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The Conformation of Human Big Endothelin-1 Favours Endopeptidase Hydrolysis of the TRP^{21}-VAL^{22} Bond

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ABSTRACT. The importance of big endothelin-1 (big ET-1) retaining a specific conformation for its conversion to ET-1 has yet to be determined. As a prelude to developing affinity labels for studying the interaction between big ET-1 and endothelin converting enzyme (ECE), the effect on biological activity of modifying human big ET-1 with the N-hydroxysuccinimide esters of 3-(p-hydroxyphenyl)propionic acid (HPP) or S-acetylthioglycolic acid (ATG) was investigated. Mono-derivatized HPP-big-ET-1 and ATG-big-ET-1, and the corresponding ET-1 molecules, were purified by HPLC. The identity of the modified big ET-1 and ET-1 molecules were confirmed by mass spectrometry. Comparison of the pressor activities with big ET-1 (1 nmol/kg) in anaesthetized rats showed the responses to equivalent doses of HPP-big-ET-1 and ATG-big-ET-1 to be reduced by 67% and 73%, respectively. In contrast, the same modifications to ET-1 had no significant effect on blood pressure responses or vasoconstrictor activity on the isolated rat thoracic aorta. To evaluate the effect of these modifications on the conversion of big ET-1 to ET-1, cultured bovine aortic smooth muscle (BASMC) and endothelial (BAEC) cells were used as sources of endothelin converting enzyme activity. After a 4-hr incubation of the modified molecules with intact cells, the quantity of ET-1 immunoreactivity generated was compared to that from unmodified big ET-1. The amount of conversion, relative to big ET-1 (1 μM), for HPP-big-ET-1 was reduced by 21% for BAEC and by 50% for BASMC. The corresponding decreases for ATG-big-ET-1 were 79% and 82%. Because of the large decreases in the level of conversion, the linear big ET-1 molecule S-carboxyamidomethylated big ET-1 (CM-big-ET-1) was prepared for comparison. Incubations of CM-big-ET-1 with BAEC and BASMC yielded only 53% and 23%, respectively, of the ET-1 immunoreactivity obtained with unmodified big ET-1. Thus, incorporation of the HPP or ATG groups, or removal of disulphide bridges decreases the ability of plasma membrane ectoenzyme ECE activities to hydrolyze the Trp^{21}-Val^{22} bond of big ET-1. This indicates that the conformation of big ET-1 is important for obtaining an optimal rate of hydrolysis by ECE activities *in vivo* and *in vitro*. Further evidence of secondary structure was obtained from studies of the crossreactivity of big ET-1 in two RIAs recognising the $ET\text{-}1_{[1-15]}$ sequence. BIOCHEM PHARMACOL 51;3:259–266, 1996.

KEY WORDS. vasoconstriction; blood pressure; endothelin converting enzyme; endothelium; vascular smooth muscle

Compared with ET-1,† big ET-1 is virtually devoid of vasoconstrictor activity on isolated vascular smooth muscle preparations [1, 2]. However, injected systemically at doses >0.5 $nmol.kg^{-1}$, big ET-1 and ET-1 are almost equipotent as vasopressor agents [3, 4]. In contrast to the effect of ET-1, the blood pressure response to big ET-1 can be blocked by pretreatment with the metalloprotease inhibitor phosphoramidon [5–9]. This finding, combined with evidence that ET-1 can be generated from big ET-1 by phosphoramidon sensitive ECE activities, as widely accepted as proof of the need for specific hydrolysis of big ET-1 by an endothelin converting enzyme to release the biologically active ET-1 molecule. The cloning of two structurally homologous ECE activities from a number of different cell sources has now been described [10–15].

Photoaffinity ligands have been used to study binding of peptide ligands with their receptors. Hence, a suitable photoaffinity ligand may prove useful for investigating the interactions of big ET-1 with ECE. Here, the effects on the enzymatic conversion of big ET-1 to ET-1 of incorporating functional groups into big ET-1 of a similar size to those commonly used for photoreactive crosslinking are described. As a model compound for phenyl-azide substituents of big ET-1, reaction with the N-hydroxysuccinimide ester of 3-(p-hydroxyphenyl) propionic acid (HPP) was chosen; this yields a group of similar size, but is sufficiently stable for the studies being performed. The results were compared with the product obtained by reacting big ET-1 with the N-hydroxysuccinimide ester of ATG acid. The effects on biological activity of these modifications to big ET-1 were tested *in vivo* by measuring blood pressure

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† Abbreviations: ET-1, endothelin-1; big ET-1, big endothelin-1; ECE, endothelin converting enzyme; RIA, radioimmunoassay; MAP, mean arterial blood pressure; HPP, 3-(p-hydroxyphenyl)propionyl-; ATG, [S-acetyl]thioglycolyl-; CM, S-carboxyamidomethyl-; BASMC, bovine aortic smooth muscle cells; BAEC, bovine aortic endothelial cells; TFA, trifluoroacetic acid.

