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Presence of functional angiotensin II receptor and angiotensin converting enzyme in the aorta of the snake *Bothrops jararaca*

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ABSTRACT

Aim: Angiotensin II (Ang II) interacts with AT_1 and AT_2 receptors and, in some vertebrates, with an Ang II binding site showing low affinity for AT_1 and AT_2 receptor antagonists. This study was carried out to characterize the Ang II receptor, and the presence of an angiotensin-converting enzyme (ACE) in the aorta of the *Bothrops jararaca* snake.

Main method: Contraction induced by Ang I or II in aortic ring from the snake was evaluated in the absence or in the presence of ACE-blocker or Ang II antagonists.

Key findings: Ang II analogs, modified at positions 1 and 5, induced vasoconstriction with differences in their potencies. The relative rank order was: [Asp^1, Val^5] Ang II = [Asp^1, Ile^5] Ang II ⋙ [Asn^1, Val^5] Ang II. ACE-like activity was detected, as well as an Ang II receptor with low affinity for AT_1 and AT_2 selective receptor antagonists (pK_B values of 5.62 ± 0.23 and 5.08 ± 0.25). A disulfide reducing agent almost abolished the Ang II effect, while an alpha adrenoceptor antagonist, or removing the endothelium, did not modify the Ang II effect. These results indicate that the *B. jararaca* aorta has an Ang II receptor pharmacologically distinct from AT_1 and AT_2 receptors, and the vasoconstrictor effect observed is independent of catecholamine or endothelium modulation. ACE and the AT receptor in the aorta of *B. jararaca* may be part of a tissue renin–angiotensin system.

Significance: The data contribute to the knowledge of the renin–angiotensin system in vertebrate species, and provide insight into the understanding of snake Ang II receptor characteristics and diversity.

Introduction

Functional and molecular studies have identified two angiotensin II (Ang II) receptors in mammalian species. They are characterized by their differential sensitivity to the selective antagonists, losartan and PD123319, which binds to AT_1 and AT_2 receptors respectively (Alexander et al. 2008). The AT_1 is involved in almost all the actions induced by Ang II, including actions that affect body fluid homeostasis, cardiovascular control, and cell growth (De Gasparo et al. 2000; Mehta and Griendling 2007). The AT_2 has been implicated in the hearing process and vascular injury, and it has an antiproliferative function (De Gasparo et al., 2000; Lemarié and Schiffrin, 2010), in contrast to the AT_1, which stimulates cell growth.

Additional Ang II receptor sites, not characterized as AT_1 or AT_2 receptors based on their pharmacological profile, have been identified in cells/tissues as neuroblastoma, heart, adrenal, brain or liver from vertebrate including rodents (Chaki and Inagami, 1992; De Oliveira et al., 1995), amphibians (Aiyar et al., 1994; Bergsma et al., 1993; Sandberg et al., 1991), birds (Brun et al., 2001; Kempf et al., 1996; Murphy et al., 1993) and fishes (Olivares-Reyes et al., 1997). This receptor has high affinity for the Ang II, and low affinity for the selective AT_1 and AT_2 receptor antagonists. A similar profile of a non-AT_1/AT_2 binding site was recently detected in human, mouse and rat brain, but unlike the other non-AT_1/AT_2 binding sites already reported, it is revealed only after pretreatment of the tissue with the protease inhibitor, p-chloromercuribenzoate (Karamyan and Speth, 2008; Karamyan et al., 2008a; Karamyan et al., 2008b).

The concept that Ang II is the unique active effector of renin–angiotensin system (RAS) has changed recently (Fyhrquist and Saijonmaa, 2008; Haulica et al., 2005). Cleavage of Ang II generates bioactive peptides as Ang 3–8 (Ang IV) and Ang 1–7, which act on their own receptors. Ang IV is the endogenous ligand for the AT_4 receptor, which was identified initially as an insulin-regulated aminopeptidase, and has a role in the regulation of local blood flow, cognitive processes, and sensory/motor functions (Chai et al., 2004). Ang 1–7 interacts with its receptor to produce vasodilation and inhibition of proliferation of vascular smooth muscle cells, and as a counter-regulatory mechanism against some AT_1 effects (Santos and Ferreira, 2007).

Several components of the RAS are present in the plasma (angiotensinogen, dipeptidyl hydrolase similar to the angiotensin

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converting enzyme) and kidney (renin) of the snake *Bothrops jararaca* (Gervitz et al., 1987; Lavras et al., 1978). Furthermore, Ang II induces a dose-dependent increase in mean arterial blood pressure and increases the plasma corticosterone concentration in this reptile (Breno and Picarelli, 1992; Breno et al., 2007; Lázari et al., 1994). *B. jararaca* has a circulating angiotensin converting enzyme (ACE) that produces the bioactive Ang II from inactive angiotensin I (Breno and Picarelli, 1992). Ang II causes a pressor response partly by a direct action and also indirectly by stimulating catecholamine release (Breno and Picarelli, 1992). The Ang II receptor in the cardiac membrane of *B. jararaca* is insensitive to the AT_1 and AT_2 antagonists losartan and PD123319, respectively (Breno et al., 2001), a pharmacological profile distinct from that characterized in mammals.

