Alterations of the renin-angiotensin system at the RVLM of transgenic rats with low brain angiotensinogen

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Baltatu, Ovidiu, Marco A. P. Fontes, Maria J. Campagnole-Santos, Sordaine Caligiorni, Detlev Ganten, Robson A. S. Santos, and Michael Bader. Alterations of the renin-angiotensin system at the RVLM of transgenic rats with low brain angiotensinogen. Am J Physiol Regulatory Integrative Comp Physiol 280: R428–R433, 2001.—The transgenic rats TGR(ASrAOGEN) (TGR) with low levels of brain angiotensinogen were analyzed for cardiovascular reactivity to microinjections of ANG II and angiotensin receptor (AT1) antagonists [CV-11974, AT1 specific; A-779, ANG-(1–7) selective; sarthran, nonspecific] into the rostral ventrolateral medulla (RVLM) of conscious rats. Microinjection of ANG II resulted in a significantly higher increase in the mean arterial pressure (MAP) of TGR than control [Sprague-Dawley (SD)] rats, suggesting an upregulation of ANG II receptors in TGR. CV-11974 produced an increase in MAP of SD but not in TGR rats. A-779 produced a depressor response in SD but not in TGR rats. Conversely, sarthran produced a similar decrease of MAP in both rat groups. The depressor effect of the AT1 antagonist may indicate an inhibitory role of AT1 receptors in the RVLM. On the other hand, ANG-(1–7) appears to have a tonic excitatory role in this region. The altered response to specific angiotensin antagonists in TGR further supports the functionally relevant decrease in angiotensins in the brains of TGR and corroborates the importance of the central renin-angiotensin system in cardiovascular homeostasis.

The rostral ventrolateral medulla (RVLM) represents the main relay for the sympathetic output and contains angiotensin receptors (12). Considered an important component of the neural circuitry regulating cardiovascular homeostasis by modulating vasomotor tone, the RVLM is situated inside the blood-brain barrier and thus receives solely locally produced angiotensins (26). The relative role of angiotensin peptides in this region for central control of blood pressure is still unclear. Microinjection of the nonspecific angiotensin antagonist sarthran into the RVLM of anesthetized animals produced a significant fall in blood pressure (2, 17, 19), suggesting an excitatory role for ANG II in this region. However, microinjection of losartan or other angiotensin receptor (AT1) antagonists into the RVLM did not change blood pressure in anesthetized animals (14, 18) and produced a pressor response in freely moving rats (13). On the other hand, microinjection of the ANG-(1–7) antagonist A-779 (29) produced significant decreases in MAP in anesthetized (14) or awake (13) rats. To further clarify the role of angiotensin peptides at the RVLM, we aimed in the present study to test the cardiovascular responsiveness to angiotensin receptor stimulation or blockade at the RVLM of conscious transgenic rats with low brain angiotensinogen (TGR(ASrAOGEN)) (TGR). TGR rats exhibit up to 90% reduced angiotensinogen levels throughout the brain, hypotension, low plasma vasopressin levels, and decreased hypertensive response to peripheral infusion of slow-pressor doses of ANG II (6, 30).

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METHODS

Animals. Adult male TGR and Sprague-Dawley (SD) Han
over rats, weighing 400 to 450 g, were obtained from the animal breeding unit of the Max Delbrück Center for Molecular Medicine. The rats were housed under a 12:12-h light-dark schedule (lights on at 0600) at 24 ± 2°C and given free access to a standard rat diet and tap water. All the experiments have been approved by the local authorities.

Surgical procedures. The detailed procedure of RVLM microinjections of conscious rats was described previously (13). To orient the microinjection needles, guide cannulas were fixed into the interpeduncular bone under chloral hydrate anesthesia (300 mg/kg ip). The rats, placed in a stereotaxic apparatus (Stoelting), were implanted with stainless steel cannulas (22 gauge) at an angle of 18–20° from the vertical plane, at 2 mm caudal from the lambda suture and 1.8 mm lateral to the midline. The cannulas, penetrating the interpeduncular bone with the tip placed just above the dura mater, were fixed with dental cement and jeweler’s screws. To avoid the blockade of the cannulas, a stainless steel trocar was placed into the cannulas. To protect the cannulas from mechanical dislocation, a polyethylene shield fixed with the dental cement was surrounding the cannulas. Four days of recovery were allowed between the stereotaxic implant and the experimental procedure. Twenty-four to forty-eight hours before the experiment, polyethylene catheters (PE-50; filled with 10 IU/ml heparinized saline) were inserted into the femoral artery and exteriorized in the interscapular area.

