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Full Paper

Oroxylin-A and its phosphonate derivative potentiate eNOS/NO-mediated relaxation and attenuate vasoconstrictor-induced contraction in the mouse aorta

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ABSTRACT

Oroxylin-A (OroA), a flavonoid isolated from *Scutellariae baicalensis*, alleviates cardiovascular dysfunction. Several procedures for synthesizing OroA have been developed but show low production yield and regioselectivity. We synthesized OroA from baicalin using a one-pot reaction to increase its overall yield. We also determined the chemical properties and mechanism of action of the synthesized OroA and OroA phosphate diethyl ester (OroA-OET) in vascular function. The induction of vascular reactivity by OroA and OroA-OET was evaluated using blood vessel myography and biochemical analysis to assess nitric oxide synthase-mediated nitric oxide production in mouse aortic arteries. OroA and OroA-OET (0.1−30 μM) induced sustained vasorelaxation, which was partly mediated by the endothelium in isolated normal arteries pre-contracted with phenylephrine. OroA and OroA-OET significantly attenuated vasoconstrictors-induced contractile responses. Dilation effects were blocked by the non-selective nitric oxide synthase inhibitor N (omega)-nitro-L-arginine methyl ester but not by tetraethylammonium or 1*H*-(1,2,4)oxadiazolo [4,3-*a*]quinoxalin-1-one. Notably, preincubation with OroA and OroA-OET potentiated acetylcholine-induced relaxation and endothelial nitric oxide production in the arteries with the endothelium. OroA and OroA-OET protected against cardiovascular dysfunction. The synthesis and lead compounds used not only improved the yield of OroA from natural sources but also potentially regulated vascular tone.

1. Introduction

The vascular system distributes oxygen and nutrients necessary for maintaining the body tissues.[1,2] Vascular endothelial cells play important roles in regulating the vascular tone by producing and releasing potent vasoactive factors, including nitric oxide (NO) and prostacyclin I_2.[1,3] NO, a well-known vasodilator, is a short-lived free radical and intercellular messenger that mediates various biological functions including vascular homeostasis, neurotransmission, antimicrobial defense, and anti-tumor activities.[1] NO produced by endothelial NO synthase (eNOS), the constitutive isoform of the enzyme, is an essential physiological regulator of blood flow in tissues and plays a critical role in normal cardiovascular function,[4]

Abbreviations: ACh, acetylcholine; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; OroA, Oroxylin-A; OroA-OET, Oroxlin-A phosphate diethyl ester; PhE, phenylephrine; SMC, smooth muscle cell; TEA, tetraethylammonium; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinoxalin-1-one; L-NAME, N(omega)-nitro-L-arginine methyl ester; sGC, soluble guanylyl cyclase; cGMP, guanosine 3′,5′-cyclic monophosphate.

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maintaining vascular patency, and protecting the intima from platelet and leukocyte adhesion.[1,5] Soluble guanylyl cyclase (sGC) and guanosine 3′,5′-cyclic monophosphate (cGMP) remarkably contribute to NO-mediated vascular tone regulation.[5–8] Drugs that elevate cAMP or cGMP levels in smooth muscle cells cause vasodilation, thus contributing to vascular homeostasis.[5,9]

Polyphenols are important components of many traditional herbal remedies and exhibit several beneficial effects, including anti-inflammatory properties and vascular tone regulation.[10,11] Oroxylin-A (OroA), an active component of the Chinese herb *Scutellariae baicalensis* (Huang Qin),[12] has been used to treat inflammation, suppurative dermatitis, allergic disease, hyperlipidemia, and arteriosclerosis.[10,12] OroA inhibits binding of the transcription factor nuclear factor-kappa B (NF-κB) to inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 promoters to exert anti-inflammatory effects.[12,13] Results of *in vivo* studies indicated that OroA improves the survival rate of endotoxemic mice,[13] prevents cerebral hypoperfusion-induced neuronal damage,[14] inhibits microglial over-activation, and protects against amyloid (Aβ)-induced memory impairment.[15] Moreover, OroA-induced relaxation in the rat uterus is mediated by opening of the uterine ATP-sensitive potassium channel or large conductance voltage- and calcium-activated potassium-channels.[16] OroA exhibits multiple protective effects, including anti-inflammatory[17] and neuroprotective effects, and can be used to treat cardiovascular dysfunction.[17,18] Thus, OroA and its derivatives show potential for use in clinical interventions.

The scarcity of natural extracts limits the potential use of OroA as a therapeutic drug. Additionally, OroA is often misidentified as its regiomer, negletein, during extraction or synthesis.[19] Componential analyses revealed that the main components of *S. baicalensis* are baicalin, wogonoside, baicalein, and wogonin. Based on high-performance liquid chromatography analysis, OroA can be obtained from the 75% methanol fraction in small amounts using a water/methanol gradient solvent system.[20] In addition, many published studies have described methods for OroA synthesis but the production yield and regioselectivity require improvement.[10] OroA is now commercially available but is costly because large quantities are required in bioassays. To further evaluate the biological activity of OroA, sufficient quantities must be obtained by isolation from natural sources or by using organic synthetic approaches. Thus, we evaluated the synthesis of OroA and its derivatives.

Here, we developed a synthetic method for increasing the overall yield and regioselectivity of OroA. According to previously published studies, compounds with little or no charge can transit through biological membranes.[21] The simplest derivative of phosphonic acid that is neutral at physiological pH may be diester-derived, such as diethyl ester.[22] We potentiated the drug-likeness of OroA by modifying its structure to a phosphate diethyl ester. We also examined the mechanisms of action of OroA and OroA phosphate diethyl ester derivative (OroA-OET) in modulating the vasodilation of aortic arteries in mice.

