, cells were treated with LA (1 mM) for 15 minutes. The phosphorylation level of Akt and eNOS was analyzed by western blotting. A representative picture (E) and the summary (F) of three independent experiments are presented. *$p<0.05$ vs. Vehicle; $^{\#}p<0.05$ vs. LA; one way ANOVA. G and H, HUVECs were pre-treated with vehicle or dorsomorphin (50 μM) for 15 minutes and then treated with LA (1 mM) for 15 minutes. The phosphorylation level of AMPK and eNOS was analyzed by western blotting. A representative picture (G) and the summary (H) of three independent experiments are presented. *$p<0.05$ vs. Vehicle; $^{\#}p<0.05$ vs. LA; one way ANOVA.

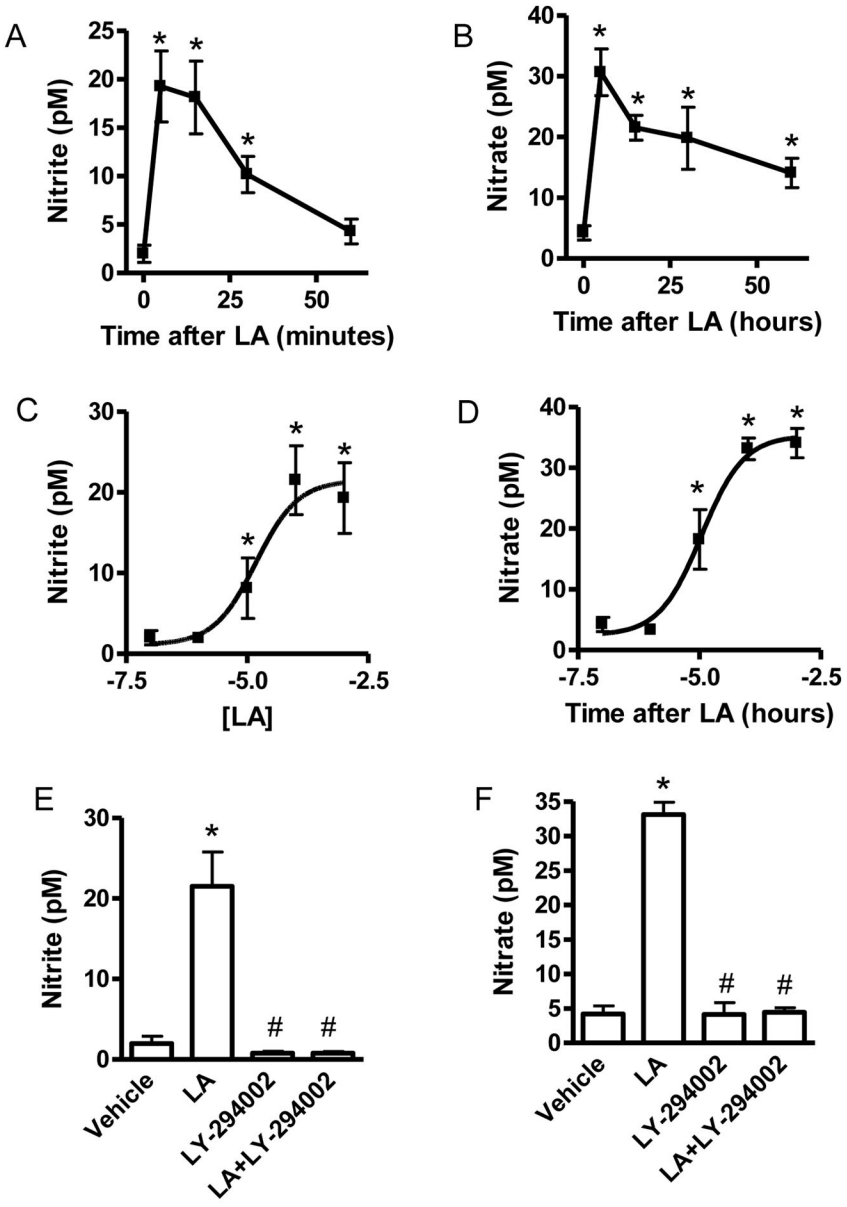

Figure 3.

LA induces NO release from HUVECs. A and B, HUVECs were treated with LA (1 mM) for the indicated time. Nitrite (A) and nitrate (B) in the medium was analyzed. n = 5. *p<0.05 vs. 0; student's t-test with bonferroni correction. C and D, HUVECs were treated with the indicated concentration of LA for 15 minutes. Nitrite (C) and nitrate (D) in the medium was analyzed. n = 5. *p<0.05 vs. Vehicle; student's t-test with bonferroni correction. E and F, HUVECs were pre-treated with LY-294002 (20 μM) for 15 minutes and then treated with LA (1 mM) for 15 minutes. Nitrite (A) and nitrate (B) in the medium was analyzed. n = 4. *p<0.05 vs. Vehicle; #p<0.05 vs. LA; student's t-test with bonferroni correction.

