Effect of an Antisense Oligodeoxynucleotide to Endothelin-Converting Enzyme-1c (ECE-1c) on ECE-1c mRNA, ECE-1 Protein and Endothelin-1 Synthesis in Bovine Pulmonary Artery Smooth Muscle Cells

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ABSTRACT
Endothelin-1 (ET-1) is secreted from endothelial and vascular smooth muscle cells (VSMC) after intracellular hydrolysis of big ET-1 by endothelin converting enzyme (ECE). The metallopeptidase called ECE-1 is widely thought to be the physiological ECE, but unequivocal evidence of this role has yet to be provided. Endothelial cells express four isoforms of ECE-1 (ECE-1a, ECE-1b, ECE-1c, and ECE-1d), but the identity of ECE-1 isoforms expressed in VSMC is less clear. Here, we describe the characterization of ECE-1 isoforms in bovine pulmonary artery smooth muscle cells (BPASMC) and the effect on ET-1 synthesis of an antisense oligodeoxynucleotide (ODN) to ECE-1c. Reverse transcriptase-polymerase chain reaction (RT-PCR) evaluation of total RNA from BPASMC showed that ECE-1a and ECE-1d were not expressed. Sequencing of cloned ECE-1 cDNA products and semiquantitative RT-PCR demonstrated that ECE-1b and ECE-1c were expressed in BPASMC, with ECE-1c being the predominant isofom. Basal release of ET-1 from BPASMC was low. Treatment for 24 h with tumor necrosis factor-α (TNFα) stimulated ET-1 production by up to 10-fold with parallel increases in levels of preproET-1 mRNA. Levels of ECE-1c mRNA were also raised after TNFα, whereas amounts of ECE-1b mRNA were decreased significantly. Treatment of BPASMC with a phosphorothioate antisense ODN to ECE-1c caused a marked reduction in ECE-1c mRNA levels and ECE-1 protein levels. However, basal and TNFα-stimulated ET-1 release were largely unaffected by the ECE-1c antisense ODN despite the inhibition of ECE-1c synthesis. Hence, an endopeptidase distinct from ECE-1 is mainly responsible big ET-1 processing in BPASMC.

Endothelin-1 (ET-1) is derived from its precursor, preproendothelin-1, by intracellular proteolytic processing (Corder et al., 1995; Harrison et al., 1995; Woods et al., 1999). The final step in its biosynthesis involves specific enzymatic hydrolysis of the intermediate, big ET-1, by an endothelin-converting enzyme (ECE) (Yanagisawa et al., 1988). ET-1 exerts wide-ranging effects on a variety of tissues and cell types through interaction with cell surface receptors (ET_A and ET_B receptors) (Douglas, 1997; Haynes and Webb, 1998). It has been implicated as a causative factor in the pathogenesis of hypertension, pulmonary hypertension, congestive heart failure, atherosclerosis, and asthma (Douglas, 1997; Haynes and Webb, 1998; Goldie and Henry, 1999). A number of highly potent ET receptor antagonists have been developed for therapeutic use. These compounds are generally selective for ET_A receptors or nonselective ET_A/ET_B antagonists (Douglas, 1997). In some tissues, most notably the airways, ET_B receptors predominate yet they are resistant to blockade by selective ET_B or nonselective ET_A/ET_B receptor antagonists (Hay et al., 1998). Therefore, specific inhibition of ET-1 synthesis with ECE inhibitors may be a better approach for attenuating the adverse effects of ET-1 excess under some conditions.

