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European Journal of Pharmacology 468 (2003) 37–45
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Induction of vasorelaxation through activation of nitric oxide synthase in endothelial cells by brazilin

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Received 29 August 2002; received in revised form 12 March 2003; accepted 21 March 2003

Abstract

The vasorelaxant activity of *Caesalpinia sappan* L., a traditional Chinese medicine, and its major component brazilin were investigated in isolated rat aorta and human umbilical vein endothelial cells. In isolated rat aorta, *C. sappan* L. extract and brazilin relaxed phenylephrine-induced vasocontraction and increased cyclic guanosine 3′,5′-monophosphate (cGMP) content. Induction of vasorelaxation of brazilin was endothelium-dependent and could be markedly blocked by pretreatment with nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME); N^G-monomethyl-L-arginine acetate (L-NMMA) and guanylyl cyclase inhibitor, methylene blue; 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and nitric oxide (NO) scavenger, hemoglobin. The increasing cGMP content induced by brazilin was also blocked by pretreatment with L-NAME, methylene blue, and the removal of extracellular Ca^{2+}. In human umbilical vein endothelial cells, brazilin dose-dependently induced an increase in NO formation and NOS activity, which were greatly attenuated by either the removal of extracellular Ca^{2+} or the chelating of intracellular Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM). Moreover, brazilin dose-dependently induced the influx of extracellular Ca^{2+} in human umbilical vein endothelial cells. Collectively, these results suggest that brazilin induces vasorelaxation by the increasing intracellular Ca^{2+} concentration in endothelial cells of blood vessels and hence activating Ca^{2+}/calmodulin-dependent NO synthesis. The NO is released and then transferred into smooth muscle cells to activate guanylyl cyclase and increase cGMP content, resulting in vasorelaxation.

Keywords: *Caesalpinia sappan* L.; Brazilin; Vasorelaxation; Endothelial cell; Aorta; Nitric oxide (NO); Nitric oxide synthase (NOS); Ca^{2+} influx

1. Introduction

Endothelium plays an important role in the vascular system, as it not only serve as a barrier between the blood and tissue, but also produces several vasoactive mediators which are important in controlling the body's normal homeostasis and in many pathological conditions (Jaffe, 1985; Vanhoutte et al., 1986). These mediators regulate immune responses, control blood coagulation states, contribute to angiogenesis and vessel repair, and modulate vascular tone. When stimulated by neurotransmitters in the cardiovascular system, hormones, substances derived from platelets and the coagulation system, can evoke vasorelaxation (Luscher and Vanhoutte, 1990; Furchgott and Zawadaki, 1980) through the production of endothelium-derived relaxing factor, also known as nitric oxide (NO) (Palmer et al., 1987). NO is synthesized from the amino acid, L-arginine, by oxidation of its terminal guanidine nitrogen via the enzyme, nitric oxide synthase (NOS) (Cobb et al., 1993; Palmer, 1993) in Ca^{2+}-dependent (Moncada et al., 1991) or Ca^{2+}-independent manners (Dimmeler et al., 1999; Fulton et al., 1999).

Caesalpinia sappan L., a traditional Chinese medicine, has been used in Oriental medicine as an analgesic and anti-inflammatory agent, to cure emmeniopathy, sprains, and convulsions (Baek et al., 2000). The extract of *C. sappan* L. has been reported to have some pharmacological activities such as being antihypercholesteremic, and having sedative and depressing effects on the central nervous system (Nagai et al., 1984), anti-hepatitis B surface antigen (HBsAg)

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doi:10.1016/S0014-2999(03)01639-X

capability (Zheng and Zhang, 1990), anti-complementary activity on the complement system (Oh et al., 1998), and an antimotility effect on human sperm (Shih et al., 1990), while *C. sappan* L. is also known to be used for treatment of diabetic complications (Moon et al., 1985) and to promote blood circulation (Xie et al., 2000).

Brazilin [7,11*b*-dihydrobenz[*b*]indeno[1,2-*d*]pyran-3,6*a*,9,10(6*H*)-tetrol], the major component of *C. sappan* L. (Hikino et al., 1977), is a natural red pigment, and usually used for histological staining (Puchtler and Sweat, 1964; Puchtler et al., 1986). It shows hypoglycemic action in experimental diabetic animals, and this hypoglycemic effect is the result of increased glucose metabolism, including glucose uptake into muscles and adipocytes (Khil et al., 1999; Moon et al., 1993). In addition, brazilin was also shown to exert many biological effects including antiplatelet aggregation (Hwang et al., 1998), inhibition of protein kinase C and insulin receptor kinase in rat liver (Kim et al., 1998), induction of immunological tolerance in mice (Mok et al., 1998), and protection of cultured hepatocytes from $BrCCl_3$-induced toxicity (Moon et al., 1992). Brazilin-induced vasorelaxation could be inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) and suggested that the mechanism by which brazilin caused vasodilation might be endothelial-dependent (Xie et al., 2000). In this study, we not only found the same effect of brazilin, but we also confirm the mechanisms causing it.

