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Nitric oxide-dependent vasodilatation by ethanolic extract of *Hancornia speciosa* via phosphatidyl-inositol 3-kinase

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Abstract

The vasodilator effect of the ethanolic extract of leaves from *Hancornia speciosa* Gomes (HSE) was studied in rat aortic rings. HSE produced a concentration-dependent vasodilatation ($pIC_{50} = 5.6 \pm 0.1$), which was completely abolished in endothelium-denuded vessels. The endothelium-dependent vasodilatation induced by HSE was abolished by L-NAME (100 μM), a nitric oxide (NO) synthase inhibitor, but not atropine (1 μM; $pIC_{50} = 5.6 \pm 0.2$), a muscarinic receptor antagonist, nor indomethacin (10 μM; $pIC_{50} = 5.4 \pm 0.2$), a cyclooxygenase inhibitor. The concentration–response curve of HSE was significantly shifted to the left by superoxide dismutase (SOD; 300 U/mL). In addition, while SOD displaced the 3-morpholino-sidnonimine (SIN-1; $P < 0.05$) concentration–effect curve to the left, HSE (50 μg/mL) had no effect. Finally, wortmannin (0.3 μM), an inhibitor of phosphatidyl-inositol 3-kinase (PI3K), dramatically reduced the vasodilator effect of HSE. Together, these findings lead us to conclude that HSE induces a NO- and endothelium-dependent vasodilatation in rat aortic preparations, likely by a mechanism dependent on the activation of PI3K.

Keywords: Nitric oxide; Endothelium; eNOS; Phosphatidyl-inositol 3-kinase; *Hancornia speciosa* Gomes

1. Introduction

1.1. Plant

Hancornia speciosa Gomes (Apocynaceae), popularly named "mangaba", is a plant species found in *cerrado*, a savanna-like vegetation in Brazil (Corrêa, 1974). The leaves of *Hancornia speciosa* were collected in São Gonçalo do Rio Preto, Minas Gerais state, Brazil, in November 1999. Dr. J.A. Lombardi, from the Botanical Department, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil, identified the species and a voucher specimen (BHCB 3565) can be found in the UFMG Herbarium.

1.2. Uses in traditional medicine

The bark of the *Hancornia speciosa* (HSE) is commonly used to treat dermatitis, diabetes, and hepatic diseases, and is also used as an anti-inflammatory agent, whereas its roots and leaves are employed to treat rheumatism and hypertension (Grandi et al., 1982; Britto and Britto, 1982; Hirschmann and Arias, 1990). Recently, we have demonstrated that the ethanolic extract of leaves from this species inhibits angiotensin I-converting enzyme (ACE) (Serra et al., 2005). This report drove us to further investigate the cardiovascular effects induced by HSE, since another plant known to inhibit ACE, *Ouratea semiserrata*, also exhibited strong vasodilator activity (Braga et al., 2000; Cortes et al., 2002).

1.3. Previously isolated class of constituents

The only constituents isolated were volatile compounds from the fruit of *Hancornia speciosa* (Sampaio and Nogueira, 2006).

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There is no report of compounds isolated from the leaves or other parts of this plant.

2. Materials and methods

2.1. Preparation of extract

After drying at 40 °C for 72 h, the plant material was powdered (10 g) and extracted with 96% EtOH (3× 30 mL) under sonication (3× 15 min). The solvent was vacuum removed in a rotavapor evaporator at 70 °C, leaving a dark residue.

2.2. Animals

Male Wistar rats (200–250 g) from the Animal Care facilities (CEBIO) at UFMG were used. They were kept at 22–25 °C in a 12 h light/dark cycle, and had free access to food and water. Animal experiments were performed according to the recommendations of the Brazilian Council for Animal Care and were approved by the Ethics Committee of the Federal University of Minas Gerais.

2.3. Rat aortic rings preparation and mounting

The descending thoracic aorta were prepared and mounted as previously described (Cortes et al., 2002).