Snakes are particularly interesting for studies related to cardiovascular function, both because their elongated shape and also because they had to adapt to wide range of habitats, gravitational influences and variable demand for metabolic energy, which requires a prompt adjustment of the blood flow (Lillywhite et al., 1997; Secor and White, 2010; Seymour and Arndt, 2004). In our laboratory, important endogenous systems related to cardiovascular homeostasis, such as autonomic (Yamanouye et al., 1992), kinin–kallikrein (Abdalla et al., 1989) and endothelin systems (Borgheresi et al., 2006), have been characterized in some South American snakes. Although their physiological function seems to be relatively well conserved, peculiarities related to ligand or receptor structure have been detected (Breno et al., 2007). Regarding the renin–angiotensin system, an Ang II receptor with a distinct pharmacological profile has been characterized in the heart of *B. jararaca* (Breno et al., 2001), however, its functionality, evaluated by the activation of phospholipase C/inositol trisphosphate (IP_3) and adenylylcyclase/adenosine 3′5′-cyclic monophosphate (AMPc) could not be found (Breno et al., 2001). Thus, this study was undertaken to characterize pharmacologically and functionally an Ang II receptor in the aorta of *B. jararaca*. It is well known that the overall actions of RAS involve a local activity, represented by the tissue renin-angiotensin system (Haulica et al., 2005), and also the circulating RAS, already detected *in vivo* in *B. jararaca* (Breno and Picarelli, 1992). To investigate a local RAS in the aorta of *B. jararaca*, two main components of the cascade were evaluated: the Ang II receptor and ACE, which is an important rate-limiting step in generating the active peptide Ang II from its inactive form Ang I (Fyhrquist and Saijonmaa, 2008). Moreover, previous studies performed in rabbit and rat arteries have shown that removing the endothelial layer modifies the contractile effect induced by Ang II (Chen et al., 1995; Le Tran and Forster, 1996), and that this peptide also induces vasoconstriction in the arteries of rat, rabbit and dog or vasopressor action in domestic fowl, partly due to the facilitation of catecholamine release (Cox et al., 1996; Guimarães et al., 2001; Nishimura, 2001; Storgaard and Nedergaard, 1997). Therefore, in order to evaluate any modulatory action of the catecholamine and/or endothelium-derived factors on the final Ang II response in *B. jararaca*, experiments were carried out in the snake aorta pretreated with catecholamine antagonist, and also in the vascular tissue without endothelium.

Materials and methods

Animals

Adult male and female *B. jararaca* snakes were captured in the wild (São Paulo, Minas Gerais and Santa Catarina States — South and Southeast regions of Brazil) and were identified by the Laboratory of Herpetology of Instituto Butantan. Animals weighing 130–300 g were kept as described by Breno et al. (1990). Water was offered *ad libitum*, and snakes were not fed before the experiments. All the procedures involving animals were in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation, and this work was also approved by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, License # 38-02001005104/2008).

Functional assay

Snakes were anesthetized with sodium pentobarbital (30 mg/kg, administered into the coelomic cavity) and euthanized. Three to eight snakes were used in the experimental treatments. A segment of almost 5 cm of the aorta, caudal to the heart (right systemic artery and after the junction between left and right systemic arteries), was removed and dissected free of connective tissue. Four aortic rings (1 cm length) were obtained from each snake aorta. The aortic ring was suspended between two L-shaped steel hooks into a 10 ml organ chamber containing a solution of the following composition (mM): NaCl 147.17, KCl 4.95, $CaCl_2 \cdot 2H_2O$ 2.75, $MgSO_4 \cdot 7H_2O$ 1.21, $NaH_2PO_4 \cdot H_2O$ 1.2, $NaHCO_3$ 29.6 and glucose 5.5 (pH 7.3–7.7); the solution was aerated with 95% O_2 and 5% CO_2 (Yamanouye et al., 1992). The rings were placed under 1.0 g resting tension for 60 min at 37°C, and the isometric contraction was recorded with a force transducer connected to a polygraph (ECB — Ampère System, São Paulo, Brazil).

