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Angiotensin-(1–7) Inhibitory Mechanism of Norepinephrine Release in Hypertensive Rats

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Abstract—Release of norepinephrine (NE) by the hypothalamic nuclei may contribute to regulation of sympathetic nervous system (SNS) activity. Angiotensin-(1–7) [Ang-(1–7)] has an antihypertensive effect and may decrease SNS activity. We tested the hypothesis that Ang-(1–7) inhibits the release of NE in hypothalami, via the Ang-(1–7) and angiotensin II type 2 (AT_2) receptors, acting through a bradykinin (BK)/NO-dependent mechanism. Hypothalami from normotensive controls and spontaneously hypertensive rats (SHR) were isolated and endogenous NE stores labeled by incubating the tissues with [^{3}H]NE. [^{3}H]NE release from the hypothalami was stimulated by KCl in the presence or absence of Ang-(1–7) alone or combined with various antagonists and inhibitors. Ang-(1–7) significantly attenuated K^+-induced NE release by hypothalami from normotensive rats but was more potent in SHR. The Ang-(1–7) receptor antagonist [D-Ala^7]Ang-(1–7), the AT_2 receptor antagonist PD 123319, and the BK B_2 receptor antagonist icatibant all blocked the inhibitory effect of Ang-(1–7) on K^+-stimulated NE release in SHR. The inhibitory effect of Ang-(1–7) disappeared in the presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester and was restored by the precursor of NO, L-arginine. The diminished NE release caused by Ang-(1–7) was blocked by a soluble guanylyl cyclase inhibitor as well as by a cGMP-dependent protein kinase (PKG). We concluded that Ang-(1–7) decreases NE release from the hypothalamus via the Ang-(1–7) or AT_2 receptors, acting through a BK/NO-mediated mechanism that stimulates cGMP/PKG signaling. In this way, Ang-(1–7) may decrease SNS activity and exert an antihypertensive effect. **(*Hypertension*. 2004;44:783-787.)**

Key Words: angiotensin ■ norepinephrine ■ receptors, angiotensin II ■ kinins ■ nitric oxide ■ angiotensin antagonist ■ bradykinin

Angiotensin-(1–7) [Ang-(1–7)] has been shown to be the most pleiotropic bioactive component of the renin-angiotensin system because it exerts effects that may be identical to, different from, or opposite from those displayed by angiotensin II (Ang II).[1] For instance, it lacks the vasoconstrictor aldosterone secretagogue or dipsogenic effects of Ang II.[1,2] However, it mimics Ang II stimulation of vasopressin and prostaglandin release[1] as well as peripheral norepinephrine (NE) outflow.[3] In contrast, Ang-(1–7) causes natriuresis, diuresis, and vasodilatation and inhibits angiogenesis and cellular growth,[1,2] suggesting that in many cases, this peptide may act as an endogenous antagonist of Ang II. In fact, Ang-(1–7) has been suggested as having an antihypertensive effect as well as counterbalancing the pressor and proliferative actions of Ang II because some of its effects that oppose those of Ang II are enhanced in rat models of hypertension.[1,4]

It has been demonstrated that the Mas proto-oncogene, originally considered to be an "orphan" G-protein–coupled receptor involved in phospholipase C activation,[5] binds Ang-(1–7) and is involved in the biological actions of this heptapeptide.[6] Genetic deletion of the Mas receptor abolishes not only binding of Ang-(1–7) to mouse kidneys but also Ang-(1–7)-induced relaxation and antidiuretic responses, suggesting that Mas is a functional receptor for Ang-(1–7).[6]

Increased sympathetic nervous system (SNS) activity plays an important role in the pathogenesis of hypertension.[7] Furthermore, release of NE by the hypothalamic nuclei contributes to regulation of blood pressure by altering SNS activity.[8,9] On the other hand, there is evidence that Ang-(1–7) has an antihypertensive effect and may decrease SNS activity.[1,2] We tested the hypothesis that Ang-(1–7) inhibits the release of NE by hypothalami of spontaneously hypertensive rats (SHR) and that this effect is mediated via the Ang-(1–7) receptor and the Ang II type 2 (AT_2) receptor. We also postulated that the inhibitory effect of Ang-(1–7) on NE release is mediated through the kinin B_2 receptor, NO and cGMP-dependent protein kinase (PKG).

