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Endothelium-derived nitric oxide is involved in the hypotensive and vasorelaxant effects induced by discretamine in rats

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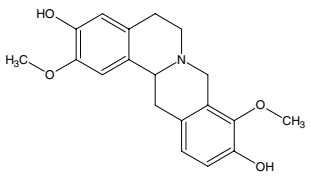
The aim of this study was to investigate the pharmacological effects of discretamine, an isoquinoline alkaloid isolated from *Duguetia magnolioidea* Maas, on the cardiovascular system, using a combined *in vivo* and *in vitro* approach. Blood pressure and heart rate measurements, as well as changes in isometric tension in rat superior mesenteric arterial rings, elicited by discretamine were recorded. In normotensive non-anaesthetized rats ($n = 6$), discretamine (0.01; 0.05; 0.1; 0.5; 1, 5 and 10 mg/kg i.v., randomly) injections produced hypotension (-5.2 ± 1.7; -5.1 ± 2.1; -7.7 ± 2; -8.9 ± 1.7; -9.6 ± 2.2; -16.8 ± 2.8 and -13.4 ± 1.3 mmHg, respectively) accompanied by tachycardia (24.2 ± 6.1; 36.8 ± 11.3; 44.2 ± 7.7; 45.9 ± 6.4; 48.2 ± 9.1; 72.1 ± 14.5 and 64 ± 17 bpm, respectively). Hypotensive and tachycardic responses were significantly attenuated after L-NAME (20 mg/kg, i.v.) administration. In isolated rat mesenteric artery rings, with endothelium intact, discretamine (10^{-12}–10^{-5} M) induced concentration-dependent relaxation of the contractions induced by phenylephrine (10 μM) [$pD_2 = 6.8 \pm 0.1$]. The effect of the discretamine on phenylephrine induced contractions was significantly attenuated after removal of the vascular endothelium [$pD_2 = 5.8 \pm 0.04$]. Similar results were obtained after pre-treatment with L-NAME 100 μM [$pD_2 = 5.8 \pm 0.04$], L-NAME 300 μM [$pD_2 = 5.9 \pm 0.06$], Hydroxocobalamin 30 μM [$pD_2 = 5.8 \pm 0.06$] or ODQ 10 μM [$pD_2 = 5.8 \pm 0.04$]. In addition, in rabbit aorta endothelial cell line, discretamine significantly increased NO_3^- levels. These results suggest that the hypotensive effect induced by discretamine is probably due to a peripheral vasodilatation, at least, in part, due to the release of NO from vascular endothelium and consequent activation of soluble guanylyl cyclase (GC) in the vascular smooth muscle cells.

1. Introduction

Several secondary metabolites derived from plants are isoquinolines alkaloids, such as coptisine, berberine and palmatine that present many biological effects (Ko et al. 2000; Cui et al. 2006; Tanabe et al. 2006; Islam et al. 2007). There are described in the literature, for example, antimicrobial, antimalarial, cytotoxic, anti-HIV activities and anti-tumor-promoting effects of some of isoquinoline type alkaloids (Iwasa et al. 2001a). Among this isoquinoline series, the protoberberine alkaloids are the most widely distributed alkaloids and are reported to exhibit several types of biological activities, such as antimicrobial (Iwasa et al. 1998b); cytotoxic (Iwasa et al. 2001b), antiproliferative effects and vasorelaxant activities (Ko et al. 2000).

Discretamine, an isoquinoline alkaloid classified as tetrahydroprotoberberine, was already isolated from plants such as *Fissistigma glaucescens* (Ko et al. 1993), *Rollinia leptopetala* (Fechine et al. 2000), *Duguetia trunciflora* Maas (Fechine et al. 2002) and in our laboratory this alkaloid was isolated from *Xylopia langsdorffiana* Saint-Hilarie & Tulasne Ann and from *Duguetia magnoliodeae* Maas. There are literature reports on their pharmacological actions such as an α-adrenoceptor and 5-HT receptor antagonistic effect with a rank order of α1 > 5-HT > α2 (Ko et al. 1993). Additionally, the removal of endothelium significantly increased the antagonistic potency of (−)-discretamine on noradrenaline or phenylephrine-induced vasoconstrictions (Ko et al. 1993). Later, Ko and colleagues (1994) showed that discretamine is a selective α1D-adrenoceptor antagonist in the vascular smooth muscle and could be a useful research tool for characterizing α1-adrenoceptors subtypes (Ko et al. 1994). In human prostatic tissues the alkaloid inhibited phenylephrine-induced contractions (Guh et al. 1999).



