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Processing of proendothelin-1 by human furin convertase

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Abstract Endothelin-1 (ET-1) is the most potent vasoactive peptide known to date. The peptide is initially synthesized as an inactive precursor (proET-1) which undergoes proteolysis at specific pairs of basic amino acids to yield bigET-1. Production of ET-1 then proceeds by cleavage of bigET-1 by the endothelin converting enzyme (ECE). Here, we demonstrate that the in vitro cleavage of proET-1 by furin, a mammalian convertase involved in precursor processing, produced bigET-1. Upon further processing, bigET-1 was converted to biologically active ET-1. Furthermore, we demonstrate that the furin inhibitor, decanoyl-Arg-Val-Lys-Arg chloromethylketone, abolished production of ET-1 in endothelial cells.

Key words: Endothelin-1; Precursor processing; Furin; Convertase; Proteolytic inhibition; Endothelial cell

1. Introduction

Endothelin-1 (ET-1), a 21 amino acid peptide with potent vasoconstricting properties, originates from a precursor (human) of 212 amino acids [1] called prepro-endothelin-1. Removal of the signal peptide in the lumen of the rough endoplasmic reticulum yields a precursor of 195 amino acids, proendothelin-1 (proET-1). The primary structure of the precursor reveals that the immediate precursor to ET-1, a 38 amino acid moiety named big-endothelin-1 (bigET-1), is flanked by pairs of basic amino acids. We can assume that biosynthesis of ET-1 would consist of an initial cleavage C-terminal to the paired basic amino acids followed by carboxy-terminal trimming of bigET-1-Lys-Arg, presumably by a carboxypeptidase H-like enzyme. Conversion of bigET-1 into the biologically active ET-1 is thought to occur on the membrane of target cells, via the endothelin converting enzyme (ECE). Recently, a strong candidate for the ECE, a membrane-bound neutral metalloprotease, has been cloned [2].

Thus, the biosynthesis of endothelin is similar to other biologically active peptides which are initially synthesized usually as large, inactive precursor proteins. These proproteins must be proteolytically cleaved at specific sites in order for the peptides to be liberated and exert their biological actions. In most processing steps, cleavage occurs on the carboxyl side of pairs of basic amino acids, usually Lys-Arg or Arg-Arg, followed by removal of the basic amino acids by carboxypeptidases [3]. Proproteins cleaved in such a manner, include those yielding a diversity of peptidergic hormones, neuropeptides, growth factors, receptors, plasma proteins, cell adhesion molecules, proteolytic enzymes and viral glycoproteins.

To date, none of the proteases responsible for the initial maturation events of the endothelin precursor have been identified. Recently however, a new family of proteolytic enzymes has been characterized which possess the specificity to process many types of precursors at pairs of basic amino acids [4–7]. These mammalian subtilisin-like proprotein convertases (SPCs) are the higher eukaryotic counterparts to the bacterial subtilisins and to the yeast Kexin enzyme, a calcium-dependent serine endoprotease specific for cleaving on the carboxyl side of pairs of basic amino acids [8]. Within the last five years, up to six members of this class of proteases have been discovered; furin [9], PC2 [10], PC1/PC3 [11], PACE 4 [12], PC4 [13] and PC5/PC6 [14]. Each enzyme possesses its own set of unique features but one common thread is their demonstrated specificity for cleaving mostly on the C-terminal end of pairs of basic amino acids.

Within this family of endoproteases, furin is the best characterized. Northern blot analysis has revealed that the fur gene is expressed at different levels, in a wide variety of cell lines and tissues [15] including endothelial cells where ET-1 is produced [16]. The furin enzyme is a calcium-dependent, serine protease predominantly localized in the trans Golgi membrane [17] but it has also been detected on the cell surface [18] and extracellularly [19]. Co-expression studies have described how furin can efficiently and correctly process a variety of proprotein precursors into the biologically active polypeptides [17,20–32]. All these precursors share the motif R-X-K/R-R- (P4-P3-P2-P1 respectively) at the processing site. In this report we show the results of in vitro digestions of proET-1 by purified furin as well as in vivo inhibition of cleavage of proET-1. Our data indicates that furin is an interesting candidate enzyme involved in the initial steps of ET-1 biosynthesis.

