Journal of Cell Science 109, 919-928 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JCS3379

Metallopeptidase inhibitors induce an up-regulation of endothelin-converting enzyme levels and its redistribution from the plasma membrane to an intracellular compartment

Kay Barnes[1], Kohei Shimada[2], Masaaki Takahashi[2], Kazuhiko Tanzawa[2] and Anthony J. Turner[1,*]

[1]Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK
[2]Biological Research Laboratories, Sankyo Co. Ltd, 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

*Author for correspondence

SUMMARY

Endothelin-converting enzyme is a phosphoramidon-sensitive membrane metallopeptidase that catalyses the final step in biosynthesis of the potent vasoactive endothelin peptides. Immunomagnetic separation technology and immunohistochemistry have been used to demonstrate the co-localisation of endothelin-converting enzyme with the established ectoenzyme, aminopeptidase N, on the surface of endothelial cells. Unlike aminopeptidase N, however, endothelin-converting enzyme is seen to associate in clusters on the plasma membrane which can be distinguished from caveolae both biochemically and immunologically. Pre-treatment of endothelial cells with the metallopeptidase inhibitors phosphoramidon or thiorphan in the range 0.01-100 μM produced a dose-dependent increase in the levels of endothelin-converting enzyme protein and its accumulation in an intracellular compartment. No corresponding change in the levels of endothelin-converting enzyme-1 mRNA was detected under these conditions, nor in the levels of the closely related metalloenzyme, endopeptidase-24.11. The phosphoramidon and thiorphan-dependent increase is not due to direct inhibition of endothelin-converting enzyme nor endopeptidase-24.11 but, rather, to an inhibition of the selective turnover of endothelin-converting enzyme protein.

Key words: Endothelin-converting enzyme, Metallopeptidase, Phosphoramidon, Thiorphan, Ectoenzyme, Plasma membrane

INTRODUCTION

The endothelin (ET) family of vasoactive peptides are produced constitutively by endothelial cells and are synthesized initially as large precursor proteins, preproendothelins, which are processed to inactive intermediates termed ‘big endothelins’ (Yanagisawa et al., 1988). Mature endothelins are then produced from big endothelins through the action of phosphoramidon-sensitive endothelin-converting enzyme (ECE) which represents a unique protein processing event (Opgenorth et al., 1992; Turner and Murphy, 1996). Two membrane-bound metallopeptidases capable of converting big endothelins have beeen cloned, ECE-1 (Ikura et al., 1994; Schmidt et al., 1994; Shimada et al., 1994, 1995a; Xu et al., 1994) and, more recently, ECE-2 (Emoto and Yanagisawa, 1995). They are homologous proteins which differ principally in their sensitivity to phosphoramidon and in pH optimum. ECE-1 is the major form in all tissues examined. ECE-1 additionally exists in two isoforms which have been termed ECE-1α and ECE-1β which differ only in their N-terminal regions through differential splicing (Shimada et al., 1995b; Turner and Murphy, 1996). The ECEs are homologous with neutral endopeptidase-24.11 (E-24.11) which is a plasma membrane ectoenzyme capable of hydrolysing a wide range of regulatory peptides (Turner and Murphy, 1996) and which is also phosphoramidon-sensitive but additionally inhibited by the compound thiorphan. ECE is considered an important target for the development of new classes of drugs regulating the cardiovascular system.

ECE was shown to be abundant in endothelial cells of rat lung from which it was first purified to homogeneity (Takahashi et al., 1993). ECE-1 has been localized to a variety of tissues by northern blot analysis (Ikura et al., 1994; Xu at al., 1994; Shimada et al., 1994), particularly high mRNA expression being demonstrated in bovine ovary, heart and lung (Ikura et al., 1994; Xu et al., 1994) and in human lung, pancreas and placenta (Schmidt et al., 1994). Using immunohistochemical analysis, ECE-1 has been localized to endothelial cells of tissues and to some secretory cells in the adrenal medulla and β-cells of pancreatic islets (Takahashi et al., 1995).

The subcellular location of the final step in endothelin processing is controversial (see Turner and Murphy, 1996, for review). Subcellular fractionation and other preliminary localization studies have produced conflicting results indicating either a plasma membrane (Waxman et al., 1994; Corder et al., 1995; Takahashi et al., 1995) or an intracellular location (Gui et al., 1993; Xu et al., 1994) for ECE. Studies on Cos-1 or Chinese hamster ovary (CHO) cells transiently transfected with ECE cDNA have also produced different interpretations

of the site of ECE action (Xu et al., 1994; Takahashi et al., 1995). Some studies using cultured endothelial cells have demonstrated that ET-1 rather than big ET-1 is the predominantly released product implicating intracellular processing (Ikegawa et al., 1990; Sawamura et al., 1991), consistent with the detection of both big ET-1 and ET-1 in a vesicle preparation isolated from bovine aortic endothelial cells (Harrison et al., 1995). On the other hand, inhibition of the conversion of big ET-1 to ET-1 in vivo by phosphoramidon has been interpreted as evidence for a plasma membrane ectoenzyme topology for ECE (Matsumura et al., 1990; McMahon et al., 1991; Pollock and Opgenorth, 1991; Corder and Vane, 1995). Clarifying the precise location of ECE on endothelial cells is therefore critical to an understanding of its physiological roles and for the design of ECE inhibitors as therapeutic agents.

