Conversion of brain angiotensin II to angiotensin III is critical for pressor response in rats

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Submitted 5 June 2002; accepted in final form 10 November 2002

Wright, John W., Elizabeth Tamura-Myers, Wendy L. Wilson, Bernard P. Roques, Catherine Llorens-Cortes, Robert C. Speth, and Joseph W. Harding. Conversion of brain angiotensin II to angiotensin III is critical for pressor response in rats. Am J Physiol Regul Integr Comp Physiol 284: R725–R733, 2003. First published November 14, 2002; 10.1152/ajpregu.00326.2002.—The present investigation measured the relative pressor potencies of intracerebroventricularly infused ANG II, ANG III, and the metabolically resistant analogs D-Asp1ANG II and D-Arg1ANG III in alert freely moving rats. The stability of these analogs was further facilitated by pretreatment with the specific aminopeptidase A inhibitor EC33 or the aminopeptidase N inhibitor PC18. The results indicate that the maximum elevations in mean arterial pressure (MAP) were very similar for each of these compounds across the dose range 1, 10, and 100 pmol/min during a 5-min infusion period. However, D-Asp1ANG II revealed significantly extended durations of pressor effects before return to base level MAP. Pretreatment intracerebroventricularly with EC33 blocked the pressor activity induced by the subsequent infusion of D-Asp1ANG II, whereas EC33 had no effect on the pressor response to subsequent infusion of D-Arg1ANG III. In contrast, pretreatment infusion with PC18 extended the duration of the D-Asp1ANG II pressor effect by about two to three times and the duration of D-Arg1ANG III's effect by ~10 to 15 times. Pretreatment with the specific AT1 receptor antagonist losartan blocked the pressor responses induced by the subsequent infusion of both analogs indicating that they act via the AT1 receptor subtype. These results suggest that the brain AT1 receptor may be designed to preferentially respond to ANG III, and ANG III's importance as a centrally active ligand has been underestimated.

THE BRAIN RENIN-ANGIOTENSIN system (RAS) appears to be separate from the peripheral system, complete with the necessary precursors and peptidases required for synthesis and degradation of active angiotensin li-
gands (for review, see Ref. 10). Central injections of octapeptide ANG II and heptapeptide ANG III elicit pronounced pressor and drinking responses, sodium appetite, the release of vasopressin and adrenocortico-
trophic hormone, and sexual behaviors with concomi-
tant increases in the release of related hormones. Two angiotensin receptors have been identified and are now referred to as the AT1 and AT2 subtypes (7, 11, 44, 47). Both the AT1 (23, 24, 33) and AT2 receptor subtypes (26, 32) have been cloned and sequenced. In rodents, the AT1 receptor subtype appears in two forms, AT1A and AT1B; both are G protein coupled (14, 23, 38). The AT1A subtype mediates the classic functions of the brain RAS, including cardiovascular control, body wa-
ter balance, cyclicity of reproductive hormones, and vasopressin and oxytocin release (for review, see Refs. 2, 3, 17, 27, 28, 37, 43, 46). Recently, the use of gene targeting has suggested that the AT1B subtype is responsible for the majority of ANG II-induced drinking in mice (8, 9). The AT2 receptor subtype also appears to be G protein coupled (26, 32) and is maximally activated by ANG III, although it also binds ANG II (for review, see Ref. 49). This subtype mediates the pro-
cesses of apoptosis, tissue regeneration, and wound healing (12, 19, 45). It is now generally agreed that conversion of brain ANG II to ANG III is primarily accomplished by aminopeptidase A (APA; EC 3.4.11.7, glutamyl aminopeptidase A, or A-like activity), whereas conversion of ANG III to the hexapeptide ANG IV is dependent on aminopeptidase N (APN; EC 3.4.11.2, membrane alanyl aminopeptidase) (for review, see Refs. 49, 55).