2. Materials and methods

2.1. Materials

Chemicals and reagents were obtained from Boehringer-Mannheim, Fisher or Sigma. The boc-Arg-Val-Arg-Arg-AMC were from Biochem Immunosystems Inc. (Laval, Canada).

2.2. Production of proendothelin-1 in Escherichia coli

The cDNA fragment encoding proendothelin-1 was cloned in the SalI/BamHI digested pQE-9 plasmid (Qiagen, Chatsworth, CA, USA). We produced and purified proET-1 protein using the QIAexpress system (Qiagen) as manufacturer's instruction. Briefly, XL-1 blue (Stratagene) were transformed with plasmids containing proET-1 insert and cultured in 2 × YT medium. Synthesis was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. Cells were harvested, centrifuged and lysed in 6 M guanidine-HCl, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0. The lysate was applied to

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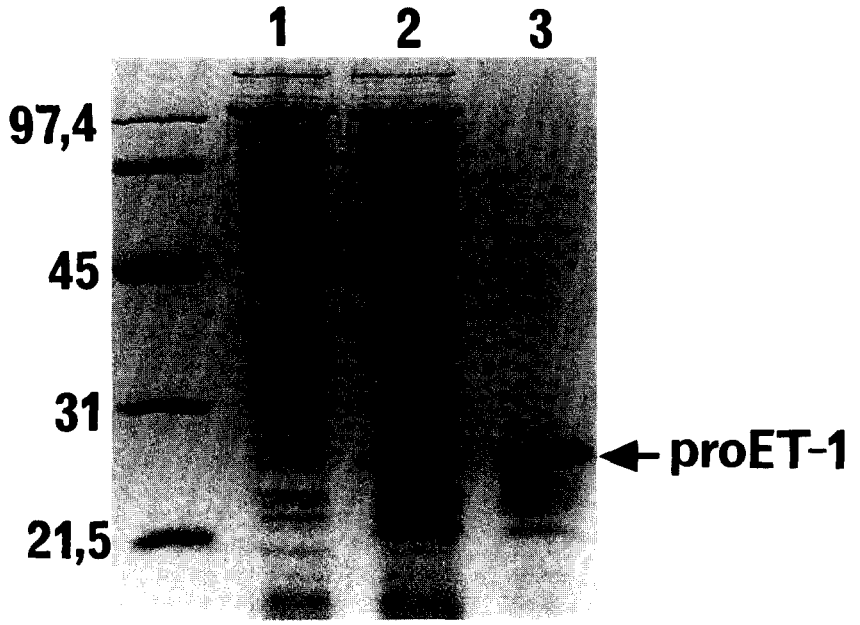

Fig. 1. SDS-PAGE of bacterial lysates and proET-1 purification. Lane 1, bacterial lysate without IPTG induction; lane 2, induced with IPTG; and lane 3, purified proET-1. Samples were applied to a 10% SDS polyacrylamide gel and stained with Coomassie blue.

a column of Ni-NTA resin and elution was according to manufacturer's instructions.

2.3. Production and purification of soluble furin

BSC-40 cells were infected with a vaccinia virus recombinant (VV:hfur713) expressing a secreted furin (hfur713t) as previously described [26]. After an 18 h infection, protein-free media (MCDB 202, Gibco/BRL) was concentrated on a Centriprep-30 (Amicon) cartridge. The concentrate was diluted 5-fold with 20 mM Tris-HCl, pH 7.4, and loaded on a Mem-Sep1000 DEAE ion exchange column (Millipore). A gradient of 0–750 mM NaCl in 20 mM Tris-HCl, pH 7.6, was used to elute the hfur713t. Fractions were tested for enzymatic activity using the boc-Arg-Val-Arg-Arg-AMC fluorogenic peptide as described [19].

2.4. In vitro digestion of proendothelin-1

In vitro digestions were performed using 3.8 μg (160 pmol) of proET-1. This was digested first with 50 U of furin (1 U = 1 pmol·h^{-1} of AMC from boc-Arg-Val-Arg-Arg-AMC) in 20 μl of digestion buffer (100 mM HEPES, pH 7.6, 1 mM $CaCl_2$, 0.5% Triton X-100) for 1 h. The digestion mixture was then adjusted to pH 4.0 and 0.1–0.2 Unit of chymosin (EC 3.4.23.4, Sigma) was used for further proteolysis (30 min). Digestion products were tested for pressor activity on rat vas deferens and rabbit carotid artery.