ABBREVIATIONS: ET-1, endothelin-1; ECE, endothelin converting enzyme; ODN, oligodeoxynucleotide; BPASMC, bovine pulmonary artery smooth muscle cells; DMEM, Dulbecco’s modified Eagle medium; BAEC, bovine aortic endothelial cells; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT, reverse transcriptase; bp, base pair(s); TNFα, tumor necrosis factor α; VSMC, vascular smooth muscle cells.
Two endothelin-converting enzyme genes have been cloned and are referred to as ECE-1 (Schmidt et al., 1994, Shimada et al., 1994; Xu et al., 1994) and ECE-2 (Emoto and Yanagisawa, 1995). ECE-1 is the most extensively studied of these two endopeptidases. It is widely expressed in many cells and tissues (Korth et al., 1999). ECE-1 was originally thought to be expressed as two isoforms, ECE-1a and ECE-1b (Valdenaire et al., 1995). More recent findings, however, have revealed two additional isoforms: ECE-1c (Schweizer et al., 1997) and ECE-1d (Valdenaire et al., 1999). The four ECE-1 isoforms result from alternative splicing at the 5'-end of a single gene (Schweizer et al., 1997; Valdenaire et al., 1999). They share a common C-terminal region (encoded by exons 4–19), that includes a transmembrane domain and the enzyme catalytic site. Studies of the distribution of the four isoforms have shown ECE-1c to have the highest relative expression (Schweizer et al., 1997; Valdenaire et al., 1999).

Based on gene deletion studies, both ECE-1 and ECE-2 have been proposed as physiologically relevant enzymes for ET-1 biosynthesis (Yanagisawa et al., 1998; Yanagisawa et al., 2000). This conclusion is derived from observations that ECE-1 gene knockout causes abnormalities in cardiac development very similar to targeted disruption of the genes for ET-1 (Kurihara et al., 1994), or the ETA receptor (Clouthier et al., 1998). Biochemical assessment of the effect of ECE-1 knockout on ET-1 synthesis is difficult to investigate in ECE-1–/– embryos because it results in midgestational lethality. ET-1 levels in gestational day 12.5 embryos show reductions of about 40% in ECE-1 null mice (Yanagisawa et al., 1998, 2000), but this may simply reflect the fact these embryos are not viable. If the physiologically essential ECE is eliminated in the ECE-1–/– mice, raised tissue levels of big ET-1 would be expected because of inhibition of its processing, but no increases were observed (Yanagisawa et al., 1998).

Thus, despite the many reports showing that ECE-1 isoforms are able to convert big ET-1 to the mature ET-1 peptide, it is still unclear whether ECE-1 plays a physiological role in big ET-1 processing. The aim of this study was to examine the role of ECE-1c in ET-1 production by using an antisense oligodeoxynucleotide (ODN) to selectively inhibit ECE-1c synthesis in cultured cells. These studies used bovine pulmonary artery smooth muscle cells (BPASMC) because initial investigations revealed ECE-1c to be the major isoform in these cells, with no expression of ECE-1a or ECE-1d. The results show that ECE-1 has only a minor role in ET-1 synthesis.

Characterization of ECE-1 Isoforms Expressed in BAEC and BPASMC by RACE and Colony Hybridization. Total RNA was extracted from confluent BAEC or BPASMC monolayers by using RNAzol B (Biogenesis, Poole, UK). Poly(A+) mRNA for BAEC and BPASMC was obtained from total RNA using poly(A) tract oligo dT-magnetic beads (Promega, Southampton, UK). After first- and second-strand synthesis and adaptor ligation, 5' rapid amplification of cDNA ends (5'-RACE) was carried out using the Marathon cDNA amplification protocol (Clontech, Basingstoke, UK). Polymerase chain reaction (PCR) was performed by using the adaptor forward primer 5'-CCATCTTAATACGACTCACTATAGGC-3' (AP1) and a reverse primer, 5'-GGGTCCTCTTTCCTGGTTATGGA-3', corresponding to a sequence common to all bovine ECE-1 isoforms (Fig. 1). For BAEC and BPASMC, cDNA from these reactions were purified, subcloned into the plasmid TA cloning vector pGEM-T Easy (Promega), used to transform competent JM109 Escherichia coli (Promega), and cultured at 37°C for 14 h. Colony hybridization to identify ECE-1 clones was performed with a 214-bp cDNA probe obtained after purification of the BglI (Promega) digest of the 253-bp ECE-1c PCR product (described below). For hybridization, 25 ng of the cDNA probe was labeled using random hexanucleotides and Klenow fragment (Promega) in a 50-μl reaction volume containing 50 μCi of [α-32P]dCTP (Amersham Pharmacia Biotech, Little Chalfont, UK) for 4 h at 37°C. Positive colonies were subcultured and further evaluated by performing PCR and restriction digests. Plasmid DNA samples containing inserts of interest were purified and sequenced by using ABI Prism BigDye Terminator Cycle sequencing in conjunction with an ABI Prism 377 sequencer (PE Biosystems, Warrington, UK).