It has been assumed that the AT1 receptor subtype is maximally activated by ANG II. However, over the past 20+ years, evidence has accumulated in support of ANG III as the primary ligand at this brain receptor

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subtype. Several lines of evidence support this hypothesis: 1) intracerebroventricular infusion of ANG II and ANG III is reasonably equipotent regarding pressor and drinking responses (51), 2) ANG III is more potent than ANG II when iontophoretically applied to hypothalamic paraventricular or suprachiasmatic organ neurons (15, 20), 3) intracerebroventricular infusion of antiserum against APA significantly reduced pressor and drinking responses induced by intracerebroventricular infusion of ANG II but failed to influence ANG III-induced drinking and pressor responses (39), and 4) specific APA inhibitor, 3-amino-4-thio-butyl-sulfonate, EC33 (6), increased the half-life of ANG II by 2.6-fold in hypothalamic tissue extracts and blocked the conversion of ANG II to ANG III (55). Intracerebroventricular pretreatment with EC33 blocked the subsequent pressor responses to intracerebroventricularly infused ANG II (35); 5) intracerebroventricular application of the specific APN inhibitor, 2-amino-4-methylsulfonyl butane thiol, PC18 (18), stimulated vasopressin release by increasing the half-life of intracerebroventricularly infused ANG III (34). These ANG III responses could be blocked by pretreatment with the AT1 receptor subtype antagonist losartan (35); 6) the major form of angiotensin released within the paraventricular nucleus following stimulation by veratradine or water deprivation was determined to be ANG III (21). 7) The minimum requirements for occupancy and activation of the AT1 receptor subtype include a heptapeptide cluster group consisting of Arg-R1-Tyr-R2-His-R3-Phe (29–31). Taken together, these findings point to an important role for ANG III in the brain RAS.

The present investigation extends recently published reports using EC33 and PC18 as specific inhibitors of APA and APN, respectively (for review, see Ref. 36), by employing the metabolically resistant analogs D-Asp1ANG II and D-Arg1ANG III (53), coupled with EC33 and PC18 to determine 1) whether EC33-induced inhibition of conversion of intracerebroventricularly infused D-Asp1ANG II to ANG III diminished the magnitude of its pressor effect, 2) whether PC18-induced inhibition of conversion of intracerebroventricularly infused D-Arg1ANG III to ANG IV prolonged its pressor effect, and 3) if the pressor responses induced by D-Asp1ANG II and D-Arg1ANG III could be blocked by the AT1 receptor antagonist losartan. The results indicate that the intracerebroventricular infusion of EC33 blocked the pressor activity induced by the subsequent intracerebroventricular infusion of D-Asp1ANG II, whereas the intracerebroventricular application of PC18 enhanced the pressor response induced by the intracerebroventricular infusion of D-Asp1ANG II and especially D-Arg1ANG III. Intracerebroventricular pretreatment with losartan blocked the pressor responses produced by both analogs, suggesting that each acts via the AT1 receptor subtype.

METHODS

The research conducted in this investigation conformed with the “Guiding Principles For Research Involving Animals and Human Beings” by the American Physiological Society.
In a previous paper (53), in vitro half-lives of the analogs D-Asp1ANG II and D-Arg1ANG III were determined, along with those of native ANG II and ANG III, by labeling them with 125I and incubating each ligand in brain homogenate at 22°C for 2 h. These half-lives suggested that the replacement of L-Asp with D-Asp at position 1 of the ANG II molecule significantly increased resistance to metabolism compared with native ANG II. Similarly, the replacement of L-Arg with D-Arg at position 1 of the ANG III molecule increased resistance compared with native ANG III. Thus, the utilization of these metabolically resistant ligands, in combination with inhibition of the specific enzymes responsible for their conversion, was anticipated to significantly reduce their rates of in vivo conversion. In turn, these reductions in conversion rates provided the opportunity to determine whether the AT1 receptor subtype is maximally activated by an ANG II analog, ANG III analog, or both ligands.

**Experiment 2: Pretreatment With EC33 or PC18 Followed by D-Asp1ANG II or D-Arg1ANG III**

Six additional groups of rats (8 each) were used. Two of these groups were used to test the influence of EC33 on the pressor responses induced by intracerebroventricularly infused D-Asp1ANG II or D-Arg1ANG III, respectively. This was accomplished by intracerebroventricularly pretreating members of the first group with EC33 (20 μg-2 μl aCSF-1 min⁻¹ for 5 min, a total of 100 μg over 5 min) followed 5 min later by D-Asp1ANG II (0, 1, 10, and 100 pmol/min for 5 min). The second group was also pretreated with EC33 followed by D-Arg1ANG III (0, 1, 10, and 100 pmol/min for 5 min). Groups 3 and 4 were similarly treated; however, they were pretreated with aCSF (2 μl/min for 5 min) followed 5 min later with D-Asp1ANG II or D-Arg1ANG III, respectively. Once again, a counterbalanced design was used in which four of the rats from each group received the above treatments in an ascending order and the other four rats in a descending order. Each dose of peptide was tested following a minimum of 24-h recovery.

