RUMEX ACETOsa L. INDUCES VASORELAXATION IN RAT AORTA VIA ACTIVATION OF PI3-KINASE/Akt- AND Ca
2+eNOS-NO SIGNALING IN ENDOTHELIAL CELLS

INTRODUCTION

The endothelium plays an important role in the control of vascular tone (1). Several endothelium-dependent vasodilators, such as bradykinin, acetylcholine and histamine, have been reported to elevate Ca\(^{2+}\) level in endothelial cells, and activate the synthesis and release of endothelium-derived relaxing factors (EDRFs), including nitric oxide (NO) and prostacyclin (PGL\(_2\)) (2), which lead to vasorelaxation (3). In blood vessels, endothelial nitric oxide synthase (eNOS) converts L-arginine to guanosine 3',5'-monophosphate, human umbilical vein endothelial cells

Rumex acetosa L. (RA) (Polygonaceae) is an important traditional Chinese medicine (TCM) commonly used in clinic for a long history in China and the aerial parts of RA has a wide variety of pharmacological actions such as diuretic, anti-hypertensive, anti-oxidative, and anti-cancer effects. However, the mechanisms involved are to be defined. The purpose of the present study was to evaluate the vasorelaxant effect and define the mechanism of action of the ethanol extract of Rumex acetosa L. (ERA) in rat aorta. ERA was examined for its vascular relaxant effect in isolated phenylephrine-precontracted rat thoracic aorta and its acute effects on arterial blood pressure. In addition, the roles of the nitric oxide synthase (NOS)-nitric oxide (NO) signaling in the ERA-induced effects were tested in human umbilical vein endothelial cells (HUVECs). The phosphorylation levels of Akt and eNOS were assessed by Western blot analysis in the cultured HUVECs. ERA induced endothelium-dependent vasorelaxation. The ERA-induced vasorelaxation was abolished by L-NAME (an NOS inhibitor) or ODQ (a sGC inhibitor), but not by indomethacin. Inhibition of PI3-kinase/Akt signaling pathway markedly reduced the ERA-induced vasorelaxation. In HUVECs, ERA increased NO formation in a dose-dependent manner, which was inhibited by L-NAME and by removing extracellular Ca\(^{2+}\). In addition, ERA promoted phosphorylation of Akt and eNOS, which was prevented by wortmannin and LY294002, indicating that ERA induces eNOS phosphorylation through the PI3-kinase/Akt pathway. Further, in anesthetized rats, intravenously administered ERA decreased arterial blood pressure in a dose-dependent manner through an activation of the NOS-NO system. In summary, the ERA-induced vasorelaxation was dependent on endothelial integrity and NO production, and was mediated by activation of both the endothelial PI3-kinase/Akt- and Ca\(^{2+}\)-eNOS-NO signaling and muscular NO-sGC-cGMP signaling.

Key words: Rumex acetosa L, aorta, PI3-kinase/Akt, endothelial nitric oxide synthase, vasorelaxation, prostaglandin, cyclic guanosine 3',5'-monophosphate, human umbilical vein endothelial cells
extract of RA (ERA) on rat aorta. The possible mechanisms of vasorelaxation were elucidated using isolated rat thoracic aortic rings and cultured endothelial cells.

**MATERIALS AND METHODS**

**Preparation of extract of Rumex acetosa L.**

The whole plant of RA was collected from the fields of the Taian, Shandong, China, in July 2012 and were identified by Professor Tongde Li, Department of traditional Chinese medicine, Taishan Medical University. Herbarium voucher specimens of RA (CH 10) were prepared and deposited in the herbarium of the Institute of Materia Medica, Taishan Medical University, Taian, Shandong, China. The powdered RA (200 g) was extracted with aqueous ethanol (70%) successively at 80°C for 3 hours, and then freeze-dried to yield the ethanol extract of RA (ERA, 30.2 g).

**Animals**

Adult male Sprague-Dawley rats (weighing 250 – 300 g) were kept under conditions of constant temperature (22 ± 2°C) with a 12 h light/12 h dark cycle and free access to food and water. All animals were cared for in compliance 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). The protocol and procedures described below were approved by the Animal Care and Use Committee of the Institute of Atherosclerosis, Taishan Medical University.

