Degradation of oxytocin by the human placenta: effect of selective inhibitors

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The hydrolysis of oxytocin by human placental subcellular fractions was studied in the presence of selective inhibitors by measuring liberated amino acids by high performance liquid chromatography (HPLC). Oxytocin degradation by microsomal and lysosomal fractions was inhibited by bestatin, amastatin and puromycin. The IC₅₀ values of these inhibitors on oxytocin degradation by both fractions were similar to those of these inhibitors on the human placental aminopeptidase M measured by L-Leu-p-nitroanilide as a substrate (LAP activity), which we reported previously. However, purified aminopeptidase M from human placental microsomal fractions could not liberate any amino acid from oxytocin. Since phosphoramidon (1 µmol/l), a putative metalloendopeptidase inhibitor, and N-benzyloxycarbonyl-γ-valyl-γ-proline (Z-Va-pro) (14 µmol/l), a selective inhibitor of post-proline endopeptidase, could not significantly influence the degradation of oxytocin by either subcellular fractions, neither enzyme seems to be actively involved in oxytocin degradation. These results strongly suggested the existence of oxytocinase(s) other than the above three enzymes in microsomal and/or lysosomal fractions of human placenta.

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The concentration of oxytocin (Cys¹-Tyr²-Ile¹-Gln⁴-Asn⁵-Cys⁶-Pro⁷-Leu⁸-Gly⁹-NH₂, OT) in the serum of pregnant woman is higher than that in non-pregnant woman (1, 2). The definitely higher concentration of OT in the arterial cord blood than in venous cord blood and maternal blood (3) suggests that OT is generated mainly in the fetus itself and destroyed within the placenta and/or by pregnancy serum.

Since Fekete first discovered the ability of human pregnancy sera to destroy oxytocin in 1930 (4), many investigators have studied (by bioassay) this inactivating enzyme called oxytocinase (5). Later, for the assay of this enzyme, various synthetic substrates such as L-cystine-dl-β-naphthylamide (6), s-benzyl-L-cysteine-p-nitroanilide (7) and L-leucine-β-naphthylamide or L-leucine-p-nitroanilide in the presence of 20 mmol/l L-methionine (placental leucine aminopeptidase, P-LAP activity (8, 9)) were used. Recently, the importance of this enzyme as an etiologic factor of diabetes insipidus in pregnancy (10) has been suggested.

On the other hand, Walter ans Schlank (11) showed that the enzyme responsible for OT degradation is the cytosolic post-proline endopeptidase (EC 3.4.21.26) in rat kidney. Despite the studies on the metabolism and degradation of OT by Walter et al. (11–13) and Burbach and Lebouille (14), little is known about its metabolism in human placenta except for old works (15, 16) and our previous reports (17, 18).

In this study we tried to show the degradation of OT by subcellular fractions of human placenta and the effects of various inhibitors on the enzyme(s) responsible for OT degradation. The degradation of OT by purified human placental microsomal leucine aminopeptidase (LAP), aminopeptidase M (EC 3.4.11.2), was also studied to elucidate the relationship between the oxytocinase and enzyme(s) responsible for OT degradation in human placenta.

Materials and methods

Normal human placentae were obtained at the Nagoya University Hospital. Synthetic OT was obtained from the Protein Research Foundation, Osaka, Japan. All other chemicals for high performance liquid chromatography (HPLC) were of analytical grade commercially available.

Tissue preparation

Human placenta (25 g) obtained after normal delivery was immediately rinsed with chilled saline. The placental tissue was rapidly dissected and homogenized in 10 vol of ice-cold 0.05 mol/l sodium phosphate buffer (pH 7.5) containing 0.15 mol/l NaCl and 0.25 mol/l sucrose, using a Polytron homogenizer at setting 5. After centrifugation at 4°C (3000 x g for 10 min) the pellet obtained was discarded and the supernatant (190 ml) was
centrifuged (1200 × g for 30 min). The resulting pellet was further subjected to differential centrifugation and sucrose density gradient centrifugation according to the methods of Ragab et al. (19) and our group (20) for isolation of lysosomes. The supernatant was centrifuged at 105,000 × g for 60 min. The resulting supernatant and pellet were used as the cytosolic and microsomal fractions, respectively. The lysosomal fraction, suspended in 0.1 mol/l sodium phosphate buffer (pH 7.0), was frozen and thawed 10 times and centrifuged at 100,000 × g for 1 h to obtain the soluble lysosomal enzymes. Protein concentrations of each subcellular fraction were as follows: microsomal (12.0 mg/ml), lysosomal (8.7 mg/ml) and cytosolic (11.25 mg/ml).