Effect of Ang II analogs, Ang II receptor antagonists and angiotensin-converting enzyme inhibitor in the snake aorta

Three Ang II analogs ([Asp^1, Ile^5] Ang II; [Asp^1, Val^5] Ang II; [Asn^1, Val^5] Ang II), with amino acid variation at positions 1 and 5, were used to evaluate their potencies in the vascular tissue of *B. jararaca*. Cumulative concentration–effect curves were obtained for each Ang II analog, and the data were expressed in g of tension. The pD_2 value (the negative logarithm of the molar concentration of Ang II required to produce 50% of the maximum effect) and E_{max} value (the maximum effect) were calculated by nonlinear regression analysis for each individual cumulative concentration–effect curve, using GraphPad Prism (GraphPad Software, San Diego, CA. USA), and are presented as the mean ± S.E.M. *n* represents the number of snakes used. To avoid desensitization, only one cumulative concentration–effect curve to Ang II was made in each aortic ring obtained from a snake, where this was considered an individual value. Except for this experimental group, all the other groups had [Asp^1, Ile^5] Ang II as the agonist peptide.

To characterize the receptor subtype, cumulative concentration–effect curves to Ang II were obtained in the absence (control) and in the presence of three concentrations of the nonselective Ang II receptor antagonist ([Sar^1, Ala^8] Ang II — 10^{-5}, 3×10^{-5}, 10^{-4} M), the selective AT_1 antagonist (losartan—3×10^{-5}, 10^{-4}, 3×10^{-4} M) and the selective AT_2 antagonist (PD 123319 — 3×10^{-6}, 10^{-5}, 10^{-4} M). Four aortic rings obtained from the same snake were used to construct the Ang II curve in the absence and in the presence of the antagonist (one antagonist concentration per ring), which was added 20 min before recording the Ang II curve. Preliminary experiments showed a similar sensitivity to Ang II among the four aortic rings obtained from the same snake (data not shown). The potency of the antagonist was expressed as the pK_B value, the negative logarithm of the dissociation constant K_B, which is equal to the molar concentration of the antagonist divided by the ratio of concentrations of the agonist that produces 50% of the maximum response in the presence and in the absence of the antagonist minus one (Besse and Furchgott 1976). Since similar pK_B values were obtained with the three different concentrations of each antagonist, they were averaged to give the reported pK_B values.

The presence of a functional tissue angiotensin-converting enzyme in the aorta was investigated in the absence (control) and in the presence of the ACE blocker captopril (10^{-6} M), applied 20 min before recording cumulative concentration–response curves to Ang I ([Asp^1, Ile^5, His^9] Ang I) or Ang II ([Asp^1, Ile^5] Ang II).

Contribution of disulfide bridge in the Ang II receptor structure, and influence of endothelium-derived factors or catecholamine to Ang II-induced vasoconstriction

The presence of a functionally important disulfide bridge in the snake Ang II receptor structure was evaluated using the reducing agent dithiothreitol. Cumulative concentration–effect curves to Ang II in aortic rings were compared after exposure to 0, 3, or 10 mM dithiothreitol for 20 min. This compound abolishes or potentiates, respectively, the response of AT_1 or AT_2 receptors to Ang II in mammalian tissues.

In order to verify the role of the endothelium layer as a modulator of Ang II response, the cumulative concentration–response curve to this peptide was compared in intact (control) and denuded-endothelium aorta from *B. jararaca* snake. The Ang II responses in denuded-endothelium aortic ring were expressed as percentage of the maximum response to this peptide obtained in intact aortic ring. Denuded rings were prepared by gently rubbing the luminal surface with cotton. Removal of the endothelium was confirmed by the absence of relaxant effect of acetylcholine (10^{-9} to 10^{-7} M) at the end of the Ang II cumulative curve. Acetylcholine is well known to produce an endothelium-dependent dilatation response in vascular tissue of vertebrates (Furchgott and Zawadzki, 1980; Knight and Burnstock, 1996).

To investigate the influence of catecholamines on Ang II vasoconstriction (Cox et al., 1996; Nishimura, 2001), concentration–effect curves to Ang II were obtained in the absence (control) and in the presence of phenoxybenzamine (10^{-7} M, 15 min). The Ang II responses in the presence of this alpha$_1$ adrenoceptor antagonist were expressed as percentage of the maximum response to Ang II obtained in aortic rings without antagonist. Phenoxybenzamine was also assayed against noradrenaline (10^{-9} to 10^{-5} M) to determine its effectiveness as a catecholamine-blocker in snake tissue.

Drugs and chemicals

[Asp^1, Ile^5, His^9] Ang I, [Asp^1, Ile^5] Ang II, [Asp^1, Val^5] Ang II, [Asn^1, Val^5] Ang II, [Sar^1, Ala^8] Ang II, captopril, and phenoxybenzamine were purchased from Sigma Chemical Co. (USA). Losartan and PD 123319 were gifts from DuPont Merck Pharmaceutical Co. (USA) and Park Davis Pharmaceutical Research Division (USA), respectively. DL-dithiothreitol was purchased from Jersey Lab. (USA). Chemicals not specified here were from Sigma Chemical (USA) or Merck (Germany).