RVLM microinjections in conscious rats. For the measurements of MAP and HR, the catheter was connected to a standard blood pressure transducer (model 101021–2, TSE, Bad Hamburg, Germany), which was connected to a data acquisition and analysis system. For microinjections, a 30-gauge needle was inserted into the RVLM through the guide cannula. At least 15 min were allowed between the placement of the needle and the microinjection. The microinjected volume was 200 nl using a Hamilton syringe connected to the needle via a PE-10 polyethylene catheter. All the experiments were performed in the afternoon when rats are in minimal activity period. The moment of microinjection was carefully chosen when the rat was in repose, with blood pressure and HR stable and in normal range. The data obtained from rats with respiratory or locomotor problems were discarded. The experimental procedures had a rate of success of ~85%.

After each experimental protocol, the rats were killed with an overdose of chloral hydrate. Then, the RVLM site of injection was verified postmortem macro- and microscopi
cally by microinjection of Alcian blue dye (2%) (15).

Pharmacological agents. All drugs were dissolved in sterile isotonic saline (NaCl 0.9%). ANG II, A-779, and Sar1-Thr1-ANG II (sarthran) were from Bachem, and CV-11974 was from Takeda (Osaka, Japan). The 25-pmol dose of ANG II used for the microinjections had been shown to produce a substantial effect on MAP when injected at the RVLM (24). The chosen dose of CV-11974 (0.2 nmol) was shown to not interact with other receptors, such as imidazoline/guanidinium receptive sites, and have no peripheral effects (20, 22). The doses of sarthran (1 nmol) and A-779 (0.2 nmol) were shown to be effective when administered locally in the brain (13, 21).

Statistical analysis. Data were extracted with the TSE Data Acquisition Software Package and analyzed with SPSS 8.0 Software. Data were analyzed for homogeneity of variance within groups of study, and independent samples t-test was used to test differences between TGR and SD rats with significance set at <0.05. Values are means ± SE.

RESULTS

The baseline levels of MAP and HR before microinjections were not significantly different between TGR and SD rats (Table 1), although there was a trend of lower MAP in TGR. The lack of a statistical difference in MAP between TGR and SD rats in this study further substantiates the importance of chronic measurements to state the MAP values for a rat strain (6, 30). Interestingly, at the moment the microinjection needle was placed into the RVLM, a transient increase in MAP was observed (Fig. 1). In fact, when this transient increase in blood pressure was not observed, the Alcian blue staining at the end of the protocol showed that the needle was outside the RVLM area.

Unilateral microinjections of ANG II (25 pmol/200 nl) into the RVLM of conscious rats produced a marked increase in MAP, and this increase was significantly higher in TGR than in SD rats (Fig. 2). The increase in MAP lasted for 10.0 ± 1.9 min for TGR and 5.0 ± 1.6 min for SD rats (P < 0.05). No significant differences in the baseline levels of HR were observed between the rat strains (Table 1). ANG II microinjection induced a consistent decrease in HR only in TGR rats (Table 2).

Microinjections of the highly specific AT1 antagonist CV-11974 (0.2 nmol/200 nl) produced an increase in MAP in SD rats. However, in TGR, the CV-11974 effect did not differ from that of vehicle (Fig. 3). The increase in MAP also lasted significantly less time in TGR than in SD rats (1 ± 0.6 vs. 5 ± 0.9 min, respectively, P < 0.05). After the CV-11974 microinjection, no significant changes in HR were observed in TGR or SD rats (Table 2).

As observed for CV-11974, the effect of A-779 was significantly changed in TGR rats. Contrasting with a significant decrease in MAP observed in SD rats, mi
table 1. Baseline levels of MAP and HR before the microinjections of drugs into the RVLM of TGR and SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>5</td>
<td>112 ± 4</td>
<td>269 ± 9</td>
</tr>
<tr>
<td>CV-11974</td>
<td>4</td>
<td>117 ± 2</td>
<td>245 ± 10</td>
</tr>
<tr>
<td>Sarthran</td>
<td>5</td>
<td>119 ± 2</td>
<td>265 ± 2</td>
</tr>
<tr>
<td>A-779</td>
<td>6</td>
<td>122 ± 4</td>
<td>271 ± 15</td>
</tr>
<tr>
<td>Saline</td>
<td>3</td>
<td>129 ± 7</td>
<td>279 ± 11</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>5</td>
<td>114 ± 4</td>
<td>328 ± 11</td>
</tr>
<tr>
<td>CV-11974</td>
<td>5</td>
<td>129 ± 2</td>
<td>294 ± 9</td>
</tr>
<tr>
<td>Sarthran</td>
<td>3</td>
<td>122 ± 6</td>
<td>327 ± 3</td>
</tr>
<tr>
<td>A-779</td>
<td>4</td>
<td>116 ± 5</td>
<td>295 ± 6</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>131 ± 3</td>
<td>279 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; RVLM, rostral ventrolateral medulla; TGR, TGR (ASrAOGEN) transgenic rat with low brain angiotensinogen; SD, Sprague-Dawley rat.
croinjection of the ANG-(1–7) antagonist in TGR rats produced an increase in MAP comparable to the microinjection of vehicle (Fig. 3). There were no statistical alterations in HR after microinjection of A-779 (Table 2).