2.5. Gel filtration and enzyme immunoassay

ProET-1 (1.4 μg, 60 pmol) was digested with furin for 6 h and sample was applied to a calibrated TSK-G3000PW gel filtration column (7.5 mm × 600 mm, TOSO) preequilibrated with 0.1% trifluoroacetate, 50% acetonitrile. Proteins were eluted in this buffer at a flow rate of 0.5 ml/min and fractions were collected every 0.5 min. Fractions were dried and dissoved in 20 mM Na-phosphate, 400 mM NaCl, 0.2% BSA, 2 mM EDTA, 10% skim milk (Block Ace Snow brand), pH 7.0. The contents of bigET-1-like immunoreactive material were measured by enzyme immunoassay as described previously (38), using an antibody which recognizes the C-terminal sequence of bigET-1 including proET-1 (unpublished results).

2.6. Pharmacological assays

Smooth muscle contractility was studied in the carotid artery (RbCA) of male New Zealand White rabbits (1.8–2.5 kg) and vas deferens (RVD) of male Wistar rats (250–350 g). RbCa and RVD were prepared according to published methods [36,37]. Briefly, the animals were killed by stunning and exsanguination. The RbCA were removed and mechanically denuded of their endothelium and cut into 5–6 mm wide rings. Two platinum self-closure wires (8–10 mm diameter) were inserted though each ring. The RVD were removed, dissected from surrounding adipose tissue and blood vessels. Segments of 15–20 mm of the prostatic portion were suspended between parallel platinum electrodes. Tissues were suspended in 10 ml baths containing oxygenated (95% O_2, 5% CO_2) Krebs' solution (composition in mM: NaCl 118; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.2; Glucose 10) at 37°C with indomethacin (1 μM). Both preparations were stretched to a resting tension of 2 g and equilibrated for 40–60 min. The mechanical responses were measured isometrically by Grass force transducers (FT03C) and recorded on a multichannel Grass polygraph (Model 7D). Following equilibration period, RVD were electrically stimulated (square-wave pulses: 50 V, 0.1 ms) at a frequency of 0.1 Hz with a Grass stimulator (Model S8).

2.7. Endothelial cell culture and proteolytic inhibition by decanoyl-Arg-Val-Lys-Arg chloromethylketone

Bovine aortic endothelial cells were cultured as previously described [16]. Cells at 80% confluency were put in serum free media (Endothelial-SFM, Gibco) and incubated with various concentrations (0–50 μM) of decanoyl-Arg-Val-Lys-Arg chloromethylketone (a generous gift of

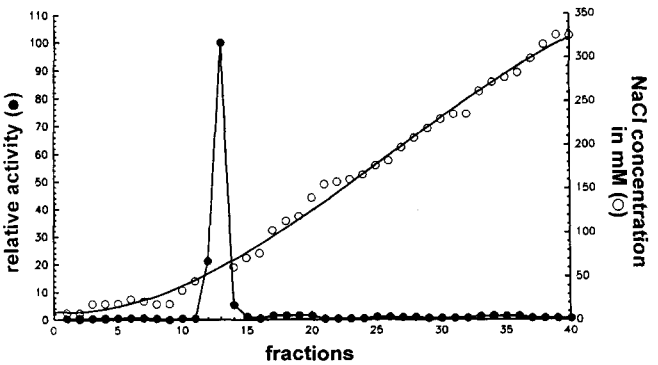

Fig. 2. Purification of hfur713t on a MemSep1000 DEAE ion exchange column. Diluted media was loaded on the column and furin was eluted by NaCl gradient (○). Enzymatic activity (●) was detected in fraction 12–13. NaCl concentration was measured by refractometry.

