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

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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 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.

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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 subfornical organ neurons (15, 20), 3) intracerebroventricular infusion of antagonist of 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.

Normotensive male Sprague-Dawley rats (300–420 g, Taconic Laboratory Animals, Germantown, NY) were maintained in an American Association for Accreditation of Laboratory Animal Care approved vivarium on a 12:12-h light-dark cycle initiated at 0700. Food and water were available ad libitum except the night before surgery when food was removed from the cage. Each animal was anesthetized with ketamine and xylazine (100 and 5 mg·kg−1·im, respectively, Phoenix Scientific, St. Joseph, MO and Mobay, Shawnee, KS). Body temperature was maintained at 37 ± 0.5°C by the use of a homeothermic blanket equipped with a rectal thermistor probe (model D28, Harvard Apparatus, Holliston, MA). The right carotid artery was isolated and catheterized (PE-60, Clay Adams, Parsippany, NJ) to permit constant monitoring of mean arterial pressure (MAP) via a blood pressure analyzer (model PBA, Micro-Med, Louisville, KY). Each animal was also fitted with an intracerebroventricular guide cannula (PE-60) as previously described (41, 51) during the same surgical session. Buprenorphine hydrochloride analgesia was administered (0.3 mg/kg im, Reckitt & Colman Pharmaceuticals, Richmond, VA) immediately following surgery. After allowing a minimum of 48-h recovery from surgery, each animal was behaviorally tested in its home cage for the accuracy of the intracerebroventricular guide cannula placement. This was accomplished by placing a preloaded 26-gauge stainless steel injector into the alert animal’s guide cannula such that it extended 2–3.5 mm beyond the tip of the guide, thus penetrating the roof of the lateral ventricle. The injector was attached to a 10-μl Hamilton syringe (model 1701, Reno, NV) by a 30-cm length of PE-20 tubing that contained ANG II (100 pmol in a total volume of 2 μl artificial cerebrospinal fluid [aCSF]). If a drinking response was not elicited within 5 min following the injection of ANG II, the animal was replaced. (One animal was replaced during this investigation.)

Experiment 1: Dose-Response Curves

A minimum of 48 h following behavioral confirmation of correct intracerebroventricular cannula placement, each animal was placed in a round metal test chamber (21-cm diameter × 30-cm tall) where the catheter was attached to a pressure transducer allowing measurement of MAP. The animal was permitted to adapt to the test chamber for 15–20 min and then a stable 5-min base level was obtained. All reported blood pressure changes were those that deviated from this established 5-min MAP base level. The blood pressure analyzer was configured to sample MAP at 5-s intervals and then provide a mean of these values at 1-min intervals. For statistical analyses, these 1-min values were averaged over 5-min intervals.

Four groups of rats (8/group) were used to measure the MAP responses induced by the 5-min intracerebroventricular infusion of 0-, 1-, 10-, and 100-pmol/min doses of ANG II, ANG III, d-Asp1ANG II, or d-Arg1ANG III. Each compound was corrected for purity, prepared in sterile aCSF, aliquoted into silicon-coated glass culture tubes, and stored at −20°C. Fresh aliquots were used for each testing session. The compounds were delivered at a rate of 2 μl/min for 5 min (model 355, Sage Instruments, Cambridge, MA). The doses of each compound were counterbalanced within members of each group such that four rats received an ascending order of doses, whereas the other four animals received a descending order. Sufficient time was permitted between doses to recover base level blood pressure. When recovery required longer than an hour, the next dose was postponed until the following day.

