

Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats

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Fontes, Marco A. P., Ovidiu Baltatu, Sordaini M. Caligorne, Maria J. Campagnole-Santos, Detlev Ganten, Michael Bader, and Robson A. S. Santos. Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats. *Physiol Genomics* 2: 137–142, 2000.—We have previously demonstrated that microinjections of the selective angiotensin-(1–7) [ANG-(1–7)] antagonist, A-779, into the rostral ventrolateral medulla (RVLM) produces a significant fall in mean arterial pressure (MAP) and heart rate (HR) in both anesthetized and conscious rats. In contrast, microinjection of angiotensin II (ANG II) AT₁ receptor antagonists did not change MAP in anesthetized rats and produced dose-dependent increases in MAP when microinjected into the RVLM of conscious rats. In the present study, we evaluated whether endogenous ANG-(1–7) and ANG II acting at the RVLM contribute to the hypertension of transgenic rats harboring the mouse renin Ren-2 gene, TGR(mREN2)27. Unilateral microinjection of A-779 (0.1 nmol) produced a significant fall in MAP (-25 ± 5 mmHg) and HR (-57 ± 20 beats/min) of awake TGR rats. The hypotensive effect was greater than that observed in Sprague-Dawley (SD) rats (-9 ± 2 mmHg). Microinjection of the AT₁ antagonist CV-11974 (0.2 nmol) produced a fall in MAP in TGR rats (-14 ± 4 mmHg), contrasting with the pressor effect observed in SD rats (33 ± 9 mmHg). These results indicate that endogenous ANG-(1–7) exerts a significant pressor action in the RVLM, contributing to the hypertension of TGR(mREN2)27 transgenic rats. The role of ANG II at the RVLM seems to be dependent on its endogenous level in this area.

rostral ventrolateral medulla; angiotensin II; angiotensin-(1–7); AT₁ antagonists; angiotensin-(1–7) antagonist; transgenic hypertensive rats

SEVERAL STUDIES HAVE SHOWN that the neurons of the rostral ventrolateral medulla (RVLM), a key region in the central regulation of blood pressure (10, 14), can be influenced by angiotensin peptides including ANG II (3, 16, 17), ANG III (36), and ANG-(1–7) (11, 12, 34). The findings that topical application or local microinjection of ANG II into the RVLM produces increases in blood pressure and renal nerve activity (3, 16, 17) are consistent with a high expression of AT₁ receptors in this

region as demonstrated by autoradiography (2, 13, 24). A physiological role for endogenous ANG II in this region has been suggested by the demonstration of a significant drop in mean arterial pressure (MAP) following topical application or microinjection of the nonselective ANG II receptor antagonist [Sar¹, Thr⁸]ANG II (3, 17, 31).

We have recently uncovered a possible role for ANG-(1–7) in this region by showing that microinjection of this heptapeptide into the RVLM increases MAP (34), whereas microinjection of its selective antagonist, A-779, produces the opposite effect (12, 29). These data, obtained first in anesthetized rats (12, 29, 34), have been confirmed in awake animals (11).

It is now well accepted that the biological effects elicited by ANG II are mediated through the interaction with AT₁ and AT₂ receptor subtypes. Several pharmacological evidences indicate that the actions of ANG-(1–7) can be mediated by different receptor or receptor subtype (29, 35). AT₁ receptor antagonists are capable of abolishing the pressor effect of ANG II at the RVLM (4, 16). However, microinjection of these antagonists alone did not change blood pressure or produce a slight increase in MAP in anesthetized animals (4, 12, 16, 29) and produced a dose-dependent increase in MAP in freely moving rats (11). These observations, especially in awake rats (11), suggest a primary inhibitory role for ANG II in this region, in normotensive animals. However, the role of angiotensin peptides in the RVLM of freely moving hypertensive animals has not been studied.

The generation of the genetic model of hypertension by insertion of the mouse Ren-2 renin gene into the genome of the Sprague-Dawley (SD) rat (26) created an important tool for investigating the pathophysiological consequences of enhanced activity of the brain renin-angiotensin system (RAS), particularly activated in this model of hypertension, TGR(mREN2)27 (8, 18, 26, 33).

