Acta Pharmacologica Sinica (2015) 36: 1318-1326
 1671-4083/15
www.nature.com/aps

Original Article

Brazilin isolated from the heartwood of *Caesalpinia sappan* L induces endothelium-dependent and -independent relaxation of rat aortic rings

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Aim: Brazilin is one of the major constituents of *Caesalpinia sappan* L with various biological activities. This study sought to investigate the vasorelaxant effect of brazilin on isolated rat thoracic aorta and explore the underlying mechanisms.
Methods: Endothelium-intact and -denuded aortic rings were prepared from rats. The tension of the preparations was recorded isometrically with a force displacement transducer connected to a polygraph. The phosphorylation levels of ERK1/2 and myosin light chain (MLC) were analyzed using Western blotting assay.
Results: Application of brazilin (10–100 μmol/L) dose-dependently relaxed the NE- or high K^+-induced sustained contraction of endothelium-intact aortic rings (the EC_{50} was 83.51±5.6 and 79.79±4.57 μmol/L, respectively). The vasorelaxant effect of brazilin was significantly attenuated by endothelium removal or by pre-incubation with *L*-NAME, methylene blue or indomethacin. In addition, pre-incubation with brazilin dose-dependently attenuated the vasoconstriction induced by KCl, NE or Ang II. Pre-incubation with brazilin also markedly suppressed the high K^+-induced extracellular Ca^{2+} influx and NE-induced intracellular Ca^{2+} release in endothelium-denuded aortic rings. Pre-incubation with brazilin dose-dependently inhibited the NE-stimulated phosphorylation of ERK1/2 and MLC in both endothelium-intact and -denuded aortic rings.
Conclusion: Brazilin induces relaxation in rat aortic rings via both endothelium-dependent and -independent ways as well as inhibiting NE-stimulated phosphorylation of ERK1/2 and MLC. Brazilin also attenuates vasoconstriction via blocking voltage- and receptor-operated Ca^{2+} channels.

Keywords: brazilin; rat aortic rings; vasorelaxation; endothelium; nitric oxide; calcium channels; ERK1/2; myosin light chain

Acta Pharmacologica Sinica (2015) 36: 1318-1326; doi: 10.1038/aps.2015.113; published online 12 Oct 2015

Introduction

Hypertension is a global health crisis that has affected over one billion people worldwide. It is also one of the key factors that contributes to cardiovascular diseases and heart attack, stroke, kidney failure and even disability and death. Currently, many anti-hypertension drugs have been used in the clinic, but they have various side effects. Traditional herbal medicines have a long history in the management of hypertension by controlling blood pressure with minimal side effects[1].

Caesalpinia sappan L is a species of flowering tree in the legume family that is known as sappanwood/sapanwood. It originates in India, Myanmar, Vietnam, the Malay Peninsula and Sri Lanka, and the dried heartwood of this tree is a traditional medicine in some Asian countries. In these areas, the heartwood is used as a folk medicine for the treatment of various diseases such as ulcers, diarrhea, epilepsy, traumatic disease and menstrual disorders[2]. Additionally, extracts of *Caesalpinia sappan* L have been shown to exhibit anti-inflammatory[3, 4], antimicrobial[5], anti-oxidation[6], and hepatoprotective[7] effects.

Brazilin [7,11b-dihydrobenz(b)indeno[1,2-d]pyran-3,6a,9,10(6H)-tetrol] (Figure 1), one of the major components isolated from the heartwood of *Caesalpinia sappan* L, is a natural red pigment largely used for histological staining. In previous studies, several biological activities of brazilin have been reported, including anti-diabetic[8, 9], anti-inflammatory[10, 11], anti-asthma[12], anti-platelet aggregation[13], anti-tumor[14], anti-

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Received 2015-03-10 Accepted 2015-05-29

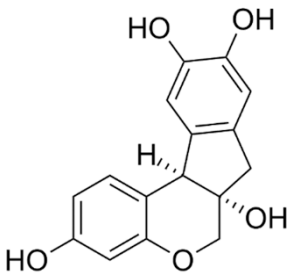

Figure 1. Chemical structure of brazilin.

oxidation[15] and anti-acne[16] effects. As a natural product, brazilin has aroused much attention, especially concerning its effect on cardiovascular diseases. Studies focused on the cardiovascular system showed that brazilin inhibits vascular smooth muscle cell proliferation and migration induced by platelet-derived growth factor (PDGF)-BB[17] and ameliorates high glucose-induced vascular inflammation in human umbilical vein endothelial cells[18]. In addition, the effects and some related mechanisms of brazilin in the vascular system have been described. Brazilin relaxed phenylephrine-induced vasoconstriction, and this response could be inhibited by N^{ω}-nitro-*L*-arginine methyl ester (*L*-NAME), N^{ω}-monomethyl-*L*-arginine acetate (*L*-NMMA), methylene blue, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and hemoglobin, suggesting that the mechanism by which brazilin caused vasodilation might be endothelium-dependent[19, 20]. However, vasoconstriction is a complicated process, involving not only endothelium but also other factors such as K^+ channels and Ca^{2+} channels. Therefore, it is not enough to explain mechanisms of brazilin-induced vasodilation from the endothelium and related factors because complete and clear mechanisms for interpretation still need to be clarified.

