Generation of human endothelin by cathepsin E

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Highly-purified cathepsin D processed human big endothelin$_{1-38}$ into endothelin-like fragments but did not appear to generate endothelin$_{1-21}$ under the conditions employed. By contrast, human cathepsin E specifically cleaved human big endothelin into endothelin$_{1-21}$ and the C-terminal fragment under identical conditions but did not degrade either product further.

Cathepsin D; Cathepsin E; Big endothelin; Endothelin generation

1. INTRODUCTION

Human endothelin (ET) is a 21-residue polypeptide produced by endothelial cells. In addition to displaying a vasoconstrictor activity more powerful than that of angiotensin II [1], the peptide produces a number of other pharmacological actions [2]. Endothelin is believed to be generated by a single proteolytic cleavage of the bond linking residues Trp-21–Val-22 (Fig. 1) in its immediate biosynthetic precursor, big ET, by a hitherto unidentified endopeptidase termed endothelin-converting enzyme. Since the vasoconstrictor activity of big ET is much less potent than that of ET [3,4], inhibition of this converting enzyme activity might have considerably therapeutic value for the treatment of hypertension.

The conversion of big ET to ET is inhibited by pepstatin, suggesting the participation of a cellular aspartic proteinase [5,6]. Pepsin, if added in high enough quantities, will convert big ET in vitro, specifically into ET plus CT at pH 2.3 [7] but, physiologically, pepsin is unlikely to be the enzyme responsible for the processing. The only known aspartic proteinases produced in human tissues (apart from the gastrointestinal and reproductive tracts) are renin, cathepsin D and cathepsin E [8]. It has been reported very recently [10,11] that a cathepsin D-like activity is capable of converting big ET into a form that is immunoreactive with antibodies which recognise ET. Under the conditions employed in the present report, we demonstrate that purified human or rat caphepsin E, but not cathepsin D, converts big ET specifically into ET and CT and might thus be considered as a likely candidate for the endothelin-converting enzyme activity.

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Abbreviations: big ET, 38 residue precursor of human endothelin-1; ET, 21 residue endothelin-1; CT, 17 residue C-terminal fragment of the big ET precursor; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

Synthetic human big ET, ET and CT were obtained from the Peptide Institute Inc. (Osaka, Japan). Big ET and CT radio-iodinated specifically at Tyr-31 (Fig. 1) were kindly prepared (by Phil Robinson in the Amersham Development Group) using a modification of the lactoperoxidase method [12]. ET and big ET labelled with ^{125}I on Tyr-13 were from Amersham plc (Bucks, UK). Two radioimmunoassays (termed **X** and **Y** for convenience of understanding) were configured. The **X** assay was the endothelin$_{1-21}$ specific ^{125}I-assay system (Amersham RPA 555) which has the following cross-reactivities: ET 100%; human big ET 0.4%; pig big ET 0.26%; human CT <0.003%. The assay has an ED_{50} of ~3 fmol/tube and permits the specific detection of ET. The **Y** assay was the rather less specific endothelin-1,2 (high sensitivity) ^{125}I-assay system (Amersham RPA 545) which has the following cross-reactivities: ET 100%; human big ET 189%; pig big ET 63%; human CT <0.003%. The assay has an ED_{50} of ~4.5 fmol/tube.

Human cathepsin D was prepared from spleen as described previously [13] and rat cathepsin D was a kind gift from Dr G. Bonelli, University of Turin, Italy. Cathepsin E of human/rat origin [14] was generously provided by Drs I.M. Samloff (V.A. Medical Centre, Sepulveda, CA, USA) and Dr K. Yamamoto (Kyushu University, Japan). Big ET (4 μg; 1 nmol), ET or CT were incubated (separately) for 1, 2 or 6 h at 37°C with individual purified proteinases (up to 0.1 μg; 0.0025 nmol) in 50 μl of 0.1 M sodium acetate or citrate buffers at the indicated pH values. Reactions were stopped by the addition of 200 μl of 5% (w/v) TFA prior to loading aliquots (200 μl) onto a C_{18} Vydac reverse phase HPLC column (Hichrom Ltd., Reading, UK). A gradient from 0 to 60% acetonitrile containing 0.1% TFA was used over 20 min for elution at a flow rate of 2 ml/min. Peak retention times were identified by chromatography of authentic standards of big ET, CT and endothelin. Fractions (0.8 ml) were collected and analysed in the **X** and **Y** RIAs. When radioiodinated peptides were employed, identical assays were performed but with the inclusion of 0.1 μCi of the appropriate labelled tracer and the use of an HPLC gradient of 0% to 40% acetonitrile plus 0.1% TFA, followed by isocratic