Here, we have used immunomagnetic separation technology in a novel way to establish the location of ECE on immunoseparated plasma membranes from cultured endothelial cells. Immunomagnetic beads have previously been used to isolate whole vascular endothelial and other cells but not their associated membranes (Jackson et al., 1990; George et al., 1992). By using double immunofluorescence and immunoblotting we additionally show that the clustered nature of plasma membrane ECE observed on endothelial cells is not due to its association with caveolin, a protein component of the endothelial cell invaginations termed caveolae (Rothberg et al., 1992) in which the endothelin ET_A receptors cluster (Chun et al., 1994). By using a combination of monoclonal antibodies to ECE, confocal microscopy and immunoblotting, we have also demonstrated the redistribution to a predominantly intracellular location and upregulation of ECE, but not the closely related E-24.11, which occurs in endothelial cell cultures treated with the metallopeptidase inhibitors, phosphoramidon or thiorphan.

MATERIALS AND METHODS

Materials

The monoclonal antibodies to ECE, AEC27-121 and AEC32-236 were generated as described by Shimada et al. (1994, 1995a). The specificities of these antibodies has been described elsewhere (Takahashi et al., 1995); they do not distinguish between the α and β isoforms of ECE (Shimada et al., 1995b). The polyclonal antibodies RP159 (to aminopeptidase-N; AP-N), RP161 (to E-24.11), RP143 (to dipeptidyl peptidase IV; DPP-IV), and RP181 (to angiotensin converting enzyme; ACE) were affinity purified as by Barnes et al. (1991). The polyclonal antibody to ACE , designated RP175K/T, was purified using the method of Williams et al. (1992) and the polyclonal antibody to C-terminal ET-1(16-21) sequence was a gift from Dr R. Corder, William Harvey Research Institute, London UK. Monoclonal antibody (B-F10) to human AP-N was obtained from Serotec, Oxford, UK and anti-caveolin from Affiniti Research Products Ltd, Nottingham, UK. Sheep anti-rabbit IgGFc and normal rabbit serum were purchased from ImmunoGen International Ltd (Dyfed, UK). Dynabeads M-280 and Dynal MPC-E, were from Dynal Ltd, Merseyside, UK. Porcine big ET-1 (1-39), ET-1 and phosphoramidon were purchased from the Peptide Institute Inc. (Osaka, Japan) and 3-[^{125}I]iodotyrosyl-ET-1, fish gelatin and enhanced chemiluminescence (ECL) western blotting kit from Amersham International PLC, Amersham, Bucks., UK. Hip-His-Leu and [D-Ala^2, Leu^5] enkephalin were from Cambridge Research Biochemicals Ltd, Northwich, Cheshire. Cell culture reagents and flasks were obtained from Gibco BRL, Life Technologies Ltd, Paisley, Scotland, UK. E-24.11 was purified to apparent homogeneity (>99% pure as judged by SDS-PAGE) by immunoaffinity chromatography as described previously (Gee et al., 1983) except that the monoclonal antibody used was GK4A9 (Gee and Kenny, 1985). ECE was purified from porcine lung membranes as described by Takahashi et al. (1993). EA.hy926 cells were kindly supplied by Dr C.-J. Edgell, University of North Carolina, USA and Simian virus 40-transformed rat lung vascular endothelial cells (TRLEC-03 cells) were donated by Dr S. Tsurufuji, Institute of Cytosignal Research, Tokyo, Japan. Human umbilical vein endothelial cells (HUVEC) were obtained as cryopreserved primary cultures from Clonetics, TCS Biologicals Ltd, Buckingham, UK. The digoxigenin (DIG) oligonucleotide tailing kit and wash buffer kit were from Boehringer Mannheim Ltd, Sussex, UK. Vectashield was from Vector Laboratories, Peterborough, UK.

Endothelial cell culture

EA.hy926 cells were cultured in plastic flasks in supplemented Dulbecco's modified Eagle medium as described by Waxman et al. (1994). HUVECs were grown in a supplemented endothelial basal medium, according to the suppliers' instructions and used for up to four passages. Porcine aortic endothelial cells were harvested by scraping cells from porcine aortas, and cultured as described for bovine aortic endothelial cells (Corder et al., 1993). TRLEC-03 cells were cultured in RPMI-1640 medium containing 10% (v/v) foetal calf serum on collagen coated flasks. All cells were maintained at 37°C in 5% CO_2 in air.