**Preparation of aortic rings**

Rats were killed by cervical dislocation and exsanguinated. For isometric tension recording, the thoracic aorta was carefully removed and placed in ice-cold Krebs solution (pH 7.4) composed of (mM) NaCl 118, KCl 4.7, MgSO$_4$ 1.1, KH$_2$PO$_4$ 1.2, CaCl$_2$ 1.5, NaHCO$_3$ 25, and glucose 10. The aorta was removed free of connective tissue and fat, and then sliced into ring segments 2 – 3 mm in length, and care was taken to avoid any damage to the endothelium. In some aortic rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the aortic ring back and forth several times with plastic tubing. Endothelial integrity or functional removal was verified by the presence or absence of the relaxant response to acetylcholine (ACH, 1 µM) on the phenylephrine (PE, 1 µM) contracted vessels.

**Record of isometric vascular tone**

The aortic rings with and without endothelium were mounted in a 10-ml organ bath containing Krebs solution by means of two stainless steel wire holders (hooks) inserted through the lumen of the ring. One of two wire holders was connected to the force-displacement transducer and the other was fixed to the bottom of the organ bath. Krebs solution was kept at 37°C, while being continuously bubbled with 95% O$_2$ – 5% CO$_2$. The baseline load placed on the aortic rings was 1.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (JH-2, Institute of Space Medical-Engineering, Beijing, China) connected to a Biological Laboratory System (Model BL-420S, Chengdu TME Technology Co, Ltd, Chengdu, China) and stored in a computer. The aortic rings were allowed to equilibrate for 90 min while changing the chamber solution at every 15-min intervals. After resting tension of each vascular specimen was stabilized, the effects of ERA on vascular tension were defined. Aortic rings were contracted with PE (1 µM) to obtain maximal response (Fig. 1A and 1B). Once the maximal response to PE had been obtained, increasing cumulative concentrations of ERA (0.1 to 100 µg/ml) were added to the bath and the responses were recorded. The interval time between additions of consecutive concentration of ERA in the organ bath was 3 min. The responses were stopped by washing the aortic rings with fresh Krebs solution. To define the mechanisms by which ERA relaxes vascular smooth muscle, another series of experiments were done on rings. The rings were exposed to various modulating agents for 20 min prior to exposure to PE, and then vascular relaxation was carried out by cumulative addition of ERA (0.1 to 100 µg/ml) to the tissue bath after PE response reached the plateau. The effect of vehicle (0.1% dimethylsulfoxide, DMSO) was also tested. Aortic rings were exposed to ERA only one time during the experiments. In the case of testing the integrity of the endothelium by using ACh, after each test, the aortic rings were washed three times with fresh Krebs solution and allowed for 30 min to equilibrate. Relaxations are expressed as the percentage of relaxation of PE-induced tone.

**Blood pressure measurement**

The changes in arterial blood pressure were measured in anesthetized rats. Male Sprague-Dawley rats (250 – 300 g) were anesthetized with urethane (1.2 g/kg, intraperitoneally). Left carotid artery and vein were catheterized for the arterial blood pressure monitoring, and injections of ERA and modulators, respectively, Trachea was catheterized to make easy respiration. Arterial blood pressure was monitored using Biological Laboratory System (Model BL-420S, Chengdu TME Technology) via pressure transducer (model PT-100, Chengdu).

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were obtained from the KeyGEN Biotech (Nanjing, China). HUVECs were cultured in DMEM medium containing 20% fetal bovine serum (Biowest, Rue de la Caille, France), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere (37°C, 5% CO$_2$).

**Determination of nitric oxide production**

HUVECs (5 x 10$^5$ cells/well) in 6-well plates were incubated with various concentrations of ERA (0 – 100 µg/ml) for 1 hour. The supernatants of conditioned cells were deproteinized by zinc-sulfate (30%, v/v) and passed through a copper cadmium reduction column to reduce NO$_2^-$ to NO$_3^-$ (18). One hundred µl of each supernatant was mixed with 100 µl of the Griess reagent (1% sulfanilamide and 0.1% N-1-naphthyl ethylenediamine) for 10 min, and then the chromophoric azo-derivative molecule’s absorbance was measured in a microplate reader at 540 nm. Fresh culture medium was used as the blank in all experiments. The NO production was expressed as µM.

