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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).