Reverse Transcription Polymerase Chain Reaction. RT-PCR measurements of mRNA levels were carried out with reagents from Promega with 100 ng of total RNA per reaction under semi-quantitative conditions so that the yield of PCR product was proportional to the quantity of RNA template (Barker et al., 1998; Corder and Barker, 1999). For preproET-1, ECE-1a, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), conditions were as described below.

Experimental Procedures

Cell Culture. BPASMC were cultured from fresh bovine pulmonary artery in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum by using the explant technique (Corder, 1996). Cultured cells, used between passages 5 and 10, exhibited characteristic smooth muscle cell morphology and stained positively for α-actin. Bovine aortic endothelial cells (BAEC) were cultured as described by Corder and Barker (1999).

Initial experiments with BPASMC evaluated the ET-1 response to TNFα (R&D Systems, Minneapolis, MN), and the effects of phosphoramidon (Peptide Institute, Osaka, Japan) on ET-1 and big ET-1 release. For both series of experiments, confluent cultures were incubated with the agents being investigated in serum-free DMEM for 24 h. The conditioned media were collected for immunoassay of ET-1 and big ET-1.
Effect of ECE-1 Antisense Oligonucleotide on ET-1 Synthesis

(Chen and Kralik, 1999). RT-PCR measurements of mRNA levels for ECE-1b, ECE-1c, and ECE-1d were all performed under the same conditions so that a comparison of their relative expression could be made. Reverse transcription with avian myeloblastosis virus RT (5 U per reaction, 48°C for 45 min), primed with isoform specific primers and oligo dT15 primer (1 μM) was followed by PCR using Taq polymerase (5 U per reaction) with the following cycling parameters: denaturation for 2 min at 94°C; 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. Isoform-specific ECE-1 primers were based on published sequences for ECE-1c, and sequences obtained during these studies from the cloned 5’-ends of ECE-1b and ECE-1d. The primers were: ECE-1b (5’-GCAGTGGCGGCGCTGTCGGGATG-3’; forward), ECE-1c (5’-GGAGGCGGCAGCGATGATG-3’, forward), ECE-1d (5’-CTTATAGGATGCGTGCTGCA-3’, forward), with a common reverse primer ECE-1b/1c/1d (5’-GGCTTCTTGTCTGCTGATTGGA-3’). These gave PCR products of 253, 253, and 269 base pairs, which were separated by electrophoresis on ethidium bromide-stained 1–12% (w/v) agarose gels. In each case, a single band of the expected size was obtained and quantified using a Gel Doc 1000 system (Bio-Rad, Hercules, CA).

Antisense ODN Experiments. The sequence chosen as the antisense ODN target was a 25-bp region around the translation start codon of bovine ECE-1c (Schmidt et al., 1994) (Fig. 1). An ODN primer corresponding to this sequence gives robust amplification of a single product by RT-PCR (Chen and Kralik, 1999). By analogy with the human ECE-1b and ECE-1c genes, 16 bp at the 3’ end of this sequence would be expected to be common to both bovine ECE-1b and ECE-1c. However, the 3’ end of the antisense ODN, which is likely to confer its specificity, is complementary only to ECE-1c. An antisense ODN corresponding to this sequence (5’-CTTGTAGGTTAGATGCGTGCTGCA-3’, antisense ODN) was used with its sense counterpart as a control (5’-GGCGGCTGTCGGGATG-3’, sense ODN). In some experiments, following recommendations of Stein and Krieg (1997), an additional scrambled control ODN was used with the same base composition as the antisense ODN (5’-CTACAGATGGCGCGCTGATGCTCTACCTACAG-3’, sense ODN). ECE-1 protein content was evaluated for ECE activity (Corder et al., 1995), protein content (detergent compatible assay; Bio-Rad), and ECE-1 protein determined by immunoblotting.

