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Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: Possible physiological significance

(neutral endopeptidase/proteolysis/receptor binding sites/inositol phospholipid turnover/structure activity relationship)

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ABSTRACT **Incubation of endothelins (ETs) with bovine kidney neutral endopeptidase (NEP) resulted in a selective two-step degradation with loss of biochemical activity. The K_m of the enzyme indicated high-affinity binding, and hydrolysis was completely inhibited by phosphoramidon. The first step was nicking of the Ser[5]-Leu[6] bond, followed by cleavage at the amino side of Ile[19]. The nicked peptide exhibited biochemical activities comparable to those of the intact peptide—i.e., binding to the ET receptor, induction of inositol phospholipid hydrolysis, and toxicity. The twice-cleaved product was inactive. The sarafotoxins (SRTXs) were more resistant than the ETs to NEP: for example, the half-time for ET-1 was ≈1 hr, while it was ≈4 hr for SRTX-b and even higher for SRTX-c. These *in vitro* findings may indicate a regulatory role of NEP (or similar enzymes) in the physiological inactivation of ETs. They might also help to explain why under certain physiological conditions ETs may be less toxic than SRTXs.**

The endothelins (ETs) and the sarafotoxins (SRTXs) are two classes of a recently discovered family of peptides containing 21 amino acids (reviewed in refs. 1 and 2). The ETs (isolated from mammalian systems) and the SRTXs (from snake venom) are among the most potent vasoconstrictor agents known (refs. 1 and 2 and references therein) and are highly lethal (3). The two classes of peptides show a considerable sequence homology: they each possess four cysteinyl residues, and ≈60% of the 21 amino acid residues are common to both. Since ET-1 was described, three other ETs have been detected and are designated ET-2, ET-3 (4), and ET-4 (Vic) (5) (Fig. 1). Also, four SRTXs—SRTX-a, SRTX-b, SRTX-c, and SRTX-d (6)—have been characterized so far (Fig. 1). The four cysteinyl residues in the ETs and in the SRTXs are interconnected by disulfide bridges between positions 1 and 15 and 3 and 11, forming an intramolecular loop structure (see diagram in Fig. 1) (7). Another characteristic of these peptides is their conserved hydrophobic C-terminal tail. Early reports (8–11) indicate that ET-1 and SRTX-b bind with high affinity to identical populations of sites in rat atrium and brain and that both peptides stimulate inositol phospholipid hydrolysis. These observations have been confirmed and extended in various other tissues and species (for review, see refs. 1, 2, and 12). More recent binding studies (1, 13, 14) as well as functional data (15–17) point to the possible existence of multiple ET/SRTX receptor subtypes.

Metallopeptidases are a group of enzymes implicated in the metabolism and regulation of a variety of hormonal, neuromodulator, and neurotransmitter peptides involved in digestion (carboxypeptidases), regulation of blood pressure [angiotensin-converting enzyme (ACE); dipeptidyl carboxypeptidase I; peptidyl-dipeptide hydrolase, EC 3.4.15.1] and other processes. Proteolysis of the ET peptides might therefore play a role in their biological function. Preliminary experiments indicated that both ET-1 and SRTX-b are insensitive *in vitro* to one of the most likely enzyme candidates, ACE. We accordingly turned our attention to neutral endopeptidase (NEP; membrane metallo-endopeptidase, EC 3.4.24.11) (18). This enzyme cleaves a variety of active peptides such as the enkephalins, substance P, and atrial rat natriuretic factor (for review, see ref. 19). The cleavage occurs at the amino side of hydrophobic amino acids. The enzyme is widely distributed in the body, occurring in specific structures in the central nervous system, kidney, lung, and intestine as well as in neutrophils and fibroblasts, but its concentration in vascular endothelial cells is very low (19). The substrates cleaved by NEP appear to be restricted to peptides of molecular mass ≈3 kDa (18, 19). These considerations prompted us to examine whether the ETs and/or the SRTXs might be good substrates for NEP. The effect of the enzyme can be evaluated by measuring enzyme-associated changes in the biochemical activities of the peptides, such as binding to the receptor, induction of inositol phospholipid hydrolysis, immunogenicity (20, 21), and lethality. As shown in this *in vitro* study, NEP inactivates ETs as a result of nicking the bond at Ser[5]-Leu[6] followed by rapid cleavage at the amino side of Ile[19]. Under identical experimental conditions, NEP also inactivated SRTXs, but at a much slower rate.

