Activation of angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis attenuates the cardiac reactivity to acute emotional stress

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Martins Lima A, Xavier CH, Ferreira AJ, Raizada MK, Wallukat G, Velloso EP, Santos RA, Fontes MA. Activation of angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis attenuates the cardiac reactivity to acute emotional stress. Am J Physiol Heart Circ Physiol 305: H1057–H1067, 2013. First published July 19, 2013; doi:10.1152/ajpheart.00433.2013.—Recent data indicate the brain angiotensin-converting enzyme/ANG II/AT1 receptor axis enhances emotional stress responses. In this study, we investigated whether the counterregulatory axis, the angiotensin-converting enzyme 2 (ACE2)/ANG-(1–7)/Mas axis, attenuate the cardiovascular responses to acute emotional stress. In conscious male Wistar rats, the tachycardia induced by acute stress (air jet 10 l/min) was attenuated by intravenous injection of ANG-(1–7) [Δ heart rate (HR): saline 136 ± 22 vs. ANG-(1–7) 61 ± 25 beats/min; P < 0.05]. Peripheral injection of the ACE2 activator compound, XNT, abolished the tachycardia induced by acute stress. We found a similar effect after intracerebroventricular injections of either ANG-(1–7) or XNT. Under urethane anesthesia, the tachycardia evoked by the beta-adrenergic agonist was markedly reduced by ANG-(1–7) [ΔHR: saline 160 ± 16 vs. ANG-(1–7) 18 ± 15 beats/min; P < 0.05]. The increase in renal sympathetic nerve activity (RSNA) evoked by isoproterenol was also abolished after the treatment with ANG-(1–7) [ΔRSNA: saline 39% vs. ANG-(1–7) –23%; P < 0.05]. The tachycardia evoked by disinhibition of dorsomedial hypothalamic neurons, a key nucleus for the cardiovascular response to emotional stress, was reduced by ~45% after intravenous injection of ANG-(1–7). In cardiomyocyte, the incubation with ANG-(1–7) (1 μM) markedly attenuated the increases in beating rate induced by isoproterenol. Our data show that activation of the ACE2/ANG-(1–7)/Mas axis attenuates stress-induced tachycardia. This effect might be either via the central nervous system reducing anxiety level and/or interfering with the positive chronotropy mediated by activation of cardiac β adrenergic receptors. Therefore, ANG-(1–7) might contribute to reduce the sympathetic load to the heart during situations of emotional stress, reducing the cardiovascular risk.

angiotensin; cardiovascular; stress

RECENT LITERATURE subdivides the renin-angiotensin system (RAS) into two distinct counterregulatory axes (21, 22, 59). The classical axis involves the formation of angiotensin (ANG) II as the product of the cleavage of ANG I by the angiotensin-converting enzyme (ACE). The main biological actions exerted by ANG II are mediated through the AT1 receptor subtype. Pharmacological interventions, such as repression of synthesis of ANG II through inhibition of ACE or by direct blockade of the AT1 receptors, have been successful in the treatment of cardiovascular disease (3). In the last decade a new alternative axis that modulates the effect of ANG II has been described. The new axis has the ANG-(1–7) as the central player. The heptapeptide, ANG-(1–7), is the substrate by the action mainly of angiotensin-converting enzyme II (ACE2) cleaving ANG II. Acting preferentially in the Mas receptor, ANG-(1–7) has been shown as a new pharmacological target to ameliorate the course of cardiovascular, renal, immunological, and neurological diseases in experimental models (3, 60).

Several previous studies indicate that peptides of the RAS may act in the brain regulating a number of physiological processes (28, 31). In particular, the brain ACE, ANG II, and AT1 receptor has been reported to have an important role in cardiovascular regulation under stress condition (2). Evidence in humans and animals show that ACE inhibitors can modulate anxiety and behavior (26, 27, 42). Pretreatment with the AT1 antagonist, candesartan, deeply modifies the response to stressors, preventing sympathetically mediated effects and the production of gastric ulcerations (52). Therefore, ANG II has been considered a major stress hormone (56) acting via AT1 receptors (15, 17, 52). Interestingly, the counterregulatory axis ACE2/ANG-(1–7)/Mas, is also involved in the stress response. Mice lacking the Mas receptor display increased anxiety (67). Besides, functional interaction between Mas and AT1 receptors was reported in the amygdala (65), a well-known limbic structure involved in the emotional responses to stress (38). In the present study, we tested the hypothesis that the activation of ACE2/ANG-(1–7)/Mas axis modifies the cardiovascular responses to emotional stress in rats. For this purpose, in different physiological preparations, we evaluated the effects of ANG-(1–7) and ACE2 activator 1-[(2-dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl)sulfonyl]oxy]-9H-xanthene-9-one (XNT) (33). Current data provide the first evidence that activation of ACE2/ANG-(1–7)/Mas axis may reduce the impact of acute stress on the cardiovascular system. Part of these results has been published in abstract form (43–46).
EXPERIMENTAL PROCEDURES