Thus, the purpose of the present study was to evaluate the relaxant effects of brazilin on norepinephrine (NE) and KCl-induced vasoconstriction in the rat thoracic aorta and to investigate its possible mechanisms from the following aspects: endothelium, K^+ channels and Ca^{2+} channels.

Materials and methods

Chemicals and reagents

NE, acetylcholine (ACh), angiotensin II (Ang II), *L*-NAME, methylene blue, indomethacin, glibenclamide, tetraethylammonium (TEA), 4-aminopyridine (4-AP) and ethylene-glycol-bis-(β-aminoethylene)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich Co (St Louis, MO, USA). All other reagents were of analytical purity. Antibodies against phospho-myosin light chain 2 (Ser19) (phospho-MLC2), MLC2, phosphorylated extracellular regulated protein kinases 1/2 (Thr202/Tyr204) (phospho-ERK1/2), ERK1/2 and GAPDH were purchased from Cell Signaling Technology Inc (Beverly, USA). Brazilin was purchased from the National Institutes for Food and Drug Control (Beijing, China). Brazilin, glibenclamide and indomethacin were dissolved in dimethyl sulfoxide (DMSO), while the other reagents were dissolved in distilled water, and further dilutions were made with distilled water. Preliminary experiments showed that DMSO kept at concentrations less than 0.2% (*v*/*v*) had no obvious effect on the development of tension in the isolated aorta.

Animals

Specific pathogen-free Sprague-Dawley (SD) rats (male, 250-300 g, Certificate No SCXK (Beijing) 2012-001) were purchased from Vital River Laboratories (Beijing, China). The SD rats were maintained in a barrier system with alternating 12-h light/dark cycles, a relative humidity of 50%±5% and at a constant temperature of 24°C. All experimental protocols involving the care and use of the SD rats were reviewed and approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

Measurements of isometric vascular tension

SD rats were anesthetized with pentobarbitone sodium (60 mg/kg, ip). The thoracic aorta was immediately excised and immersed in Krebs-Henseleit (K-H) solution at 37°C with the following composition (mmol/L): NaCl 120, KCl 4.8, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.4 and ethylenediaminetetraacetic acid (EDTA) 0.01. After tissue debris was removed, and the aorta was cut into strips (2–3 mm in length). For endothelium-denuded aortae, the endothelium was removed by gently rubbing the inner surface with a wet cotton ball.

The aortic rings were suspended by a pair of stainless steel pins in a well-oxygenated (95% O_2–5% CO_2) bath containing 8.0 mL K-H solution at 37 °C and stabilized for 60 min equilibration under a resting tension of 1.2 g, and K-H solution was changed every 20 min during the equilibration period. The tension of aortic rings was recorded isometrically with a force displacement transducer connected to a BIO-PAC polygraph (MP100A)[21].

Before the experiments, the aortic rings were given two successive stimulations with high KCl (60 mmol/L) K-H solution. The endothelial integrity was confirmed by eliciting relaxation with ACh (10 μmol/L) after contraction induced by NE (0.1 μmol/L). Only endothelium-intact rings exhibiting more than 60% relaxation to ACh were used for the experiments. In endothelium-denuded rings, the relaxation to ACh was less than 5%.

Measurements of the effects of brazilin on contractions induced by NE, high KCl or Ang II

To evaluate the effects of brazilin on contractions induced by NE, high KCl or Ang II in aortic rings, three different experimental protocols were used[22, 23].

Protocol 1: The endothelium-intact aortic rings were precontracted with high KCl (60 mmol/L) or NE (1 μmol/L). Once the plateau was attained, brazilin was added cumulatively (10–100 μmol/L) to obtain the concentration-response

curves.

Protocol 2: The endothelium-intact aortic rings were pretreated with brazilin (25, 50 or 100 μmol/L) for 20 min and then contracted by adding KCl (10–60 mmol/L) or NE (10^{-9}–10^{-6} mol/L) cumulatively and respectively to obtain the concentration response curve for KCl or NE, with the maximum contraction induced by the second administration of 60 mmol/L KCl considered to represent 100%.

Protocol 3: The endothelium-intact or denuded aortic rings were pretreated with brazilin (25, 50 or 100 μmol/L) for 20 min, and then the rings were contracted with Ang II (100 nmol/L). The transient constrictor effects were tested, with the maximal contraction induced by NE (0.1 μmol/L) considered to represent 100%.

Brazilin-mediated aortic relaxation and the role of endothelium

To demonstrate the role of the endothelium, dose-responses of brazilin were examined in endothelium-intact and endothelium-denuded rings pre-contracted with NE (1 μmol/L).

To determine which endothelial mediators are related to the vasorelaxant effect of brazilin, the nitric oxide synthase (NOS) inhibitor *L*-NAME (100 μmol/L), the guanylate cyclase inhibitor methylene blue (10 μmol/L) and the cyclooxygenase inhibitor indomethacin (5 μmol/L) were used. The endothelium-intact aortic rings were pre-incubated with these inhibitors for 20 min before NE (1 μmol/L) was added to the bath, and then brazilin (10–100 μmol/L) was added cumulatively.

Brazilin-mediated relaxation and K^+ channels

To illustrate whether K^+ channels are involved, endothelium-denuded aortic rings were pre-incubated with calcium-activated K^+ channel blocker TEA (5 mmol/L), the ATP-sensitive K^+ channel blocker glibenclamide (10 μmol/L) and the voltage-dependent K^+ channel blocker 4-AP (100 μmol/L), respectively, for 20 min before NE (1 μmol/L) was added to the bath, and then brazilin (10–100 μmol/L) was added cumulatively.

