Expression of an angiotensin-(1-7)-producing fusion protein in rats induced marked changes in regional vascular resistance

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The heptapeptide angiotensin-(1-7) [ANG-(1-7)] is now considered one of the biologically active end products of the renin-angiotensin system (RAS) (8, 14, 17, 44, 45). It can be formed from ANG II through hydrolysis by angiotensin-converting enzyme 2, prolylendopeptidase, or prolylcarboxypeptidase (8, 10, 17, 20, 44, 57) or directly from ANG I through converting enzyme 2, prolylendopeptidase, or prolylcarboxypeptidase (8, 17, 20, 44, 57). ANG-(1-7) was shown to produce relaxation of aortic rings of Sprague-Dawley (SD) (53) and mRen-2 transgenic rats (29), canine (4) and porcine coronary arteries (39), canine middle cerebral artery (23), piglet pial arterioles (33), fetal systemic vasculature (38), rabbit renal afferent arterioles (40), and mesenteric microvessels of normotensive and hypertensive rats (16, 37). In anesthetized rats ANG-(1-7) induces vasodilation in various vascular beds and an increase in stroke volume and cardiac index (41).

Many studies have shown that the vascular actions of ANG-(1-7) appear to involve increased production of vasodilators, prostanoids, nitric oxide (NO) and endothelium-derived hyperpolarizing factor (15, 27, 36, 39, 42) and that the relative importance of these mechanisms is dependent on the vessel diameter, the vascular regional bed, and the species (44). Moreover, some vascular effects involve simultaneous participation of these vasodilator mediators. A cross talk between ANG-(1-7) receptors and other receptors such as kinin B2 and ANG II type 2 receptors may also activate these pathways in blood vessels (45). In addition, the vascular actions of ANG-(1-7) could involve modulation of ANG II-induced changes in vascular resistance (45).

There are few studies examining the effect of chronic increases in plasma ANG-(1-7) concentration (2, 3, 52, 59). In these studies ANG-(1-7) was administrated using osmotic mini-pumps for periods no longer than 15 days (2, 3, 59). Thus information on the effect of chronic increase in ANG-(1-7) is still missing. Data in this regard are particularly important considering that several pharmacological and nonpharmacological measures for treating hypertension and other cardiovascular diseases produce chronic increases in plasma ANG-(1-7) concentration (7).

It has been recently described that peptides can be directly released within specific tissues from an engineered fusion protein by proteolytic action of the furin enzyme (34, 35). This technique provided a possibility to increase the release of peptides in defined tissues or a particular cell line (35). With this strategy, transgenic mice have been produced expressing an ANG II-producing fusion protein exclusively in cardiac myocytes or astrocytes (31, 55). We have recently described a transgenic rat expressing an ANG-(1-7)-producing fusion pro-
tein in testis. In these rats, TGR(1-7)3292 (TGR), there is a 2.5-fold increase in plasma ANG-(1-7) concentration (19, 46).

Sampaio et al. (41) demonstrated that acute infusion of ANG-(1-7) changed the regional blood flow distribution, increased the cardiac output (CO), and decreased total peripheral resistance (TPR) in Wistar rats. Considering these data, we hypothesized that chronic increase in ANG-(1-7), as observed in transgenic rats, could also change the systemic and regional hemodynamic. To test this hypothesis, we performed systemic and regional hemodynamic measurements in TGR rats using fluorescent microspheres.

MATERIALS AND METHODS

Animals and Surgical Preparation

Male SD and TGR rats 3–4 mo old were housed in cages and maintained in a temperature-regulated room (22–24°C) on a 12-h:12-h light-dark cycle (lights on at 6:00 AM). ANG-(1-7) plasma levels in SD and TGR are 17.6 ± 0.9 and 43.6 ± 13.1 pg/ml, respectively (47).

