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Endothelium-derived factors and K^+ channels are involved in the vasorelaxation induced by *Sida cordifolia* L. in the rat superior mesenteric artery

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The vasorelaxantion of the aqueous fraction of the hydroalcoholic extract of the *Sida cordifolia* leaves (AFSC) was evaluated in this work. In rat superior mesenteric artery, AFSC (3–1000 μg/mL) induced relaxation of phenylephrine-induced contractions. This effect was significantly attenuated after removal of the endothelium, after atropine (1 μM), L-NAME (100 μM), indomethacin (10 μM), high K^+ (20 mM), tetraethylammonium (1 μM), a K_{Ca} blocker, apamin (1 μM), a SK_{Ca} blocker and ChTX (0.1 μM), a BK_{Ca} blocker, however, it was not affected after glibenclamide (10 μM), an K_{ATP} blocker, and 4-aminopyridine (1 μM), a K_v blocker. ChTX (0.1 μM) was able to induce an additional inhibition of the vasorelaxation induced by AFSC in the presence of L-NAME plus indomethacin. The vasorelaxation induced by AFSC in the presence of L-NAME plus indomethacin plus ChTX was not different from that induced by AFSC in rings without endothelium. In conclusion, the results show that endothelium-derived factors (mainly NO, PGI_2) and K^+ channels (BK_{Ca} and SK_{Ca}) play a crucial role in the vasorelaxation induced by AFSC in the rat superior mesenteric artery.

1. Introduction

Sida cordifolia L. (Malvaceae), popularly known as "Malva Branca", is a medical plant native of the Brazilian Northeast. It is used in folk medicine for several purposes: antirheumatic, antipyretic (Muzaffer et al. 1991), laxative, diuretic, antiinflammatory, analgesic (Kanth 1999; Franzotti el al. 2000), hypoglycaemic (Kanth 1999), antiasthmatic and in the treatment of nasal congestion (Ghosh and Dutt 1930).

A phytochemical screening of the hydroalcoholic extract of the leaves of *S. cordifolia* demonstrated the presence of alkaloids, steroids, flavonoids and saponins. Chemical studies of the leaves of this plant revealed the presence of ephedrine, pseudoephedrine (a potent vasoconstrictor), vasicinone (Ghosal et al. 1975), vasicine and vasicinol (bronchodilators) (Gunatilaka et al. 1980).

In a preliminary pharmacological study we demonstrated that the aqueous fraction of the hydroalcoholic extract of the leaves of *Sida cordifolia* L. (AFSC) induced a marked hypotension associated with bradycardia. The hypotensive effect was attenuated after atropine, an antagonist of the muscarinic receptors, and L-NAME, an inhibitor of NO-synthase. This led us to investigate whether AFSC could exert its effect through the decrease of peripheral vascular resistance by vasorelaxation.

Considering that not any pharmacological study related to the activity of this plant in rat resistance arteries was found in the literature, this work aimed to evaluate the role of the endothelium-derived factors and K^+ channels in the vasorelaxant response induce by AFSC in isolated rat superior mesenteric artery rings.

2. Investigations and results

In pre-contracted rings of superior mesenteric artery, AFSC (3, 10, 30, 100, 300 and 1000 μg/mL) elicited concentration-dependent vasorelaxations, which were significantly attenuated after the endothelium has been removed and after the incubation of the preparations with atropine, N^Wnitro-L-arginine methyl ester (L-NAME) or Indomethacin (Indo) ($p < 0.05$; $n = 6$). When compared with the control, the maximal responses were significantly reduced from 85 ± 3 to 21 ± 2, 69 ± 5, 47 ± 4 and $46 \pm 3\%$, respectively (Fig. 1). Furthermore, relaxation induced by AFSC 3000 μg/mL was not different from that induced by 1000 μg/mL (data not shown).