2.4. Vasorelaxant activity in precontracted rat aortic rings

The determination of vasorelaxant activity was performed in aortic rings with or without functional endothelium, precontracted to the same tension (1.5 g) with submaximal concentrations of phenylephrine (0.3 or 0.1 μM, respectively). HSE was added in increasing cumulative concentrations once the response to phenylephrine had stabilized. To evaluate the participation of endothelium-derived products and muscarinic receptors in the relaxant effect of HSE, experiments were carried out in the presence of L-NAME (100 μM), indomethacin (10 μM), atropine (1 μM) or superoxide dismutase (SOD, 300 U/mL), added to the bath 20 min prior to the addition of phenylephrine. In another set of experiments, the participation of the PI3K in the vasorelaxant effect of HSE was investigated by pre-treatment of aortic rings for 30 min with wortmannin (0.3 μM). The concentration–response curves to 3-morpholinosidnonimine (SIN-1) were performed in endothelium-denuded rat aortic rings.

2.5. Drugs

Acetylcholine chloride, atropine sulphate, indomethacin, L-NAME, L-phenylephrine chloride, SIN-1, SOD and wortmannin were purchased from Sigma (St. Louis, MO, USA). Indomethacin was dissolved in 0.5% (w/v) sodium bicarbonate and the other drugs were dissolved in distilled water at a concentration of 10 mM. All subsequent dilutions were made with

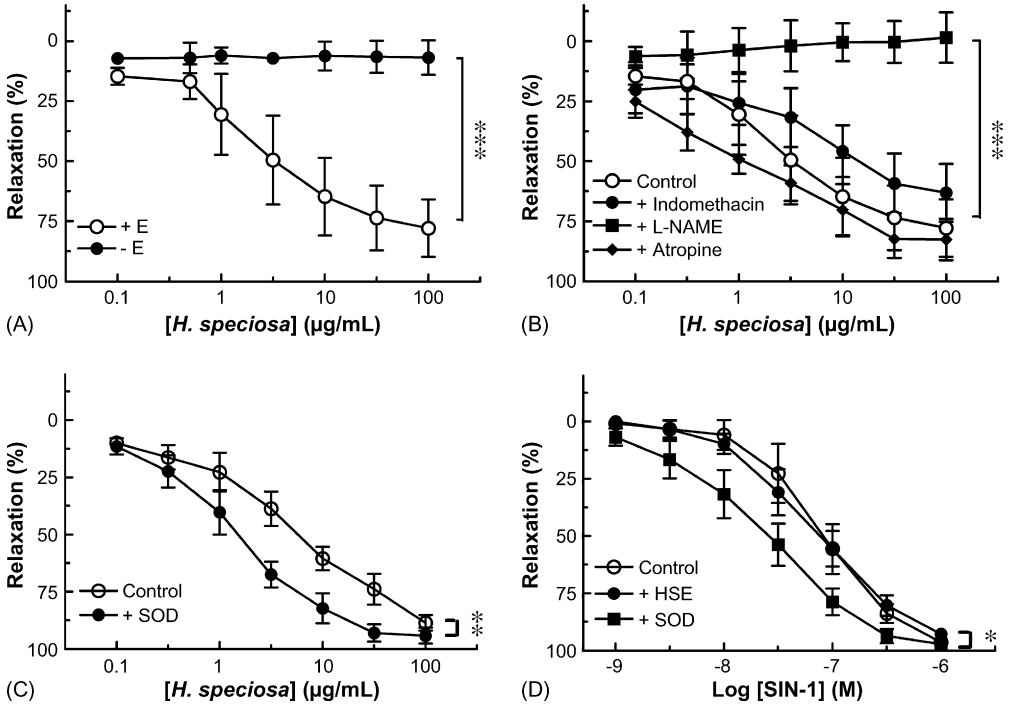

Fig. 1. Concentration–response curves of HSE (A–C) and SIN-1 (D) on phenylephrine-precontracted rat aortic rings in the presence (+E) or absence (−E) of a functional endothelium. The curves of HSE were generated in the absence (control) or presence of L-NAME (100 μM), indomethacin (10 μM) or atropine (10 μM) (B) and of SOD (300 U/mL) (C) in arteries with a functional endothelium. The curves of SIN-1 (D) were performed in the absence (control) or in the presence of HSE (50 μg/mL) or SOD (300 U/mL) in arteries without a functional endothelium. The results are expressed as mean ± S.E.M. of at least five experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ compared to the control curve and $^{*}P < 0.05$ for values in the presence of SOD compared to control.

