0026-895X/01/5902-163–169$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:163–169, 2001

Vol. 59, No. 2
511/880960
Printed in U.S.A.

ACCELERATED COMMUNICATION

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

STEWART BARKER, NOORAFZA Q. KHAN, ELIZABETH G. WOOD, and ROGER CORDER

Department of Experimental Therapeutics, William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, Charterhouse Square Campus, London, EC1M 6BQ, UK

Received August 22, 2000; accepted October 18, 2000

This paper is available online at http://molpharm.aspetjournals.org

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 isoform. 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 two subtypes of 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.

This work was supported by the Institut de Recherches Internationales Servier and The William Harvey Research Foundation.

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 ET_A 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.

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.

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′-CCATCCTAATACGACTCACTATAGGGC-3′ (AP1) and a reverse primer, 5′-GGCGTTCTTGTCTGGTATTGGA-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 *Bgl*I (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-^{32}P]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

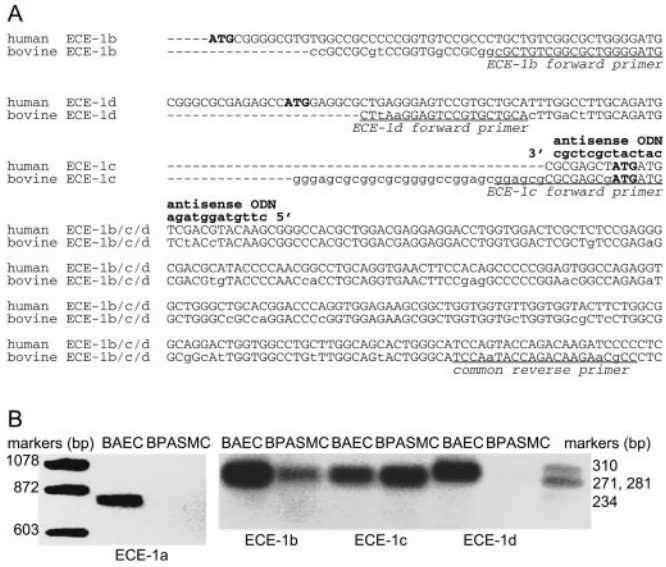

Fig. 1. A, comparison of the nucleotide sequences for bovine ECE-1b, ECE-1c, and ECE-1d obtained in these studies with published sequences of the corresponding human isoforms. Differences are shown as lower case letters in the bovine sequences. The translation start codon for each isoform is shown in bold print. The positions of RT-PCR primer sequences are underlined. The antisense ODN sequence is shown in a 3′ to 5′ orientation complementary to the cDNA sequence of ECE-1c. B, comparison of the relative expression by semiquantitative RT-PCR of the four ECE-1 isoforms in BAEC and BPASMC using 200 ng and 400 ng of total RNA, respectively. Markers are ΦX174 RF DNA/*Hae*III fragments from Life Technologies (Paisley, Scotland).

(Corder and Barker, 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 *Tfl* 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 3 0s, 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′-CGCTGTCGGCGCTGGGGATG-3′, forward), ECE-1c (5′-GGAGCGC-GCGAGCGATGATG-3′, forward), ECE-1d (5′-CTTAAGGAGTCCG-TGCTGCA-3′, forward), with a common reverse primer ECE-1b/1c/1d (5′-GGCGTTCTTGTCTGGTATTGGA-3′). These gave PCR products of 253, 253, and 269 base pairs, which were separated by electrophoresis on ethidium bromide-stained 1–1.2% (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 (Corder and Barker, 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′-CT-TGTAGGTAGACATCATCGCTCGC-3′; antisense ODN) was used with its sense counterpart as a control (5′-GCGAGCGATGATGTC-TACCTACAAG-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′-CTACAGATGCGCTCGCTAGATGTTC-3′; scrambled ODN). The antisense, sense, and scrambled ODNs were synthesized as full phosphorothioate ODNs and supplied as the high-performance liquid chromatography-purified products (Eurogentec, Seraing, Belgium).