**Western blotting assay**

After washing twice with cold PBS, HUVECs were lysed with ice-cold lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Na-DOC, 0.2 mmol/L PMSF, and protease inhibitor cocktails) for 30 min on ice, lysates were centrifuged at 12,000 x g for 20 min and the supernatants were used as total cell lysates. Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). A quantity
of 50 µg total proteins per lane was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% milk powder in 0.05% Tween-TBS, incubated with the specific antibodies such as mouse anti-β-actin (1:1000), rabbit anti-Akt (1:1000), anti-p-Akt (Ser473,1:1000), anti-eNOS (1:400), anti-p-eNOS (Ser1177,1:1000). Detection of the target proteins on the membranes was performed using the ECL Western blotting detection reagents.

Cell viability by MTT assay

The MTT assay is a colorimetric assay for assessing cell viability. Briefly, cells (5.0 × 10^3/well) were grown using DMEM culture media in a 96-well flat-bottomed culture plate at 37°C in a humidified mixture of air (95%) and CO_2 (5%). At 24 h post treatment of ERA, 20 µl of the MTT (5 mg/ml) was added to each well and the plate was incubated for a further 4 hours. The absorbance of the samples at 490 nm was measured using a microtiter plate reader (model 550, BIO-RAD Laboratories, Inc., Hercules, CA, USA).

Reagents

Acetylcholine chloride, phenylephrine HCl, NG-nitro-L-arginine methylester (L-NAME), 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ), indomethacin, atropine, and (±)-propranolol HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Wortmannin and LY294002 were purchased from Biomol (Plymouth Meeting, PA, USA). Rabbit anti-Akt, p-Akt (Ser473), eNOS, p-eNOS (Ser1177) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Western Lightning Plus ECL was purchased from Perkinelmer (Boston, MA, USA). All other agents of cell culture were obtained from Sigma Chemical. ERA was dissolved in DMSO and the final concentration of DMSO was less than 0.1% for all experiments. Working solutions were made in Krebs solution. Control experiments demonstrated that the concentration of DMSO (0.1%) had no effect on vascular tone.

Statistical analysis

Relaxant responses are expressed as a percentage relaxation of PE (1 µM) precontraction levels unless otherwise described in the figure legends. Results were expressed as means ± S.E.M. The concentration of vasorelaxation, giving a half-maximal response (EC_{50}), was obtained by fitting four-parameter sigmoidal concentration - response curves using GraphPad Prism (version 3.0, San Diego, CA, U.S.A.). Significant difference between the group means was determined with ANOVA repeated for cumulative concentration-response curves and Student’s t-test for the production of NO, the expression and phosphorylation of eNOS and Akt using GraphPad Prism. Statistical significance was defined as P < 0.05.

RESULTS

Vasorelaxant effects of extract of Rumex acetosa L. on aortic rings

To define the vasorelaxant property, ERA was tested under the vasoconstriction by PE. As shown in Fig. 1A and 1B, PE contracted aortic rings in a concentration-dependent manner (A), and the contraction reached maximum response with PE (1 µM) and the tension maintained (B). At the late phase of PE contraction, the tension slightly declined by 8.67 ± 1.86%. To identify the effect of ERA on vascular tension, aortic rings were
exposed to cumulative doses of ERA. At 0.1 – 100 µg/ml, ERA relaxed PE-precontracted endothelium-intact aortic rings in a concentration-dependent manner (Fig. 1Cb and 1D). The maximum relaxation induced by ERA was 96.72 ± 0.94% (vs. PE contraction) at a concentration of 100 µg/ml (Fig. 1D). In contrast, denudation of the functional endothelium abolished ERA-induced vasorelaxation (Fig. 1Ca and 1D). This finding indicates that ERA induces vasorelaxation via activation of the endothelium-dependent signaling pathway.