2. Materials and methods

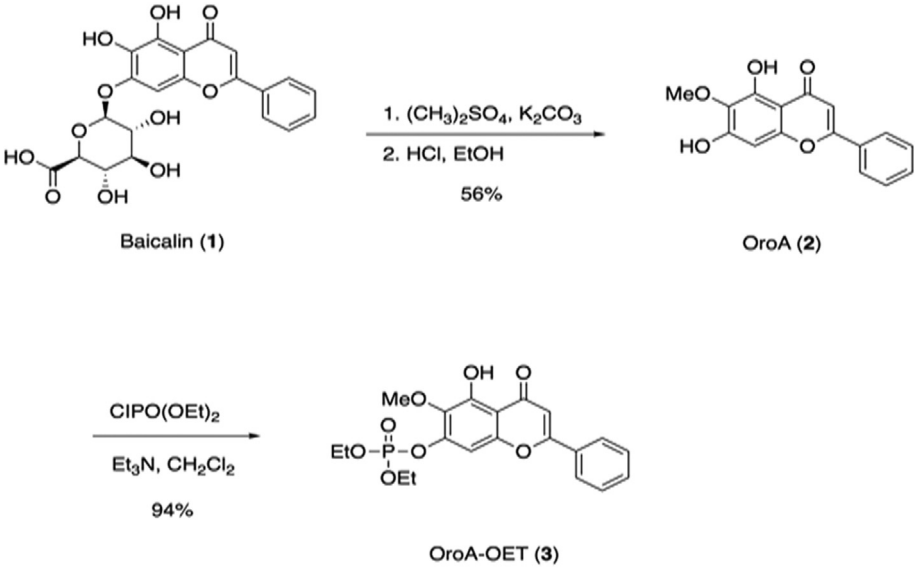
2.1. Synthesis of OroA and OroA-OET

OroA (**2**) was synthesized in 56% yield by methylating baicalin (**1**) with dimethyl sulfate, followed by a deglucuronidation reaction with HCl-ethanol solution in a one-pot reaction. The reaction of OroA with diethyl chlorophosphite produced phosphate esters (**3**) in 94% yield (shown in Scheme 1). The successful synthesis of OroA and OroA-OET was confirmed using high-performance liquid chromatography. All synthesis steps and methods have been patented (process for preparing oroxylin-A; US patent 2019. US10239855B2).

2.1.1. Synthesis of OroA (*2*)

Baicalin (223 mg, 0.50 mmol) was mixed with dimethyl sulfate (1.26 g, 10.0 mmol) and potassium carbonate (345 mg, 2.5 mmol) in a reaction flask. The mixture was stirred at room temperature for 24 h. A solution of HCl/ethanol (10 mL, HCl/ethanol = 1:9) was added, and the mixture was heated to 80 °C and stirred for 12 h. After cooling, the solution was concentrated, diluted in ethyl acetate, and washed with water. Concentrated and silica gel column chromatography yielded OroA (80 mg, 56%).

^{1}H-NMR (700 MHz, $CDCl_3$): δ13.00 (s, 1H), 7.89–7.88 (m, 2H), 7.56–7.52 (m, 3H), 6.67 (s, 1H), 6.63 (s, 1H), 6.62 (s, 1H), 4.05 (s, 3H). **^{13}C-NMR (175 MHz, $CDCl_3$)**: δ183.1, 164.1, 155.2, 153.2, 152.1, 131.9, 131.2, 130.4, 129.2, 129.1, 126.4, 126.3, 105.9, 105.2, 93.5, 60.9.



Scheme 1. Synthetic procedure for OroA and its phosphonate derivative (OroA-OET) All synthetic steps and methods have been patented (process for preparing oroxylin-A; US patent 2019. US10239855B2).

*2.1.2. Synthesis of OroA-OET (**3**)*

To a solution of OroA (**2**) (142 mg, 0.50 mmol) in CH_2Cl_2 (5 mL), triethylamine (253 mg, 2.5 mmol) and diethyl chlorophosphate (129 mg, 0.75 mmol) were added at room temperature. After 2 h, the reaction mixture was diluted in ethyl acetate. The solution was washed twice with water (20 mL), dried over Na_2SO_4, filtered, and concentrated under a vacuum. The mixture was purified using silica gel column chromatography to yield compound **3** (198 mg, 94%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ12.89 (s, 1H), 7.91–7.82 (m, 2H), 7.56–7.51 (m, 3H), 7.13 (s, 1H), 6.71 (s, 1H), 4.34–4.27 (m, 4H), 3.98(s, 3H), 1.43–1.39 (m, 6H). **^{13}C-NMR (100 MHz, $CDCl_3$)**: δ183.1, 164.8, 154.2, 151.8, 149.3, 135.1, 132.1, 131.0, 129.1, 126.4, 109.0, 105.4, 99.6, 65.2, 65.1, 61.0, 16.1, 16.0.

2.2. Animals and experimental procedures

Six-week-old male C57BL/6 mice, weighing 20–25 g, were purchased from BioLASCO Co., Ltd. (Taipei, Taiwan). The animals were housed at the Animal Quarter of Tzu Chi University under standard conditions. The mice were quarantined for seven days before laboratory examination. All animals had *ad libitum* access to standard food and water. The mice were maintained under pathogen-free conditions with a 12-h light/dark cycle. All experimental procedures and protocols were performed according to the 3Rs guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of Tzu Chi Hospital in Taiwan (approval no: 109-53). The experiments were designed to compare the results of the two treatments with at least four to five animals per treatment, with each animal considered as an experimental unit. All animals in different cages were shuffled or randomized by lab technicians using random ID numbers. The mice were anesthetized under isoflurane inhalation (initiation concentration 0.5% for 5 min), and then sacrificed by injecting an overdose of urethane (0.5 g/kg, intraperitoneal). Selected aortic arteries and blood samples were collected and examined to determine their reactivity and NO concentrations.[23]

2.3. In vitro blood vessel myography for evaluating vascular tension reactivity

The reactivity of the isolated arterial ring segments (length, 4 mm) was evaluated using blood vessel myography, as described previously.[24] The presence of functional endothelial cells was verified by assessing acetylcholine (1–10 μM)-induced concentration-dependent relaxation of the arteries which had been pre-contracted with phenylephrine (PhE; 1 μM). After washing with Krebs' solution under an active muscle tone re-induced with PhE (1 μM), KCl (90 mM), serotonin (5-HT, 1 μM), or norepinephrine (1 μM), the segments were treated with OroA (2) (0.1–30 μM), OroA-OET (3) (0.1–30 μM), the NOS inhibitor N (omega)-nitro-L-arginine methyl ester (L-NAME; 300 μM), sGC inhibitor 1*H*-(1,2,4) oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ; 10 μM), non-selective potassium channel blocker tetraethylammonium (TEA; 100 mM), or solvent (dimethyl sulfoxide [DMSO]; 0.1% in Krebs' solution) in the chamber. All pharmacological inhibitors and specific blockers were dissolved in an appropriate solvent DMSO or normal saline (solvent alone as a negative control), and incubated for 15 min. A similar magnitude of active muscle tone was induced again by adding PhE (1 μM). Following PhE-induced active muscle tone, OroA or OroA-OET was added to induce a relaxation response for 15 min. The response to treatment with 120 mM KCl was defined as 100% contraction and used to determine the percent changes in contraction in response to other treatments.[25]