Methods

Chemicals

L-[7-^{3}H]NE (specific activity 13.7 Ci/mmol) and losartan were purchased from DuPont. PD 123319 was a gift from Dr Jack Hodges at

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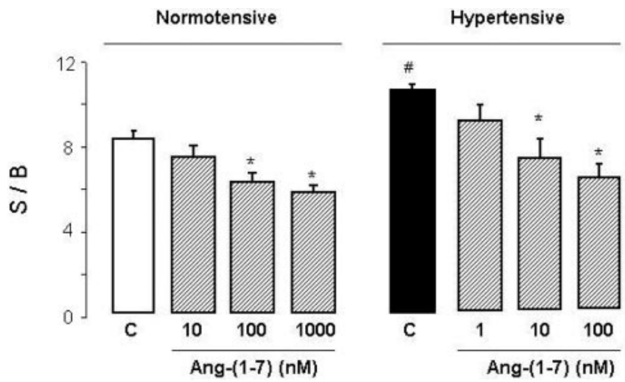

Figure 1. Effect of Ang-(1–7) on K^+-evoked [^{3}H]NE release by hypothalami isolated from normotensive and hypertensive rats. S/B represents the ratio between K^+-stimulated tritium release (S) and radioactivity released during the 2-minute period before high K^+ stimulation (B). Values are mean±SEM (n=8 to 10). #$P<0.05$ compared with the normotensive group; *$P<0.05$ compared with control.

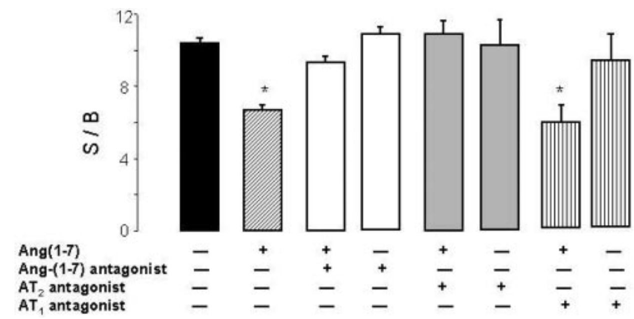

Figure 2. Effect of Ang-(1–7) antagonist, AT_2-receptor antagonist, and AT_1-receptor antagonist on the inhibitory effect of Ang-(1–7) on K^+-evoked [^{3}H]NE release by hypothalami isolated from SHR. S/B represents the ratio between K^+-stimulated tritium release (S) and radioactivity released during the 2-minute period before high K^+ stimulation (B). When indicated, 1 μmol/L Ang-(1–7) antagonist (D-Ala^7), 1 μmol/L AT_2-receptor antagonist (PD 123319), or 1 μmol/L AT_1 receptor antagonist (losartan) was added alone or simultaneously with 100 nmol/L Ang-(1–7). Values are mean±SEM (n=8 to 10). *$P<0.05$ compared with control.

Parke Davis (Detroit, Mich). (Rp)-8-(para-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate (RP8) and (Sp)-8-(para-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate were obtained from Alexis. Ang-(1–7) and [D-Ala^7]Ang-(1–7) were synthesized in our laboratory by the Merrifield solid-phase procedure.[3] The purity of Ang-(1–7) was 98% as verified by mass spectrometry. 1H-(1,2,4)oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) and the other reagents were purchased from Sigma-Aldrich.

Experimental Protocol

[^{3}H]NE release was measured as described previously, with slight modifications.[10] Briefly, minced hypothalami isolated from either 12-week-old Wistar rats or 12-week-old SHR were incubated at 37°C for 30 minutes in Krebs solution. We used Wistar rats as the normotensive control group because they are genetically homogeneous, whereas the genetic homogeneity of Wistar-Kyoto rats is in doubt.[11] Furthermore, to our knowledge, no difference between Sprague-Dawley and Wistar-Kyoto rats has been reported with regard to K^+-stimulated NE release from the paraventricular hypothalamic nucleus.[12]

NE stores were labeled with 0.1 μmol/L [^{3}H]NE (1.5 μCi/mL) during a 30-minute incubation period. Eighteen consecutive 5-minute washes with Krebs solution were performed, and then 6 consecutive 2-minute samples were collected. Tissues were incubated for 2 minutes in Krebs solution containing 25 mmol/L KCl. Ang-(1–7) and inhibitors were present 2 and 4 minutes before, respectively, and during the incubation of tissues in high-K^+ medium. [^{3}H]NE release was measured in each sample as the amount of radioactivity present in the incubation medium. Results are expressed as the ratio of K^+-stimulated tritium release (S) to spontaneous outflow (B), which is the radioactivity released in the 2-minute period before high K^+ incubation.