Groups 5 and 6 were used to test the influence of pretreatment with PC18 on the pressor responses induced by intracerebroventricularly infused D-Asp1ANG II and D-Arg1ANG III. The doses of this inhibitor and peptides were as described above. These doses of EC33 and PC18 are equivalent with those previously employed (35).

**Experiment 3: Pretreatment With Losartan Followed by D-Asp1ANG II or D-Arg1ANG III**

Finally, two additional groups of rats (8 each) were used to determine whether pretreatment with losartan blocked the subsequent pressor response to D-Asp1ANG II and D-Arg1ANG III. Rats of the first group were intracerebroventricularly pretreated with aCSF (2 μl/min for 5 min) or losartan [20 nmol (~10 μg)-2 μl aCSF-1 min⁻¹ for 5 min], followed 5 min later by D-Asp1ANG II (100 pmol-2 μl aCSF-1 min⁻¹ for 5 min). Four animals received pretreatment with aCSF first, followed by losartan, separated by a minimum of 24 h, whereas the other four rats received pretreatment with losartan first and aCSF second. Members of the second group were treated equivalently; however, they received D-Arg1ANG III (100 pmol-2 μl aCSF-1 min⁻¹ for 5 min).

**Compounds**

The structure, molecular weight, peptide content, and source of each compound used in these experiments are provided in Table 1. ANG II and ANG III were purchased from Peninsula Laboratories (catalog #7002 and 7003, respectively, Belmont, CA). The analogs D-Asp1ANG II and D-Arg1ANG III were synthesized by J. W. Harding using a Vega amino acid synthesizer (Coupler 250, Dupont, Wilmington, DE), purified by reverse-phase HPLC, followed by amino acid analysis. The purity by weight of the compounds was determined to be 85 and 82% for ANG II and ANG III, respectively, as supplied by Peninsula, and 72 and 68% for D-Asp1ANG II and D-Arg1ANG III, respectively, as measured by HPLC and mass spectroscopy. Peptide purity ranged from 98 to 100% while acetate represented the major contributor of L-Asp with D-Asp at position 1 of the ANG II molecule significantly increased resistance compared with native ANG III. Thus, the utilization of these metabolically resistant ligands, in combination with inhibition of the specific enzymes responsible for their conversion, was anticipated to significantly reduce their rates of in vivo conversion. In turn, these reductions in conversion rates provided the opportunity to determine whether the AT1 receptor subtype is maximally activated by an ANG II analog, ANG III analog, or both ligands.

**Statistical Analyses**

**Experiment 1.** The data set from the first experiment concerning maximum MAP induced by each compound (ANG II, ANG III, D-Asp1ANG II, and D-Arg1ANG III) at each dose (0, 1, 10, and 100 pmol) was analyzed by a 4 (ligands) × 4 (doses) ANOVA, with repeated measures on the second factor. The magnitude of each pressor response was calculated by subtracting the corresponding MAP base level from the maximum pressor change induced by each treatment. The duration of each dose was similarly evaluated and was defined as the time from termination of infusion until MAP returned to base level (i.e., nonstatistically different from base level).

**Experiment 2.** The data set from the second experiment concerned with the influence of pretreatment with EC33, PC18, or aCSF on subsequent pressor responses induced by D-Asp1ANG II was analyzed by a 3 (pretreatment condi-

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**Table 1. Structure, molecular weight, peptide content, and source of each peptide and inhibitor examined**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Molecular Weight</th>
<th>Content, %wt</th>
<th>Source</th>
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</thead>
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<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
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<td>85</td>
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<tr>
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<td>931</td>
<td>82</td>
<td>Peninsula</td>
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<td>72</td>
<td>Harding</td>
</tr>
<tr>
<td>D-Arg1ANG III</td>
<td>D-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>931</td>
<td>68</td>
<td>Harding</td>
</tr>
<tr>
<td>EC33</td>
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<td>243.5</td>
<td>98</td>
<td>Roques</td>
</tr>
<tr>
<td>PC18</td>
<td>2-amino-4-methylsulfonyl butane thiol</td>
<td>187.5</td>
<td>98</td>
<td>Roques</td>
</tr>
<tr>
<td>Losartan</td>
<td>2-buty1-4-chloro-1-f-(o-1H-tetrazol-5-phenylbenzyl)-imidazole-5-methanol monopotassium salt</td>
<td>481</td>
<td>98</td>
<td>Dupont-Merck</td>
</tr>
</tbody>
</table>