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responses to bolus i.v. injections and by comparison with ET-1 modified in a similar manner. In addition, as most studies of systemically administered big ET-1 have concluded that it is converted locally in the vasculature to ET-1 [4, 9, 16], most probably by ECE activities present on endothelial or vascular smooth muscle cells [17–20], we have determined the rate of conversion to ET-1 of these modified molecules *in vitro*. This was performed by incubating the modified big ET-1 molecules with intact cultured BAEC and BASMC, followed by measurement of the ET-1 generated [17–19]. To develop further the concept of big ET-1 having a specific conformation that favours hydrolysis of its Trp^{21}-Val^{22} bond, we also studied the *in vitro* generation of ET-1 immunoreactivity using the linear molecule, S-carboxyamidomethylated big ET-1 (CM-big-ET-1). Some of these findings have been presented to the Federation of American Societies for Experimental Biology [21].

MATERIALS AND METHODS

Preparation of Modified Big ET-1 and ET-1 Molecules

The N-hydroxysuccinimide esters of HPP acid or ATG acid (Sigma, Poole, Dorset, U.K.) were dissolved in dry acetonitrile and then reacted with human big $ET\text{-}1_{[1-38]}$ or ET-1 (Peptide Institute Inc., Osaka, Japan) in 0.1 M sodium bicarbonate at a peptide concentration of 65–130 μM for 2 hr at 4°C followed by 30 min at room temperature. For the preparation of each modified peptide, the standard molar ratio of active ester to peptide was 1.25:1. The products were acidified with HCl and purified by HPLC using a column of Techogel wide pore (300 Å) octadecasilyl silica (5 μm, 4.6 × 250 mm, HPLC Technology Ltd., Macclesfield, Cheshire, U.K.) connected to a Pharmacia model 2249 gradient pump. The column was eluted at a flow rate of 1 mL/min using a gradient of acetonitrile in 0.1% TFA as follows: 0 to 24% acetonitrile over 5 min, followed by 24% to 48% over 40 min. Column effluent was monitored continuously (A_{280}) using a Pharmacia variable wavelength model 2141 spectrophotometer, and 0.5 min fractions were collected throughout. Between samples, the column was flushed for 5 min with 0.1% TFA in 80% acetonitrile, and re-equilibrated with 0.1% TFA for 10 min. With each reaction two peaks predominated, unreacted peptide and the mono-derivatized product. Confirmation that only one HPP group or ATG group was incorporated per big ET-1 or ET-1 molecule was obtained by mass spectrometry (Table 1).

For further comparison with the *in vitro* conversion of modified big ET-1 molecules, a linear big ET-1 molecule was prepared by reduction of big ET-1 with dithiothreitol followed by carboxyamidomethylation with iodoacetamide. One mL big ET-1 (50 μM) was diluted with 1 mL 0.1 M Tris-HCl pH 8.0 containing 2 mM EDTA, 0.2 mL, 0.1 M dithiothreitol was added, and the mixture was incubated at 37°C for 1 hr in the dark. The reaction mixture was then cooled on ice and 0.22 mL 0.21 M iodoacetamide was added. After a further 30 min at 0°C, the mixture was acidified with HCl, and the product (CM-big-ET-1) was purified by HPLC. The identity of the product was confirmed by mass spectrometry.

Big ET-1 and ET-1 molecules were quantified using RIAs for big $ET\text{-}1_{[22-38]}$ or $ET\text{-}1_{[16-21]}$, respectively [22], in combination with the HPLC peak area recorded from the UV absorbance (A_{280}). For each of the modified molecules, samples were retained from the HPLC fractions of the major product for mass spectrometry, as well as from HPLC fractions containing unreacted ET-1 or big ET-1. The remainder of the peak fractions were used to prepare stock solutions for use *in vivo* and *in vitro*. After removal of acetonitrile with a stream of N_2, peak fractions were neutralised with a minimum volume of 0.3 M sodium bicarbonate and diluted with 0.9% saline containing 0.2% rat albumin to a concentration of 4 μM. Further dilutions in the same vehicle solution were made for *in vivo* studies. For *in vitro* studies, the peptide solutions were further diluted in serum free Dulbecco's modified Eagle medium DMEM. Reference preparations of big ET-1 and ET-1 were prepared in the same manner from the peak fractions of unreacted peptide (the identity of the unreacted peptide was confirmed by mass spectrometry).