Addition of phosphoramidon and thiorphan to endothelial cell cultures

For some immunofluorescence and immunoblotting experiments, cells were incubated with phosphoramidon or thiorphan, ranging from 0.01 μM to 200 μM final concentration, in the culture medium. The inhibitors were routinely added two days prior to fixation for staining or harvesting for immunoblotting. In a time course experiment, 100 μM phosphoramidon was incubated with EA.hy926 cell cultures for 2 hours, 12 hours and 48 hours prior to harvesting.

In situ hybridisation of EA.hy926 cells

ECE-1 mRNA was detected by hybridisation with four oligonucleotides (4× 36mer) which corresponded to bases 742-777, 799-834, 892-927 and 1,033-1,070 of the rat ECE-1 cDNA (Shimada et al., 1994). The oligomers were labelled using a DIG oligonucleotide tailing kit and visualised with an alkaline phosphatase conjugate followed by an enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium salt. The specificity of the oligonucleotides is indicated from in situ hybridization studies with Cos-1 cells expressing ECE-1 cDNA; only the transfected Cos-1 cell cultures gave a positive signal (data not shown).

EA.hy926 cells were cultured on coverslips for two days, washed rapidly three times in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 minutes. After washing in PBS for 5 minutes, the cells were permeabilised with 0.1% Triton X-100 (v/v) and 0.025% Nonidet-P40 (v/v) in PBS for 10 minutes. The coverslips were washed 2× 5 minutes in PBS and immersed in 20% aqueous acetic acid for 15 seconds at 4°C. Following a quick wash in PBS, incubation in 20% aqueous glycerol for 30 minutes at room temperature and two rinses in standard saline-citrate (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) the cells were hybridised overnight with 70 μl/coverslip of the ECE DIG-tailed probes (approx. 0.1 ng/μl) diluted in standard 25% formamide hybridisation buffer. Cells were rinsed twice for 10 minutes at room temperature in 2× SSC and once in 1× SSC for 5 minutes.

Immunological detection was performed according to the instructions accompanying the DIG nucleic acid detection kit and wash buffer set, except that cells were blocked with 3% normal goat serum. Normal goat serum (1%) in maleic acid buffer with 3% Tween-20

(v/v) was used to dilute the anti-DIG conjugate (1/500). The coverslips were mounted in 95% glycerol in PBS. In control sections, coverslips were incubated with unlabelled probes overnight, briefly washed in 2× SSC, followed by incubation with labelled probes, prior to enzyme-linked detection.

Preparation of EA.hy926 and HUVEC membranes

Confluent EA.hy926 cells were washed 3 times in PBS and scraped into 50 mM Tris-HCl, 100 mM NaCl, 18 mM $CaCl_2$, pH7.4, and homogenized in a Parr cell disruption bomb using N_2 (800 psi at 4°C for 10 minutes). After centrifugation at 1,000 ***g*** for 10 minutes, the resultant supernatant was further centrifuged at 100,000 ***g*** for 90 minutes. The membrane pellet was resuspended in 50 mM Tris-HCl, 100 mM NaCl (Tris-buffered saline; TBS), pH 7.4. HUVEC were harvested at 80% confluency and membranes prepared as above, except that the cells and membranes were resuspended in 25 mM 2-[*N*-morpholino] ethane sulphonic acid (Mes) buffer, pH 6.5, containing 0.15 M NaCl and a cocktail of protease inhibitors (200 μM phenylmethanesulphonyl fluoride, 2 μM pepstatin A and 10 μM leupeptin), was included in the buffers.

Binding secondary antibodies to magnetic beads for immunomagnetic separation

Magnetic beads coupled to sheep anti-mouse immunoglobulin G were resuspended and washed, 3× 10 minutes, in PBS containing 0.8% (w/v) bovine serum albumin and 0.1% fish gelatin (v/v), pH 7.4. Beads were collected between washings using a magnet, Dynal MPC-E, and resuspended to their original volume in the above buffer. Washed sheep anti-mouse beads were rotated with a human anti-AP-N, 20 μl antibody to 100 μl beads, for 2-3 hours at 4°C, followed by washing, 4× 30 minutes, with buffer. Sheep anti-rabbit beads coated with anti-ACE (RP175K/T, 20 μl antibody to 100 μl bead, were used as a control.

Immunomagnetic separation of EA.hy926 membranes

Aliquots of anti-AP-N and anti-ACE immunobeads, 5-100 μl, were incubated overnight at 4°C with 200 μl EA.hy926 membranes, 1 mg/ml, which had been previously blocked with the above buffer for 30 minutes. The beads and adsorbed membranes were collected with the magnet and washed, 4× 30 minutes as before and resuspended with 200 μl 1% Triton X-100 in buffer, prior to being assayed.