**Effects of L-NAME, ODQ and indomethacin on extract of Rumex acetosa L.-induced vasorelaxation**

To identify the roles of endothelium-derived relaxing factors in the ERA-induced vasorelaxation, the effects of L-NAME (10 µM), an inhibitor of NOS, ODQ (10 µM), an inhibitor of sGC, and indomethacin (Indo; 10 µM), an inhibitor of cyclooxygenases were examined. As shown in Fig. 2, the ERA-induced vasorelaxation was significantly attenuated by pretreatment of endothelium-intact aortic rings with L-NAME or ODQ (P < 0.01; n = 6; Fig. 2A), but not by indomethacin (n = 8; Fig. 2B). These findings indicate that ERA induces vasorelaxation via an activation of NO-sGC-cGMP signaling.

**Role of PI3-kinase/Akt pathway in extract of Rumex acetosa L.-induced vasorelaxation**

Because ERA induces vasorelaxation via endothelium-dependent NO-sGC-cGMP signaling pathway, the involvement of PI3-kinase/Akt signaling was tested. As shown in Fig. 3, pretreatment of endothelium-intact aortic rings with wortmannin (0.1 µM), a PI3K/Akt inhibitor, significantly attenuated the ERA-induced vasorelaxation (as for EC\textsubscript{50}, 35.95 ± 4.64 for wortmannin + ERA, n = 6, vs 3.57 ± 0.68 µg/ml for vehicle + ERA, n = 9; P < 0.01). In addition, the same result was found by pretreatment of aortic rings with LY294002 (30 µM), an inhibitor of PI3K/Akt (as for EC\textsubscript{50}, 12.50 ± 2.07 for LY294002 + ERA, n = 6, vs 3.57 ± 0.68 µg/ml for vehicle + ERA; P < 0.01), which suggests that ERA induces vasorelaxation via an activation of PI3-kinase/Akt-eNOS signaling pathway.

**Extract of Rumex acetosa L. induces nitric oxide release in HUVECs**

To define the effects of ERA on the NO signaling system, experiments were performed in the vascular endothelial system, HUVECs culture cells. As shown in Fig. 4, exposure to ERA (for
24 hours) shows no toxic effect on HUVECs culture system. To confirm the role of the eNOS activation in the responses to ERA, NO production was measured in cultured medium of HUVECs.

As shown in Fig. 5, when HUVECs were treated with various concentrations of ERA (3, 10, 30, and 100 µg/ml) for 1 hour, the NO production, dose-dependently, increased in HUVECs. As shown in Table 1, the ERA-induced NO release was significantly inhibited by pretreatment with L-NAME, an inhibitor of NOS, implying that the effect was dependent on the activation of eNOS. In addition, the ERA-induced NO production was completely inhibited by removal of intercellular Ca\(^{2+}\) by treatment with EGTA. These results suggest that influx of extracellular Ca\(^{2+}\) is also involved in the ERA-activated eNOS pathway.

In parallel, to further characterize the eNOS activation by ERA, the change in the PI3-kinase/Akt pathway was traced in HUVECs. The phosphorylation levels of Akt and eNOS were assessed in HUVECs using Western blot analysis. First, we examined the activation status of Akt and eNOS after ERA stimulation. As shown in Fig. 6, ERA time-dependently increased phosphorylation of Akt at Ser473 (Fig. 6A and 6B) and eNOS at Ser1177 (Fig. 6C and 6D) in HUVECs. The levels of phosphorylation were significantly increased as early as 2 min after treatment with ERA, and peaked at 5 min. Thereafter, the signal persisted for at least 20 min for Akt whereas the phosphorylation levels of eNOS returned to baseline at 20 min. Fig. 7 depicts that ERA concentration-dependently increased the

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**Table 1.** Effects of ERA on the levels of NO release and inhibition by L-NAME and Ca\(^{2+}\) depletion in HUVECs.