The goal of this study was to further investigate the underlying mechanism of vasodilation induced by an extract of *C. sappan* L. and brazilin using isolated rat aorta and human umbilical vein endothelial cells. In this study, we found that *C. sappan* L. induced vasorelaxation in both endothelium-dependent and -independent manners, but brazilin only caused an endothelium-dependent vasorelaxing effect.

2. Materials and methods

2.1. Drugs and chemicals

The dried heartwood of *C. sappan* L. (200 g) was purchased from a traditional Chinese herb store, chopped into small pieces, and extracted with 2 l of boiling water for 1 h. The combined aqueous extract was first concentrated using a Yamato vacuum rotary evaporator (Japan) at 45 °C and then freeze-dried at −80 °C in a vacuum freeze-dryer (MicroModulyo, Savant Instruments, USA). Normally, 30 g of dried powder can be obtained from 200 g of heartwood. Brazilin was purchased from ICN Pharmaceuticals (Irvine, CA, USA). Cell culture reagents including M-199 medium, L-glutamine, penicillin, streptomycin, and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, USA). A cyclic guanosine 3′,5′-monophosphate (cGMP) enzyme immunoassay kit was purchased from Cayman Chem. (Ann Arbor, MI, USA). Arginine,L-[2,3,4-^{3}H]monohydrochloride and $^{45}Ca^{2+}$ were purchased from Amersham Life Sciences (Arlington Heights, IL, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of aortic rings and tension recording

Male Wistar rats weighing about 250–300 g were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. The thoracic aorta of rats was carefully removed after the rat was killed by exsanguinations of the head, and fat and tissue were dissected away in normal Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1, and $CaCl_2$ 2.5; pH 7.4). The aorta was cut into rings about 5 mm in length in a 10-ml organ bath and constantly gassed with 95% O_2 + 5% CO_2 at 37 ± 0.5 °C. Two "L" type stainless steel hooks were inserted into the aortic lumen; one was fixed in the bottom bath and the other was connected to a force transducer. The aortic rings were equilibrated in Krebs solution and maintained under an optimal tension of 1 g for 60 min, with three changes of the Krebs solution, before the experimental procedures were begun. Contractions were recorded isometrically via a force-displacement transducer (Grass FT.03) connected to a MacLab/8e (ADIntruments, Australia) recorder. In denuded aorta, the endothelium was removed by rubbing with a cotton ball and the absence of acetylcholine-induced relaxation was taken as an indicator of successful denudation.

2.3. Rat aorta cGMP measurement

Rat aorta cyclic nucleotide was determined as described by Kauffman et al. (1987). After replacing the Ca^{2+}-free Krebs (EGTA 2.5 mM) buffer or pretreatment inhibitors of L-NAME (50 μM) and methylene blue (10 μM) for 10 min, test compounds, such as sodium nitroprusside (10 μM), acetylcholine (10 μM), and brazilin (0.3 mM), were added for a further 2 min. After incubation with the test compound, the rat aortic rings were rapidly frozen in liquid nitrogen and stored at −70 °C until homogenized in 0.5 ml of 10% trichloroacetic acid using a Potter glass homogenizer. The homogenate was centrifuged at 10,000 × *g* for 5 min, and the supernatant was removed and extracted four times with three volumes of ether. The cGMP content was then assayed using enzyme immunoassay kits. The precipitate was used for the protein assay (Lowry et al., 1951).

2.4. Human umbilical vein endothelial cells isolation and culture

Human umbilical cords were obtained from the Hospital of National Taiwan University, and human umbilical vein endothelial cells were isolated by enzymatic digestion as described (Rosenkranz-Weiss et al., 1994). After a 15-min incubation with 0.1% collagenase at 37 ± 0.5 °C, umbilical cord vein segments were perfused with 30 ml of medium 199 containing 10 U/ml penicillin and 100 μg/ml strepto-

mycin for the collection of cells. After centrifugation for 8 min at 900 × *g*, the cell pellet was resuspended in previous medium supplemented with 20% heat-inactivated fetal bovine serum, 30 μg/ml endothelial cell growth supplement, and 90 μg/ml heparin. Confluent primary cells were detached by trypsin–EDTA (0.05%:0.02%, v/v), and passages between three and five were used in the experiments. Cultures had typical cobblestone morphology and stained uniformly for human von Willebrand factor (vWF) (Janel et al., 1997) as assessed by indirect immunofluoresence.