2.4. Griess reaction for measuring NO in conditioned medium with aortic arteries

The nitric oxide (NO) concentration in the conditioned medium was measured using the Griess reaction.[13] Briefly, arterial segments with endothelium or without endothelium (by mechanical denudation as a negative control) were incubated in Krebs' solution (37 °C) containing OroA (**2**) or OroA-OET (**3**) (10 μM). OroA or OroA-OET dissolved in DMSO was added to the mixture in the presence or absence of acetylcholine (ACh; 1 μM) for 12 h. The conditioned medium (1 mL) was collected from each experimental group, and NO release was measured. Frozen media were thawed and deproteinized by incubation with 95% ethanol (Sigma, St. Louis, MO, USA) at 4 °C for 60 min and centrifuged for 10 min at 12,235×*g*. The supernatant (100 μL) was incubated for 5 min with 100 μL Griess reagent (containing 0.1% N-1-napthylethylenediamine dihydrochloride, 10% sulfanilamide in 5% phosphoric acid, from Sigma in 96-well plates. Standard nitrite salt (Sigma) solutions (0–100 μM) were used. The absorbance of the reaction products was measured at 550 nm. The nitrite concentration (μM) in the conditioned media was calculated from a standard curve.

2.5. Western blotting

The protein concentration was determined using a standard Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of the extracts were diluted in a 1:4 ratio with sample buffer (0.25 M Tris–Cl pH 6.8, 2% 2-mercaptoethanol, 8% sodium dodecyl sulfate, 0.02% bromophenol blue, and 40% glycerol) and boiled for 5 min at 100 °C. The concentrations of all protein extracts were analyzed simultaneously to ensure equal loading of the samples. Protein extracts were separated on 10% sodium dodecyl sulfate polyacrylamide gels (30 μg of protein per lane) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) by semidry electroblotting (Amersham Biosciences, Buckinghamshire, UK) for 60 min. The blots were blocked for 2 h at room temperature in 5% non-fat milk in Tris-buffered saline containing 0.25% Tween 20 (TBS-T) and then washed in TBS-T. The blot was incubated with an anti-eNOS mouse antibody (1:500, BD Transduction, San Diego, CA, USA) and anti-phospho-eNOS Ser^{1177} rabbit antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) in TBS-T buffer solution overnight at 4 °C. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies or horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:2000, KPL, Washington DC, USA) for 1 h at 25 °C, and immunoreactivity was visualized using an enhanced chemiluminescence detection system (PerkinElmer, Waltham, MA, USA). After stripping, the blots were stained with a mouse anti-actin antibody (1:4000, Thermo Fisher Scientific, Waltham, MA, USA) for normalization to ensure equal protein loading. After scanning the blots on a computer, individual band intensities were analyzed using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). Data from independent blots were quantified.[23]

2.6. Normalization

The data were log-transformed. Data obtained by examining the reactivity of isolated aortic arteries, following vasoconstriction or dilation, were normalized to the maximum response induced by KCl (120 mM) or sodium nitroprusside (100 μM), respectively. Because high variation exist in different regions of the arteries, both KCl and sodium nitroprusside are widely used for normalization.[25]

Table-1
The predicted properties for the synthesized OroA (2) and OroA-OET (3).

Compounds	MW[a]	Solubility	cLogP[b]	Drug-Score
OroA (2)	284.27	−3.17	2.61	0.79
OroA-OET (3)	420.35	−3.77	3.06	0.4

[a] MW: molecular weight.
[b] cLogP calculated by OSIRIS Property Explorer.

3. Drugs

The effects of OroA (**2**) and OroA-OET (**3**) dissolved in DMSO (0.1%) on isolated arteries were examined using blood vessel myography. NaCl, PhE, norepinephrine, KCl, ACh, L-NAME, sodium nitroprusside, DMSO, and ethanol were purchased from Sigma. TEA and ODQ were purchased from TOCRIS Bioscience (Bristol, UK).

3.1. Statistical analysis

The experimental data are presented as the mean ± standard error of the mean (SEM). All data were analyzed using GraphPad Prism 8.0 software (GraphPad, Inc., San Diego, CA, USA). Statistical analyses were performed using one-way analysis of variance. *Post hoc* comparisons between groups were performed using the Bonferroni multiple comparison test. A *P*-value less than 0.05 was considered to indicate statistically significant results.

4. Results

4.1. Synthesis of OroA (*2*) and OroA-OET (*3*)

OroA is an O-methylated flavone with a wide range of biological activities.[26] The critical step in the synthesis of OroA is regioselective methylation at the C-6 position.[26] We used baicalin as the starting material. Baicalin is a glycosyloxyflavone, the 7-O-glucuronide of baicalein. Baicalin is an active and major ingredient in *S. baicalensis* and can be used to produce OroA in a semi-synthetic process. Structurally, baicalin shows intramolecular hydrogen bonding between C4-carbonyl and C5—OH; C7—OH is glucuronidated, making C-6 the only possible methylation position. Using dimethyl sulfate followed by deglucuronidation with an HCl-ethanol solution, OroA was produced in an efficient one-pot regioselective reaction in high yield (Scheme 1).

4.2. Predicted properties of OroA (*2*) and OroA-OET (*3*)

We further enhanced and potentiated the drug-likeness properties of OroA by modifying its structure to form its phosphate diethyl esters. The molecular weight (less than 500 Da) and clogP (in the 2–5 range) met Liponski's rule of five,[27] suggesting good bioavailability. As shown in Table 1, the cLogP in OroA (**2**) and OroA-OET (**3**) increased from 2.61 to 3.06, and lipophilicity and cell membrane permeability significantly increased.

4.3. OroA (*2*) induced concentration-dependent dilatation response in the aortic rings of mice

To determine whether OroA (**2**) can cause vasorelaxation as demonstrated previously,[28] the C57/B6 mouse aortic artery was isolated. After PhE (1 μM) induction, the contractile response of the aortic rings with endothelium was drastically decreased following treatment with OroA (0.1–30 μM) in a concentration-dependent manner (Fig. 1A and B). OroA (EC_{50}: 9.74 μM) significantly facilitated vasorelaxation (Fig. 1A and B). The peak-tonic or substantial contraction in the control groups (0.1% DMSO) remained at normal levels throughout the experiment (Fig. 1B).