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In a previous paper (53), in vitro half-lives of the analogs D-Asp\(^1\)ANG II and D-Arg\(^1\)ANG III were determined, along with those of native ANG II and ANG III, by labeling them with \(^{125}\)I 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 AT\(_1\) 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-Asp\(^1\)ANG II or D-Arg\(^1\)ANG 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-Asp\(^1\)ANG II or D-Arg\(^1\)ANG III, respectively. This was accomplished by intracerebroventricularly pretreating members of the first group with EC33 (20 mg·kg\(^{-1}\)·h\(^{-1}\) ± 1 mL aCSF \(\times 1\) min \(^{-1}\) for 5 min, a total of 100 \(\mu\)g over 5 min) followed 5 min later by D-Asp\(^1\)ANG II (0, 1, 10, and 100 pmol/min for 5 min). The second group was also pretreated with EC33 followed by D-Arg\(^1\)ANG 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 \(\mu\)L/min for 5 min) followed 5 min later with D-Asp\(^1\)ANG II or D-Arg\(^1\)ANG 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-Asp\(^1\)ANG II and D-Arg\(^1\)ANG 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-Asp\(^1\)ANG II or D-Arg\(^1\)ANG III**

Finally, two additional groups of rats (8 each) were used to determine whether pretreatment with losartan blocked the subsequent pressor response to D-Asp\(^1\)ANG II and D-Arg\(^1\)ANG III. Rats of the first group were intracerebroventricularly pretreated with aCSF (2 \(\mu\)L/min for 5 min) or losartan (20 nmol (~10 \(\mu\)g)·2 \(\mu\)L aCSF \(\times 1\) min \(^{-1}\) for 5 min), followed 5 min later by D-Asp\(^1\)ANG II (100 pmol·2 \(\mu\)L aCSF \(\times 1\) 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-Arg\(^1\)ANG III (100 pmol·2 \(\mu\)L aCSF \(\times 1\) 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-Asp\(^1\)ANG II and D-Arg\(^1\)ANG 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-Asp\(^1\)ANG II and D-Arg\(^1\)ANG III, respectively, as measured by HPLC and mass spectroscopy. Peptide purity ranged from 98 to 100% while acetate represented the major contributor to the decreased purity by weight. These peptides were adjusted for purity such that the doses reflected actual peptide delivered in mols. EC33 and PC18 were synthesized in the laboratory of B. P. Roques according to previously described procedures (6, 18), with purities determined to be 98%. Losartan was obtained as a gift from Dr. R. D. Smith, DuPont-Merck, Wilmington, DE.

**Statistical Analyses**

**Experiment 1.** The data set from the first experiment concerning maximum MAP induced by each compound (ANG II, ANG III, D-Asp\(^1\)ANG II, and D-Arg\(^1\)ANG 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-Asp\(^1\)ANG II was analyzed by a 3 (pretreatment condi-
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. 24.
did not differ. Infusion of each dose (1, 10, and 100 pmol/min) of angiotensin analog to evaluate the subsequent change in MAP induced by D-Arg1ANG D-Asp1ANG II compared with the two other groups that produced by D-Asp1ANG II compared with both aCSF and great reduction in recovery times collapsing across doses \[ F(2,21) = 239.41, P < 0.0001 \]. Post hoc analyses indicated that pretreatment 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 \( \times \) 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-Arg1ANG 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 2B represents the influence of pretreatment with aCSF, EC33, or PC18 on MAP changes from base level induced by the subsequent infusion of D-Arg1ANG 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-Arg1ANG III. Thus, there was a significant overall difference comparing pretreatment groups collapsed across doses \[ F(2,21) = 331.02, P < 0.0001 \]. Post hoc analyses established that members of the group pretreated with PC18 revealed MAP levels not different from 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-Arg1ANG II. There was also an unexpected 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 \( \times \) doses interaction was significant \[ F(6,63) = 30.24, P < 0.0001 \]. Pretreatment with EC33 significantly decreased recovery time at each dose of D-Arg1ANG 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-Arg1ANG 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 \( \times \) dose interaction was

![Figure 2](https://www.ajpregu.org/database/)