We performed all experiments on male Wistar rats (250–320 g) bred at the animal facilities of the Biological Sciences Institute (CEBIO, UFMG, Belo Horizonte, MG, Brazil). Experimental procedures were approved by our local institutional animal welfare committee (CETEA/UFMG protocol number 137/2006 and 244/2011). All experiments were performed in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. We made all efforts to minimize the number of animals used. In all surgical procedures the adequacy of anesthesia (administered ip) was verified by the absence of a withdrawal response to nociceptive stimulation of a hindpaw and by a stable baseline arterial pressure, heart rate, and renal sympathetic nerve activity (RSNA) in experiments using urethane.

Experiments in Conscious Rats

Experiment 1: Effects of intravenous injection of ANG-(1–7) or XNT on heart rate (HR) and mean arterial pressure (MAP) in rats subjected to acute stress. Under tribromoethanol anesthesia (250 mg/kg ip), catheters were implanted into femoral artery and vein for recording cardiovascular parameters and drug injection, respectively. Rats were then allowed to recover in their cages for at least 24 h before starting experiments.

We used three different groups of animals for intravenous bolus injection. After 20 min of baseline HR and blood pressure (BP) monitoring, animals received intravenous injection of I) ANG-(1–7) [2.5 nmol/kg (n = 8)]; 2) XNT [1 µmol/kg (n = 4)]; or 3) saline [vehicle, 0.9% NaCl (n = 8)]. After 5 min of the intravenous injection, rats were placed into a plastic restrainer and subjected to a 10-min air jet stress, a stream of air (10 l/min) directed to the head. After the air jet the animals remained in the cage for additional 15 min for a recovery period.

Experiment 2: Effects of central administration of ANG-(1–7) or XNT on HR and MAP in rats subjected to acute stress. Rats were anesthetized with tribromoethanol (250 mg/kg ip), and the head was placed in a stereotaxic frame (Stoelting, IL) with the tooth bar fixed at −3.3 mm below the interaural line. A small craniotomy was made near the bregma to allow the insertion of a guide cannula intracerebroventricularly (icv) (1.2 mm posterior, 1.5 mm lateral, 4.0 mm ventral). The cannula was fixed to the skull by dental acrylic cement anchored with stainless steel screws (69). After 5 days recovery, the animals were again anesthetized to allow femoral artery and vein catheterization.

Eight different groups of animals were used for injections into the central nervous system. After 20 min of baseline HR and BP monitoring, animals received injection (2 µl icv) of I) three different concentrations of ANG-(1–7) [50, 100, or 200 pmol (n = 5 each)]; 2) three different concentrations of XNT [350, 500 (n = 5 each), or 1,000 pmol (n = 6)]; 3) A-779 (Mas receptor antagonist) plus ANG-(1–7) [200 and 100 pmol, respectively (n = 5)]; or 4) saline [0.9% NaCl (n = 6)]. Immediately after, we exposed the animals to air jet stress as described above (experiment 1). At the end of the experiments, icv injection of ANG II (1,000 pmol) was performed to confirm the position of the central cannula, by observing the drinking response and pressor effect extensively described in scientific literature (1, 16, 24, 51, 63). Histological confirmation using microinjection of alcin blue dye (2 µl/2%) was also performed.

Experiment 3: Effects of peripheral administration of ANG-(1–7) on cardiovascular responses evoked by peripheral β-adrenergic stimulation in conscious rats. Under tribromoethanol anesthesia (250 mg/kg ip), catheters were implanted into femoral artery and vein. After 24 h, two different groups of animals received intravenous bolus injection of I) ANG-(1–7) [2.5 nmol/kg (n = 4)] or 2) saline [0.9% NaCl (n = 6)]. After 5 min, the animals received an intravenous injection of isoproterenol (1 µg/kg), a nonselective β-adrenergic agonist. After the isoproterenol injection, the animals remained in the cage for an additional 15 min.