Immunoblotting. Solubilized protein samples (25 μg) were mixed with an equal volume of 62.5 mM Tris-HCl, pH 6.8, containing 10 mM dithiothreitol, 25% glycerol, 2% SDS, and 0.01% bromphenol blue and heated for 2 min at 100°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5% gels) and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Blots were blocked with 1-block (Tropix, Bedford, MA) and then incubated overnight with rabbit anti-ECE-1 IgG (1.5 μg/ml). Second antibody was a horseradish peroxidase–goat anti-rabbit IgG conjugate, and detection of bands was achieved by using the chemiluminescence substrate SuperSignal West Pico (Pierce, Rockford, IL). ECE-1 antisera was raised in rabbits against the C-terminal sequence of human ECE-1 residues 737 to 753 (Schmidt et al., 1994) by Neosystem (Strasbourg, France). ECE-1 specific IgG was purified on GSPM-NPPHKAEEVW (Genosys, Cambridge, UK) coupled to CNBr-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, UK).

Materials. Unless otherwise indicated all chemicals and standard reagents were obtained from either Sigma (Poole, UK) or Merck (Lutterworth, UK). Tissue culture medium and reagents were from Sigma or Life Technologies (Paisley, UK). PCR primers were obtained from Eurogentec.

Analysis of data. Results are expressed as mean ± S.E.M. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and compared by ANOVA with Fisher’s protected least-significant difference post hoc test or Student’s t test using Statview software (SAS Institute, Cary, NC).

Results

ECE-1 Isoform Expression in BPASMC. Using RACE combined with colony hybridization to identify cloned cDNAs, ECE-1c was the most common ECE-1 isoform sequence obtained from BPASMC mRNA. It was identified by the double ATG at the 5’-end and a short, conserved 5’-flanking sequence (Fig. 1A) that corresponded to the established bovine ECE-1c sequence encoded by exon 1c (Schmidt et al., 1994; Valdenaire et al., 1999). Another ECE-1 sequence of lower frequency was identified in the cDNA from BPASMC. On sequencing, this was found to be homologous with the exon 1b of human ECE-1b (Fig. 1A) and hence represents bovine ECE-1b. Although the full exon 1b sequence was not obtained, sufficient sequence information was acquired to allow the design of a specific forward primer for bovine ECE-1b. Neither the ECE-1a nor ECE-1d isoforms were identified in the cloned ECE-1 cDNAs from BPASMC. For comparison, experiments were also performed using cloned cDNA from BAEC; ECE-1a was found to be the most abundant transcript. ECE-1b and ECE-1c were also present and confirmed the sequences obtained from the BPASMC cDNA. In addition, a single colony was identified from the cloned BAEC cDNA with ECE-1d specific sequence. This was used to design a specific primer for ECE-1d RT-PCR (Fig. 1A).
The specific primers for the ECE-1b, ECE-1c, and ECE-1d isoforms were used in combination with the common reverse primer in semiquantitative RT-PCR studies to compare the expression of ECE-1 isoforms in BPASMC and BAEC. To emphasise the differences in levels of mRNA for the specific isoforms for each reaction 200 ng total RNA from BAEC was compared with 400 ng BPASMC RNA (Fig. 1B). This showed that under basal conditions ECE-1c was the predominant isoform expressed in BPASMC, ECE-1b was also present at ~35% of the level of ECE-1c mRNA, but neither ECE-1a nor ECE-1d were detectable in BPASMC RNA. This contrasted with results using total RNA from BAEC where all four ECE-1 isoforms were expressed.