Mass spectrometry of modified ET-1 molecules was performed by continuous flow FAB mass spectrometry using a VG-analytical 70 VSEQ mass spectrometer (Fisons Instruments, Manchester, U.K.). The identities of modified big ET-1 molecules were confirmed by electrospray mass spectrometry using a VG Biotech Trio-2 mass spectrometer (Fisons Instruments, Manchester, U.K.) with flow injection into a 50:50:1 (v/v/v) mixture of water:acetonitrile:acetic acid at 5 μL/min. The mass scale was corrected internally using authentic human big ET-1 as a reference (Table 1).

Vasoactive Properties of Modified Big ET-1 and ET-1

Pressor effects of mono-derivatized big ET-1 and ET-1 were compared with unmodified molecules in anaesthetised rats.

TABLE 1. HPLC elution times and mass spectrometry data for modified ET-1 and big ET-1 molecules

Peptide product	HPLC elution time (min)	Theoretical M_r	FAB MH^+	Electrospray M_r
HPP-ET-1	39.7	2640	2640.8	–
ATG-ET-1	40.0	2608	2609.5	–
HPP-big ET-1	38.6	4431	–	4430.4
ATG-big ET-1	38.9	4399	–	4399.0
CM-big ET-1	35.7	4515	–	4515.1

Male Wistar rats (250–330 g; A. Tuck & Son, Battlebridge, Essex, U.K.) were anaesthetised with sodium thiopentone (120 $mg \cdot kg^{-1}$, i.p.; Intraval, RMB Animal Health Care, Dagenham, U.K.). Intravascular catheters were inserted into the right jugular vein for peptide administration by bolus injection, and the left carotid artery for continuous recording of mean arterial blood pressure (MAP). MAP was recorded using a Spectramed P23XL blood pressure transducer connected to a Grass 7D polygraph (Grass Instruments, Quincy, MA, U.S.A.). To calculate blood pressure responses expressed as an area in arbitary units, the mean change in arterial blood pressure (mmHg) between each time point was multiplied by the time between the observations (min) and summed to give the total area of the depressor and pressor responses in each rat (area units = AU).

An additional comparison of the biological activity of HPP-ET-1 with ET-1 was obtained by measuring vasoconstrictor responses using rings of isolated thoracic aorta from the rat [23].

Cell Culture and Evaluation of In Vitro *Conversion of Big ET-1*

Conversion of modified big ET-1 molecules was studied using confluent cultures (6 × 35 mm well plates) of BAEC or BASMC as sources of ECE activity [17, 19]. BAEC were cultured as previously described [22]. Cultured BASMC were prepared from the medial layer of bovine aorta using the explant procedure [24]. Small sections of aorta were gently removed as strips from the medial layer. These were rinsed thoroughly with sterile Hanks' balanced salt solution, and then placed in 6-cm culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glutamine, penicillin (100 IU/mL), streptomycin (0.1 μg/mL) and amphotericin (2.5 μg/mL), and supplemented with 10% foetal calf serum (FCS). Smooth muscle cells migrating from the explants were allowed to grow for 7–10 days at 37°C in an atmosphere of 5% CO_2 in air. Primary cultures were treated with trypsin (0.05%), grown to confluence in T-75 flasks, and further subcultured with cells from the 5th–10th passages being used in these studies.

The modified big ET-1 peptides were incubated for 4 hr at the concentrations indicated with BAEC or BASMC. ET-1 formation was measured by specific RIA [22]. To confirm that the ET-1 immunoreactivity generated was authentic ET-1, the products of representative incubations of big ET-1 with BAEC and BASMC were subjected to HPLC analysis combined with RIA [30]. This RIA shows 100% crossreactivity with $ET\text{-}1_{[16-21]}$ and ET-1. Crossreactivity of ET-1 in the RIA was unaffected by incorporation of HPP or ATG groups. Crossreactivity of the following peptides in the ET-1 RIA were for big ET-1 <0.015%, $ET\text{-}1_{[19-21]}$ (Ile-Ile-Trp) 0.0003%, and big $ET\text{-}1_{[19-35]}$ (Ile-Ile-Trp-Val-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser) 0.0008%.

In addition to comparing the conversion by BAEC and BASMC of modified big ET-1 molecules, the effect of phosphoramidon on the ECE activities of both cell types was determined. Elsewhere it has been reported that fragments of big ET-1 that span the cleavage sequence can be used to study ECE activity [25]. Here we have tested the degree of competitive substrate inhibition of the ECE activities of BAEC and BASMC that could be obtained by coincubation of big ET-1 (1 μM) with 1, 10, or 100 μM human big $ET\text{-}1_{[19-35]}$ (Zinsser Analytic U.K. Ltd., Maidenhead, Berkshire, U.K.). To facilitate comparison of results from several different experiments, results in each experiment were expressed as % of control activity (fmol ET-1 generated per hr from 1 μM big ET-1).