Experiments in Anesthetized Rats

Experiment 4: Effects of peripheral administration of ANG-(1–7) on cardiovascular response and sympathetic reactivity evoked by peripheral β-adrenergic stimulation in anesthetized rats. Rats were anesthetized with urethane (1.2–1.4 g/kg ip), and catheters were implanted in a femoral artery and vein. The animals were prepared for recording of RSNA activity as previously described (69). The noise level of the RSNA recording system was determined postmortem and subtracted from initial RSNA values.

After 20 min of baseline RSNA, HR, and BP monitoring, two different groups of animals were administered intravenous bolus injection of I) ANG-(1–7) [2.5 nmol/kg (n = 5)] or 2) saline [0.9% NaCl (n = 7)]. After the injection procedure, the venous cannula was flushed using 0.2 ml of saline. After 5 min, the animals received an intravenous injection of isoproterenol (1 µg/kg). Thirty minutes later, the animals were euthanized with an intravenous injection of urethane (0.1 g/kg, 0.5 ml).

Experiment 5: Effects of peripheral administration of ANG-(1–7) on cardiovascular response evoked by activation of the dorsomedial hypothalamus in anesthetized rats. Since the DMH is a key region in the descending pathways involved in the cardiovascular response to stress (25, 40), this experimental strategy was adopted because DMH activation results in a nonpharmacological stimulation of cardiac β-adrenergic receptor (24). Under urethane anesthesia (1.2–1.4 g/kg ip) rats were prepared for central microinjections into the dorsomedial hypothalamic neurons (DMH: 3.1 mm posterior, 0.6 mm lateral, 8.5 mm ventral) as previously described (69). Catheters were placed into a femoral artery and vein. After 20 min of baseline HR and BP monitoring, an injection of saline (0.9% NaCl) was administered intravenously. After 5 min, DMH stimulation was induced by unilateral microinjection (right side) of the GABA_A receptor antagonist, bicuculline methiodide (BMI, 20 pmol/100 nl). After 30 min, the same experimental group of animals (n = 4) received an intravenous injection of ANG-(1–7) (2.5 nmol/kg). After 5 min, a new stimulation of the DMH was evoked. At the end of the experiments, the animals were killed with an intravenous injection of urethane (0.1 g/kg, 0.5 ml), and a microinjection of alcin blue dye (100 nl/2%) was performed for histological confirmation.

Experiment 6: Effect of preincubation with ANG-(1–7) on basal contraction rate and contraction rate induced by adrenergic stimulation in spontaneously beating cardiomyocytes. Isolation and culture of neonatal cardiomyocytes were performed as described in detail previously (66). Briefly, Wistar rats (1–3 days old) were decapitated and single cells were dissociated from the minced ventricles with a 0.2% solution of crude trypsin and were cultured as monolayer with a density of 800 cells/mm² in the medium SM 20-1 at 37°C (18). The medium contained 10% heat-inactivated neonatal calf serum and 2 µmol/l fluorodeoxyuridine, the latter to prevent the proliferation of the nonmyocytes. Six selected cultures or synchronously contracting cell cluster per culture flask (Falcon 12.5 cm²) were counted visually for 15 s on the heated stage (37°C) of an inverted microscope. This procedure was repeated twice in different cultures to obtain results representing a total of up to 18 cells or cell clusters. The basal contraction rate of the spontaneously beating cardiomyocytes was 156 ± 8 beats/min. We performed two different protocols in this experimental series. In the first, we preincubated cardiomyocytes with ANG-(1–7) (1 µM) or ANG-(1–7) plus A-779 (1 µM + 1 µM). In the second, we preincubated cardiomyocytes with different concentrations of isoproterenol (10^-8 up to 10^-5 M) in the presence or not of ANG-(1–7) (1 µM). The change of contraction rate was measured by 10.220.33.3 on August 26, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/ by 10.220.33.3 on August 26, 2017
**Data Analysis**

The baseline values of MAP and HR were measured as the average values of these variables over the 5-min period immediately preceding the experimental procedure. In all experimental groups, stress-induced values of MAP and heart rate changes were measured as the average for these variables over a 1-min period at the steady-state time (~8 min after beginning of the stress trial). In the isoproterenol and bicuculline groups, measurements were made during the peak changes. Comparisons among the vehicle-treated group and the drug-treated groups were performed by one-way analysis of variance followed by the Newman-Keuls test. Changes evoked by isoproterenol were assessed by unpaired t-test. Changes from baseline evoked by activation of the DMH were assessed by paired t-test. Comparisons between the cardiomyocytes vehicle-treated group and the cardiomyocytes drug-treated groups for each concentration point were performed by two-way analysis of variance (factors: drug and concentration) followed by Bonferroni’s post hoc test. We analyzed all data using GraphPad Prism Software (San Diego, CA). A value of $P < 0.05$ was considered to indicate statistical significance. All values are presented as means ± standard error of the mean (SE).