Enzyme and protein assays

ECE was assayed using big ET-1 (1 μM) as substrate with 10 μl samples from the immunoseparations in 50 mM Tris-HCl, 0.1% (w/v) BSA, 0.1% (w/v) Triton X-100, pH 7.4 (Greenhough et al., 1994). Samples were pre-incubated with a proteinase inhibitor cocktail (pepstatin A, 10 μM; E-64 (trans-epoxysuccinyl-L-leucylamide (4-guanidino)butane), 10 μM; amastatin, 10 μM; 3,4-dichloroisocoumarin, 100 μM; diisopropylfluorophosphate, 100 μM; thiorphan, 10 μM) at 37°C for 1 hour. The ET-1 product was quantified by radioimmunoassay (Harrison et al., 1993). ECE activity was defined by its sensitivity to 100 μM phosphoramidon. E-24.11 and AP-N were assayed by HPLC methods with [D-Ala^2, Leu^5] enkephalin (Matsas et al., 1983) and [Leu] enkephalin (Matsas et al., 1985), respectively, as substrate. For enzyme assays 1 unit of enzyme activity is defined as 1 nmol of product/minute. Protein was determined by the bicinchoninic acid method, with bovine serum albumin (1 mg/ml) as standard (Smith et al., 1985).

Antibody dilutions for immunofluorescence and immunoblotting

The monoclonal antibodies, AEC27-121 (4 mg/ml) and AEC32-236 (4 mg/ml) to ECE were diluted 1/10 or 1/20 for immunohistochemistry and at 1/200 for immunoblotting. The antibody to AP-N (clone BF-10) was diluted 1/5 and 1/50 for immunofluorescence and immunoblotting, respectively. The polyclonal antibodies, RP161 to E-24.11, RP181 to ACE and RP143 to DPP-IV (all 1 mg/ml) were diluted 1/500. Anti-caveolin (0.25 mg/ml) was used at 1/50 for immunohistochemistry and 1/250 for immunoblotting. The secondary conjugated antibodies, anti-mouse and anti-rabbit fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRIC) and anti-mouse biotin, were all diluted 100-fold and the streptavidin complex was diluted 400-fold.

Immunofluorescence of EA.hy926 cells and HUVEC

Cells were seeded onto coverslips, cultured for 2/3 days and immunostained prior to obtaining confluence (Barnes et al., 1994). Cells were fixed routinely in a 1:1 (v/v) methanol/acetone mixture for 10 minutes. For demonstration of plasma membrane ECE, fixation was in 4% paraformaldehyde for 20 minutes. After washing, cells were blocked with 0.2% gelatin and 1% normal goat serum (w/v/v) for 30 minutes. Primary antibodies were applied to the coverslips and incubated for 2 hours at room temperature or overnight at 4°C. The primary antibody was omitted or replaced with pre-immune serum for control coverslips. After washing, cells were incubated with the appropriate anti-mouse or anti-rabbit fluorescent conjugate for 30 minutes. Alternatively, the anti-mouse biotin/streptavidin FITC system was used. Coverslips were mounted in Vectashield and viewed either with a Zeiss light microscope or a Leitz confocal microscope.

Double labelling immunofluoresence

In double immunolabelling experiments, the cells were treated concomitantly with the primary antibodies. At the appropriate stage in the staining procedure the secondary antibodies were applied simultaneously. In some instances wheat germ agglutinin conjugated to TRIC, diluted 1/500 in 0.2% gelatin/TBS (w/v), was added together with the anti-mouse FITC or streptavidin-FITC.

Electrophoresis and immunoblotting for ECE, E-24.11 and caveolin in HUVEC and EA.hy926 cell membranes

For immunoblotting, EA.hy926 and HUVEC membranes were solubilized in 1% Triton in TBS (v/v) or 25 mM Mes/0.15 M NaCl, pH 6.5 (v/v) for 45 minutes at 4°C, followed by centrifugation at 100,000 ***g*** for 90 minutes. The resultant pellet was resuspended in 1% Triton in TBS (v/v) , pH 7.4. Dissociation buffer was added to the supernatant and pellet prior to their storage at −20°C.

Samples of Triton-solubilised membranes and the Triton-insoluble pellet from HUVEC and EA.hy926 cells were thawed, and 5% mercaptoethanol was added. For immunoblot analysis of ECE and E-24.11, proteins were separated by SDS-PAGE on 7.5% gels and transferred to 0.45 μm poly(vinylidene difluoride) membranes (Millipore) which were washed in 0.1% Tween-20 in 10 mM Tris-HCl, pH 7.4, and blocked with the same buffer containing 5% milk powder for 1 hour at room temperature, or overnight at 4°C. The antibodies were diluted in the blocking buffer and the membrane incubated in primary antibody overnight at 4°C and in secondary horseradish peroxidase conjugates, diluted 1/5,000, for 1 hour at room temperature. For immunoblot analysis of caveolin, 10% gels were used for SDS-PAGE followed by transfer to 0.2 μm poly(vinylidene difluoride) membranes (Bio-Rad Transblot). For caveolin blots, 2% gelatin in TBS (w/v) was used for blocking and for diluting the antibodies. After copious washing, the proteins were visualized by using the Amersham ECL western blotting kit. Triton-solubilised membranes from EA.hy926 cells were treated similarly for immunoblotting.