Discretamine

Additionally, the chemical structure of discretamine presents a great similarity with berberine that elicits a diversity of pharmacological actions such as antibiotic (Hahn and Cuak 1975), antitumor (Nishino et al. 1996), antimotility properties (Yamamoto et al. 1993), positive inotropic and negative chronotropic effects in isolated guinea-pig atria (Shaffer 1985) and relaxation in isolated vascular preparations (Chiou et al. 1991), among other effects.

Pharmacological screening realized in our laboratory showed that discretamine induced hypotensive and vasorelaxant effects in isolated rat superior mesenteric artery rings.

The aim of this study was to evaluate the effects induced by discretamine on the arterial pressure and heart rate in normotensive and non-anaesthetized rats and to elucidate its mechanism of action with special emphasis to the vascular endothelium mediated responses.

2. Investigations and results

2.1. Systemic hemodynamic effects elicited by discretamine

After the cardiovascular parameters had stabilized, the MAP and HR were recorded before (baseline values) and after i.v. administration of discretamine 0.01; 0.05; 0.1; 0.5; 1; 5 and 10 mg/kg, i.v., randomly). Successive injections were separated by a time interval sufficient to allow full recovery of arterial pressure, usually 20–30 min. In five non-anaesthetized rats, baseline values of mean arterial blood pressure and heart rate were 107 ± 1 mmHg and 378 ± 5 bpm, respectively. Discretamine administration (0.01; 0.05; 0.1; 0.5; 1; 5 or 10 mg/kg, i.v., randomly) induced hypotension associated to an increase in the heart rate (Fig. 1). These hypotensive and tachycardic responses were significantly attenuated after acute blockade of the NO-synthase (NOS) with L-NAME 20 mg/kg, showing the importance of the NOS-activation in the systemic effects induced by discretamine (Fig. 1).

