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Degradation of angiotensin I in the endothelium and smooth muscle of the rat aorta

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Abstract—In homogenates of the endothelium and smooth muscle cum adventitia of the rat aorta, exogenous angiotensin (ANG) I was found to be degraded to des-aspartate-ANG I (des-Asp-ANG I) instead of ANG II. ANG II and ANG III were not detectable in either of the homogenates after 5, 10 and 30 min of incubation with the decapeptide. However, both the homogenates were able to catalyse hippuryl-L-histidyl-L-leucine (HHL) to hippuric acid and the catalysis was completely inhibited by 3 μM captopril. The data show that the angiotensin converting enzyme (ACE) present in the homogenates of rat aorta, prepared by normal laboratory procedures, is not able to hydrolyse ANG I to ANG II. This finding has important consequences in the study of vascular ACE as the assay of the enzyme is often carried out using crude homogenate and HHL or other artificial substrates. In addition, the aminopeptidase that degraded ANG I to des-Asp-ANG I was not inhibited by either amastatin or bestatin, indicating that it was not aminopeptidase A or B. Together with the recent findings of other investigators which show that the *de novo* production of ANG II in vascular tissues is stimulated and inhibited by β- and α-agonists, respectively, our present data may suggest that production of vascular ANG II occurs only in intact tissues and is probably under adrenergic regulation.

The importance of tissue angiotensin converting enzyme (ACE*), rather than its serum counterpart, in determining long-term response to ACE inhibitors has been demonstrated by several investigators [1–3]. These studies carried out on hypertensive rats [2, 3] showed that the magnitude and duration of the response appeared to correlate better with the inhibition of ACE activity in certain critical tissues, e.g. aorta and kidney, than with the inhibition of serum enzyme activity. However, the correlation is based on an assumption, namely that, in these tissues, the major pathway of angiotensin (ANG) I degradation is its conversion by ACE to ANG II. To ascertain if this assumption is correct and to obtain a better understanding of the other possible degradation pathways of vascular ANG I, we investigated the fate of exogenous ANG I in the homogenates of the endothelium and smooth muscle cum adventitia of the rat aorta in the presence and absence of ACE and aminopeptidase inhibitors. The endothelium and smooth muscle cum adventitia were employed separately because there is a paucity of information regarding the fate of ANG I in the separated tissues and also because ACE activity in endothelial homogenate measured with ANG I as substrate has not been reported before.

Materials and Methods

Preparation of tissue homogenates. Eight to 10-week-old male Sprague–Dawley rats (230 ± 10 g) were obtained from the local University Animal Centre. Rats were paralysed by cervical dislocation and killed immediately by decapitation. Each descending aorta was removed and placed in cold saline solution. The preparation of endothelial and smooth muscle cum adventitia homogenate of each aorta was carried out as described previously [4]. Briefly, the aorta was trimmed free of fat, blood clot and connective tissue, and weighed. The aorta was then everted over a pasteur pipette, cut into two sections and immersed in 2 vol. of 0.1 M phosphate buffer pH 7.5 or 8.3 containing 0.1 M NaCl in a test-tube placed in an ice vial. The endothelium of pooled everted sections of aortas from three rats were then ruptured by ultrasound sonication for 30 min at maximum speed in a Vibra Cell sonicator to obtain the endothelial homogenate. Selective rupture of the endothelium by this method had been confirmed histologically. The remainder of the sonicated aortas was minced with a pair of fine scissors and homogenized with a teflon Potter–Elvejhem tissue grinder in 2 vol. of the same phosphate buffer to obtain the smooth muscle cum adventitia homogenate. Of each homogenate 300 μL were then introduced into a dialysing tubing (10,000 M_r cutoff) and dialysed against 1000 mL of 0.1 M phosphate buffer pH 7.5 or 8.3 for 2 hr.

Incubation with ANG I and ANG II. Of each homogenate, 105 μL were added to a 210 μL solution of ANG I in 0.1 M potassium phosphate buffer pH 7.5 or 8.3 containing 300 μM ANG I and 0.1 M NaCl in a final volume of 315 μL. Incubation was carried out at 37° and three sequential aliquots of 100 μL of this incubation solution were pipetted into three separate vials containing 100 μL of 0.5 M perchloric acid at 5, 10 and 30 min. These three solutions were then centrifuged at 100,000 *g* and 2° (Beckman TL100) for 2 hr. The angiotensins in each supernatant were then separated and quantitated by capillary electrophoresis (Waters Quanta 4000 Capillary Electrophoresis System). The details of the electrophoresis protocol are given in the legend of Fig. 1. Similar incubations were also carried out in the presence of 100 μM of each of ACE and the following aminopeptidase inhibitors: captopril, enalaprilat, perindoprilat, pGlu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro, amastatin and bestatin. The possible effects of the following drugs on the degradation of ANG I were also similarly investigated: isoproterenol (5 μM), norepinephrine (5 μM), phenylephrine (50 μM) and dibutyryl cyclic AMP (5, 20 μM). In control incubations, the perchloric acid was added at zero time and three sequential 200 μL aliquots were pipetted into separate vials at similar time intervals. These incubation mixtures were also centrifuged and the angiotensins in each supernatant similarly determined by capillary electrophoresis. Three separate determinations were also carried out using undialysed homogenates. In another three determinations, the assay of ACE with ANG I was carried out in 0.1 M Tris–HCl buffer, pH 7.5, using homogenates that were prepared in the same Tris–HCl buffer.

