**Keynote Address**

Pathways of Angiotensin Formation and Function in the Brain

Carlos M. Ferrario, Karen L. Barnes, Christine H. Block, K. Bridget Brosnihan, Debra I. Diz, Mahesh C. Khosla, and Robson A.S. Santos

New findings from this laboratory suggest that fragments of angiotensin derived from the amino (N-)terminus are biologically active end products of the renin-angiotensin system. In vitro and in vivo experiments revealed that the heptapeptide angiotensin-(1-7) [Ang-(1-7)] is a major endogenous product of the renin-angiotensin system cascade in the brains of rats and dogs. Additional studies with enzyme inhibitors showed that Ang-(1-7) is produced directly from angiotensin I by an enzyme other than the angiotensin converting enzyme. Immunocytochemical analysis revealed the presence of specific staining for immunoreactive Ang-(1-7) in neurons and fibers within the hypothalamo-neurohypophyseal vasopressinergic system of the rat. Although Ang-(1-7) is as potent as angiotensin II (Ang II) in stimulating release of vasopressin from superfused hypothalamo-neurohypophyseal explants, the heptapeptide has no dipsogenic or vasoconstrictor activity. In contrast, Ang-(1-7) mimics the effects of Ang II in augmenting the intrinsic discharge rate of neurons within the vagal-solitary complex and in causing monophasic depressor responses after microlnjection into the medial region of the nucleus tractus solitarii. The evidence obtained in these experiments suggests novel mechanisms for the generation of angiotensin peptides in the brain. Additionally, the findings suggest that some of the biological actions ascribed to Ang II might be conveyed by the endogenous production of other angiotensin peptides that are generated by enzymatic pathways alternate to those described in the peripheral circulation. (Hypertension 1990;15(suppl I):I-13–I-19)

Angiotensin II (Ang II) is a potent pressor agent when given into either the brain ventricles or the cerebral circulation of mammals. The peptide also stimulates the release of pituitary hormones and triggers thirst behaviors. These effects correlate with the presence of specific high-affinity Ang II binding sites in the endocrine hypothalamus and circumventricular organs. Ang II neuroreceptors, however, are not confined to the sites where the blood–brain barrier (BBB) is permeable. Because Ang II receptors are also associated with neuronal elements situated within the BBB, the peptide might be produced locally. This possibility has been confirmed by a number of findings. Immunocytochemistry shows binding of Ang II–directed antibodies to neuronal perikarya and fibers of the hypothalamus and lower brainstem. Furthermore, the brain also contains the genes that specify the composition of the enzymes and precursors of Ang II. Because experiments showed that most other organs also express the messenger RNAs for both renin and angiotensinogen (Aogen), these diverse locations of angiotensin precursor proteins suggest the existence of a paracrine renin-angiotensin system (RAS) in the kidney, heart, blood vessels, and of course, the brain. These tissue systems might complement the known functions of circulating Ang II in cardiovascular regulation.

In this article, we examine the implications of a recent discovery regarding pathways for the processing of angiotensin peptides by the brain. The analysis is based on the discovery that, in the brain, the heptapeptide angiotensin-(1-7) [Ang-(1-7)] is not an inert metabolite of Ang II and that it can be formed directly from angiotensin I (Ang I). Indeed, these findings suggest that tissues might process Aogen by pathways alternate to those described in the blood. Based on this information, we offer a new concept regarding the functional significance of tissue RASs and also propose that Ang II is a member of a family of biologically active angiotensin peptides. Although our studies were carried out in the brain, the information is applicable to other organs that contain a functional RAS. It is quite possible...
that the production of biologically active angiotensin peptides in various tissues is accounted for by mechanisms that diverge from the accepted RAS cascade described in the peripheral circulation.4

Biological Actions of Angiotensin II: A Critique of the Obvious?

A brimming literature on the biochemical pathways and physiological actions of the RAS has established that the octapeptide Ang II is the principal active end product of this system.7-9 Moreover, previous studies suggest that both precursors and metabolites of Ang II (other than angiotensin III [Ang III]) are weak agonists.7,9 This finding is surprising because the RAS should share with most other endocrine systems the property that multiple active forms of peptide hormones are derived from their precursors. But investigators that probed for this possibility found no evidence because biological responses mediated by Aogen or Ang I are readily explained by the presence of the Asp'-Phe7 amino acid sequence of Ang II within their molecules.9 Although one carboxyl (C-)terminal fragment of Ang II, the heptapeptide Ang III [Ang-(2-8)], is a potent aldosterone secretagogue and neuronal stimulant,8,10 its actions are comparable with those produced by Ang II. Other C-terminal fragments of Ang II are weak pressor agents, whereas those fragments lacking an amino acid in the ultimate position of Ang II have no myotropic activity.7 Page’s observation that “Ang II has a function and probably multiple ones”8 justifies the present formulation of our thinking with regard to these general principles. It is, indeed, conceded by most investigators that any specialization of the RAS in blood pressure regulation is dictated by the selective expression of specific receptors on target cells.8