Statistical Analysis

All values are mean±SEM. Data were submitted to 1-way ANOVA followed by a Student–Newman-Keuls test. The effect of Ang-(1–7) on normotensive rats and SHR was evaluated by 2-way ANOVA. $P<0.05$ was considered significant.

Results

The evoked NE overflow induced by K^+ was significantly greater in hypothalami from SHR than in those from normotensive rats. Increasing concentrations of Ang-(1–7) resulted in significant attenuation of K^+-induced NE release, showing higher potency in hypothalami from SHR (Figure 1).

To examine the receptor subtypes involved in the inhibitory effect of Ang-(1–7) on NE overflow in SHR, specific antagonists were added. PD 123319 (1 μmol/L), an AT_2 receptor antagonist, and [D-Ala^7]Ang-(1–7) (1 μmol/L), an Ang-(1–7)-specific antagonist,[13] prevented the inhibitory effect of 100 nmol/L Ang-(1–7) on NE release evoked by K^+, whereas losartan (1 μmol/L), an AT_1 receptor antagonist, did not (Figure 2). The antagonists, per se, did not modify this mechanism.

Because several effects elicited by Ang-(1–7) have been shown to be mediated by NO,[14] its possible involvement in the inhibitory response of Ang-(1–7) was investigated. We found that the diminished K^+-induced NE release caused by 100 nmol/L Ang-(1–7) was abolished by 1 μmol/L N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NO synthesis, and was restored when L-Arg (10 μmol/L), the precursor of NO synthesis, was simultaneously present (Figure 3). L-NAME, L-Arg, or L-NAME plus L-Arg did not modify the enhanced K^+-evoked NE release (S/B 11.7±0.9, 9.8±0.4, and 9.3±0.7, respectively, versus 10.4±0.3 for controls). The inhibitory action of Ang-(1–7) was also blocked in the presence of 1 μmol/L ODQ, a specific soluble guanylyl cyclase (sGC) inhibitor, as well as by 1 μmol/L RP8, a PKG inhibitor (Figure 4). ODQ or RP8 by itself did not modify NE release (S/B 9.1±0.5 and 11.1±0.4, respectively, versus 10.4±0.3 for controls).

To test whether the NO/kinin system is involved in the inhibitory response to K^+-evoked NE release elicited by Ang-(1–7), we assayed the effect of the peptide in the presence of icatibant, a B_2 receptor antagonist. A total of 10 μmol/L icatibant blocked the inhibition of NE release caused by 100 nmol/L Ang-(1–7) but did not modify NE release by itself (Figure 5).

Discussion

High blood pressure in hypertension is associated with and probably caused by increased SNS activity.[7,15] A central disturbance in NE release by the hypothalamus is related to

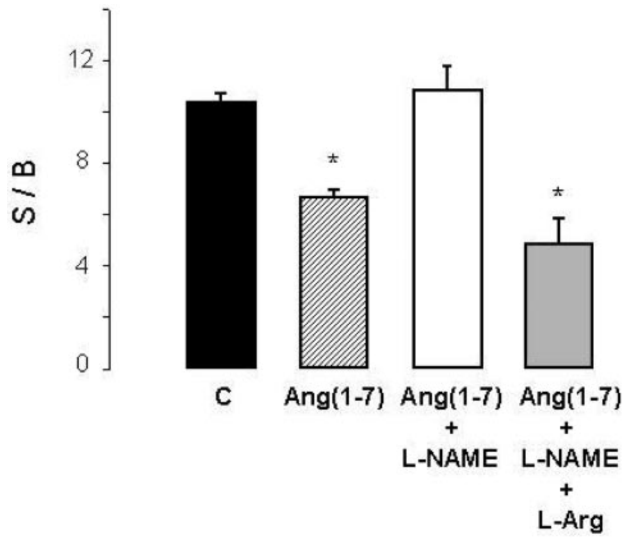

Figure 3. Effect of L-NAME and L-NAME plus L-arginine (L-Arg) on the inhibitory effect of Ang-(1–7) on K^+-evoked [^{3}H]NE release by hypothalami isolated from SHR. S/B represents the ratio between K^+-stimulated tritium release (S) and radioactivity released during the 2-minute period before high K^+ stimulation (B). When indicated, 1 μmol/L L-NAME or 1 μmol/L L-NAME plus 10 μmol/L L-Arg was added simultaneously with 100 nmol/L Ang-(1–7). Values are mean±SEM (n=8 to 10). *$P<0.05$ compared with control.