Crossreactivity of Big ET-1 in Loop Region Specific ET-1 RIAs

Crossreactivity of big ET-1 was evaluated in two ET-1 RIAs showing a high degree of selectivity for $ET\text{-}1_{[1-15]}$. These were ET-1 assay kit RPA 555 (Amersham International plc., Little Chalfont, Bucks, U.K.) and the ET-1 antiserum 14198-v (Peptide Institute Inc., Osaka, Japan). Both assays are reported to have crossreactivities of <1% with human big ET-1. For the purpose of these evaluations, both assays were performed as follows: Dilutions of ET-1 or big ET-1 (200 μl) were incubated overnight with the antisera (50 μL) in assay buffer (0.05 M sodium phosphate pH 7.4 containing 0.15% bovine serum albumin and 0.005% Triton X-100); the following day [^{125}-I]-ET-1 was added (10,000 cpm, 50 μL). After a further 24-hr incubation, bound [^{125}-I]-ET-1 was separated from free using 300 μL Amerlex-M donkey anti-rabbit separation reagent.

Statistical analysis were performed by one-way analysis of variance or Student's *t*-test, as appropriate.

RESULTS

Preparation of Modified Big ET-1 and ET-1

Under the conditions described, the HPLC elution times for ET-1 and big ET-1 were 33.5 and 33.0 min, respectively. Using the reaction procedure outlined, unreacted peptide typically represented 40%–60% of the total. Confirmation that the unreacted ET-1 and big ET-1 had not been affected by the reaction and HPLC conditions was obtained from mass spectrometry (MH^+ 2493 for ET-1 and estimated M_r for big ET-1 4283). To obtain the best comparison with the modified molecules, the unreacted ET-1 and big ET-1 in these HPLC fractions were used as a reference for *in vivo* and *in vitro* studies. The reaction of active esters with ET-1 and big ET-1 yielded in each case one major product and a number of minor product peaks; RIA and HPLC showed the major peak to represent 60%–80% of the total product peaks. The HPLC elution times of monoderivatized products and their molecular masses are indicated in Table 1. Reduction and carboxyamidomethylation resulted in a 100% yield of CM-big ET-1 as confirmed by HPLC, RIA, and mass spectrometry.

Effect of Modifications to Big ET-1 and ET-1 on Vasoconstrictor Activity

The pressor responses in anesthetised rats expressed as areas in arbitrary units (AU) during the 30-min period following i.v. bolus administration (1 nmol/kg) showed no significant dif-

ference between the response to big ET-1 (1257 ± 88 AU) and ET-1 (1303 ± 98 AU) (Fig. 1a and b). However, modification of big ET-1 by the introduction of the HPP or ATG groups reduced the blood pressure response by 67% (409 ± 88 AU) and 73% (339 ± 77 AU), respectively. In contrast, the same modifications to ET-1 had little or no effect on its vasoactive properties. The increases in blood pressure for HPP-ET-1 and ATG-ET-1 were only 6% (1221 ± 65 AU) and 8% (1202 ± 91 AU) lower than that obtained with ET-1; and the ET_B receptor mediated acute vasodilator responses, seen as a fall in blood pressure, were not significantly different from ET-1 (−5 ± 1 AU) with either HPP-ET-1 (−9.7 ± 4.1 AU) or ATG-ET-1 (−8.8 ± 2.2 AU). CM-big ET-1 was not tested *in vivo* because the product of Trp^{21}-Val^{22} hydrolysis, CM-ET-1, has only limited vasoconstrictor activity [26, 27].

Comparison of the vasoconstrictor activity of HPP-ET-1 with ET-1, using the isolated rat thoracic aorta showed that the vasoconstrictor response to HPP-ET-1 was unaffected by the modification (Fig. 2).