Synthesis of Ang II analogues has proven useful in decoding that the aromatic amino acid phenylalanine in position 8 is required for biological activity.7,9 Additionally, binding of Ang II to a receptor depends on the presence of the aromatic side chains in positions 4 and 6, the guanido group in position 2, and the C-terminal carboxyl.9 Conformation studies suggest that the full biological activity of Ang II agonists and antagonists is retained by those peptides that most resemble the backbone and side-chain structure of Ang II.11 Fragments of Ang II derived from the amino (N-)terminus have no direct vasoconstrictor activity and negligible effects on renin and aldosterone secretion.9,12 These findings support the view that receptors mediating angiotensin end-organ responses require the presence of an amino acid in position 8 of the molecule.7 The diversified biological actions of Ang II in the control of cardiovascular function, however, raise the question of whether differential effects of the peptide in various organ systems depend solely on the presence of target receptors. Alternatively, specific functions of angiotensins in the tissue can be controlled by the expression of singular forms of angiotensin peptides. Our data suggest that there exist, in tissues such as the brain, other active angiotensin peptides with unique actions. We will review here new findings that support this probability.

Angiotensin-(1-7) Is a Biologically Active N-Terminal Fragment of Angiotensin II

Expression of Angiotensin-(1-7) in the Brain

The discovery that an N-terminal fragment of Ang II is bioactive13 originated from studies directed to establish the role of Ang II in the regulation of blood pressure. The complete story is told elsewhere.14 Briefly, the research was first prompted by the need to verify that the presence of Ang II in the cerebrospinal fluid of bilaterally nephrectomized dogs was not accounted for by the existence of other immunoreactive fragments that cross-reacted with the Ang II antibody.15,16 As a component of a research strategy, we initiated an analysis of the hydrolysis products of Ang I metabolism in the canine brain. In these seminal experiments, homogenates obtained from micropunches of the dorsal medulla oblongata were incubated with picomolar amounts of either [125I]Ang I or [125I]Ang II.17 The products of hydrolysis were characterized by a high-performance liquid chromatography (HPLC) technique perfected in this laboratory and described elsewhere.18 Figure 1 shows that both Ang II and the heptapeptide Ang-(1-7) were the two major products of [125I]Ang I metabolism in canine medulla oblongata homogenates. Furthermore, Ang-(1-7) was also produced when [125I]Ang II was incubated with homogenates from the medulla oblongata.17 Pretreatment of the
Because we interpreted these findings as potentially important for the understanding of the function of brain Ang II, we took additional steps to determine whether Ang-(1-7) is an endogenous product of the angiotensin system in the brain. Specific polyclonal antibodies were raised in the rabbit against a synthetic form of Ang-(1-7). Immunocytochemistry\textsuperscript{19} revealed intense Ang-(1-7) immunofluorescence of neurons and fibers in the supraoptic (Figure 2) and paraventricular nuclei of the hypothalamus in the rat. A similar intense staining was found in the internal zone of the median eminence and the neural lobe of the hypophysis. In all but the neurohypophysis, the fluorescence was more intense than that obtained with an Ang II antibody (C.H. Block, unpublished observations). In parallel studies, Chappell et al\textsuperscript{20} determined the relative proportions of angiotensin peptides in the rat brain by the technique of HPLC combined with radioimmunoassay (RIA). The elution of angiotensin peptides into clearly separated regions of the chromatogram permitted a quantitative analysis of Ang-(1-7), Ang II, and Ang I with three separate antisera in HPLC-coupled RIAs.\textsuperscript{18} Additionally, each antibody recognized a different epitope in the angiotensin molecules. We also determined that the Ang-(1-7) antibody is directed to the C-terminal sequence of Ang-(1-7) because it also recognized Ang-(2-7) and Ang-(3-7). The specificity of the Ang-(1-7) antisera was verified by showing that the antibody had less than 0.05% cross-reactivity with Ang II, Ang I, and their related fragments. Moreover, the Ang-(1-7) antibody showed less than 0.001% cross-reactivity with other neuropeptides containing proline at the C-terminus. Armed with these tools, we measured the content of angiotensin peptides in several regions of the rat brain. In the hypothalamus, we found that Ang-(1-7), Ang II, and Ang I existed in similar amounts (Figure 3). Ang II and Ang-(1-7) amounted to 81% and 76% of the concentration of Ang I, respectively. Additionally, the chromatographic profile from the hypothalamus showed the presence of Ang-(2-10), Ang-(3-10), Ang-(3-8), and Ang-(3-7). These fragments existed in concentrations averaging less than one third of the congeners from which they were derived. A similar concentration profile was found in the medulla oblongata and in samples of tissue obtained from the amygdala.\textsuperscript{20} In these other two regions of the rat brain, however, levels of Ang-(1-7) exceeded those recorded for Ang I. In contrast, HPLC fractions obtained from extracts of either the cerebral cortex or the cerebellum contained no immunoreactive angiotensin peptides. Current studies by this laboratory further suggest that Ang-(1-7) is not restricted to the neu-
rational microenvironment of the brain. In the anesthetized dog, measures of angiotensin peptides by HPLC coupled with RIA detected the presence of Ang-(1-7) in blood from the aorta, the coronary sinus, and the right atrium. 21