4.4. Endothelium partially enhanced OroA-OET (*3*)-induced relaxation after PhE-induced contraction in arteries

We further investigated whether the lipidic phosphate diethyl ester (OET) structure on OroA (**2**) is a critical functional group that potentiates vascular bioactivity. Different concentrations OroA-OET (**3**) (0.1–30 μM) markedly induced relaxation of aortic rings with intact endothelium and that had been pre-contracted by PhE (Fig. 2A and B). The peak-tonic or substantial contractions in the control groups remained unchanged (Fig. 2A and B). OroA with the OET structure caused potent vasorelaxation, similar to the effects of OroA.

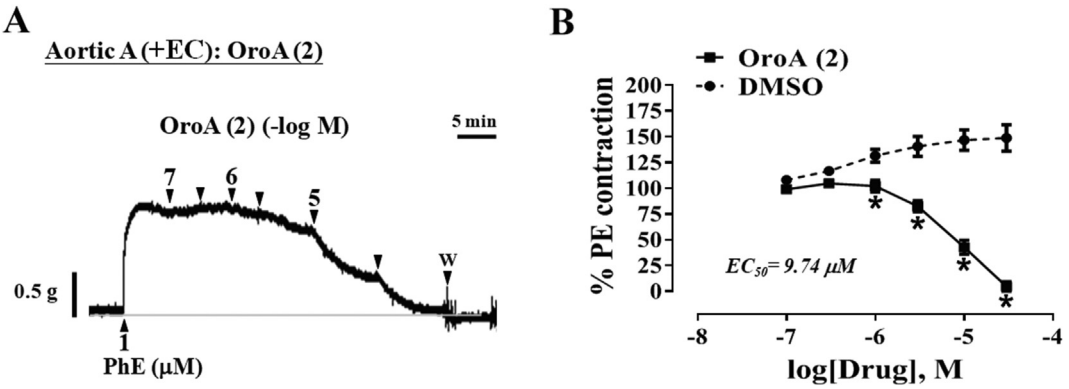


Fig. 1. Synthesized oroxylin-A (OroA)-induced relaxation of PhE pre-contracted aortic arterial rings. The experiments were performed in intact endothelium isolated aortic arterial rings from mice in the presence of active muscle tone induced by phenylephrine (PhE, a α1-adrenal receptor agonist). Representative tracing (A) indicates that synthesized OroA (**2**) (0.1, 0.3, 1, 3, 10, and 30 μM) relaxes PhE (1 μM)-pre-contracted isolated aortic arteries in a concentration-dependent manner. After washing (W), the artery pre-contracted with PhE was administered DMSO as a control (B). The percentage of OroA-induced vasorelaxation was estimated using PhE (3 μM)-induced maximal vasoconstriction as 100%. Relaxations of aortic arteries with preserved endothelial cells (EC) were calculated and summarized (B). Data are presented as the mean ± SEM. *P < 0.05, significant difference relative to the respective DMSO controls. Five experiments were performed.

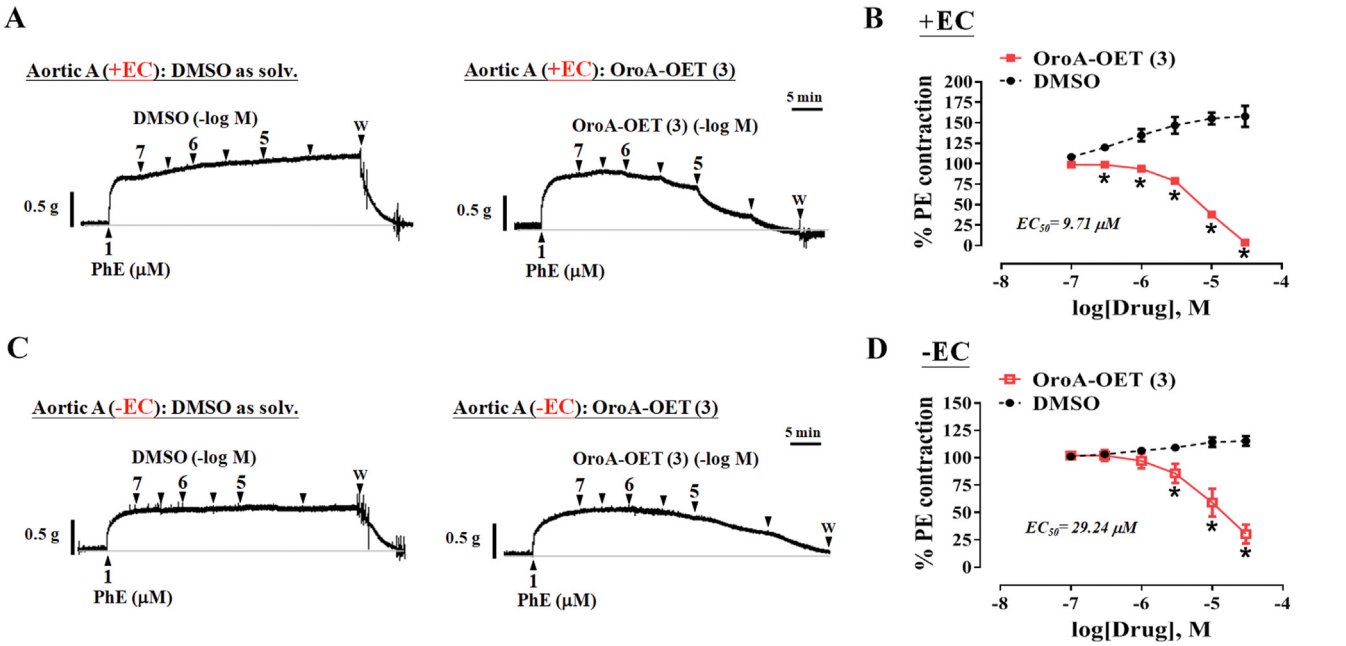


Fig. 2. Endothelium mediated partly relaxation in arterial rings by OroA-OET treatment. Representative tracings showing the presence of intact endothelium (+EC, **A**) and denuded endothelium (−EC, C) of arteries from normal control mice, and endothelial function verified by ACh-induced vasorelaxation (A and C). After washing (W), the artery pre-contracted with phenylephrine (PhE, 1 μM) was administered a serial concentration of DMSO as a control (A and C). OroA-OET (**3**) (dissolved in 0.1% DMSO) induced significant vasorelaxation (original tracing in panel A) with EC_{50} = 9.71 μM (n = 5, summary in panel B) in a concentration-dependent manner. Isolated normal control aortic artery denuded of the endothelium (−EC), as verified by the lack of ACh-induced vasorelaxation, and OroA-OET induced a lesser degree of vascular relaxation (**C**). These OroA-OET-induced vasodilations are summarized in panels B and D. Data are presented as the mean ± SEM. *P < 0.05, a significant difference relative to the DMSO control group. OroA-induced vasorelaxation was estimated as the percentage of maximum contraction induced by PhE. Five experiments were performed.