**RESULTS**

Table 1 shows the baseline values of MAP and HR for all experimental groups used in this study. There are no significant differences among the groups for the basal values of HR and MAP.

**Effects of Intravenous Injection of ANG-(1–7) or XNT on HR and MAP in Rats Submitted to Acute Stress**

Intravenous injections of ANG-(1–7) (2.5 nmol/kg) or saline did not change baseline cardiovascular values. Intravenous injection of XNT (1 μmol/kg) evoked an acute fall in HR and BP that lasted ~1 min. In the saline group, acute stress exposure markedly increased HR (mean maximum changes, $\Delta$ 136 ± 22 beats/min) and MAP (Δ 18 ± 4 mmHg) that lasted throughout the stress trial and returned gradually to baseline during the recovery period (Fig. 1). Compared with control, the tachycardia induced by stress was significantly attenuated by intravenous injection of ANG-(1–7) [mean maximum changes $\Delta$HR, saline 136 ± 22 vs. ANG-(1–7) 61 ± 25 beats/min; $P < 0.05$]. ANG-(1–7) did not significantly alter the changes in MAP evoked by acute stress (Fig. 1). Peripheral injection of XNT completely abolished the tachycardia induced by air jet stress and a bradycardic effect was observed instead ($\Delta$HR: saline 136 ± 22 vs. XNT -44 ± 7 beats/min; $P < 0.001$) (Fig. 1). A similar pattern was observed in the BP response, in which an abolition in BP pressure response was observed in rats injected with XNT during the stress trial ($\Delta$MAP: saline 18 ± 4 vs. XNT -8 ± 11 mmHg; $P < 0.05$) (Fig. 1). Interestingly, at the end of the recovery time (15th minute), the animals treated with ANG-(1–7) or XNT showed HR values closer to baseline compared with saline group.

**Effects of Central Administration of ANG-(1–7) or XNT on HR and MAP in Rats Submitted to Acute Stress**

Figure 2 shows that icv injection of ANG-(1–7) (100 pmol/2 μl) attenuated the tachycardia induced by stress compared with the control group ($\Delta$HR: saline 112 ± 22 vs. ANG-(1–7) 50 ± 9 beats/min; $P < 0.05$; Fig. 2A). In a separate group of experiments we observed that concomitant administration of