**Fig. 2.** A: change in MAP due to the intracerebroventricular infusion of D-Asp1ANG II (1, 10, and 100 pmol/min for 5 min) following an intracerebroventricular pretreatment infusion of aCSF (2 μL/min for 5 min), EC33 (20 μg·2 μL aCSF –1·min –1 for 5 min), or PC18 (20 μg·2 μL aCSF –1·min –1 for 5 min). B: above pretreatments were also used to evaluate the subsequent change in MAP induced by D-Arg1ANG III (100 pmol/min for 5 min). The pretreatment infusion and the infusion of each dose (1, 10, and 100 pmol/min) of angiotensin analog were separated by 5 min. C: durations required for the change in MAP produced by each treatment to return to base level. \#P < 0.05 level of significance; \*P < 0.01 level of significance.

These results indicate that pretreatment with EC33 greatly decreased the subsequent pressor effects induced by D-Asp1ANG II compared with both aCSF and PC18 pretreatment. Specifically, there was a significant overall difference comparing the pressor effects induced by D-Asp1ANG 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 \( \times \) 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-Asp1ANG II compared with the other two groups that did not differ.

As shown in Fig. 2A, right, pretreatment with EC33 at the 20-μg dose failed to completely block the pressor response induced by the 100-pmol/min dose of D-Asp1ANG II. Pretreatment with a higher dose of EC33 (30 μg·2 μL aCSF –1·min –1 for 5 min) was successful in blocking the subsequent pressor response induced by D-Asp1ANG II at the 100-pmol dose (data not shown).
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 \( \text{d-Arg}^1 \text{ANG II} \) and \( \text{d-Arg}^1 \text{ANG III} \).

**Experiment 3: Pretreatment With Losartan**

The influence of pretreatment with losartan or aCSF on subsequent MAP changes from base level induced by \( \text{d-Asp}^1 \text{ANG II} \) or \( \text{d-Arg}^1 \text{ANG III} \) is presented in Fig. 3A. These results indicate that pretreatment with losartan blocked the pressor effects of both \( \text{d-Asp}^1 \text{ANG II} \) and \( \text{d-Arg}^1 \text{ANG III} \). 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 \( \text{d-Asp}^1 \text{ANG II} \) following pretreatment with losartan or aCSF \( (t_7 = 6.61, P < 0.001) \). A similar pattern was observed for \( \text{d-Arg}^1 \text{ANG III} \) \( (t_7 = 9.63, P < 0.001) \).

The influence of pretreatment with losartan or aCSF on the subsequent durations of the pressor effects induced by \( \text{d-Asp}^1 \text{ANG II} \) or \( \text{d-Arg}^1 \text{ANG III} \) 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 \( \text{d-Asp}^1 \text{ANG II} \) or \( \text{d-Arg}^1 \text{ANG III} \) following losartan or aCSF \( (t_7 = 9.48 \text{ and } 10.76, \text{ respectively, } P < 0.001) \).

**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 amastatin and bestatin (25, 42) and the carboxypeptidase inhibitor Plummer’s (4) to slow the metabolism 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 given the present observation 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-Asp\(^1\)ANG II to recover to \(\sim 2.5\) h and the time to recover from D-Arg\(^1\)ANG 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 AT\(_1\) 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 AT\(_1\) receptor, why are there so many reports of ANG II binding at the AT\(_1\) 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 AT\(_1\) receptor. Such a conversion would not necessarily be detected by standard in vitro radioreceptor binding assays or autoradiography techniques. Thus, the published AT\(_1\) receptor affinities for ANG II and ANG III may be misleading. Our laboratory is presently attempting to address this issue. 2) There may be as yet undiscovered angiotensin receptor with high affinity for ANG III that mediates blood pressure regulation along with the AT\(_1\) 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 AT\(_{1A}\), 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-Arg\(^1\)ANG III-induced pressor responses argues that ANG III acts at the AT\(_1\) subtype; thus the utilization of losartan (Cozaar) as a specific AT\(_1\) 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.

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