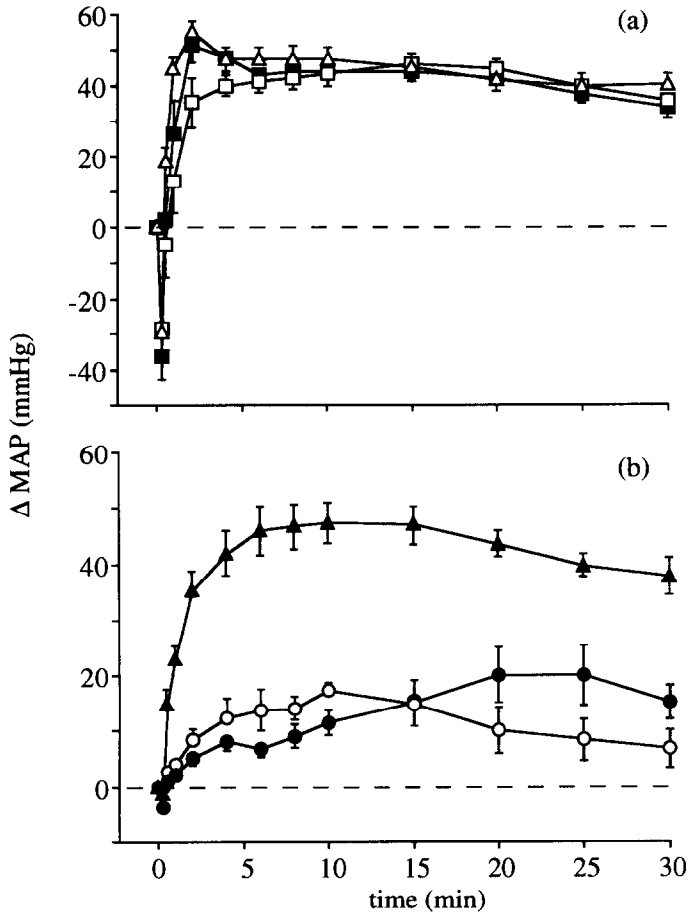

FIG. 1. (a) Comparison of blood pressure responses to ET-1 and modified ET-1 molecules, 1 nmol/kg (△, ET-1 basal MAP 103 ± 3 mmHg, *n* = 6; ■, HPP-ET-1 basal MAP 109 ± 4, *n* = 3; □, ATG-ET-1 basal MAP 96 ± 6 mmHg, n = 3). (b) Comparison of pressor responses to big ET-1 and modified big ET-1 molecules, 1 nmol/kg (▲, big ET-1 basal MAP 111 ± 3 mmHg *n* = 5; ● HPP-big-ET-1 basal MAP 116 ± 12, *n* = 3, ○, ATG-big-ET-1 basal MAP 109 ± 5 mmHg, *n* = 3).

Conversion of Big ET-1 to ET-1 by Cultured Endothelial or Smooth Muscle Cells

Incubation of big ET-1 with BAEC or BASMC resulted in the formation of ET-1. HPLC of the products confirmed that >95% of the ET-1 immunoreactivity generated was present as authentic ET-1. Phosphoramidon-sensitive ECE activities of intact endothelial or vascular smooth muscle cells may be involved in the conversion *in vivo* of big ET-1 to ET-1 to yield a pressor response [9]. Consistent with this idea, conversion by intact BAEC and BASMC was inhibited by phosphoramidon with IC_{50}s of approximately 5 and 20 μM, respectively (Fig. 3). In agreement with the reduced pressor activity *in vivo*, the conversion rates estimated by measurement of ET-1 immunoreactivity generated were reduced for HPP-big-ET-1 (1 μM) compared to big ET-1 (1 μM) by 21% and 50% for BAEC and BASMC (Fig. 4). The corresponding decreases in conversion for ATG-big-ET-1 were 79% and 82%, respectively. Similarly, the linear molecule CM-big-ET-1 showed reductions in conversion on BAEC and BASMC of 47% and 77%.

Coincubation of big ET-1 (1 μM) with 1, 10, or 100 μM big $ET\text{-}1_{[19-35]}$ resulted in significant reductions in the formation of ET-1, expressed as a percentage of control, only at the 100 μM concentration (−34.7 ± 5.7% and −34.4 ± 3.0% for BAEC and BASMC, $P < 0.05$ compared to control). Lower concentrations had no significant effect (values for control, 1, and 10 μM big $ET\text{-}1_{[19-35]}$ were for BAEC 100 ± 4.4, 110.6 ± 7.3, and 90.6 ± 3.8; and for BASMC 100 ± 3.0, 103.7 ± 3.9, and 100.0 ± 4.3% of control activity, $n = 6$ or more for each).

Crossreactivity of Big ET-1 in Specific ET-1 RIAs

In the two ET-1 RIAs tested, which recognise the $ET\text{-}1_{[1-15]}$ loop region, big ET-1 showed non-parallel crossreactivity (Fig. 5). The percentage crossreactivity with the Amersham antibody at the IC_{80}, IC_{50}, and IC_{20} concentrations of big ET-1 were 0.1%, 1.2%, and 23%; the corresponding values for the Peptide Institute antibody were 0.4%, 8%, and 23.5%.