**Table 1. Basal values for heart rate and mean arterial pressure**

<table>
<thead>
<tr>
<th>Protocols</th>
<th>Preinjection</th>
<th>Prestress</th>
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<tbody>
<tr>
<td></td>
<td>HR, beats/min</td>
<td>MAP, mmHg</td>
<td>HR, beats/min</td>
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<td>MAP, mmHg</td>
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<tr>
<td><strong>Intravenous</strong></td>
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<tr>
<td>Saline (0.9% NaCl)</td>
<td>346 ± 11</td>
<td>106 ± 3</td>
<td>359 ± 9</td>
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<tr>
<td>ANG-(1–7) (2.5 nmol/kg)</td>
<td>361 ± 5</td>
<td>110 ± 4</td>
<td>360 ± 8</td>
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<tr>
<td>XNT (1 μmol/kg)</td>
<td>333 ± 15</td>
<td>106 ± 6</td>
<td>333 ± 14</td>
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<td>Intracerebroventricular</td>
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<tr>
<td>Saline (0.9% NaCl)</td>
<td>344 ± 13</td>
<td>108 ± 2</td>
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<tr>
<td>XNT (350 pmol)</td>
<td>325 ± 9</td>
<td>106 ± 3</td>
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<tr>
<td>XNT (500 pmol)</td>
<td>347 ± 3</td>
<td>107 ± 5</td>
<td></td>
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<tr>
<td>XNT (1,000 pmol)</td>
<td>368 ± 6</td>
<td>106 ± 2</td>
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<tr>
<td>ANG-(1–7) (50 pmol)</td>
<td>351 ± 6</td>
<td>107 ± 4</td>
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<tr>
<td>ANG-(1–7) (100 pmol)</td>
<td>352 ± 6</td>
<td>111 ± 4</td>
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<tr>
<td>ANG-(1–7) (200 pmol)</td>
<td>345 ± 16</td>
<td>114 ± 1</td>
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<tr>
<td>A-779 (200 pmol) + ANG-(1–7) (100 pmol)</td>
<td>340 ± 16</td>
<td>109 ± 1</td>
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<td><strong>Isoproterenol (nonanesthetized)</strong></td>
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<tr>
<td>Saline (0.9% NaCl)</td>
<td>357 ± 11</td>
<td>116 ± 4</td>
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<tr>
<td>ANG-(1–7) (2.5 nmol/kg)</td>
<td>375 ± 24</td>
<td>116 ± 11</td>
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<tr>
<td><strong>Isoproterenol (anesthetized)</strong></td>
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<tr>
<td>Saline (0.9% NaCl)</td>
<td>339 ± 5</td>
<td>90 ± 5</td>
<td></td>
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<tr>
<td>ANG-(1–7) (2.5 nmol/kg)</td>
<td>370 ± 9</td>
<td>103 ± 3</td>
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<tr>
<td><strong>Bicuculline (anesthetized)</strong></td>
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<tr>
<td>Saline (0.9% NaCl)</td>
<td>349 ± 15</td>
<td>99 ± 8</td>
<td></td>
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<tr>
<td>ANG-(1–7) (2.5 nmol/kg)</td>
<td>368 ± 18</td>
<td>95 ± 5</td>
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Values are means SE. HR, heart rate; MAP, mean arterial pressure.
the Mas antagonist (61), A-779 (58), completely reversed the effect produced by ANG-(1–7) on the stress-evoked tachycardia [ΔHR: saline 112 ± 22 vs. ANG-(1–7) (100 pmol) + A-779 (200 pmol) 120 ± 32 beats/min (data not shown). Central injection of XNT (500 pmol/2 μl) also attenuated the tachycardia evoked by air stress [ΔHR: saline 112 ± 22 vs. XNT 10 ± 8 beats/min; P < 0.01; Fig. 2B]. Neither ANG-(1–7) nor XNT administered icv altered the pressor response evoked by air stress (Fig. 2, A and B). As observed in experiments performed with intravenous injections, at the end of the recovery time (15th minute), the animals treated with ANG-(1–7) or XNT centrally showed heart rate values closer to the baseline compared with saline group. A representative histological analysis of injection site into the rat cerebral ventricle is shown in Fig. 2C.

As expected, icv injection of ANG II resulted in a pressor effect (ΔMAP 29 ± 1 mmHg) accompanied by bradycardia (ΔHR −28 ± 3 beats/min) and drinking response (8 ± 0.2 ml/10 min). The observation of this cardiovascular effect was helpful to further confirm the correct placement of the guide into the lateral ventricle. These results are similar to findings previously reported (10, 36, 37, 48, 49, 55).

Effects of Peripheral Administration of ANG-(1–7) on Cardiovascular and Sympathetic Responses Evoked by Peripheral β-Adrenergic Stimulation in Conscious and Anesthetized Rats

Figure 3A (left column) shows that the increase in HR evoked by isoproterenol was markedly attenuated by intravenous injection of ANG-(1–7) in conscious rats [ΔHR: saline 141 ± 18 vs. ANG-(1–7) 28 ± 18 beats/min; P < 0.01]. In the same group, the treatment with ANG-(1–7) also potentiated the isoproterenol-induced depressor response [ΔMAP: saline −11 ± 3 vs. ANG-(1–7) −23 ± 4 mmHg; P < 0.05]. Figure 3B (right column) shows that in anesthetized rats, peripheral injection of isoproterenol produced a substantial increase in HR [ΔHR 100 ± 16 beats/min) and RSNA (ΔRSNA 39%), accompanied by a significant fall in MAP (Δ mmHg −16 ± 9). As observed in conscious rats, treatment with ANG-(1–7) abolished the HR changes evoked by isoproterenol in anesthetized rats [ΔHR: saline 100 ± 16 vs. ANG-(1–7) 18 ± 15 beats/min; P < 0.01]. The increase in RSNA evoked by isoproterenol was also abolished in rats previously treated with ANG-(1–7) [ΔRSNA: saline 39% vs. ANG-(1–7) −23%; P < 0.05]. In anesthetized rats, ANG-(1–7) did not alter the hypotensive effect evoked by isoproterenol compared with the control group [ΔMAP: saline −16 ± 9 vs. ANG-(1–7) −10 ± 8 mmHg; Fig. 3B].