To investigate whether the endothelium contributed to OroA-OET-induced vasorelaxation, the aortic endothelium was mechanically removed. We observed 50% vasorelaxation in the denuded endothelium treated with a high concentration of OroA-OET (**3**) (≥10 μM) (Fig. 2C and D). We also observed significant relaxation properties in the denuded endothelium of the arterial rings (Fig. 2C and D). As shown in Fig. 2B, 30 μM OroA-OET induced approximately 100% relaxation only in the endothelium-intact arterial rings. Moreover, the EC_{50} values in the OroA-OET-induced relaxations were 9.71 ± 0.52 and 29.24 ± 0.93 μM for arteries with endothelium (Fig. 2B) and denuded-endothelium (Fig. 2D), respectively. Notably, the OroA-OET-induced vascular relaxation was most pronounced in the presence of endothelium.

4.5. OroA-OET (*3*) augments select vasoconstrictor responses

Well-established contracting agents were used to evaluate the pharmacological mechanism underlying OroA-OET (**3**)-attenuating vasoconstriction. First, we determined whether OroA-OET-induced relaxation was caused by blockade of the alpha (α)-1 receptor or beta (β) receptor in smooth muscle cells; the α1 receptor agonist PhE and α/β receptor agonist norepinephrine (NE) were used after pre-incubation with OroA-OET. OroA-OET significantly blocked PhE-induced contraction by 75% (Fig. 3A and B) and attenuated norepinephrine-induced vasoconstriction by at least 45% (Fig. 3C and D). We further investigated whether the 5-HT2A receptor agonist 5-HT (1 μM) and potassium channel operator KCl (90 mM) were blocked by pretreatment with OroA-OET. OroA-OET did not attenuate 5-HT- (Fig. 3E and F) or KCl-induced vasoconstriction (Fig. 3G and H). Pretreatment with 0.1% DMSO as a control did not affect PhE- (Fig. 3A and B), norepinephrine-(Fig. 3C and D), 5-HT- (Fig. 3E and F), or KCl-induced tonic contraction (Fig. 3G and H) in the isolated arteries, as verified at a maximal contraction of 120 mM KCl. These results indicate that the α1 and β receptors but not the 5-HT2A receptor or potassium channel in VSMCs participated in the mechanism of OroA-OET-induced vasorelaxation.

4.6. OroA-OET (*3*)-induced relaxation of aortic arteries is blocked by non-selective NOS inhibitor L-NAME but not inhibited by specific sGC inhibitor ODQ and potassium channel blocker TEA

As a primary regulator, the sGC/cGMP pathway[9] modulates the relaxation of VSMCs upon treatment with OroA-OET (**3**). ODQ (10 μM), an sGC inhibitor, prevents OroA-OET-induced relaxation (Fig. 4A and B). OroA-OET caused significant relaxation of the aortic rings with intact endothelium (Fig. 4A and B); however, these effects were not blocked upon pre-incubation with ODQ (Fig. 4A and B). In the presence of 10 μM ODQ, OroA-OET-induced vasorelaxation was more remarkable than that induced by DMSO treatment alone (Fig. 4B). Dilation of the artery treated with 10 μM OroA-OET was not inhibited by pretreatment with a high concentration (100 mM) of the potassium channel blocker TEA (Fig. 4C and D). The sGC/cGMP and potassium channel pathways were not involved in OroA-OET-induced vasorelaxation. As shown in Fig. 2, OroA-OET-induced relaxation was observed in the endothelium. Additionally, OroA-OET, which is involved in eNOS-induced vasorelaxation, was further investigated. OroA-OET-induced relaxation was inhibited by L-NAME (Fig. 4E and F). These results suggest that the NOS-generated NO production pathway is partly mediated OroA-OET-induced vasorelaxation.

