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Potent antihypertensive effect of *Hancornia speciosa* leaves extract

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ABSTRACT

Background: Hancornia speciosa Gomes is an herb traditionally used in Brazil for blood pressure control.
Purpose: The present work investigated the antihypertensive effect of an extract from *Hancornia speciosa* leaves (SFH) and analyzed its underlying mechanisms of action.
Methods: Hypertension was induced in mice by surgical removal of a kidney and by subcutaneous administration of a pellet with deoxycorticosterone. Vasodilatation was measured in mesenteric arteries with a wire myograph. Nitrites were measured by fluorescence with 2,3-diaminonaphthalene and H_2O_2 was measured with carbon microsensors.
Results: SFH (0.03, 0.1 or 1 mg/kg; po) induced a dose-dependent, long-lasting reduction in the systolic blood pressure in conscious DOCA-salt hypertensive mice (DOCA). Administration of SFH produced a significant increase in the plasmatic level of nitrites. The systemic inhibition of nitric oxide synthase by L-NAME (20 mg/kg) reduced its antihypertensive effect. SFH also induced a concentration-dependent vasodilatation of mesenteric resistance arteries contracted with phenylephrine, which was more potent in arteries from DOCA mice. Removal of the endothelium or pretreatment with L-NAME or catalase reduced the vasodilator response for SFH. The nitrite production induced by SFH was significantly bigger in mesenteric arteries from DOCA than in SHAM mice. However, the production of H_2O_2 induced by SFH was twice higher in DOCA mice.
Conclusion: Altogether, our results point to an antihypertensive effect of SFH due to a reduction in peripheral resistance through the production of NO and by a mechanism involving an increased production of H_2O_2 in the mesenteric arteries from hypertensive mice. These findings are further evidence to support the use of *Hancornia speciosa* by traditional medicine as an antihypertensive drug.

Introduction

Hypertension is a major risk factor that predisposes to cardiovascular disorders and is responsible for a large morbidity and mortality in patients (Lawes et al. 2008). Hypertension is closely related to the development of an impaired vascular function associated with an endothelial dysfunction and oxidative stress (Endemann and Schiffrin 2004). In this sense, several animal models of hypertension present a similar profile of impaired vascular function associated with an endothelium dysfunction (Ghiadoni et al. 2012).

One of the main problems related to the management of hypertension is the adherence to the treatment, which is estimated as 57% of the patients (Naderi et al. 2012). The interruption of treatment is highly associated with the failure in restoring the physiological blood pressure, mostly resulting from resistant hypertension that reaches approximately 20% of treated individuals (Cushman et al. 2002). The side effects related to the treatment are the primary reason for non-adherence to the treatment (Naderi et al. 2012). Therefore, there still are spaces for the development of new drugs with the ability to improve the vascular function by acting on targets that regulate the vascular smooth muscle contraction and revert or reduce the endothelial dysfunction (Fu et al. 2014). Phytochemicals consumed on a diet or as traditional medicines have been associated with a reduction in cardiovascular diseases (Hertog et al. 1993) and reduction of blood pressure

Abbreviations: (SFH), extract from *Hancornia speciosa* leaves; (NO), nitric oxide; (H_2O_2), hydrogen peroxide; (DOCA), deoxycorticosterone; (DAN), 2,3-diaminonaphthalene; (SBP), systolic blood pressure; (NOS), nitric oxide synthase.

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(Faraji and Tarkhani 1999). Therefore, the investigation of plants used in traditional medicine for the treatment of hypertension may lead to the development of new drugs.

Hancornia speciosa Gomes (Apocynaceae), commonly known as "mangabeira", is a plant species found in savanna-like vegetation in Brazil. The chemistry of the ethanolic extract from *H. speciosa* leaves comprises flavonoids, chlorogenic acid and L-(+)-bornesitol (Endringer et al. 2009; Ferreira et al. 2007). This extract inhibited the angiotensin-converting enzyme (ACE) (Serra et al. 2005) and promoted vasorelaxant effects in rat aorta (Ferreira et al. 2007). More recently, our group demonstrated that a refined dry extract from *H. speciosa* leaves (SFH) has a hypotensive effect in mice by a mechanism dependent on inhibition of ACE and increased production of NO in mice (Silva et al. 2011).

