Mode of cleavage of pig big endothelin-1 by chymotrypsin

Production and degradation of mature endothelin-1

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Pig endothelin-1 [ET-1-(1–21)] seems to be produced via proteolytic processing between Trp-21 and Val-22 of an intermediate form consisting of 39 amino acid residues, termed big ET-1-(1–39), by a chymotrypsin-like proteinase. We examined the chymotryptic-cleavage sites of big ET-1-(1–39) by reverse-phase h.p.l.c. and sequence analysis, and found that chymotrypsin cleaved initially the Tyr-31–Gly-32 bond of big ET-1-(1–39), followed by cleavage between Trp-21 and Val-22. Furthermore, chymotrypsin hydrolysed the generated ET-1-(1–21), producing a single major product that had the same amino acid sequence as ET-1-(1–21) with a cleavage between Tyr-13 and Phe-14. The disulphide bridge between Cys-1 and Cys-15 remained intact. These results indicate that the conversion of big ET-1-(1–39) into ET-1-(1–21) catalysed by chymotrypsin requires hydrolysis of the Tyr-31–Gly-32 bond before that of the Trp-21–Val-22 bond, an event followed by cleavage between Tyr-13 and Phe-14 within the loop of ET-1-(1–21). Thus a chymotrypsin-like proteinase might be involved not only in the production but also in the degradation of ET-1-(1–21) *in vivo*.

INTRODUCTION

Endothelin-1 [ET-1-(1–21)], isolated from culture medium of pig aortic endothelial cells, is a potent vasoconstrictor peptide consisting 21 amino acid residues with two intramolecular disulphide bonds [1]. Yanagisawa *et al.* [1] proposed a possible biosynthetic pathway for the production of the mature ET-1-(1–21), i.e. the precursor form is initially processed by dibasic-pair specific proteolysis to produce a 39-amino acid-residue intermediate form, termed big ET-1-(1–39), and the big ET-1-(1–39) is converted into the mature form by an unusual proteolytic processing between Trp-21 and Val-22. Recent studies [2,3] demonstrated that the big ET-1-(1–39) and its *C*-terminal fragment consisting of 18 amino acid residues, termed big ET-1-(22–39), are present in the culture medium of endothelial cells, as is ET-1-(1–21). This suggests that ET-1-(1–21) is generated from big ET-1-(1–39) via a single cleavage between Trp-21 and Val-22, by a putative big-ET-1-(1-39)-converting enzyme, in vascular endothelial cells.

Yanagisawa *et al.* [1] also suggested that an endopeptidase with chymotrypsin-like proteinase specificity is presumably involved in the proteolytic processing between Trp-21 and Val-22 of big ET-1-(1–39). McMahon *et al.* [4] found that chymotrypsin-treated big ET-1-(1–40) produced an ET-1-(1–21)-like contraction of isolated rat aortic rings, and a characteristic ET-1-(1–21)-like effect on blood pressure *in vivo*. However, the mode of conversion of big ET-1-(1–39) into ET-1-(1–21) by chymotrypsin is less well understood.

We have now examined amino acid sequences of the cleavage products obtained from big ET-1-(1–39) by chymotrypsin treatment, and obtained evidence that chymotrypsin hydrolyses the Tyr-31–Gly-32 bond in the big ET-1-(1–39) molecule before the production of ET-1-(1–21) by cleavage of the Trp-21–Val-22 bond. In addition, we found that chymotrypsin cleaves the Tyr-13–Phe-14 bond in the ET-1-(1–21) molecule.