Effects of Peripheral Administration of ANG-(1–7) on Cardiovascular Response Evoked by Activation of Dorsomedial Hypothalamus

The disinhibition of DMH by injection BMI (20 pmol/100 nl), in conscious rats, produced marked increases in HR. The magnitude of the DMH-evoked tachycardia was reduced by ∼45% in rats submitted to intravenous injection of ANG-(1–7) [ΔHR: saline 69 ± 13 vs. ANG-(1–7) 31 ± 10 beats/min; P < 0.05] (Fig. 4A). In the group treated with ANG-(1–7), the range of the pressor response evoked by injection of BMI into the DMH did not differ from control [ΔMAP: saline 8 ± 3 vs. ANG-(1–7) 5 ± 2 mmHg] (Fig. 4B). Histological analysis of injection sites into the DMH is shown in Fig. 4C.

Effect of Preincubation of ANG-(1–7) on Basal Contraction Rate and Contraction Rate Induced by Adrenergic Activation in Spontaneously Beating Cardiomyocytes

In the experiments performed in cell culture, the basal beating rate of the spontaneously beating cardiomyocytes was 156 ± 8 beats/min. Figure 5A shows that the incubation with ANG-(1–7) markedly reduced the spontaneously beating rate (BR) of the cardiomyocytes (Δ BR −30 ± 2 beats/min; P < 0.001 compared with baseline). The incubation with A-779...
completely blocked the reduction in the beating rate induced by ANG-(1–7) [ΔBR: −2 ± 1 beats/min; P < 0.001 vs. ANG-(1–7)]. The adrenergic stimulation with isoproterenol induced a significant increase in beating rate in cardiomyocytes. This effect was concentration dependent [ΔBR after isoproterenol: 10^{-8} M (6 ± 1 beats/min); 10^{-7} M (15 ± 1 beats/min); 10^{-6} M (40 ± 1 beats/min); 10^{-5} M, (50 ± 1 beats/min)]. The incubation with ANG-(1–7) (1 μM) markedly attenuated the beating rate induced by isoproterenol. This effect was observed in all concentrations of isoproterenol [BR: Δ 10^{-8} M (−9 ± 1 beats/min); 10^{-7} M (5 ± 2 beats/min); 10^{-6} M (12 ± 2 beats/min); and 10^{-5} M (14 ± 1 beats/min)] (Fig. 5B).

**DISCUSSION**

Our data indicate that peripheral or central injections of ANG-(1–7) can modulate the cardiac component of the acute stress response. The BP response to stress was not significantly altered by peripheral or central injection of ANG-(1–7). However, a tendency toward attenuation was observed in several groups, possibly as a result of the attenuation in the stress-evoked tachycardia.

Acute emotional stress is characterized by activation of the sympathetic nervous system (50), and we first investigated a possible action of ANG-(1–7) on sympathetic-mediated responses. In the present study, ANG-(1–7) markedly attenuated the tachycardia evoked by intravenous injections of the β-adrenergic agonist isoproterenol in conscious and anesthetized rats. The ANG-(1–7) also reduced the chronotropic effect induced by isoproterenol in cardiomyocytes. All together these findings indicate that ANG-(1–7) may act peripherally, interfering with the chronotropic response mediated by β-adrenergic receptor activation. However, other mechanisms need to be...
considered regarding the negative chronotropic effects produced by peripheral ANG-(1–7). In our experiments we observed a decrease of the spontaneous beating of cardiomyocytes treated with ANG-(1–7) in the absence of isoproterenol, and this effect is Mas receptor mediated. One possibility is the interaction between Mas receptor activation and ionic channels leading to a change in ionic currents. Liu et al. (41) showed that ANG-(1–7) prevented the changes in the transient outward current, voltage-dependent L-type Ca\(^{2+}\) current, and in the expression of Kv4.3 potassium channel induced by atrial fibrillation (41). This finding strongly suggests that ANG-(1–7) can act directly in cardiomyocytes, remodeling ionic current and changing the expression of ionic channels. In addition, ANG-(1–7) may block the sympathetic input to target organs in other physiological conditions, beyond emotional stress. Since ANG-(1–7) reduces the norepinephrine release in sympathetic vascular terminals of spontaneously hypertensive rats (11), a presynaptic action of ANG-(1–7) on cardiac sympathetic terminals should be considered and further investigated.