DISCUSSION

Previous studies of structure-activity relationships for ET-1 have shown that N-terminal acetylation abrogates the vasoconstrictor activity of ET-1 [27], whereas substituion of Lys^9 by Ala or Leu had little effect on the vasoconstrictor activity or pressor response to ET-1 [27, 28]. Similarily, ET-1 that has been biotinylated at Lys^9 retains high affinity for ET_A receptors [29]. HPP-ET-1 and ATG-ET-1 were potent pressor agents, and HPP-ET-1 was equipotent with ET-1 on the rat thoracic aorta. This indicates that the reaction conditions favoured incorporation onto Lys^9 rather than N-terminal Cys^1. However, the pressor activities of the big ET-1 molecules with HPP– or ATG– groups incorporated were markedly attenuated. The level of conversion by BAEC or BASMC of these modified big ET-1 molecules was also reduced. Thus, the *in vivo* and *in vitro* results suggest that the modifications to big ET-1 affect the ability of ECE activities to cleave the Trp^{21}-

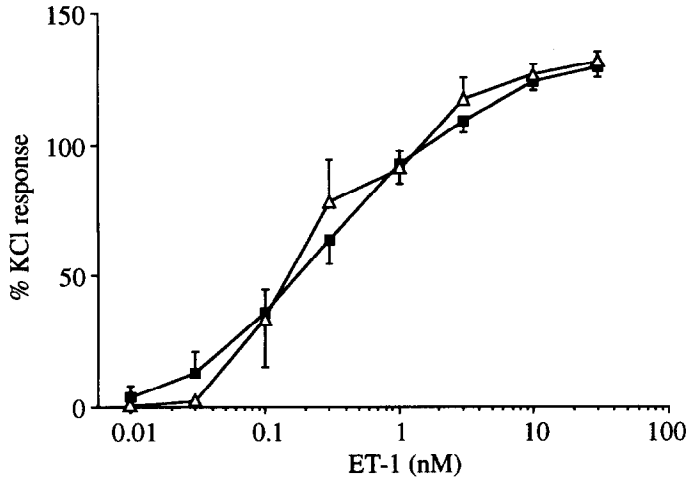

FIG. 2. Comparison of the vasoconstrictor activity of HPP-ET-1 (n = 4, △) with ET-1 (n = 15, ■) on the rat thoracic aorta.

Val^{22} bond. Hence, incorporation of an active group into Lys^9 is not a viable approach for developing photoaffinity ligands for big ET-1 to study the interaction with ECEs. Moreover, although it was not unexpected that reduction and S-carboxyamidomethylation to generate a linear big ET-1 molecule markedly reduced its level of conversion by the ECE activities of endothelial and smooth muscle cells, it was surprising that incorporation of a single group, probably on Lys^9, resulted in a similar or greater reduction in conversion of ATG-big ET-1. Consistent with the results obtained with CM-big ET-1, the linear fragment big $ET\text{-}1_{[19-35]}$ was a very poor competitive substrate inhibitor of big ET-1 hydrolysis. Hence, it may be concluded that the secondary structure of big ET-1 is important for its hydrolysis, and that this structure is stabilised by an interaction between the C-terminal sequence of big ET-1 and either Lys^9 or an adjacent amino acid. The marked effect on the pressor response and *in vitro* hydrolysis of incorporating the HPP– and ATG– groups into big ET-1 could then be explained by steric hindrance of this interaction.

Further support for big ET-1 maintaining a secondary structure was obtained by studying the crossreactivity of big ET-1 in two RIAs that recognise the 1–15 loop-region of ET-1. Big ET-1 crossreacted only poorly at high concentrations (<1%) but, as concentrations decreased, there was a progressively greater crossreactivity. Although aggregation of big ET-1 at high concentrations cannot be excluded, a more plausible explanation for this phenomenon can be obtained from considering an equilibrium between an unfolded conformation recognised by the endothelin antibodies, and a folded conformation where the C-terminal sequence of big ET-1 folds over the 1–15 region, preventing access of the antibodies. If the equilibrium at physiological pH results in a mainly folded conformation, at high concentrations of big ET-1 the proportion in