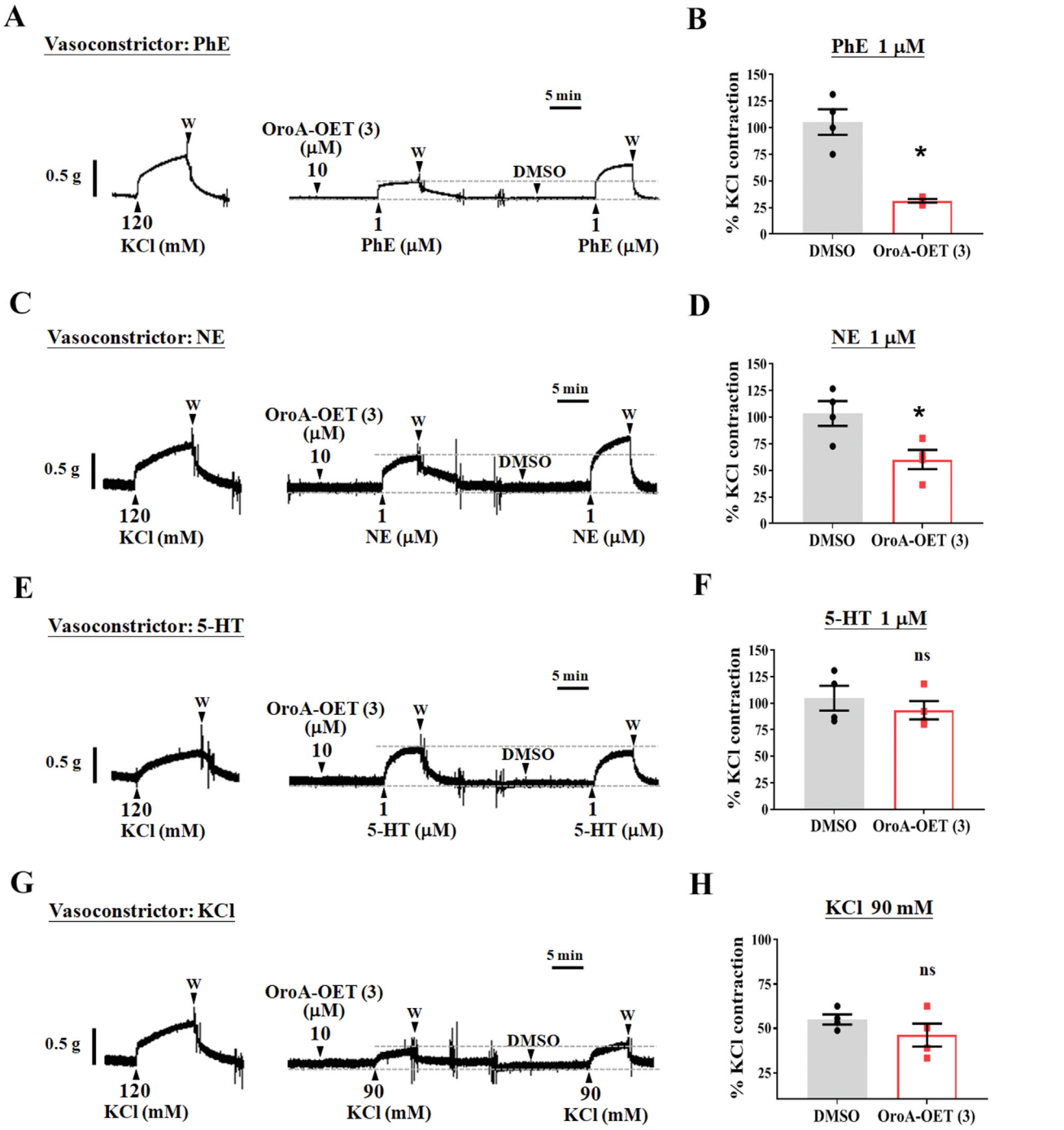


Fig. 3. OroA-OET blocks alpha (α)-1 receptor or beta (β) receptor-mediated constriction in the smooth muscle cells. Representative tracings showing the presence of intact endothelium of arteries from normal control mice pre-incubated with 10 μM OroA-OET (**3**) for 15 min, and vasoconstrictors, including phenylephrine (PhE, 1 μM, panel A), norepinephrine (NE, 1 μM, panel C), serotonin (5-HT, 1 μM, panel E), and potassium chloride (KCl, 90 mM, panel E) to induce vasoconstriction (A, C, E, and G). After washing (W), the responses following pre-incubation with 0.1% DMSO for 15 min as the control in each group were estimated to evaluate OroA-OET-inhibiting contractile responses. OroA-OET suppression of vasoconstriction agent-induced vascular responses assessed as the percentage of maximum contraction induced by KCl (120 mM). These results are summarized in panels B, D, F, and H. A dot symbol of at least five in each column represents the number of mice examined. Data are presented as the mean ± SEM. *P < 0.05, a significant difference relative to the respective DMSO control group. ns: not significant.

*4.7. OroA (**2**) and OroA-OET (**3**) contribute to enhanced ACh-induced relaxation in mouse aortic rings*

We evaluated whether OroA-OET (**3**) induced relaxation of the blood vessels by stimulating the response of endothelial NO synthase based on the results shown in Fig. 4E. We predicted that OroA (**2**) and OroA-OET influence ACh-induced relaxation and contribute to vascular reactivity. OroA, OroA-OET, or 0.1% DMSO (10 μM) were pre-incubated with PhE-induced contracted aortic rings; ACh was applied at the peak contraction of PhE induction