The inhibition of K^+ efflux with high concentrations of KCl (20 mM), significantly attenuated the relaxations induced by AFSC ($p < 0.05$; $n = 6$) and the maximal response was significantly reduced from 85 ± 3 to $49 \pm 3\%$ (Fig. 2). In the presence of K^+ channels blockers, tetraethylammonium (TEA) (1 μM), apamin (1 μM) or Charybdotoxin (ChTX) (0.1 μM), the relaxations induced by AFSC were significantly attenuated ($p < 0.05$; $n = 6$) and the maximal response was significantly reduced from 85 ± 3 to 56 ± 5; 69 ± 5 and $52 \pm 6\%$, respectively

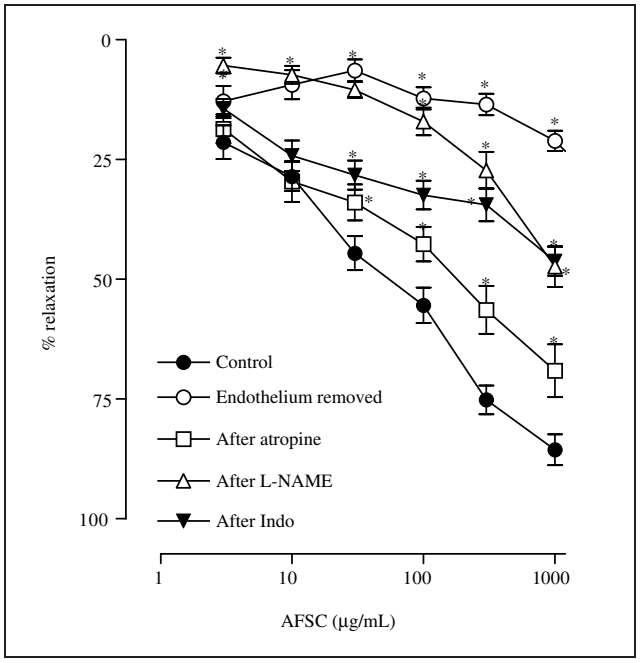

Fig. 1: Concentration-response curves to AFSC in intact isolated rat superior mesenteric arteries pre-contracted with 10 µM Phe, before (control) and after 1 µM atropine, 100 µM L-NAME or 10 µM Indo or after endothelium removed. Values represent mean ± S.E.M. of six experiments. * $p < 0.05$ vs control

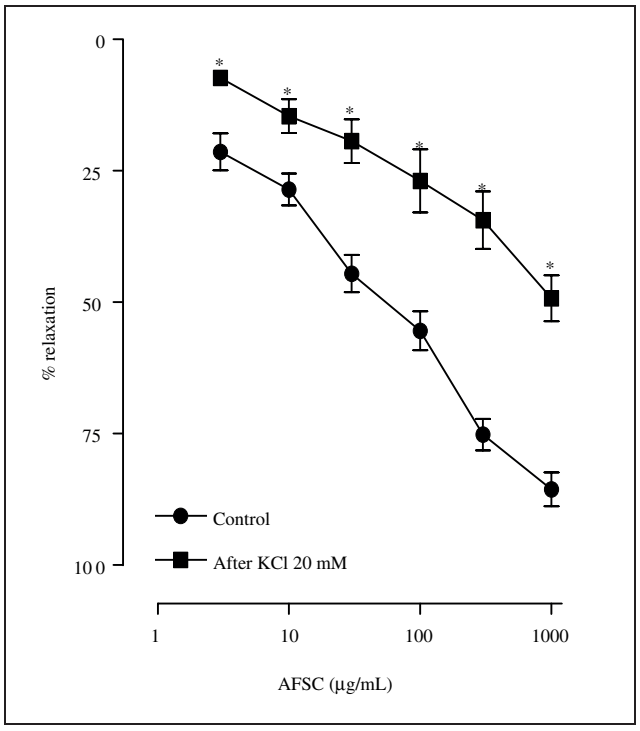

Fig. 2: Concentration-response curves to AFSC in intact isolated rat superior mesenteric arteries pre-contracted with 10 µM Phe before (control) and after high K^+ (KCl 20 mM). Values represent mean ± S.E.M. of six experiments. * $p < 0.05$ vs control

(Figs. 3a and 3b). However, in the presence of 4-aminopyridine (4-AP) (100 µM) or glibenclamide (Glib) (10 µM), these responses only were significantly affected in the concentrations of 300 and 1000 µg/mL, respectively (Fig. 3a).