Contents adapted from Refs. 49, 50, 53.
tion) × 4 (doses) ANOVA, with repeated measures on the second factor. The data set resulting from pretreatment with EC33, PC18, or aCSF followed by D-Arg1ANG III was also analyzed using a 3 (pretreatment condition) × 4 (doses) ANOVA, again with repeated measures on the second factor. The durations of the pressor responses produced by each dose of D-Asp1ANG II following each pretreatment condition EC33, PC18, or aCSF were evaluated by a 3 (pretreatment condition) × 4 (doses) ANOVA, with repeated measures on the second factor. The data sets concerned with these same pretreatment conditions followed by the doses of D-Arg1ANG III were similarly analyzed.

Experiment 3. Finally, the data sets from the third experiment concerned with maximum changes in MAP and duration involving pretreatment with losartan or aCSF followed by D-Arg1ANG II or D-Arg1ANG III were each analyzed by a 2 (pretreatment condition) × 2 (peptide) ANOVA. Similar analyses were applied to these dependent measures resulting from pretreatment with losartan or aCSF. Similar analyses were applied to these dependent measures resulting from D-Arg1ANG III infusion. The level of significance for all tests was set at 0.05.

RESULTS

Experiment 1: Dose-Response Curves

There were no differences among the groups infused with ANG II, ANG III, D-Asp1ANG II, and D-Arg1ANG III with respect to base level MAP before treatment \( [F(3,28) = 1.49, P > 0.10; \text{means } \pm \text{SE} = 123.0 \pm 1.5, 122.3 \pm 2.0, 120.2 \pm 1.6, 120.4 \pm 1.6 \text{ mmHg, respectively}] \). Figure 1, A and B, presents the mean ± SE changes in MAP from base level due to the influence of each dose of ANG II, ANG III, D-Asp1ANG II, and D-Arg1ANG III. There were no differences among these compounds with regard to maximum MAP collapsing across doses \( [F(3,28) = 0.96, P > 0.10] \). However, there was an expected dose effect \( [F(3,84) = 2.77, P < 0.05] \). Post hoc analyses indicated that each increment in dose produced significantly greater pressor responses than the previous dose. There was no significant ligands × doses interaction.

Figure 1C presents the mean ± SE durations required for the pressor effects induced by each ligand at each dose to return to a level not statistically different from base level. There were differences among the four compounds collapsing across doses \( [F(3,28) = 13.28, P < 0.0001] \). Specifically, D-Asp1ANG II revealed the longest durations, followed by ANG II, D-Arg1ANG III, and ANG III. As expected, there was a significant dose-response relationship \( [F(3,84) = 45.60, P < 0.0001] \), with the 100-pmol dose revealing the longest durations, followed by the 10- and 1-pmol doses. All doses produced significantly longer durations than those following aCSF infusion. There was also a significant compound × dose interaction \( [F(9,84) = 2.41, P < 0.02] \). Each dose of D-Asp1ANG II revealed significantly longer durations of effect than comparable doses of the other compounds. In turn, the 10- and 100-pmol/min doses of ANG II displayed longer durations than comparable doses of D-Arg1ANG III or ANG III. D-Arg1ANG III revealed a longer duration than ANG III at the 1- and 100-pmol doses.

On the basis of the increased duration of effects evidenced by D-Asp1ANG II over ANG II, and D-Arg1ANG III over ANG III, these two compounds were selected for further investigation.