Krebs–Henseleit solution with the following composition (mM): NaCl 110.8, KC1 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, NaH_2PO_4 2.33 and glucose 11.51; immediately before use.

2.6. Statistical analysis

The experimental data are expressed as mean ± standard error mean (S.E.M.) of at least five experiments. Statistical analyses were performed using unpaired Student's *t*-test. Concentration–response curves were compared by two-way ANOVA plus Bonferroni's post-test. Results were considered significantly different when $P < 0.05$. The values of pIC_{50} represent the −log of the values of the concentrations (in grams for HSE and in molar for SIN-1) that induce 50% reduction of the sustained contraction induced by phenylephrine.

3. Results and discussion

HSE elicited a concentration-dependent relaxation in aortic rings with functional endothelium (Fig. 1A; Table 1). This effect was completely abolished in the absence of a functional endothelium (Fig. 1A), indicating that the vasodilator effect of HSE is dependent on endothelium-derived relaxing factors. To evaluate the participation of NO in the vasodilator effect, aortic rings were treated with L-NAME, a classical NO synthase inhibitor. In this experimental condition, the HSE-induced vasodilatation was completely abolished (Fig. 1B), similar to what was observed in endothelium-denuded aortic rings, suggesting that NO is the main endothelium-derived factor involved in HSE activity. On the other hand, vasodilatation was not modified in aortic rings treated with indomethacin (Fig. 1B; Table 1), a cyclooxygenase inhibitor, at a concentration which inhibited contraction by arachidonic acid (data not shown). In addition, atropine, a selective muscarinic antagonist, was also unable to inhibit HSE-induced vasodilatation (Fig. 1B; Table 1), although it strongly inhibited vasodilatation by 1 μM ACh (data not shown). These findings not only demonstrate that prostanoids are likely not involved in HSE-induced vasodilatation, but also that the HSE effect is not due to activation of muscarinic receptors, a possible mechanism in NO- and endothelium-dependent vasodilatation. When the vessels were pre-treated with superoxide dismutase (SOD) a shift to the left in the concentration–response curve of HSE was observed (Fig. 1C; Table 1), demonstrating that dismutase of superoxide anions induce a protective action on the NO-dependent vasodilator effect of HSE. Furthermore, HSE did not modify the concentration–response curve from morpholino-sidnonimine (SIN-1), a nitric oxide donor, in aortic rings without a functional endothelium, but SOD induced a significant shift to the left (Fig. 1D; Table 1). Therefore, these data suggest that the effect of HSE is probably not due to an increase in the bioavailability of NO as consequence of its antioxidant effect.

Endothelium-derived NO plays an important role in the control of vascular homeostasis. NO modulates vascular tone, growth of vascular smooth muscle cells, and decreases platelet adhesion and aggregation. It also decreases the adherence of other blood components (Moncada et al., 1991; Scott-Burden and Vanhoutte, 1994). A decrease in NO production by vascular endothelial cells is closely associated with endothelial dysfunction or injury, which is thought to be an important factor in pathologies such as atherosclerosis, restenosis and hypertension (Lüscher, 1994; Busse and Fleming, 1996). Therefore, the development of new therapeutic agents capable of increasing production or bioavailability of NO is extremely relevant for the treatment of several cardiovascular related diseases.

