Inactivation of Endothelin-1 by an Enzyme of the Vascular Endothelial Cells

Herbert L. Jackman, Paul W. Morris, Sara F. Rabito, Gerd B. Johansson, Randal A. Skidgel, and Ervin G. Erdös

We previously investigated the inactivation of endothelin-1 by deamidase (lysosomal protective protein), present in many cells, including vascular smooth muscle cells. This enzyme, which we originally purified from human platelets, preferentially hydrolyzes peptides at the C-terminus with hydrophobic amino acids in the P₁ or P₁' position or both and thereby inactivates endothelin-1, which has a C-terminal sequence of Ile₁⁰-Ile₁⁰-Trp₂¹-OH. We tested for the presence of deamidase in cultured bovine aortic endothelial cells. The final supernatant of the homogenized cells (S₃) cleaved the deamidase substrate dansyl-Phe-Leu-Arg at a rate of 1-3 nmol/min per 10⁶ cells at pH 5.5 at 37°C. Endothelin-1 was completely inactivated by the S₃ fraction as determined on rat thoracic aorta strips. The major site of inactivation was the Ile₁⁰-Trp₂¹ bond, established by high performance liquid chromatography and by amino acid analysis where the main product was des-Trp₂¹- endothelin-1. The hydrolysis of endothelin-1 (5.9 nmol/min per milligram of protein at pH 5.5 at 23°C) by S₃ was blocked mainly by inhibitors of deamidase, including diisopropyl fluorophosphate, but not by inhibitors of some other peptidases. This is the first report of a novel pathway of endothelin-1 metabolism in endothelial cells. Thus, endothelial cells, besides being the source of endothelin-1, contain an enzyme that inactivates it.

(Hypertension 1993;1:925-928)

Key Words • cathepsin A • deamidase • endothelium • high performance liquid chromatography • vasoconstriction • carboxypeptidases • aorta • bioassay

Endothelin-1 (ET-1) is the most potent naturally occurring peptide that contracts vascular smooth muscle, causing a long-lasting increase in blood pressure. Because ET-1 is synthesized in endothelial cells and is released in a preferentially polar direction toward underlying smooth muscle, a role for ET-1 in the control of blood pressure has been hypothesized.

Recently, we isolated and characterized an enzyme from human platelets that has deamidase, carboxypeptidase, and esterase activities. We named the enzyme deamidase because it deamidates peptides such as tachykinins, converting Met₁⁰-NH₂ to Met-OH and thereby inactivating them. Although deamidase and esterase activities are highest at neutral pH, the optimal pH for carboxypeptidase activity lies in the acid range as shown with substrates such as angiotensin I and bradykinin. Partial sequencing of this human serine carboxypeptidase revealed that it is identical with the so-called lysosomal protective protein and that its physical and enzymatic characteristics are similar to those reported for cathepsin A.

Deamidase preferentially cleaves naturally occurring substrates where the P₁ and/or P₁' amino acid is hydrophobic and synthetic substrates with hydrophobic amino acids in the P₁ and P₂ or P₁ and P₁' positions. Because the structure of ET-1 combines these elements at its C-terminus (-Ile₁⁰-Ile₁⁰-Trp₂¹), we tested it with the purified enzyme and found ET-1 to be an excellent substrate for deamidase, yielding the inactive des-tryp- tophan₂¹-ET-1 as the major product.

Here we report that ET-1, which is primarily synthesized in endothelial cells, is inactivated by deamidase present in these cells.

Methods

Materials

- 5-Dimethylaminonaphthalene-1-sulfonyl-L-phenylalanin-l-leucyl-l-arginine (dansyl-Phe-Leu-Arg) was synthesized by coupling dansyl-Phe to Leu-Arg using standard techniques.
- Diisopropyl fluorophosphate (DFP), trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane (E-64), p-chloromercuribenzenesulfonate (PCMS), N-tosyl-Phe-chloromethyl ketone (TPCK), and other laboratory reagents were obtained from Sigma Chemical Co., St. Louis, Mo., and benzoxycarbonyl (Z)-Gly-Leu-Phe-CH₂Cl (CK8) was from Enzyme Systems Products, Livermore, Calif.

Cell Preparation

Bovine aortic endothelial cells (BAE) were established as a primary cell culture and subcultured before use. The BAE were then harvested, homogenized by sonication, and subjected to a subcellular fractionation.
The final soluble fraction (S3) after centrifugation at 100,000g for 60 minutes was used as the source of deamidase.

When the total activity in cells was measured, BAE were harvested, counted, homogenized by sonication, and treated with 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate (CHAPS) detergent (1%). The supernatant after centrifugation at 16,000g for 10 minutes was then the source of deamidase.