Statistical analysis

The data were expressed as mean ± S.E.M. The mean pD_2, E_{max} and pK_B parameters were analyzed by analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons, or by two-tailed Student's *t*-test to compare two mean data. *P* values <0.05 were accepted as significant.

Results

Effect of Ang II analogs, Ang II receptor antagonists, and angiotensin converting enzyme inhibitor in the snake aorta

Three Ang II analogs that varied at amino acid positions 1 and 5 produced concentration-dependent contraction in aortic rings of the *B. jararaca* snake. The maximum effect of these analogs was similar. [Asn^1, Val^5] Ang II was the least potent Ang II analyzed, with pD_2 being significantly different from that of the two other Ang II analogs ($P<0.05$). The relative order of the potency for angiotensin analogs in snake aorta was: [Asp^1, Val^5] Ang II = [Asp^1, Ile^5] Ang II ⋙ [Asn^1, Val^5] Ang II. E_{max} and pD_2 values are shown in Table 1.

Table 1
pD_2 and E_{max} values for Ang II analogs in the aorta isolated from the *Bothrops jararaca* snake. Data are mean ± S.E.M.; the number of snakes used is indicated in parentheses.

Ang II analog	pD_2	E_{max}
[Asp^1, Ile^5] Ang II	6.76 ± 0.20 (6)	1.43 ± 0.20
[Asp^1, Val^5] Ang II	7.11 ± 0.11 (8)	1.71 ± 0.21
[Asn^1, Val^5] Ang II	5.92 ± 0.09 (4)*	1.44 ± 0.23

* Significantly different from the other two Ang II values, $P<0.05$.

[Sar^1, Ala^8] Ang II (nonselective Ang II receptor antagonist), losartan (selective AT_1 receptor antagonist) and PD123319 (selective AT_2 receptor antagonist) shifted the concentration–effect curve to [Asp^1, Ile^5] Ang II to the right and reduced the maximum effect (Fig. 1A, B and C). The potency of these antagonists, expressed as pK_B values, is summarized in Table 2. The selective AT_1 and AT_2 antagonists produced an Ang II-curve displacement only at high concentrations, suggesting that the angiotensin receptor in the snake aorta is not similar to the AT_1 and AT_2 receptors.

Captopril (10^{-6} M), an angiotensin-converting enzyme inhibitor, shifted the Ang I curve to the right (pD_2 value: 6.04 ± 0.10 to 4.94 ± 0.15, n = 5; Fig. 2A) but not the Ang II curve (pD_2 value: 6.80 ± 0.11 to 6.79 ± 0.16, n = 5; Fig. 2B). These results suggest the presence of ACE-like activity in the snake aorta. Differences between Ang I and Ang II pD_2 values in the absence of captopril may be indicative of an incomplete conversion of the first peptide.

Effect of the disulfide reducing agent dithiothreitol on the vascular response to Ang II

Dithiothreitol (3 or 10 mM) blocked contractions induced by almost all [Asp^1, Ile^5] Ang II concentrations (Fig. 3). However, higher concentrations of Ang II (above 10^{-6} M) induced a gradual response. The two concentrations of dithiothreitol had similar effects on Ang II concentration–effect curve, which could indicate the presence of a disulfide bond in the Ang II receptor structure.

Influence of catecholamine and removal of the endothelium on Ang II-induced contraction

The irreversible alpha$_1$ adrenoceptor antagonist phenoxybenzamine (10^{-7} M) did not modify the Ang II concentration–effect curve (Fig. 4A). However, this antagonist concentration was able to block noradrenaline responses (10^{-9} to 10^{-5}) in this tissue, indicating its effectiveness as a pharmacological tool (data not shown), while there was no involvement of catecholamine in the vasoconstriction induced by Ang II.

The removal of the endothelial layer also did not modify the concentration–effect curves to Ang II (Fig. 4B), but abolished the vasodilation induced by acetylcholine (10^{-9} to 10^{-7} M, data not shown), which is an endothelium-dependent agonist. Endothelium-derived factors do not seem to contribute to Ang II response in the snake aorta.