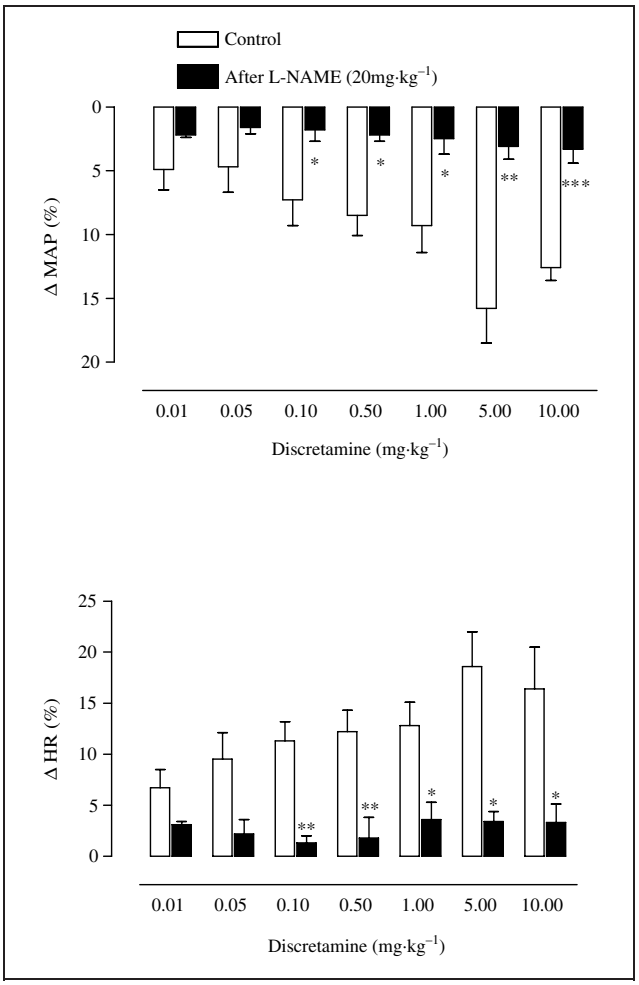


Fig. 1: Bar graph showing changes in mean arterial pressure (MAP, %) (A) and in heart rate (HR, %) (B) induced by the acute administration of increasing doses of discretamine in non-anaesthetized normotensive rats, before (control) and after acute NO-synthase inhibition (L-NAME). Results are means ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control (n = 6)

After stabilization of MAP and HR, discretamine (0.01; 0.05; 0.1; 0.5; 1; 5 or 10 mg/kg, i.v., randomly) was administered. Following a 60 min period, a bolus injection of L-NAME (20 mg/kg, i.v.) was given, and 30 min later, the administration of discretamine was repeated. Changes in MAP and HR induced by alkaloid were compared before (control) and after L-NAME administration.

2.2. Vasorelaxant effect of discretamine and EDRF participation

After equilibration period, the rings with or without functional endothelium were pre-contracted with the agonist and once the response to the second administration of phenylephrin (PHE, 10 μM) had reached the plateau, increasing cumulative concentrations of discretamine (10^{-12} to 10^{-5} M) were added to the bath. The relaxations were measured by comparing the developed tension before and after addition of discretamine.

Initially, the relaxant effect induced by discretamine was investigated in rat superior mesenteric arteries rings, without endothelium. To assess the role of endothelium factors in the relaxation induced by discretamine in mesenteric artery, tissues were pretreated with the L-NAME (100 or 300 μM); the NO scavenger, hydroxocobalamin (30 μM) (Kruszyna et al. 1998); the soluble guanylate cyclase inhibitor, ODQ (10 μM) (Garthwaite et al. 1995) or the non-selective COX inhibitor, indomethacin (1 μM), separately. To evaluate the influence of the muscarinic activation, mesenteric rings were pretreated with atropine (1 μM). Inhibition was calculated by comparing the responses induced by discretamine before and after the addition of the inhibitor or antagonist.

In isolated rat superior mesenteric rings with intact endothelium pre-contracted with phenylephrine (10 μM), increasing concentrations of discretamine (10^{-12} to 10^{-5} M) induced, in a concentration dependent manner, vasorelaxation (Fig. 2A and Table). After removal of vascular endothelium, the relaxant response induced by discretamine was significantly shifted to the right when compared with intact endothelium rings (Fig. 2A and Table).

Since discretamine induced both endothelium-dependent and -independent relaxation in rat isolated mesenteric arteries, an attempt was made to investigate the endothelium-derived vasoactive factors implicated in this relaxation.

The vasorelaxant response induced by increasing concentrations of discretamine (10^{-12} to 10^{-5} M) was significantly rightward shifted in presence of NO-synthase inhibition (L-NAME 100 or 300 μM) (Fig. 2B and Table). Interestingly, in the presence of hydroxocobalamin (30 μM) or ODQ (10 μM), the relaxation induced by increasing concentrations of discretamine (10^{-12} to 10^{-5} M) was significantly shifted to the right in a similar proportion to that obtained in endothelium denuded or L-NAME pre-incubated rings (Fig. 2C and Table).