The finding that the circulating RAS activity is normal in TGR(mREN2) rats and the effectiveness of drugs interfering with the RAS to lower blood pressure in these animals (25) indicate a major role for tissue RAS in the pathogenesis of this genetic model of hypertension. Interference with the brain RAS by intracerebroventricular administration of ANG II anti-

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body decreases blood pressure in TGR(mREN2) rats (25), demonstrating a role for brain RAS in the maintenance of high blood pressure in TGR. However, the sites within the brain where the increased production of angiotensins would influence peripheral sympathetic activity increasing blood pressure have not yet been identified. Considering that the RVLM is a key region in the central nervous system to determine peripheral sympathetic activity, we addressed the hypothesis that one of the mechanisms determining hypertension in TGR rats is an increased angiotensinergic activity in this region. Thus in this study, we used the improved technique for microinjections into the RVLM of freely moving rats (11), in TGR(mREN2)27 rats, to further elucidate the role of endogenous angiotensin peptides at the RVLM.

METHODS

Experiments were carried out in 12 heterozygous adult male TGR(mREN2)27 (TGR) and 9 male SD (300–350 g) from the Max-Delbrück-Center For Molecular Medicine (Berlin-Buch, Germany). All the procedures followed with the animals were in accordance with the guidelines recommended by the institution.

Surgical procedures. Under chloral hydrate anesthesia (300 mg/kg ip), the animals were positioned on a stereotaxic frame (Stoelting, Wood Dale, IL) with the tooth bar fixed 11 mm below the level of the interaural line. Guide cannulas were fixed in the interparietal bone according to a modification of a procedure previously described (19). Briefly, bilateral guide cannulas (22 gauge) held in a micromanipulator were inclined at an angle of 18–20° from the vertical plane and fixed with dental cement and jeweler's screws in the interparietal bone with the tip oriented caudally. The tip of the guide cannulas were positioned just above the dura mater (~1 mm below the skull surface). Each guide cannula was positioned 2 mm caudal to the lambdoid suture and 1.8 mm lateral to the midline. A polyethylene catheter was inserted under ether anesthesia, into the abdominal aorta through the femoral artery, 24 h before the experiment. The catheter was tunneled through the subcutaneous tissue, exteriorized at the back of the neck of the animals, and secured by suture. MAP and heart rate (HR) were continuously recorded with a solid-state strain gauge transducer coupled to a data acquisition system (model BP14; Technical & Scientific Equipment, Bad Homburg, Germany) or to a Nihon-Kohden polygraph (Japan).

Microinjections. For microinjections, 4 days after guide cannula implantation, a 30-gauge needle (22 mm long), was slowly lowered through the guide cannula into the RVLM. The placement of the injection needle or microinjection of vehicle (200 nl) into the RVLM produces a transient increase in MAP. This effect was not elicited when the needle was positioned in adjacent areas and for this reason was helpful to confirm that the injection needle was in the desired site. In addition, microinjection of angiotensins or antagonists into adjacent areas such as the inferior olivary nucleus did not change blood pressure. A minimum of 15 min was allowed between the positioning of the injection needle and the microinjection. The rats were healthy and did not show any signal of discomfort during the microinjection procedures.

Experimental protocol. In 21 animals, we measured the cardiovascular effects produced by unilateral microinjection of saline (200 nl: TGR, $n = 4$; SD, $n = 4$), the selective AT₁ angiotensin antagonist, CV-11974 (0.2 nmol: TGR, $n = 4$; SD,

$n = 5$), or the selective ANG-(1–7) antagonist, A-779 (0.1 nmol: TGR, $n = 4$; SD, $n = 4$). All microinjections were made in a total volume of 200 nl over a 20-s period using a Hamilton syringe connected to the injection needle through a polyethylene tubing (PE-10). Each rat received only one microinjection in each side of the medulla. During and after placement of the injector needle into the RVLM, the rats remained undisturbed, without external signals of discomfort. Excluding the transient change in MAP produced by the placement of the injection needle at the RVLM, the blood pressure and HR remained within the normal range for the particular rat strain.