In the presence of L-NAME plus Indo, AFSC induced relaxations significantly different from the control and that

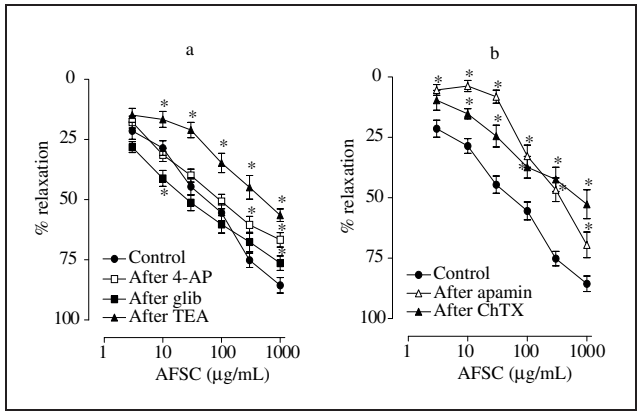

Fig. 3: Concentration-response curves to AFSC in intact isolated rat superior mesenteric arteries pre-contracted with 10 µM Phe before (control) and after: **(a)** 1 µM 4-AP, 10 µM Glib or 1 µM TEA; and **(b)** 1 µM apamin or 0.1 µM ChTX. Values represent mean ± S.E.M. of six experiments. * $p < 0.05$ vs control

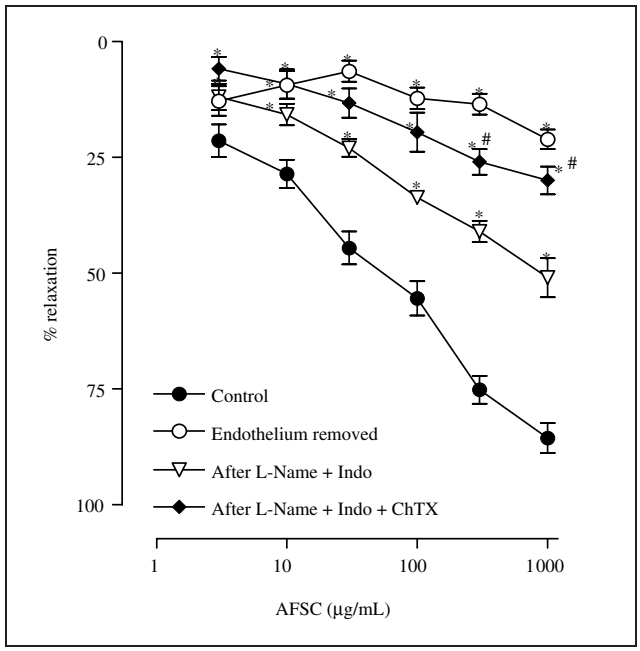

Fig. 4: Concentration-response curves to AFSC in intact isolated rat superior mesenteric arteries pre-contracted with 10 µM Phe before (control) and after 100 µM L-NAME plus 10 µM Indo and L-NAME plus Indo plus 0.1 µM ChTX, and in rings in which the endothelium was removed (endothelium removed). Values represent mean ± S.E.M. of six experiments. * $p < 0.05$ vs control; # $p < 0.05$ vs endothelium removed

were significantly affected after 0.1 µM ChTX (Fig. 4). As shown in Fig. 4, maximal response to AFSC in the presence of the three blocking drugs was reduced from $51 \pm 4\%$ (L-NAME + Indo) to $30 \pm 3\%$ ($p < 0.05$; $n = 6$). Interestingly, the relaxations induced by AFSC in the concentrations of 3, 10, 30 and 100 µg/mL after incubation with the three blocking drugs (L-NAME + Indo + ChTX), were similar to the relaxations induced by AFSC in endothelium-denude rings (Fig. 4).

3. Discussion

The major finding of this study is that AFSC induces vasorelaxation in a concentration-dependent manner in isolated rat superior mesenteric rings, which involve the vascular endothelium.

The endothelium is an important regulator of vascular tone and it is well established that in most vascular beds, muscarinic receptors activation in the endothelial cells induces vasorelaxation by release of endothelium-derived relaxant factors (EDRFs), including NO and PGI_2 (Furchgott and Zawadzki 1980; Moncada and Higgs 1993). These EDRFs diffuses to adjacent smooth muscle cells and cause them to relax (Vanhoutte et al. 1995). Several studies using affinities of selective muscarinc antagonists have indicated that the receptor mediating these effects is the M_3 subtype (reviewed by Eglen and Whiting 1990; Caufield 1993). In order to verify the role of muscarinic receptors in this response, we performed experiments in the presence of atropine, a non-selective antagonist of muscarinic receptor. Under these conditions, the vasorelaxant effect of AFSC was significantly attenuated, suggesting that AFSC could act via muscarinic receptor activation. To determine whether this vasorelaxant response involves the release of NO and PGI_2, we incubated the preparations with L-NAME, an inhibitor of the NOS, and Indo, an inhibitor of COX, separately. In the presence of both inhibitors, the AFSC-induced vasorelaxations were significantly attenuated, but not completely abolished. It is interesting to note that the response induced by AFSC in presence of atropine was less inhibited when compared to that in the presence of L-NAME or Indo. These results indicate that EDRFs play an important role in the AFSC-induced vasorelaxant response, nevertheless they further indicate that muscarinic activation seems not to completely account for EDRFs release from the vascular endothelium.