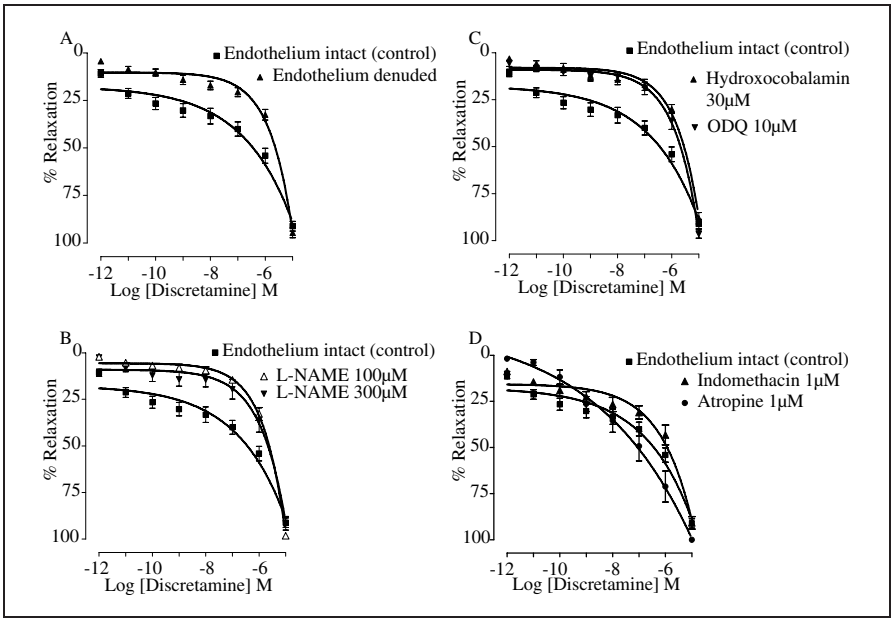


Fig. 2:
Line plot graph showing the effects of increasing concentrations of discretamine (10^{-12} a 10^{-5} M) in phenylephrine (10 μM) induced contractions in rat mesenteric superior artery rings. 3A, with (n = 13) or without (n = 7) functional endothelium; 3B, rings with functional endothelium, in the absence (Control, n = 13) or in the presence (n = 7) of L-NAME 100 or 300 μM; 3C, in the absence (Control, n = 13) or in the presence of hydroxocobalamim (n = 7) 30 μM or ODQ (n = 7) 10 μM; 3D, in the presence of indomethacin 1 μM (n = 8) or atropine 1 nM (n = 7). Values are expressed mean ± s.e.m

Table: Comparison of the E_{max} (% of relaxation) and pD_2 values of discretamine in relation to the tonic contractions induced by FEN (10 M) in superior mesenteric artery rings

Experimental conditions	E_{max} (%)	pD_{50}	n
Endothelium intact	91.2 ± 2.6	6.8 ± 0.1	13
Endothelium denuded	94.7 ± 2.6	5.8 ± 0.04***	9
L-NAME 100 μM	98.2 ± 1.2	5.8 ± 0.04***	6
L-NAME 300 μM	91.5 ± 3.6	5.9 ± 0.06***	7
Hydroxocobalamin 30 μM	88.1 ± 3.0	5.8 ± 0.06***	6
ODQ 10 μM	96.5 ± 2.2	5.8 ± 0.04***	6
Atropine 1 μM	100 ± 0.0	7.2 ± 0.2	7
Indomethacin 1 μM	90.9 ± 3.4	6.4 ± 0.1	6

Values are expressed as mean ± SEM. ***p < 0,001 vs. Endothelium Intact. n, number of experiments