Drugs. All drugs were dissolved in sterile saline (NaCl 0.9%). A-779 was obtained from Bachem (batch 513666). CV-11974 was obtained from Takeda Chemical Industries (Osaka, Japan). Based in preliminary experiments, the doses of CV-11974 (21) and A-779 (11, 29) used in this study were found sufficient to block the effects of pressor doses of ANG II (25 pmol) and ANG-(1–7) (25 pmol), respectively.

Histological analysis. At the end of each experiment, the animals were anesthetized with ether and a microinjection of Alcian blue dye (200 nl, 2.5%) was made. The brain was then carefully removed, and the deposition of the dye was macroscopically and microscopically examined to further confirm the location of the injection site in the RVLM. Microinjection site was referred to standard anatomic structures of the lower brain stem according to the atlas of Paxinos and Watson (28).

Statistical analysis. The results are expressed as means \pm SE. Comparisons between two observations in the same animal were evaluated by Student's *t*-test for paired observations, and comparisons between two groups were analyzed by Student *t*-test. Differences were considered significant for $P < 0.05$.

RESULTS

Figure 1 shows the averaged basal values of MAP and HR of all conscious transgenic ($n = 12$) and SD ($n = 9$) rats used in this study, 15 min after the positioning of the microinjection needle into the RVLM. As expected, the MAP baseline values were significantly higher in TGR compared with normal SD rats (174 ± 2 mmHg and 123 ± 4 mmHg, respectively; $P < 0.0001$, Student's *t*-test). Baseline values of HR were greater in TGR rats (345 ± 16 beats/min) compared with SD rats (284 ± 6 beats/min).

As shown in Fig. 2, unilateral microinjection of the AT₁ antagonist, CV-11974 (0.2 nmol), into the RVLM of awake SD rats (baseline MAP = 129 ± 2 mmHg, $n = 5$) produced a marked increase in MAP (33 ± 9 mmHg) that lasted for 5 ± 0.9 min. In contrast, microinjection of CV-11974 into the RVLM of TGR rats (baseline MAP = 170 ± 5 mmHg, $n = 4$) produced a significant fall in MAP (-14 ± 4 mmHg, Fig. 2). This depressor response lasted for 13 ± 3 min. No significant changes in HR were observed after CV-11974 microinjection in both normal SD (baseline HR = 294 ± 9 beats/min) or transgenic conscious rats (baseline HR = 326 ± 16 beats/min) (see Fig. 2).

The microinjection of the ANG-(1–7) antagonist, A-779 (0.1 nmol), in the RVLM of conscious SD rats (baseline MAP = 116 ± 5 mmHg, $n = 4$) produced a significant decrease in MAP (-9 ± 2 mmHg, $P < 0.05$; Fig. 2). In TGR rats (baseline MAP = 177 ± 5 mmHg, $n = 4$), a greater hypotensive effect after A-779 microin-

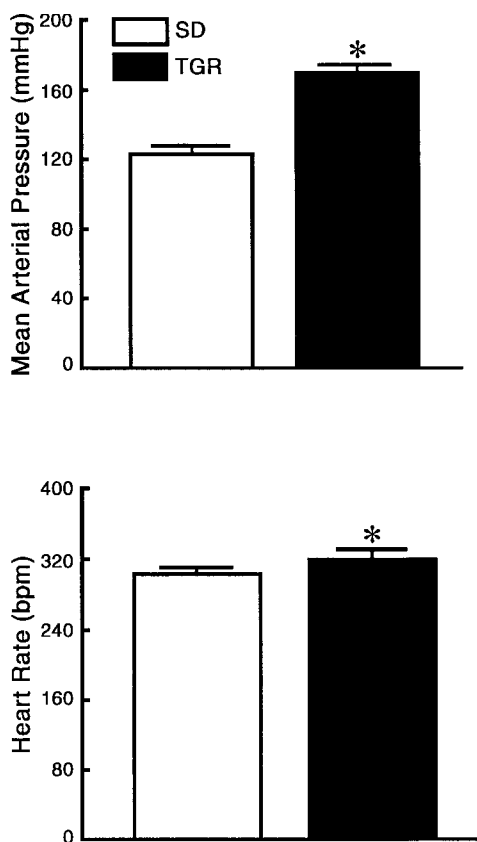


Fig. 1. Averaged baseline values of mean arterial pressure (MAP) and heart rate (HR) of Sprague-Dawley (SD, $n = 9$) and TGR-(mREN2)27 transgenic hypertensive rats (TGR, $n = 12$). * $P < 0.05$ compared with SD rats (Student's t -test).