MATERIALS AND METHODS

ET-1, ET-2, ET-3, and ET-4 (Vic) were purchased from American Peptide (Santa Clara, CA). SRTX-b and SRTX-c were those used in a previous study (20). ^{125}I-labeled ET-1 (^{125}I-ET-1) was prepared by iodination with Enzymobeads (Bio-Rad) and was purified as described (8). Bovine kidney NEP (0.4 mg/ml) was purified by Triton X-100 extraction followed by DEAE-Sepharose Fast Flow, concanavalin A-Sepharose, Q-Sepharose, and hydroxyapatite chromatographic procedures (22). Hippuryl-His-Leu and ACE were obtained from Sigma. The synthetic peptide corresponding to the C-terminal fragment His-Gln-Asp-Val-Ile-Trp of SRTX-a, -b, and -c was prepared by solid-phase synthesis on an Applied Biosystems peptide synthesizer (model 480A) in the Unit of Chemical Services at the Weizmann Institute of Science (Rehovot, Israel). The purity of all peptides was >97% as determined by HPLC analysis using gradient I as described below.

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Abbreviations: ET, endothelin; SRTX, sarafotoxin; NEP, neutral endopeptidase; ACE, angiotensin-converting enzyme; TFA, trifluoroacetic acid.

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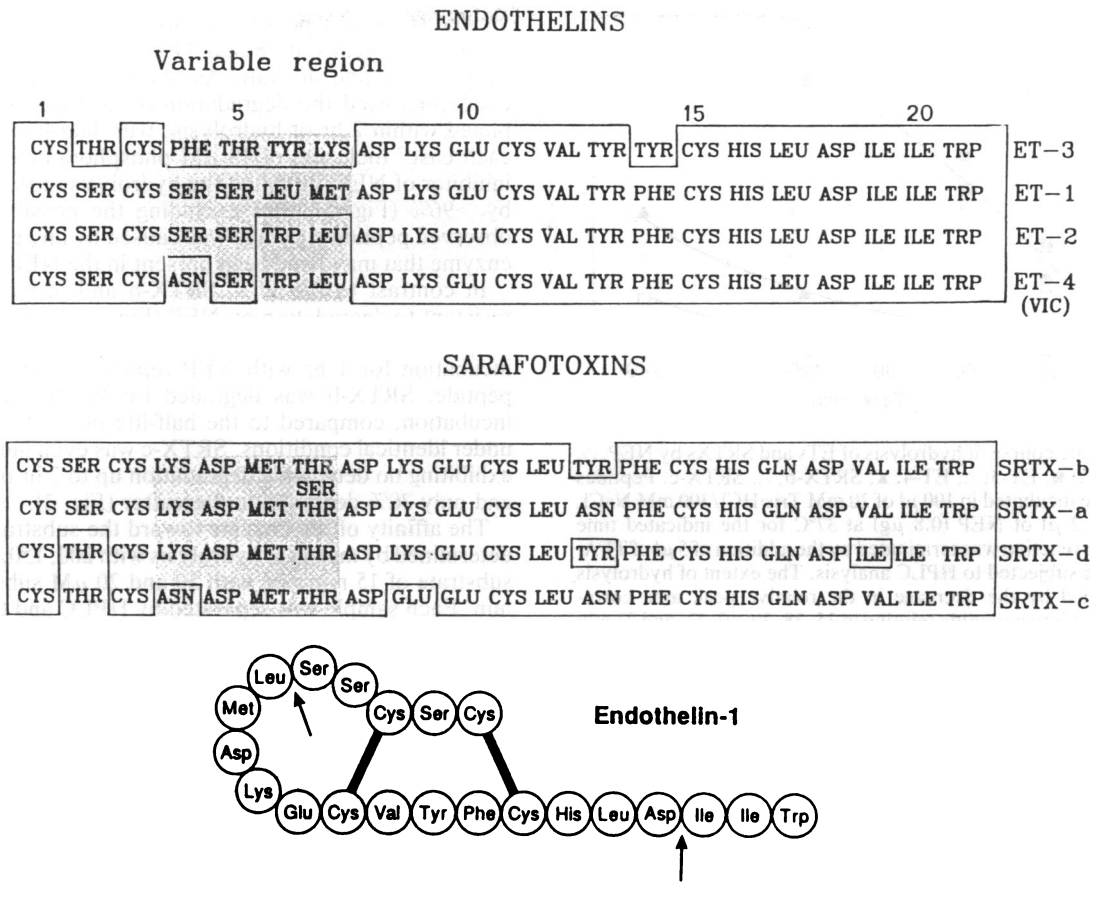

FIG. 1. Structure of ETs (ref. 2 and references therein; ref. 5) and SRTXs (ref. 6 and references therein). Arrows indicate the points of cleavage.