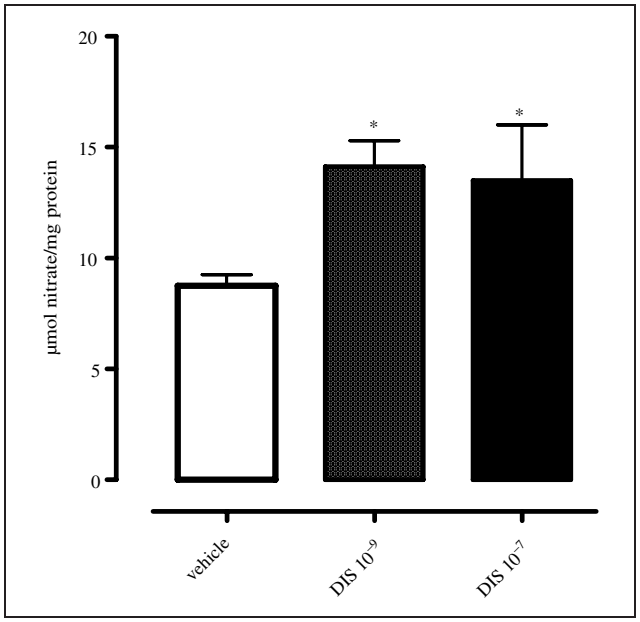


Fig. 3: Bar graph depicting the effect of increasing concentractions of discretamine (DIS, 10^{-9} and 10^{-7} M) on the nitrate concentration in rabbit aorta endothelial cells line. Values are expressed mean ± s.e.m. *p < 0.05 vs. control (vehicle), n = 3

However, relaxing effect induced by the alkaloid was not significantly altered after pre-incubation of endothelium-intact rings with indomethacin (1 μM), or muscarinic receptor blockade (atropine 1 μM), suggesting that COX-derived products and muscarinic activation are not involved in the relaxation induced by the alkaloid (Fig. 2D).

2.3. Influence of discretamine on NO levels

Rabbit aortic endothelial cells (REC) line were incubated with discretamine (10^{-9} and 10^{-7} M) or vehicle (cremophor 0.01%) during 18 h. Later, after incubation period, the culture medium of the cells treated with discretamine or vehicle was used to evaluate the production of nitric oxide. In rabbit endothelial cells discretamine (10^{-7} M), in a concentration close to the molar concentration of discretamine that produces 50% of the maximal possible effect, was able to increase NO_3^- levels in the culture medium, when compared to the vehicle administration (Fig. 3).

3. Discussion

The major finding of this work was that in conscious rats, the acute administration of discretamine induced a decrease in arterial pressure, followed by a significant tachycardia. Since discretamine presented a vasorelaxant effect in isolated rat superior mesenteric artery rings, this hypotensive effect seems to be mediated by decreased peripheral vascular resistances.

The endothelium plays a pivotal role in the control of vascular tone and blood pressure. In response to a variety of physiological stimuli such as acetylcholine, substance P and shear stress, endothelial cells release vasodilator substance, among these, there are three main components: nitric oxide (NO), prostacyclin and endothelium derived hyperpolarizing factor (EDHF) (Chauhan et al. 2003). Nitric Oxide, synthesized by NO synthase (NOS), is a major factor in the cardiovascular system. Biosynthesis of NO involves a two step oxidation of L-arginine to L-citrulline by NOS, with concomitant production of NO (Andrew and Mayer 1999). The NO formed by enzyme NOS can be inhibited by several substituted L-arginine analogues such as L-NAME and L-NMMA.

In intact rat superior mesenteric rings, increasing concentrations of discretamine induced a potent concentration-dependent relaxant effect. This effect was significantly rightward shifted in the absence of the vascular endothelium, indicating that relaxing factors released by the endothelium participate in the vasorelaxant response evoked by discretamine. Furthermore, discretamine action may involve two basic mechanisms, the first dependent and the second one independent of the presence of the vascular endothelium. These results contrast with a report on aorta tissue where discretamine increased its antagonistic potency on noradrenaline- or phenylephrine-induced vasoconstriction after removal of vascular endothelium (Ko et al. 1993). However, our results corroborate those observed by Chun et al. (1979) reporting that berberine, a compound presenting close chemical similarity with discretamine, was able to induce hypotensive action in rats and both endothelium-dependent and -independent relaxations in rat superior mesenteric rings. In addition it was observed that nitric oxide, but not other endothelium-derived factors, were involved in these endothelium-dependent relaxations (Ko et al. 2000).

Based on the fact that NO is important in the regulation of the vascular tonus we decided to further evaluate the participation of the L-arginine – NO pathway in the effect induced by discretamine. In the presence of a competitive NOS inhibitor (L-NAME) (Ress et al. 1990), in different concentrations (100 and 300 μM), the relaxation induced by discretamine was significantly attenuated, with decrease of the pharmacological potency.