Differing from the data obtained with the selective antagonists, microinjection of the nonspecific ANG II antagonist sarthran (1 nmol/200 nl) led to a fall in MAP in TGR and SD rats, which was similar in extent (Fig. 3) and duration (11 ± 3 min in TGR and 7 ± 1.7 min in SD rats; $P > 0.05$). The HR changes after sarthran microinjections were not different in TGR compared with SD rats (Table 2).

Saline microinjection, used to control the specificity of the effects of the drugs, produced only slight and transient changes in MAP and HR (Fig. 2, Table 2).

Table 2. Alteration of HR produced by the microinjections of drugs into the RVLM of TGR and SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Change in HR from Baseline Levels, beats/min</th>
<th>Duration, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>5</td>
<td>$-32 \pm 13^{*}$</td>
<td>11 ± 3.3</td>
</tr>
<tr>
<td>CV-11974</td>
<td>4</td>
<td>13 ± 9.4</td>
<td>4 ± 3.4</td>
</tr>
<tr>
<td>Sarthran</td>
<td>5</td>
<td>28 ± 5.1</td>
<td>14 ± 6.4</td>
</tr>
<tr>
<td>A-779</td>
<td>6</td>
<td>19 ± 22</td>
<td>13 ± 5.3</td>
</tr>
<tr>
<td>Saline</td>
<td>3</td>
<td>9 ± 9</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>5</td>
<td>11 ± 18</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td>CV-11974</td>
<td>4</td>
<td>14 ± 8.1</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>Sarthran</td>
<td>3</td>
<td>17 ± 20</td>
<td>16.0 ± 7.0</td>
</tr>
<tr>
<td>A-779</td>
<td>4</td>
<td>$-6 \pm 15$</td>
<td>11.0 ± 3.4</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>4 ± 7</td>
<td>1 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. *$P < 0.05$ compared with values before injection.
DISCUSSION

Microinjections of ANG II into the RVLM of conscious rats produced a considerable increase in MAP in both TGR and SD rats. These data confirm and extend the conclusions of previous studies that ANG II elicits a pressor response when applied to the RVLM of both anesthetized (2, 16, 28) and conscious animals (13). Moreover, it was suggested that increased activity of the RAS at the RVLM contributes to the hypertension of spontaneously hypertensive rats (25, 33). In TGR, an animal with reduced AOGEN concentration in the brain, we observed a significantly higher reactivity of the RVLM to ANG II when the blood pressure effects were measured. Importantly, these results indicate that a permanent reduction of AOGEN in the brain can lead to an overreactivity of ANG II receptors to exogenous ANG II. The RVLM contains a high density of binding sites of the AT1 receptor subtype (16). Accordingly, we have observed increased levels of AT1 receptors in several brain regions of TGR by radioligand-binding studies (23). These observations together with the exaggerated blood pressure effect of ANG II microinjected into the RVLM can indicate that the concentration of the available ANG II may be an important factor whether ligand binding occurs at inhibitory or stimulatory neurons.

The high levels of AT1 receptors at the RVLM suggest that endogenously produced angiotensins act in this region to modulate blood pressure. To test the function of locally generated ANG II in the RVLM, we blocked its effects by the highly specific AT1 antagonist CV-11974. Interestingly, the AT1-specific antagonist produced a clear increase in MAP in SD rats, which is apparently a contradiction to the same effect of the agonist ANG II. On the other hand, the increase in blood pressure observed in TGR rats did not differ from that observed with vehicle microinjection. The absence of effect of CV-11974 in TGR indicates that the pressor response observed in SD rats is dependent on a normal angiotensinergic activity in this region. The clear effect of the AT1 antagonist on blood pressure in conscious rats, which could not be observed in the anesthetized situation (14, 18), can be first explained by the fact that in our experiments we avoided the possibility of anesthesia-induced alterations of the response (3, 13, 31). In fact, in agreement with recently published reports (7, 13, 18), the data suggest an inhibitory role of endogenous angiotensins acting on AT1 receptors at the RVLM, at least in basal conditions. In support of a differential role of endogenous angiotensins in normal and pathophysiological situations are our recent experiments on the hypertensive rats TGR(mREN2)27 with overactive brain RAS. Opposite to the present results, unilateral microinjections of CV-11974 in this hypertensive model produced a decrease in MAP (15). On the basis of the pressor effect of exogenous ANG II at the RVLM and our data in TGR(mREN2)27 rat, it is reasonable to hypothesize that in normal conditions endogenous angiotensins have access to AT1 receptors present in inhibitory neurons, whereas the increased levels in pathophysiological situations would operate on additional excitatory neuronal mechanisms and/or pathways, normally not accessible by low levels of angiotensins. One might also argue that the contralateral RVLM is activated when the AT1 antagonist is microinjected unilaterally in conscious rats. This less than likely possibility needs to be tested by bilateral microinjections into the RVLM, although technically this experiment is very demanding.