Biochemical Assays. Binding of ^{125}I-ET-1 to intact rat heart myocyte cultures was carried out as described (20). The binding of unlabeled peptides to intact myocytes was measured by competition with 2 nM ^{125}I-ET-1 as described (20).

Inositol phospholipid hydrolysis, as indicated by the formation of [^{3}H]inositol phosphate, was assayed in intact myocytes as described (20). Assays were initiated by the addition of the indicated peptide at 1 μM. Mixtures were incubated for 15 min (at 37°C).

RIA of ET and its proteolytic cleavage products was carried out by using the Amersham system with an Amertex-M magnetic separation kit (Amersham). Sensitivity and reproducibility were according to the data supplied by the manufacturer.

Lethality was assessed after i.v. injection of a freshly prepared solution of peptide into ICR mice (males; 20 g). The LD_{50} values obtained for ET-1 and SRTX-b were both $\approx$15 ng per g of body weight (3).

HPLC Analysis. Analysis of peptides [10–100 μg in 0.1 ml of 0.1% trifluoroacetic acid (TFA) in water] was performed as described (21). The column (Lichrosorb RP-18; 250 × 4 mm; Merck) was developed at 1 ml/min with one of two gradient programs. Program I consisted of a 5-min wash with buffer A (0.1% TFA), followed by a linear gradient of 0–100% buffer B (80% acetonitrile in 0.1% TFA) over 35 min. The column was then washed with buffer B for 10 min and with buffer A for an additional 10 min. Program II consisted of three steps of linear gradients of 0–30% buffer B over 5 min, 30–60% buffer B over 30 min, and 60–100% buffer B over 5 min. The column was then washed with buffer B for 10 min and with buffer A for an additional 10 min. Elution profiles were monitored at 230 and 280 nm.

Reduction and Carboxymethylation. Reduction and carboxymethylation of the peptide mixtures were carried out with dithiothreitol in 8 M urea and 0.2 M Tris·HCl (pH 8.3) for 1 hr, followed by treatment with iodo[^{14}C]acetamide for an additional 1 hr.

Sequence Analysis. Sequence analysis was performed by the Protein Analysis Group at the Hebrew University (Hadassah Medical School, Jerusalem) with a 470A gas phase protein sequencer with an on-line 120A HPLC-AA analyzer (Applied Biosystems).

Concentration of Peptides. Concentration was determined by amino acid analysis carried out by Aminolab (Weizmann Science Park, Rehovot, Israel).

RESULTS

Both ET-1 and SRTX-b were found to be resistant to cleavage by 10 μg of ACE (50 mM Hepes/300 mM NaCl, pH 7.4) even at prolonged incubation periods (up to 4 hr). Moreover, ET-1 at concentrations up to 50 μM also failed to inhibit hydrolysis of the synthetic substrate hippuryl-His-Leu by ACE. In contrast to the ETs and the SRTXs, the synthetic hexapeptide His-Gln-Asp-Val-Ile-Trp (corresponding to the C-terminal sequence of the SRTXs) was readily degraded under the above conditions, giving rise to two peptide products that were separated by HPLC: one eluted in 23.7 min and the other eluted in 24.7 min. The intact peptide eluted in 26 min. Amino acid sequencing of the two fragments revealed a single cleavage point at the Val^4-Ile^5 peptide bond.