Discussion

The RAS is present in vertebrates throughout the phylogenetic scale, and plays an important physiological role (Brown et al., 2005; Nishimura, 2001). A previous study from our laboratory has shown that Ang II produces a dose-dependent increase in carotid blood pressure in the *B. jararaca* snake, due to a direct action on its receptor and an indirect action resulting from catecholamine release (Breno and Picarelli, 1992; Breno et al., 2007). Outside the cardiovascular system, Ang II induces a concentration-dependent contraction in isolated uterus and increases plasma corticosterone concentration in *B. jararaca* (Lázari et al., 1994). A specific and saturable Ang-II binding site, with low

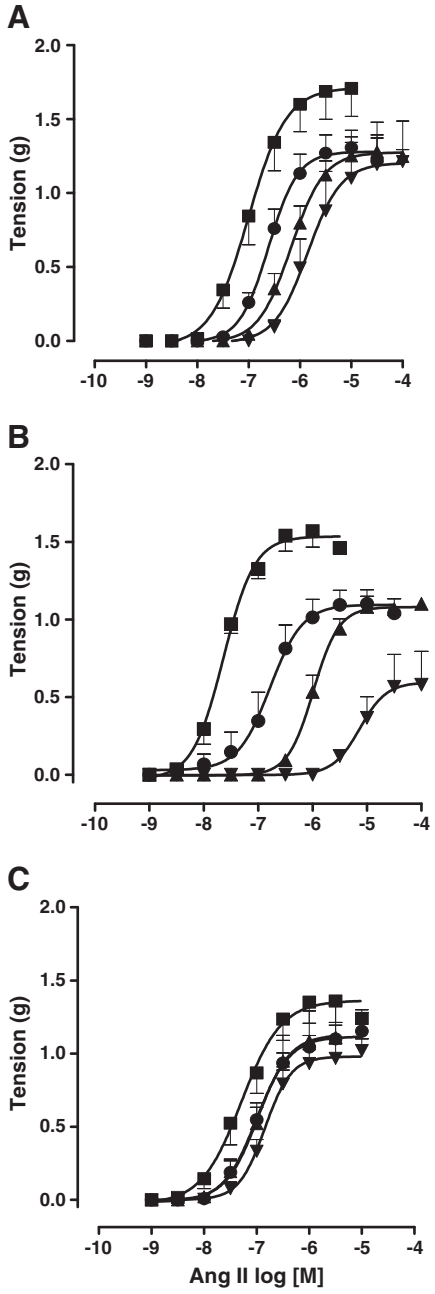

Fig. 1. Cumulative concentration–response curves to Ang II obtained in aortic rings from *Bothrops jararaca*, in the absence (control, ■) and in the presence of [Sar^1, Ala^8] Ang II (A — nonselective antagonist, ● 10^{-5} M; ▲ 3×10^{-5} M; ▼ 10^{-4} M; $n=7$), Losartan (B — selective AT_1 antagonist, ● 3×10^{-5} M; ▲ 10^{-4} M; ▼ 3×10^{-4} M; $n=4$) and PD123319 (C — selective AT_2 antagonist, ● 3×10^{-6} M; ▲ 10^{-5} M; ▼ 10^{-4} M; $n=5$). Each point and each vertical line represent the mean ± S.E.M., n = number of snakes used.

affinity for AT_1 and AT_2 receptor antagonists, has also been detected in the heart of the *B. jararaca* (Breno et al., 2001). However, there is no information in the literature about the presence of a functional Ang II receptor and the enzyme responsible for generating Ang II, ACE, in the vascular tissue of this snake.

Many species of fishes have [Asn^1, Val^5] Ang II, and tetrapods have [Asp^1, Val^5] Ang II endogenously (Nishimura, 2001; Takei et al., 2004), but the plasma of *B. jararaca* contains [Asp^1, Ile^5] Ang II and [Asp^1, Val^5, Tyr^9] Ang I (Borgheresi et al., 1996). Previous studies in *B. jararaca* showed reduced potency for [Asn^1, Val^5] Ang II compared to [Asp^1, Ile^5] Ang II and [Asp^1, Val^5] Ang II at increasing arterial pressure and in uterine and cardiac tissue assays of this snake (Breno and Picarelli, 1992; Breno et al., 2001; Lázari et al., 1994). The present results showed a concentration-dependent contraction response for [Asp^1, Ile^5] Ang II, [Asp^1, Val^5] Ang II, and [Asn^1, Val^5] Ang II, with a similar E_{max}, but potency varied. A contraction effect for Ang II was also observed in reptile, such as turtle (*Pseudemys scripta*, Stephens, 1984) and snake (*Naja naja* and *Ptyas korros*, Yung and Chiu, 1985), but in fowl, Ang II induces an endothelium-dependent relaxation response (Hasegawa et al., 1993; Yamaguchi and Nishimura, 1988). In rabbit aorta and fowl blood pressure, [Asp^1, Ile^5] Ang II and [Asp^1, Val^5] Ang II were also equipotent as in the aorta of *B. jararaca*, and [Asn^1, Val^5] Ang II was the least potent analog (Helmer, 1964; Nakamura et al., 1982; Nishimura et al., 1982). Nevertheless, [Asn^1, Val^5] Ang II, an endogenous angiotensin in some fish species (Takei et al., 2004), was equipotent to [Asp^1, Ile^5] Ang II in calcium mobilization, inositol trisphosphate (IP_3) formation, and [^{125}I] Ang II displacement in hepatocytes of the catfish *Ictalurus punctatus* (Olivares-Reyes et al., 1997). Thus, amino acid variation at position 1,