It is well reported in the literature that EDRFs induce vasorelaxation by activating the K^+ channels and consequently closing voltage-dependent calcium channels in the vascular smooth muscle (Nelson and Quayle 1995; Campbell et al. 1996). Four major types of K^+ channels appear to be involved in vascular regulation. They include: large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}), voltage-activated K^+ channels (K_v) and ATP-sensitive K^+ channels (K_{ATP}) (Adeagbo, 1999; Jackson 2000). In order to investigate the involvement of K^+ channels in the vasorelaxant effect of AFSC, we performed experiments in the presence of high concentrations (20 mM) of K^+. This procedure partially prevents the efflux of K^+ through the plasmatic membrane (Campbell et al. 1996), inhibiting the relaxations mediated by opening of K^+ channels (Clark and Fuchs 1997). Under these conditions, the vasorelaxant effect of AFSC was significantly attenuated, suggesting an involvement of K^+ channels in this response. To identify the type of K^+ channels that could to be involved in the vasorelaxation, we incubated the preparations with 4-AP, TEA, Glib, apamin or ChTX. This set of experiments revealed that AFSC-induced vasorelaxations were significantly attenuated after TEA, apamin or ChTX, but were not affected after Glib or 4-AP, suggesting that the effect induced by AFSC appears to be due to the opening of Ca^{2+}-activated K^+ channels, both subtypes BK_{Ca} and SK_{Ca}.

In many species, endothelium-dependent relaxations that were not mediated by NO and PGI_2 have been demonstrated in a variety of blood vessels, and this response has been proposed to be mediated by EDHF (Zygmunt et al. 1994; Garland et al. 1995; Urakami-Harasawa et al. 1997). Other studies have demonstrated that EDHF-induced hyperpolarization and subsequent relaxation are insensitive to K_{ATP}-blockers, as Glib, but sensitive to BK_{Ca}-blockers, as ChTX (Van de Voorde et al. 1992; Weston and Edwards 1992; Garland et al. 1995; Campbell and Harder 1999). Finally, in order to evaluate the role of BK_{Ca} in the NO- and PGI_2-independent vasorelaxations induced by AFSC, we incubated the preparations with L-NAME and Indo, simultaneously. Under these conditions, AFSC-induced vasorelaxations were attenuated, but not abolished. The residual vasorelaxant effect was blocked after ChTX. Interestingly, in the presence of the three inhibitors, the AFSC -induced vasorelaxations were not different from those observed in endothelium-denuded rings. We therefore suggest that the residual vasorelaxant effect observed after NO-synthase and COX inhibition is possibly linked to the release of EDHF.

In conclusion, the results obtained in this study show that endothelium-derived factors (at least, NO and PGI_2) and K^+ channels (BK_{Ca} and SK_{Ca}) play a major role in the vasorelaxation induced by the aqueous fraction of the hydroalcoholic extract of the leaves of *Sida cordifolia* L. in rat superior mesenteric arteries, and this effect appears to contribute for the AFSC-induced hypotensive response observed in rats.

4. Experimental

4.1. Preparation of the hydroalcoholic extract of the leaves of S. cordifolia L.

S. cordifolia leaves (voucher specimen no. 30171, deposited in the Department of Biology of the University of Sergipe, Brazil) were dried at 40 °C and pulverized. The powder was extracted with 70% ethanol in water at room temperature (25–30 °C), for 72 h. The resulting extract was dried at 60 °C using a rotavaporator. A portion of this extract was dissolved in distilled water, filtered and known volumes were dried to determine the water-soluble fraction (72%). This factor was used to calculate the final concentration of the AFSC. When required, the extract was dissolved in a distilled water/cremophor solution and diluted to desired concentrations. The final concentration of cremophor in the bath never exceeded 0.01% and was without effect when tested in control preparations (data not shown).

