Pergamon

Biochemical Pharmacology, Vol. 48, No. 5, pp. 1043–1046, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00246-0

SHORT COMMUNICATIONS

Degradation of angiotensin I to [des-Asp^1]angiotensin I by a novel aminopeptidase in the rat hypothalamus

(*Received* 3 *December* 1993; *accepted* 16 *May* 1994)

Abstract—The particulate enzyme that degrades angiotensin I (ANG I) to [des-aspartate^1]angiotensin I ([des-Asp^1]ANG I) in the hypothalamic homogenate of the rat has been established as a specific aminopeptidase. The major characteristic is its resistance to inhibition by 10^{-4} M amastatin, bestatin and EDTA. Among the four amino acyl-β-naphthylamides (aspartyl-, glutamyl-, arginyl- and leucyl-β-naphthylamide), aspartyl-β-naphthylamide is the most susceptible substrate of the enzyme; being degraded at twice the rate of arginyl- and leucyl-β-naphthylamide, and six times that of glutamyl-β-naphthylamide. Although the precise role of this aminopeptidase has yet to be determined, its presence establishes the existence of a specific pathway for the degradation of ANG I that bypasses the formation of ANG II. The relationship between degradation and hypertension is shown by our recent findings that the formation of [des-Asp^1]ANG I form ANG I in the hypothalamic homogenate of the spontaneously hypertensive rat (SHR) is significantly enhanced, and the findings of other investigators that the production of ANG II by neuronal cultures of the SHR is significantly decreased.

Key words: aminopeptidases; [des-aspartate^1]angiotensin I; angiotensin I/II; aspartyl-β-naphthylamide; amastatin; bestatin

It has been known for some time that mammalian tissue degrades ANG I* to [des-Asp^1]ANG I besides ANG II [1, 2]. [des-Asp^1]ANG I has also been detected in the rat [3] and human [4] circulation. However, the importance of this pathway and the specific aminopeptidase involved in the formation of [des-Asp^1]ANG I has remained unknown. The paucity of information is probably due to the fact that, unlike ANG II, [des-Asp^1]ANG I is a relatively inactive peptide. Studies with [des-Asp^1]ANG I tend to indicate that it has pressor and steroidogenic actions, but that a major portion of these actions is dependent on its conversion to ANG III [5–8]. In a recent study, we found that homogenates of the rat aorta [9] and hypothalamus [10] degrade exogenous ANG I to mainly [des-Asp^1]ANG I instead of ANG II and that the enzyme involved in the degradation was an uncharacterized aminopeptidase. The activity of this aminopeptidase in the hypothalamus of two models of hypertensive rats has also been found to be significantly higher than in the corresponding normotensive controls [10]. The present study established the hypothalamic enzyme to be a specific aminopeptidase that is involved in the formation of [des-Asp^1]ANG I. For ease of reference throughout the text, this aminopeptidase is referred to as either hypothalamic aminopeptidase or aminopeptidase.

Materials and Methods

Preparation of tissue homogenates. Three to four month old male Sprague–Dawley rats (body weight between 250 and 300 g) were obtained from the local University Animal Centre. The animals were killed by decapitation. Each brain was removed and placed in cold saline solution. The hypothalamus was dissected according to the method of Glowinski and Inversen [11]. Four pooled hypothalami were sonicated in 4 volumes (v/w) of 0.1 M Tris–HCl buffer pH 7.5 (5 min at maximum speed in a Vibra Cell sonicator) to obtain the homogenate. Three hundred microlitres of homogenate were introduced into dialysing tubing (10,000 Da molecular mass cutoff) and dialysed against 1000 mL of the same Tris buffer for 2 hr. The remainder of the homogenate was then centrifuged at 100,000 *g* and 4° for 60 min using the Beckman TL 100 ultra centrifuge. The supernatant was decanted and the pellet resuspended in equal volume of buffer. Both the supernatant and resuspended pellet were dialysed as with the homogenate. The protein concentration in the tissue solutions was determined by the method of Lowry *et al.* [12].