EXPERIMENTAL

Materials

Synthetic big ET-1-(1–39) and ET-1-(1–21) were purchased from the Peptide Institute (Osaka, Japan), dissolved in sterile water at a concentration of 0.1 mM and stored at −85 °C until use. Chymotrypsin (bovine pancreas; type VII) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), dissolved in 1 mM-HCl and stored at −85 °C until use. All other chemicals were either h.p.l.c. grade or analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

Chymotrypsin treatment

A 50 μl portion of chymotrypsin solution (20 μg/ml) diluted with 50 mM-Tris/HCl buffer, pH 8.0, was prewarmed with 40 μl of the same buffer at 37 °C for 5 min, and then 10 μl of 0.1 mM-big ET-1-(1–39) or -ET-1-(1–21) was added. After incubation at 37 °C for various periods, the reaction was stopped by the addition of 10% (v/v) trifluoroacetic acid and the preparation was then subjected to reverse-phase h.p.l.c. The control was performed by the addition of the above buffer instead of chymotrypsin solution.

Analytical procedures

Reverse-phase h.p.l.c. analyses were performed with a Waters apparatus consisting of a model 600E multi-solvent delivery system, a model U6K injector and a model 990J photodiode-array detector. The column used was Capcell-Pak $5C_{18}$-SG300 (4.6 mm × 250 mm) from Shiseido (Tokyo, Japan). Elution was performed with 0.02% (v/v) trifluoroacetic acid in acetonitrile (solvent A). The gradient consisted of a linear one of 0–35% (v/v) solvent A in 15 min, followed by isocratic elution at 35% (v/v) solvent A for 15 min and a linear gradient of 35–63% (v/v) solvent A in 15 min. The flow rate was 0.5 ml/min. Eluates were

Abbreviation used: ET-1, endothelin-1.

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monitored by the absorbance at 215 nm, and peak areas were calculated with an NEC PC-9801 VX personal computer (Tokyo, Japan) coupled to the detector. Automated Edman degradation was performed with a model 477A pulsed-liquid-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The resulting phenylhydantoin derivatives were identified by an Applied Biosystems phenylhydantoin analyser (model 120A), linked on-line with the sequencer.

RESULTS AND DISCUSSION

Reverse-phase h.p.l.c. profiles of big ET-1-(1–39) and ET-1-(1–21) treated with chymotrypsin as a function of time are presented in Fig. 1. In this experiment intact big ET-1-(1–39) was eluted at a retention time of 41 min, as described previously [5]. After only 0.2 min of incubation the big ET-1-(1–39) had practically disappeared, and five cleavage products were detected, at retention times of 32, 36, 41.5, 42 and 43 min; we designated these as peaks 1, 2, 3, 4 and 5, in the order of elution. Subsequently, we incubated big ET-1-(1–39) with chymotrypsin for several periods up to 30 min, separately applied their reaction mixtures to a C_{18} column and collected each peak for sequence analysis. On the other hand, when synthetic ET-1-(1–21) was applied to the column, one major and one minor peak were detected, at retention times of 43 min and 41.5 min respectively. The major peak is an intact form of ET-1-(1–21), as described previously [5]. The minor peak is probably a Met-7 sulphoxide form of ET-1-(1–21), which is known to be eluted slightly earlier than the intact form by reverse-phase h.p.l.c. on a C_{18} column [6,7]. The intact ET-1-(1–21) began to disappear after 0.2 min of incubation with chymotrypsin, and after 30 min the decrease in the peak area was nearly 80 %. Concomitant with the progressive decline in intact ET-1-(1–21), the peak eluted at 41.5 min increased in area. However, it is unlikely that an increase in the peak area is caused by an increment in a production of the sulphoxide form of ET-1-(1–21), because the sulphoxide form was not produced by incubation of synthetic ET-1-(1–21) alone for 30 min (result not shown). This finding was confirmed by determination of the amino acid sequence of the newly appearing component with a retention time of 41.5 min, as described below. These results suggest that chymotrypsin hydrolyses big ET-1-(1–39), producing five cleavage products, and also degrades ET-1-(1–21) to a single major product.