<table>
<thead>
<tr>
<th>Nitrite (µM)</th>
<th>Blank</th>
<th>ERA (30 µg/ml)</th>
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<tr>
<td>Blank</td>
<td>1.58 ± 1.02</td>
<td>11.52 ± 1.14</td>
</tr>
<tr>
<td>L-NAME (10 µM)</td>
<td>1.73 ± 0.96</td>
<td>3.47 ± 0.63**</td>
</tr>
<tr>
<td>Ca(^{2+})-free</td>
<td>1.61 ± 0.78</td>
<td>1.95 ± 0.56**</td>
</tr>
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</table>

HUVECs were incubated with ERA for 1 h, and then the culture medium was collected for NO detection. L-NAME (10 µM) was pretreated the HUVECs for 20 min before adding ERA. Nominally Ca\(^{2+}\)-free medium contained 2.5 mM EGTA. Results are shown as the means ± S.E.M. of 4 separate experiments. "P < 0.01 indicates a significant difference from ERA alone (Blank).

Fig. 4. Effects of ERA on human umbilical vein endothelial cells (HUVECs) viability. ERA up to 300 µg/ml showed non-toxic effect in HUVECs. *P < 0.01, #P < 0.05 vs. control (C) group.

Fig. 5. Effects of ERA on NO production by human umbilical vein endothelial cells (HUVECs). Cells were treated with various concentrations of ERA (3, 10, 30, and 100 µg/ml) for 1 h. Medium was then collected for NO detection. Results are shown as the means ± S.E.M. of 5 separate experiments. *P < 0.05, **P < 0.01 vs. control group.

Extract of Rumex acetosa L. induces phosphorylation of Akt and eNOS in HUVECs

In parallel, to further characterize the eNOS activation by ERA, the change in the PI3-kinase/Akt pathway was traced in HUVECs. The phosphorylation levels of Akt and eNOS was assessed in HUVECs using Western blot analysis. First, we examined the activation status of Akt and eNOS after ERA stimulation. As shown in Fig. 6, ERA time-dependently increased phosphorylation of Akt at Ser473 (Fig. 6A and 6B) and eNOS at Ser1177 (Fig. 6C and 6D) in HUVECs. The levels of phosphorylation were significantly increased as early as 2 min after treatment with ERA, and peaked at 5 min. Thereafter, the signal persisted for at least 20 min for Akt whereas the phosphorylation levels of eNOS returned to baseline at 20 min. Fig. 7 depicts that ERA concentration-dependently increased the

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*Note: The diagrams and figures are not included in the text.*
levels of phosphorylation of Akt (Fig. 7A) and eNOS (Fig. 7B). When the endothelial cells were exposed to ERA (30 µg/ml) for 5 min, the phosphorylation levels of Akt and eNOS were increased 2.67 ± 0.03-fold and 3.65 ± 0.05-fold, respectively. However, the effects of ERA on the phosphorylation levels of Akt and eNOS was abolished by pretreatment of cells with PI3-kinase/Akt inhibitors, wortmannin (WT; 0.1 µM) and LY294002 (LY; 30 µM) (Fig. 8A and 8B), supporting that PI3-kinase/Akt pathway mediates ERA-induced eNOS phosphorylation.

**Extract of Rumex acetosa L. decreases arterial blood pressure**

To identify the acute effects of ERA on arterial blood pressure, ERA was injected in urethane-anesthetized rats. Intravenously administered ERA (0.5 mg/kg) decreased arterial blood pressure (Fig. 9A). The effect of ERA on the changes in arterial blood pressure was dose-dependent (Fig. 9B). To identify the roles of the NO system in the ERA-induced hypotensive effects, experiments were performed in the presence of L-NAME. Pretreatment with L-NAME (20 mg/kg, i.p.) increased arterial blood pressure (upper tracing of Fig. 9A) and blocked the ERA-induced decrease in arterial blood pressure.

**DISCUSSION**

In the present study, we demonstrate that ERA induces vasorelaxation via endothelium-dependent NO-cGMP pathway through activation of the PI3-kinase/Akt- and Ca²⁺-eNOS-NO signaling in the endothelial cells and then subsequent stimulation of the NO-sGC-cGMP signaling in the vascular smooth muscle cells. The ERA induced accentuation of NO
production is due to the PI3-kinase/Akt-dependent activation of eNOS by phosphorylation. Our results provide a molecular mechanism of action of ERA on the cardiovascular system.

