Angiotensin-(1-7) Reduces Norepinephrine Release Through a Nitric Oxide Mechanism in Rat Hypothalamus

Mariela M. Gironacci, Marcelo Vatta, Martín Rodríguez-Fermepín, Belisario E. Fernández, Clara Peña

Abstract—Angiotensin (Ang)-(1-7) elicits a facilitatory presynaptic effect on peripheral noradrenergic neurotransmission, and because biological responses to the heptapeptide on occasion are tissue specific, the present investigation was undertaken to study its action on noradrenergic neurotransmission at the central level. In rat hypothalamus labeled with [3H]-norepinephrine, 100 to 600 nmol/L Ang-(1-7) diminished norepinephrine released by 25 mmol/L KCl. This effect was blocked by the selective angiotensin type 2 receptor antagonist PD 123319 (1 μmol/L) and by the specific Ang-(1-7) receptor antagonist [D-Ala5]Ang-(1-7) (1 μmol/L) but not by losartan (10 nmol/L to 1 μmol/L), a selective angiotensin type 1 receptor antagonist. The inhibitory effect on noradrenergic neurotransmission caused by Ang-(1-7) was prevented by 10 μmol/L Nω-nitro-L-arginine methylester, an inhibitor of nitric oxide synthase activity, and was restored by 100 μmol/L L-arginine, precursor of nitric oxide synthesis. Methylene blue (10 μmol/L), an inhibitor of guanylate cyclase considered as the target of nitric oxide action, as well as Hoe 140 (10 μmol/L), a bradykinin B2-receptor antagonist, prevented the inhibitory effect of the heptapeptide on neuronal norepinephrine release, whereas no modification was observed in the presence of 0.1 to 10 μmol/L indomethacin, a cyclooxygenase inhibitor. Our results indicate that Ang-(1-7) has a tissue-specific neuromodulatory effect on noradrenergic neurotransmission, being inhibitory at the central nervous system by a nitric oxide–dependent mechanism that involves angiotensin type 2 receptors and local bradykinin production. (Hypertension. 2000;35:1248-1252.)

Key Words: angiotensin ■ norepinephrine ■ nitric oxide ■ angiotensin antagonist ■ bradykinin ■ prostaglandins

Angiotensin (Ang)-(1-7) is considered a bioactive end product of the renin-angiotensin system formed from Ang I metabolism through an enzymatic pathway independent of the angiotensin-converting enzyme.1 It can be formed either from Ang I or Ang II by a prolyl-endopeptidase that cleaves the Pro-Phe bond.1,2 Ferrario and coworkers2 demonstrated the presence of Ang-(1-7) in several regions of the rat brain such as the hypothalamus, amygdala, and medulla oblongata but not in the cerebral cortex and cerebellum. In addition, type 1 (AT1) and type 2 (AT2) Ang receptors were described in several regions and nucleus of the central nervous system, including the hypothalamus.3,4 Although Ang-(1-7) is not an agonist in terms of activating vasoconstriction,5 stimulating thirst,6 or promoting aldosterone secretion,5 the heptapeptide causes neuronal excitation in the paraventricular nucleus of hypothalamus and dorsal vagal complex of the medulla oblongata,7 facilitates the noradrenergic neurotransmission,8 and stimulates prostaglandin9–11 and vasopressin release12 with potency comparable to that of Ang II. Conversely, some of the effects of Ang-(1-7) are opposite to those elicited by Ang II, that is, it displays an antiproliferative action on vascular smooth muscle cells,13 produces natriuresis14 and diuresis15 as well as vasodilation,16,17 and facilitates the baroreflex activity.7,18 Several studies indicated that Ang-(1-7) effects are tissue-specific, that is, the heptapeptide activates Na+ K+-ATPase in rat brain synaptosomal membranes, whereas a biphasic effect on this enzymatic system is observed in rat renal membranes.19 Furthermore, Gironacci et al8 have reported a facilitatory effect of the heptapeptide on the sympathetic neurotransmission in rat atri. Conversely, this stimulatory action was not detected in the rabbit vas deferens.11 Because of the suggested tissue-specific activity and the presynaptic effect of Ang-(1-7) on peripheral noradrenergic neurotransmission,8 the present study was performed to assess the effect of Ang-(1-7) on K+-evoked neuronal release of norepinephrine (NE) in rat hypothalamus.