2.5. NO determination

Human umbilical vein endothelial cells cultured in 12-well plates were washed twice with Hanks balanced salt solution (HBSS) and then incubated at 37 ± 0.5 °C in the same buffer for 30 min with various concentrations of brazilin. Acetylcholine (30 μM) was used as a positive control. Supernatants were collected and then injected into a nitrogen purge chamber containing vanadium(III) chloride in hydrochloric acid at 91 ± 0.5 °C. All NO metabolites can be liberated as gaseous NO and reacted with ozone to form activated nitrogen dioxide that is luminescent in red and infrared spectra. The chemiluminescence was detected using a nitric oxide analyzer (NOA280, Sievers Instruments, Boulder, CO, USA) (Ewing and Janero, 1998). For calibration, the area under the curve was converted to nanomolar NO using a $NaNO_3$ standard curve, and the final data were expressed as pmol/mg protein.

2.6. NOS activity measurement

Endothelial nitric oxide synthase (eNOS) activity in human umbilical vein endothelial cells was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline which was sensitive to L-NAME (Bredt and Synder, 1990). Confluent human umbilical vein endothelial cells in 60- or 100-mm dishes were washed twice with HBSS buffer and incubated in 500 μl HBSS buffer supplemented with arginine,L-[2,3,4-^{3}H]monohydrochloride (1 μCi/ml) at 37 ± 0.5 °C for 30–60 min with or without calcium. In the same time, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) (20 μM) was used to chelate intracellular calcium. Extracellular Ca^{2+} was removed by replacing the medium with Ca^{2+}-free medium in the presence of 2.5 mM EGTA. Test compounds (brazilin, 0.01–0.3 mM) were then added and incubated for another 30 min at 37 ± 0.5 °C (acetylcholine, 30 μM; histamine, 50 μM, or A23187, 5 μM, was used as a positive control) (Lee et al., 1998). The supernatant was aspirated and washed twice with cold stop buffer (HEPES 50 mM, EDTA 5 mM) to stop the reaction. Cells were denatured with 100 μl 95% ethanol. After evaporating the ethanol, the soluble components were dissolved in 1 ml of stop buffer. The L-citrulline and L-arginine in the supernatants were separated using a Dowex AG 50WX-8 (Na^+ form) column and eluted with 2 ml of stop buffer. Eluted solution (1 ml) was mixed with 3 ml of scintillation cocktail, and the radioactivity was determined with a β-counter (Model 2200; Beckman, Palo Alto, CA, USA). Cells were lysed with 400 μl 0.5 N NaOH, and protein was determined by the Bradford (1976) method. The final data were expressed as pmol/g protein/min.

2.7. $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ influx measurement was modified from Meisheri et al. (1980). Confluent human umbilical vein endothelial cells cultured in six-well plates were washed twice with HBSS, then incubated in HBSS containing $^{45}Ca^{2+}$ (10 μCi/ml), and treated with various concentrations of brazilin (0.001–0.1 mM) for 5 min. The supernatants were aspirated and washed three times with cold HBSS with 10 mM $LaCl_3$. Acetylcholine (30 μM), histamine (50 μM), or A23187 (5 μM) was used as a positive control. Cells were lysed with 0.01 N NaOH, and the $^{45}Ca^{2+}$ uptake by cells was measured with a scintillation counter (Model 2200; Beckman).