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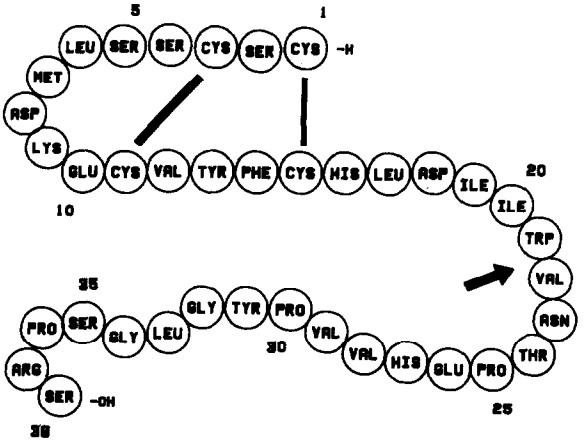

Fig. 1. Structure of human big endothelin. The site required to be cleaved to generate endothelin and release the C-terminal fragment is indicated by an arrow.

elution at 40% acetonitrile and 1 ml/min. Radioactive peaks were monitored by means of an in-line loop held within a Roberts tube connected to a ratemeter (ESI Nuclear, Ruislip, UK) and receptor.

Sequencing was performed by automated Edman degradation with an Applied Biosystems Model 473 sequencer.

3. RESULTS AND DISCUSSION

Initial experiments were carried out by incubating the precursor, big ET (4 μg), for 1, 2 and 6 h at pH 4.7 with homogeneous human cathepsin D (0.1 μg). The reaction products were analysed by HPLC (Fig. 2a) and RIA (Fig. 2b) but only the 6 h data are illustrated since the other time points of analysis showed similar patterns of digestion. None of the products observed on reverse phase HPLC corresponded to authentic synthetic standard ET. Furthermore, no immunoreactivity was observed in any of the newly-generated peaks using the X RIA which is highly specific for ET (Fig. 2b). By contrast, the much less discriminatory Y RIA indicated ET-like immunoreactivity in the fractions eluting between the positions of CT and big ET. The peak that eluted first on the gradient (Fig. 2a) was not precisely identical with the elution position of authentic CT. Its area was estimated to be less than 20% of the expected peak size if complete conversion to CT itself had occurred. Experiments performed with [^{125}I]big ET labelled at position 133 (Fig. 1) confirmed the production of one major and one minor labelled ET-like fragment by human cathepsin D but no peak of radioactivity corresponding to ET was detected in the elution profile (data not shown).

When highly-purified cathepsin D (0.1 μg) from rat was incubated for 2 h with big ET (4 μg), multiple degradation products were produced (Fig. 2c) but, once again, no formation of ET was observed. Thus, it would appear that big ET is digested by cathepsin D in a more complex manner than the specific generation of ET. However, the possibility remained that the conversion of big ET to ET + CT was slow by comparison with the subsequent further degradation of ET/CT. This was investigated by incubation of synthetic ET and CT, each at 2 μg (separately), for 2 h with human cathepsin D (0.1 μg). Fig. 3a shows that ET is indeed degraded readily by human cathepsin D whereas CT was not affected (Fig. 3b). It is noteworthy that residual big ET was still present even after 6 h incubation with cathepsin D (Fig. 2a) whereas all of the ET added initially was destroyed in less than 2 h (Fig. 3a) after addition to cathepsin D. Thus, if cathepsin D were to be involved in the conversion of big ET to ET in vivo, this reaction would not appear to be of particular benefit biosynthetically, since the destruction of the newly-formed ET would be faster than its generation.