To determine the mode of cleavage of big ET-1-(1–39) by chymotrypsin, the amino acid sequences of the five products were analysed (Table 1). The products in peaks 1 and 2 comprised eight and ten amino acid residues respectively, each of which was detected as a single amino acid phenylthiohydantoin derivative. Comparison of these amino acid sequences with that of big ET-1-(1–39) revealed that the products in peaks 1 and 2 are identical with big ET-1-(32–39) and big ET-1-(22–31) respectively. In addition, the amino acid sequences of the products in peaks 4 and 5 correspond to those of big ET-1-(1–31) and ET-1-(1–21) respectively. The unidentified amino acid residues at positions 1, 3, 11 and 15 correspond to cysteine residues present at the same positions in the authentic big ET-1-(1–39) and ET-1-(1–21) sequences. The cysteine phenylthiohydantoin derivative was undetectable as we did not use pyridylethylated samples for sequence analysis. These sequence data indicate that ET-1-(1–21) is produced by chymotryptic hydrolysis of big ET-1-(1–39), although chymotrypsin is by no means specific for the Trp-21–Val-22 bond of big ET-1-(1–39).

Automated Edman degradation of the product in peak 3 showed that only single amino acids were identified at the first three cycles and the last five cycles, except for the eleventh, in which no amino acid phenylthiohydantoin derivative was detected. The analysis also revealed the presence of two amino acids during each of five cycles from the fourth to the eighth.

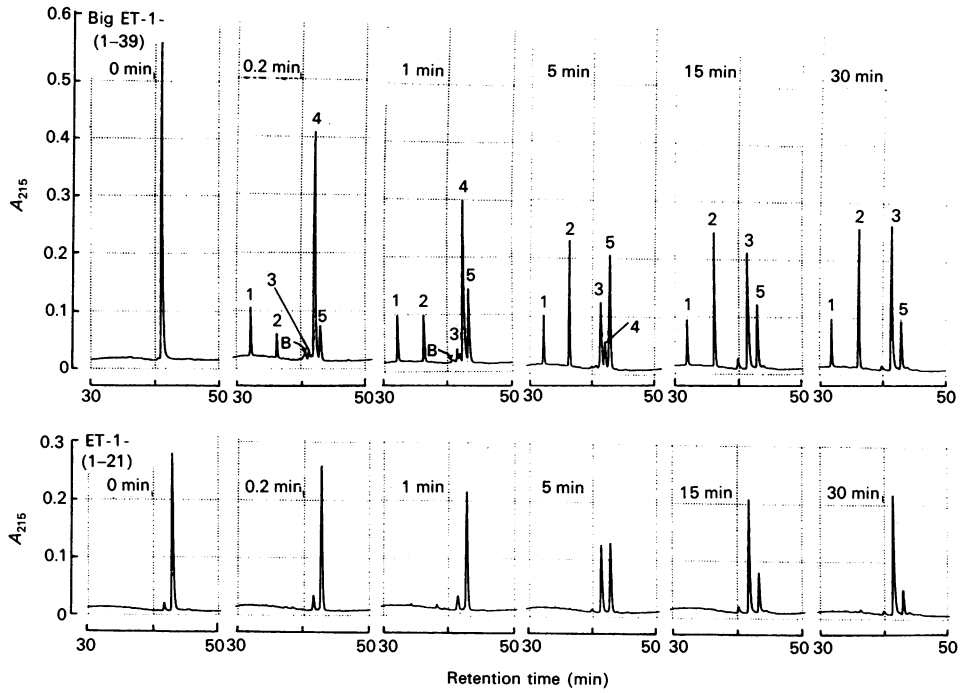

Fig. 1. Reverse-phase h.p.l.c. of big ET-1-(1–39) and ET-1-(1–21), with or without chymotrypsin treatment

Big ET-1-(1–39) (4.4 μg, 1 nmol) or ET-1-(1–21) (2.5 μg, 1 nmol) was incubated with chymotrypsin (1 μg) for the various periods of time indicated. Each sample was then applied to a Capcell-Pak C_{18} column. Details of the elution conditions are given in the text. Peak B represents big ET-1-(1–39); peaks 1–5 represent the cleavage products of big ET-1-(1–39) by chymotrypsin, in the order of elution.