Table 2
pK_B values for Ang II receptor antagonists in the aorta isolated from the *Bothrops jararaca* snake. Data are mean ± S.E.M.; the number of the snakes used is in parentheses.

Ang II antagonist	pK_B
[Sar^1, Ala^8] Ang II (7)	5.10 ± 0.08
Losartan (4)	5.62 ± 0.23
PD123319 (5)	5.08 ± 0.25

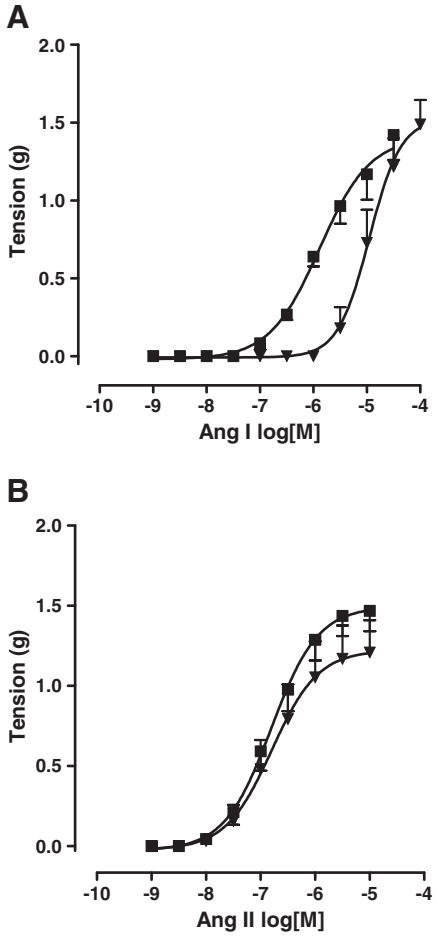

Fig. 2. Cumulative concentration–response curves to Ang I (A) and Ang II (B) obtained in aortic rings from *Bothrops jararaca*, in the absence (control, ■) and in the presence of captopril (▼ 10^{-6} M; $n=5$). Each point and each vertical line represent the mean ± S.E.M., n = number of snakes used.

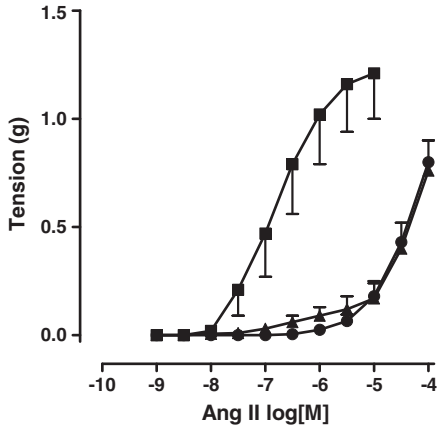

Fig. 3. Cumulative concentration–response curves to Ang II obtained in aortic rings from *Bothrops jararaca*, in the absence (control, ■) and in the presence of dithiothreitol (● 3 mM, $n=8$; ▲ 10 mM, $n=3$). Each point and each vertical line represent the mean ± S.E.M., n = number of snakes used.