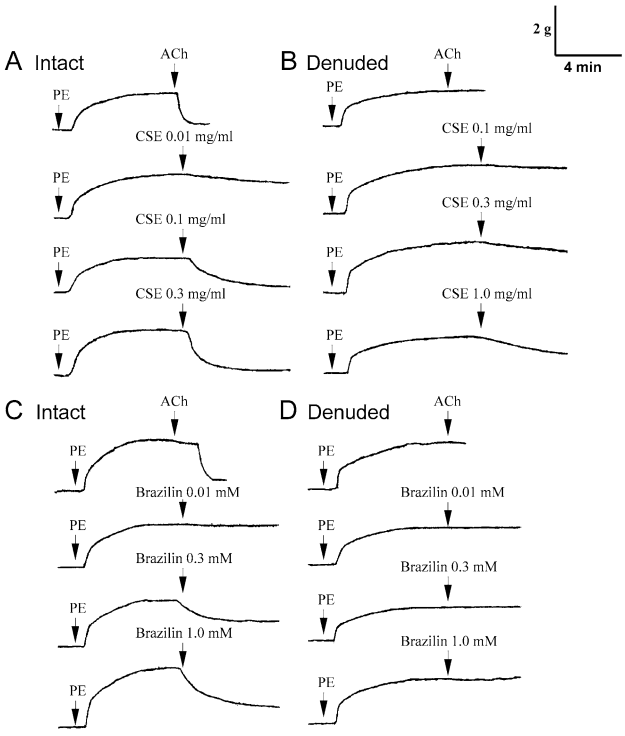

Fig. 1. Relaxation effect of *C. sappan* L. (CSE) and brazilin in isolated thoracic rat aorta. Phenylephrine (PE, 3 μM) induced a transient phasic contraction followed by tonic contraction, which lasted at least 15 min in intact or denuded endothelium rat aorta. Acetylcholine (ACh, 10 μM) induced relaxation in intact (A, upper trace) but not denuded (B, upper trace) aorta. *C. sappan* L. and brazilin dose-dependently relaxed phenylephrene-precontracted intact aorta (A and C), but partial or no relaxation was seen with denuded one (B and D). Each experiment was repeated more than three times.

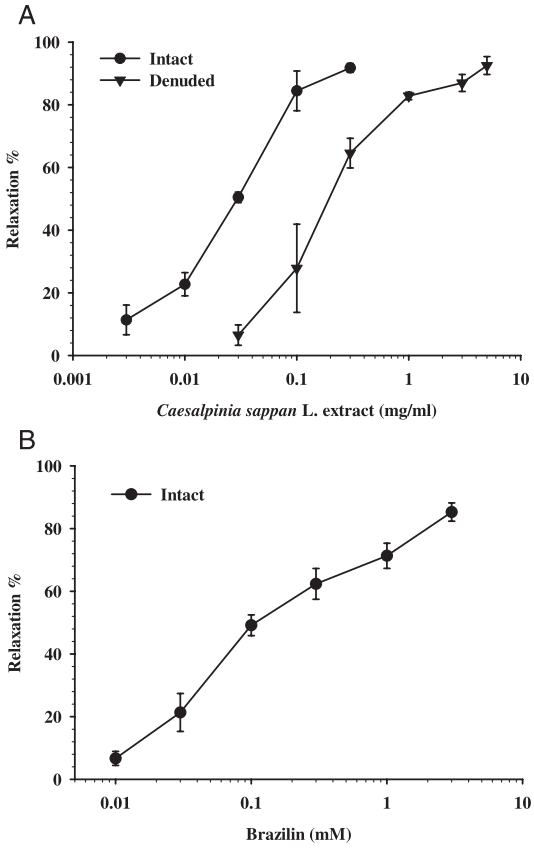

Fig. 2. Dose–response curve of vasorelaxation caused by *C. sappan* L. extract and brazilin on phenylephrine-precontracted rat aorta. (A) Various concentrations of the aqueous extract of *C. sappan* L. were added to the sustained contraction induced by phenylephrene (3 μM) at 37 °C in normal Krebs solution in order to induce relaxation in intact (●) and denuded (▼) aorta. (B) Brazilin (0.01–3 mM) was added to the sustained contraction induced by phenylephrine (3 μM) in order to induce relaxation in intact (●) aorta. Data are expressed as the means ± standard error of the mean (S.E.M.) for six individual determinations.

2.8. Statistical analysis

Data are expressed as the mean ± S.E.M. for the number of experiments indicated. Statistical analysis of the data was performed using Student's *t*-test, and $P < 0.05$ was considered significantly different. Values of IC_{50} were calculated and obtained from five regression lines; each regression line was constructed from three to five points. The values of inhibition of these points ranged between 20% and 80%.

3. Results

3.1. C. sappan L.- and brazilin-induced vasorelaxation in rat aorta

The effects of *C. sappan* L. and brazilin on blood vessels were studied using thoracic aorta isolated from rats. Phenylephrine (3 μM) induced contraction in aorta with (intact, Fig. 1A, upper trace) or without (denuded, Fig. 1B, upper trace) endothelium. Addition of acetylcholine (10 μM) caused relaxation in intact but not denuded aorta. *C. sappan* L. extract dose-dependently relaxed phenylephrene-induced contraction in both intact (0.03–0.3 mg/ml) (Fig. 1A) and denuded (0.1–1.0 mg/ml) (Fig. 1B) aorta with IC_{50} values of 34.17 ± 1.65 and 260.25 ± 66.98 μg/ml, respectively (Fig. 2A).