It has been observed in Wistar rats (13) that microinjection of ANG-(1–7) produced a significant increase in MAP. More importantly, as also observed in the present study, microinjection of its putative specific antagonist decreased blood pressure, suggesting an excitatory role for this heptapeptide at the RVLM. In keeping with this hypothesis, the depressor effect of A-779 was completely abolished in TGR rats. Also meaningfully, the altered A-779 effect in the TGR rats indicates an inadequacy in ANG-(1–7) production.

Most of the data available in the literature about the role of ANG II at the RVLM was obtained with nonspecific angiotensin antagonists. Thus we aimed to compare the effects of the specific AT1 antagonist with the nonspecific angiotensin antagonist sarthran. Unilateral microinjections of sarthran into the RVLM of conscious rats produced a decrease in MAP in both SD and TGR rats. Unexpectedly, the magnitude of this decrease was not significantly different between strains. This observation has important implications because several studies have suggested physiological roles for endogenous ANG II at the RVLM and other brain regions based on the results obtained with sarthran or sarthran-related peptides (13, 19, 25). Our data also raise the possibility that sarthran can bind to a nonangiotensin receptor-binding site blocking the action of a nonangiotensin peptide with a tonic excitatory role at the RVLM. Although apparently controversial, the results obtained from both the specific AT1
antagonist and the nonspecific angiotensin antagonist further stress the necessity to solve the pending question on the relative contribution of different angiotensin species acting on specific receptors in various pathophysiological situations (9).

In summary, this study shows that a permanent reduction of brain AOGEN leads to an upregulation of ANG II receptors causing an enhanced response to exogenous ANG II at the level of the RVLM. The altered blood pressure response at both the AT$_1$ or ANG-(1–7) antagonist in TGR compared with SD rats further supports the functionally relevant decrease in angiotensins in the brains of TGR and corroborates the importance of the central RAS in cardiovascular homeostasis. The unilateral microinjections of the specific AT$_1$ antagonist, but not the nonspecific antagonist sarthran, in the RVLM of conscious SD rats also increased blood pressure, suggesting an inhibitory role of endogenous ANG II acting on AT$_1$ receptors. Moreover, an excitatory role for endogenous ANG-(1–7) at the RVLM is suggested.

**Perspectives**

After the pioneering studies of Andreattta et al. (2) and Allen et al. (1), experimental evidences provided by several groups have contributed to establish the RVLM as an important site for the action of the RAS in the brain (9, 13, 16, 18, 19, 24, 28). Most of the experimental data showing an excitatory role for the RAS at the RVLM was obtained using angiotensin peptides or nonspecific angiotensin antagonists. More recent studies performed with selective antagonists indicate that the influence of the RAS at the RVLM is far more complex than formerly suspected (13, 15, 18, 25). Our previous studies in freely moving rats suggested that in basal conditions, ANG II would primarily have an inhibitory action at the RVLM, whereas ANG-(1–7) would act as an excitatory peptide at this site (13, 14). Our current work adds further support to this hypothesis by indicating that the pressor effect of an AT$_1$ antagonist or the depressor effect of the ANG-(1–7) antagonist A-779 at the RVLM of freely moving rats is dependent on an operating local RAS. Further studies should address the question where and how within the RVLM neuronal network ANG II conveys its inhibitory role in basal conditions. A similar question should be addressed for the excitatory role of ANG-(1–7) at this site. On the other hand, we (15) and others (32) have shown that endogenous ANG II can also have an excitatory role at the RVLM when the local RAS is activated. Studies directed to elucidate the dual influence of ANG II at the RVLM are warranted for determining more precisely the physiological and pathophysiological role of the RAS at the RVLM.

The observation that the nonspecific angiotensin antagonist Sar$^1$-Thr$^8$-ANG II (sarthran) produced similar falls in blood pressure in TGR and SD rats introduces concerns regarding conclusions about the role of the RAS in the brain and possibly other sites based on the effects produced by nonspecific angiotensin antagonists. On the other hand, studies using Sar$^1$-Thr$^8$-ANG II or other nonspecific angiotensin antagonists can lead to identification of a new and important non-angiotensinergic modulator of the sympathetic activity at the RVLM.

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