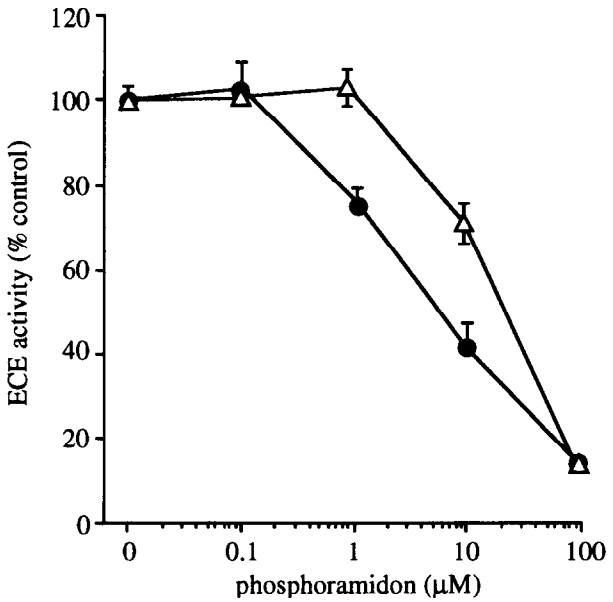

FIG. 3. Phosphoramidon inhibits the conversion of exogenous big ET-1 (1 μM) to ET-1 by BAEC (●) and BASMC (△). Control ECE activity with 1 μM big ET-1 was 1566 ± 69 fmol ET-1/h/35 mm well for BAEC, and 3957 ± 452 fmol ET-1/h/35 mm well for BASMC.

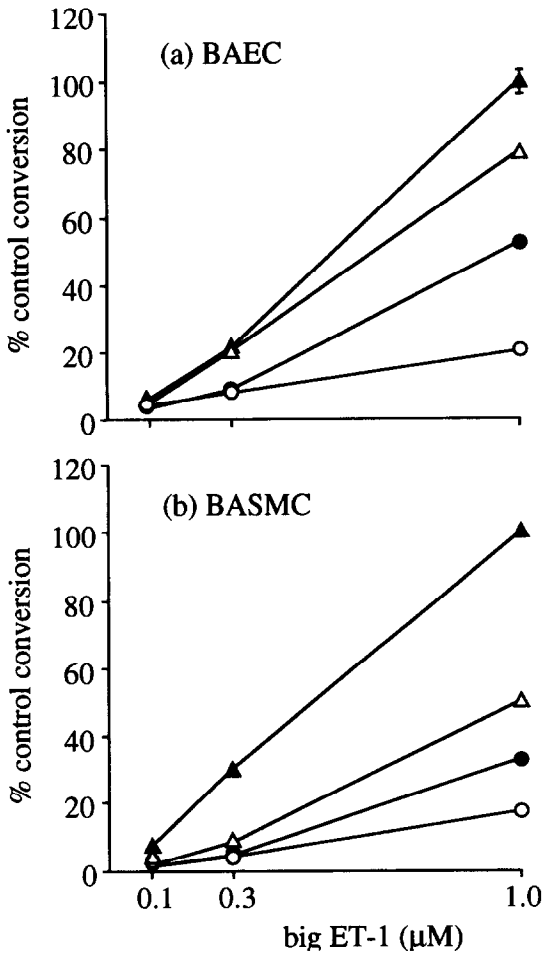

FIG. 4. Conversion of big ET-1 and modified big ET-1 molecules by (a) BAEC and (b) BASMC (control ECE activity with 1 μM big ET-1 was 1994 ± 148 and 2878 ± 201 fmol/h/well, respectively). ▲, big ET-1; △, HPP-big-ET-1; ●, CM-big-ET-1; ○, ATG-big-ET-1.

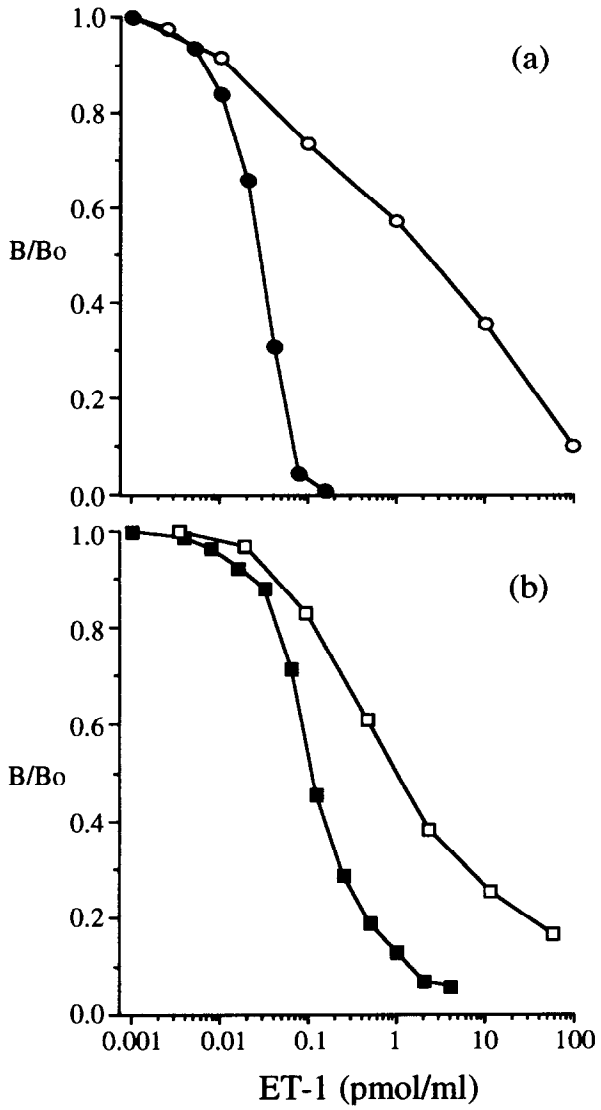

FIG. 5. Comparison of the crossreactivity of big ET-1 in two different ET-1 radioimmunoassays (a) from Amersham International plc and (b) from Peptide Institute, Inc. (solid symbols ET-1, open symbols big ET-1).