jection in the RVLM was observed (-25 ± 5 mmHg, $P < 0.05$; Fig. 2). The duration of the depressor observed in TGR rats was also significantly greater than that observed in SD rats (47 ± 12 and 12 ± 5 min, respectively; $P < 0.05$). In addition, A-779 microinjection produced a significant bradycardic effect (-57 ± 20 beats/min, $P < 0.05$; Fig. 2) in TGR rats (baseline HR = 317 ± 17 , $n = 4$). However, the bradycardic effect was not statistically different from that observed after A-779 microinjection in SD rats (-6 ± 15 beats/min, Fig. 2).

Saline microinjection into the RVLM produced small and transient increases in MAP (5 ± 0.75 in SD and 4 ± 1.6 mmHg in TGR; Fig. 2). These MAP changes were not accompanied by significant changes in HR (3.5 ± 7.2 in SD and 0 ± 0 beats/min in TGR; Fig. 2).

The histological examination of the brain of the animals considered in this study, showed that the microinjections reached the ventral area of the RVLM ~ 1.8 to 2.2 mm rostral to the obex, corresponding to the lateral paragigantocellular nucleus and the ventral portion of the rostroventrolateral reticular nucleus (Fig. 3).

DISCUSSION

The present study showed that microinjection of the AT₁ ANG II antagonist, CV-11974, or the ANG-(1-7)

antagonist, A-779, into the RVLM of awake TGR-(mREN2)27 transgenic rats produced significant reductions in MAP. As reported previously for the AT₁ blockers losartan and L-158,809 (11), microinjection of CV-11974 into the RVLM of SD rats produces a significant increase in MAP, in freely moving rats. Microinjection of A-779 in the RVLM of SD rats produced a small but significant decrease in MAP.

Based on results obtained with the exogenous administration of ANG II and with the use of nonspecific ANG II antagonists, especially [Sar¹,Thr⁸]ANG II, an excitatory role for endogenous ANG II in the RVLM has been proposed by several groups (3, 17, 31). However, following observations in anesthetized rats (4, 12) and rabbits (16) that microinjection of AT₁ antagonist into the RVLM produces increases rather than decreases in MAP, an excitatory role for ANG II at the RVLM in basal conditions was questioned (12, 15, 16). Our recent study in freely moving rats showing a dose-dependent increase in MAP produced by microinjection of the AT₁ antagonist, losartan, in this region (11) was a further

