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PARTIAL CHARACTERIZATION OF A METALLOENDOPEPTIDASE ACTIVITY PRODUCED BY CULTURED ENDOTHELIAL CELLS THAT REMOVES THE COOH-TERMINAL TRIPEPTIDE FROM ^{125}I-ATRIAL NATRIURETIC FACTOR

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Summary: The presence of the COOH-terminal region of human atrial natriuretic factor-(99-126) (hANF) is necessary for the full expression of its biological activity. Here, we report on the partial characterization of a proteolytic activity in the conditioned medium from cultured bovine aortic endothelial cells that cleaves the Ser^{123}-Phe^{124} bond of ^{125}I-hANF generating the COOH-terminal tripeptide. The concentrated conditioned medium was fractionated by gel filtration high performance liquid chromatography and fractions were assayed for the ability to generate the COOH-terminal tripeptide from ^{125}I-hANF. This analysis indicated that the protein responsible for this activity had an approximate molecular weight of 200,000 daltons. Of 16 protease inhibitors tested, only 1,10 phenanthroline, EDTA, EGTA and N-ethylmaleimide significantly inhibited the endopeptidase activity. Thus, we conclude that cultured bovine aortic endothelial cells produce a potentially novel phosphoramidon-insensitive metalloendopeptidase that removes the COOH-terminal tripeptide from ^{125}I-hANF.

Atrial natriuretic factor (ANF) , initially discovered by de Bold and coworkers (1), is a potent peptide hormone which induces natriuresis, diuresis, inhibition of aldosterone secretion and vasorelaxation (for review, see Ref. 2). The COOH-terminal region of ANF, running from Phe^{124} to Tyr^{126}, is essential for the full expression of its bioactivity (3-5) and in cultured bovine aortic endothelial and smooth muscle cells is necessary to generate intracellular cyclic GMP (6,7). At present, two cell surface receptors for ANF have been identified. One is coupled to the generation of cyclic GMP and has a high affinity for intact ANF, but not for truncated ANF molecules lacking the COOH-terminal tripeptide (6-9). The other receptor has a high affinity for both intact ANF

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The Abbreviations used are: ANF, atrial natriuretic factor-(99-126); ^{125}I-hANF, monoiodinated human atrial natriuretic factor-(99-126); BAECs, bovine aortic endothelial cells; CMF-DPBS, calcium, magnesium-free Dulbecco's phosphate buffered saline; TFA, trifluoroacetic acid.

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and truncated ANF molecules lacking the COOH-terminal tripeptide, but is apparently not coupled to the generation of cyclic GMP (silent or clearance receptor) (6-10). Thus, a peptidase that can remove the COOH-terminal tripeptide from ANF may be involved in the regulation of its biologic effects. We have previously observed the generation of the COOH-terminal tripeptide from ^{125}I-hANF by cultured smooth muscle (11) and endothelial cells (12) via an extracellular proteolytic event. Here, we report on the partial characterization of a phosphoramidon-insensitive metalloendopeptidase in the conditioned medium from cultured bovine aortic endothelial cells (BAECs).

MATERIALS AND METHODS

Materials- ^{125}I-hANF was purchased from Amersham. Calcium, magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS) and all cell culture reagents were purchased from GIBCO. Bovine serum albumin (fraction V, protease free), bestatin, amastatin, carboxypeptidase inhibitor (potato tuber), 1,10 phenanthroline, EGTA, N-ethylmaleimide, phenylmethylsulfonyl fluoride, phosphoramidon, pepstatin A, soybean trypsin inhibitor, leupeptin and aprotinin were purchased from Sigma. Alpha$_2$-macroglobulin, antipain, cystatin, calpain inhibitor I, E-64, beef liver catalase and ferritin were purchased from Boehringer Mannheim. EDTA was purchased from Fisher Biotech. Captopril was supplied by the Medicinal Chemistry Department, Schering-Plough Research. A gel filtration standard containing thyroglobulin, gamma globulin, ovalbumin, myoglobin and vitamin B-12 was purchased from Bio-Rad.