Drugs. ANG I, ANG II, ANG III, pGlu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro, amastatin and bestatin were

* Abbreviations: ANG, angiotensin; des-Asp-ANG I, des-aspartate-angiotensin I; HHL, hippuryl-L-histidyl-L-leucine; ACE, angiotensin converting enzyme.

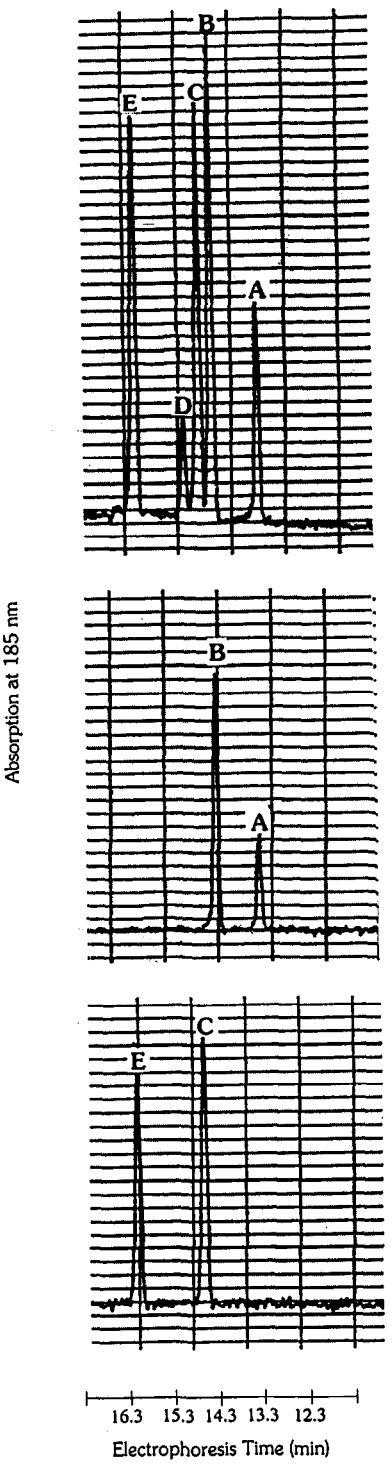

Fig. 1. Capillary electrophoresis of angiotensins. A fraction was hydrostatically sampled (for 10 sec) from a 40 μL standard solution or centrifuged incubation mixture. The fraction was then subjected to electrophoresis at 10 kV for a duration of 20 min using a solution of 0.1 M phosphoric acid having a pH of 1.95 as electrolyte. The angiotensins were detected by their UV absorbance at 185 nm. Top panel: standard solution containing 100 μM of ANG I, 120 μM ANG II, 100 μM ANG III, 80 μM des-Asp-ANG I and 40 μM ANG-(1-7). Middle panel: centrifuged endothelial homogenate that had been incubated with 300 μM ANG I for 10 min. Bottom panel: centrifuged endothelial homogenate that had been incubated with 300 μM ANG II for 10 min. (A) des-Asp-ANG I, (B) ANG I, (C) ANG III, (D) ANG-(1-7), (E) ANG II.

purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). des-Aspartate-angiotensin I (des-Asp-ANG I) was purchased from Bachem Feinchemikalien AG. ANG-(1-7) was a gift from Dr Mahesh C. Khosla, The Cleveland Clinic Foundation. Captoril, enalaprilat and perindoprilat were gifts from E. R. Squibb & Sons, Merck (Darmstadt, Germany) and Servier, respectively.