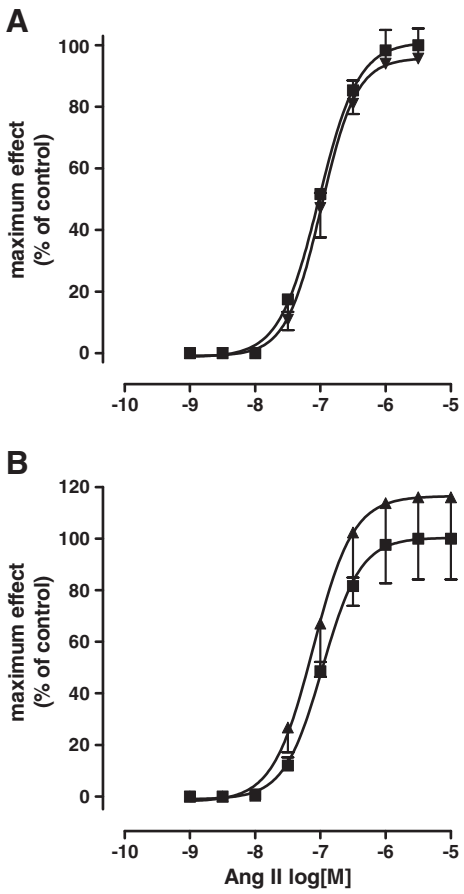

Fig. 4. Cumulative concentration–response curves to Ang II obtained in aortic rings from *Bothrops jararaca*. A — Rings with intact (control, ■) and denuded-endothelium (▲, $n=5$). B — Rings in the absence (control, ■) and in the presence of phenoxybenzamine (10^{-7} M, ▼, $n=3$). Each point and each vertical line represent the mean ± S.E.M., n = number of snakes used.

more than at position 5, seems to affect the potency of these Ang II analogs in *B. jararaca*.

The angiotensin molecule is phylogenetically ancient and well conserved (Takei et al., 2004). Most variation occurs at positions 1, 5 and 9. Residue 1 of the Ang I is Asn in fish and Asp in species from amphibians to mammals, although there are exceptions, as Asp in the holostean fish *Amia calva* (Takei et al., 1998), and Asn in the amphibian *Xenopus laevis*. Residue 5 is Val in nonmammalian, and Ile in mammals, although there are exceptions here as well: dogfish (*Triakis scyllia*, Takei et al., 1993a), flounder (*Platichthys flesus*, Balment et al., 2003) and amphibians (*X. laevis*, Takei et al., 2004) have Ile, and cattle have Val. Residue 9 has the largest amino acid variation. In the plasma of *B. jararaca*, residue 1 is Asp, and both forms with Val and Ile at position 5 are present (Borgheresi et al., 1996). Based on our results in the aorta, and those obtained with blood pressure and uterine/cardiac tissues of the same snake, it appears that the spatial arrangement of [Asp^1, Ile^5] Ang II and [Asp^1, Val^5] Ang II offers a better adjustment to the *B. jararaca* receptor binding pocket than that of [Asn^1, Val^5] Ang II.

Angiotensin converting enzyme is a dipeptidyl-carboxypeptidase that is a limiting step for generation of active Ang II from the inactive Ang I. The ACE inhibitor captopril inhibited contraction in the aorta of *B. jararaca* induced by Ang I, but not by Ang II, indicating that ACE-like activity is present in the snake aorta, and that Ang II is the active molecule of RAS. Similar result was obtained in the carotid artery of this snake (data not shown). Interestingly, exogenous Ang I, used in the current work and in an *in vivo* assay with *B. jararaca* (Breno and Picarelli, 1992), has a histidine at position 9 instead of a tyrosine reported for endogenous Ang I in *B. jararaca* (Borgheresi et al., 1996). This amino acid replacement does not seem to alter the enzymatic action of ACE in the aorta, as Ang I and Ang II had close pD_2 values in the absence of the ACE-blocker. A vasopressor response to Ang I was also reported for alligator (*Alligator mississippiensis*), quail (*Coturnix coturnix japonica*) and rat, independent of the ninth residue in the peptide molecule (Takei et al., 1993b).

AT_1 and AT_2 are classical Ang II receptors that have been characterized extensively by molecular and pharmacological methods (Alexander et al., 2008). Losartan and PD123319 are used as pharmacological tools to characterize both receptors, but they also aided in the identification of an additional high-affinity Ang II-binding site in some vertebrates, which has a low affinity for both selective antagonists (Nishimura, 2001). To characterize the Ang II receptor in the aorta of *B. jararaca*, we used nonselective and selective Ang II receptor antagonists. [Sar^1, Ala^8] Ang II shifted the Ang II concentration–effect curve to the right and reduced the maximum effect, indicating the presence of a functional Ang II receptor in the snake. The pK_B (5.10 ± 0.08 $n=7$) is relatively close to the pK_i (6.28 ± 0.32 $n=6$) parameter obtained for this antagonist in competition-binding studies using the cardiac membrane of the same snake (Breno et al., 2001). However losartan and PD123319, selective antagonists, shifted the Ang II curves to the right and reduced the maximum effect at high concentrations, which are out of the nanomolar range used to characterize the AT_1 and AT_2 in mammalian (De Gasparo et al., 1998). The potencies of these antagonists, expressed by the pK_B (Table 2), indicate a low affinity for losartan and PD123319, as reported for the Ang II receptor identified in turkey adrenal and amphibian myocardium (Aiyar et al., 1994; Murphy et al., 1993; Sandberg et al. 1991). Losartan also attenuated but did not completely block the pressor response to native Ang II in the reptile *Caiman crocodilus* (Butler, 2006). Thus, our data suggest the presence of functional Ang II receptor in the aorta of *B. jararaca*, with a distinct pharmacological profile compared to the classical AT_1 and AT_2, but similar compared to the Ang II receptor previously reported in the cardiac membrane of this snake. Lack of sensitivity for both selective Ang II antagonists was also shown for Ang II receptor in brain and kidney of gerbil (De Oliveira et al., 1995; Moriuchi et al., 1998), adrenal of turkey (Murphy et al., 1993) and heart of frog (Sandberg and Ji, 2001), in which there was almost 60% amino acid homology with the AT_1 (Sandberg and Ji, 2001). In this context, an important step for understanding the losartan-Ang II receptor interaction was made by mutating losartan-insensitive Ang II receptor from the amphibian *X. laevis* (Ji et al., 1995). Replacement of thirteen amino acids with the