Table 1. Sequence analyses of products of big ET-1-(1–39) cleavage by chymotrypsin

Each peak fraction, which showed the highest absorbance at 215 nm in Fig. 1, was collected, peaks 1 and 4 after 0.2 min, peaks 2 and 5 after 5 min and peak 3 after 30 min incubation with chymotrypsin. Details of the incubation conditions are given in the text. —, Not identified.

		Amino acid phenylthiohydantoin derivative (pmol)				
Cycle	Peak no....	1	2	3	4	5
1		Gly (636)	Val (762)	Phe (107)	—	—
2		Leu (898)	Asn (325)	Ser (665)	Ser (128)	Ser (120)
3		Gly (634)	Thr (247)	His (25)	—	—
4		Ser (204)	Pro (446)	Ser (376), Leu (243)	Ser (108)	Ser (76)
5		Pro (342)	Glu (290)	Ser (408), Asp (146)	Ser (93)	Ser (33)
6		Ser (140)	His (84)	Leu (56), Ile (36)	Leu (320)	Leu (138)
7		Arg (51)	Ile (383)	Met (52), Ile (38)	Met (260)	Met (96)
8		Ser (69)	Val (320)	Asp (65), Trp (11)	Asp (71)	Asp (16)
9			Pro (290)	Lys (32)	Lys (194)	Lys (27)
10			Tyr (38)	Glu (28)	Glu (76)	Glu (19)
11				—	—	—
12				Val (14)	Val (169)	Val (58)
13				Tyr (15)	Tyr (91)	Tyr (24)
14					Phe (195)	Phe (86)
15					—	—
16					His (11)	His (3)
17					Leu (153)	Leu (63)
18					Asp (40)	Asp (14)
19					Ile (129)	Ile (52)
20					Ile (203)	Ile (78)
21					Trp (23)	Trp (28)
22					Val (66)	
23					Asn (3)	
24					Thr (26)	
25					Pro (37)	
26					Glu (11)	
27					His (3)	
28					Ile (39)	
29					Val (22)	
30					Pro (41)	
31					Tyr (5)	

These data are indicative of two peptide strands being sequenced simultaneously. The amino acid composition of the product in peak 3 was identical with that of the product in peak 5, containing 17 amino acid residues of ET-1-(1–21), except for four cysteine residues, which were not identified in this work. Examination of the sequence of the product in peak 3, in comparison with that of the product in peak 5, suggests that chymotryptic cleavage occurs between Tyr-13 and Phe-14. The observation that peak 3 was detected as only a single peak during reverse-phase h.p.l.c. elution indicates that a disulphide bridge between Cys-1 and Cys-15 remained intact. From these findings, the product in peak 3 was found to have the same sequence as that in peak 5, i.e. ET-1-(1–21), with a cleavage of the Tyr-13–Phe-14 bond. Accordingly, the product in peak 3 is referred to as Tyr-13-nicked ET-1-(1–21) in the present study. The sites of cleavage by chymotrypsin that we identified are shown in Fig. 2.

The time courses for the production from big ET-1-(1–39) of big ET-1-(32–39), big ET-1-(1–31), big ET-1-(22–31), ET-1-(1–21) and Tyr-13-nicked ET-1-(1–21) are plotted in Fig. 3, on the basis of the experiment illustrated in Fig. 1. Big ET-1-(1–31) and big ET-1-(32–39) appeared after 0.2 min of incubation with chymotrypsin. The former peptide decreased gradually, whereas the latter one remained at a fixed level. ET-1-(1–21) and big ET-1-(22–31) were also present at the earliest time examined. ET-1-(1–21) increased during 5 min of incubation and then decreased progressively. Big ET-1-(22–31) attained the approximate maximal level after 5 min. The Tyr-13-nicked ET-1-(1–21) slowly increased in peak area throughout the experiment. When the integrated peak areas of the five peptides formed at each time period were summed, the values were almost the same as for intact big ET-1-(1–39). These data indicate that with chymotrypsin treatment big ET-1-(1–39) is rapidly converted into big ET-1-(1–31) by removal of big ET-1-(32–39), and then to mature ET-1-(1–21) by removal of big ET-1-(22–31). In addition, the ET-1-(1–21) generated was found to be transformed into Tyr-13-nicked ET-1-(1–21) by chymotrypsin, although both the big ET-1-(32–39) and big ET-1-(22–31) liberated from big ET-1-(1–39) remained without further cleavage.