Cell Culture- Cultured bovine aortic endothelial cells (BAECs) were prepared as described by Schwartz (13) and grown in 10% calf serum, 1% penicillin (5,000 u/ml)-streptomycin (5,000 mcg/ml) solution, 1% 200 mM L-glutamine in RPMI 1640. Cell monolayers were grown to confluence in 100 mm diameter plates in a 5% CO_2 atmosphere at 37°C. Cells in passages 6-9 were used for the experiments.

Preparation and Concentration of Conditioned Medium-The confluent cell monolayers were washed 3 times with 7 ml of calcium, magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS). Five ml of CMF-DPBS containing 0.1% bovine serum albumin were then added to the cells, and the plates were placed in the 5% CO_2 incubator for 10 min at 37°C. The conditioned medium was collected, filtered through 0.2 micron filters and placed on ice. The conditioned medium was then concentrated approximately 20-fold by centrifugation (5,000 x g) at 4°C using a Centricon-10 Microconcentrator (Amicon). This concentrated conditioned medium was either immediately injected upon the HPLC system or frozen in liquid nitrogen, dried on a Savant Speed Vac Concentrator and stored at -20°C for later use.

Fractionation of Conditioned Medium by Gel Filtration High Performance Liquid Chromatography (HPLC)-The concentrated conditioned medium (100 µl) was fractionated by gel filtration HPLC using a Zorbax BioSeries GF-250 (DuPont) column on a Beckman System Gold HPLC system. The system was equilibrated at ambient temperature with CMF-DPBS (pH 7.2) at a flow rate of 1 ml/min and fractions were collected at 0.5 min intervals, starting 6.3 min after injection of the sample (corresponding to the void volume of the system).

Assay for the Generation of COOH-Terminal Tripeptide from ^{125}I-hANF- Assays were performed by combining 10 µl of 1 mM bestatin,1 mM amastatin, 100 µM carboxypeptidase inhibitor (potato tuber) in CMF-DPBS (pH 7.2), 30 µl of CMF-DPBS, 10 µl of 2 nM ^{125}I-hANF in CMF-DPBS and 50 µl of the appropriate fraction collected off the HPLC. The assay mixture was incubated at 37°C for 15 min followed by acidification to pH 2 with trifluoroacetic acid (TFA). Generation of the COOH-terminal tripeptide was quantified by reverse phase HPLC on a C18 resin as described by Johnson et al. (11) using a linear gradient of 15 to 35% acetonitrile (0.1% TFA) over 38 min.

Protease Inhibitor Studies- The conditioned medium was fractionated by gel filtration HPLC and the inhibitor studies were performed on the endopeptidase-containing fraction collected between 7.3 and 7.8 min after injection of the sample. Assays were performed by combining 10 µl of a 10X stock solution of inhibitor in CMF-DPBS (or just CMF-DPBS for control), 10 µl of 1 mM bestatin, 1 mM amastatin, 100 µM carboxypeptidase inhibitor (potato tuber) in CMF-DPBS, 20 µl of CMF-DPBS and 50 µl of the endopeptidase-containing fraction. This mixture was then allowed to incubate for 1 hour at room temperature followed by the addition of 10 µl of 2 nM ^{125}I-hANF. The reaction mixture was incubated for 15 min at 37°C followed by acidification to pH 2 with TFA. Production of the COOH-terminal tripeptide was quantified as previously described.

RESULTS

Estimation of Molecular Weight of Endopeptidase- The molecular weight of the endopeptidase was estimated by fractionating the concentrated conditioned medium derived from cultured BAECs by gel filtration HPLC and assaying the collected fractions for the ability to generate the COOH-terminal tripeptide from ^{125}I-hANF (Fig.1). The conditioned medium was fractionated in, and assays were performed in calcium, magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS) at pH 7.2. The aminopeptidase inhibitors bestatin (100 µM), amastatin (100 µM) and the carboxypeptidase inhibitor from potato tuber (10 µM) were included in the assay to block any potential breakdown of the tripeptide product due to exopeptidase action. The amount of the COOH-terminal tripeptide produced in the assay was quantified by reverse phase HPLC (11). The elution profile of the activity in fractions collected off the HPLC