The various ETs and SRTXs were then incubated with 0.8 μg of NEP. Fig. 2 depicts the time course of the hydrolysis. After 1 hr, two major peptide peaks were obtained by HPLC from the partially degraded ET-1 preparation, one eluting at

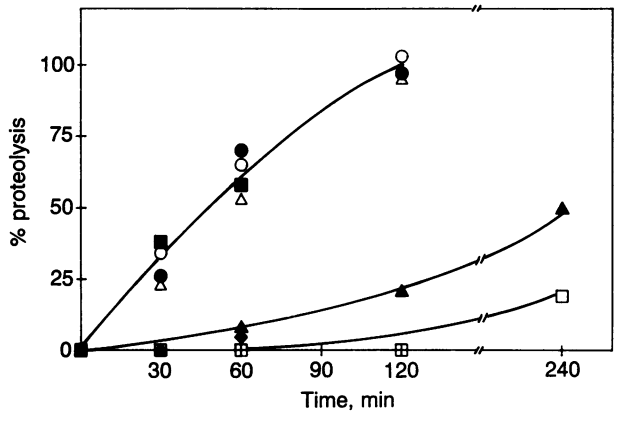

FIG. 2. Time course of hydrolysis of ETs and SRTXs by NEP. ○, ET-1; ●, ET-2; ■, ET-3; △, ET-4; ▲, SRTX-b; □, SRTX-c. Peptides (12.5 μg) were incubated in 100 μl of 20 mM Tris·HCl/300 mM NaCl, pH 7.4, with 2 μl of NEP (0.8 μg) at 37°C for the indicated time intervals. The reaction was terminated by the addition of 5 μl of TFA. Samples were subjected to HPLC analysis. The extent of hydrolysis was determined by the decrease in the relative peak area corresponding to the intact peptide (eluting in 35, 38, 39, 40, 22, and 21 min for ET-1, ET-2, ET-3, ET-4, SRTX-b, and SRTX-c, respectively) and the increase in the relative peak area representing their products (eluting in 18, 19, 18, 19, 18, and 18 min, respectively): e.g., for ET-1, % hydrolysis = [area of product peak (after 18 min)]/[area of product peak (after 18 min) + area at parent peak (after 35 min)] × 100. ◆, Hydrolysis of ET-1 in the presence of phosphoramidon (18 μM).

18 min and the other (corresponding to the intact ET-1) eluting at 34.6 min (Fig. 3). Similarly, cleavage of ET-2

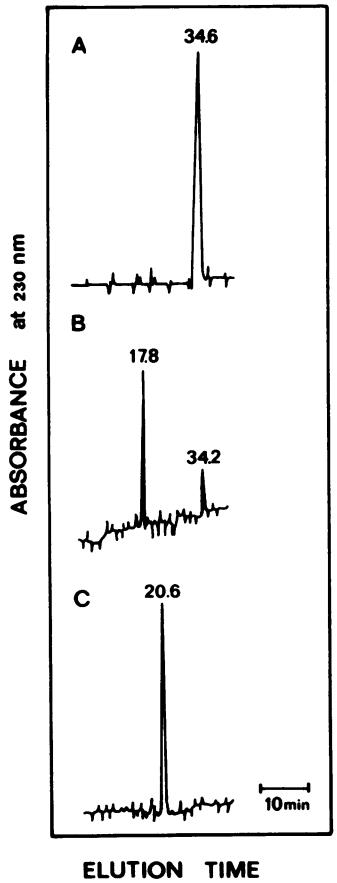

FIG. 3. HPLC profile of intact ET-1 (*A*) and ET-1 and SRTX-c subjected to enzymatic hydrolysis (*B* and *C*, respectively) for 1 hr at 37°C under the experimental conditions outlined for Fig. 2. Intact SRTX-c elutes in 20.6 min.

yielded two major peaks at 19 and 38 min, cleavage of ET-3 yielded two peaks at 18 and 34 min, and ET-4 yielded two peaks at 19 and 40 min. As shown in Fig. 2, under the conditions used the degradation of all four ETs was completed within 2 hr of hydrolysis, with half-lives of ≈1 hr in each case. Inclusion of 18 μM phosphoramidon, a specific inhibitor of NEP, inhibited the hydrolysis of the endothelins by >96% (Fig. 2), thus excluding the possibility that the observed peptide cleavage was caused by any contaminating enzyme that may have been present in the NEP preparation.

In contrast to the ETs, SRTX-b and -c were relatively resistant to degradation by NEP (Fig. 2). Sequence analysis confirmed that the peaks obtained from SRTX-b following incubation for 1 hr with NEP represent mainly the intact peptide. SRTX-b was degraded by 40–50% after 4 hr of incubation, compared to the half-life of ≈1 hr for the ETs under identical conditions. SRTX-c was even more resistant, exhibiting no detectable degradation up to 2 hr of incubation and only 20% degradation thereafter (Fig. 2).