We are not the first to report the existence of amino terminal fragments of Ang II in organ systems. Production of Ang-(1-7) from Ang II was first shown by Yang et al 22 in swine kidney and human urine. Similar findings were obtained by Regoli et al 23 in vascular smooth muscle and by Allard et al 24 in cultured mouse spinal cord cells. Tonnaer et al 25 also reported the generation of an unidentified peptide resembling Ang-(1-7) in preparations of synaptic membranes from the rat brain. Urata et al 26 also found Ang-(1-7) in lysosomes of the rat adrenal cortex. These investigators, however, suggested that the internalization of Ang-(1-7) in lysosomes is consistent with an intracellular pathway for the degradation of Ang II by a process of receptor-mediated endocytosis. We believe that this conclusion is not in keeping with alternative interpretations regarding intracellular mechanisms for the processing of bioactive neuropeptides. 27

The observation that Ang-(1-7) is produced directly from Ang I led us to evaluate the putative nature of the enzymes that might participate in the endogenous generation of this heptapeptide. Enzymes capable of cleaving the Pro-Phe bond of Ang I or Ang II include prolyl endopeptidase (EC 21.34), 28,29 enkephalinase (neutral endopeptidase 24.11), 30 angiotensinase C, 22 and membrane-bound proline endopeptidase. 31,32 Because prolyl endopeptidase is widely distributed in the brain, it has been implicated in the inactivation of hypophyseal peptides such as thyrotropin. 29 An insight into the possible mechanisms that account for the generation of Ang-(1-7) is derived from studies with enzymatic inhibitors, especially by the use of N-benzylxoxycarbonyl-prolyl-prolinol (Z-PP), which is a specific inhibitor of prolyl endopeptidase. 33 Welch et al 34 found that addition of Z-PP (1 μM) to canine homogenates of the hypothalamus caused blockade of the formation of Ang-(1-7) from labeled Ang I by about 60–80%. Recently, Chappell and colleagues 35 from our laboratory showed that other endopeptidases might contribute to the production of Ang-(1-7) in the NG-108 neuronal cell line. In these studies, Z-PP inhibited the production of Ang-(1-7) from labeled Ang I by approximately 40%. Addition of difluorophosphate (DFP) caused no further inhibition of Ang-(1-7) production. In contrast, coinubcation of the neuronal cell line with p-chloromercuribenzenesulphonic acid and labeled Ang I resulted in a greater than 90% reduction in Ang-(1-7) production. 36 These data further illustrate the potential diversity of the enzymatic pathways that contribute to the generation of angiotensin peptides in target tissues.

**Actions of Angiotensin-(1-7) in the Brain**

For many years there has been evidence suggesting that the heptapeptide Ang-(1-7) had no vasactive or dipsogenic properties. 9,22,36 These data led to the assumption that Ang-(1-7) was an inactive product of Ang II catabolism. When Schiavone et al 13 compared the agonistic activities of Ang-(1-7) and Ang II as vasopressin (AVP) secretagogues, this idea was refuted. The study was done in a functional unit of the rat hypothalamus (the hypothalamic-neurohypophyseal explant system [HNS]), in isolation from the multiple influences inherent in experiments in vivo. In the perfused HNS explants we found that the N-terminal heptapeptide Ang-(1-7) was as potent as Ang II in stimulating AVP secretion. 13 These new studies provided the first evidence that a fragment of Ang II lacking an amino acid in position 8 is biologically active.