Methods

Animals and Chemicals
Male Sprague-Dawley rats weighing 250 to 300 g were used. dl-[7,8-3H]norepinephrine (specific activity 32 Ci/mmol) was purchased from Amersham Life Science; cocaine hydrochloride was kindly supplied by Dr Edda Villaamil (Cátedra de Toxicología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires);...
Hoe 140 was kindly supplied by Dr Martiarena (Cátedra de Control de Medicamentos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires); hydrocortisone, pargyline, indomethacin, methylene blue, D-arginine, and N\-nitro-D-arginine methylester were from Sigma Chemical Co. Ang-(1-7) and [D-Ala\(^7\)]Ang-(1-7) were synthesized in our laboratory by the Merrifield solid-phase procedure, as previously described.8

**Experimental Protocol**

\([\text{\textsuperscript{3}H}]\text{NE} \) release was measured according to the technique described by Vatta et al.\(^6\) with slight modification. Briefly, minced rat hypothalami were incubated at 37°C for 30 minutes in 2 mL of standard Krebs solution. Monoamine-oxidase activity and extraneuronal NE uptake were inhibited by the addition of 0.1 mmol/L pargyline and 0.1 mmol/L hydrocortisone, respectively. NE stores were labeled with 2.5 \(\mu\)Ci/mL \([\text{\textsuperscript{3}H}]\text{NE} \) (100 nmol/L) during a 30-minute incubation period. After 8 consecutive 5-minute washes with Krebs solution, tissues were then incubated during a period of 4 minutes in high-potassium Krebs solution containing 0.1 mmol/L cocaine to inhibit neuronal NE uptake, 0.1 mmol/L hydrocortisone, and 0.1 mmol/L pargyline. Two consecutive samples of incubation medium were collected every 2 minutes (S\(_1\) and S\(_2\)), and \([\text{\textsuperscript{3}H}]\text{NE} \) in the medium 15 minutes before sample collection. Results were assessed as the ratio of the radioactivity released by the second period to that of the first period (S\(_2\)/S\(_1\)).

**Results**

To determine Ang-(1-7) effect on K\(^+-\)evoked neuronal NE release in hypothalamus, different concentrations of the heptapeptide were tested. Data given in Figure 1 show that 100, 300, and 600 nmol/L Ang-(1-7) diminished neuronal NE release evoked by K\(^+\).

To study the Ang-receptor subtypes coupled to the inhibitory activity of Ang-(1-7) on neuronal NE release, the effects of selective antagonists for AT\(_1\)- and AT\(_2\)-receptor subtypes were assessed. Results showed that losartan (10 nmol/L to 1 \(\mu\)mol/L), a selective AT\(_1\)-receptor antagonist, diminished neuronal NE release evoked by 25 mmol/L KCl (data not shown). In addition, the reduction of evoked NE release produced by 100 nmol/L Ang-(1-7) was not blocked by losartan (10 nmol/L to 1 \(\mu\)mol/L) (Figure 2). On the other hand, the role of AT\(_2\)-receptor reduction of evoked NE release in hypothalamus was studied in the presence of PD 123319, a selective AT\(_2\)-receptor blocker, which, at levels >1 \(\mu\)mol/L, abolished Ang-(1-7)-inhibitory effects (Figure 2). Simultaneous addition of losartan and PD 123319 did not modify the stimulated NE release (data not shown).

The selective Ang-(1-7) antagonist [D-Ala\(^7\)]Ang-(1-7) (1 \(\mu\)mol/L) partially blocked the effect of Ang-(1-7) on NE release (Figure 2). PD 123319 and D-Ala\(^7\)-Ang-(1-7) each had no effect by itself on NE release (data not shown). It has been demonstrated that Ang-(1-7) exerts several effects through the NO pathway.\(^{16,21-23}\) To assess the role of NO as possible mediator of Ang-(1-7) effect on neuronal NE release evoked by K\(^+\) in rat hypothalamus, we investigated the effect of Ang-(1-7) in the presence of N\-nitro-D-arginine methyl ester (L-NAME), an inhibitor of NO synthase activity. The reduction of evoked NE release produced by 100 nmol/L Ang-(1-7) was prevented by the addition of 10 \(\mu\)mol/L L-NAME and was restored when L-arginine (100 \(\mu\)mol/L), the precursor of NO synthesis, was simultaneously present (Figure 3). Moreover, 10 \(\mu\)mol/L methylene blue, an inhibitor of guanylate cyclase considered as the target of NO action, prevented the inhibitory effect of Ang-(1-7) on NE release (Figure 3). L-NAME and methylene blue did not alter K\(^+-\)evoked neuronal NE output by itself (data not shown).

It has been reported that NO formation after AT\(_2\)-receptor stimulation is due to the activation of local bradykinin (BK) production.\(^{24,25}\) Therefore, we investigated the effect of Hoe 140, a kinin B\(_2\)-receptor antagonist, on the inhibitory effect of Ang-(1-7) on NE release. As shown in Figure 4, 10 \(\mu\)mol/L Hoe 140 completely blocked the induced reduction of K\(^+\)-evoked neuronal NE release caused by the heptapeptide in rat hypothalamus. The antagonist had no direct effect. To assess if the prostaglandin pathway is involved in Ang-(1-7) effects, experiments were performed in the presence of 0.1 to 10 \(\mu\)mol/L indomethacin, a cyclooxygenase.
inhibitor. No differences in the effect elicited by 100 nmol/L Ang-(1-7) were observed (Figure 5). Indomethacin (0.1 to 10 μmol/L) failed to modify by itself the K⁺-evoked neuronal NE release (data not shown).