Immunoblotting of TRLEC-03 cells

For immunoblotting experiments, TRLEC-03 cells were seeded into 24-well plates at 1×10^5 cells/well. After 12 hours culture, phosphoramidon or thiorphan, ranging from 0.01 to 100 μM at the final concentration, and/or 1 μM of ET-1, -2, or -3 were added to the culture medium. After 48 hours, cells were washed twice with PBS and were solubilized with 100 μl of SDS-PAGE sample buffer containing 1% β-mercaptoethanol. Each 10 μl of the solubilized sample was boiled

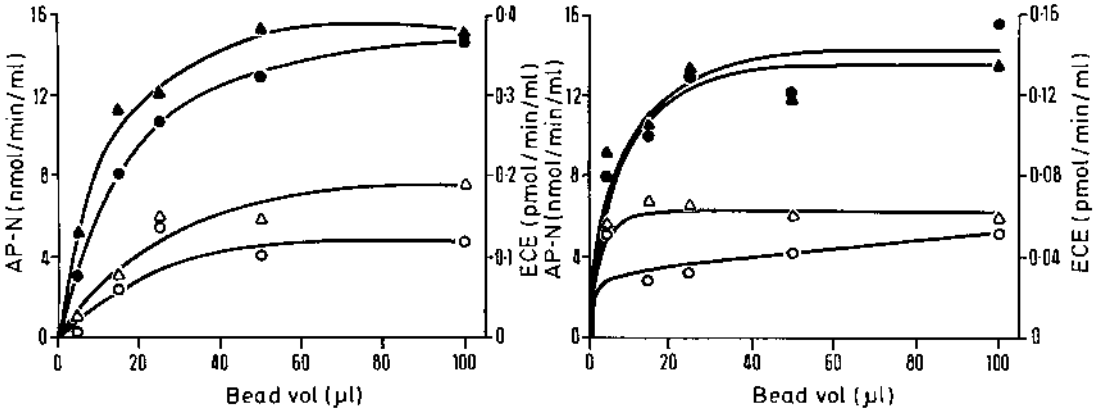

Fig. 1. Immunomagnetic separation of EA.hy926 plasma membranes. For details of the procedure, see Materials and Methods. Membranes were separated by using either anti-AP-N (▲,●) or anti-ACE (△,○) coated magnetic beads. The separated, solubilized membranes were then assayed for AP-N (▲,△) and for ECE (●,○) activities.

for 5 minutes and subjected to immunoblotting using mAb AEC27-121 as in the method of Takahashi et al. (1995).

The expression level of ECE-1 mRNA

The effect of phosphoramidon on the expression level of ECE-1 mRNA was examined by reverse transcription-PCR. From 2 µg of total RNA prepared from TRLEC-03 cells which had been treated with 100 µM of phosphoramidon for 0-48 hours, cDNAs were synthesized using random 9mer primer in a total volume of 20 µl. Aliquots of 2 µl of this reaction were subjected to the PCR. Since TRLEC-03 cells do not express ECE-1β mRNA, ECE-1α specific primers (sense primer: ATGATGTCATCCTACAAGCGGGCC and antisense: GGGTCCATGGAGTTTAGGATGGAGCTGGT) were used as described previously (Shimada et al., 1995b). As a control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA was amplified using rat G3PDH control amplimer set (Clontech).

RESULTS

Immunoseparation of EA.hy926 plasma membranes

Plasma membranes from EA.hy926 cells were separated from a heterogeneous mixture of membranes by overnight incubation with magnetic beads coated with the anti-AP-N antibody. Since ACE is absent from the plasma membranes of EA.hy926 cells (Greenhough et al., 1994), the enzyme activities on membranes adsorbed to anti-AP-N beads were compared to those adhering non-specifically to anti-ACE beads. The AP-N and ECE activities on membranes adsorbed to the immunobeads were found to be comparable in two independent immunoseparations and significantly greater than the activities on membranes extracted by ACE beads (Fig. 1). Another plasma membrane marker, E-24.11, showed a similar distribution to AP-N and ECE (data not shown).

Expression of peptidases on membranes of EA.hy926 cells, HUVEC and PAEC

In situ hybridisation demonstrated the presence of ECE-1 mRNA in EA.hy926 cells (Fig. 2), thus confirming that the endothelin-converting activity present in this transformed cell line is attributable to ECE described by other authors (Schmidt et al., 1994; Shimada et al., 1995a).