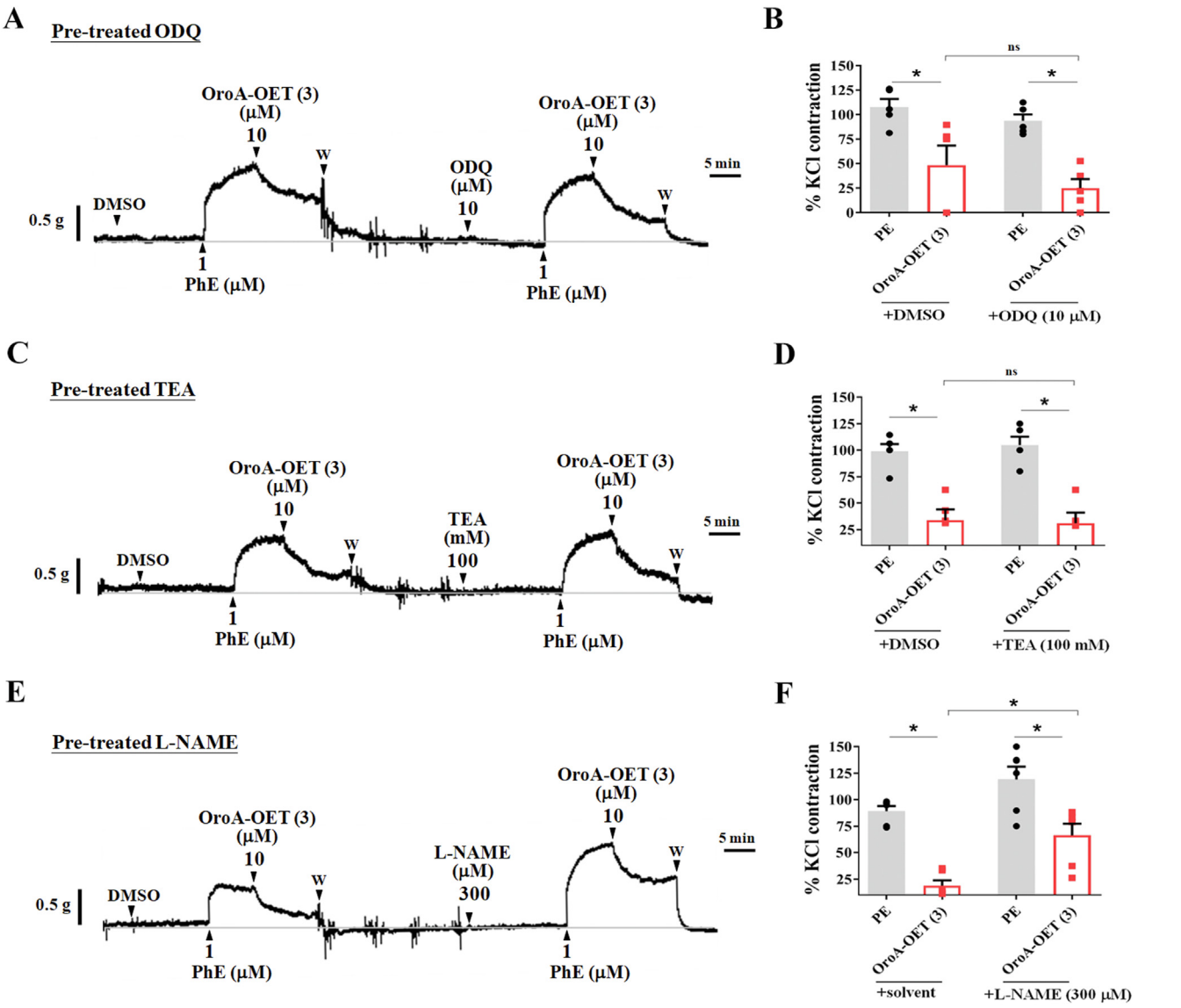


Fig. 4. OroA-OET-induced relaxation is sensitive to NOS inhibitor blockade. OroA-OET (**3**) (10 μM) significantly attenuates phenylephrine (PhE)-induced contraction, and these effects are reproducible (A, C, and E). Pretreatment with the soluble guanylyl cyclase inhibitor ODQ (10 μM) (A and B) or potassium channel blocker TEA (100 mM) (C and D) did not abolish OroA-OET-induced vasorelaxation. Panel E shows that OroA-OET-induced relaxation reverses upon pre-incubation with the non-selective NOS inhibitor L-NAME (300 μM). These results are summarized in panel F. A dot symbol of at least five samples in each column represents the number of mice examined. Data are presented as the mean ± SEM. *P < 0.05 indicates a significant difference among the compared groups. ns: not significant.

(Fig. 5A and C). ACh caused significant relaxation of the aortic rings with intact endothelium during PhE-induced contraction (Fig. 5A and C); these effects were markedly enhanced upon pre-incubation with OroA (Fig. 5A and B) and OroA-OET (Fig. 5C and D). Immunoblot analysis demonstrated that the control aortic arteries minimally constitutively expressed eNOS, eNOS-Ser^{1177} phosphorylation, and showed basal levels of NO production (Fig. 5E–H). The expression of eNOS induced by OroA and OroA-OET treatment in these arteries was significantly higher than that in the respective control arteries (Fig. 5E and F). In parallel, OroA and OroA-OET treatment significantly increased eNOS phosphorylation at Ser^{1177} (Fig. 5E and G). Simultaneously, NO release was increased significantly in OroA- and OroA-OET-treated isolated aortic arteries with endothelium (Fig. 5G). These results suggest that the synthesized OroA and OroA-OET facilitated relaxation and that the vasodilation effect occurred through OroA and OroA-OET stimulation of eNOS-generation of NO production in the aortic arteries of mice.

5. Discussion

Oriental herbs such as Huang Qin and *S. baicalensis* are traditionally used to treat inflammation and high blood pressure.[10,13,18,23,29] However, the limited availability of natural extracts results in variability in functional biochemical assays and limits the application of OroA (**2**). We developed a synthetic method for increasing the overall yield and regioselectivity of OroA and its derivative. We further predicted the mechanism of action of OroA and OroA-OET (**3**) derivatives in modulating the vascular function of the aortic arteries in mice.

Poor solubility is a critical problem during drug development.[30] We modified OroA to its phosphate diethyl ester, OroA-OET, to potentiate its drug-likeness and identify therapeutic candidate drugs. The simplest derivative of phosphonic acid that is neutral at physiological pH may be diester-derived, such as diethyl ester.[22] Drugs containing phosphates (phosphonates or phosphinates)

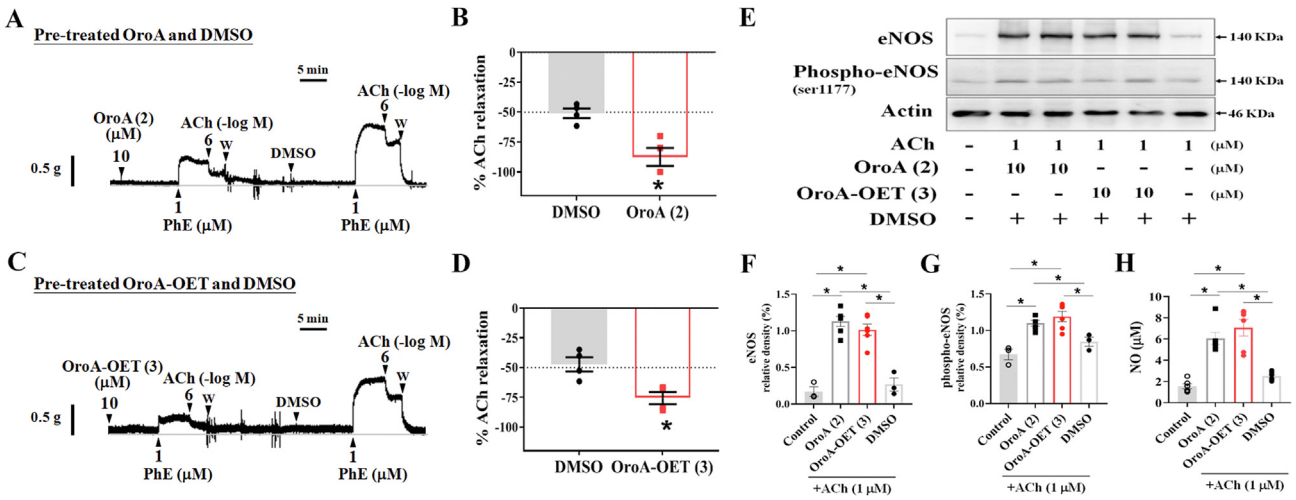


Fig. 5. OroA and OroA-OET enhance ACh-induced relaxation through eNOS-generated NO production in endothelium-intact aortic arteries. A representative tracing showing the presence of intact endothelium (EC+) of an arterial ring from normal mice as verified by ACh-induced vasorelaxation, and *in vitro* incubation of isolated arteries in the presence of OroA (**2**) and OroA-OET (**3**) (1 μM) for 15 min (**A** and **C**). After washing (W), responses following pre-incubation with 0.1% DMSO for 15 min as the control in each group were estimated for comparing OroA and OroA-OET-mediated ACh-inducing relaxation of arteries (A and C). All treatment-enhanced ACh-induced vasodilatation was estimated as the percent of maximum contraction induced by KCl (120 mM). These endothelium-dependent, ACh-induced vasorelaxations are summarized in panels B and D. Western blotting results showing constitutive eNOS expression and eNOS phosphorylation at Ser^{1177}in the aortae from mice after 10 μM OroA and OroA-OET treatment (E). Increased eNOS expression and eNOS phosphorylation are summarized in panel F and G, respectively. OroA and OroA-OET-induced NO release in the medium of aortic organ culture (H). The dot symbol of at least 5 in each column represents the number of mice examined. Data are presented as the mean ± SEM. *P < 0.05 indicates a significant difference relative to the respective DMSO control group.

can effectively promote their uptake and release with minimal toxicity.[22] Based on Lipinski's rule, the molecular weight (<500 Da) and clogP (in the 2–5 range) are used to evaluate bioavailability.[27] According to our results (Table 1), cLogP increased significantly in OroA-OET, further increasing lipophilicity and cell membrane permeability. The effects of OroA and OroA-OET on the regulation of vascular function remain unclear. We showed that OroA and OroA-OET, via various mechanisms, regulate the vascular smooth muscle tone (graphical abstract). (i) OroA and OroA-OET induced endothelium-independent relaxation of the aortic arterial rings in mice. (ii) OroA and OroA-OET attenuated PhE- and norepinephrine-induced constriction by blocking the α1-and β1-adrenergic receptors in muscle cells. (iii) OroA-OET-induced dilatation of the aortic arteries is mediated by eNOS-generated NO production but does not stimulate sGC, thus mediating the opening of potassium channels via cGMP activation. These results suggest that OroA and OroA-OET (**3**) structure are beneficial for protecting vascular endothelial function, maintaining arterial tension, and regulating vasodilatation.