On the other hand, brazilin, the major component of *C. sappan* L., induced vasorelaxation only in intact but not denuded aorta (Fig. 1C and D). The effect was dose-dependent, and the IC_{50} value was 0.183 ± 0.03 mM (Fig. 2B). These results suggest that brazilin might be responsible for the endothelium-dependent vasorelaxation induced by *C. sappan* L. at lower concentrations.

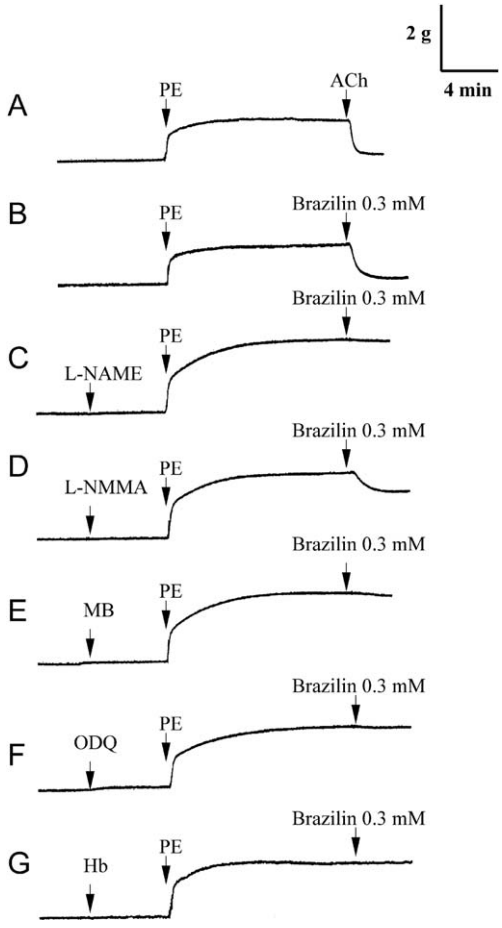

Fig. 3. Effect of nitric oxide synthase inhibitor, guanylyl cyclase inhibitor, and nitric oxide scavenger on brazilin-induced relaxation in isolated rat aorta. Acetylcholine (ACh, 10 μM) and brazilin (0.3 mM) were treated after phenylephrine (PE, 3 μM)-induced vasocontraction reached maximal force in intact aorta (traces A and B). Prior to addition of 0.3 mM brazilin, the aortic rings were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 50 μM) (trace C), N^G-monomethyl-L-arginine acetate (L-NMMA, 100 μM) (trace D), methylene blue (MB, 10 μM) (trace E), 1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxaline-1-one (ODQ, 10 μM) (trace F), and hemoglobin (Hb, 3 μM) (trace G) for 5 min followed by addition of phenylephrene. Each experiment was repeated at least six times.

3.2. Brazilin-induced vasorelaxation was inhibited by NOS inhibitor, guanylyl cyclase inhibitor, and NO scavenger

The involvement of NO in brazilin-induced vasorelaxation was further investigated. It has been shown that blockers of NO-related pathway, at higher concentrations, will affect the basal tension of the aorta (Romano et al., 2002; Ma et al., 1996; Kim and Greenberg, 2000), therefore, lower concentrations of blockers that exert minimal effects on the basal tension were chosen. As seen in Fig. 3, acetylcholine (10 μM, trace A) and brazilin (0.3 mM, trace B) relaxed phenylephrine-induced vasocontraction in endothelial intact aorta. Pretreatment of the aorta with either nonselective NOS inhibitor, L-NAME (50 μM; trace C), and selective eNOS inhibitor N^G-monomethyl-L-arginine acetate (L-NMMA, 100 μM; trace D), guanylyl cyclase inhibitor (methylene blue, 10 μM; trace E), and 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ, 10 μM; trace F) (Deply et al., 1996; Verde et al., 1999), or the NO scavenger, hemoglobin (3 μM; trace G), inhibited brazilin (0.3 mM)-induced vasorelaxation (Fig. 3). As seen in Fig. 3D, 0.1 mM L-NMMA cannot completely abolish 300 μM brazilin-induced vasorelaxation, but higher concentration of L-NMMA displayed 95% of relaxation inhibition (data not shown). These results suggest that activation of NOS and guanylyl cyclase was involved in brazilin-induced vasorelaxation.

3.3. Brazilin increased cGMP formation in rat aorta

As shown in Table 1, acetylcholine (10 μM) and sodium nitroprusside (10 μM) significantly increased cGMP content to 2.52 ± 0.76 and 2.83 ± 0.93 pmol/mg protein, respectively. Brazilin, at 0.3 mM, caused an increase in cGMP in intact but not denuded aorta. The effect was blocked by pretreatment with L-NAME (50 μM) and methylene blue (10 μM). Removal of extracellular Ca^{2+} (Ca^{2+}-free medium + 2.5 mM EGTA) also inhibited the effect of brazilin. These data further supported that, in isolated rat aorta, NOS activity, guanylyl cyclase activity, and extracellular Ca^{2+} were involved in brazilin-mediated vasorelaxation.