BPASMC were seeded at a density of $\approx 5 \times 10^4$ cells/well in 6 × 35 mm well plates to reach 70 to 80% confluence at the beginning of the transfection procedure. Treatment of BPASMC with phosphorothioate ODNs (400 nM) was performed with the cationic lipid transfectant Tfx-50 (Promega) at a charge ratio of 3:1 (Tfx-50/ODN) in serum-free DMEM. Pilot experiments showed this was the maximum concentration that could be used without cytotoxicity. After 1 h, medium was replaced with DMEM containing 10% fetal bovine serum. The treatment with ODN was repeated 24 later. After a further 24 h, cells were transferred to serum-free DMEM with or without TNFα (30 ng/ml) to study ET-1 synthesis over the following 24 h. At the end of this period, conditioned media were collected for ET-1 and big ET-1 immunoassay. For RT-PCR, cells were harvested in 1 ml of RNAzol B and stored at −80°C. For immunoblotting and measurement of ECE activity, cells were scraped into ice-cold PBS and centrifuged to generate cell pellets that were stored at −80°C for later analysis.

Assays. ET-1 was measured by sandwich immunoassay (R&D Systems). Big ET-1 was measured by 96-well plate sandwich immunoassay with an affinity purified anti-ET-1 rabbit IgG as the plate-coating antibody. The detection system was biotinylated anti-human big ET-$1_{[22–38]}$ IgG with ^{125}I-streptavidin. Big ET-1 IgG was affinity-purified from a rabbit antiserum (raised against human big ET-$1_{[22–38]}$) by using VNTPEHVVPYGLGS (Zinsser Analytic, Maidenhead, UK) coupled to CNBr-Sepharose (Amersham Pharmacia Biotech). This peptide sequence is homologous in human and bovine big ET-1 sequences and hence yielded affinity purified IgG suitable for bovine big ET-1 measurements. Synthetic porcine big ET-1 was used as standard.

ECE activity was extracted from cell pellets by lysis on ice in 10 mM Tris-HCl pH 8.5 containing 100 μM PMSF, 100 μM leupeptin, 100 μM chymostatin, 10 μM pepstatin, and 10 μM thiorphan. Pellets were vortexed, subjected to a cycle of freeze-thawing, and centrifuged at 14,000*g* for 15 min at 4°C to obtain a pellet depleted of soluble proteolytic activity. Membrane-bound ECE activity was solubilized by incubation of this pellet on ice for 1 h with 10 mM Tris-HCl, pH 8.5, containing the inhibitors indicated above and 1% Triton X-100. After centrifugation to remove insoluble material, solubilized protein 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 I-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 antiserum 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-NPPHKAEVW (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 IC_{50} 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).