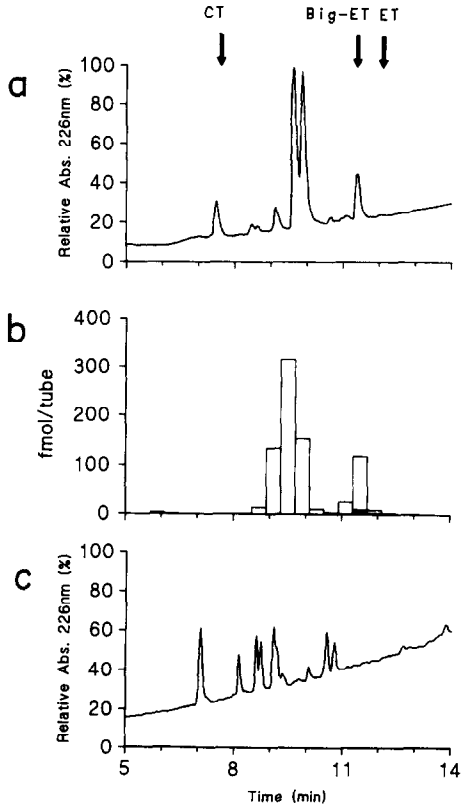

Fig. 2. Effect on human big ET of human (a) and rat (c) cathepsin D at pH 4.7 and 37°C. Fractions collected from the reverse phase HPLC column in (a) were analysed (b) by the X (closed bars) and Y (open bars) radioimmunoassays. The positions of elution of authentic CT, big ET and ET applied to the HPLC column as markers were as indicated.

Attention was therefore focussed on the other known intracellular aspartic proteinase, cathepsin E. Samples of big ET (4 μg) were incubated for 1 and 6 h with the homogeneous human enzyme (0.1 μg; a molar ratio of 400:1). The reaction resulted in the generation of two peaks (Fig. 4a), the elution positions of which coincided exactly with those of CT and ET. Close to complete conversion was observed within 2 h. Diminution of the

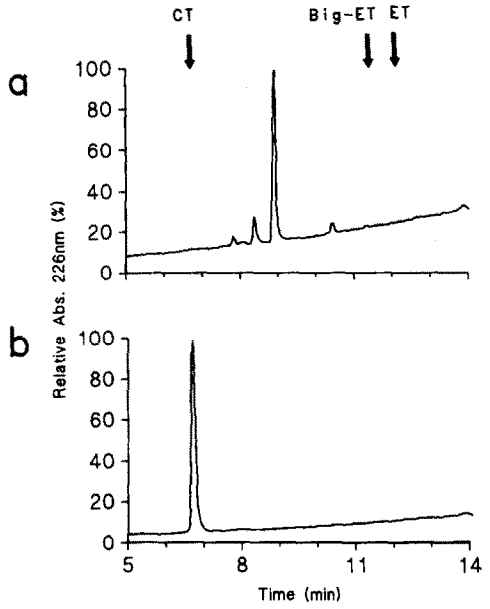

Fig. 3. Effect of human cathepsin D on human ET (a) and CT (b) at pH 4.7 and 37°C.

amount of cathepsin E included in the incubation mixture to 0.05 or 0.01 μg (i.e. molar ratios of big ET/cathepsin E of 800:1 or 4000:1) resulted in the conversion of approx. 80% and 15%, respectively, of big ET in 2 h incubation at 37°C. In order to confirm the identity of the product corresponding in elution position to the authentic C-terminal standard (Fig. 4a), the peak was collected and subjected to sequence analysis. The complete sequence of this product was:

Val-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser-Pro-Arg-Ser (repetitive yield = 87.3%)

thus indicating specific cleavage by human cathepsin E at the site in human big ET (Fig. 1) proposed for the endothelin-converting enzyme [1].