Aminopeptidase assay. The assay of hypothalamic aminopeptidase was carried out using ANG I, arginyl-β-naphthylamide, glutamyl-β-naphthylamide, lucyl-β-naphthylamide and aspartyl-β-naphthylamide as substrates. The assay protocol using ANG I as substrate was as described previously [10]. Briefly, 105 μL of each homogenate, supernatant or resuspended pellet were added to a 210 μL solution of ANG I in 0.1 M Tris–HCl buffer, pH 7.5, containing 300 μM ANG I 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 15 min, respectively. These three solutions were then centrifuged at 100,000 *g* and 4° for 2 hr. The angiotensins in each supernatant were then separated and quantitated by capillary electrophoresis, as described previously [10, 11]. The assay of hypothalamic aminopeptidase was also carried out in the presence of 10^{-4} M amastatin, bestatin and EDTA.

Preliminary experiments showed that the homogenate and resuspended pellet, but not the supernatant, degraded ANG I to [des-Asp^1]ANG I, and that the degradation was not affected in the presence of a combination of 10^{-4} M amastatin, bestatin and EDTA. This indicates that hypothalamic aminopeptidase is a particulate enzyme that

* Abbreviations: ANG, angiotensin; [des-Asp^1]ANG I, [des-aspartate^1]angiotensin I; SHR, spontaneously hypertensive rat.

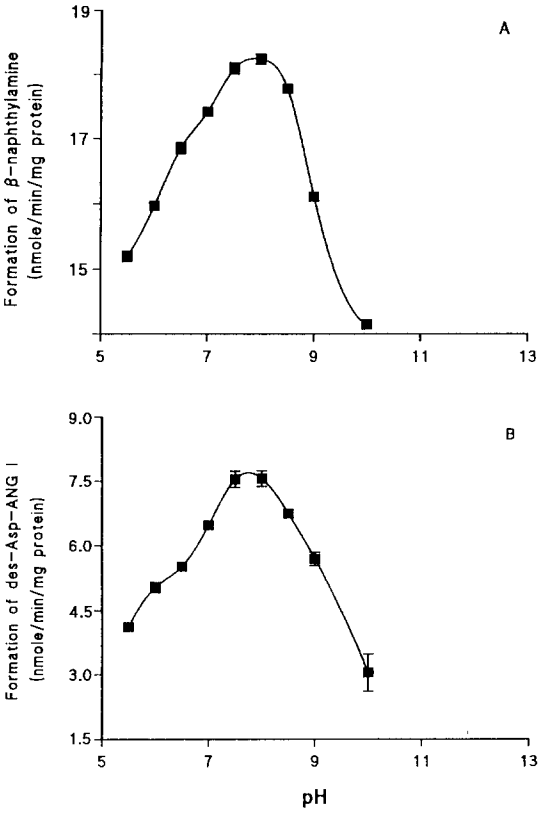

Fig. 1. pH optima of aminopeptidase X. The enzyme was assayed as described in the text. Each point is the mean value of four separate sets of determination. The vertical bars represent SEM. Sodium phosphate buffer was used for pH 5.5 to 7 and Tris–HCl for pH 7.5 to 10.

is resistant to the two common aminopeptidase inhibitors and EDTA. Hence, substrate characterization of the enzyme i.e. assay of the enzyme using and the four amino acyl-β-naphthylamides was carried out using only the hypothalamic homogenate. As amino acyl-β-naphthylamides are substrates of a large number of aminopeptidases, the incubation was carried out in the absence and presence of a combination of 10^{-4} M amastatin, bestatin and EDTA to determine the activity of total aminopeptidases and aminopeptidase X, respectively. The protocol for the assay of total hypothalamic aminopeptidases was as follows. Fifty microlitres of homogenate was added to 2.45 mL of a Tris–HCl buffer, pH 7.4, containing 750 μM of one of the four amino acyl-β-naphthylamides in the final volume of 2.5 mL. For assay of hypothalamic aminopeptidase, the homogenate was incubated with 10^{-4} M amastatin, bestatin and EDTA for 10 min prior to the addition of the substrate. Incubation was carried out at 37° and three sequential aliquots of 800 μL of the mixture were then transferred to separate stoppered tubings at 0, 20, 40 and 60 min, respectively. The tubings were then immersed in boiling water for 10 min to kill the enzyme and the mixture centrifuged for 10 min at 5000 *g*. Each supernatant was then transferred to a quartz cuvette and the amount of β-naphthylamine formed was measured by its absorption at 336 nm. The reading of the 0 min sample was used as the control. The conversion of aspartyl-β-naphthylamide to β-naphthylamine by soluble aminopeptidases was followed spectrophotometrically in an earlier experiment to determine the extinction coefficient of the conversion.