The vasculature meets metabolic needs and provides necessary resources to support biological processing and maintain homeostasis.[31] The arterial tone is mainly dependent on the reactivity of smooth muscle cells, which are closely regulated by endothelial cells and perivascular adipose tissue.[32] The peripheral vasculature disrupts hemodynamic stability, leading to endothelial and smooth muscle cell dysfunction, hypertensive responses, and tissue ischemia.[2,31,33] Therefore, it is essential to protect vascular endothelial and smooth muscle regulation as well as appropriate vasodilation and vasoconstriction to maintain arterial wall regulation. The key signal transduction enzyme sGC, which is activated by NO, is crucially involved in regulating vascular homeostasis and organ functions.[5,9] NO is synthesized from L-arginine in the presence of NO synthase (NOS).[1] We found that both OroA and OroA-OET pre-incubation significantly elevated and potentiated ACh-induced relaxation of the endothelium in isolated arteries (Fig. 5). The mechanisms of vascular relaxation caused by OroA and OroA-OET may involve stimulating NOS to produce NO in endothelial cells or synthesized compounds enabled direct activation of vascular smooth muscle sGC to induce vascular relaxation. L-NAME (a non-selective NOS inhibitor, 300 μM) attenuated the vasodilatory effects of OroA-OET, suggesting that OroA-OET stimulated eNOS-generated NO production in endothelial cells without influencing sGC and cGMP activation. Therefore, OroA-OET-induced vasorelaxation of the aortic arteries may have resulted from enhanced endothelial NO synthesis and/or release. Notably, a high concentration of L-NAME did not completely inhibit vascular relaxation caused by OroA-OET. Therefore, the synthesized compounds have other mechanisms of action that affect vascular activity. Although we reported (2016)[25] that OroA may potentiate the vasoconstrictor-evoked contractile response by inhibiting endothelial NO formation and/or release from lipopolysaccharide-pretreated rat mesenteric arteries, others studies showed that OroA-induced relaxation of the normal rat aorta rings occurs partly because of the release of endothelial NO.[28] Pretreatment with N_{ω}-Nitro-L-arginine (L-NNA; another NOS inhibitor) did not completely block OroA-induced relaxation *in vitro* (unpublished data). These results suggest that OroA- and OroA-OET-induced sustained vascular relaxation and gain-on ACh-induced NO production in endothelial cells were partly caused by activation of the NOS pathway or NO-related metabolites.

Potassium channels on the smooth muscle cell membrane are functionally pivotal in regulating circulation.[34,35] The opening of activated potassium channels facilitates a negative feedback mechanism to reverse vasoconstriction and can be regulated via phosphorylation by several kinases, including protein kinase A, cGMP-dependent protein kinase, and protein kinase C.[34–37] The ATP-sensitive potassium channel is widely distributed across tissues and cell types, including vascular smooth muscle, cardiomyocytes, and skeletal muscle.[34,35] ATP-sensitive potassium channels in the peripheral arteries play essential roles in mediating endothelium-independent vascular tone regulation.[38] These channels and downstream protein kinases are activated

by membrane depolarization and increase intracellular calcium during vasoconstriction.[35,39] Pre-incubation of the arterial rings with the non-selective potassium channel blocker TEA did not attenuate vasorelaxation induced by OroA-OET relative to that of DMSO-pretreated arteries (Fig. 4). However, vasorelaxation was not regulated by potassium channel activation (Fig. 4C and D).

The sGC and cGMP pathways and their activities are involved in vascular tone regulation.[5] NO, sodium nitroprusside, and atrial natriuretic factor stimulate sGC activation, causing cGMP accumulation and vasorelaxation.[5] Moreover, endogenous NO release from the endothelium aggravates the activation of sGC, leading to the relaxation of smooth muscle cells.[34] However, we found that OroA-OET-induced relaxation was not blocked by ODQ. OroA-OET induced significant relaxation of isolated arteries with endothelium. These results suggest that OroA and OroA-OET exert specific effects to protect cardiovascular function. OroA and OroA-OET induce specific vasodilating substance(s), which are absent or present in low concentrations in the normal arteries of animals. Endothelium-dependent vasodilator(s) are explicitly released by OroA and OroA-OET, causing prompt relaxation of VSMCs. Additionally, both contractile responses were induced by α1-and β1-adrenergic receptor agonists; however, these effects were blocked by OroA-OET in the aortic arteries, suggesting that OroA-OET can modulate the α1 and β1 receptors on the smooth muscle cells of arteries. These results indicate the dual mechanisms of vasorelaxation induced by OroA and OroA-OET direct inhibition of the reactivity of smooth muscle cells to vasoconstrictors (Fig. 3) and stimulation of eNOS-mediated NO release from endothelial cells (Fig. 5).

In summary, we developed an efficient process for producing OroA in high overall yield and regioselectivity to overcome the limitations of obtaining OroA from natural sources. OroA and OroA-OET induce aortic arterial relaxation under normal physiological conditions by stimulating the eNOS-NO pathway and blocking adrenergic receptor-mediated vasoconstriction induced by the vasoconstrictors phenylephrine and norepinephrine. These effects of OroA may be valuable for preventing vascular dysfunction.

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Authors contributions

1) Substantial contributions to conception and design: TL Tseng, WY Ho, JZ Liao, KH Lee.
2) Acquisition of data or analysis and interpretation of data: TL Tseng, WY Ho, PJ Huang, JZ Liao, KH Lee.
3) Drafting the article or revising it critically for important intellectual content: TL Tseng, KH Lee.
4) Final approval of the version to be published: KH Lee.

Declaration of competing interest

The authors declare no potential competing interests.

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