Enzyme Assays

Deamidase activity with ET-1 as substrate was determined by high performance liquid chromatography (HPLC) with a 30-minute gradient of 15% to 45% acetonitrile containing 0.05% trifluoroacetic acid. Reaction mixtures contained BAE-S3 fraction, 10 mmol/L ET-1, 100 mmol/L buffer (sodium acetate at pH 5.5 or 4-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] [HEPES] at pH 7.4), and water to 100 µL. Reactions were carried out at 23°C for 10 or 30 minutes and stopped with an equal volume of ethyl alcohol. Inhibitors were preincubated in the reaction mixture at 23°C for 15 minutes before the addition of ET-1. Deamidase activity is taken as the difference in activity in the presence and absence of its inhibitor, 1 mmol/L DFP.

The carboxypeptidase activity of deamidase was further assessed with dansyl-Phe-Leu-Arg. Each reaction tube contained BAE (either S3 or total cell preparation), 100 mmol/L sodium acetate, pH 5.5, 200 µmol/L dansyl-Phe-Leu-Arg, and water to 250 µL final volume. Reaction mixtures were incubated at 37°C for 30 minutes and stopped with 150 µL of 1 M citric acid, pH 3.1. The reaction product was extracted into 1 mL of chloroform, and the fluorescence was measured at 340 nm excitation and 495 nm emission wavelengths. Deamidase activity was taken as the difference in fluorescence between reactions done in the presence and absence of its inhibitor, 1 mmol/L DFP.

Amino Acid Analysis of Des-Trp21-Endothelin-1

Enzymatic reactions using BAE-S3 and ET-1 (35 µmol/L) were run in the presence and absence of 1 mmol/L DFP as described above. Aliquots were fractionated by HPLC, and the product and substrate peaks were collected. Amino acid analysis by coupled 6N HCl hydrolysis/phenylisothiocyanate derivatization was performed on an Applied Biosystems Inc. 420H/130 instrument. Hydrolysis was done in the presence of 1-dodecanethiol scavenger to afford tryptophan recovery ranging from 45% to 55% at 250 pmol input.

Bioassay

The inactivation of ET-1 by the S3 fraction of BAE at pH 5.5 was determined in a bioassay using rat aortic strips denuded of endothelial cells. The enzymatic reaction was carried out as described for ET-1, but it was stopped by cooling to 4°C. Aliquots of the reaction mixtures were added to the tissue bath (250 pmol/L of ET-1) containing the rat aortic strip, and isotonic contractions were recorded; parallel samples of the reaction mixture were analyzed in the HPLC system.

Results

Cleavage of Endothelin-1

The S3 fraction, the final supernatant of homogenized BAE, cleaved ET-1 at a considerable rate. At 23°C and pH 5.5, 5.9±1.2 nmol was hydrolyzed in 1 minute by 1 mg protein as established by the decrease in the authentic ET-1 substrate peak in HPLC (Figure 1). The hydrolysis of ET-1 at pH 7.4 was less than 10% of the activity seen at pH 5.5. The enzymatic activity was characterized further by using inhibitors (Table 1). DFP (1 mmol/L) completely abolished the appearance of a new peak, attributed to the product des-Trp21-ET-1, and inhibited the reaction.

Table 1. Inhibition of Endothelin-1 Hydrolysis by Bovine Endothelial Cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mmol/L)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td>E-64</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PCMS</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>CK8</td>
<td>0.1</td>
<td>99</td>
</tr>
<tr>
<td>CaCl2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>0.01</td>
<td>32</td>
</tr>
<tr>
<td>TPCK</td>
<td>0.1</td>
<td>84</td>
</tr>
</tbody>
</table>

DFP, diisopropyl fluorophosphate; E-64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; EDTA, ethylenediaminetetraacetic acid; PCMS, p-chloromercuribenzenesulfonate; CK8, benzoyloxy carbonyl (Z)-Gly-Leu-Phe-CH2Cl; TPCK, N-tosyl-Phenylmethyl ketone.
Bioassay of the Inactivation of Endothelin 1 (ET1) by Endothelial Cell S3 Fraction

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (nmol/min per 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM DFP</td>
<td>[Average values]</td>
</tr>
<tr>
<td>ET1</td>
<td>1.3 ±0.3</td>
</tr>
<tr>
<td>Endothel. Cell S3</td>
<td></td>
</tr>
<tr>
<td>ET1 + 1 mM DFP</td>
<td>0.1 ±0.1</td>
</tr>
</tbody>
</table>

Figure 2. Recording on isolated rat aortic strip shows the effect of the S3 fraction of homogenized endothelial cells on endothelin-1 (ET1). Incubation mixtures and control diisopropyl fluorophosphate (DFP) were injected into the isolated tissue bath as indicated by arrows. Endothelial cell extract inactivated ET1, very likely by cleaving Trp21 as shown in Figure 1. Added DFP inhibited the inactivation, and ET1 caused a long-lasting contraction.