Brazilin-induced relaxation and Ca^{2+} channels

To determine whether the inhibition of extracellular Ca^{2+} influx was related, the effect of brazilin was tested on contractions in membrane depolarized endothelium-denuded rings[24]. First, aortic rings were washed with Ca^{2+}-free solution twice (approximately 10 min) containing EGTA (1 mmol/L) and then rinsed with Ca^{2+}-free solution (without EGTA) containing high KCl (60 mmol/L). Then, in the absence of brazilin (vehicle group) or after a 20-min incubation with brazilin (25, 50 or 100 μmol/L), $CaCl_2$ (0.1, 0.5, 1, 1.5, 2, and 2.5 mmol/L) was added cumulatively to obtain concentration-response curves. With the maximum contraction induced by the second administration of 60 mmol/L KCl, considered to represent 100%, concentration-response curves for the added Ca^{2+} were constructed.

To elucidate whether the inhibition of intracellular Ca^{2+} release was involved in brazilin-induced relaxation, the experiments were carried out in Ca^{2+}-free K-H solution[25]. The endothelium-denuded aortic rings were washed as described above. After being incubated with or without brazilin (25, 50 or 100 μmol/L) for 20 min, NE (1 μmol/L) was added to stimulate the release of intracellular Ca^{2+} and the contraction was recorded. With the maximum contraction induced by NE (1 μmol/L) considered to represent 100%, the contraction for Ca^{2+} release was obtained.

Tissue extracts for Western blotting

After being pretreated with brazilin (25, 50 or 100 μmol/L) for 20 min, the endothelium-intact or denuded aortic rings were contracted with NE (1 μmol/L) for 15 min, and the vessels were immediately immersed in liquid nitrogen[26]. The rings were homogenized in ice-cold RIPA lysis buffer, and the soluble proteins were quantified by the bicinchoninic acid protein assay as described by Li[27]. After being mixed with loading buffer and boiled for 10 min, the phosphorylation levels of ERK1/2 and MLC were determined by a Western blotting assay[23]. The bands were quantified by Quantity One software (Bio-Rad, Richmond, CA, USA) and normalized to GAPDH as an internal control.

Statistical analysis

All data are expressed as the mean±SEM. The significance of the differences between groups was determined by one-way ANOVA followed by Dunnett's multiple comparison test. A P value less than 0.05 was significantly different. The images in this article were created using GraphPad Prism5 (GraphPad Software Inc, La Jolla, CA, USA).

Results

Brazilin inhibits the contractions induced by NE or KCl in endothelium-intact aortic rings

Brazilin inhibited the NE (1 μmol/L)-induced sustained contraction in the rat aortic rings in a dose-dependent manner; the 50% effective concentration (EC_{50}) was 83.51±5.6 μmol/L, and the maximal relaxant effect (E_{max}) reached 66.51%±7.54% at a concentration of 100 μmol/L (Figure 2A, B). Brazilin also relaxed aortic rings pre-contracted with KCl (60 mmol/L) in a similar way (EC_{50}=79.79±4.57 μmol/L, E_{max}=75.01%±5.8%, n=6) (Figure 2C, D).

Brazilin inhibits the concentration-response curves of NE and KCl in endothelium-intact aortic rings

In endothelium-intact aortic rings, pre-incubation with brazilin inhibited the concentration-response contraction of NE in a nonparallel fashion, and E_{max} declined with higher concentrations of brazilin. Brazilin (25, 50 and 100 μmol/L) depressed E_{max} to 127.22%±6.96%, 59.38%±10.17% and 0.91%±1.56%, respectively (*vs* control group 133.68%±5.58%, n=6) in endothelium-intact aortic rings (Figure 3A, B). We also observed that brazilin (25, 50 and 100 μmol/L) shifted the concentration-response curves of KCl to the right in a nonparallel fashion and depressed E_{max} to 86.66%±6.33%, 30.55%±4.68%, and 6.33%±1.51%, respectively (*vs* control group 90.37%±3.37%, n=6) in endothelium-intact aortic rings (Figure 3C, D). Compared with the above results (Figure 2), these results indicated