Immunostaining of paraformaldehyde-fixed, non-permeabilised EA.hy926 cells with anti-AP-N and anti-ECE (AEC32-236) monoclonal antibodies revealed bright surface membrane fluorescence (Fig. 3a and b, respectively), whereas

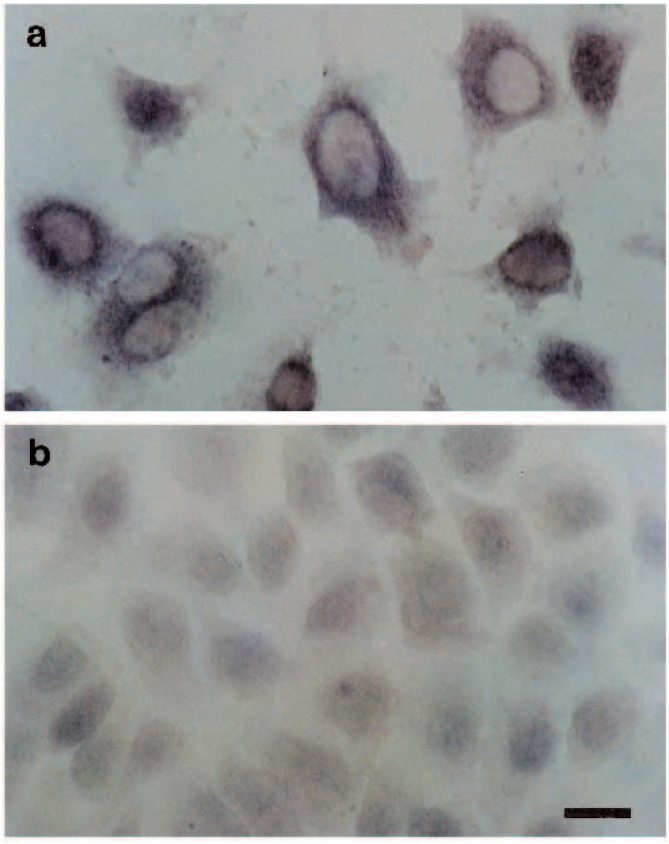

Fig. 2. In situ hybridization to ECE-1 mRNA in the transformed endothelial cell line, EA.hy 926. A cocktail of digoxigenin-tailed oligonucleotides related to ECE-1 mRNA were applied and visualized using an alkaline phosphatase immunological detection system. For details of procedure see Materials and Methods.
(a) EA.hy926 cells expressing mRNA for ECE. (b) EA.hy926 cells were hybridized with an unlabelled cocktail of oligonucleotides against ECE-1 mRNA, prior to application of labelled probes. No mRNA was detected. Bar, 20 µm (a,b).

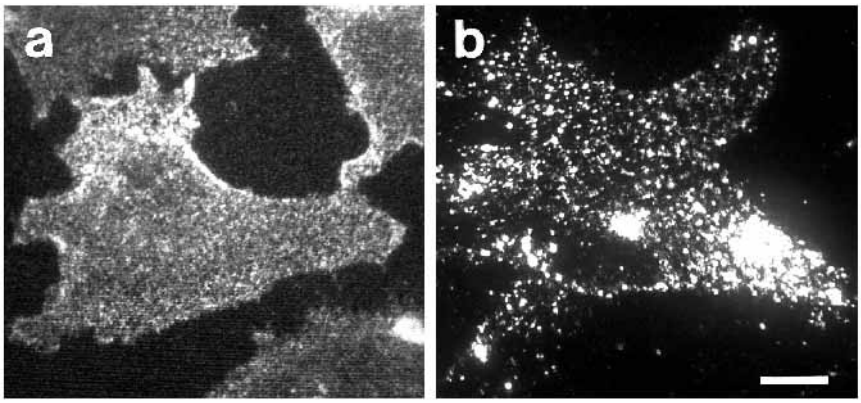

Fig. 3. Immunofluorescence staining of EA.hy926 cells for AP-N and for ECE-1. (a and b) Composite images taken with a confocal microscope. The cells were fixed with 4% paraformaldehyde prior to immunostaining by the monoclonals, BF-10 to AP-N (a) and antibody AEC32-236 to ECE-1 (b), followed by anti-mouse FITC. Compare the clustered plasma membrane immunostaining of ECE (b) with the more evenly distributed, punctate immunostaining of AP-N (a). Bar, 10 μm (a,b).

the antibody raised to E-24.11 revealed only weak staining (data not shown). Staining with antibodies raised to either ACE or DPP-IV did not produce fluorescence. The AP-N was seen as punctate dots (Fig. 3a), evenly distributed over the plasmalemma, whereas ECE was present as larger patches clustered on the cell surface (Fig. 3b). A similar localization for ECE was observed in HUVEC (see Fig. 5a) and in porcine aortic endothelial cells (not shown). No intracellular AP-N could be detected in permeabilised cell preparations, endorsing its plasma membrane location.