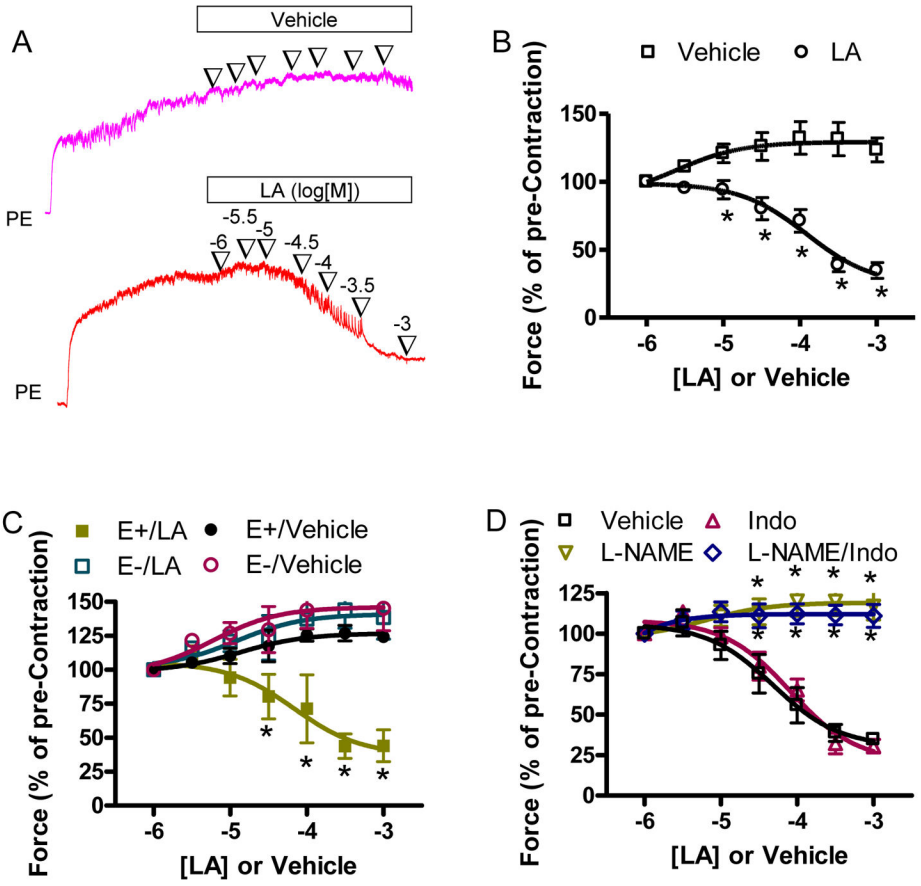

Figure 4.

LA relaxes vasoconstriction through eNOS-dependent mechanism. Aortic segments were pre-contracted with PE (0.3 μM), and then the responses to indicated concentration of LA were recorded. A, a representative response to LA or Vehicle. B, the quantitation of responses to LA or Vehicle. 2 segments/mouse. n = 9. *p<0.05 vs. Vehicle; two way ANOVA. C, Endothelium of mouse aortic segments were denuded and mounted on myography. The responses to LA or Vehicle were recorded and quantified. E−, endothelium denuded; E+, endothelium intact. 2 segments/mouse. n = 5. *p<0.05 vs. E+/Vehicle; two way ANOVA. D, in the presence of the NO synthase inhibitor (L-NAME, 100 μM) and/or an inhibitor of prostanoid synthesis (indomethacin, 10 μM), the response of mouse aortic segments to LA or vehicle were analyzed. 2 segments/mouse. n = 4. *p<0.05 vs. Vehicle; two way ANOVA.