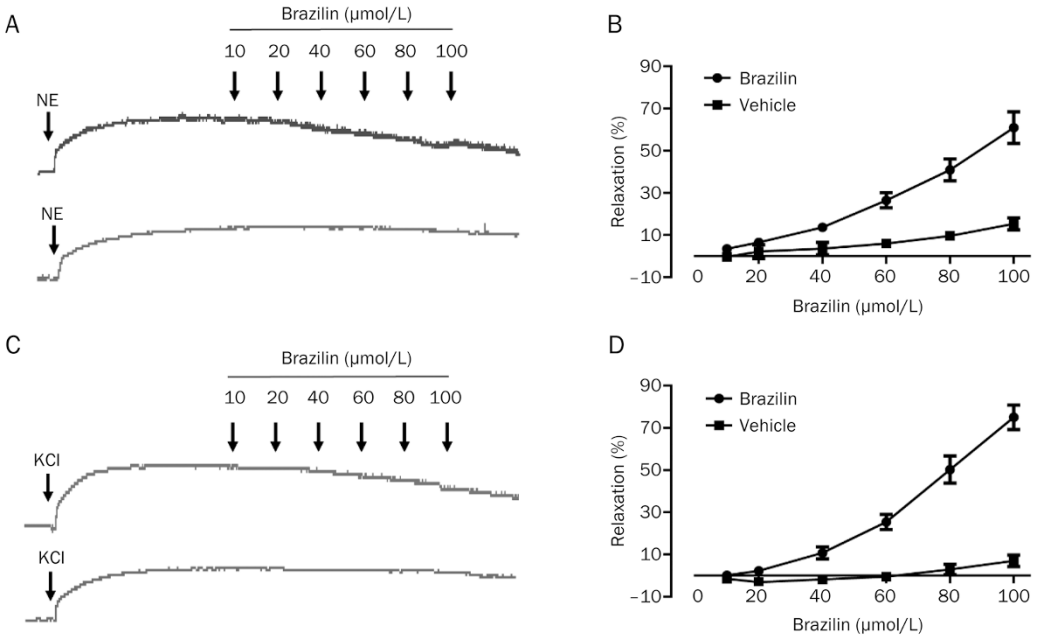

Figure 2. Vasorelaxant effects of brazilin on endothelium-intact thoracic aorta rings pre-contracted with NE (1 μmol/L) or KCl (60 mmol/L). Brazilin dose-dependently relaxed NE (A and B) or KCl (C and D)-precontracted intact aorta. The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to NE (1 μmol/L) (B) or KCl (60 mmol/L) (D). Data are expressed as mean±SEM. *n*=6.

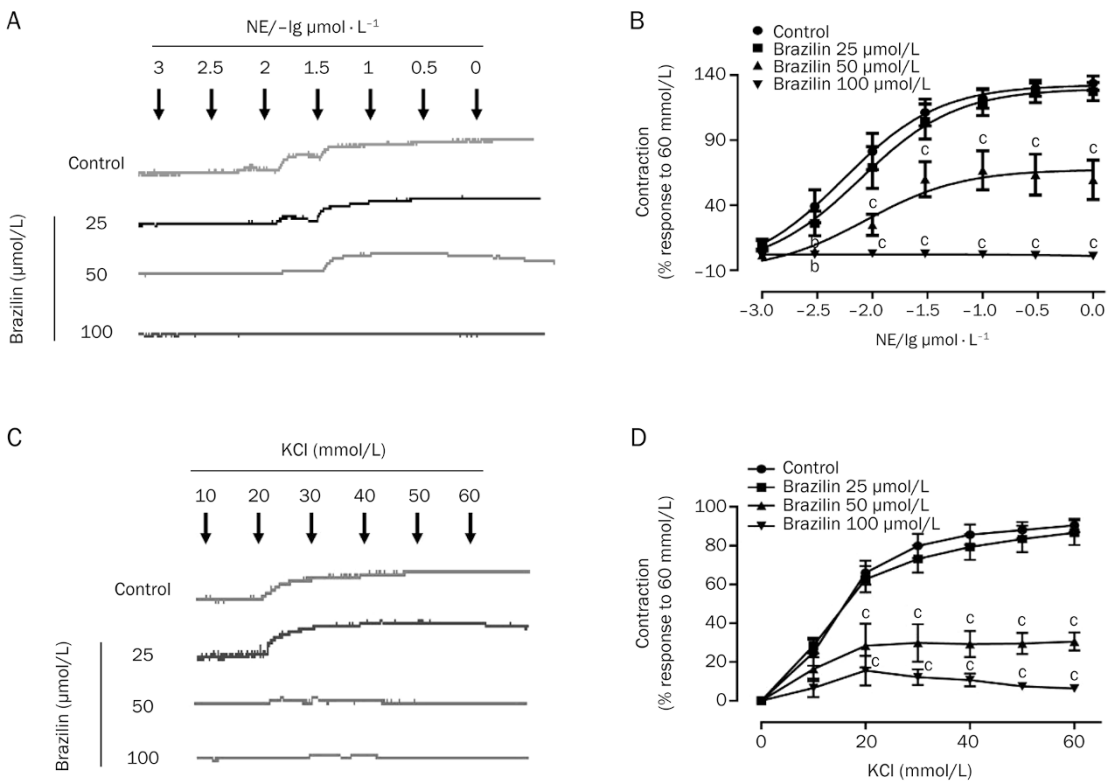

Figure 3. Inhibitory effects of brazilin (25, 50, and 100 μmol/L) on concentration-response curves of NE (10^{-9}–10^{-6} mol/L) (A and B) and KCl (10–60 mmol/L) (C and D) in endothelium-intact aortic rings. The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to the second time of KCl (60 mmol/L). Data are expressed as mean±SEM. *n*=6. $^{b}P<0.05$, $^{c}P<0.01$ compared with control.

that the relaxant effect of brazilin with pre-treatment is more potent than the effect with post-treatment.