After its synthesis and release NO diffuses to adjacent smooth muscle cells and acts through activation of soluble guanylyl cyclase, thereby increasing the intracellular cGMP concentration and promoting relaxation (Bredt et al. 1992). In preparations pre-treated with hydroxocobalamine (30 μM) and ODQ (10 μM), NO scavenger and sGC inhibitor respectively, the vasorelaxant effect evoked by discretamine was significantly attenuated, further suggesting that the relaxant response elicited by the alkaloid involves L-arginine – NO/sGC pathway. Surprisingly, our results demonstrate that the vasorelaxant response induced by discretamine was reduced to virtually the same extent in the following conditions: endothelium denuded rings, L-NAME, hydroxocobalamin or ODQ (Table), and suggest a closed relationship between L-arginine – NO – sGC pathway and endothelium-dependent relaxation induced by discretamine.

It is reported that endothelium-dependent vasorelaxant response may also involve the release of COX-derived products, such as prostacyclin (PGI_2), via cyclooxygenase pathway (Moncada et al. 1979). Nitric oxide release, as well as prostacyclin, can be mediated, in most vascular beds, by muscarinic activation (M_3) located on the endothelial cells (Moncada et al. 1991), despite the lack of vascular cholinergic innervation. Under our experimental conditions, neither inhibition of the cyclooxygenase with indomethacin nor blockade of the muscarinic receptors with atropine were capable to significantly alter the relaxant response induced by discretamine, suggesting that COX-derived products and muscarinic activation are not involved in the relaxation induced by the alkaloid.

To clearly demonstrate that NO release from vascular endothelium is involved in the vasorelaxant response induced by discretamine, a biochemical assay using a very sensitive technique for NO analysis (Leite et al. 2003) was performed. Our data showed that, in rabbit aorta endothelial cell line, NO_3^- levels significantly increased in response to increased concentrations of discretamine. These results reflect the involvement of the NO released by endothelium in the relaxation induced by discretamine in isolated rat superior mesenteric artery. However, it remains to be examined how discretamine would stimulate nitric oxide release in the endothelial cells. Nevertheless, in the present study we were not able to elucidate the initial step involved in the NOS activation and further appropriated studies are necessary to explain this mechanism.

In addition, in non-anaesthetized rats, discretamine-induced hypotension and tachycardia also were practically abolished after NO-synthase inhibition, suggesting that NO released play an important role in the hypotensive and vasorelaxant responses induced by discretamine. Taken together, these results further corroborate the hypothesis that discretamine could, at least, in part, induce activation of the NO leading to vasorelaxation and hypotension.

Interestingly, it has been reported in the literature that discretamine induced α1-adrenoceptor antagonist activity (Ko et al. 1993, 1994), however, when compared to another classic nonselective α-adrenoceptor antagonist, prazosin, some differences can be found. For example, in anaesthetized rat, prazosin did not produce a reflex tachycardia, but a sustained hypotension, about 30 min, accompanied by a significant decrease in heart rate (Orallo et al. 2003). Furthermore, the presence of the endothelial system did not significantly modify the vasorelaxant effects of prazosin in rat aortic rings (Orallo et al. 2003), which suggests that these effects are not due to an indirect and/or a direct action of this compound on the endothelium.

In conclusion, the present study, using a combined approach (*in vivo* and *in vitro* experiments), demonstrated that discretamine markedly lowers arterial pressure and increases heart rate in conscious unrestrained rats. The results shown here suggest that the hypotensive action of discretamine can be a consequence of the decrease in peripheral vascular resistance, at least in part, due to release of NO by vascular endothelium.

4. Experimental

4.1. Animals

Male Wistar rats (300–350 g) were used for all experiments. Animals were housed under conditions of controlled temperature (21 ± 1 °C) and lighting (lights on: 06 : 00–18 : 00 h). In addition, they had free access to food (PURINA-Brazil) and tap water *ad libitum*. All the protocols used in this study followed the guidelines of the Animal Care and Use Committees of the Federal University of Paraiba.