The present study aimed at investigating the antihypertensive effect of the SFH and its respective mechanism of action in hypertensive mice.

Materials and methods

Hancornia speciosa *leaves extraction*

The leaves of *Hancornia speciosa* Gomes (checked with http://www.theplantlist.org) were collected in São Gonçalo do Rio Preto, Minas Gerais state, Brazil, in October 2003 (voucher specimen BHCB 49895, deposited at the Herbarium of the Universidade Federal de Minas Gerais – UFMG). After drying at 40 °C for 72 h in a ventilated oven, the leaves were grinded in a knife mill. The extract of *Hancornia speciosa* leaves (SFH) was prepared by a two-step process: 250 g of the ground leaves were exhaustively percolated with 96% EtOH (total volume: 17.5 l) at room temperature. The ethanol extract was evaporated under reduced pressure to give 69 g of a dark residue. It was then submitted to column chromatography over silica gel (0.2–0.5 mm; 37.0 × 6.8 mm i.d.) sequentially eluted with (500 ml each) *n*-hexane, dichloromethane, dichloromethane / ethyl acetate (1:1 v/v), ethyl acetate and ethyl acetate / methanol (1:1 v/v). Following, the dichloromethane / ethyl acetate (1:1) eluate (500 ml) was evaporated under vacuum to furnish 31.4 g of the dry extract SFH. According to EMA guideline (European Medicines Agency (HMPC) 2010), the extract is "other herbal preparation" declared as: refined dry extract from *Hancornia speciosa* Gomes, leaves (DER = 8:1). Extraction solvent: ethanol 96% v/v.

Since bornesitol and flavonoids are related to the biological activity of the extract (Endringer et al. 2014; Pereira 2012; Ferreira et al. 2007), they were chosen as analytical markers. The content of bornesitol (7.75 ± 0.78% w/w) was determined using a HPLC method developed by us (Pereira et al. 2012), whereas total flavonoids (14.52 ± 0.44% w/w) were quantified by a spectrophotometric method (Pereira 2012). Both methods were validated according to ICH guideline (International Conference on Harmonization (ICH) 1996).

Animals

All experimental protocols were performed in accordance with guidelines for the humane use of laboratory animals at our Institute and were approved by local ethics committee (protocol # 227/08, UFMG). Male Swiss mice (12–15 week-old) were used. All animals were obtained from CEBIO (Centro de Bioterismo – Instituto de Ciências Biológicas, UFMG). Free access was allowed to the standard diet (Labina, São Paulo, Brazil), and tap water was supplied ad libitum. All mice were maintained at eight per cage at a constant temperature (24 °C), with 12-h dark/light cycle.

Induction of hypertension

Mice (25–30 g) were unilaterally nephrectomized under anesthesia using a solution containing ketamine (500 mg/ml) and xylazine (20 mg/ml), administered once by intraperitoneal route. The skin over the left flank was shaved, and a 1.5 cm incision was made through the skin and underlying muscle caudal to the rib cage. The left kidney was externalized and removed after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon, Inc, Somerville, NJ, USA). The muscle and skin layers were then closed separately with 4-0 silk sutures. A small area between the shoulder blades was shaved, and a 1 cm incision was made for implanting DOCA pellets to provide a dose of 1 mg/kg. The DOCA-salt mice were given water containing 0.9% NaCl and 0.2% KCl. The sham mice were also unilaterally nephrectomized, but they did not receive a DOCA pellet and were given tap water. All mice were placed on standard pellet rodent chow. After recovery, the mice were housed under standard conditions for 4 weeks, after which their systolic BP was determined by the tail-cuff method (Silva et al. 2013).