The affinity of the enzyme toward the substrate ET-1 was determined by means of hydrolysis with 200, 150, and 100 μM substrate of 15 min and with 50 and 20 μM substrate for 30 min. Each sample was separated by HPLC and the extent of hydrolysis was determined from the corresponding peak areas. A Lineweaver–Burk plot of the data yielded a K_m value of 30 ± 10 μM.

Sequence analysis of the two peaks (Fig. 3*B*) obtained after hydrolysis of ET-1 for 1 hr revealed two component peptides in each case. The peak eluting at the position of the native peptide (34.6 min) contained, in addition to the intact peptide (70%), a peptide (30%) nicked at the Ser^5-Leu^6 bond. The latter peptide was not separable from the peak corresponding to the intact ET-1. The material eluting at the first peak (18 min) contained ET-1 cleaved at two sites, Ser^5-Leu^6 and Asp^{18}-Ile^{19}, with a small quantity of material cleaved also at Cys^{11}-Val^{12}. There was no evidence of a peptide nicked at Asp^{18}-Ile^{19} only. Interestingly, all our efforts to isolate the tripeptide Ile-Ile-Trp formed by the second cleavage failed, even when this was attempted in the presence of 8 M urea, thus clearly indicating a strong association between the peptide first nicked at Ser^5-Leu^6 and the C-terminal tripeptide resulting from the subsequent cleavage at Asp^{18}-Ile^{19}. Separation could be achieved only after reduction and carboxymethylation of the reduced peptides (data not shown). The nature of the interaction of the tripeptide Ile-Ile-Trp with the remainder of the molecule is not clear; it may be related to the tripeptide's hydrophobic character.

The binding properties of the components of the two peaks (Fig. 3) were determined by using ^{125}I-ET-1. The peptide eluting at 18 min showed no binding capacity at all, while the one eluting at 34.6 min exhibited binding characteristics similar to those of the intact peptide—i.e., B_{max} = 280 ± 10 fmol per mg of protein (300 ± 16 fmol per mg of protein for intact peptide) and K_d = 0.6 nM for both modified and native peptides. The other biochemical assays yielded similar response patterns: the components of the peak that eluted at 18 min did not induce hydrolysis of inositol phospholipids, showed no response in the RIA, and in the lethality test exhibited an LD_{50} more than 50 times higher than that of the intact peptide (which is 15 ng per g of body weight). The components of the peak eluting at 34.6 min exhibited induction of inositol phospholipid hydrolysis similar to that obtained with the native peptide, ≈70% of the value for the native peptide in the RIA assay, and their LD_{50} in the lethality test closely resembled that of the intact peptide.

DISCUSSION

This *in vitro* study demonstrates that ETs are susceptible to hydrolysis by NEP in a two-step process: initially the Ser^5-

Leu^6 bond, which is part of the intramolecular loop structure (see diagram in Fig. 1), is nicked, and this is followed by cleavage at Asp^{18}-Ile^{19}, resulting in formation of the C-terminal tripeptide Ile^{19}-Ile^{20}-Trp^{21}. The possibility of an initial cleavage at Asp^{18}-Ile^{19} followed by rapid cleavage at Ser^5-Leu^6 is contraindicated by the fact that HPLC did not yield a peptide nicked at Asp^{18}-Ile^{19} only; moreover, if the final cleavage site was at Asp^{18}-Ile^{19}, our analysis would have failed to reveal a peptide nicked at Ser^5-Leu^6 only.

As a result of the observed cleavage, ETs are apparently converted to a biologically inactive form, as indicated by four biochemical assays: binding to the ET/SRTX receptors, induction of inositol phospholipid hydrolysis, immunogenicity, and lethality. Thus, in view of the high affinity of the enzyme for ETs, enzymatic control might represent an important means of physiological regulation of the activity of ETs.

Some interesting points emerge from the findings of this study.