To test whether the degradation of ANG II to ANG III in the same hypothalamic homogenate reported in our earlier study [10] was brought about by hypothalamic aminopeptidase or aminopeptidase A, an assay using 300 μM ANG II as substrate was carried out in the presence of 10^{-4} M of the three inhibitors. In addition, hypothalamic aminopeptidase activity was tested after 60 min pre-incubation with 80 mM $MnCl_2$ (to compare it to a soluble aminopeptidase from the dog kidney, see Discussion for details). In this assay only amastatin and bestatin, but not EDTA, were included in the assay mixture.

Effect of pH. The assay of hypothalamic aminopeptidase using ANG I and aspartyl-β-naphthylamide as the substrate was also conducted at different pHs (see Fig. 1 for details). In an earlier experiment, the UV absorption of β-naphthylamine at the same pHs was also determined. This was carried out to establish that within the pH range the UV absorption of the amine remained constant.

Effect of Triton X-100, trypsin and 0.5 M NaCl. To test whether this particular enzyme is a bona fide integral membrane protein, the homogenate was treated with Triton X-100 (0.1–0.2%, w/v), trypsin (0.01–0.025%, w/v), 0.5 M NaCl and a combination of Triton X-100 and trypsin, respectively. It was then centrifuged at 100,000 *g* and the enzyme activity in the 100,000 *g* supernatant was determined.

Drugs. ANG I, ANG II, ANG III, amastatin, bestatin, the amino acyl-β-naphthylamides, β-naphthylamine and trypsin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [des-Asp^1]ANG I was purchased from BACHEM Feinchemikalien AG (Switzerland).

Results

The maximum UV absorption of β-naphthylamine at 336 nm does not vary with pH (from pH 5.5 to 10). Similar

Table 1. Hydrolysis of synthetic substrates by particulate aminopeptidase X from the rat hypothalamus

Substrate	Aminopeptidase activity (nmol/min/mg protein) Total*	Aminopeptidase X†
Arginyl-β-naphthylamide	34.2 ± 2.0	8.0 ± 0.04
Aspartyl-β-naphthylamide	35.7 ± 1.5	18.5 ± 0.11
Glutamyl-β-naphthylamide	33.8 ± 1.8	3.0 ± 0.01
Lucyl-β-naphthylamide	21.9 ± 1.2	9.5 ± 0.07

The values are the means ± SEM of three separate determinations. Assayed in the *absence and †presence of 10^{-4} M amastatin, bestatin and EDTA.

findings have also been reported by Lee *et al.* [13]. The ε_{336} and ε_{340} for the formation of β-naphthylamine from aspartyl-β-naphthylamide were found to be 1850 and 1775, respectively. The latter is close to the value of 1780 reported by Lee *et al.* [13].

Table 1 shows that hypothalamic aminopeptidase exhibits substrate specificity; having aspartyl-β-naphthylamide as the most susceptible substrate. Figure 1 shows that hypothalamic aminopeptidase has a pH optima of 7.8. The value remained constant, irrespective of whether ANG I or aspartyl-β-naphthylamide was used as the substrate. This, together with the fact that the assay was carried out in the presence of amastatin, bestatin and EDTA indicates that it is hypothalamic aminopeptidase that degrades the two substrates. The degradation of ANG II to ANG III in the hypothalamic homogenate was not inhibited by 10^{-4} M amastatin, bestatin and EDTA indicating that hypothalamic aminopeptidase also degrades ANG II to ANG III. However, compared with the rate of formation of [des-Asp^1]ANG I, the rate of ANG III formation is about 65% of the former. Preincubation with 80 mM $MnCl_2$ for 60 min inhibited hypothalamic aminopeptidase activity by about 20% (from 7.3 ± 0.5 to 5.8 ± 0.4 nmole/min/mg protein).