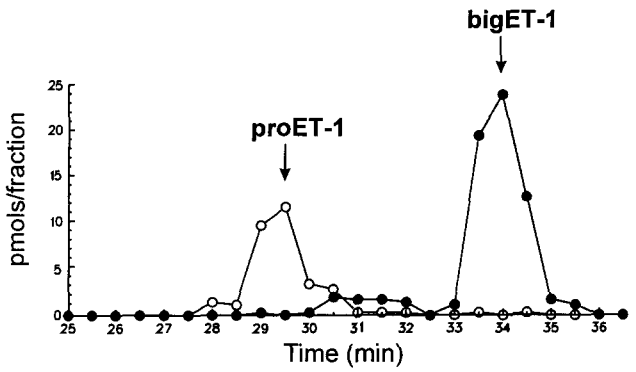

Fig. 3. Gel filtration and enzyme immunoassay of furin-cleaved (●) and uncleaved (○) proET-1. Arrows indicate fractions where proET-1 and bigET-1 eluted from the column. Column was calibrated with synthetic ET-1 (2.5 kDa), bigET-1 (4.3 kDa), lysozyme (14.3 kDa), and chymotrypsinogen (25 kDa).

Drs Herbert Angliker and Elliott Shaw, Friedrich Miescher-Institut, Basel) for 18 h. Conditioned media was harvested and ET-1 specific radioimmunoassay (Amersham) was performed as previously described [16].

3. Results

3.1. *In vitro digestions of proET-1 by furin and chymosin*

In order for us to perform in vitro digestions of proET-1, sufficient quantities of the precursor had to be produced. We used a prokaryotic expression system which facilitated purification of the biosynthesized proteins. Milligram amounts of 70% pure proET-1, having a molecular weight of 25,000 Da as determined by SDS-PAGE, were obtained (Fig. 1). Our source of furin relied on a vaccinia virus recombinant expressing a soluble form of the enzyme, hfur713t, engineered to be secreted in media of infected BSC-40 cells. It was previously shown that this protein maintains all of native furin's enzymatic properties [26]. In order to exclude any other proteolytic activity present in the conditioned media, the secreted furin was purified by ion exchange chromatography. Following overnight incubation of infected cells in protein-free media (MCDB 202), concentrated media containing soluble furin was diluted in 20 mM Tris, pH 7.4, and chromatographed on a DEAE anion exchange column. Fig. 2 illustrates that enzymatic activity, measured by release of fluorescence using the Boc-Arg-Val-Arg-Arg-AMC substrate, eluted in fractions 12–13. When conditioned media from wild-type infected cells were chromatographed under identical conditions, no enzymatic activity was detected using either the fluorogenic substrate or the proET-1 as a substrate in all elution fractions.

We then tested furin's capacity to cleave proET-1 in vitro. Following proET-1 digestion by furin, products were chromatographed on a TSK-G3000PW gel filtration column and fractions were assayed with an antiserum recognizing bigET-1 and proET-1. Fig. 3 shows that, under our conditions, proET-1 is completely converted to a bigET-1-like peptide eluting at a