The endothelium plays a vital role in maintaining cardiovascular homeostasis via production and release of NO and endothelin (ET). ET-1 is a potent endothelium-derived vasoconstrictor, relate to endothelial dysfunction (19). It is well known that NO counteracts the vasoconstriction of ET-1. The present study shows that an accentuation of the production of NO in the endothelial cells is the first step for the vasorelaxation by ERA. The Ca²⁺-dependent endothelial step of NO production by ERA is controlled by the PI3K/Akt signaling. That is, ERA activates PI3K/Akt-eNOS-NO signaling pathway in the endothelial cells (endothelial step). Next, in the vascular smooth muscle cells, diffused NO activates the NO-sGC-cGMP signaling to induce vasorelaxation (smooth muscle step). It is known that the PI3-kinase/Akt pathway is an important upstream mediator of NO production and is Ca²⁺-dependent (4, 20).

Activation of PI3-kinase leads to phosphorylation of membrane phosphatidylinositol 3, 4-biphosphate, which recruits Akt to the cell membrane leading to phosphorylation and activation of Akt (21). Activated Akt elevates eNOS expression and NO production in endothelial cells by directly phosphorylating eNOS at Ser1177 (20, 22). Our data show that ERA time- and dose-dependently increased the phosphorylations of Akt at Ser473 and eNOS at Ser1177, which was markedly blocked by PI3-kinase/Akt
inhibitors, supporting that ERA activates eNOS through activation of the upstream PI3-kinase/Akt signaling pathway.

Our study showed that propranolol and atropine, inhibitors of β-adrenoceptor and muscarinic receptor, had no significant effect on ERA-induced vasorelaxation (data not shown), indicating that this relaxation was not related to activation of muscarinic receptor and β-adrenoceptors in aortic rings. Considering that the ACh-induced activation of the NO-cGMP signaling is through the muscarinic receptor, the present finding showing the absence of the muscarinic signaling pathway of the ERA-induced vasorelaxation suggests that ERA and ACh induce the effects by different mechanisms of action.

The vasorelaxants are useful for treatment of cerebral vasospasm and hypertension, and also improvement of peripheral circulation. Many researches focused on the bioactive compounds from natural resources as potential substances for the treatment of hypertension. Although previous study has shown the antihypertensive effects of the aerial parts of Rumex acetosa (17), the exact mechanisms of action are in close relation with the NO system, eicosanoid system, and K+ channel functions (23-26). The present study indicates that an activation of the NO system, but not prostaglandin pathway, is involved in the ERA-induced vasorelaxation.

Previous studies have reported that the aerial parts of Rumex acetosa contain hyperin, protoanomycin, and phloroglucinol derivative beside different flavonoids, such as emodin and chrysophanol (12-14), some of which exhibited many pharmacological effects including diuretic, anti-oxidative, anti-hypertensive, antimicrobial, and anticancer effect (13-17). Further work is necessary to characterize the active components of RA and their actions on endothelial and smooth muscle cells.

In conclusion, the present study demonstrated that ERA induces vasorelaxation via endothelium-dependent signaling, which involves two-step signaling: first is an activation of the PI3-kinase/Akt- and Ca2+-eNOS-NO signaling in the endothelial cells and then subsequent stimulation of the NO-sGC-cGMP signaling in the vascular smooth muscle cells. The present results support the ethnomedical application of this plant in handling of cases of hypertension.

Limitation of our present study is that we do not have pure compound to explain the vasorelaxation. However, experiments with ERA show clear acute hypotensive effect in anesthetized rats. Intravenous ERA decreased arterial blood pressure in NO- and dose-dependent manner. The finding provides rationale for the prescription of Rumex acetosa in clinics.

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Conflict of interests: None declared.

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**Fig. 9.** Acute effects of ERA on arterial blood pressure and modulation by L-NAME.

(A) Effects of ERA on arterial blood pressure and inhibition by L-NAME. *P < 0.01 vs. control time. L-NAME pretreatment blocked the ERA-induced decrease in arterial blood pressure. Number of experiments, n = 6 for each group.

(B) Dose-dependent effects of ERA on arterial blood pressure. *P < 0.01, **P < 0.001 vs. vehicle. *P < 0.01, vs. ERA (0.1 mg/kg) group. Number of experiments, n = 6 for each group.

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