Experiment 2: Pretreatment With EC33 or PC18 Followed by D-Asp1ANG II or D-Arg1ANG III

There were no differences in base level MAP among the groups pretreated with aCSF, EC33, or PC18 followed by D-Asp1ANG II or D-Arg1ANG III \( [F(5,42) = 0.49, P > 0.10] \). Base level MAP for those groups pretreated with aCSF, EC33, or PC18 followed by D-Asp1ANG II was mean ± SE = 123.1 ± 2.1, 121.7 ± 2.1, and 124.4 ± 2.7 mmHg, respectively. Base level MAP for those groups pretreated with aCSF, EC33, or PC18 followed by D-Arg1ANG III was 123.6 ± 1.6, 122.2 ± 2.5, and 124.2 ± 1.3 mmHg, respectively. The influence of intracerebroventricular pretreatment with aCSF, EC33, or PC18 on MAP changes from base level induced by D-Asp1ANG II is presented in Fig. 2A.
response induced by the 100-pmol/min dose of D-Asp\(^1\)ANG II. Pretreatment with a higher dose of EC33 (30 \(\mu\)g-2 \(\mu\)l aCSF^{-1}\cdot \text{min}^{-1} for 5 min) was successful in blocking the subsequent pressor response induced by D-Asp\(^1\)ANG II at the 100-pmol data not shown).

Figure 2B represents the influence of pretreatment with aCSF, EC33, or PC18 on MAP changes from base level induced by the subsequent infusion of D-Arg\(^2\)ANG III. These results indicate that pretreatment with aCSF or EC33 resulted in similar patterns of MAP responses, whereas pretreatment with PC18 elevated and prolonged the MAP responses induced by D-Arg\(^2\)ANG III. Thus, there was a significant overall difference comparing pretreatment groups collapsed across doses \(F(2,21) = 239.41, P < 0.0001\). Post hoc analyses indicated that the group pretreated with PC18 was different from the other two groups. There was an expected dose effect \(F(3,63) = 69.58, P < 0.0001\). Post hoc analyses revealed that each increment in dose yielded a significantly greater MAP. Finally, the groups × doses effect was also significant \(F(6,63) = 8.32, P < 0.0001\). Post hoc analyses established that members of the group pretreated with EC33 revealed MAP levels not different from aCSF pretreatment at each dose of D-Arg\(^2\)ANG III. Members of the group pretreated with PC18 displayed significant elevations above those levels displayed by the groups pretreated with aCSF or EC33 at the 10- and 100-pmol doses.

Figure 2C presents the durations required for each analog-induced elevation in MAP to return to base level at each dose. Overall, the recovery times for D-Asp\(^1\)ANG II-induced pressor responses to return to base level were reduced by pretreatment with EC33. A different pattern emerged with recovery times for D-Arg\(^2\)ANG III in that pretreatment with PC18 greatly extended recovery times, especially at the 10- and 100-pmol doses, whereas EC33 had no effect on recovery times compared with aCSF pretreatment. Specifically, there was a significant group effect \(F(2,21) = 74.30, P < 0.0001\). Pretreatment with EC33 resulted in the greatest reduction in recovery times collapsing across doses, compared with aCSF pretreatment, whereas pretreatment with PC18 induced a significant increase in the duration required to recover following infusion with D-Asp\(^1\)ANG II. There was also an expected significant dose effect \(F(3,63) = 134.80, P < 0.0001\), with each increment in dose producing an increase in the duration required for recovery. Furthermore, the groups × doses interaction was significant \(F(6,63) = 30.24, P < 0.0001\). Pretreatment with EC33 significantly decreased recovery time at each dose of D-Asp\(^1\)ANG II. Once again, there was a significant group effect \(F(2,21) = 331.02, P < 0.0001\) regarding pretreatment with aCSF, EC33, or PC18 followed by D-Arg\(^2\)ANG III. Pretreatment with PC18 significantly extended the duration required for recovery compared with the other two groups that did not differ. There was a dose effect \(F(3,63) = 196.96, P < 0.0001\) with each increase in dose producing an increase in the duration of action. The groups × dose interaction was

These results indicate that pretreatment with EC33 greatly decreased the subsequent pressor effects induced by D-Asp\(^1\)ANG II compared with both aCSF and PC18 pretreatment. Specifically, there was a significant overall difference comparing the pressor effects induced by D-Asp\(^1\)ANG II following pretreatment with EC33, PC18, or aCSF collapsed across doses \(F(2,21) = 239.41, P < 0.0001\). Post hoc analyses indicated that those animals pretreated with EC33 revealed significantly suppressed MAP responses compared with members of the two groups pretreated with aCSF or PC18. There was an anticipated overall dose effect \(F(3,63) = 69.58, P < 0.0001\) with each successive dose yielding a greater increase in MAP. There was also a significant groups × doses effect \(F(6,63) = 8.32, P < 0.0001\). Post hoc analyses indicated that members of the group pretreated with EC33 revealed significantly lower maximum MAP levels at each dose of D-Asp\(^1\)ANG II compared with the two other groups that did not differ.