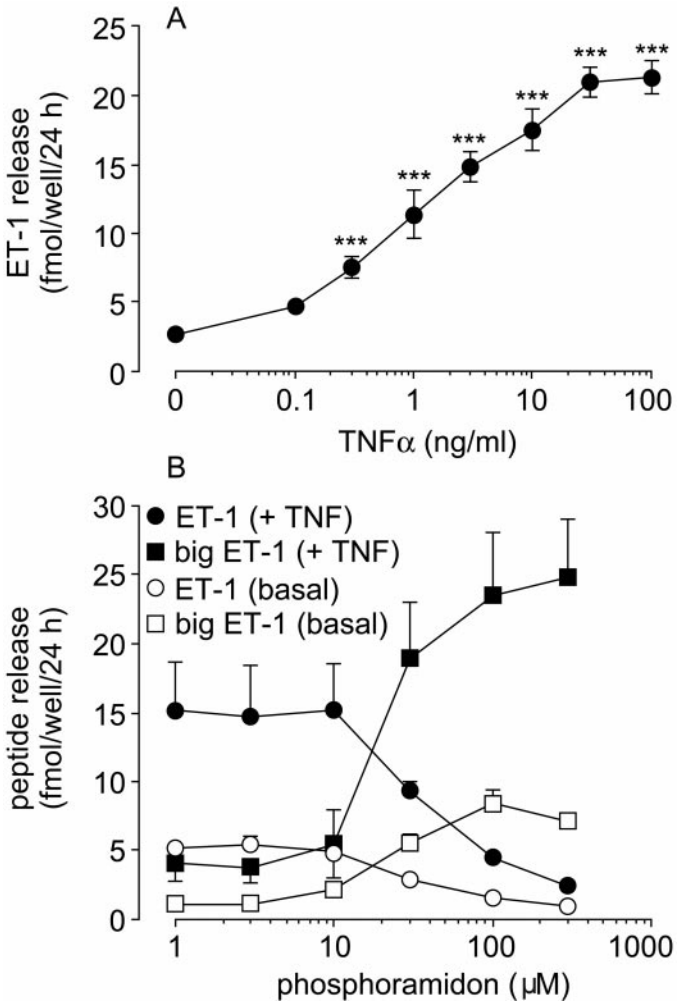

Fig. 2. Characterization of ET-1 synthesis by BPASMC. Experiments were performed using confluent cultures in 35-mm well plates. For A and B, results are mean ± S.E.M. from two separate experiments performed in triplicate. A, effect of TNFα on ET-1 release. ***$p < 0.001$ compared with basal release. B, comparison of the effect of phosphoramidon on basal and TNFα-stimulated accumulation of ET-1 and big ET-1. Release of ET-1 in the absence of phosphoramidon was 4.8 ± 0.4 and 14.3 ± 2.9 fmol/well/24 h under basal conditions and with TNFα respectively. The corresponding values for big ET-1 were 0.6 ± 0.1 and 2.9 ± 0.8 fmol/well/24 h. ET-1 and big ET-1 were significantly different from values without phosphoramidon at concentrations of 30 μM or greater ($p < 0.01$).

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

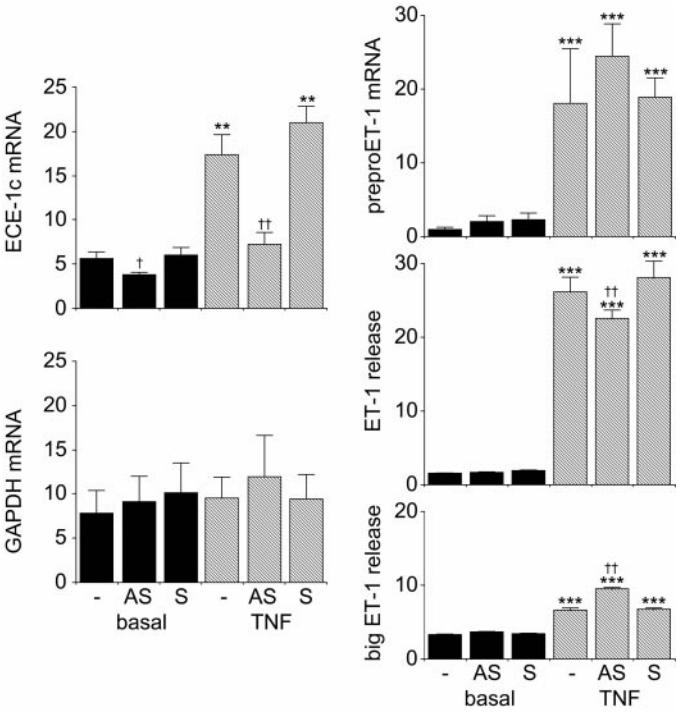

Fig. 3. Effect of treatment with an ECE-1c antisense ODN on ET-1 synthesis in BPASMC under basal conditions or with TNFα stimulation for 24 h. Results are mean ± S.E.M. from two experiments performed in triplicate. −, wells not subjected to ODN treatment; AS, ECE-1c antisense ODN; S, sense ODN control. Left panels show a comparison between levels of ECE-1c mRNA and GAPDH mRNA levels. Right panels show the corresponding changes in ET-1 synthesis. Levels of mRNA determined by semiquantitative RT-PCR are expressed in arbitrary units. ET-1 and big ET-1 release are shown as femtomoles per well per 24 h. **$p < 0.01$, ***$p < 0.001$ compared with untreated basal; †$p < 0.05$, ††$p < 0.01$ compared with corresponding sense ODN treatment.