Discussion

Deamidase is a two-chain serine peptidase, which has three different types of activity.\(^5\) At neutral pH it hydrolyzes ester bonds and deamidates C-terminal amino acids; for example, it converts substance P (Met¹-NH₂) to substance P-free acid (Met¹-OH) or enkephalinamide to enkephalin. It is also a carboxypeptidase that can cleave free and protected C-terminal amino acids; for example, it releases Gly⁴-NH₂ of oxytocin.\(^5\) The carboxypeptidase activity is more effective at an acid pH.

After deamidase was purified from human platelets and the first 25 amino acids of each chain were sequenced, the identity with the so-called lysosomal proteolytic protein was established.\(^5,\)\(^7\) Although this protein can form a high molecular weight complex with β-galactosidase and neuramidase in lysosomes, a noncomplexed form is released by thrombin from human platelets.\(^5\) The lack of the mature protein can cause a genetically determined disease, β-galactosidosis. Besides being present in platelets\(^5\) or fibroblasts,\(^7\) the enzyme is present in other cells, for example, in smooth muscle cells.\(^6\) These experiments demonstrated that 70% to 80% of the inactivation of endothelin by the final supernatant of endothelial cells can be attributed to deamidase. The additional enzymes that contribute to the residual activity were not characterized further.

Deamidase also shares properties in common with cathepsin A, and as a serine carboxypeptidase, belongs to a family of enzymes together with yeast carboxypeptidase Y, KEX-1 gene product,\(^5,\)^7 plant carboxypeptidases,\(^9\) and human prolylcarboxypeptidase.\(^10\)

The current and previous experiments\(^6\) show that deamidase inactivates ET-1 by removing a single amino acid, Trp²¹. The newly revealed C-terminal Ile²⁵. Ile⁶-OH seems to be resistant to further degradation under our conditions.

The goal of this investigation was to determine if deamidase is present in endothelial cells. The identity of the inactivator of ET-1 in endothelial cells with deamidase was shown by HPLC analysis and by amino acid analysis of the reaction products, by using inhibitors, and by the cleavage of a fluorescent substrate of the enzyme. Because of the affinity of deamidase for hydrophobic amino acids, especially in the P₁ position, it is inhibited by some but not all inhibitors of chymotrypsin-type enzymes such as cathepsin G.\(^4\) Thus, chymostatin or (Z)-Gly-Leu-Phe-CH₂Cl inhibits the deami-
dase, but lima bean trypsin inhibitor does not. Dansyl-Ph-Leu-Arg is cleaved rapidly by endothelial cell deamidase, and the inhibition pattern of this reaction is similar to that of ET-1 hydrolysis.

Vascular endothelial cells as the source of ET-1 inactivator are of special interest, because besides releasing endothelins,3,4,8 they also contain receptors for ET-1.8,11 Because the inactivation of ET-1 proceeds at an acidic pH (5.5) much faster than at neutral pH,6 deamidase, as a lysosomal enzyme, probably inactivates ET-1 intracellularly after it is taken up by receptor-mediated endocytosis.8,12 On the other hand, it could also be present on the cell membrane of endothelial cells, as we have noticed with granular cathepsin G, which is also active on the plasma membrane of granulocytes.13

Neutral endopeptidase 24.11 or enkephalinase (NEP) also inactivates ET-1 by cleaving at two internal peptide bonds14,15 different from the bond of the C-terminal amino acid split by deamidase. Under our conditions, NEP did not participate in the inactivation of ET-1, as suggested by the fact that its inhibitor, phosphoramidon, did not block the reaction observed (not shown) at an acidic or neutral pH—the latter is optimal for NEP. Furthermore, EDTA did not block ET-1 hydrolysis as reported here. We have demonstrated in other studies that the concentration of NEP in vascular endothelial cells is very low.16 NEP as a plasma membrane-bound enzyme could contribute to ET-1 inactivation at the subendothelial level, for example, on the vascular smooth muscle cells.

In conclusion, vascular endothelial cells, besides being a site of origin for ET-1 and containing its receptors, also have a potent inactivator of the peptide, deamidase, which removes its C-terminal tryptophan.6,17

References
Inactivation of endothelin-1 by an enzyme of the vascular endothelial cells.
H L Jackman, P W Morris, S F Rabito, G B Johansson, R A Skidgel and E G Erdös

Hypertension. 1993;21:925-928
doi: 10.1161/01.HYP.21.6.925

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/21/6_Pt_2/925

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/