Brazilin inhibits the contractions induced by Ang II in endothelium-intact and denuded aortic rings

Pre-incubation with brazilin inhibited the transient contraction induced by Ang II (100 nmol/L) in both endothelium-intact and denuded aortic rings. In endothelium-intact aortic rings, brazilin (25, 50 and 100 μmol/L) was observed to produce a significant reduction in the maximal contractile response to 64.34%±0.35%, 55.06%±2.16% and 45.75%±3.97%, respectively (*vs* control group 67.9%±3.66%, n=6) (Figure 4A). We also observed that in endothelium-denuded aortic rings, brazilin (25, 50 and 100 μmol/L) blocked the contractile response and E_{max} decreased to 63.32%±2.59%, 61.10%±5.06% and 47.53%±2.75%, respectively, (*vs* control group 62.84%±2.55%, n=6) (Figure 4B). As the vasorelaxant effects of brazilin in endothelium-intact and -denuded aortic rings exhibited some differences, a further study was designed to demonstrate the role of endothelium in brazilin-induced aorta relaxation.

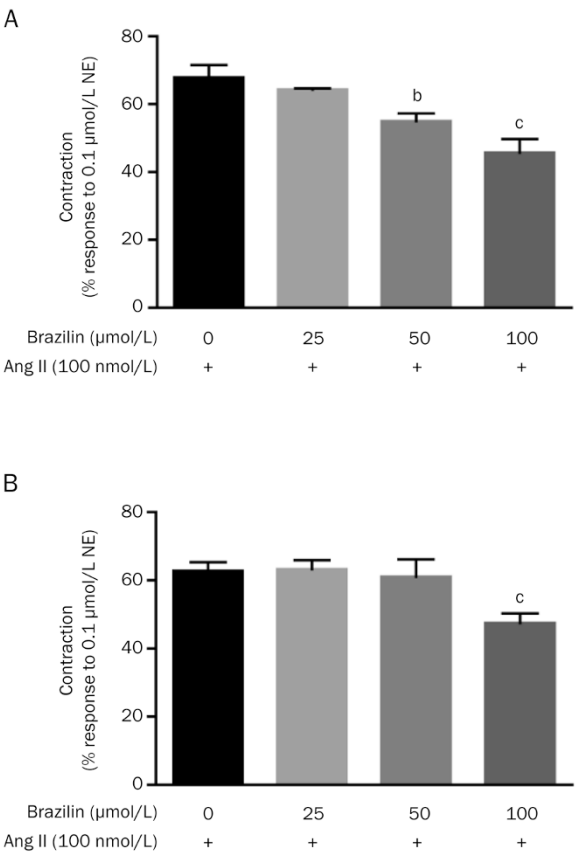

Figure 4. Inhibitory effects of brazilin (25, 50, and 100 μmol/L) on the contraction induced by Ang II (100 nmol/L) in endothelium-intact (A) and denuded aortic rings (B). The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to NE (0.1 μmol/L). Data are expressed as mean±SEM. n=6. ^bP<0.05, ^cP<0.01 compared with control.

Role of endothelium in brazilin-induced aortic relaxation

Brazilin exhibited a stronger vasorelaxant effect on endothelium-intact aortic rings than that on endothelium-denuded aortic rings. In endothelium-denuded rings, brazilin produced a partial relaxation with E_{max} of 49.96%±7.1% (*vs* endothelium-intact group 75.01%±5.8%, n=6) (Figure 5A).

As the vasorelaxant effect of brazilin on endothelium-denuded rings and endothelium-intact rings differed, we investigated which endothelium-derived vasoactive factors contributed to brazilin-induced relaxation. Pre-incubation with *L*-NAME (100 μmol/L), methylene blue (5 μmol/L) and indomethacin (5 μmol/L) significantly reduced the brazilin-induced relaxation of endothelium-intact rings, with E_{max} of 44.08%±2.47%, 54.45%±6.79%, and 57.4%±3.92%, respectively (*vs* control group 75.01%±5.8%, n=6, Figure 5B).

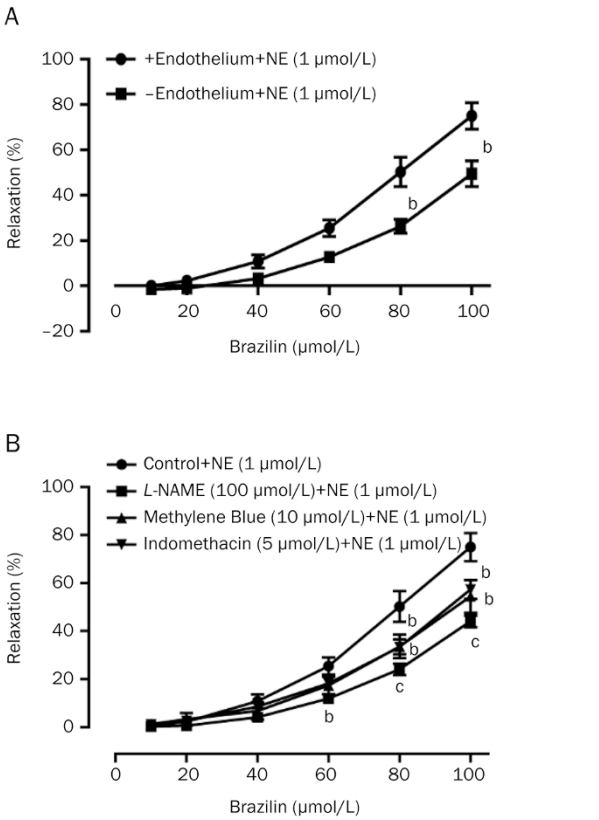

Figure 5. Role of endothelium in brazilin-induced aorta relaxation. (A) The vasorelaxant effect of brazilin (25, 50, and 100 μmol/L) on the contraction induced by NE (1 μmol/L) was attenuated in the aortic rings without endothelium compared with those with endothelium. (B) Vasorelaxation of brazilin was reduced by pre-incubation of *L*-NAME (100 μmol/L), methylene blue (10 μmol/L) and indomethacin (5 μmol/L) in endothelium-intact aorta contraction induced by NE (1 μmol/L). The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to NE (1 μmol/L). Data are expressed as mean±SEM. n=6. ^bP<0.05, ^cP<0.01 compared with endothelium-intact aorta.