the extended form will be low; hence, the low crossreactivity. However, in dilute solution the ratio to the antibodies changes, so that the antibodies, by binding the unfolded form, are able to drive the equilibrium in favour of further unfolding. This results in a greater proportion of big ET-1 in the extended form recognised by the antibody; hence, a higher crossreactivity.

Studies with endopeptidase 24.11 (E-24.11) also support the hypothesis that big ET-1 adopts a conformation that favours hydrolysis of the Trp^{21}-Val^{22} bond. Thus, while ET-1 and the C-terminal peptide of big ET-1 (big $ET\text{-}1_{[22-39]}$) are rapidly degraded by E-24.11 [30], when these peptides are joined as in big ET-1, they become comparatively resistant to degradation [30]. Hence, the cleavage sites in ET-1 and the C-terminal sequence become inaccessible or protected in big ET-1, presumably as a result of a particular conformation in which the C-terminal sequence is folded over the ET-1 moiety. Moreover, when cleavage of big ET-1 by E-24.11 occurs, it is primarily through hydrolysis of the Trp^{21}-Val^{22} bond [30], indicating that the conformation of big ET-1 not only protects against general proteolysis, but also favours the specific cleavage of the Trp^{21}-Val^{22} bond.

Whether this conformation is also present in big ET-2 and big ET-3 is uncertain. Nevertheless, it should be noted that besides the high degree of sequence homology in the ET-1, ET-2, and ET-3 sequences, certain structural features are conserved in the C-terminal sequence of all three big endothelin molecules. The 23–26 and 31–33 sequences are fully conserved, including Pro^{25} and Pro^{30}, which may be of particular significance for the formation of secondary structure. In addition, there is an Arg residue at 37 or 38 in all big ET molecules [31, 32]. However, at present it seems unlikely that a similar conformation is formed by big ET-2 or big ET-3, because ECE-1 shows a remarkable degree of selectivity for big ET-1 compared to the other big endothelins [10, 11, 13, 14].

The results described here and the hypothesis that big ET-1 has a specific conformation are largely consistent with one theoretical attempt to model the structure of big ET-1 [33], but contradict results from two NMR studies [34, 35]. The reasons for this are unclear, but the pH at which NMR studies were performed may have disrupted any intramolecular electrostatic interactions. Interestingly, X-ray crystallography of ET-1 has revealed features not observed by NMR [36]. Studies of the secondary structure of endothelin-1 by X-ray crystallography and nuclear magnetic resonance (NMR) have shown that it has a well defined globular structure stabilised by two disulphide bridges [36]. The importance of this secondary structure for receptor binding and interactions leading to a biological response is demonstrated by findings from a number of investigations of structure activity relationships.

The need for peptide prohormones and biosynthetic intermediates to maintain specific conformations during processing to generate biologically active molecules from inactive precursors is less well understood [37, 38]. However, it has been suggested that at cleavage sites a particular type of loop structure is formed in prohormones as a means of restricting proteolysis to just those residues required to release specifically the biologically active peptide [37, 38]. A similar mechanism may operate with proendothelin-1 processing at double basic residues, and this may also be the basis for selective hydrolysis of big ET-1. The original hypothesis that formation of ET-1 from the intermediate big ET-1 involves a novel selective endopeptidase [39] has been substantiated by the purification and cloning of two structurally related isoforms of an enzyme referred to as ECE-1 [10–15]. However, initially the isolation of ECE proved technically difficult for a number of reasons, not least the fact that numerous proteases display apparently selective ECE activity even though their functional importance remained unclear [40]. The evidence presented here suggests that the conformation of big ET-1 favours hydrolysis of the Trp^{21}-Val^{22} bond, and this may in part account for why so many peptidases cleave big ET-1 with apparent selectivity, and give the impression of being specific endothelin converting enzymes.

In conclusion, the results described provide strong evidence that the conformation of big ET-1 is important for optimal ECE activity, and this conformation appears to be stabilised by the disulphide bridges and probably by an interaction of Lys^9 or a nearby amino acid with the C–terminal sequence of big ET-1.

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