4.2. Preparation of isolated rat superior mesenteric artery rings

Male Wistar rats (200–300 g) were killed by stunning and bled. The superior mesenteric artery was removed and cleaned from connective tissue and fat. Rings (1–2 mm) were obtained and suspended by cotton threads in organ baths containing 10 ml of Tyrode's solution (composition mM: NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0 and glucose, 5.6), maintained at 37 °C and gassed with 95% O_2 and 5% CO_2. Then, they were stabilized under a resting tension of 0.75 g for 1 h. During this time the solution was changed every 15 min to prevent accumulation of the metabolites. The isometric tension was recorded by a force transducer (FORT-10, WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI, Sarasota, EUA). The endothelium was removed by gently rubbing the intimal surface of the vessels. Removal of the endothelium was verified by the loss of the relaxation to acetylcholine (10 μM) in vessels pre-contracted with phenylephrine (10 μM).

4.3. Drugs

The drugs used were: cremophor, L-phenylephrine chloride (Phe), acetylcholine chloride (Ach), atropine sulfate, N^w-nitro-L-arginine methyl ester (L-NAME), indomethacin (Indo), 4-aminopyridine (4-AP), glibenclamide (Glib), tetraethylammonium (TEA), apamin and charybdotoxin (ChTX), all from SIGMA. Indo was dissolved in 0.5% w/v sodium bicarbonate. The other compounds were freely dissolved in distilled water and kept at 4 °C.

4.4. Experimental protocols

4.4.1. Effect of AFSC on sustained contractions induced by Phe (10 μM) in isolated rat superior mesenteric artery rings with or without endothelium

After the stabilization period, the rings with or without endothelium were pre-contracted with Phe (10 μM) and different concentrations of AFSC (3, 10, 30, 100, 300 and 1000 μg/mL) were added cumulatively to the bath. The relaxations were measured by comparing the developed tension before and after addition of AFSC.

4.4.2. Verification of the role of the muscarinic receptors and endothelium-derived factors on the AFSC-induced vasorelaxant response

Phe-induced sustained contractions were obtained in preparations incubated with atropine (1 μM), a non-selective antagonist of the muscarinic receptors, L-NAME (100 μM), an inhibitor of the NO-synthase (NOS) (Moncada and Higgs 1993), and Indo (10 μM), an inhibitor of the cyclooxygenase (COX) (Clark and Fuchs 1997), separately. Then, concentration-response curves to AFSC were obtained. Atropine was added 15 min before the contraction with Phe, and L-NAME and Indo were added 30 min before. The effectiveness of muscarinic blockade was verified by the loss of relaxing responses to 1 μM Ach against Phe-induced contractions (data not shown).

4.4.3. Effect of high concentration of K^+, 4-AP, Glib, TEA, apamin or ChTX on the AFSC-induced vasorelaxant responses

Phe-induced sustained contractions were obtained in preparations incubated with high concentration of K^+ (20 mM), an inhibitor of K^+ efflux (Garland et al. 1995; Edwards and Weston, 1998), 4-AP (100 μM), a blocker of the voltage – activated K^+ channels (K_v) (Lagaud et al. 1999), Glib (10 μM), a blocker of the ATP-sensitive K^+ channels (K_{ATP}) (Lagaud et al. 1999), TEA (1 μM), in this concentration is selective blocker to Ca^{2+}-activated K^+ channels (K_{Ca}) (Langton et al. 1991; Lagaud et al. 1999), apamin (1 μM), a blocker of the small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) (Jiang et al. 2000) and ChTX (0.1 μM), a blocker of the large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), separately. Then, concentration-response curves to AFSC were obtained. All blocking drugs were added 30 min before the contractions with Phe.

4.4.4. Effect of ChTX on the AFSC-induced vasorelaxant responses in the presence of L-NAME plus Indo

Phe-induced sustained contractions were obtained in preparations incubated with L-NAME plus Indo or L-NAME plus Indo plus ChTX. Then, concentration-response curves to AFSC were obtained. All blocking drugs were added 30 min before the contractions with Phe.