In addition to the tachycardia, it is known that an intravenous injection of isoproterenol evokes a substantial fall in MAP due to reductions in peripheral vascular resistance. This fall in MAP is likely mediated via activation of β2 adrenergic receptors dilating resistance arteries (68). It is also known, when MAP decreases, RSNA increases in sigmoidal fashion, a baroreflex-mediated response (30). Thus the increase in RSNA evoked by isoproterenol reported in the present study was likely a consequence of the fall in MAP. In our experiments ANG-(1–7) blocked the RSNA response induced by isoproterenol. Therefore, it could be argued that this effect results from an interference of ANG-(1–7) on baroreflex function. Although we cannot discard this possibility it seems unlikely because peripheral infusion of ANG-(1–7) does not change baroreflex sensitivity (12). One plausible explanation could be the central action of peptides from RAS when injected peripherally (20, 75). Xiao and colleagues (70) showed that subcutaneous infusion of Mas receptor antagonist, A-779, increases renal sympathetic nerve activity in mice with central overexpression of ACE2 during chronic heart failure. This result supports our hypothesis that most likely ANG-(1–7), even injected intravenously, can target the blood-brain barrier-deficient circumventricular organs, attenuating the sympathetic flow. Undoubtedly, the mechanisms involved need to be further evaluated in a novel study.
The activation of DMH neurons increases cardiac sympathetic outflow, resulting in HR increases (13). The portion of the DMH (rostral and anterior) targeted in our experiments is known for exerting predominant control on the cardiac function (64). This adjoining area of the DMH, named the dorsal hypothalamic area (DA) (25), sends dense projections to the cardiac presympathetic neurons located at the rostral ventromedial medulla. Therefore, considering that raphe pallidus-projecting neurons in the DA mainly mediate DMH-induced tachycardia, a small effect of the disinhibition on BP is not surprising (57). Moreover, the tachycardia evoked by activation of DMH is completely abolished by blockade of β-adrenergic receptors (24). In our study, the intravenous injection of ANG-(1–7) reduced the tachycardia produced by activation of DMH neurons. Together with the data obtained in the experiments with isoproterenol we can strongly suggest that, at least peripherally, the mechanism of action of ANG-(1–7) in attenuating the tachycardia evoked by acute stress seems to involve β adrenergic receptors.

A previous study from our laboratory showed that ANG-(1–7) infused subcutaneously did not alter the range of HR or BP evoked by restraint stress, even though a sustained bradycardia was observed after ANG-(1–7) infusion in baseline conditions (9). This raises the question as to why ANG-(1–7) did not attenuate the tachycardia in the restraint stress model. In this regard, the specificity of stress responses and stress models is an extensive matter of discussion in the scientific literature. Basically, different types of stress stimuli can evoke different patterns of central nervous system activation evoking different physiological responses (50). Despite the difference between stress models, in our study ANG-(1–7) was injected in a different route of administration (bolus intravenous injection) and this possibly could result in a more effective ANG-(1–7) action.

The mechanisms involving the attenuation of the tachycardia produced by central administration of ANG-(1–7) need further investigation. One possibility is that ANG-(1–7) might exert an inhibitory effect over the central circuits controlling the stress-induced sympathetic outflow. ANG-(1–7) and Mas receptors are present in the rodent brain, including in areas involved with cardiovascular regulation (5, 8, 47). Brain overexpression of ACE2 prevents the development of hypertension induced by ANG II in mice (20). Kar and colleagues (35) also demonstrated that central ANG-(1–7) inhibits sympathetic outflow, increasing vagal outflow in rabbits with chronic heart failure. In addition, recent evidence suggests that, in catecholaminergic neurons, ANG-(1–7) inhibits neuronal excitation by activating outward K\(^+\) current through a Mas nNOS-NO signaling pathway (73). Considering that our injections were performed into the lateral ventricle, there are several possibilities regarding the specific site of action of ANG-(1–7) in the brain in attenuating the tachycardia to stress. One possibility could be the dorsomedial hypothalamus; however, preliminary experiments from our laboratory demonstrated that injections of ANG-(1–7) into this region do not attenuate the tachycardia evoked by stress. Nevertheless, injection of ANG-(1–7) into the basolateral amygdala greatly reduced the tachycardia produced by acute stress (29). Recent evidence also raises the periaqueductal gray (PAG) as another possible target. Recently, Xing and colleagues (71) demonstrated the presence of Mas receptors in the dorsolateral PAG, and ANG-(1–7) inhibits neuronal activity in this region. Of note, the dorsolateral PAG, an important synaptic relay in the descending pathways controlling the tachycardia evoked by acute stress (4), is located around the cerebral aqueduct (53). Despite that, a previous study already described the involvement of ANG-(1–7) on behavioral responses (34). More recently, Bild and colleagues (7) showed that central