(*i*) The two-step process, in which the C-terminal tripeptide is cleaved off only after the formation of a nick between positions 5 and 6 in the intramolecular loop, suggesting that the ETs assume a compact structure within which the tail is hidden and is unavailable for enzymatic cleavage. A synthetic peptide corresponding to positions 16–21 (21) can be hydrolyzed by both ACE and NEP (data not shown), indicating that it is not the specific bond itself that resists hydrolysis but rather its inaccessibility. The similar chromatographic behavior of the four digested ETs indicates that all of them are susceptible to the enzyme, thus suggesting that the process is a general one. The likelihood of a compact structure is indicated by the strong tendency of the fragments to remain together in spite of attempts to separate them by HPLC, even in the presence of 8 M urea. Further corroboration comes from a recent NMR study (23), which showed that in dimethyl sulfoxide ET assumes a compact conformation with the C-terminal hexapeptide closely associated with the bicyclic part of the molecule. Such a structure might explain the nonsusceptibility of the ETs to ACE, which catalyzes the hydrolytic release of dipeptides from the C terminus of oligopeptide substrates (24).

(*ii*) Biochemical assays of the peak eluting at 34.6 min (Fig. 3), which consisted of 70% native peptide and 30% nicked peptide (between positions 5 and 6), revealed reduced immunogenicity of the nicked peptide relative to the native one, with no change in the ^{125}I-ET-1 binding capacity or induction of inositol phospholipid hydrolysis. The lack of change in the latter two activities might indicate that the nicked peptide is still biologically active and, if so, that the presence of disulfide bonds and of the C-terminal peptide (23) keeps the ETs in the active conformation. Since only 30% of the peptide is in a nicked form, these conclusions should be regarded with caution. Nevertheless, they are supported by previous experiments (21) in which CNBr treatment of SRTX-b yielded a modified peptide (cleaved at Met^6) with properties of binding and inositol phospholipid hydrolysis induction similar to those of the active peptide, thus indicating that in spite of cleavage the biologically active conformation is retained, probably because of the disulfide bonds.

The structure–activity relationship data reported here are in agreement with other recent findings: Kimura *et al.* (25) suggested that both the C-terminal Trp and the intramolecular loop structure (illustrated in Fig. 1) are especially important for the vasoconstrictor activity of ET-1; Nakajima *et al.* (26) concluded that the terminal amino and carboxyl groups, the carboxyl groups of Asp^8 and Glu^{10}, as well as the aromatic moiety of Phe^{14} are important for binding the ET molecule to its receptor. It was also concluded that it is not essential for the ET family of peptides to possess two disulfide bridges to act as vasoconstrictor agents; the presence of Cys^1-Cys^{15} enables the molecule to retain some of these activities but with reduced potency (7, 26, 27).

Another major finding of the study is that SRTXs are more resistant than ETs to enzymatic hydrolysis by NEP. Comparison between the sequences of ETs and SRTXs (Fig. 1) indicates that the presence of the charged amino acids Lys^4-Asp^5 is the likely reason for the relatively lower susceptibility of SRTXs to NEP. Asp alone (i.e., when not preceded by Lys) does not appear to be sufficient for this reduced sensitivity to NEP, since Asp^{18} does not prevent the hydrolysis of ETs at Ile^{19}. The higher resistance of SRTX-c than of SRTX-b might be related to the net charges of the loop structure (1). SRTX-b has two positive and three negative charges, resulting in a net charge of −1 within the loop, while SRTX-c has a net charge of −4 (Fig. 1). Assuming that these *in vitro* data represent a mechanism analogous to *in vivo* conditions, the low susceptibility of SRTXs to NEP can provide an explanation for their relatively high toxicity—i.e., under physiological conditions, the ETs may be less lethal than SRTXs because they are inactivated in certain biological target tissues and/or cells by NEP or NEP-like enzymes, while the SRTXs remain intact and can cause the cascade of reactions leading to lethality. It should be noted that i.v. injection of both ETs and SRTXs to mice is lethal above a certain dose (3). This phenomenon may be attributable to the inability of the proteolytic enzyme(s) to digest the high, nonphysiological quantities of ETs at a high enough rate, thus rendering them lethal. The proposed role of NEP as a modulator of ETs is in accord with its regional distribution (18, 19), which fits very well with the known target cells and tissues in which the ETs are active (2, 12).

Thus, the level of ETs can be regulated at two levels. On the one hand, ET production is regulated at the posttranscriptional level (2); on the other hand, its level can be regulated by the metabolic activity of enzymes in a tissue-specific manner. Any dysfunction of these regulatory processes could lead to various pathological conditions, some of which (coronary and cerebral vasospasm and hypertension) are already known (for review, see ref. 2), and some of which remain to be identified and explored.

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