Blood pressure measurements

SBP and HR were measured by the tail-cuff method (Gross and Luft 2003) using the XBP1000 series rat tail blood pressure system (Kent Scientific, Torrington, USA). Conscious rats were conditioned in restraints in a warming chamber controlled at 37 °C for no more than 5 min. Thereafter, an integrated sensor cuff was placed at the tail and used to take at least 7 different pressure measurements from SBP. Measurements were taken every 15 or 30 min for 3 h and recorded using a DI-194RS data acquisition system (Dataq, Akron, USA) connected to a personal computer. After the measurement of the basal SBP, each animal randomly received 0.03, 0.1, 1 mg/kg of SFH or 100 mg/kg captopril by oral route.

Nitrite dosage in the serum

Nitrite (NO_2^-) was measured by using the Griess reaction with modifications (Grisham et al. 1996; Silva et al. 2011). The animals were treated by intragastric gavage with 1 mg/kg SFH or 20 mg/kg L-NAME or saline. One hour after, the animals were sacrificed by decapitation. Briefly, an aliquot (100 μl) of mice serum was added to a microtiter plate and the enzymatic treatment was started by adding 10 UL/ml *Aspergillus* nitrate reductase (Sigma, São Paulo, Brazil), 1 M HEPES buffer (pH 7.4), 0.1 mM FAD, 1 mM NADPH. After homogenization, the mixture was incubated for 30 min, at 37 °C. 1500 U/ml lactate dehydrogenase and 100 mM pyruvic acid were then added and mixed for 10 min at 37 °C. Following the above enzymatic treatment steps, 500 μl of the sample was added to each freshly prepared 500 μl Griess. The absorbance of each sample was then determined at 540 nm, and total nitrite concentrations were calculated from the slope of the standard curves established using known concentrations of nitrite. The water was used as blank solution.

Myograph studies

Mice were euthanized by decapitation. The abdomen was immediately opened and the mesenteric arcade removed. The branch II of the mesenteric resistance arteries in the mice were cleaned of fat and connective tissue, and a segment of approximately 1.6–2.0 mm in length was removed as previously described (Silva et al. 2013). The segments were then mounted in physiological salt solution (PSS) of the following composition (mmol/l): 119 NaCl; 4.7 KCl; 0.4 KH_2PO_4; 14.9 $NaHCO_3$; 1.17 $MgSO_4$; 2.5 $CaCl_2$, and 5.5 glucose. Mechanical activity was recorded isometrically

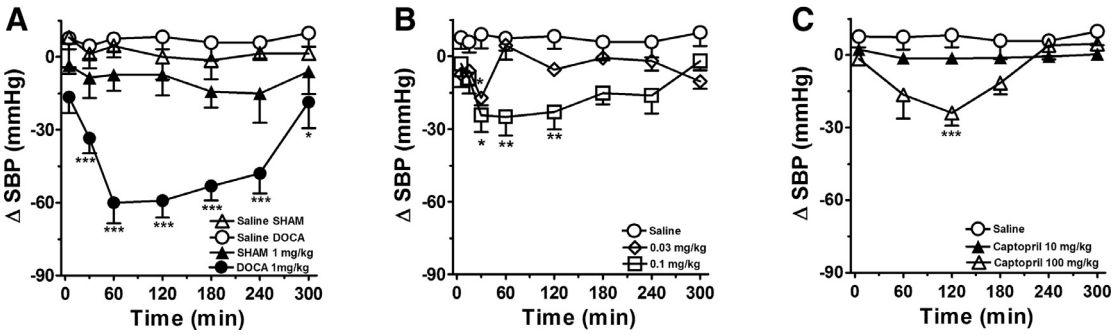

Fig. 1. Effect of the *Hancornia speciosa* leaves extract (SFH; A and B) and captopril (C) in the systolic blood pressure (SBP) of normotensive SHAM (A) and hypertensive DOCA (A, B and C) mice. The first point represents the effect observed five minutes after the oral administration of saline or SFH. The values are the mean ± SEM of five experiments $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus saline DOCA.

using a wire myograph (DMT, Aarhus, Denmark). Once in the myograph, the artery was stretched to a length that yielded a circumference equivalent to 90% of that given by the internal pressure of 100 mmHg; this required a load of approximately 200 mg. The vessel was maintained for an equilibration period of 60 min. Concentration-response curves of either freshly prepared SFH (0.1–300 g/l) were conducted in vessels pre-contracted to the same tension level (approximately 2.5 mN/mm) with submaximal concentrations of phenylephrine (0.03–0.1 mmol/l). Changes in the isometric tension were analyzed using PowerLab software (ADInstruments, Bella Vista, Australia).