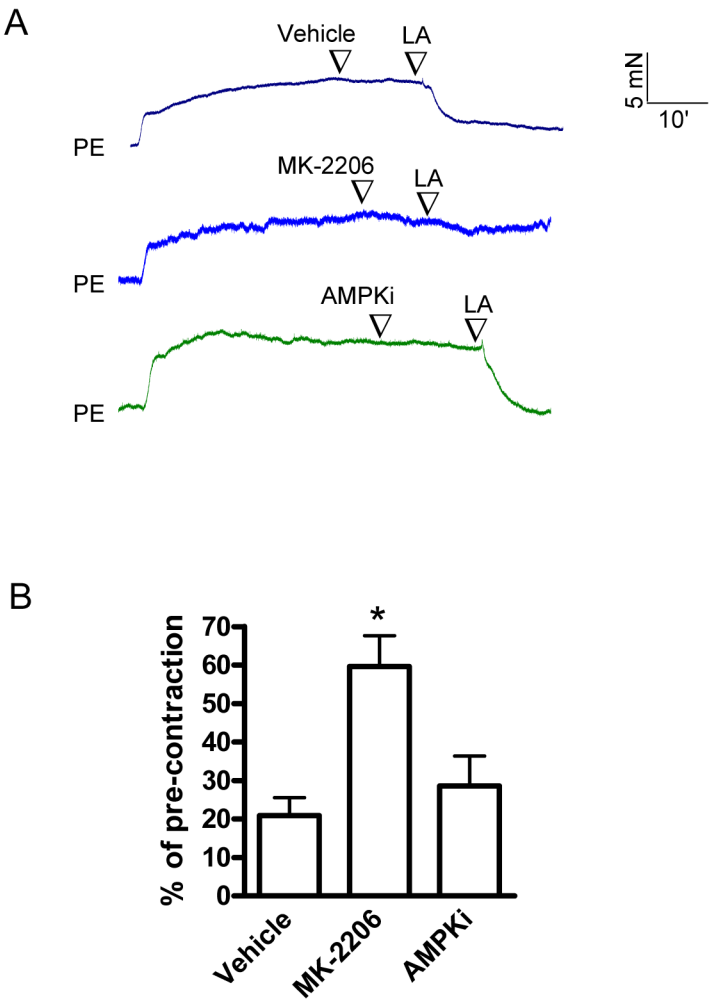

Figure 5.

The vasodilator action of is mediated by Akt. Thoracic aortas were isolated from C57Bl/6j mice, and mounted onto myograph. After pre-contraction with PE (0.3 μM), MK-2206 (10 μM) or dorsomorphin (50 μM) were added, followed by LA (1mM). A, the representative response. B, the quantitation of responses. 2 segments/mouse. n = 4. *p<0.05 vs. Vehicle; one way ANOVA.

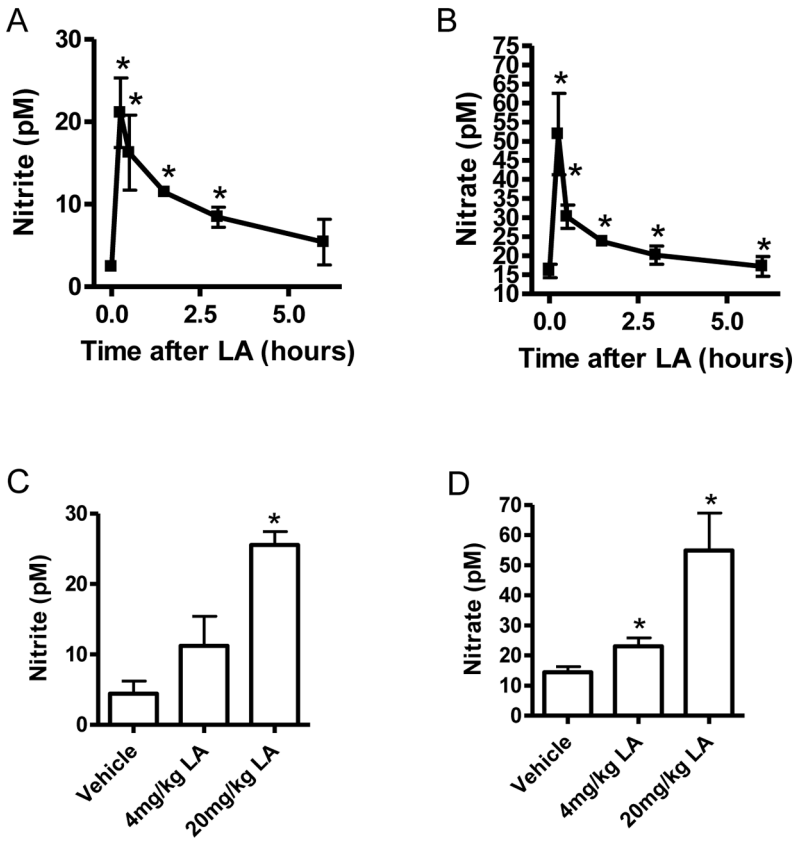

Figure 6.

LA induces NO release in C57bl/6 mice. A and B, after the indicated time of ip injection with LA (20 mg/kg), mice were humanly killed, and plasma were collected. Nitrite (A) and nitrate (B) were then analyzed. n = 5/time point. *p<0.05 vs. 0; student's t-test with bonferroni correction. C and D, after 30 minutes of ip injection with the indicated dose of LA, mice were humanly killed, and plasma were collected. Nitrite (C) and nitrate (D) were then analyzed. n = 4/time point. *p<0.05 vs. Vehicle; student's t-test with bonferroni correction.