As shown in Fig. 2A, right, pretreatment with EC33 at the 20-\(\mu\)g dose failed to completely block the pressor...
also significant \( F(6,63) = 152.73, P < 0.0001 \). Pretreatment with PC18 significantly extended recovery times at the 10- and 100-pmol doses of d-Arg1ANG III.

**Experiment 3: Pretreatment With Losartan**

The influence of pretreatment with losartan or aCSF on subsequent MAP changes from base level induced by d-Asp1ANG II or d-Arg1ANG III is presented in Fig. 3A. These results indicate that pretreatment with losartan blocked the pressor effects of both d-Asp1ANG II and d-Arg1ANG III. Specifically, there was a difference comparing pretreatment with losartan or aCSF across the ligands \( F(1,14) = 115.09, P < 0.0001 \). Post hoc analyses using a priori established dependent t-tests indicated significant differences comparing the maximum MAP induced by d-Asp1ANG II following pretreatment with losartan or aCSF (\( t_7 = 6.61, P < 0.001 \)). A similar pattern was observed for d-Arg1ANG III (\( t_7 = 9.63, P < 0.001 \)).

The influence of pretreatment with losartan or aCSF on the subsequent durations of the pressor effects induced by d-Asp1ANG II or d-Arg1ANG III is presented in Fig. 3B. Losartan significantly reduced the duration of the pressor effect induced by each ligand. Specifically, ANOVA indicated no group or interaction effects, but there was a difference comparing pretreatment with losartan or aCSF across ligands \( F(1,14) = 62.75, P < 0.0001 \). Post hoc analyses revealed significant differences comparing the durations of effects induced by d-Asp1ANG II or d-Arg1ANG III following losartan or aCSF (\( t_7 = 9.48 \) and 10.76, respectively, \( P < 0.001 \)).

**DISCUSSION**

Some years ago our laboratory (50–52) and others (16) investigated the possibility that ANG III is the active ligand of the brain RAS. At that time, we used aminopeptidase inhibitors such as bestatin and aminopeptidase P inhibitors have been synthesized (6, 18). Intracerebroventricular injection of EC33 has been shown to block subsequent intracerebroventricular ANG II-induced pressor responding, whereas the intracerebroventricular injection of PC18 increased blood pressure presumably by increasing the half-life of endogenously synthesized ANG II and ANG III, thus extending their half-lives. We also synthesized ANG II and ANG III analogs in an effort to increase resistance to degradation (53). Although these efforts resulted in the suggestion that ANG III is equipotent with ANG II as a centrally active ligand involved in drinking and blood pressure regulation, the results were inconclusive primarily due to the nonspecificity of the available inhibitors. Recently, very specific and selective APA and APN inhibitors have been synthesized (6, 18). Intracerebroventricular injection of EC33 has been shown to block subsequent intracerebroventricular ANG II-induced pressor responding, whereas the intracerebroventricular injection of PC18 increased blood pressure presumably by increasing the half-life of endogenously formed ANG III (35). The authors concluded that conversion of ANG II to ANG III may be a prerequisite to angiotensin-induced pressor response (34–36) and vasopressin release (34, 55). Song and colleagues (39) came to the same conclusion concerning both blood pressure and dipsogenic responses using an antibody against APA.

These previous results are very important with regard to identifying the active ligand of the brain RAS, however, several procedural shortcomings became apparent. The shortcomings included the following. 1) A major portion of this previous work with EC33 and PC18 was conducted using anesthetized rats, thus increasing the risk of dampened pressor responding. The present investigation used alert rats. 2) Bolus intracerebroventricular injections were used rather than intracerebroventricular infusions, as presently employed, that provide a more stable pressor response. 3) Native ANG II and ANG III were used as ligands. It has been previously reported that ANG II and ANG III
are rapidly metabolized in vivo (13, 22). Metabolically resistant ANG II and ANG III analogs were presently used. 4) Intracerebroventricular injection of the specific AT1 receptor antagonist losartan was previously found to block subsequent PC18-induced elevations in blood pressure. Losartan blockade of the pressor responses induced by ANG II and ANG III was not tested. 5) In addition, the emphasis of this previous work with EC33 and PC18 focused on the spontaneously hypertensive rat (SHR) model rather than normotensive rats, although Wistar-Kyoto (WKY) normotensive rats were also examined. The present investigation employed Sprague-Dawley normotensive rats, a strain frequently used in blood pressure investigations. Thus, the present joint investigation was designed to correct these deficiencies and extend previous findings.