Role of K^+ channels in the vasodilatory effect of brazilin

In endothelium-denuded rings, pretreatment with TEA (5 mmol/L), glibenclamide (10 μmol/L) and 4-AP (100 μmol/L) did not remarkably affect brazilin-induced vasorelaxation, with E_{max} of 46.63%±7.25%, 62.1%±1.9%, and 58.7%±2.5%, respectively (*vs* control group 51.53%±5.66%, *n*=6, Figure 6).

Role of Ca^{2+} channels in brazilin-induced aortic relaxation

In the Ca^{2+}-free solution containing 60 mmol/L KCl, the cumulative addition of $CaCl_2$ (0.1–2.5 mmol/L) induced a stepwise tension increase of aortic rings because of extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs). Pre-incubation with brazilin (25, 50 and 100 μmol/L) for 20 min significantly inhibited the concentration-response contraction of $CaCl_2$ (E_{max} was 56.64%±7.24%, 4.97%±1.64% and 0.53%±4.21%, respectively, *vs* control group 101.33%±6.92%, *n*=6) (Figure 7A), suggesting that brazilin reduced the influx of Ca^{2+}.

In the Ca^{2+}-free solution, NE (1 μmol/L) induced a transient contraction due to the release of intracellular Ca^{2+} via receptor-operated Ca^{2+} channels (ROCCs). Pre-incubation with brazilin (25, 50 and 100 μmol/L) for 20 min significantly reduced the contraction induced by NE (1 μmol/L), and E_{max} decreased to 23.12%±4.03%, 21.32%±5.83% and 18.17%±5.11%, respectively (*vs* control group 34.8%±3.15%, *n*=6) (Figure 7B), implying that brazilin attenuated the release of sarcoplasmic reticulum Ca^{2+}.

Inhibitory effect of brazilin on the phosphorylation levels of ERK1/2 and MLC induced by NE

NE (1 μmol/L) evokes the phosphorylation of Thr202/Tyr204 on ERK1/2 and Ser19 on MLC, contributing to smooth muscle cell contraction. Western blot analysis showed that pre-incubation with brazilin (50 or 100 μmol/L) inhibited the increases of ERK and MLC phosphorylation induced by NE in both endothelium-intact and -denuded rat aortic rings to different degrees (Figure 8).

Discussion

Brazilin, one of the major constituents of *Caesalpinia sappan* L, displays a broad range of pharmacological actions. This study demonstrated that brazilin showed a vasorelaxant effect in isolated rat aortic samples, and this effect was achieved through both endothelium-dependent and endothelium-independent mechanisms. Brazilin also relaxed the aortic rings by blockage of the entry of extracellular Ca^{2+} via VDCCs and the release of intracellular Ca^{2+} via ROCCs. Furthermore, the vasorelaxant effect of brazilin was related to the inhibition of the phosphorylation of ERK1/2 and MLC.

Vascular endothelium occupies the location between circulating blood and vascular smooth muscle and is considered to be important for the regulation of vascular tone via the actions of several vasodilators, including nitric oxide (NO), prostaglandin I_2, and endothelium-derived hyperpolarizing factor[28, 29]. Hu *et al*[20] previously reported that brazilin induced vasorelaxation only in intact but not denuded aorta, suggesting that the vasorelaxant effect of brazilin was depen-

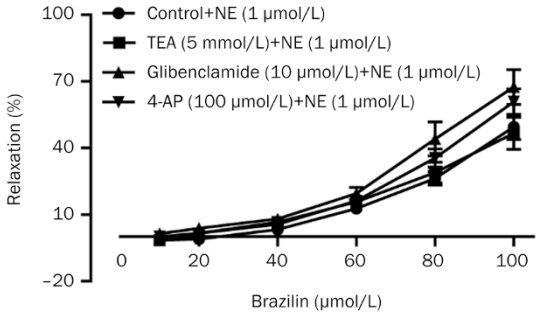

Figure 6. Role of K^+ channels in the vasodilatory effect of brazilin in endothelium-denuded aortic rings. Pre-incubation of glibenclamide (10 μmol/L), TEA (5 mmol/L) and 4-AP (100 μmol/L) did not have significant effect on brazilin induced relaxation in endothelium-denuded aorta rings pre-contracted by NE (1 μmol/L). The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to NE (1 μmol/L). Data are expressed as mean±SEM. *n*=6.