**Assays**

LAP activity was measured according to our method (21). One ml SI unit of LAP activity was defined as the amount of enzyme that liberated 1 nmol of synthetic substrate per min at 37°C. Protein was determined according to the method of Lowry et al. (22) with BSA as a standard.

**Purification of enzymes**

Aminopeptidase M was purified from human placental microsomal fraction according to our method described previously (21, 23). The method of purification of aminopeptidase M was as follows; in brief, human placenta obtained at normal full-term deliveries were homogenized in 1.15% KCl. After isolation of the postmitochondrial membrane fraction, the enzyme was solubilized with Triton X-100 and trypsin digestion from the membrane fractions. It was purified after zinc sulfate fractionation by chromatographies on DE-52 (Whatman, Kent, UK), hydroxyapatite (Seikagaku Kogyo, Tokyo, Japan) and Bio gel A-50 (Bio Rad, Richmond, CA). Further purification was achieved by affinity chromatographies using Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with bestatin and with anti-human placental aminopeptidase M which was raised in a rabbit against the purified enzyme (21). The specific activity of the purified aminopeptidase M was 597.9 SI unit per mg protein.

**Incubations**

Each subcellular fraction or enzyme (10 μl) was incubated with 20 nmol of OT at 37°C in 40 μl of 50 mmol/l sodium phosphate buffer (pH 7.5) containing 150 mmol/l NaCl. At various time intervals following the start of incubation, the reaction was stopped by adding 70 μl of ethanol. Each inhibitor was incubated with the enzyme preparation for 10 min before the addition of OT (in the case of N-benzyloxycarbonyl-valyl-prolinal (Z-Val-proinal): 30 min).

**Determination of amino acids by HPLC**

An LC-4A pump, fluorometric detector type RF-530, and a Chromatopac C-R 2A recorder (Shimazu Co Ltd, Kyoto, Japan) were used. A packed column with 7.5 μm strongly acidic cation exchange resin of styrene-divinyl benzene copolymer (4 × 15 cm ID, Shimazu Co Ltd, Kyoto, Japan) was used. Chromatography was performed essentially according to the method of Ishida et al. (24). By this system Gln and Asn, constituent amino acid amides of OT, could not be quantitated. Since crude enzymes themselves produce amino acids during incubation, incubations with crude enzyme and purified enzyme preparations without OT were performed as a control in every experiment. Amounts of those amino acids were subtracted from the quantities of identified amino acids in each experiment.

**Results**

Table 1 shows the velocity of amino acid liberation from OT by the subcellular fractions of human placenta. While microsomal fractions liberated actively Tyr, Ile, cystine 1, 6 and Leu 8 and lysosomal fractions liberated Tyr 2 and Ile 3 from OT, cytosolic fractions did not liberate any amino acid from OT under the conditions of our incubations.

In order to differentiate between individual enzymes present in placental microsomal and lysosomal fractions, various inhibitors of peptidases were included in the incubation medium. Previously we showed the effects of various inhibitors on purified oxytocinase (P-LAP) from retroplacental serum and aminopeptidase M from human placenta (25). IC50 values of serum oxytocinase

| Table 1. Amino acid liberation with human placental subcellular fractions and aminopeptidase M. |
|---------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|
| Enzyme preparation              | Velocity of amino acid liberation (μmol/min/mg protein) | Mean ± SE       |
| Microsomal fractions (N = 10)   | Tyr  | 83.14 ± 14.72 | 71.31 ± 10.97 | 15.84 ± 4.11 | 37.03 ± 7.40 |
| Lysosomal fractions (N = 5)     | Ile  | 28.70 ± 3.00  | 14.91 ± 2.18 | 0              | 0              |
| Cytosolic fractions (N = 5)     | Cystine | 0             | 0              | 0              | 0              |
| Aminopeptidase M (N = 5)        | Leu  | 0             | 0              | 0              | 0              |

* The degradation of OT was carried out for 120 min at 37°C.
The degradation of OT was carried out for 60 min at 37°C. The values are the means of three determinations in duplicate.