**Discussion**

In agreement with previous studies in rat medulla, the present findings indicate that Ang-(1-7) attenuates the K⁺-evoked neuronal [³H]NE release from rat hypothalamus. The inhibitory effect induced in rat hypothalamus differs from that produced in the peripheral nervous system, in which the heptapeptide acts presynaptically, increasing NE released by nerve stimulation. It has been reported that Ang-(1-7) elicits a tissue-specific neuromodulatory action. In this regard, a facilitatory effect on noradrenergic neurotransmission in rat atria was observed, but no effect in rabbit vas deferens was described.

Biological activity of Ang-(1-7) is distinguishable from that of Ang II, and frequently contrasting effects were observed: For example, Ang-(1-7) facilitates the baroreflex as well as induces vasodilation, effects not produced by Ang II. There is evidence that the renin-angiotensin system regulates several functions through multiple-level feedback mechanisms. Contrasting activities of Ang-(1-7) and Ang II would further confirm the hypothesis that the renin-angiotensin system may limit Ang II effects through generation of the heptapeptide. In agreement, the present results show that Ang-(1-7) opposes the enhancement on K⁺-induced [³H]-NE release from rat hypothalamus caused by Ang II.

The decreased NE release caused by Ang-(1-7) was blocked by the AT₂-receptor antagonist PD 123319 and not by the AT₁-receptor antagonist losartan, suggesting that AT₂ receptors are involved in such response. In fact, central responses of Ang-(1-7) appear to be more sensitive to inhibition by AT₂-receptor antagonists. For example, prostaglandin synthesis in human astrocytes and substance P release in rat hypothalamus as well as neuronal excitation in the paraventricular nucleus induced by Ang-(1-7) were blocked by AT₂-receptor antagonists.

Interestingly, both AT₁ and AT₂ receptors appear to have antagonistic roles on central noradrenergic neurotransmission, since Ang II–facilitated NE release is mediated by AT₁ receptors whereas Ang-(1-7) inhibitory action on NE release is coupled to AT₂ receptors (present results). Several reports have suggested that these receptors mediate opposite physiological effects.

Furthermore, the inhibitory action of Ang-(1-7) on NE release was partially prevented by [d-Ala²]Ang-(1-7), suggesting that Ang-(1-7) receptors are also involved. Likewise, the stimulation of specific Ang-(1-7) receptors accounts for the Ang-(1-7)–induced excitation of paraventricular neurons in rat and [³H]arachidonic acid release in rabbit aortic smooth muscle cells. In this latter case, both PD 123319 and [d-Ala²]Ang-(1-7) were required to fully block the response.

Because Ang II increases NE release through AT₁-receptor activation and not through AT₂ receptors, the opposite response induced by losartan itself may result from blockade of
endogenous Ang II binding to AT1 receptors, as it was previously suggested.\(^{35,36}\) We disregarded this possibility in our results because the addition of losartan plus PD 123319 did not modify the stimulated NE release, suggesting that losartan may unmask the binding of endogenous either Ang-(1-7) or Ang II to AT1 sites.

Ang-(1-7) is a potent stimulator of prostaglandin release in neural and vascular cells.\(^9\)\(^{11}\) Furthermore, vasodilatation of cerebral arteries as well as the natriuresis and depressor activities produced by Ang-(1-7) could be abolished by indomethacin, suggesting that these effects are mediated by prostaglandins.\(^{37}\) Despite their inhibitory role on NE release, the attenuation on sympathetic neurotransmission caused by Ang-(1-7) in the rat hypothalamus (present results) was not prevented by indomethacin, excluding prostaglandin involvement.

Because the neurmodulatory effect of Ang-(1-7) on NE release was blocked by L-NAME and methylene blue, an NO-mediated mechanism is suggested in accordance with previous reports that demonstrated NO dependence in various Ang-(1-7) effects.\(^{16,21\text{-}23}\) Moreover, NO formation induced by Ang-(1-7) (present data) after AT1-receptor stimulation appears to be due to the activation of local BK production, since the inhibitory effect of the heptapeptide on NE release disappeared in the presence of Hoe 140, a B2-receptor antagonist. Accordingly, Seyedi et al\(^{38}\) have shown that the increased aortic NO production induced by Ang-(1-7) resulted from the activation of AT1 receptors and also involved local BK production. In fact, it recently has been shown that mice lacking AT1 receptors have low renal BK and NO production,\(^{25}\) suggesting that these receptors mediate BK and NO formation. It should be pointed out that Ang-(1-7) interaction with kinins is a receptor-mediated event and not simply attributable to angiotensin-converting enzyme inhibition, which may possibly prevent BK degradation\(^{39}\) or down-regulation of the B2 receptor.\(^{40}\)

In conclusion, Ang-(1-7) has a tissue-specific neurmodulatory effect on noradrenergic neurotransmission, being inhibitory at the central level by a NO-dependent mechanism that involves AT2 receptors and local BK generation.

Acknowledgments

This work was supported by grants from UBACYT FA134 and TB58, CONICET PID4566, PIP0492, and ANPCYT P116. The excellent technical assistance of Adriana Turiaci is gratefully acknowledged.

References

Clara Peña

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*Hypertension*. 2000;35:1248-1252
doi: 10.1161/01.HYP.35.6.1248

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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