increased SNS activity.[8] Accordingly, augmented NE release and catecholamine synthesis as well as tyrosine hydroxylase gene expression have been reported at central sites related to blood pressure regulation in adult SHR.[12,16–18] We found that K^+-induced NE release was greater in hypothalami from SHR than normotensive controls. It has been suggested that activation of the renin-angiotensin system is a mechanism involved in sympathetic hyperactivity in hypertension because Ang II has been documented to enhance sympathetic outflow.[7] In contrast, and supporting the antihypertensive role

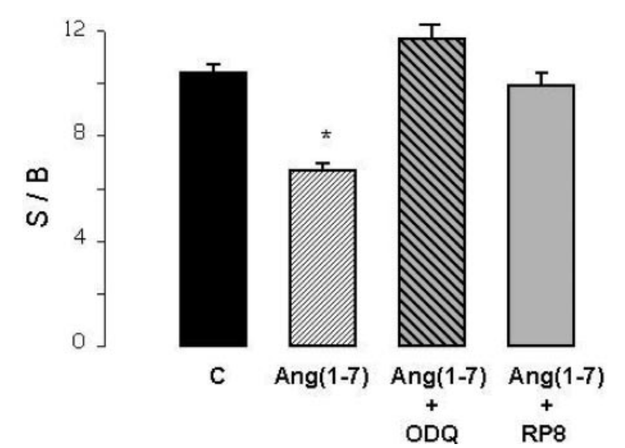

Figure 4. Effect of the guanylate cyclase inhibitor ODQ or the PKG inhibitor RP8 on the inhibitory effect of Ang-(1–7) on K^+-evoked [^{3}H]NE release by hypothalami isolated from SHR. S/B represents the ratio between K^+-stimulated tritium release (S) and radioactivity released during the 2-minute period before high K^+ stimulation (B). When indicated, 1 μmol/L ODQ or 1 μmol/L RP8 was added simultaneously with 100 nmol/L Ang-(1–7). Values are mean±SEM (n=8 to 10). *$P<0.05$ compared with control.

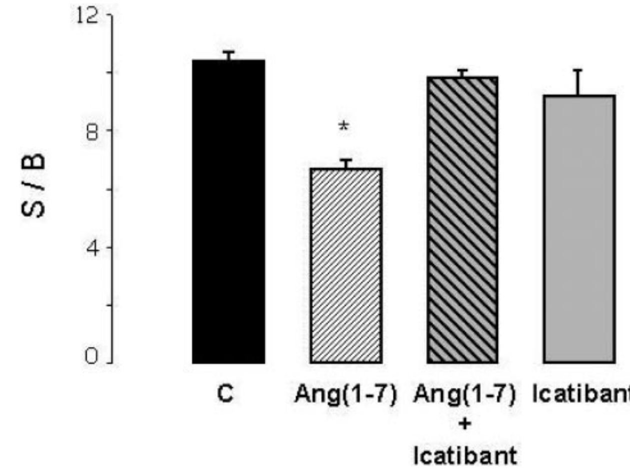

Figure 5. Effect of the B_2 receptor antagonist icatibant on the inhibitory effect of Ang-(1–7) on K^+-evoked [^{3}H]NE release by hypothalami isolated from SHR. S/B represents the ratio between K^+-stimulated tritium release (S) and radioactivity released during the 2-minute period before high K^+ stimulation (B). When indicated, 1 μmol/L icatibant was added simultaneously with 100 nmol/L Ang-(1–7). Values are mean±SEM (n=8 to 10). *$P<0.05$ compared with control.

suggested for Ang-(1–7),[1] our present results demonstrate that Ang-(1–7) decreases K^+-induced NE release more so in hypothalami from SHR than the normotensive group (Figure 1), and in this way, may affect SNS activity. Nevertheless, the inhibitory effect of Ang-(1–7) on NE release may also result from a blocking action on the increased NE release elicited by Ang II. In accord with this possibility, we have shown previously that Ang-(1–7) not only diminished NE release but also blocked Ang II-enhanced NE outflow from hypothalami isolated from hypertensive rats with aortic coarctation.[19]

Our present results show that the inhibitory effect of Ang-(1–7) on K^+-induced NE release was blocked by both an AT_2 antagonist and an Ang-(1–7) antagonist, suggesting that this effect is mediated by the AT_2 and Mas receptors. However, in the central nervous system, Mas mRNA has been shown to be present in limbic, thalamic, and cortical structures but not in hypothalamus,[20,21] so we cannot rule out the possibility that the antagonist for Ang-(1–7) may recognize AT_2 sites in the rat hypothalamus.