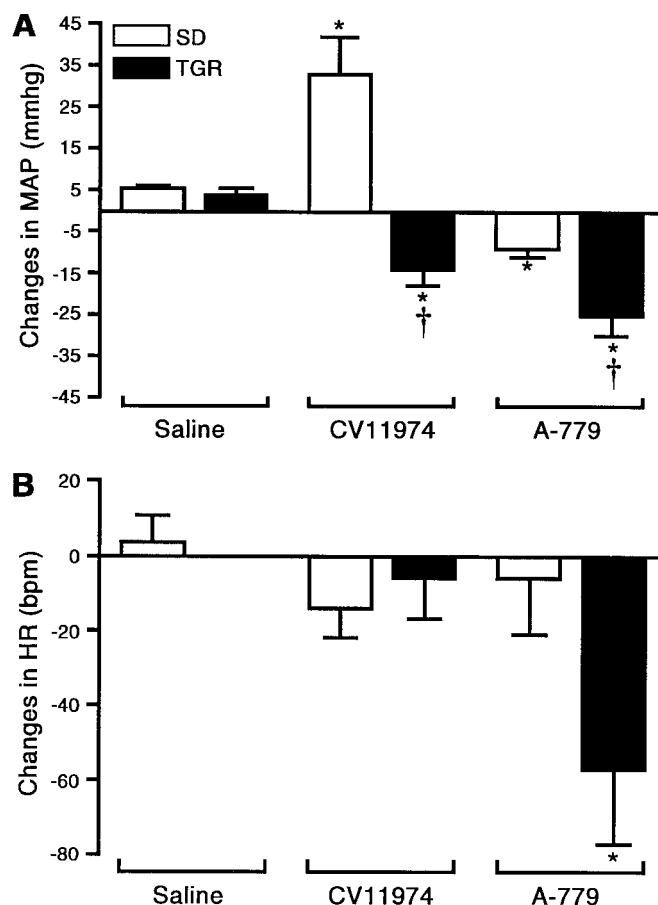


Fig. 2. Averaged peak changes in MAP or HR produced by microinjection of saline (200 nl), the AT₁ receptor antagonist, CV-11974 (0.2 nmol), or the ANG-(1-7) antagonist, A-779 (0.1 nmol), into the rostral ventrolateral medulla (RVLM) of awake transgenic hypertensive (TGR, $n = 4$ each) or normotensive (SD, $n = 4-5$) rats. Microinjection of antagonists were made 15 min after placement of the injector needle in the RVLM. * $P < 0.05$ compared with effect obtained with saline microinjection (Student's t -test). † $P < 0.05$ compared with effect obtained for the same antagonist in SD rats (Student's t -test).