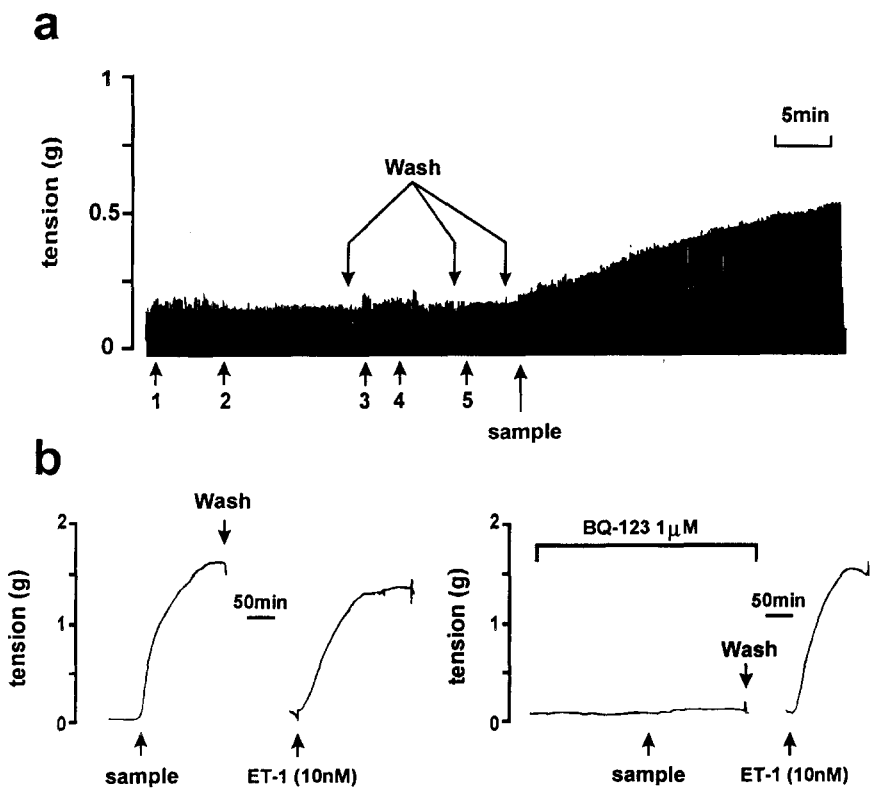

Fig. 4. Constriction activity of proET-1 digested with furin and chymosin (sample). (A) Contraction-response of rat vas deferens. Controls (indicated by arrows) were (1) digestion buffer alone, (2) chymosin and furin, (3) proET-1 alone, (4) proET-1 cleaved by furin alone, (5) proET-1 cleaved by chymosin alone. (B) Contraction of rabbit carotid artery with (right panel) or without (left panel) the ET_A selective antagonist BQ-123. Control reactions also showed no pressor activity on this preparation (not shown).

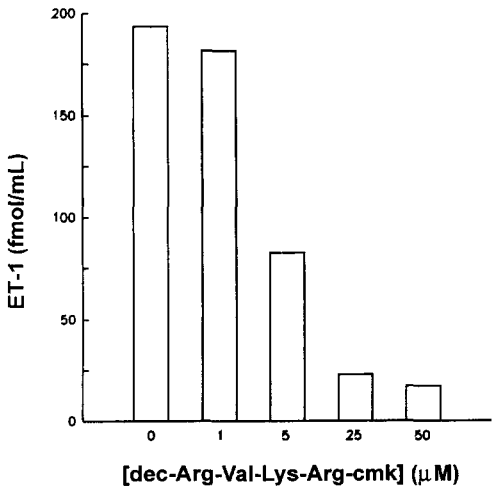

Fig. 5. Effect of dec-RVKR-cmk on production of ET-1 in cultured endothelial cells. Levels (fmol/ml) of ET-1 was measured in conditioned media of cells that were incubated for 18 h in the presence of varying concentrations of dec-RVKR-cmk. Cell viability was unchanged when exposed to this inhibitor (not shown).

position corresponding to this peptide's molecular weight on the gel permeation column and recognized by the antibody.

To confirm production of a bigET-1-like peptide, the digestion products of proET-1 by furin were incubated with chymosin, an aspartyl protease previously shown to efficiently convert bigET-1 into ET-1. We verified for the production of biologically active ET-1 by pharmacological assay on the rabbit vas deferens and the rat carotid artery. We initially subjected the tissues with various controls. As depicted in Fig. 4, the following samples failed to elicit contraction of the tissue: (i) digestion buffer alone, (ii) proET-1 alone, (iii) proET-1 digested with furin alone, (iv) proET-1 digested with chymosin alone, (v) chymosin and furin. Only when chymosin digestion was performed on furin-cleaved proendothelin-1, did we obtain a significant pharmacological response in the rat vas deferens and the rabbit carotid artery preparations. We next asked whether this response was blocked by the ET_A antagonist, BQ-123. Incubation of the tissue with this compound prevented pharmacological response and thus confirmed the specific interaction of the produced peptide with the ET_A receptor in this preparation.

3.2. *Inhibition of ET-1 synthesis by dec-RVKR-cmk in endothelial cells*

We had previously detected furin mRNA in cultured endothelial cells, the major producers of ET-1. To test whether a chloroalkylketone could inhibit endogenous convertase activity in endothelial cells, the effect of dec-RVKR-cmk was analyzed. As depicted in Fig. 5, ET-1 synthesis was suppressed by increasing concentrations of the compound. Levels were at approximately 40% of mock-treated cells (no inhibitor) with 5 μM and were 9% of maximum at 50 μM. These high concentrations (50 μM) of dec-RVKR-cmk had no deleterious effects on these cells when viability was assessed (not shown). Thus, the results suggest that this chloroalkylketone could block cleavage of proET-1 in endothelial cells, possibly due to inhibition of endogenous furin proteolytic activity.