Results

Figure 1 shows that ANG I was degraded mainly to des-Asp-ANG I in the endothelium homogenate. ANG I in the homogenate of the smooth muscle cum adventitia was similarly degraded (data not shown in Fig. 1). ANG II and ANG III were not detected in either of the homogenates after 5, 10 and 30 min of incubation at pH 7.5 and 8.3. The two pHs were used because the pH optimum of ACE had been reported to be 7.5 and 8.3 for ANG I and hippuryl-L-histidyl-L-leucine (HHL), respectively [5]. However, when HHL was added to both homogenates, formation of hippuric acid occurred within 5 min of the start of incubation. The formation of hippuric acid was completely inhibited by 3 μM captopril showing that the ACE activity present in the homogenates could be measured with the substrate HHL (as has been reported previously [4, 6]) but not with ANG I. In incubations using undialysed homogenates, ANG II was also not detected indicating that the lack of hydrolysis of ANG I to ANG II was not due to the removal of important co-factor(s) during the dialysis. Similarly, incubations using 0.1 M Tris–HCl instead of potassium phosphate buffer also failed to produce any detectable ANG II. This shows that phosphate ions which have been reported to inhibit the hydrolysis of model synthetic substrate [7] were not responsible for the lack of hydrolysis of ANG I to ANG II.

The adrenergic drugs, isoproterenol, norepinephrine and phenylephrine, and dibutyryl cyclic AMP were without effect on the degradation of ANG I or the formation of des-asp-ANG I. When 300 μM ANG II instead of ANG I were incubated with the homogenates, ANG II was degraded to ANG III (see Fig. 1 for the data obtained with the endothelial homogenate). No other angiotensins were detected during the three periods of incubation. On the other hand, when 300 μM ANG III were similarly incubated, the heptapeptide was not degraded, i.e. only ANG III was detected and its concentration remained constant throughout the three periods of incubation (data not shown). These data confirmed that ANG II was not formed when ANG I was incubated with the homogenates as its formation would have been detected *per se* or as ANG III. In addition, des-Asp-ANG I has also been shown to be a substrate of ACE, the product being ANG III [8].

The aminopeptidase(s) that converted ANG I to des-Asp-ANG I was not inhibited by 100 μM of either amastatin or bestatin. Inhibition of the enzyme (23 ± 2%, mean ± SEM of four separate determinations) occurred only when the homogenates were preincubated (10 min) with 100 μM of both amastatin and bestatin. Of the four ACE inhibitors, only captopril inhibited the formation of des-Asp-ANG I by 13 ± 1.8% (mean ± SEM of four separate determinations). The inhibition was concentration dependent with 27.4 ± 4% (mean ± SEM of four separate determinations) inhibition with 400 μM.

Discussion

Mammalian tissues contain aminopeptides with a great variety of substrate specificities. One such enzyme, aminopeptidase A, is responsible for the rapid destruction

of ANG II in the plasma by hydrolytic removal of the N-terminal aspartic acid residue [9, 10]. This enzyme is inhibited by amastatin and a recent study reported an over 60% inhibition at 100 μM of the inhibitor [11]. However, the aminopeptidase(s) that was responsible for degrading ANG I to des-Asp-ANG I in the two aortic homogenates was not inhibited by 100 μM of either amastatin or bestatin indicating that it is not aminopeptidase A. In addition, this aminopeptidase is a particulate enzyme (unpublished data) while aminopeptidase A is non-particulate.

Of the four ACE inhibitors investigated, only captopril inhibited the formation of des-Asp-ANG I. It is of interest to note that a recent study on the pig kidney aminopeptidase P [12] showed that the enzyme was inhibited by captopril and four of the other 10 ACE inhibitors investigated. The authors concluded that such an inhibition may account for some of the effects or side effects noted with the clinical use of ACE inhibitors. However, whether the inhibition noted in our study is of any significance to the overall therapeutic action of captopril remains to be investigated. No noticeable inhibition of the enzyme was seen at 100 μM enalaprilat, perindoprilat or pGlu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro.