Results from experiment 1 indicated reasonably comparable pressor responses induced by the intracerebroventricular infusion of native ANG II and ANG III with respect to maximum MAP achieved at each dose examined. However, at the highest dose of 100 pmol/min, the duration of the pressor response produced by ANG II was longer compared with ANG III. A similar pattern of responding was measured comparing pressor responses induced by the intracerebroventricular infusion of d-Asp1ANG II and d-Arg1ANG III. The duration advantage of d-Asp1ANG II over d-Arg1ANG III could be seen at each of the three doses used. This was somewhat surprising since the metabolic resistance offered by substitution of L-Arg with D-Arg at position 1 of the ANG III molecule was expected to increase the duration of pressor effect. We have no ready explanation for these results. However, at this point in the investigation, two potential explanations could be offered. 1) Native ANG II, and especially the metabolic resistant analog d-Asp1ANG II, may bind at the AT1 receptor, and then be converted to ANG III and continue to activate the AT1 receptor. Under these circumstances, prolonged response duration could be expected. The intensity of this phenomenon would be amplified with increasing doses that yield a larger pool of ligand to be converted to ANG III. 2) Both ANG II and d-Asp1ANG II must be converted to ANG III in order for activation of the AT1 receptor to occur. The more resistant the ligand to conversion, the longer is the anticipated duration but the lower the maximum elevation of the pressor effect. Under both scenarios, it is assumed that the primary metabolic pathway for ANG II and ANG III ligands is via NH2-terminal degradation (1, 55).

The results of experiment 2 helped clarify the above issue in that pretreatment with EC33, but not PC18, completely blocked the subsequent d-Asp1ANG II-induced pressor response at the lower doses and blunted the response at the highest dose. A higher dose of EC33 was successful in completely blocking the 100-pmol/min dose of d-Asp1ANG II as well. PC18 failed to influence d-Asp1ANG II-induced pressor responding presumably because it acts on APN rather than APA, thus permitting the formation of ANG III (55). In contrast, pretreatment with PC18 significantly increased the duration of the d-Arg1ANG III-induced pressor response especially at the 10- and 100-pmol/min doses, whereas pretreatment with EC33 had very little influence on subsequent d-Arg1ANG III-induced pressor responding. The pressor durations provoked by the combination of PC18 followed by d-Arg1ANG III infusion are the longest that we observed at any dose of ligand. These results are consistent with the notion that APN is responsible for cleaving arginine from the ANG III molecule, not APA (34, 55). Thus, the application of a specific APA inhibitor would not be expected to alter the potency of an exogenously applied ANG III analog. In contrast, inhibition of APN would be expected to prolong the half-life of an ANG III analog and extend the duration of its effect. However, a similar prediction holds for ANG II and ANG II analogs. The present investigation extended previous observations that these compounds appear to act via conversion to ANG III. Although we did measure a significant increase in duration to ~2.5 h with the infusion of PC18 followed by d-Asp1ANG II, we expected to see a duration approximately equivalent with PC18 followed by d-Arg1ANG III. This prediction rests on the assumption that ANG II and ANG II analogs are converted directly to ANG III and no other metabolic pathways exist. Previous work from our laboratory indicates that ANG II can be converted to ANG III (2-7) and shorter fragments (1). Thus, although the primary step in the metabolism of ANG II is the formation of ANG III, other fragments are also formed from ANG II.