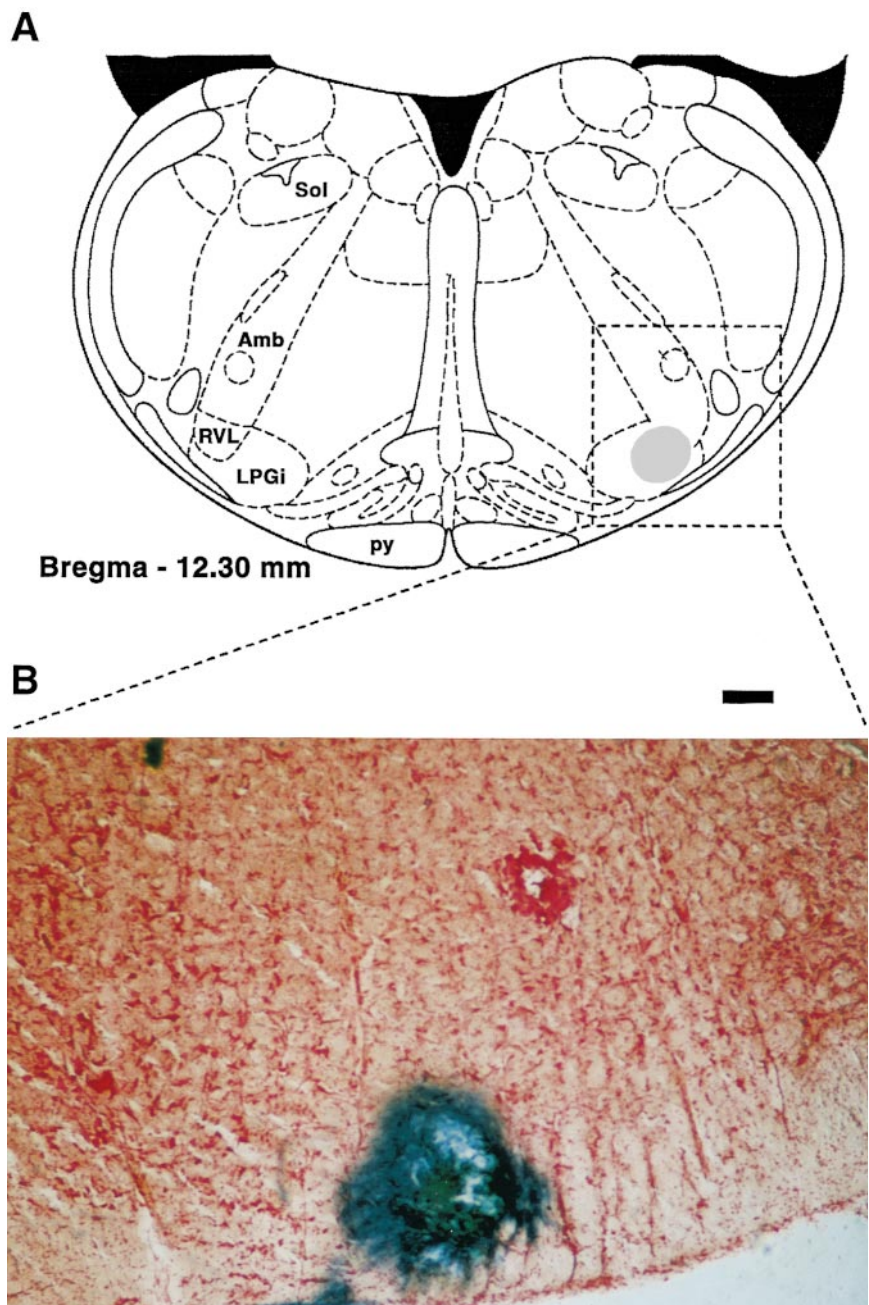


Fig. 3. *A*: diagram of a coronal section of the rat medulla according to the stereotaxic coordinates of the atlas of Paxinos and Watson (28), showing the location of the center of the microinjections into the RVLM. *B*: photomicrograph of a coronal section of the rat medulla showing a typical site of microinjection into the RVLM marked by the deposition of Alcian Blue dye. Sol, nucleus tractus solitarius; Amb, nucleus ambiguus; RVL, rostromedullary reticular nucleus; LPGi, lateral paraganglionic nucleus; py, pyramidal tract. Bar = 200 μ m.

indication that in normotensive animals ANG II could play an inhibitory role in this key region for the central control of blood pressure (10, 14). In the present study, we reinforced this possibility, using the AT₁ antagonist CV-11974. One may argue that the effects obtained with CV-11974 could be due to a nonselective action. Although we cannot completely exclude this possibility, it is unlikely. First, other AT₁ blockers (losartan, L-158,809) also produced pressor responses when microinjected in this region in normotensive rats (11). Second, CV-11974 has been proven to be a highly specific AT₁ antagonist with no interaction with thromboxane A₂ receptors, as shown for losartan (21), or with imidazole/guanidinium binding sites in the brain (20). In addition, it is unlikely that a nonspecific pressor effect

in normotensive rats could turn in a hypotensive effect in hypertensive rats as shown for the effects of CV-11974 in this study.

Conversely to that observed in normotensive SD rats, microinjection of CV-11974 into the RVLM of TGR(mREN2)27 rats produced a small but significant decrease in MAP, indicating that in these animals, endogenously synthesized ANG II acting on AT₁ receptors contributes to the hypertensive levels of MAP. At present, we have no data to explain the apparently opposite role of AT₁ receptors in normotensive vs. hypertensive rats. However, taking into account the data obtained by Senanayake et al. (33), which showed increased levels of ANG II in the medulla of TGR(mREN2)27 rats and the pressor effect produced

by exogenous administration of ANG II in the RVLM (3, 31, 32, 34), it is reasonable to suppose that increased levels of ANG II in the RVLM could facilitate the effect of the peptide through AT₁ receptors located in sympathetic excitatory neurons. Whether this could be due to factors such as differential sensitivity of inhibitory vs. excitatory sympathetic neurons or access of the peptides to subregions within the RVLM remains to be established. On the other hand, in basal conditions ANG II would be produced and/or released preferentially close to AT₁ receptors associated with interneuronal inhibitory pathways and/or in neuronal terminals projecting from the caudal ventrolateral medulla.

Microinjection of A-779 into the RVLM of SD or TGR(mREN2)27 rats produced decreases in MAP. The fall in MAP was greater and of longer duration in TGR rats. One may argue that the greater decrease in MAP produced by A-779 in the hypertensive rats could be only due to the higher levels of MAP. However, even taking into account the basal levels of MAP in both groups, the averaged percentile of decrease in MAP calculated from the data presented in Fig. 2 was higher in the hypertensive group (14.1%) than in SD rats (7.7%). These results indicate that ANG-(1-7) or an ANG-(1-7)-related peptide acting at the RVLM plays an important role in the maintenance of hypertension in TGR(mREN2)27 rats, specially considering that the reductions in MAP were obtained with unilateral microinjections in freely-moving animals.