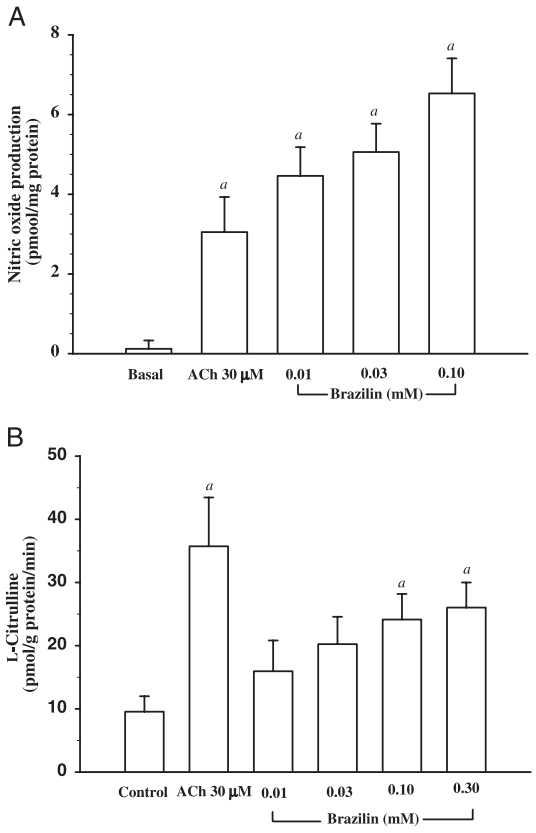

Fig. 4. Brazilin increased NO production and NOS activity in isolated human umbilical vein endothelial cells. (A) Brazilin (0.01–0.10 mM) and acetylcholine (30 μM) were incubated with cultured human umbilical vein endothelial cells for 30 min. Medium was then collected for NO detection with a nitric oxide analyzer as described in Materials and methods. (B) NOS activity was determined as the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. After treated with acetylcholine (30 μM) or brazilin (0.01–0.30 mM), the amount of [^{3}H]L-citrulline was counted by β-counter as described in Materials and methods. Data are expressed as the means ± standard error of the mean (S.E.M.) ($n = 6$); [a] $P < 0.05$, significantly different from respective control value by unpaired Student's *t*-test.

3.4. Effect of brazilin on NO formation and NOS activity

The direct effect of brazilin on endothelium was investigated in human umbilical vein endothelial cells.

Table 1
Effect of brazilin on cyclic guanosine 3′,5′-monophosphate (cGMP) formation in rat aorta

	Cyclic GMP (pmol/mg protein)				
	Intact	Denuded	L-NAME (50 μM)	Methylene blue (10 μM)	Ca^{2+}-free medium
Control	0.41 ± 0.04	ND	ND	ND	ND
Sodium nitroprusside (10 μM)	2.83 ± 0.93[a]	ND	ND	ND	ND
Acetylcholine (10 μM)	2.52 ± 0.76[a]	0.64 ± 0.19	0.48 ± 0.06	0.50 ± 0.03	0.44 ± 0.01
Brazilin (0.3 mM)	0.93 ± 0.13[a]	0.44 ± 0.09	0.51 ± 0.03	0.50 ± 0.02	0.56 ± 0.03

Rat aorta rings were first incubated in Krebs solution with 3-isobutyl-1-methylxanthine (IBMX, 10 μM) for 5 min and then with indicated drugs for 2 min. Reactions were stopped by immersing the aorta rings into liquid nitrogen and cGMP contents were measured as described in Materials and methods. Data are expressed as the means ± standard error of the mean (S.E.M.) for six individual experiments.
ND: not determined.
[a] $P < 0.05$: significantly different from control value by paired Student's *t*-test.