4. Discussion

Within the last four years, the discovery and characterization of enzymes belonging to a new family of endoproteases which cleave on the C-terminal of pairs of basic amino acids, brought to light a long-standing question as to the identification of some of the key players involved in production of biologically active peptides. There are presently six distinct members within this family, all possessing particular features with regards to their specificity, sub-cellular localization and tissue distribution. Two sub-groups, each having 3 members, can be readily identified on the basis of their distribution. The furin, PACE 4 and PC5/PC6 enzymes are more ubiquitously distributed while the PC2, PC1/PC3 and PC4 enzymes are localized to endocrine/neuroendocrine cells and testicular spermatids (PC4). Thus, we can link processing of precursors, which follow the regulated pathway of secretion, to the PC2, PC1/PC3 and PC4 enzymes while constitutively secreted proteins would be cleaved by furin, PACE 4 and PC5/PC6.

For proET-1, which contains multiple paired basic residues constituting processing motifs, the precursor probably goes through the endothelial cell's constitutive secretory pathway and is cleaved, initially, by endoproteases which have specificity for Lys-Arg or Arg-Arg sites. In the human proET-1 there are 4 of these sites along with multiple single arginines which can also serve as cleavage signals.

We recently described the presence of furin mRNA in cultured bovine endothelial cells which suggested a possible role in cleaving the ET-1 precursor [16]. Thus we needed to assess furin's specificity towards this precursor. Two furin consensus motifs (Arg-Ser-Lys-Arg at both the N- and C-terminal) border the bigET-1 peptide. Furin's ability to cleave at a Arg-X-Lys/Arg-Arg site within proET-1 would suggest production of bigET-1-Lys-Arg. Thus, processing of the N-terminal cleavage motif would correspond to the intact endothelin-1 N-terminal while the C-terminal would have to undergo carboxyterminal trimming prior to cleavage by the ECE. However, when examining the amino acid sequence of proET-1, there exists multiple furin cleavage signals and other similar cleavage sites like Arg-Arg or single Arg. One of these is located at the N-terminal processing site of bigET-1, where there is an overlapping furin cleavage motif (-Arg-Leu-Arg-Arg^{49}-Ser-Lys-Arg^{52}-bigET-1). Thus, if cleavage was to occur upstream (at Arg^{49}) of the known cleavage site (Arg^{52}) full biological activity would not follow either because of the failure of the endothelin-converting enzyme to produce intact ET-1 or because the Ser-Lys-Arg-endothelin-1 would not interact with its receptor. Thus, furin is very specific with regards to its recognition of the substrate's cleavage site.

Since digestion of proET-1 by furin putatively produced bigET-1-Lys-Arg, we needed to artificially produce the ET-1 peptide. This because conversion of bigET-1 into ET-1 by the ECE in vivo necessitates the intact C-terminal end of bigET-1 [33]. It had been reported that the aspartyl protease chymosin specifically converted bigET-1 into biologically active and intact ET-1 [34]. Production of a peptide indistinguishable from ET-1 as measured by pharmacological assay confirmed correct cleavage of the precursor by furin.

To further authenticate furin's role in proET-1 conversion we used the dec-RVKR-cmk in endothelial cells. Our results are similar to those reported for the inhibition of processing of the

glycoprotein gp160 of HIV-1 [28]. It was observed that the most potent chloromethylketone (dec-REKR-cmk) inhibited cleavage by more than 70% at concentrations of 5 μM. At the same concentrations we observed a 60% inhibition in the production of ET-1 in endothelial cells.

Our data suggest that furin may play a role in the biosynthesis of the ET-1 peptide. Although it remains to be determined if other members of this family are also involved in ET-1 biosynthesis, recent findings showing presence of furin and PC5/PC6 convertases in endothelial cells narrows the possibilities that the other SPCs are involved in proET-1 processing [35]. Thus, although there is presently no direct physiological role for furin in the biosynthesis of ET-1, it is currently the strongest candidate. Clearly, further work will be necessary to identify bona fide proET-1 processing endoprotease(s) in endothelial cells and in other cell types where isoforms of endothelins (ET-1, -2 and -3) have been detected.

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