4.2. Compounds

The compounds used were: cremophor, dimethyl sulphoxide (DMSO), L-phenylephrine chloride, acetylcholine chloride, atropine sulfate, N^G-nitro-l-arginine methyl esther (L-NAME), indomethacin, 1 H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ) and hydroxocobalamin (all from Sigma Chemical Co., Saint Louis, MO, USA). Stock solutions were prepared in distilled water and kept at −4 °C. Discretamine was isolated from *Duguetia magnolioidea* Maas according to the method described by Fechine et al. (2002) and was solubilized in a mixture of distilled water/chremophor (for *in vivo* approach mixture of saline/chremophor) and diluted to the desired concentrations with distilled water just before the use. The final concentration of chremophor in the bath never exceeded 0.01% and has shown to be without effect when tested in control preparations (data not shown). Indomethacin was dissolved in 0.5% w/v sodium bicarbonate and ODQ was dissolved in DMSO (100%). The other compounds were freely dissolved in distilled water.

4.3. Measurement of arterial pressure (AP) and heart rate (HR) in non-anaesthetized rats

For measurement of AP and HR the procedure was similar to that previously described by Guedes et al. (2002). Briefly, rats were anaesthetized

using sodium thiopental (45 mg/kg, i.p.), and polyethylene catheters were inserted into the lower abdominal aorta and inferior vena cava through left femoral artery and vein, respectively. The arterial catheter was connected to a pre-calibrated pressure transducer (Statham P23 ID; Gould, Cleveland, OH, USA) for the measurements of arterial pressure (AP) and heart rate (HR). The systemic effects of discretamine in non-anaesthetized rats were evaluated after haemodynamic parameters had stabilized. Different doses of discretamine (0.01; 0.05; 0.1; 0.5; 1, 5 and 10 mg $\cdot$ kg^{-1}, randomly, i.v.) were administered and changes in MAP and HR induced by alkaloid were analyzed.

4.4. Preparation of isolated rat superior mesenteric artery rings

For measurement of isometric tension the rats were killed by cervical dislocation and superior mesenteric artery was removed and cleaned from connective tissue and fat. When appropriated the endothelium was removed by gently rubbing of the intimal surface of the vessels. Rings (1–2 mm) were obtained and placed in physiological Tyrode's solution, maintained at 37 °C, gassed with 95% O_2 and 5% CO_2 and maintained at pH 7.4, under a resting tension of 0.75 g for 1 h. The force of contraction was isometrically recorded by a force transducer (FORT-10, WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI, Sarasota, FL, USA) and to a personal computer equipped with an analog – to – digital converter board. Concentrations of discretamine (10^{-12} to 10^{-5} M) were cumulatively added after contractile responses induced by phenylephrine (10 μM).

4.5. Cell culture and nitrate levels in culture medium

Rabbit aorta endothelial cells (REC) were grown in 24-well plates on F12 medium (F12 Coon's modification) and supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). After reaching confluence, REC were incubated with discretamine (10^{-9} and 10^{-7} M) or vehicle (cremophor 0.01%) during 18 h.
Subsequently, nitrate levels were measured in culture medium of the cells treated with discretamine or vehicle. The total amount of nitrate in the medium was determined by a modification of the procedure described by Leite et al. (2003), where 10 μL aliquots were injected into Sievers chemiluminescence analyzer (model 280) to react with the solution saturated of VCl_3 and 1 M HCl (at 95 °C) as reductants**,** converting NO_3^- (nitrate) to NO, which was then detected by ozone-induced chemiluminescence. NO_3^- concentrations were calculated by comparison with standard solution of sodium nitrate. Data were collected and normalized for protein concentration, assessed through technique described by Bradford (Bradford 1976).

4.6. Data analysis

Values are expressed as mean ± S.E.M. When appropriate, statistical significance was examined with Student's t-test and one-way ANOVA following Bonferroni, using Graph Pad Prism TM 3.02 software. The pD_2 value (The negative logarithm to base 10 of the EC_{50} of an agonist) was calculated by nonlinear regression of individual concentration-response curves and $p < 0.05$ was considered significant.

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