Table 2
Effects of calcium on brazilin-induced L-citrulline formation in human umbilical vein endothelial cells

	L-Citrulline (pmol/g protein/min)					
	Resting	Acetylcholine (30 μM)	Histamine (50 μM)	A23187 (5 μM)	Brazilin (0.1 mM)	Brazilin (0.3 mM)
Normal HBSS	9.55 ± 2.45	35.72 ± 7.73[a]	28.06 ± 3.51[a]	46.91 ± 3.46[a]	24.14 ± 4.04[a]	26.02 ± 3.97[a]
Ca^{2+}-free medium	− 0.68 ± 1.08[b]	1.06 ± 1.63[b]	4.46 ± 2.44[b]	11.18 ± 4.06[b]	− 1.12 ± 1.83[b]	0.18 ± 2.14[b]
BAPTA-AM	0.8 ± 1.70[b]	7.89 ± 1.49[b]	− 19.91 ± 1.94[b]	− 6 ± 2.70[b]	5.78 ± 1.83[b]	8.63 ± 4.26[b]

L-Citrulline formation was detected with chemical-treated cells according to procedures outlined in Materials and methods. Extracellular Ca^{2+} was removed by replacing the medium with Ca^{2+}-free medium in the presence of 2.5 mM EGTA. BAPTA-AM (20 μM) was used to chelate intracellular Ca^{2+}. Data are expressed as the means ± standard error of the mean (S.E.M.) for six individual experiments.

[a] $P < 0.05$: significantly different from resting value by paired Student's *t*-test.

[b] $P < 0.05$: significantly different from control value by paired Student's *t*-test.

Both NO production and NOS activity, as judged by the formation of L-citrulline, were both significantly induced by brazilin in a dose-dependent manner reaching a maximum at 0.3 mM (Fig. 4). In NO formation, acetylcholine (30 μM) significantly increased NO content relative to the control. Brazilin (0.01–0.10 mM) dose-dependently increased NO production (Fig. 4A). The effects of higher concentrations of brazilin (0.3 mM) were not determined due to interference by the color of brazilin on the NO determination.

In the NOS activity measurement, as shown in Table 2, acetylcholine (30 μM), histamine (50 μM), and A23187 (5 μM) significantly activated NOS compared to the control. The effect of brazilin (0.01–0.30 mM) on NOS activity was increased dose-dependently (Fig. 4B) and attenuated by either removal of extracellular Ca^{2+} or chelation of the intracellular Ca^{2+} with BAPTA-AM (Table 2). These data indicate that the influx of extracellular Ca^{2+} plays an important role in the brazilin-activated NOS pathway.

Table 3
Effect of brazilin on $^{45}Ca^{2+}$ influx in isolated human umbilical vein endothelial cells

	Percent increase of $^{45}Ca^{2+}$ influx
Resting	0
Acetylcholine (30 μM)	30.27 ± 10.8[a]
Histamine (50 μM)	35.49 ± 5.1[a]
A23187 (5 μM)	33.75 ± 10.26[a]
Brazilin	
0.001 mM	16.66 ± 0.98[a]
0.003 mM	25.95 ± 7.35[a]
0.01 mM	28.60 ± 7.96[a]
0.03 mM	36.00 ± 9.49[a]
0.1 mM	35.30 ± 3.74[a]

The brazilin-induced Ca^{2+} influx was measured in cultured human umbilical vein endothelial cells as described in Materials and methods using $^{45}Ca^{2+}$ as tracer. The percent increase was calculated by subtracting the radioactivity measured for the untreated resting aorta. Data are expressed as the means ± standard error of the mean (S.E.M.) for six individual experiments.

[a] $P < 0.05$: significantly different from resting value by paired Student's *t*-test.

3.5. Brazilin-induced Ca^{2+} influx

The influx of extracellular Ca^{2+} induced by brazilin was investigated by measuring $^{45}Ca^{2+}$. As shown in Table 3, acetylcholine (30 μM), histamine (50 μM), and A23187 (5 μM) significantly increased $^{45}Ca^{2+}$ influx as positive controls. Ca^{2+} influx was greatly enhanced upon addition of brazilin at a concentration of as low as 1 μM. These results demonstrated that brazilin can induce extracellular Ca^{2+} influx, and then further activate NOS activity.

4. Discussion

In this study, we found that the water extract of *C. sappan* L., at lower concentrations, and its major component, brazilin, dose-dependently induced vasorelaxation in phenylephrene-precontracted aorta in an endothelium-dependent manner. Higher concentrations of *C. sappan* L. extract also induced endothelium-independent vasorelaxation, suggesting a direct effect on smooth muscle cells.