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_A-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).

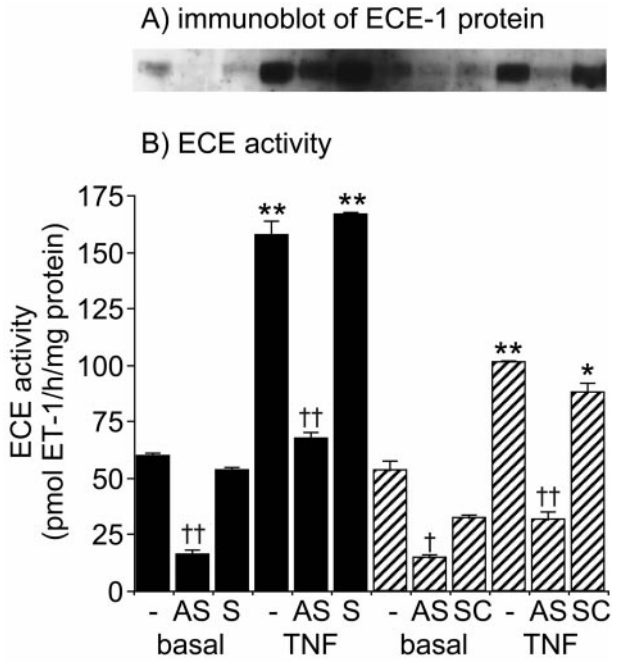

Fig. 4. Effect of ODN treatment of BPASMC on ECE-1 protein levels measured by immunoblotting (A) and ECE activity in solubilized membrane samples (B). Immunoblotting with a C-terminal specific ECE-1 antibody identified a single protein band of 140 kDa. The immunoblot in A was performed on the same samples used for ECE activity measurements in B. Solid columns and hatched columns represent two separate experiments performed in duplicate. Abbreviations are as indicated in the legend to Fig. 3 with the addition that SC corresponds to a scrambled OND control. $*p < 0.05$, $**p < 0.01$ compared with basal levels; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with corresponding control ODN.

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 $\approx$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. Despite 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% inhibition 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 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 mediators the regulation of prepropeptide genes generally occurs in parallel with the relevant processing enzymes (Corder et al., 1998). This enables appropriate quantities of enzyme to be present with the peptide precursor in the *trans*-Golgi network and secretory vesicles during peptide synthesis. 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 Davenport, 1999a,b), but this in itself does not prove a role in ET-1 processing. Earlier studies of BAEC have shown that expression of ECE-1 isoforms is not coordinated with the regulation of preproET-1 mRNA levels (Corder and Barker, 1999). Similarly, 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 identified it on the cell surface and at intracellular sites including a substantial proportion that is colocalized with α-actin filaments (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 disulfide bonds decreases the amount of ET-1 formed and also results in hydrolysis of other peptide bonds besides Trp^{21}-Val^{22} (Corder, 1996; Fahnoe et al., 2000). If specific processing is so dependent on the conformation of big ET-1, then the evidence that it is the physiological 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 activate ET-1 in vivo (Yanagisawa et al., 1998, 2000). The experiments 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 knockout (Yanagisawa et al., 2000). Although these studies of BPASMC seem to exclude a role for ECE-1 in ET-1 biosynthesis, 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.

Acknowledgments

We wish to acknowledge the expertise and assistance of Dr. Louise Jones from the Medical Oncology Department for DNA sequencing. We would also like to thank Dr. Canet and colleagues at Institut de Recherches Internationales Servier for providing the ECE-1 antiserum used for Western blotting.

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Send reprint requests to: Prof. Roger Corder, Department of Experimental Therapeutics, William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, Charterhouse Square Campus, London, EC1M 6BQ, UK. E-mail: r.corder@mds.qmw.ac.uk