Our findings show for the first time the degradation of ANG I to mainly des-Asp-ANG I in homogenates of the endothelium and smooth muscle cum adventitia of the rat aorta. The rate of formation of the nanopeptide in the two homogenates was 152 ± 16 and 40 ± 6 pmol/min/mg aorta (mean $\pm$ SEM of four separate determinations), respectively. Our data also show that it is not possible to assay the ACE present in either of the homogenates using ANG I as substrate. Although the exact cause was not known, it was not due to the limit of detection of our capillary electrophoresis system as ANG II present in a 1 μM solution could be accurately determined. It was also not due to the rapid degradation of ANG I to des-Asp-ANG I by aminopeptidase(s) as the ACE present in the homogenate of the smooth muscle cum adventitia (measured using HHL [6]) was 3-fold higher than the activity of the aminopeptidase(s) present in similar homogenates (211 ± 8.5 pmol/min/mg aorta for ACE vs 40 ± 4 pmol/min/mg aorta for aminopeptidase). However, the rapid hydrolysis of HHL by the homogenates could be due to the fact this artificial tripeptide is an extremely good substrate for ACE having a V_{max} that is 22 times greater than the natural substrate for the purified rabbit lung ACE [5]. In this connection, it is of interest to note that Johnson and Drummer [13] also reported inability of rat aortic and lung homogenates to form ANG II from ANG I despite the fact that HHL is converted to hippuric and histidyl-leucine (HL) by the homogenates. Probably because they did not dialyse the homogenate to remove interfering endogenous peptides, they were not able to differentiate the formed des-asp-ANG I from the other peptides. However, the recent findings of two groups of investigators may provide a plausible explanation. Nakamura *et al.* [14, 15] showed that secretion of ANG II by the mesenteric artery into the perfusate was stimulated by isoproterenol and Tang *et al.* [16] found that production of angiotensins (including ANG II) by cultured endothelial cells was stimulated by isoproterenol via β_2-receptors (mimicked by forskolin) and inhibited by phenylephrine. These findings together with our present data (i.e. the inability of the homogenates to form ANG II from ANG I and the absence of any effects of the adrenergic drugs and dibutyryl cyclic AMP on the degradation of ANG I) may indicate that: (i) formation of vascular ANG II is under adrenergic control and occurs only in intact tissues, and (ii) vascular ACE when extracted by normal laboratory procedures is present in a conformation capable of hydrolysing HHL but has either negligible or low catalytic action on ANG I. Our present findings also have important consequences in the study of vascular ACE as the assay of the enzyme is often carried out using crude tissue homogenates and HHL or other artificial substrates.

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REFERENCES

1. Cohen ML and Kurz KD, Angiotensin converting enzyme inhibition in tissues from spontaneously hypertensive rats after treatment with captopril or MK-421. *J Pharmacol Exp Ther* **220**: 63–69, 1982.
2. Unger T, Ganten D, Lang RE and Scholkens BA, Is tissue converting enzyme inhibition a determinant of the antihypertensive efficacy of converting enzyme inhibitors? Studies with the two different compounds, Hoe398 and MK421, in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **6**: 872, 880, 1984.
3. Unger T, Ganten D, Lang RE and Scholkens BA, Persistent tissue converting enzyme inhibition following treatment with Hoe498 and MK421 in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **7**: 36–41, 1985.
4. Sim MK, Angiotensin converting enzyme in the endothelium and smooth muscle cum adventitia of the normo- and hypertensive rat aorta. *Clin Exp Hypertens* **A12**: 1107–1113, 1990.
5. Cushman DW and Cheung HS, Studies *in vitro* of angiotensin-converting enzyme of lung and other tissues. In: *Hypertension '72* (Eds. Genest J and Koiw E), pp. 532–541. Springer, New York, 1972.
6. Sim MK and Chan CS, Effect of experimentally-induced hypertension on angiotensin converting enzyme activity in the aortic endothelium and smooth muscle cum adventitia of the Sprague Dawley rat. *Life Sci* **50**: 1821–1825, 1992.
7. Bunning P, Holmquist B and Riordan JF, Substrate specificity and kinetic characteristics of angiotensin converting enzyme. *Biochemistry* **22**: 103–110, 1983.
8. De Rio CG, Smellie WSA and Morton JJ, des-Asp-Angiotensin I: its identification in rat blood and confirmation as a substrate for converting enzyme. *Endocrinology* **108**: 406–412, 1981.
9. Khairallah PA, Bumpus FM, Page IH and Smeby RR, Angiotensinase with a high degree of specificity in plasma and red cells. *Science* **140**: 672–674, 1962.
10. Regoli D, Riniker B and Brunner H, The enzymatic degradation of various angiotensin II derivatives by serum, plasma or kidney homogenate. *Biochem Pharmacol* **12**: 637–646, 1963.
11. Ahmad S and Ward PE, Role of aminopeptidase activity in the regulation of the pressor activity of circulating angiotensins. *J Pharmacol Exp Ther* **252**: 643–650, 1990.
12. Hooper NM, Hryszko J, Oppong SY and Turner AT, Inhibition by converting enzyme inhibitors of pig kidney aminopeptidase P. *Hypertension* **19**: 281–285, 1992.
13. Johnson H and Drummer OH, Hydrolysis of angiotensin I by peptidase in homogenates of rat lung and aorta. *Biochem Pharmacol* **37**: 1131–1136, 1988.
14. Nakamura M, Jackson EK and Inagami T, Role of

vascular angiotensin II released by beta-adrenergic stimulation in rats. *J Cardiovasc Pharmacol* **8** (suppl 10): S1–S5, 1986.
15. Nakamura M, Jackson EK and Inagami I, Beta-adrenoceptor-mediated release of angiotensin II from mesenteric arteries. *Am J Physiol* **250**: H144–H148, 1986.
16. Tang SS, Stevenson L and Dzau J, Endothelial renin-angiotensin pathway: adrenergic regulation of angiotensin secretion. *Circ Res* **66**: 103–108, 1990.