Indirect measurements of NO production in small mesenteric artery

Nitrite amount were assessed by a fluorescent method using 2,3-diaminonaphthalene (DAN; Sigma, USA) with modifications (Misko et al. 1993). Mesenteric artery segments were removed as described above, incubated in tubes containing PSS, and maintained at 37 C. Vessels were stimulated with SFH (1 μg/ml or 3 μg/ml) and 150 μl of perfusate taken 5 min later. Briefly, 20 μl of 0.05 mg/ml DAN were added to 50 μl of supernatant. After 10 min incubation at 37 °C protected from light, the reaction was stopped with 10 μl of 2.8 mol/l NaOH. Formation of fluorescent product was measured using a fluorescent plate reader (Cary Eclipse Microplate reader; Varian, Palo Alto, USA) with excitation at 360 nm and emission read at 440 nm with a gain setting at 100%.

H_2O_2 measurements

H_2O_2 production in the mesenteric artery was measured using carbon microsensors with an H_2O_2 permeable membrane (ISO-HPO100, World Precision Instruments Inc., Sarasota, USA). Mesenteric artery segments were removed as described above, incubated in tubes containing PSS, and maintained at 37 °C. Vessels were stimulated with SFH (1 μg/ml or 3 μg/ml). Carbon microsensors were stabilized for at least 1 h in PSS and then placed next to the lumen of the vessels prior to SFH (1 μg/ml or 3 μg/ml) treatment. Currents (nA) were measured by microsensors, and H_2O_2 concentrations were determined by calibration curves of known concentrations of H_2O_2 (1 nmol/l to 10 μmol/l).

Statistical analysis

Data are expressed as mean ± S.E.M. Two-way ANOVA with Bonferroni multiple comparisons post-test was used to compare SBP and concentration-response curves obtained in small mesenteric arteries. One-way ANOVA with Bonferroni multiple comparisons post-test was used in the other experiments.

Results and discussion

Antihypertensive effect on DOCA hypertension

The systolic blood pressure (SBP) of SHAM mice was 124.0 ± 2.0 mmHg ($n = 6$) and remained constant throughout the period of investigation, while in DOCA mice the SBP was 178.0 ± 4.0 mm Hg ($n = 6$) four weeks after the surgical procedure, reflecting the installation of hypertension.

As illustrated in Fig. 1A, oral administration of SFH (1 mg/kg) induced a significant antihypertensive effect only in DOCA mice. The maximal reduction in the SBP was reached after 60 min in DOCA (−60.0 ± 8.5 mmHg; $P < 0.01$). In DOCA mice, oral administration of 0.03 and 0.1 mg/kg of the SFH also induced a significant reduction in the SBP, as can be observed in Fig. 1B. However, the reduction of the SBP induced by 0.03 mg/kg lasted for a short period, with a significant effect observed only after 30 min, while the dose of 0.1 mg/kg reduced SBP significantly up to 120 min after the administration of SFH. These results suggest that SFH has a favorable bioavailability by the oral route. Rutin, bornesitol, quinic acid and chlorogenic acid are the main compounds found in SFH (Endringer et al. 2009). Quinic acid and bornesitol have no reported effect as antihypertensive drugs. The hypotensive effect of rutin has been previously demonstrated (Lapa et al. 2011) and ascribed to an increase in the plasmatic level of quercetin in mice (Perez-Vizcaino et al. 2009). The antihypertensive effect of chlorogenic acid has been also reported (Suzuki et al. 2006).