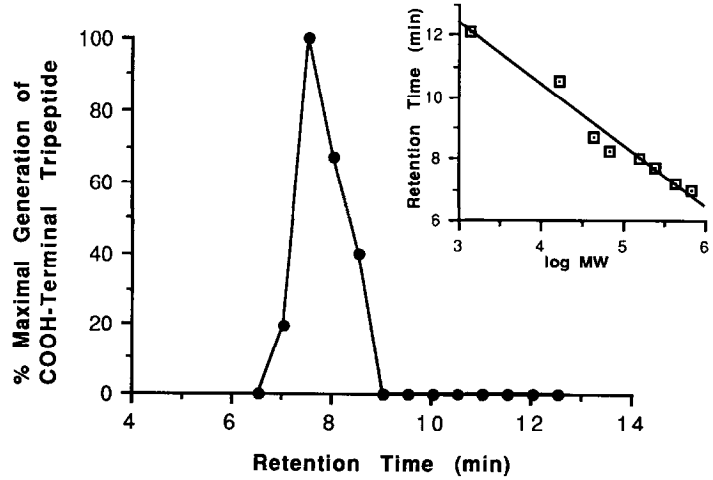

Figure 1: Fractionation of Conditioned Medium from Cultured Bovine Aortic Endothelial Cells by Gel Filtration HPLC. Conditioned medium was concentrated, fractionated at pH 7.2 by gel filtration HPLC and 0.5 minute fractions were assayed for the ability to generate the COOH-terminal tripeptide from ^{125}I-hANF as described in MATERIALS AND METHODS. Inset: Standard Curve for the Elution of Proteins by Gel Filtration HPLC. Standards used were thyroglobulin (molecular weight of 670 kilodalton (kD)), ferritin (450 kD), beef liver catalase (240 kD), bovine gamma globulin (158 kD), bovine serum albumin (67 kD), chicken ovalbumin (44 kD), horse myoglobin (17 kD) and vitamin B-12 (1.35 kD).

TABLE I

Effect of Various Protease Inhibitors on the Generation of the COOH-Terminal Tripeptide from ^{125}I-hANF by the Endopeptidase Produced by BAECs

Inhibitor (Concentration)	% Inhibition
Phenylmethylsulfonyl fluoride (0.2 mM)	0
Soybean trypsin inhibitor (0.1 mg/ml)	1
Aprotinin (2 ug/ml)	2
Leupeptin (0.5 µg/ml)	3
Antipain (50 µg/ml)	0
Pepstatin A (1 µM)	0
Phosphoramidon (10 µM)	1
Captopril (10 µM)	0
EDTA (1 mM)	30
EGTA (1 mM)	41
1,10 phenanthroline (1 mM)	100
N-ethylmaleimide (1 mM)	68
E-64 (1 mM)	0
Cystatin (1 µg/ml)	0
Calpain inhibitor I (17 µg/ml)	0
alpha$_2$-macroglobulin (1 µg/ml)	0

The conditioned medium was concentrated and fractionated by gel filtration HPLC. The fraction eluting between 7.3 and 7.8 min (See figure 1) was collected and utilized for inhibitor studies. Prior to addition of ^{125}I-hANF to a final concentration of 0.2 nM, the endopeptidase-containing fraction was preincubated for one hour at room temperature in the presence (or absence for control) of the inhibitor. Assays were performed in CMF-DPBS (pH 7.2) at 37°C as described in MATERIALS AND METHODS. % inhibition was calculated relative to the generation of the COOH-terminal tripeptide in the absence of inhibitor.

revealed a peak of activity centered at approximately 7.8 min, corresponding to a protein with an approximate molecular weight of 200,000 daltons (Fig.1).

Classification of Endopeptidase using Various Protease Inhibitors- Peptidases can be classified as either serine, cysteine, aspartic or metallopeptidases based upon their sensitivities to various protease inhibitors (14). The effects of these various protease inhibitors were tested on the endopeptidase-containing fraction collected off the HPLC and centered at approximately 7.55 min (see Fig.1). Prior to the addition of substrate, this endopeptidase-containing fraction was preincubated for 1 hour at room temperature in the presence (or absence for control) of the inhibitor (14). Inhibitors of serine proteinases (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, aprotinin), aspartic proteinases (pepstatin A) and cysteine proteinases (E-64, cystatin, calpain inhibitor I) had little or no effect upon the generation of the COOH-terminal tripeptide from ^{125}I-hANF (Table I). Other less group-specific protease inhibitors, such as antipain, leupeptin and alpha$_2$-macroglobulin also had no significant inhibitory effect. The metalloproteinase inhibitors EDTA, EGTA and 1,10 phenanthroline did significantly inhibit the proteolysis, indicating that this activity can be attributed to a metalloendo-