Several lines of evidence demonstrate that function and signaling of AT_2 receptors are quite different from AT_1 sites and that these receptors may exert opposite effects in terms of cell growth and blood pressure regulation.[22,23] In addition, a counter-regulatory role in catecholamines synthesis by adrenal medullary chromaffin cells has been reported.[24] Supporting this hypothesis, we suggest that AT_2 sites counter-regulate the facilitatory actions of Ang II on NE release at its type 1 receptors because the AT_2 sites are involved in the decreased NE release caused by Ang-(1–7) (present results).

Accumulating evidence suggests that the effects of Ang-(1–7) are mediated by NO.[14] In our study, pretreatment with L-NAME abolished Ang-(1–7)-induced attenuation of NE release, which was restored when L-Arg was simultaneously added, suggesting that this process depends on NO synthesis. In fact, when sGC, the NO target, was inhibited with ODQ, Ang-(1–7) failed to modify K^+-induced NE release, again supporting NO participation through a cGMP-dependent mechanism (present results). In contrast, the blocking effect of Ang-(1–7) on Ang II-enhanced NE release by hypothalami from hypertensive rats with aortic coarctation is also mediated by NO release but in a cGMP-independent manner, involving γ-aminobutyric acid participation (M.M. Gironacci et al., 2003, unpublished data), suggesting that at the central level, the mechanism of action of Ang-(1–7) on NE release may depend on the hypertensive process.

NO stimulates sGC and increases cytosolic cGMP concentration, leading to activation of PKG.[25,26] In accord with this signal transduction cascade, our study shows that the attenuation of K^+-induced NE release caused by Ang-(1–7) is blocked by RP8, a specific inhibitor of PKG. This suggests involvement of PKG, probably by phosphorylation of either voltage-dependent calcium channels, resulting in their inhibition,[26] or of synaptic vesicle proteins associated with neurotransmitter release, as suggested previously.[27]

It has been observed that activation of AT_2 receptors results in bradykinin (BK)-dependent stimulation of NO release.[28,29] In addition, formation of NO in response to Ang-(1–7) is caused by activation of local kinin production.[30] Accordingly, the inhibitory effect of Ang-(1–7) on K^+-induced NE release disappears when the B_2 receptors are blocked with the antagonist icatibant (present results), suggesting that an NO/BK mechanism plays a critical role in the inhibition of NE release caused by Ang-(1–7). The mode of interaction between AT_2 and B_2 receptors remains to be elucidated. Tsutsumi et al[29] have reported that in aortic smooth muscle cells, AT_2 receptors activation causes intracellular acidosis through inhibition of amiloride-sensitive Na^+/H^+ exchanger activity, resulting in enhanced kininogenase activity. Another possibility is that AT_2 and B_2 receptors on the plasma membrane may interact directly through heterodimer formation, as demonstrated previously for AT_1 and B_2,[31] because it is generally accepted that G-protein–coupled receptors exist and could function as dimers or higher oligomers.[32] Further studies are under way in our laboratory to clarify the interactions between these receptors.

Perspectives

The SNS is involved in maintaining high blood pressure. Our results suggest that Ang-(1–7) may be an important neuromodulator of this system at the central level in SHR by inhibiting NE release through an NO-related mechanism, which in turn, maintains low NE outflow. The inhibitory effect of Ang-(1–7) may be mediated by the Ang-(1–7) or AT_2 receptors in a BK/NO-dependent manner through the cGMP/PKG pathway. In this context, Ang-(1–7) opposes the action of Ang II and contributes to blood pressure regulation, supporting the antihypertensive role suggested previously.[1] Thus, clarification of the mechanism of action of Ang-(1–7) could improve not only our understanding of its role in the pathogenesis of hypertension and cardiovascular and renal diseases but also their treatment. This is even more important at a time when more selective antagonists of the renin-angiotensin system, and also newer agents such as angiotensin receptors and angiotensin-converting enzyme inhibitors, are being widely used for treatment of hypertension, heart failure, and cardiovascular and renal diseases.

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