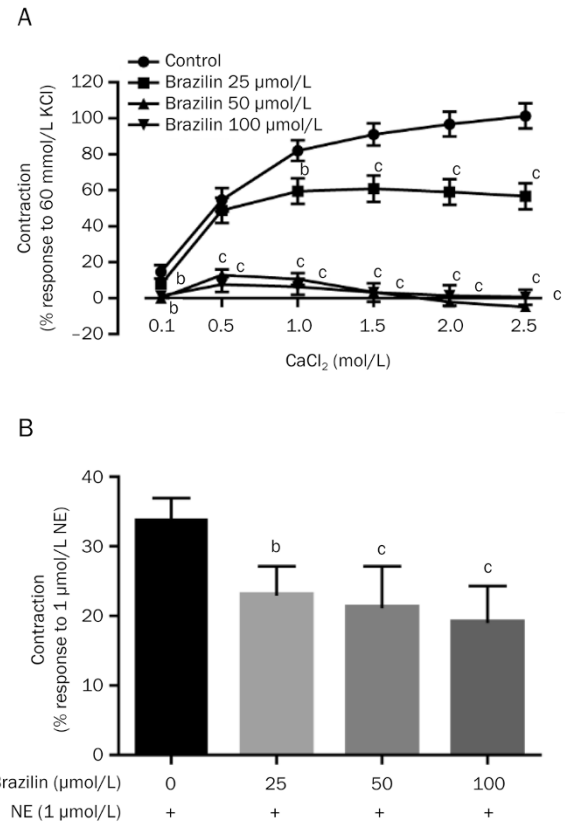

Figure 7. Role of Ca^{2+} channels in brazilin induced endothelium-denuded aorta relaxation. (A) Brazilin had inhibitory effect on the cumulative-contraction curve dependent on extracellular Ca^{2+} influx induced by KCl (60 mmol/L) in Ca^{2+}-free solution. (B) Inhibitory effect of brazilin on the NE (1 μmol/L) induced transient contraction in Ca^{2+}-free solution. The relaxant effects of brazilin on isolated rat aortic rings were calculated as a percentage of the contraction in response to the second time of KCl (60 mmol/L) (A) or NE (1 μmol/L) (B). Data are expressed as mean±SEM. *n*=6. $^bP<0.05$, $^cP<0.01$ compared with control.

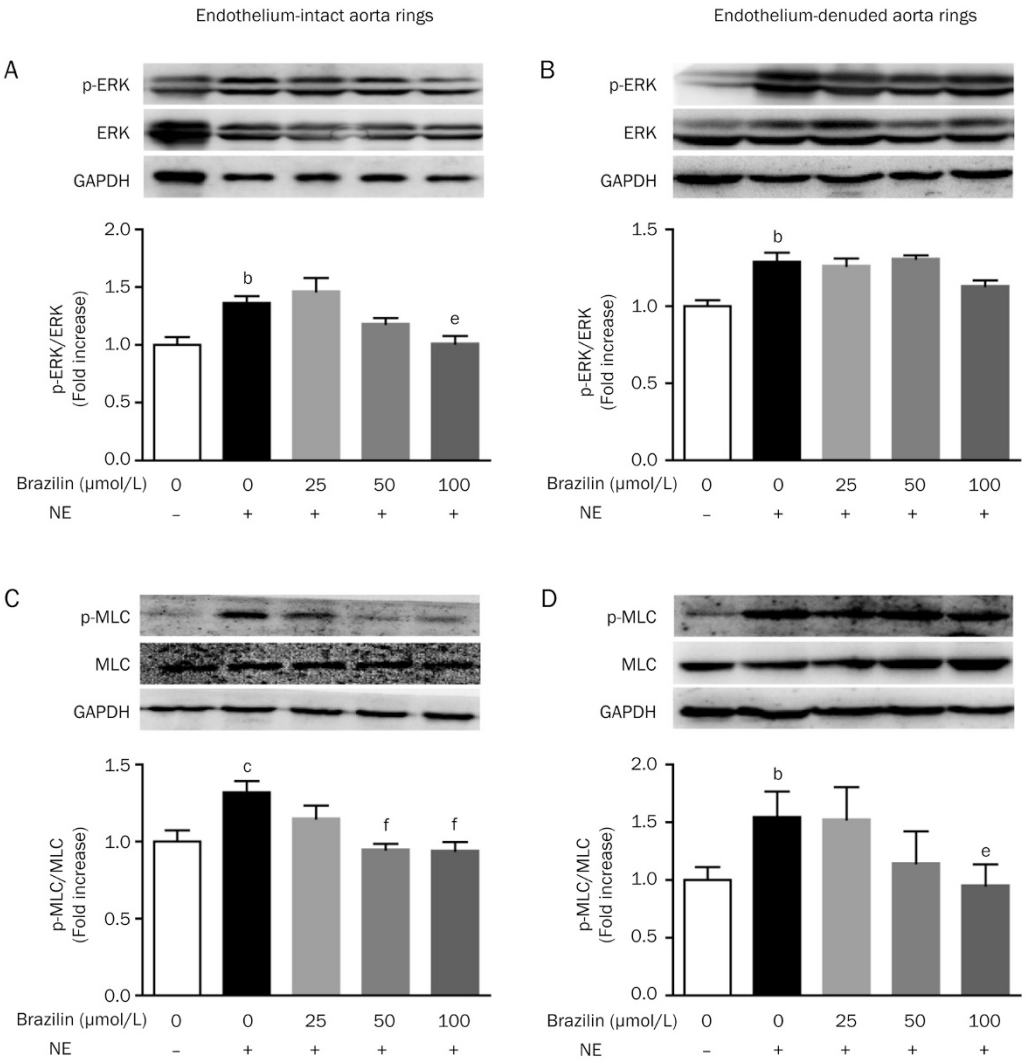

Figure 8. Inhibitory effects of brazilin on phosphorylation levels of ERK (A, B) and MLC (C, D) induced by NE in endothelium-intact (A, C) or endothelium-denuded (B, D) aorta rings. Data are expressed as mean±SEM. n=6. ^{b}P<0.05, ^{c}P<0.01 compared with control. ^{e}P<0.05, ^{f}P<0.01 compared with model.