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**Fig. 4.** Maximal changes in cardiovascular parameters (A and B) from baseline evoked by unilateral injection of bicuculline methiodide (BMI) into DMH of rats (n = 4) treated with iv injection of 1) saline (0.9% NaCl) or 2) 2.5 nmol/kg ANG-(1–7). C: histological sections showing the location (large arrow) of the microinjection needle inserted into the DMH. DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; mt, mammillothalamic tract; f, fornix. *P < 0.05 compared with saline (Student’s t-test paired).
administration of ANG-(1–7) induces anxiolytic-like effects in elevated plus maze.

In the experiments using icv injection, the doses chosen were based on previous studies that demonstrated physiological actions for ANG-(1–7) in the brain using picomolar concentrations (14, 62). Pesquero and colleagues (54) showed that the pressor effect evoked by icv injection of ANG II results from direct stimulation of central periventricular structures and not via systemic circulation (54). Considering that ANG-(1–7) has similar chemical structure to ANG II (1 amino acid difference from the other) and in this study we used low doses compatible to other studies (32, 35), we conclude that ANG-(1–7) effects obtained by central injections were restricted to the central nervous system.

Previous studies using site-specific injections indicated that ANG-(1–7) plays a sympathoexcitatory role in the rostral ventrolateral medulla and PVN (19, 23, 39, 62). Both brain nuclei play a pivotal role in the tonic and reflex control of sympathetic activity (16). Taking into account that current data show ANG-(1–7) attenuating the sympathetic response to stress, our findings seem contradictory. However, the exact effect of ANG-(1–7) in the brain is still unclear and cannot be interpreted based only on acute, site-specific injections. First it is necessary to consider that, in the present study, we performed central injections (icv). Using a similar methodology, it was previously reported that icv injections of ANG-(1–7) induced a significant improvement in baroreceptor control of heart rate in conscious rats (12). Furthermore, recent studies show that the chronic actions of ANG-(1–7) may interfere in the central nervous system in a different way. For example, overexpression of ACE2 in the RVLM of spontaneously hypertensive rats caused a long-lasting hypotensive effect (72). In addition, contrasting the studies using acute microinjections, the increased expression and interaction of ACE2 and nNOS within the PVN leads to a reduction in sympathetic outflow in the chronic heart failure (74). Finally, the physiological or physiopathological condition needs to be considered. For example, intravenous injections of ANG-(1–7) have a hypotensive effect in hypertensive but not in normotensive rats (6).

The ACE2 activator, XNT, is a synthetic molecule identified in a virtual screening among 140,000 molecules (33). This is the first report in the literature showing a central effect produced by an ACE2 activator compound. No study has shown the pharmacokinetics and pharmacodynamics of XNT. However, using a different approach (molecular biology techniques), studies described the central effects of ACE2 attenuating the cardiovascular changes associated with hypertension and chronic heart failure (20, 70, 74). On the cardiovascular response to stress, our results demonstrated similar effects produced between XNT and ANG-(1–7) injection. Therefore, it is reasonable to speculate that at least part of the effect produced by XNT is due to ANG-(1–7) formation as a result of ACE2 activation. Our results showed that central XNT markedly attenuated the stress-induced tachycardia. Although XNT and ANG-(1–7) provoked similar effects in our experiments, the intravenous injection of XNT was able to evoke wide cardiovascular responses. One possibility is that XNT can modify the balancing between ANG II and ANG-(1–7). This
suggested that XNT would achieve its effects not by forming only ANG-(1–7), but also by degrading ANG II, which might explain such a discrepancy observed in our study. A direct action of XNT in the heart or direct change in the autonomic activity, increasing vagal or decreasing sympathetic tonus, also needs to be considered (33).

Our study opens a new possibility for exploring the role of ACE2-ANG-(1–7)–Mas axis components on the cardiovascular reactivity to emotional stress, suggesting that ANG-(1–7) can interfere with cardiac β-adrenergic effects. Finally, other ANG II-derived peptides devoid of phenylalanine in position 8, including ANG-(1–7), result in psychotropic activity (34) and deserve future investigation on emotional stress effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

ACE2/ANG-(1–7)/Mas ATTENUATES CARDIAC REACTIVITY TO STRESS