The DOCA model of hypertension is not related to an increased circulating level of angiotensin II (Lockette et al. 1986). In this sense, captopril (10 mg/kg, po) did not reduce SBP in DOCA mice (Fig. 1C), as previously observed in normotensive mice (Silva et al. 2011), but at the higher dose of 100 mg/kg it reduced SBP significantly with a peak effect 120 mins after administration (Fig. 1C). However, the duration and intensity of the effect induced by captopril was smaller than observed with SFH. These results confirm the non-involvement of angiotensin II in this model of hypertension. In addition, the obtained results also suggest that in DOCA mice the antihypertensive effect of SFH is rather related to the production of NO than to the inhibition of ACE, as previously reported in normotensive mice (Silva et al. 2011).

Nitric oxide production and systolic blood pressure

NO plays an important role in the cardiovascular system. It modulates vascular tone, decreases platelet adhesion and aggregation, and inhibits the growth of vascular smooth muscle cells (Radomski et al. 1987; Vallance et al. 1989). Impairment of NO production by vascular endothelial cells is associated with vascular dysfunction, which is known to be an important factor

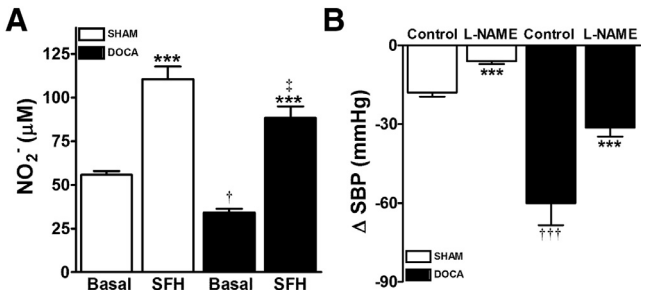

Fig. 2. Effect of the *Hancornia speciosa* leaves extract (SFH; 1 mg/kg) in the plasma concentration of nitrite (A) and the effect of L-NAME in the reduction of the systolic blood pressure (SBP) induced by SFH, shown as control (B) in normotensive SHAM and hypertensive DOCA mice. All results are expressed as mean ± S.E.M. of five experiments. $^{***}P < 0.001$ versus respective control or basal values. $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ versus control or basal values in SHAM mice and $^{\ddagger}P < 0.05$ versus nitrite value of SHAM mice.

in pathologies such as atherosclerosis, restenosis, hypertension and ischaemic events in coronary and cerebral vascular bed (Shimokawa and Tsutsui 2010). The treatment with SFH (1 mg/kg) significantly increased the plasma level of nitrite in both SHAM and DOCA mice (Fig. 2A). Conversely, the basal as well as the increase in the plasma level of nitrite induced one hour after oral administration of SFH was smaller in DOCA than in SHAM mice (Fig. 2A). However, the amount of nitrite produced by SFH after subtraction of the basal level is similar in the plasma of DOCA and SHAM mice (54.1 ± 4.4 and 54.8 ± 7.0 μM, respectively). Pretreatment with L-NAME (20 mg/kg, ip), a non-selective inhibitor of NOS, partially inhibited the effect of SFH (1 mg/kg) in SHAM and DOCA. Moreover, the inhibitory effect was significantly higher ($P < 0.01$) in normotensive (67.0 ± 4.4%) than in hypertensive mice (46.7 ± 2.7%; Fig. 2B). These results suggest that the potent antihypertensive effect of SFH in DOCA mice is not entirely related to the production of NO, as observed in the present work and previously described in normotensive mice (Silva et al. 2011). Actually, these results are in agreement with reports which demonstrated an impaired production or bioavailability of NO in DOCA hypertensive mice (Landmesser et al. 2003).