dent on endothelium, whereas in our study, different phenomena were observed. Brazilin dose-dependently relaxed NE and Ang II induced contraction in both intact and denuded aortic samples. Endothelium removal partially inhibited brazilin-induced vasodilation, indicating that brazilin-mediated vasorelaxation contained both endothelium-dependent and -independent components. Inhibition of the brazilin response by NOS inhibitor *L*-NAME was comparable to that by endothelium removal, and the guanylate cyclase inhibitor methylene blue also inhibited the brazilin response to some extent, which suggested that the NO-cGMP-mediated pathway might be involved in endothelium-dependent relaxation. The cyclooxygenase inhibitor indomethacin attenuated brazilin-induced vasodilation, implying that its relaxant effect might occur via prostaglandin synthesis. However, the difference between endothelium-intact and endothelium-denuded (or inhibited) arteries was not very significant. Thus, the relaxant effect is likely to develop through other pathways.

K^+ channels are important in the regulation of smooth muscle contraction and vascular tone. The opening of K^+ channels in the vascular smooth muscle cells causes hyperpolarization membrane potential, which dilates arteries[30, 31]. In this study, the calcium-activated K^+ channel blocker TEA, ATP-sensitive K^+ channel blocker glibenclamide and voltage-dependent K^+ channel blocker 4-AP did not significantly affect the brazilin response, implying that the activation of K^+ channels might not be involved.

The influx and release of Ca^{2+} play important roles in the excitation-contraction coupling of smooth muscle. There are two types of Ca^{2+} channels in VSMCs: VDCCs and ROCCs[32]. KCl is a membrane depolarizing agent that is generally believed to induce smooth muscle contraction mainly by open-

ing L-type VDCC (L-VDCC)[33, 34]. However, in the absence of extracellular Ca^{2+}, NE induces fast-onset, non-sustainable contractions by stimulating the formation of inositol 1,4,5-triphosphate (IP_3), which binds to and opens specific IP_3-receptor-operated channels in the sarcoplasmic reticulum membrane and induces intracellular Ca^{2+} release through ROCCs[35]. In the present experiments, brazilin (50 and 100 μmol/L) was able to inhibit high K^+-induced extracellular Ca^{2+} influx and NE-induced intracellular Ca^{2+} release in endothelium-intact aortic rings in a nonparallel fashion, indicating that brazilin might interfere with both VDCCs and ROCCs. Taken together, these results indicate that at higher concentrations, brazilin may act as a Ca^{2+} antagonist.

It is well known that mitogen-activated protein kinase (MAPK) plays an important role in vascular smooth muscle functions[36]. It was demonstrated that the activation of ERK1/2 is tightly associated with augmented vascular contraction and modulation of VSMC contractile machineries[37]. The calcium- and calmodulin-dependent phosphorylation of the MLC has clearly been shown to be a major regulatory step in the activation of smooth muscle contraction[38]. The present study showed that brazilin inhibited the NE (1 μmol/L)-induced sustained contraction in a dose-dependent manner in endothelium-intact and endothelium-denuded arteries, so we collected artery rings treated with different doses of brazilin and NE (1 μmol/L) and analyzed the phosphorylation levels of ERK and MLC by Western blotting. NE evokes the activation of ERK1/2 and MLC in vascular smooth muscle. The results demonstrated that the phosphorylation levels of ERK1/2 and MLC stimulated by NE were significantly reduced by brazilin.

In conclusion, brazilin induced relaxation in rat aortic rings through both endothelium-dependent and -independent pathways. The NO-cGMP-mediated pathway may be involved in the endothelium-dependent relaxation due to brazilin. Brazilin also inhibited extracellular Ca^{2+} influx by interacting with VDCCs and the release of intracellular Ca^{2+} by blocking ROCCs. The vasorelaxant effect of brazilin was related to the inhibition of the phosphorylation of ERK1/2 and MLC. As a traditional herbal medicine, brazilin has the advantages of low toxicity and low side effects. Vascular tone is an important determinant of peripheral resistance and blood pressure. Brazilin has vasorelaxant effect on the rat aortic rings, and it may therefore be useful in the prevention and/or treatment of hypertension.

Acknowledgements

This study was supported by grants from the National Science and Technology Major Project (No 2013ZX09103-001-008 and 2012ZX09103-101-078) and the National Natural Science Foundation of China (No 81102444 and 81202538).

Author contribution

Yu YAN designed the experiments and drafted the manuscript; Yu-cai CHEN and Yi-huang LIN prepared the reagents and thoracic aortae and measured isometric vascular tension; Li LI and Zi-ran NIU participated in the Western blotting assay; Jing GUO and Shou-bao WANG carried out the statistical analysis; Guan-hua DU and Lian-hua FANG measured the isometric vascular tension, participated in experimental design and drafted the manuscript.

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