Two recent studies [2,3] revealed that big ET-1-(22–39) and

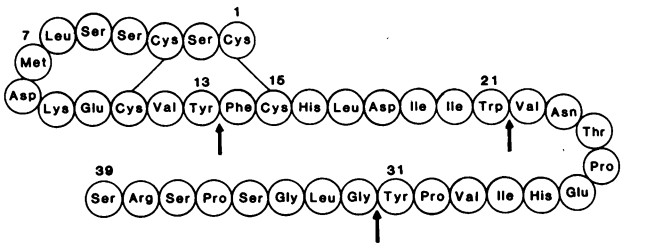

Fig. 2. Sequence of big ET-1-(1–39) and identification of cleavage sites by chymotrypsin

The positions of the peptide bonds cleaved by chymotrypsin are shown by arrows.

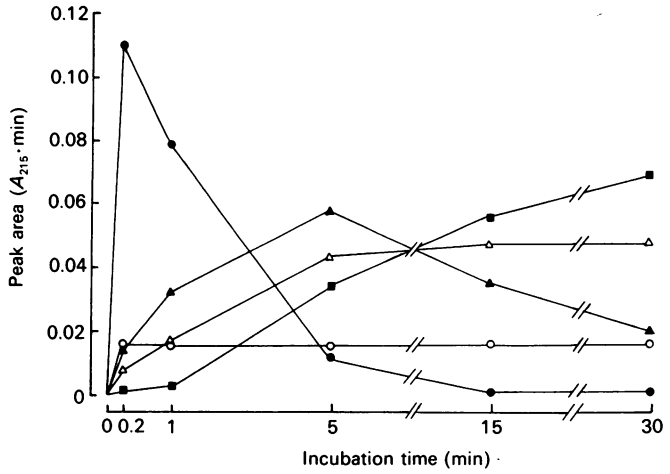

Fig. 3. Time course for the production of five peptides from big ET-1-(1–39) by chymotrypsin

○, Peak 1 [big ET-1-(32–39)]; ●, peak 4 [big ET-1-(1–31)]; △, peak 2 [big ET-1-(22–31)]; ▲, peak 5 [ET-1-(1–21)]; ■, peak 3 [Tyr-13-nicked ET-1-(1–21)]. Data indicate the integrated peak areas of five peptides shown in Fig. 1. Each point represents the mean of two separate experiments.

ET-1-(1–21) were present in culture medium of vascular endothelial cells, thereby suggesting that the conversion of big ET-1-(1–39) into ET-1-(1–21) occurs only by cleavage between Trp-21 and Val-22. On the basis of the finding that chymotrypsin cleaves not only the Trp-21–Val-22 bond but also the Tyr-31–Gly-32 bond of big ET-1-(1–39), a chymotrypsin-like proteinase may not physiologically contribute to the proteolytic processing of big ET-1-(1–39) in vascular endothelial cells. It should be noted, however, that ET-1-(1–21) has also been detected in non-endothelial cells, such as tracheal and renal epithelial cells or tumour cells with an epithelial-like morphology [8–10]. Taken together with the finding that ET-1-(1–21) is produced from big ET-1-(1–39) by chymotrypsin, the possibility that a chymotrypsin-like proteinase is responsible for the production of ET-1-(1–21) in non-endothelial cells cannot be excluded.

In conclusion, the present study has demonstrated that chymotrypsin produces mature ET-1-(1–21) from big ET-1-(1–39) by cleavage at the Tyr-31–Gly-32 bond followed by the Trp-21–Val-22 bond, and then degrades the generated ET-1-(1–21) to Tyr-13-nicked ET-1-(1–21). Our results provide insight into a possible mode of big ET-1-(1–39) metabolism involving a chymotrypsin-like proteinase *in vivo*.

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