Characterization of ET-1 synthesis by BPASMC. ET-1 release under basal conditions was low (Fig. 2). Treatment of BPASMC for 24 h with TNFα caused concentration-dependent increases in ET-1 release with a significant rise at 0.3 ng/ml (p < 0.001) and maximum effect at 30 ng/ml (Fig. 2A). TNFα-stimulation increased ET-1 secretion by up to 10 fold with similar rises in levels of preproET-1 mRNA (Fig. 3). TNFα also augmented the secretion of big ET-1 by 2- to 5-fold. Under basal conditions and during TNFα-stimulation, big ET-1 release represented ~20% of total endothelin production by BPASMC (Fig. 2B). Synthesis of ET-1 under basal and TNFα-stimulated conditions was inhibited by phosphoramidon with IC50 values of 29 ± 3 μM and 43 ± 14 μM, respectively (Fig. 2B). Inhibition of ET-1 synthesis with phosphoramidon increased big ET-1 levels by 8- to 10-fold compared with the corresponding values obtained without phosphoramidon (p < 0.001; Fig. 2B).

Effect of TNFα on ECE-1 Isoform Expression in BPASMC. Under basal conditions, levels of ECE-1c mRNA were more abundant than ECE-1b mRNA (4.0 ± 0.7 compared with 1.6 ± 0.3 arbitrary densitometric units, n = 4 experiments, p = 0.021). Treatment of BPASMC with TNFα caused a 3-fold increase in ECE-1c mRNA levels (Fig. 3). In comparison, amounts of ECE-1b mRNA were significantly lower in BPASMC after treatment with TNFα (expressed as a percentage of untreated samples, ECE-1b mRNA levels were reduced to 24.8 ± 10%, n = 3, p < 0.01). Expression of mRNAs for both ECE-1a and ECE-1d were still undetectable after TNFα treatment.

Effects of ECE-1c Antisense ODN on BPASMC. Treatment of BPASMC with the ECE-1c antisense ODN reduced
basal and TNFα-stimulated ECE-1c mRNA levels by 37% and 65% compared with sense ODN control ($p < 0.05, p < 0.01$; Fig. 3). Levels of preproET-1 and GAPDH mRNA were unaffected by the ECE-1c antisense ODN.

The production of ET-1 and big ET-1 under basal conditions were not altered by treatment with the sense or antisense ODNs. After TNFα stimulation, ET-1 release was 19% lower from cells treated with ECE-1c antisense ODN when compared with the sense ODN control ($p < 0.01$, Fig. 3), but this change was not significantly different from release with TNFα alone. Consistent with the reduction in ET-1 levels with the ECE-1c antisense ODN, big ET-1 levels were increased by 42% compared with the sense ODN control ($p < 0.001$, Fig. 3).

In agreement with changes in ECE-1c mRNA levels, TNFα increased by more than 2-fold the level of membrane-bound ECE activity measured by immunoblotting and enzyme assay ($p < 0.01$, Fig. 4). Treatment with the ECE-1c antisense ODN reduced ECE-1 protein and enzyme activity by at least 60% under basal and TNFα-stimulated conditions (Fig. 4). In comparison, neither sense ODN nor scrambled ODN controls had any significant effects on ECE-1 protein levels. These results are consistent with changes observed on ECE-1c mRNA levels and demonstrate the effectiveness of the antisense ODN treatment.