4.5. Statistics

Values are expressed as mean ± SEM. When appropriate, *student's t-test* or ANOVA were done to evaluate the significance of the differences between means, by using Graph Pad Prism™ 3.0 software.

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References

Adeagbo ASO (1999) 1-Ethyl-2-benzimidazolinone stimulates endothelial K_{Ca} channels and nitric oxide formation in rat mesenteric vessels. Eur J Pharmacol 379: 151–159.

Campbell WB, Gebremedhin D, Prait PF, Herder DR (1996) Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ Res 78: 415–423.

Campbell WB, Harder DR (1999) Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. Circ Res 84: 484–488.

Caulfield MP (1993) Muscarinic receptors: characterization, coupling and function. Pharmacol Ther 58: 323–331.

Clark SG, Fuchs LC (1997) Role of nitric oxide and Ca^{++}-dependent K^+ channels in mediating heterogeneous microvascular responses to acetylcholine in different vascular beds. J Pharmacol Exp Ther 282: 1473–1479.

Edwards G, Weston AH (1998) Endothelium-derived hyperpolarizing factor – a critical appaisal. Prog Drug Res 50: 107–133.

Eglen RM, Whiting R (1985) Determination of the muscarinic receptor subtype mediating vasodilatation. Br J Pharmacol 84: 3–5.

Franzotti EM, Santos CVF, Rodrigues HMSL, Mourão RHV, Andrade MR, Antoniolli AR (2000) Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-Branca). J Ethnopharmacol 72: 273–278.

Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373–376.

Garland C, Plane F, Kemp B, Cocks T (1995) Endothelium-dependent hyperpolarization: a role in the control of vascular tone. Trends Pharmacol Sci 16: 23–30.

Ghosal S, Chauhan RBPS, Mehta R (1975) Chemical constituents of Malvaceae. Part I. Alkaloids of *Sida cordifolia*. Phytochemistry 14: 830–832.

Ghosh S, Dutt A (1930) The chemical examination of *Sida cordifolia*. J Indian Chem Soc 7: 825.

Gunatilaka A, Sotheeswaran S, Balasubramaniam S, Chandrasekara AI, Badra Sriyani HT (1980) Studies on medicinal plants of Sri Lanka. III. Pharmacologically important alkaloids of some *Sida* species. Planta Med 39: 66–72.

Jackson WF (2000) Ion channel and vascular tone. Hypertension 35 [part 2]: 173–178.

Jiang F, Li CG, Rand MJ (2000) Mechanisms of nitric oxide-independent relaxations induced by carbachol and acetylcholine in rat isolated renal arteries. Br J Pharmacol 130: 1191–1200.

Kanth VR, Diwan PV (1999) Analgesic, antiinflammatory and hypoglycaemic activities of *Sida cordifolia*. Phytotherapy Res 13: 75–77.

Lagaud GJ, Skarsgard PL, Laher I, van Breemen C (1999) Heterogeneity of endothelium-dependent vasodilation in pressurized cerebral and small mesenteric resistance arteries of the rat. J Pharmacol Exp Ther 290: 832–839.

Langton PD, Nelson MT, Huang Y, Standen NB (1991) Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 260: H927–934.

Moncada S, Higgs EA (1993) The L-arginine – nitric oxide pathway. N Engl J Med 29: 2002–2012.

Muzaffer A, Joy S, Usmanu Ah S (1991) Screening of *Sida cordifolia* L., *Sida rhomboidea* L. and *Triumfetta rotundifolia* Lam. for anti-inflammatory and anti-pyretic activities. Indian Drugs 28: 397.

Nelson MT, Quayle JM (1995) Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol 268(4Pt1): C799–822.

Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A (1997) Importance of endothelium-derived hyperpolarizing factor in human arteries. J Clin Invest 100: 2793–2799.

Van de Voorde J, Vanhell B, Leusen J (1992) Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. Circ Res 70: 1–8.

Vanhoutte PM, Boulanger CM, Mombouli JV (1995) Endothelium-derived relaxing factors and converting enzyme inhibition. Am J Cardiol 76: 3E–12E.

Weston AH, Edwards G (1992) Recent progress in potassium channel opener pharmacology. Biochem Pharmacol 43: 47–54.

Zygmunt PM, Waldeck K, Hogestatt ED (1994) The endothelium mediates a nitric oxide-independent hyperpolarization and relaxation in the rat hepatic artery. Acta Physiol Scand 152: 375–384.