ECE and caveolin do not co-localise

Immunoblots of HUVEC with antibody AEC32-236 to ECE revealed the antigen to be present predominantly in the Triton X-100 solubilized fraction (Fig.4a, lane 3) of the membrane preparation, not in the Triton-insoluble pellet (Fig. 4a, lane 4). In contrast, anti-caveolin immunoblots of the HUVEC revealed the resuspended Triton X-100-insoluble pellet to be rich in caveolin (Fig. 4a, lane 2) and the Triton-solubilized fraction to be a poor source of the protein (Fig. 4a, lane 1). When the HUVEC were concomitantly immunostained for the two antigens, co-localization of caveolin and ECE was not observed (Fig. 4b and c, respectively). Both ECE and E-24.11 could be also clearly resolved from detergent-insoluble membrane microdomains containing caveolin which were prepared from pig lung membranes by sucrose gradient cen-

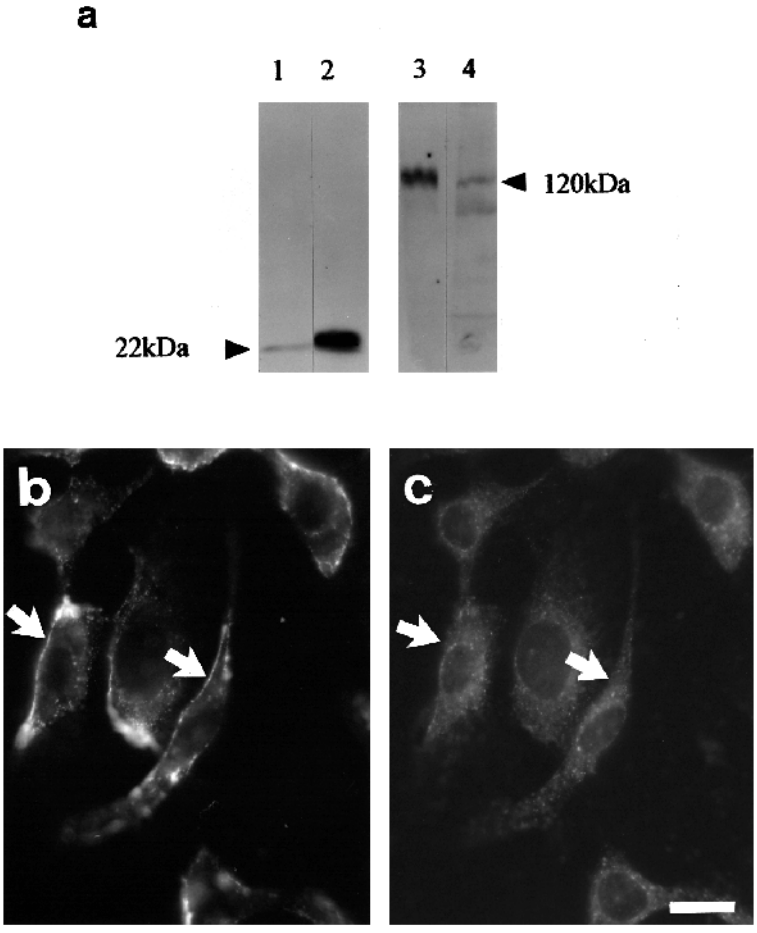

Fig. 4. Immunoblot and double immunofluorescence staining for ECE-1 and for caveolin on HUVEC cells. (a) Samples of membrane protein (10 μg) were prepared and analysed as described in Materials and Methods. Following electrophoretic transfer of proteins, the PVDF membranes were immunoblotted with antibody AEC32-236 to ECE-1 and with anti-caveolin. For details see Materials and Methods. Lanes 1, 2, caveolin; lanes 3, 4, ECE. Lanes 1 and 3, Triton X-100 solubilized fraction of cells; lanes 2 and 4, the resuspended Triton X-100 insoluble pellet. (b and c) HUVEC which were fixed in methanol/acetone 1:1 (v/v) and double immunostained for caveolin (b) and ECE-1 (c). The bright rhodamine fluorescence for caveolin (b) does not co-localize with the fluorescein fluorescence seen in c (arrows). Bar, 20 μm (b,c).