Rats were anesthetized with urethane (1.2 g/kg ip; Sigma). A tracheostomy was performed to keep airway patency. Body temperature, continually monitored with a rectal probe, was kept at 36–37°C by a heating pad. The left braquial artery was cannulated with polyethylene tubing (PE-10) for recording of blood pressure and heart rate (Power Lab 4/20 ADInstruments/Bridge Amp Panlab S.J., Spain). For administration of fluorescent microspheres, the right carotid artery was exposed, and a PE-50 cannula was guided through the common carotid artery into the left ventricle. The positioning of the cannula in the left ventricle was confirmed by recording the typical ventricular pressure. The right femoral artery was cannulated and connected to a pump (Minipuls 3, Gilson; Villiers le Bel, France) for blood withdrawal. The right femoral vein was also cannulated for infusion of vehicle (0.9% NaCl). The experimental procedures used in this study are in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were approved by the Animal Committee of the Federal University of Minas Gerais (CETEA).

Determination of Systemic and Regional Hemodynamics

Systemic hemodynamics and regional blood flow were determined using 15-µm fluorescent polystyrene microspheres (FluoSpheres Blood Flow Determination, Molecular Probes; Eugene, OR) as previously reported (25, 26, 41). Briefly, two different colors of fluorescent microspheres were used for two consecutive measurements to control for the reproducibility of the measurement. The color of microspheres was selected to avoid the spillover between the colors (26). To prevent aggregation, the microspheres suspension was vortexed for 2 min, followed by sonication for an additional 60 s. After the mixing was completed, 300,000 fluorescent microspheres were infused into the left ventricle over a 10-s period. To calculate the blood flow, arterial blood was withdrawn at a rate of 0.85 ml/min through the right femoral artery. Blood for reference blood sample was withdrawn for 90 s starting 10 s before the microsphere injection. The same procedure was repeated with the second color 10 min after the first one. At the end of the experiment the animals were euthanized with an overdose of anesthetic, and the tissues (kidneys, brain, mesentery, adrenals, spleen, abdominal skin, gastrocnemius muscle, lungs, testis, left ventricle, brown and white fat) were dissected, weighed, and stored in individual vials. Both tissue and reference blood samples were digested using a solution of 4 M ethanolic KOH containing 2% Tween 80 in a hot bath (50°C) overnight. At the end of the digestion period the microspheres were recovered by the sedimentation method (56), and the dye was extracted in 4 ml of the organic solvent ethyl acetate. The fluorescence intensity of the dye was measured using a spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer/Varian). The following parameters were calculated: CO (ml/min), cardiac index (ml·min⁻¹·100 g⁻¹) and stroke volume (ml), TPR (mmHg·ml⁻¹·min⁻¹·100 g), regional blood flow (ml·min⁻¹·g⁻¹), and regional vascular resistance (mmHg·ml⁻¹·min⁻¹·g⁻¹) as previously described (25, 26, 56).

Data Analysis

Data are presented as means ± SE. Statistical comparisons were assessed by Student’s t-test. Values of P < 0.05 were considered significant.

RESULTS

Systemic Hemodynamic of SD and TGR Rats

Figure 1 shows the averaged values of cardiac index, stroke volume, and TPR of urethane-anesthetized SD (n = 20) and TGR (n = 19). The TGR showed a cardiac index (24.59 ± 0.91 ml·min⁻¹·100 g⁻¹) and stroke volume (0.29 ± 0.01 ml) significantly higher than that observed in SD rats (21.98 ± 0.65 ml·min⁻¹·100 g⁻¹ and 0.25 ± 0.01 ml, respectively; P < 0.05, Student’s t-test). Consequently, a significant smaller TPR was observed in TGR (3.95 ± 0.13 mmHg·ml⁻¹·min⁻¹·100 g) in comparison to SD rats (4.55 ± 0.13 mmHg·ml⁻¹·min⁻¹·100 g; P < 0.05, Student’s t-test). Mean arterial pressure was similar between the two groups of rats (TGR = 99 ± 3 mmHg and SD = 101 ± 3 mmHg). However, the heart rate was slightly smaller in TGR (326 ± 7 beats/min) in comparison to SD (359 ± 6 beats/min) rats. These differences remained the same after the second measurement, repeated 10 min after the first one (data not shown).