peptidase (14-16). Of all the protease inhibitors tested, only 1,10 phenanthroline completely blocked (100% inhibition) the peptidase activity. Phosphoramidon (an inhibitor of many metalloproteinases) and the angiotensin converting enzyme inhibitor, captopril (17), had no significant effect upon this metalloendopeptidase activity. Interestingly, the sulfhydryl-reacting reagent, N-ethylmaleimide (1 mM), was found to inhibit the endopeptidase activity by approximately 68%.

DISCUSSION

In previous studies from our laboratory, we have observed the extracellular proteolytic generation of the COOH-terminal tripeptide from ^{125}I-hANF by cultured vascular cells (11,12). Since the COOH-terminal region of ANF is critical to the generation of intracellular cyclic GMP in cultured vascular cells (6,7), our observations suggest that this peptidase may play a role in ANF metabolism in vivo. It is important to note that the findings reported here involve an endopeptidase activity and not a homogeneous purified enzyme. To specifically address such issues as substrate specificity and structure, it will be necessary to purify the peptidase.

Using inhibitors of various classes of proteases, we have concluded that a metalloendopeptidase is responsible for the observed generation of the COOH-terminal tripeptide from ^{125}I-hANF. The results indicate that the endopeptidase has the characteristics of a metalloendopeptidase and not a metal-activated endopeptidase (15,16). Metalloendopeptidases, in general, contain zinc as the essential metal and most often cleave bonds adjacent to hydrophobic residues (15). Therefore, it is not surprising that we have found that a metalloendopeptidase cleaves the peptide bond between Ser^{123} and Phe^{124} of ^{125}I-hANF. The metal can play a structural role or a catalytic role (at active site) in the action of a metalloendopeptidase (15).

It was interesting to observe that the sulfhydryl-reacting reagent, N-ethylmaleimide, inhibited the enzymatic activity of the peptidase. It is not unusual for sulfhydryl reagents to affect the activity of a peptidase by reacting with cysteine residues at sites other than the active site (16). However, specific sulfhydryl-reacting reagents such as methylmethanethiosulfonate and p-hydroxymercuriphenylsulfonate have been used to displace Zn^{2+} from metalloproteins by reacting with sulfhydryl groups necessary for the chelation of the metal (18,19). Generally, the inhibitory action of N-ethylmaleimide on the hydrolytic action of the peptidase studied in this report suggests that one or more cysteine residues play a critical role in the structure and/or activity of this peptidase.

Two other metalloendopeptidases have been documented to catalyze cleavage between Ser^{123} and Phe^{124} of ANF. One, rich in the renal brush border, is endopeptidase 24.11 (enkephalinase) (20) while there is also one derived from atrial tissue, atrial dipeptidyl carboxyhydrolase (21). The metalloendopeptidase which we have partially characterized in this report is neither of these enzymes, since it was not inhibited by phosphoramidon or captopril. Based upon our limited knowledge about the structure and

substrate specificity of this metalloendopeptidase, it is not possible to conclude whether this is a novel enzyme or one that has been previously characterized.

At the present, we do not know whether the peptidase is secreted from inside the cell or is released from the surface of the cell. Peptidases can be bound to the surface of cells through hydrophobic membrane-spanning segments (15), through glycosyl-phosphatidyl inositol moieties (22) or be loosely associated with the plasma membrane (16). Soluble forms of membrane bound peptidases are often observed, as in the case of endopeptidase 24.11 (23), angiotensin converting enzyme (24) and rat brain metalloendopeptidase 24.15 (25). Nevertheless, cultured bovine aortic endothelial cells produce a significant amount of a soluble metalloendopeptidase that removes the COOH-terminal tripeptide from ^{125}I-hANF, which should aid in future studies assessing the peptidase's potential role in the metabolism of ANF in vivo.

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