Vasodilator effect of SFH

The vascular tone of small arteries and arterioles underlies the maintenance of peripheral resistance in the circulation and plays a significant role in the control of blood pressure (Christensen and Mulvany 1993). Decreased endothelium-dependent vasodilatation, which is associated with endothelial dysfunction, is a hallmark of most forms of hypertension (Nakazono et al. 1991). Endothelial dysfunction seems to precede the structural alterations in arterial walls, and it has been suggested as a key event in the establishment and development of vascular disorders (Mombouli and Vanhoutte 1999). In the present work, mesenteric arteries from DOCA hypertensive mice presented an impaired vasodilatation to acetylcholine, when compared with normotensive SHAM mice (not shown), demonstrating that DOCA mice presented an endothelium dysfunction as previously reported (Landmesser et al. 2003). On the other hand, in endothelium-intact mesenteric arteries precontracted with phenylephrine (3 μM), SFH produced a concentration-dependent vasodilatation (Fig. 3A) in normotensive and hypertensive mice. As depicted in Fig. 3, the concentration-response curve for SFH was significantly shifted to the left in mesenteric arteries from DOCA mice, when compared to those from SHAM (pIC_{50} = 4.50 ± 0.05 and 5.30 ± 0.04 for SHAM and DOCA, respectively; $P < 0.001$). In endothelium-denuded vessels, the concentration-response curve to SFH was shifted to the right, but the vasodilator effect of SFH was still significantly higher in mesenteric arteries from DOCA than in SHAM mice (Fig. 3A). Pretreatment of endothelium-intact vessels with L-NAME (300 μM) shifted to the right the concentration-response curve for SFH, in both DOCA and SHAM mice (Fig. 3B). Curiously, in the presence of L-NAME the concentration-response curves to SFH in SHAM and DOCA were overlapped. Catalase (2.400 IU/ml), an enzyme that decomposes H_2O_2 into oxygen and water, also shifted the concentration-response curve of SFH to the right and blunted the difference between SHAM and DOCA (Fig. 3C). These results suggest the participation of both NO and H_2O_2 in the vasodilator effect of SFH, both are well known EDRFs in mesenteric arteries (Fujimoto et al. 2001; Graves and Poston 1993).

Measurements of nitrite and H_2O_2 in small mesenteric artery

The above results suggest the participation of NO and H_2O_2 in the vasodilator effect of SFH. For this reason, the next step was the measurement of nitrite and H_2O_2 in mesenteric arteries stimulated with SFH (3 μg/ml). As illustrated in Fig. 4, SFH induced a significant increase in the level of nitrite and H_2O_2 in mesenteric arteries from SHAM and DOCA mice. The basal level and the total production of nitrite induced by SFH were significantly lower in DOCA than in SHAM mice (Fig. 4A). However, when the basal level of nitrite was subtracted from the total amount produced in the presence of SFH the level of nitrite in DOCA (38.6 ± 1.5 μM) was significantly higher ($P < 0.01$) than observed in SHAM mice (29.2 ± 1.8 μM). The production of H_2O_2 induced by SFH was significantly higher in DOCA than in SHAM