In experiment 3, pretreatment with the specific AT1 receptor antagonist losartan significantly decreased subsequent d-Asp1ANG II- and d-Arg1ANG III-induced pressor responses. These results suggest that both analogs act at the AT1 receptor subtype. Our laboratory has been conducting binding studies comparing native ANG II and ANG III with d-Asp1ANG II and d-Arg1ANG III, respectively, in brain and liver. Preliminary results indicate that the ligand being stripped from the AT1 receptor is ANG III, or d-Arg1ANG III, not ANG II or d-Asp1ANG II. Although preliminary, these findings support the notion that ANG II must be converted to ANG III to bind at this receptor subtype. Taken together, these results confirm, and significantly extend, previous findings using EC33 and PC18 (35, 36). In agreement with this earlier work, pretreatment with EC33 prevented any pressor response to the infusion of ANG II (presently an ANG II analog), whereas EC33 had no effect on ANG III-induced (presently an ANG III analog) pressor responding. Pretreatment with PC18 significantly potentiated the pressor effect and duration of ANG III (presently an ANG III analog). The present investigation extended previous work by testing whether pretreatment with PC18 also potentiated the pressor effect of an ANG II analog, which it did not, although it did significantly extend the duration of the pressor effect. And it was presently demonstrated that losartan blocked the pressor response induced by both ANG II and ANG III analogs. We also measured significantly greater pressor responses to reasonably equivalent doses of native ANG.
II and ANG III in alert rats compared with those previously reported using anesthetized SHR and WKY rats (35). It should be noted that alert SHRs were previously used to compare the hypotensive effects of intracerebroventricular vs. intravenous injections of EC33. All other conclusions were based on results taken from anesthetized rats. The presently noted increased responsiveness is illustrated by the observation that pretreatment with PC18 extended the time required for the pressor response resulting from the infusion of D-Asp1ANG II to recover to \( \sim 2.5 \) h and the time to recover from D-Arg1ANG III infusion to more than 7 h. This compares with durations of \( \sim 15 \) min for recovery from ANG III in anesthetized SHR and WKY rats (35).

The present results, coupled with recent reports (5, 34–36, 39, 55), support the hypothesis that the active ligand in the brain RAS is ANG III. There is general agreement that the AT1 subtype mediates the classic functions of the brain RAS (for review, see Refs. 10, 48, 49, 54). However, debate continues over the important issue of whether ANG II and/or ANG III is the endogenous ligand at this receptor subtype. The present results support ANG III. Even so, at least two questions remain. Why is the brain RAS designed such that ANG II must be converted to ANG III? And, if it is necessary for ANG II to be converted to ANG III to activate the AT1 receptor, why are there so many reports of ANG II binding at the AT1 receptor subtype? Although there is presently no ready explanation, at least four possibilities are worth considering. 1) Rapid conversion of ANG II to ANG III could be occurring locally at the AT1 receptor. Such a conversion would not necessarily be detected by standard in vitro radioreceptor binding assays or autoradiography techniques. Thus, the published AT1 receptor affinities for ANG II and ANG III may be misleading. Our laboratory is presently attempting to address this issue. 2) There may be an as yet undiscovered angiotensin receptor with high affinity for ANG III that mediates blood pressure regulation along with the AT1 receptor subtype. This may explain why losartan failed to completely block the increase in blood pressure induced by PC18 alone in SHR and WKY rats (35). However, it should be noted that AT1A receptor-deficient mice do not respond to intracerebroventricular injections of ANG II (8, 9). 3) Recent studies, coupled with the present results, suggest that ANG II may not be an agonist in the brain and thus may be an antagonist (40). 4) Perhaps the brain RAS is designed to provide at least two active ligands, ANG III and ANG IV (49), and both ANG I and ANG II are precursor molecules. Consistent with this notion is the observation that intracerebroventricular infusion of EC33 alone induced a significant reduction in blood pressure in SHR (35, 36) presumably by inhibiting the formation of ANG III. Resolution of these issues must await further investigation.

### Perspectives

Debate continues concerning the identity of the active form(s) of angiotensin in the brain RAS. Results from the present investigation confirm and extend earlier reports indicating an important role for ANG III. Identification of the active ligand within the brain angiotensin system is of considerable clinical importance especially with regard to the development of antihypertensive compounds. The present finding that losartan significantly attenuated D-Arg1ANG III-induced pressor responses argues that ANG III acts at the AT1 subtype; thus, the utilization of losartan (Cozaar) as a specific AT1 receptor antagonist in the treatment of essential hypertension associated with activation of brain angiotensin receptors appears appropriate. However, the availability of a very specific inhibitor of APA may offer an independent or additional therapeutic approach in the control of essential hypertension.

We thank R. Day for excellent secretarial help in preparing this manuscript. We also thank E. S. Murphy for assistance with the preparation of the figures.

This research was supported by the Edward E. and Lucille I. Laing Endowment for Alzheimer’s Research, funds provided for medical and biological research by the State of Washington Initiative Measure No. 171, and Washington State University.

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