The possibility that the A-779 effect could be due to an interference with other peptides cannot be completely ruled out. However, this possibility is unlikely for several reasons: First, the molecular structure of A-779 is very close to ANG-(1-7). This compound is an analog of ANG-(1-7) presenting a D-alanine at the carboxy terminus substituting for proline (1, 29). Second, it is devoid of agonistic activity in several preparations (6, 27, 29). Third, A-779 has been characterized as a selective ANG-(1-7) antagonist based on the observation that it antagonizes several actions of ANG-(1-7), including its antidiuretic effect in water-loaded rats (29, 30), the bradykinin-potentiating activity of ANG-(1-7) in awake rats (22), the changes in MAP produced by ANG-(1-7) microinjection into the dorsomedial (29) or ventrolateral medulla (11, 12, 29), the facilitatory effect of ANG-(1-7) on the baroreflex sensitivity (7, 27), and the ANG-(1-7) stimulatory effect on the neuronal activity in the paraventricular nucleus (PVN) (1). Fourth, A-779 did not influence the ANG II effects in the rostral ventrolateral (11, 12, 29) or dorsomedial (29) medulla, and it did not change the myotropic actions of bradykinin, substance P, ANG II, and ANG III in the rat ileum (29) or the antidiuretic effect of vasopressin in water-loaded rats (29, 30). Furthermore, A-779, which has a very low affinity for AT₁ or AT₂ receptors (29), has been reported to compete with high affinity for the binding of ANG-(1-7) to endothelial cells membranes (35) and kidney slices (23).

A role for ANG-(1-7) in the central mechanisms controlling MAP has been additionally suggested by the observation that intracerebroventricular infusion

(7) or microinjection of this angiotensin into the nucleus tractus solitarii (nTS) (9) facilitates baroreflex, whereas intracerebroventricular infusion or nTS microinjection of its antagonist, A-779, produces the opposite effect (6, 9, 27). Additionally, microinjection of ANG-(1-7) into the RVLM (11, 12, 29) or the caudal ventrolateral medulla (34) produced increases or decreases in MAP, respectively. ANG-(1-7) has also been reported to increase the neuronal activity in the PVN (1). In accordance with this last observation, an intense immunolabeling of ANG-(1-7) in PVN neurons and other hypothalamic subnuclei has been described in SD rats (5) and in TGR(mREN2)27 transgenic rats (18). In this study, we used the TGR rats originally reported by Mullins et al. (26) to further evaluate the role of ANG-(1-7) in the central regulation of blood pressure. Incorporation of the mouse submandibular gland renin gene (mRen-2) into the rat genome resulted in a line that develops fulminant hypertension associated with increased levels of brain angiotensins (8, 18, 33). A role for central angiotensins in the development of hypertension in TGR(mREN2)27 rats has been suggested by the decrease in blood pressure produced by intracerebroventricular administration of a monoclonal antibody to ANG II (25). Conversely to our observation in the RVLM, intracerebroventricular administration of a selective ANG-(1-7) monoclonal antibody increases MAP and HR (25). However, the use of homozygous female rats on lifetime lisinopril therapy (25), instead of male, untreated, heterozygous rats as used in our study, precludes a more elaborated discussion regarding the blood pressure effect differences. It is important to point out, however, that despite the marked differences in the experimental conditions, the opposite cardiovascular responses obtained would not be surprising, considering the important functional differences between periventricular neuronal structures and the brain stem nuclei involved in central blood pressure control, such as the ones comprising the RVLM in the rat (paragigantocellular nuclei and rostroventrolateral reticular nuclei) (10, 14).

In conclusion, this study gained new insights in the function of angiotensins in the RVLM. Our findings are consistent with an action of angiotensins in the RVLM contributing to the maintenance of hypertension in TGR(mREN2)27 rats. In addition, the different pattern of cardiovascular responses to the AT₁-receptor and ANG-(1-7) antagonists further corroborates the complexity of angiotensin peptides actions. Although ANG-(1-7) seems to act at the RVLM to increase blood pressure, the direction of the effect of ANG II via the AT₁ receptor depends on the levels of this peptide and/or of blood pressure. Specific receptor subtypes might be involved in the effects of ANG-(1-7). Further studies are pending to elucidate the functionality of different receptor subtypes for the different angiotensin peptides in normal and pathophysiological situations.

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