Less than 10% of hypothalamic aminopeptidase activity was detected in supernatant of homogenate treated with 0.1–0.2% Triton X-100. Fifty per cent of activity was detected in supernatant of homogenate treated with 0.015% trypsin but higher concentrations of trypsin inactivated the enzyme. A combination of 0.2% Triton X-100 and 0.015% trypsin yielded a supernatant containing 75% of enzyme activity. The low activity in Triton X-100-treated preparations could be due to the association of the detergent form of the enzyme with sedimentable cytoskeleton. This association was probably prevented by trypsin which converted the detergent to the protease form of the solubilized enzyme. The enzyme was not solubilized by 0.5 M NaCl, indicating that hypothalamic aminopeptidase was not a cytosolic protein that had associated itself with membrane fragments.

Discussion

The present data show that the degradation of ANG I to [des-Asp^1]ANG I in the rat hypothalamic homogenate is brought about by a specific aminopeptidase that has not been described before. As with most aminopeptidases, the hypothalamic aminopeptidase appears to be an integral membrane protein. The currently known particulate aminopeptidases that are able to degrade ANG I and aspartyl-β-naphthylamine to their respective [des-Asp] compounds include the ubiquitous aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7) and aminopeptidase W (EC 3.4.11.-) [14]. However, these enzymes are susceptible to inhibition by amastatin and or bestatin, having K_i or IC_{50} values in the nano to lower μmolar range [14, 15], whilst hypothalamic aminopeptidase is not affected by 100 μM of the two inhibitors. In addition, the substrate specificity of these three particulate aminopeptidases [14] is different from that of hypothalamic aminopeptidase, which degrades aspartyl-β-naphthylamide at six times the rate of glutamyl-β-naphthylamide. However, there are two reports of an aminopeptidase which readily hydrolysed the aspartyl residue from ANG I. One is a soluble aminopeptidase from dog kidney which is also not inhibited by EDTA but is stimulated by $MnCl_2$ [16], and another is detected in the intact perfused rat lung which has been shown to hydrolyse ANG I but not ANG II [17]. Hypothalamic aminopeptidase is unlikely to be the soluble dog kidney aminopeptidase as 60 min preincubation with 80 mM $MnCl_2$ (which increased the activity of the kidney enzyme by 200%, [16]) inhibited the activity of hypothalamic aminopeptidase by about 20%. Hypothalamic aminopeptidase is also unlikely to be the lung enzyme as it hydrolyses ANG II to ANG III while the lung enzyme does not. However, definitive identification of these enzymes requires further study.

Hypothalamic aminopeptidase together with angiotensin converting enzyme and aminopeptidase N may, thus, be part of a specific pathway for the degradation of ANG I that bypasses the formation of ANG II. The importance of such a pathway has yet to be determined. However, based on our recent finding that the hypothalamus of the SHR exhibits enhanced level of this aminopeptidase [10], it is possible to theorise that the degradation of ANG I in certain critical tissues of the SHR is shunted in favour of the [des-Asp^1]ANG I pathway. In such a scenario the formation of the pressor ANG II would be curtailed while its degradation enhanced. This hypothesis may then account for the findings that the production of ANG II in neuronal cultures of the SHR as compared to that of the WKY decreased by 71% [18] and that intracerebroventricularly-administered losartan, an AT_1 antagonist, is not effective in lowering the blood pressure of the SHR [19].

Acknowledgements—This study was supported by a Grant RP880351 from the National University of Singapore.