Discussion

Once ECE-1 had been cloned (Schmidt et al., 1994; Shimada et al., 1994; Xu et al., 1994), it became generally accepted as the endopeptidase responsible for the physiological conversion of big ET-1 to ET-1 (Turner et al., 1998). Four isoforms of ECE-1 have been identified (Valdenaire et al., 1999), but the relative importance of each isoform for endogenous ET-1 synthesis has not been defined. Importantly, targeted disruption of the ECE-1 gene to generate ECE-1$^{−/−}$ mice did not prevent ET-1 synthesis in these animals (Yanagisawa et al., 1998, 2000). In heterozygous ECE-1$^{+/−}$ embryos, there was no significant change in ET-1 peptide levels despite a 27% reduction in membrane-bound ECE activity. Characterization of homozygous ECE-1$^{−/−}$ embryos showed ECE activity to be largely eliminated, yet the quantity of ET-1 was decreased by only 48% without any increase in the levels of big ET-1. Based on these observations it could have been inferred that the reduced ET-1 content of ECE-1$^{−/−}$ embryos was not the result of inhibition of ET-1 synthesis, but simply because of the perturbed development. However, because the phenotype of ECE-1$^{−/−}$ mice resembled that observed after deletion of genes for ET-1 (Kurihara et al., 1994) or the ET$\alpha$-receptor (Clouthier et al., 1998), it was concluded that the phenotype, rather than the ET-1 measurements, confirmed the physiological role of ECE-1 in ET-1 biosynthesis (Yanagisawa et al., 1998, 2000).

Until now there have been no specific investigations of the role of ECE-1 in ET-1 biosynthesis at the cellular level. Where ECE inhibitor studies have been performed, and big ET-1 has been measured to confirm ECE inhibition, the agents tested have shown low potency and therefore a low degree of peptidase specificity (Ikegawa et al., 1990; Corder et al., 1995; Woods et al., 1999). Future studies with highly specific ECE-1 inhibitors may well clarify whether ECE-1 plays a significant role in ET-1 biosynthesis.

Specific inhibition of ECE-1 synthesis using an antisense approach did not seem feasible if all four isoforms needed to be eliminated. However, in contrast to endothelial cells, where the four ECE-1 isoforms are expressed (Valdenaire et al., 1999), here we have shown that under basal conditions, BPASMC express predominantly ECE-1c with a ~2.5-fold lower level of the ECE-1b isoform. This difference is further accentuated by treatment with TNFα, which induced a 3-fold increase in ECE-1c mRNA levels and, conversely, reduced ECE-1b mRNA to 25% of its basal level. This indicates that TNFα can regulate the switch between the first and second ECE-1 gene promoters (Valdenaire et al., 1999) so that activation of transcription is driven mainly through the dominant ECE-1c promoter (Funke-Kaiser et al., 2000). Because ECE-1a and ECE-1d were not expressed in BPASMC, ECE-1c mRNA represented >95% of total ECE-1 mRNA after TNFα stimulation, hence providing a suitable cell line to examine the role of ECE-1 in ET-1 synthesis using an antisense ODN to ECE-1c.

Treatment with the ECE-1c antisense ODN specifically reduced ECE-1c mRNA levels. This effect was particularly marked in cells stimulated with TNFα. The reduction in ECE-1c mRNA was associated with a substantial reduction in membrane-bound ECE-1 protein and ECE activity. Both the immunoblotting procedure, using a C-terminal specific ECE-1 antibody, and the enzyme assay measure the contribution of all ECE-1 isoforms to these variables. Hence, the effect of the antisense ODN confirmed that ECE-1c expression was the main source of ECE-1 activity in these cells.