corresponding amino acids of the rat AT_1 generated a mutant that was sensitive to the losartan. Thus, despite of both receptors binding the Ang II with high affinity, specific epitopes are important for the antagonist interaction. We can speculate that similar epitopes could be present in the Ang II receptor of *B. jararaca* based on its pharmacological profile.

AT_1 and AT_2 receptors can also be differentiated by their sensitivity to the sulfhydryl-reducing agent dithiothreitol, which reduces/potentiates responses mediated, respectively, by AT_1 and AT_2 (Heerding et al., 2001; Ohyama et al., 1995; Zhang et al., 1994). The Ang II receptor in the aorta of *B. jararaca* behaves like the AT_1, since dithiothreitol abolished the response to Ang II. However, concentrations above 10^{-6} M still increased tension in the aorta treated with dithiothreitol. It suggests that the Ang II receptor in *B. jararaca* could contain a functionally important disulfide bond. A mutagenesis study carried out on AT_1 and AT_2 receptors demonstrated disulfide bonds that confer, respectively, DTT inhibition/potentiation in AT_1 and AT_2 (Heerding et al., 2001). Molecular analysis of the amphibian angiotensin II receptor showed cysteine residues located at similar positions, composing disulfide bridges important for Ang II binding (Ji et al., 1993; Sandberg and Ji, 2001). We speculate the existence of such disulfide bonds in the snake Ang II receptor, as the Ang II responses were almost completely abolished by dithiothreitol.

Ang II induces vasoconstriction or vasopressor response partly due to facilitation of catecholamine release in vertebrate species (Cox et al., 1996; Guimarães et al., 2001; Nishimura, 2001; Storgaard and Nedergaard, 1997). We reported that the hypertensive effect of Ang II in *B. jararaca* was partly due to catecholamine release (Breno and Picarelli, 1992). In the present study, we did not find the participation of catecholamine in the vasoconstriction induced by Ang II, indicating a direct action on its own receptor. Similar reports with vascular preparations of fish (*T. scyllia*, Hamano et al., 1998), snake (*N. naja*, *P. korros*, Yung and Chiu, 1985) and turtle (*Pseudemys scripta elegans*, Stephens, 1984) also pointed to an Ang II vasoconstriction independent of catecholamine release, as we observed in the aorta of *B. jararaca*.

It is known that endothelial cells modulate vascular reactivity through the production of vasodilating and vasoconstricting factors (Chen et al., 1988; Ignarro et al., 1987; Palmer et al., 1987). Removal of the endothelial layer abolishes the relaxant response to acetylcholine (Furchgott and Zawadzki, 1980), and modifies the contractile effect of Ang II in the rabbit aorta, and rat arteries (Chen et al., 1995; Le Tran and Forster, 1996). However, removal of the endothelium did not affect the vasoconstriction to Ang II in the aorta of *B. jararaca*, but abolished the vasodilatation response to acetylcholine showing the effectiveness of the procedure (data not shown). Therefore, endothelium-derived factors did not contribute to the Ang II response in this snake, and the endothelial layer has no Ang II receptor involved in the contraction effect. This differs from fowl aorta, where Ang II produces an endothelium-dependent relaxation, associated with a rise in cGMP (Nishimura, 2001; Yamaguchi and Nishimura, 1988).

Conclusion

These results show the presence of a functional Ang II receptor in the aorta of *B. jararaca*, that has a low affinity for the selective AT_1 and AT_2 receptor antagonists. It behaves like those Ang II receptors pharmacologically distinct from the classical AT_1 and AT_2 receptors. The Ang II-vasoconstrictor effect observed in this tissue is independent of catecholamine or endothelium modulation, and the snake aorta contains an effective ACE-like activity. ACE and the AT receptor in the aorta of *B. jararaca* may be part of a tissue renin–angiotensin system of this snake. Our data contribute to the knowledge of the renin–angiotensin system in vertebrate species, and provide insight into the understanding of snake Ang II receptor characteristics and diversity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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