Discussion

Until now, three main enzymes on the degradation of oxytocin have been reported: (1) Oxytocinase which degrades the cyclic structure of OT and liberates Tyr², Ile³, Gln⁴ and Asn⁵ (17, 18, 26). Ferrier et al. showed that hydrolytic cleavage of the ring of OT at the peptide bond between residues Cys¹ and Tyr² drastically reduces its biological activity (27). (2) Post-proline endopeptidase which releases the carboxyterminal dipeptide, Leu-Gly-NH₂ from OT (11-14). (3) Metalloendopeptidase, endopeptidase-24,11 (EC 3.4.24.11) which cleaves bonds including the amino groups of hydrophobic amino acid residues (28). However, the physiological importance of these enzymes for OT degradation is obscure, because these enzymes have been mainly studied in purified preparations obtained from organs containing few, if any, enzymes for OT degradation.

Our present data suggest that the enzyme(s) involved in OT degradation are metalloproteases (Table 2) to which, it is known, both aminopeptidase M and serum oxytocinase belong (21, 29). It is known that bestatin and amastatin are the potent and specific inhibitors of exopeptidases such as LAP and aminopeptidase M (21, 23, 30). Since microsomal fractions degraded OT actively and the similarity between effects of the inhibitors on OT degradation (25) and IC₅₀ values of the inhibitors on aminopeptidase M suggested the possible involvement of aminopeptidase M in OT degradation. However, our purified aminopeptidase M could not hydrolyze OT (Table 1).

At 5 nmol/l, Z-Val-proinal is considered as a selective
inhibitor of post-proline endopeptidase (31) and, at 1 μmol/l, phosphoramidon as that of metalloendoproteinidase (32). Therefore our present data suggest that neither of the enzymes is actively engaged in OT degradation (Table 2). In contrast to the data by Walter and Shlank (13), Tate (33) and ourselves (17) reported that OT is not cleaved by the bovine brain and human placental post-proline endopeptidase, although it acts as a potent competitive inhibitor of post-proline endopeptidase (33).

Concerning the roles of post-proline endopeptidase and aminopeptidase M in the degradation of OT we reported that the combination of placental post-proline endopeptidase with porcine kidney aminopeptidase M commercially available (Sigma) or human placental aminopeptidase M actively degraded OT into constituent amino acids, cystine1,6, Tyr2, Ile3, Pro7 and Leu8 (17). However, porcine (17) and human (Table 1) aminopeptidase M themselves could not liberate any amino acid from OT. Thus, in the conditions using purified enzyme preparations, OT is degraded first by post-proline endopeptidase followed by the action of aminopeptidase(s). However, in the microsomal and lysosomal fractions, the former enzyme seems not to play an essential role in the OT degradation.

Previously we showed that human placental post-proline endopeptidase releases specifically C-terminal Phe from angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (34). Tate (33) reported that post-proline endopeptidase is identical to TRH deamidase. Therefore, post-proline endopeptidase would act as carboxypeptidase and carboxyamidase at the carboxyl side of Pro. With the appearance of Leu8 as a cleavage product of OT, detected in this study, there are two possibilities of inactivating pathways. First, it seems that the post-proline endopeptidase liberates dipeptide Leu-Gly-NH2 and that this peptide is consequently split by the aminopeptidases. The alternative route might include another enzyme system of carboxyamidase and carboxypeptidase which attacks the OT molecule from the carboxyl terminal; first carboxyamidase liberates glycynamide and then the liberation of leucine by carboxypeptidase occurs (11, 12). Since Leu-Gly-NH2, the cleaved product of OT by post-proline endopeptidase, could not be identified by our HPLC system. Further studies are necessary to ascertain whether this fragment would be released or not.

Since cytosolic fractions could not degrade OT during 3 h incubations (Table 1), cytosolic enzymes seemed to contribute little to OT degradation in the human placenta. Post-proline endopeptidase is believed to be a cytosolic enzyme (35, 36). Going back to the potential participation of the post-proline endopeptidase in the degradation of OT, we should keep in mind that post-proline endopeptidase is SH-enzyme (35). Therefore, its function is dependent on the state of its SH groups, which can be reactivated by sulfhydryl substances such as cysteine or glutathione. In either case, a cytosolic post-proline endopeptidase seemed not to play a role in OT degradation in the human placenta.

Rychlik (37) suggested two-step inactivation routes in tissues for OT (vasopressin), in which the reduction of the disulphidic bridge resulting in abolition of the cyclic structure of the peptide, which enables the non-specific aminopeptidases to attack the peptide bonds. This report supports our results on the involvement of bestatin, amastatin-sensitive metallopeptidase in OT degradation.

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