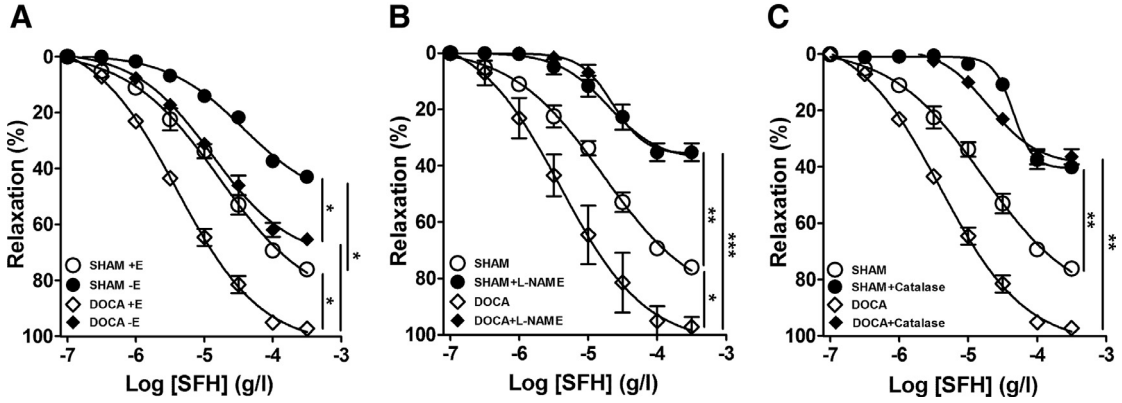

Fig. 3. Vasodilator effect of the *Hancornia speciosa* leaves extract (SFH) in mesenteric arteries from normotensive SHAM and hypertensive DOCA mice in the presence (+E) and in the absence (−E) of a functional endothelium (A), in the absence and in the presence of L-NAME (B), and in the absence and in the presence of catalase (C). All results are expressed as mean ± S.E.M. of at least five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

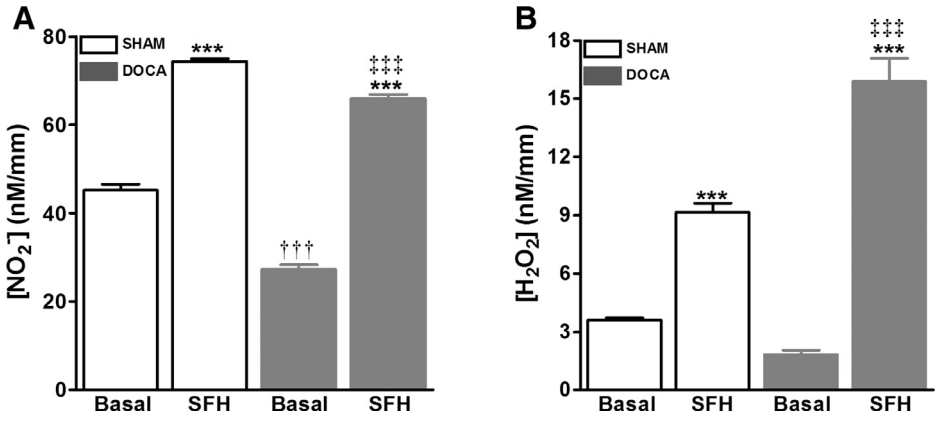

Fig. 4. Measurement of nitrite (A) and H_2O_2 (B) in mesenteric arteries from normotensive SHAM and hypertensive DOCA mice in the absence (basal) and in the presence of the *Hancornia speciosa* leaves extract (SFH). All results are expressed as mean ± S.E.M. of five experiments. $^{***}P < 0.001$ versus basal values. $^{\dagger\dagger\dagger}P < 0.001$ versus basal level from SHAM mice. $^{\ddagger\ddagger\ddagger}P < 0.001$ versus nitrite or H_2O_2 values from SHAM mice.

mice while the basal level was not different (Fig. 4B). When the basal values were subtracted from the total amount of H_2O_2 produced in the presence of SFH, the values observed in DOCA mice (13.5 ± 1.3 nM/mm) were more than twice ($P < 0.001$) of those in SHAM mice (5.5 ± 0.5 nM/mm). The present results demonstrate that the vasodilator effect of SFH is related to the production of both NO and H_2O_2 in the mesenteric arteries from both SHAM and DOCA mice. They also suggest that the increased sensitivity of arteries from DOCA mice to SFH is related to the increased production of NO and H_2O_2. It is noteworthy the difference in the production of H_2O_2, which is well known as responsible for the EDH in mesenteric arteries from mice (Matoba et al. 2000), after the administration of SFH in hypertensive mice. The origin of this H_2O_2 seems to be endothelial, but its production by the vascular smooth muscle cannot be ruled out.

In conclusion, the present work demonstrates that SFH has an antihypertensive action by a mechanism dependent on a systemic production of NO and on its vasodilator effect in resistance arteries. This vasodilator effect is related to the release of EDRFs, such as NO and H_2O_2. This last factor seems to be the main responsible for the increased sensitivity of arteries from hypertensive mice to the vasodilator effect of SFH.

Conflict of interest

The authors declared that there is no conflict of interest.

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