Endothelium-derived relaxing factor has been identified as NO, which is produced from L-arginine by binding of Ca^{2+}/calmodulin to NOS (Moncada et al., 1991; Cobb et al., 1993; Palmer, 1993b; Sessa, 1994), and the NOS could be inhibited by L-NAME and L-NMMA (Ishikawa et al., 1995; Chan et al., 2000). Generally, the release of NO from endothelial cells will stimulate soluble guanylyl cyclase, which leads to an increasing production of cGMP in vascular smooth muscle (Rapoport and Murad, 1983), and methylene blue and ODQ can inhibit the activation of guanylyl cyclase (Ignarro, 1989; Wu et al., 1998). In addition, the NO could be scavenged by hemoglobin (Rioux et al., 1994: Kolpakov et al., 1995). In this study, we have found that brazilin relaxed the precontracted aorta in an endothelium-dependent manner, and that the effect could be inhibited by NOS inhibitor (L-NAME, L-NMMA), guanylyl cyclase inhibitor (methylene blue, ODQ), and the nitric oxide scavenger, hemoglobin. These data indicate that the formation of NO and cGMP through the activation

of NOS and guanylyl cyclase, respectively, might be involved. This is further supported by the fact that brazilin caused an increase in cGMP accumulation in rat aorta, and this effect could also be inhibited by L-NAME and methylene blue. The inability of the eNOS specific inhibitor L-NMMA to completely block the brazilin-induced vasorelaxation even at high concentration might suggest that NOS other than eNOS might also be activated during the brazilin treatment. On another view, protein kinase C-mediated feedback inhibition of phosphoinositide hydrolysis in endothelial cells may cause in numerous altered physiologic response. For instance, ATP and ADP could release NO, since the release of NO in response to these agonists is inhibited by protein kinase C activation (De Nucci et al., 1988; Smith and Lang, 1990; Oriji and Keiser, 1996). Brazilin had been previously reported to regulate blood glucose in diabetic mice, which may be through inhibition of protein kinase C activity and insulin receptor serine kinase (Moon et al., 1993). Therefore, the ability of protein kinase C inhibition by brazilin and aqueous extract probably involved its relaxing action. In addition, we found that the increase in cGMP content induced by brazilin was inhibited when the extracellular Ca^{2+} in the medium was removed, suggesting that the vasorelaxing effect induced by brazilin might be caused by an influx of extracellular Ca^{2+}.

The Ca^{2+} signal pathway is important in the regulation of many cellular processes, including the release and secretion of many kinds of chemical mediators. In endothelial cells, NO synthesis by NOS is activated by an increase in the intracellular Ca^{2+} concentration (Furchgott, 1983; Lopez-Jaramillo et al., 1990). The possible involvement of Ca^{2+} in the above effects seen with brazilin on isolated aorta was then further investigated using primary cultured endothelial cells from human umbilical veins. In the present study, we found that in cultured human umbilical vein endothelial cells, brazilin dose-dependently increased both NOS activity and NO formation. As seen with the aorta, the effect of brazilin-induced cGMP formation could be attenuated by removal of extracellular Ca^{2+} or by chelating the intracellular Ca^{2+} with BAPTA-AM. These data suggest that brazilin can promote Ca^{2+} influx from the extracellular space and cause an increase in intracellular Ca^{2+} followed by NO system activation. Khil et al. (1997) reported that the hypoglycemic effect of brazilin could be inhibited by quin 2-AM, an intracellular Ca^{2+} chelator, and by trifluoperazine, a Ca^{2+}/calmodulin complex inhibitor. Hwang et al. (1998) also showed that brazilin can inhibit platelet aggregation by reducing the activity of phospholipase A_2 and the elevation of intracellular Ca^{2+}. Our data on $^{45}Ca^{2+}$ measurement further support brazilin either inducing the opening of Ca^{2+} channels or increasing the Ca^{2+} permeability in endothelial cells. Results from this study show that the increase in intracellular Ca^{2+} induced by brazilin might be responsible for all the effects seen in isolated aorta and primary cultured human umbilical vein endothelial cells.

The water extract of *C. sappan* L. exerts both endothelium-dependent and -independent responses in a dose-dependent manner with an EC_{50} of 34.17 ± 1.65 and 260.25 ± 66.98 μg/ml, respectively. These data suggest that *C. sappan* L. might possess a multiple biochemical and pharmacological actions, only part of which involve the content of brazilin. Induction of vasorelaxation by *C. sappan* L. in denuded aorta at higher concentrations indicates that *C. sappan* L. might have direct effects on smooth muscle. The detailed mechanism still awaits further investigation.

C. sappan L. and brazilin have long been used in Oriental folk medicines to promote blood circulation with little scientific evidence. According to our data, *C. sappan* L. extract was shown to have an effective dose around 0.01–0.1 mg/ml, which is the dose easy to reach in vivo. Finally, the results from this study provide a better understanding of the effect of *C. sappan* L. and brazilin on blood circulation and their pharmacological mechanism.

Acknowledgements

This study was supported in part by a grant from the National Science Council, Taiwan (NSC-89-2320-B-038-074).

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