Activation of endothelial nitric oxide synthase (eNOS) depends on the formation of calcium–calmodulin complex (Fleming and Busse, 2003). However, eNOS can also be activated by phosphorylation with PI3K, a calcium-independent mechanism (Dimmeler et al., 1999). The PI3K-dependent activation of eNOS by wine-derived polyphenolic compounds has been recently reported (Ndiaye et al., 2005). A preliminary analysis of the extract suggested the presence of polyphenols in HSE (not shown). Therefore, the participation of the PI3K pathway was evaluated as a mechanism involved in HSE-induced vasodilatation. For this purpose, the aortic rings were pre-treated with wortmannin, an inhibitor of PI3K, 15 min before precontraction with phenylephrine. Wortmannin strongly reduced HSE-induced vasodilatation (Fig. 2), suggesting that PI3K-dependent activation of eNOS is an important underlying mechanism in the vasodilator effect of HSE. In addition, we also observed that wortmannin inhibited the relaxation induced by 10 μM insulin,

Table 1
pIC_{50} values for the vasodilator effect induced by HSE and SIN-1 in the presence of different pharmacological agents

	Indomethacin	Atropine	SOD	HSE
HSE				
Control	5.6 ± 0.1	5.6 ± 0.1	5.1 ± 0.2	–
Treatment	5.4 ± 0.2	5.6 ± 0.2	5.8 ± 0.1**	–
SIN-1				
Control	–	–	7.0 ± 0.2	7.0 ± 0.2
Treatment	–	–	7.6 ± 0.2*	7.1 ± 0.2

All values are given as mean ± S.E.M.—drugs not tested. The vasodilatation effect of HSE was observed in endothelium-intact aortic rings, while that of SIN-1 was performed in endothelium-denuded aortic rings.
* $P < 0.05$ as compared to control.
** $P < 0.01$ as compared to control.

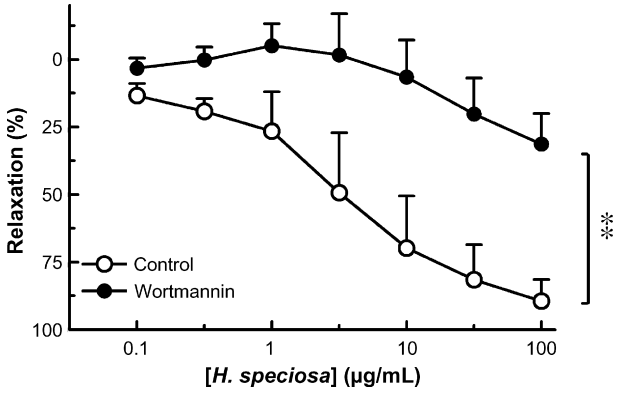

Fig. 2. Concentration–response curve of HSE on phenylephrine-precontracted rat aortic rings with a functional endothelium, in the absence (control) or in the presence of wortmannin (0.3 μM). The results are expressed as mean ± S.E.M. of five experiments. ** $P < 0.01$ compared to control.

but not with 1 μM ACh (data not shown). Ndiaye et al. (2005) reported that an increase in the production of superoxide was responsible for redox-sensitive activation of PI3K by polyphenolic compounds of wine, leading to subsequent activation of eNOS. Conversely, Krotz et al. (2005) reported an increase in ROS production through activation of a NAD(P)H-oxidase-dependent mechanism via PI3K-dependent phosphorylation in human umbilical vein endothelial cells, resulting in reduction of NO bioavailability. Both works describe the relevance of the PI3K pathway in the endothelium-mediated control of vascular tone. If, in rat aortic endothelial cells, the activation of PI3K increased the production of ROS, the inhibition by wortmannin of the vasodilator effect of HSE could be related with its inability to further protect the basal production of NO. If this were true, it would mean that HSE was acting as an ROS scavenger or as inhibitor of production of ROS. However, in the present work, we observed that HSE was not able to modify the concentration–response curve of SIN-1 as observed with SOD, using a method previously described as a valuable pharmacological protocol for characterization of antioxidant substances in isolated arteries (Andriantsitohaina et al., 1999). In addition, SOD also induced a significant leftward shift in the concentration–response curves of HSE, suggesting that even in the presence of HSE, ROS were produced. Consequently, an antioxidant effect of HSE may not be the mechanism involved on its NO- and endothelium-dependent vasodilator effect. For the above reasons, our results suggest that wortmannin is selectively inhibiting only the NO-dependent vasodilatation induced through activation of eNOS by PI3K (Sowers, 1997).

In conclusion, in the present study we demonstrated that HSE induces potent vasodilatation in rat aorta through activation of a mechanism dependent on NO production, likely via activation of PI3K.

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