We have further probed whether other receptors recognize angiotensin peptides lacking a residue in the ultimate position of Ang II. The demonstration that Ang II inhibits baroreceptor transmission within the vagal-solitary complex that contains specific high-affinity Ang II binding sites suggested that we compare the actions of Ang II with those of Ang-(1-7) in neuronal pathways of the dorsal medulla. In experiments with rats, Campagnole-Santos et al 17 found that the microinjection of small amounts of Ang-(1-7) into either the medial region of the nucleus tractus solitarii (NTS) or the dorsal motor nucleus of the vagus nerve produced a monophasic depressor response. The hemodynamic effects of Ang-(1-7) were comparable with those obtained with injections of equivalent amounts of Ang II into the same brain areas. 37 Moreover, Barnes et al 38 showed that Ang-(1-7) has direct actions on neuronal systems. In these important experiments, application of Ang-(1-7) onto perfused slices of the canine dorsal medulla oblongata increased the discharge rate of neurons in the NTS and the dorsal motor nuclei of the vagus. 38 Again the neuronal response was similar to that obtained with Ang II. Therefore, these new data pose important questions regarding the potential diversity of the neuronal receptors that convey functional signals from angiotensin peptides in the brain.

**New Concepts of Angiotensin Function**

From studies of Ang II antagonists, 9 it has been concluded that replacement of the aromatic amino acid in position 8 with other aliphatic amino acids gives rise to antagonist compounds that have a high affinity for Ang II receptors. Because Ang II stimulates thirst, raises blood pressure, and causes secretion of hypophyseal hormones, 29 it has been assumed that the octapeptide is the principal or even the sole effector substance of the brain RAS. Our data suggest, however, that some of the varied actions of the RAS in the brain might be caused by other congeners of Ang II.
The discovery of an angiotensin peptide that is as potent as Ang II in stimulating AVP secretion but has no direct pressor or dipsogenic properties raises the provocative possibility that the production of angiotensin peptides with selective biological properties might be influenced by the enzymatic composition within the tissue microenvironment. Although our research is at an early stage, it is our thesis that the multiple actions of Ang II within the neuronal microenvironment of the brain are conveyed by the direct production of more selective N- and C-terminal fragments derived from Aogen, Ang I, or both. It has long been evident that the processing and metabolism of angiotensin peptides might involve alternate mechanisms. Although angiotensin converting enzyme plays a crucial role in the production of Ang II, other peptidases can also play a role. Thus, production of angiotensin peptides in the cellular microenvironment of cardiovascular tissues can be regulated by a variety of biotransformation steps that result from the specific availability of enzymes in these organ systems. According to this new concept, cellular receptors might discriminate between signals generated by either the circulating or local tissue RAS on the basis of the primary structure of the angiotensin peptide produced endogenously (Figure 4). These ideas provide a plausible explanation for the existence of separate enzymatic pathways that produce bioactive angiotensin peptides.

There are other reasons to further investigate this new concept. Ang I is a linear peptide having no repeating amino acids. According to Ryan, Ang I has 54 possible combinations of lower homologues. If we consider potential products from end-group modifications of Ang II only, the number of possible fragments is reduced to no less than eight additional homologues for each end modification at either the C- or N-terminal end of the molecule. Previous studies suggested that the complete unmodified structure of the C-terminal is required for the biological activity of Ang II. This is, apparently, not a valid conclusion. Ang-(1-7) lacks an amino acid residue at position 8, but in the brain, Ang-(1-7) is a potent secretagogue and excitatory transmitter. Furthermore, our studies suggest that the activities of this heptapeptide are comparable with those of Ang II for some but not all of the diverse actions that Ang II possesses. Ang-(1-7) differs from Ang II in that it lacks both direct pressor and dipsogenic effects. Thus, the problem with previous concepts regarding mechanisms for the expression of biologically active angiotensin peptides is that conclusions were derived solely from measures of peripheral effector responses. The data described here suggest that N-terminal fragments are biologically relevant.
active products of the RAS and might participate in conveying the specific signals that are shared by Ang II. By extension of this thesis, the angiotensin peptide family might eventually challenge the opiate and tachykinin families in both variety and complexity. Further investigation of the putative functions of Ang-(1–7) will provide additional insights into the selective functions that the proposed family of angiotensin peptides accomplishes in the control of blood pressure by the brain and, possibly, other organ systems.

References


KEY WORDS • angiotensin • angiotensin II • blood pressure • converting enzyme • essential hypertension • sympathetic nervous system • renin-angiotensin system • vasomotor centers • vasopressin • peptides
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