The inverse molar relationship between ET-1 and big ET-1...
secretion during treatment with ECE inhibitors is a well-
accepted characteristic of inhibition of the physiologically
relevant ECE. Similarly, the relative amount of ET-1 and big
ET-1 released from a cell population indicates their capacity
to process big ET-1, and this indirectly reflects the level of
functional ECE activity. Here, big ET-1 release represented
~20% of total endothelin output from BPASMC, implying
that the level of intracellular ECE activity was already less
than that required for complete processing of big ET-1. De-
spite this apparent lack of redundancy in the level of enzyme
activity, when ECE-1c antisense ODN was used to reduce
ECE-1 protein by more than 60%, big ET-1 output did not
alter under basal conditions and was only increased by 0.4-
fold in the presence of TNFα. In marked contrast, a 70%
hibition of ET-1 biosynthesis with phosphoramidon (100
μM) caused an 8- to 10-fold increase in big ET-1 output.
When the effects of the ECE-1c antisense ODN are compared
with the effects of phosphoramidon, this shows that ECE-1c
plays a little part in big ET-1 processing in BPASMC. A role
for ECE-1b also seems unlikely because its expression decreased
during stimulation with TNFα, whereas ET-1 production
increased. Despite the lack of evidence for ECE-1 processing
of big ET-1 in BPASMC, it remains a possibility that it makes
a greater contribution in endothelial cells where the various
ECE-1 isoforms are more highly expressed.

To ensure efficient intracellular processing of peptide me-
diators the regulation of prepropeptide genes generally oc-
curs in parallel with the relevant processing enzymes (Cord-
er et al., 1998). This enables appropriate quantities of enzy-
me to be present with the peptide precursor in the trans-
Golgi network and secretory vesicles during peptide synthe-
sis. A number of studies have localized ECE-1 and ECE-2 to
secretory vesicles, sometimes with colocalization of ET-1
(Barnes et al., 1998; Turner et al., 1998; Russell and Daven-
port, 1999a,b), but this in itself does not prove a role in ET-1
processing. Earlier studies of BAEC have shown that expres-
sion of ECE-1 isoforms is not coordinated with the regulation
of preproET-1 mRNA levels (Corder and Barker, 1999). Sim-
ilarly, in vivo after angioplasty when the endothelium as a
source of ECE-1 has been removed, ECE-1 expression in vascular
smooth muscle does not have the same time course as
ET-1 up-regulation, indicating that the likely function of
ECE-1 is not linked to big ET-1 processing (Wang et al.,
1996). Subcellular localization of ECE-1 in VSMC has iden-
tified it on the cell surface and at intracellular sites including
a substantial proportion that is colocalized with α-actin fil-
aments (Barnes & Turner, 1999). Because ET-1 synthesis in
VSMC is negligible in the basal state, it was also suggested
that ECE-1 has other functions in these cells (Barnes &
Turner, 1999).

ECE-1 has a fairly broad specificity; it will hydrolyze a
variety of peptide substrates, so a role other than that of an
ECE is quite conceivable (Hoang and Turner, 1997; Johnson
et al., 1999). Moreover, hydrolysis of big ET-1 by ECE-1 is
highly dependent on the secondary structure of big ET-1 as
removal of intramolecular disulphide bonds decreases the
amount of ET-1 formed and also results in hydrolysis of other
peptide bonds besides Trp Val (Corder, 1996; Fahnoe et
al., 2000). If specific processing is so dependent on the con-
formation of big ET-1, then the evidence that it is the phys-
iological substrate of ECE-1 may not be as strong as so far
presumed.

The observation that substantial quantities of ET-1 were
found after disruption of the genes for ECE-1 and ECE-2 has
already lead to the conclusion that other proteases can acti-
vate ET-1 in vivo (Yanagisawa et al., 1998, 2000). The exper-
iments described here have not examined the contribution of
ECE-2 to ET-1 processing in BPASMC, so a functional role
cannot be excluded. But it should be noted that deletion of
the ECE-2 gene has no effect on ET-1 synthesis in mouse
embryos either alone or when combined with ECE-1 knock-
out (Yanagisawa et al., 2000). Although these studies of
BPASMC seem to exclude a role for ECE-1 in ET-1 biosyn-
thesis, preliminary investigations have identified a soluble
secreted ECE from both endothelial and vascular smooth
muscle cells that is synthesized in parallel with ET-1 (Corder
et al., 1998). Future investigations are likely to identify other
functions for ECE-1 as well as other ECEs that play a greater
part in ET-1 biosynthesis in VSMC and other cell types.

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