Department of Pharmacology M. K. SIM*
Faculty of Medicine M. H. H. CHOO†
National University of Singapore; X. S. QIU
†*Division of Cardiology*
Cardiac Department
National University Hospital
Singapore 0511

REFERENCES

1. Darracott E, Ackerly JA, Lantz GH, Glen AW and Peach MJ, Formation and circulating level of (des-Asp^1)-angiotensin (ANG) I in the rat. *Circulation* (Suppl 3) **56**: 215, 1977.
2. Ackerly JA, Felger TS and Peach MJ, Des-Asp^1-angiotensin I: A metabolite of angiotensin I in the perfused feline adrenal. *Eur J Pharmacol* **38**: 113–121, 1976.
3. Del Rio CG, Smellie WSA and Morton J. des-Asp-angiotensin I: its identification in rat blood and confirmation as a substrate for converting enzyme. *Endocrinology* **108**: 406–412, 1981.
4. Nussberger J, Brunner DB, Waeber B and Brunner HR, Specific measurement of angiotensin metabolites and *in vitro* generated angiotensin II in plasma. *Hypertension* **8**: 476–482, 1986.
5. Larner A, Vaughan ED Jr, Tsai BS and Peach MJ, Role of converting enzyme in the cardiovascular and adrenal cortical responses to [des-Asp^1]-angiotensin I. *Proc Soc Exp Biol Med* **152**: 631–634, 1976.
6. Cambell WB, Schmitz JM and Itoskovitz HD, [Des-Asp^1]-angiotensin I: A study of its pressor and steroidogenic activities in conscious rat. *Endocrinology* **100**: 46–51, 1977.
7. Vaughan ED Jr, Peach MJ, Ackerly JA, Tsai BS and Larner A, Pressor and steroidogenic actions of [des-Asp^1]angiotensin I. *Circ Res* **40** (Suppl 1): I-94–I-97, 1077.
8. Sexton JM, Britton SL, Beierwaltes WH, Fiksen-Olsen MJ and Romerco JC, Formation of angiotensin III from [des-Asp^1]angiotensin I in the mesenteric vasculature. *Am J Physiol* **237**: H218–H223, 1979.
9. Sim MK, Degradation of angiotensin I in the endothelium and smooth muscle of the rat aorta. *Biochem Pharmacol* **45**: 1524–1527, 1993.
10. Sim MK and Qiu XS, Formation of [des-Asp^1] angiotensin I in the hypothalamic extract of normo- and hypertensive rats. *Blood Pressure*, in press.

* Corresponding author.

11. Glowinski J and Inversen LL, Regional studies of catecholamines in the rat brain. 1. The deposition of [^{3}H]dopamine and [^{3}H]dopa in various regions of the brain. *J Neurochem* **13**: 655–659, 1966.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Lee HJ, LaRue JH and Wilson IB, A simple spectrophotometric assay for amino acyl arylamindases (naphthylamindases, aminopeptidases). *Anal Biochem* **41**: 397–401, 1971.
14. Kenny AJ, Stephenson SL and Turner AJ, Cell surface peptidases. In:*Mammalian Ectoenzymes* (Eds. Kenney AJ and Turner AJ), pp.169–210. Elsevier, Amsterdam, 1987.
15. Rich DH, Moon BJ and Harbeson S, Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding process. *J Med Chem* **17**: 417–442, 1984.
16. Chueng HS and Cushman DW, A soluble aspartate aminopeptidase from dog kidney. *Biochim Biophys Acta* **242**: 190–193, 1971.
17. Ackerly JA, Peach MJ, Vaughan ED Jr and Glenn AW, Formation of [des-Asp^1]angiotensin I by the perfused rat lung. *Endocrinology* **107**: 1699–1704, 1980.
18. Raizada KM, Stenstrom B, Phillips MI and Summers C, Angiotensin II in neuronal cultures from brains of normotensive and hypertensive rats. *Am J Physiol* **247** (*Cell Physiol*) **16**: C115–C119, 1984.
19. DePasquale MJ, Fossa AA, Holt WF and Mangiapone ML, Central DuP 753 does not lower blood pressure in spontaneously hypertensive rats. *Hypertension* **19**: 668–671, 1992.