Regional Blood Flow and Vascular Resistance of SD and TGR Rats

Table 1 and Fig. 2 present the values of blood flow and vascular resistance, respectively, in the different regions stud-
ied in SD and TGR rats. As shown in Fig. 2, the vascular resistance in the kidney, adrenals, testis, brain, lung, spleen and brown fat were substantially smaller in the TGR rats compared with their age-matched controls. There were no statistically significant differences in the vascular resistance in the left ventricle, muscle, skin, mesentery and white fat (Fig. 2).

The changes in vascular resistance were paralleled by an increase in blood flow in lung, spleen, renal, adrenals, cerebral, testis, and brown fat territories of the TGR rats compared with control rats (Table 1). The blood flow differences between SD and TGR rats remained the same in a second measurement repeated 10 min after the first one (data not shown).

### DISCUSSION

Several studies have demonstrated that the blood vessels are an important site for the formation and actions of ANG-(1-7) (41, 43, 44, 48). Our results, which are in keeping with a previous study in Wistar rats (41), indicate that ANG-(1-7) has an effective and important role in the tonic control of blood flow distribution.

Chronic increase in ANG-(1-7) observed in TGR rats produced pronounced changes in regional blood flow, resulting in an increase in vascular conductance in the kidneys, lungs, adrenals, spleen, brain, testis and brown fat tissue. The potent and selective effect of ANG-(1-7) in several territories confirms and extends previous observations using acute infusion of ANG-(1-7) (41). The cardiac index of TGR rats was 12% higher than that of SD rats, which can be explained, at least in part, by the smaller TPR (13%) of TGR rats.

We have found that the chronic increase in endogenous ANG-(1-7) in TGR rats produced a higher blood flow in the brain. The blood flow was 17% higher and vascular resistance was 26% smaller in the TGR brain as compared with age-matched control SD rats. This observation is in agreement with a previous study by Meng and Busija (33) demonstrating a vasodilatory effect of ANG-(1-7) in piglet pial arterioles.

### Table 1. Regional blood flow in urethane-anesthetized SD and TG rats

<table>
<thead>
<tr>
<th>Region</th>
<th>Blood Flow, ml·min⁻¹·g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.70±0.25</td>
</tr>
<tr>
<td>Lung</td>
<td>0.93±0.14</td>
</tr>
<tr>
<td>Adrenal</td>
<td>15.60±1.29</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>10.71±1.31</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.41±0.21</td>
</tr>
<tr>
<td>Mesentery</td>
<td>0.44±0.05</td>
</tr>
<tr>
<td>Brain</td>
<td>1.27±0.08</td>
</tr>
<tr>
<td>Skin</td>
<td>0.11±0.009</td>
</tr>
<tr>
<td>Gastrocnemius muscle</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td>Testis</td>
<td>0.19±0.017</td>
</tr>
<tr>
<td>Brown fat tissue</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>White fat tissue</td>
<td>0.08±0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. SD, Sprague-Dawley (n = 15–20 rats); TG, transgenic (n = 15–19 rats). *P < 0.05, Student’s t-test.
Feterik et al. (23) also found that ANG-(1-7) produces relaxation of canine middle cerebral arteries through release of NO from endothelial cells, and Sampaio et al. (41) demonstrated that acute infusion of low doses (110 fmol/min) of ANG-(1-7) in Wistar rats caused an increase in blood flow and a decrease in vascular resistance in the brain vascular bed.

The TGR rats also presented a smaller vascular resistance and a higher blood flow than SD rats in testis. This observation is in agreement with previous studies showing the presence of the RAS in the reproductive organs and its involvement in the physiology of this reproductive system (9, 30). Additionally, Alenina et al. (1) demonstrated a well-controlled regulation of Mas expression in testis beginning 18 days after birth and increasing until 6 mo of age. This continuous increase in Mas mRNA level indicated that Mas is not just involved in the maturation process of testis but may also be implicated in the function of the mature organ. Our results suggest that one of the roles of Mas in testis is the modulation of blood flow.