The peaks from the HPLC elution were also analysed using both RIA methods employing the ET-specific **X** and the less-specific **Y** RIAs (Fig. 4b). The latter recognised both the residual big ET (second peak) and the newly-generated third peak (ET) whereas the ET-specific **X** RIA reacted only with the third peak, thus identifying it as ET. The availability of the ET specific **X** antibody permits such an unequivocal identification of ET consisting of residues 1-21. Such a well-characterised specific and sensitive assay was not employed for precise immunological identification of reaction products in previous reports (e.g. [10]). Additional experiments were also carried out in 0.1 M sodium citrate buffers at pH 5.5 and 6.2. (in the presence of 6 mM ATP to stabilise the activity of cathepsin E [15]). Identical elution patterns were observed to that depicted for pH 4.7 in Fig. 4a with con-

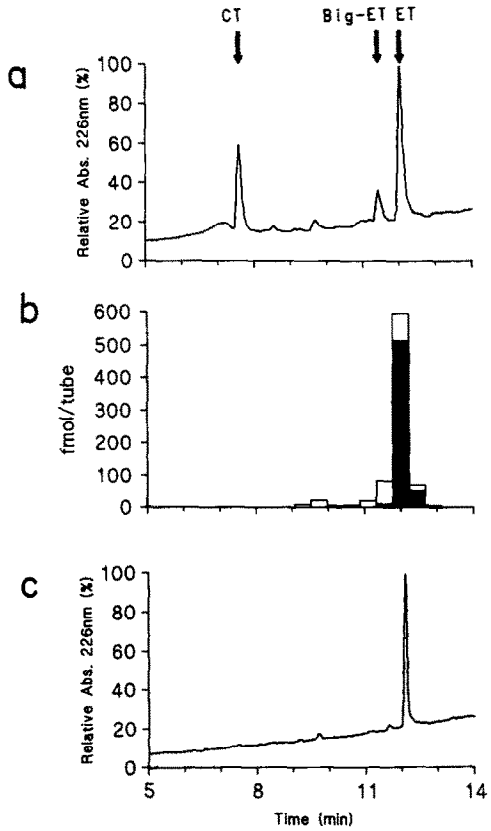

Fig. 4. Effect of human cathepsin E on human big ET (a) and ET (c) at pH 4.7 and 37°C. Fractions (0.8 ml) collected from the reverse phase HPLC column in (a) were analyzed (b) by both the **X** (closed bars) and **Y** (open bars) radioimmunoassays.

version of approx. 50% and 15%, respectively, of the precursor in 2 h by 0.1 μg of cathepsin E.

Incubation of aliquots of synthetic ET (Fig. 4c) or CT (not shown) with human cathepsin E resulted in no proteolysis of either peptide. It would thus appear that human cathepsin E does specifically convert big ET into ET plus CT (Fig. 1) with no further degradation thereafter. Identical results were also obtained with cathepsin E of rat origin (data not shown). When big ET radioiodinated at position 13 (Fig. 1) was incubated with human cathepsin E, the label was detected on radio-HPLC only in the elution position corresponding to that of ET. Similarly, when the enzymic digestion was performed with the precursor radiolabelled at position 31 (Fig. 1), the conversion by human cathepsin E paralleled that observed with the non-labelled substrate and the isotype was detected by radio-HPLC this time in an identical elution position to that of authentic [^{125}I]CT.

Finally, overnight incubation at 37°C of human big ET (4 μg) with human renin (up to 1 μg) resulted in no conversion of the precursor whatsoever. Renin is believed to function as an extracellular aspartic proteinase whereas cathepsin E and cathepsin D (with the exception of transformed cells [16]) are not secretory en-

zymes but are both located, albeit in different compartments, within cells (I.M. Samloff, personal communication). Thus, it would appear that if a (known) aspartic proteinase is to be responsible for the specific conversion of big ET to ET as has been suggested [10,11], then the reaction ought to take place intracellularly. The work described herein has focussed primarily on human (and to a lesser extent, rat) enzymes and it should be emphasised that earlier reports with extracts of cells/tissues have been performed with material of bovine or porcine origin (e.g. [5,6,10,11]). It is not clear at present whether the same enzyme is responsible for endothelin conversion in all species but since blood pressure regulation in man is of considerable pharmaceutical interest, our ongoing studies are directed towards identification of the proteinase in endothelial and other [8] cells that catalyses this important reaction.

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