The discovery of a local adipose-tissue RAS has allowed the detection of a pathophysiological role for the RAS in obesity-associated disturbances and insulin resistance, as well as a function in the secretion of adipocytokines-derived products (13). Additionally, ANG I, ANG II and ANG-(2-8) have been detected in rat brown adipose tissue (BAT) (5, 6, 13, 50), suggesting endogenous production, mainly of ANG II. In this study, we found a marked difference between blood flow in BAT of TGR and SD rats. BAT blood flow was 150% higher and vascular resistance 53% smaller in TGR rats. This observation indicates that BAT is a major target for ANG-(1-7). Whether the differences in blood flow are secondary to an effect of ANG-(1-7) in BAT metabolic rate, a direct effect on BAT microvessels or a combination of both factors remains to be established.

The influence of ANG-(1-7) in the renal functions is complex and depends on the hydroelectrolytic status, the renal sympathetic nervous activity, and the level of activity of the RAS (19, 22, 48, 51). Accumulating evidence suggests that apart from ANG II, ANG-(1-7) plays a significant role in renal function. ANG-(1-7) increases renal blood flow in anesthetized rats (41) and produces afferent arteriolar relaxation through specific receptor-mediated NO release in isolated kidneys of rabbits (40). In addition to the effects on renal blood flow, studies in vitro and in awake animals have suggested that ANG-(1-7) can act as a natriuretic/diuretic or antidiuretic hormone depending of the hydroelectrolytic status (28, 48).

Our results extend these observations and suggest that ANG-(1-7) can importantly modulate renal blood flow. It should be pointed out that in a recent study, van der Wouden et al. (54) did not observe an effect of ANG-(1-7) on renal blood flow in freely moving rats. However, the doses used by these authors (133, 333, and 667 ng kg⁻¹·min⁻¹) were substantially higher than that used by Sampaio et al. (41) (330 fmol kg⁻¹·min⁻¹, i.e., ~0.3 ng kg⁻¹·min⁻¹). Indeed, with a dose more close to that used by van der Wouden et al. (~32 ng kg⁻¹·min⁻¹), Sampaio et al. (41) observed a decrease in renal blood flow. Apart from that, other factors such as anesthesia may account for the differences observed.

Sampaio et al. (41) observed that acute infusion of ANG-(1-7) in Wistar rats increased the blood flow to skin and did not change the blood flow to spleen, adrenal and lung. In contrast, we observed that the chronic increase in ANG-(1-7) levels in the TGR produced a significant increase in the blood flow to adrenal, spleen and lung without changing the blood flow to skin. These discrepant findings may be related to many factors including acute versus chronic changes in plasma ANG-(1-7), differences between rat strains or the possibility of changes in the expression of enzymes and/or receptors, which may be involved in regional blood flow distribution, produced by chronic increase in circulating ANG-(1-7).

Another interesting observation with the chronic increase in ANG-(1-7) levels was the smaller TPR of TGR rats, which may have contributed to the increase in stroke volume leading to an increase in CO. On the other hand, the opposite changes in TPR and CO could explain the absence of substantial changes in blood pressure in response to ANG-(1-7) as described previously (2, 3, 41). The higher CO of TGR rats is probably also related to the slightly smaller heart rate combined with a possible increase in venous return, which, in turn, could be the consequence of the vasodilatation in several territories, including cerebral, renal, spleen, BAT, adrenal, pulmonary and testis vascular beds. However, a direct inotropic effect of ANG-(1-7) cannot be excluded. Our recent observations in rats and in Mas-deficient mice are in keeping with this later possibility (46, 47).

**Perspectives**

Our results indicate that discrete changes in circulating ANG-(1-7) may produce important variations in the regional and systemic hemodynamic. More importantly, our results indicate that the hemodynamic changes produced are sustained, suggesting a hitherto unsuspected important physiological role for ANG-(1-7) in the tonic control of blood flow.

Many of the cardiovascular effects of ANG-(1-7) are completely blocked by the selective ANG-(1-7) antagonist A-779, suggesting that the effects of ANG-(1-7) on the blood vessels are mediated by a specific receptor sensitive to A-779 (23, 24). Recently, Santos et al. (49) reported that ANG-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Future studies should address the possibility of the involvement of the ANG-(1-7) receptor Mas in the hemodynamic effects of ANG-(1-7) by using A-779 and receptor Mas knockout mice. In addition, the local contribution of NO, prostaglandins, and endothelium-derived relaxing factor as well as the central nervous system to the changes in blood flow observed should be explored in future studies.

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