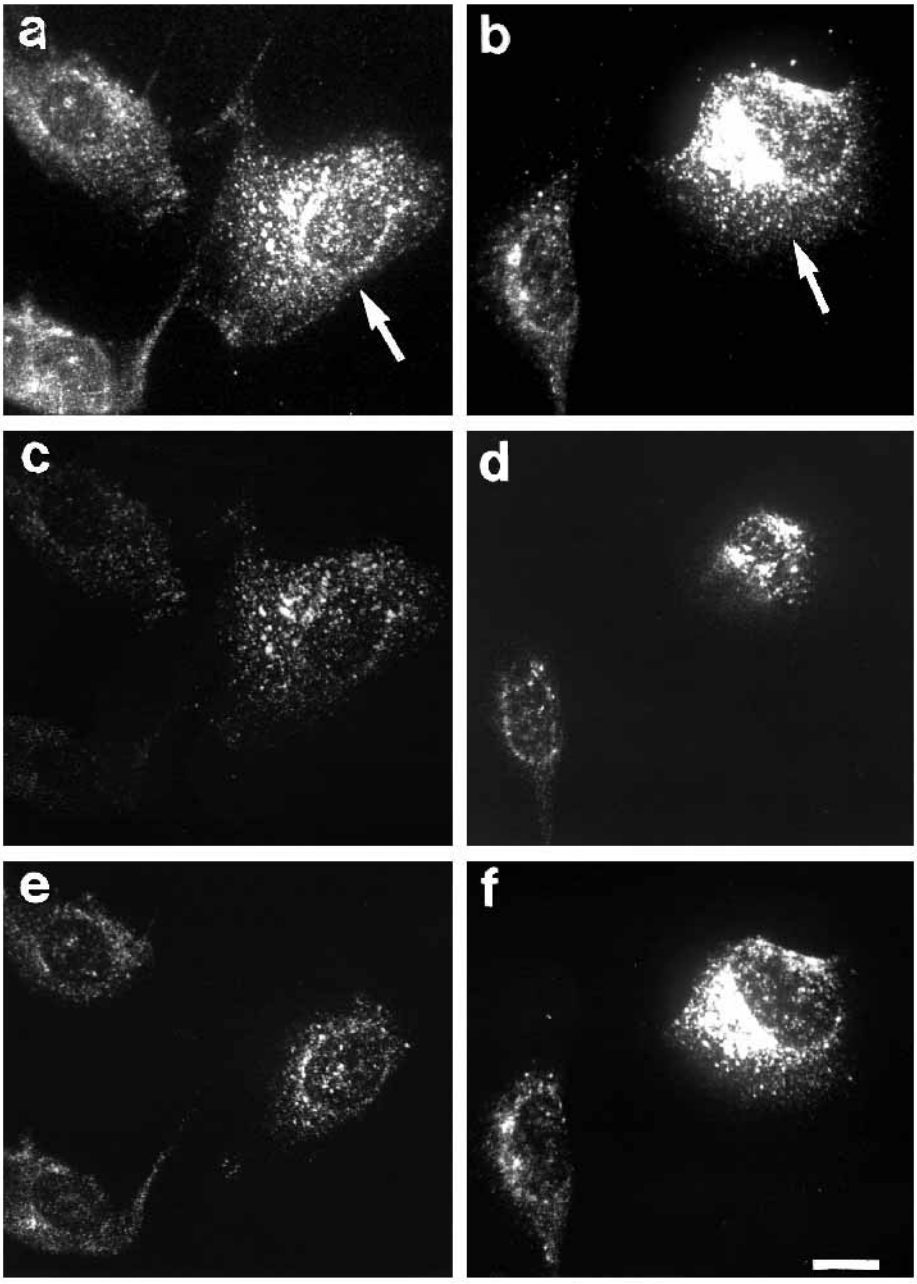

Fig. 5. Confocal microscopy of phosphoramidon-treated HUVEC immunostained for ECE-1. (b,d,f) Cells treated with 100 μM phosphoramidon for 48 hours prior to harvesting; (a,c,e) cells grown in medium minus phosphoramidon. The cells were fixed in acetone/methanol 1:1 (v/v) prior to application of antibody AEC32-236 to ECE-1, followed by biotin/streptavidin-FITC. The composite image from seven focal planes illustrates the intense intracellular immunofluorescence immunostaining for ECE-1 in the Golgi region (b, arrow), whereas the ECE clusters are more evenly distributed in untreated cells (a, arrow). (c to f) Images taken from a single focal plane at the cell surface (c and d) and below (e and f). Clusters of plasma membrane ECE are present in untreated (c) and treated (d) cells. The Golgi staining is, however, far more intense in the treated cells (f). Bars, 10 μm (a-f).

trifugation according to the method of Lisanti et al. (1994) (data not shown).

The metallopeptidase inhibitors, phosphoramidon and thiorphan, increase ECE levels in EA.hy926, HUVEC and TRLEC-03 cells

EA.hy926 and HUVEC cells which had been growing on coverslips for 24 hours were incubated with 100 or 200 μM phosphoramidon in the medium for a further two days and then fixed with methanol/acetone 1:1 (v/v), prior to immunostaining for ECE. The majority of cells, both HUVEC (Fig. 5) and EA.hy926 (Fig. 6), grown in the presence of phosphoramidon (Fig. 5b,d,f), showed a marked increase in the intensity of immunofluorescence in the perinuclear region (Fig. 5b,f) compared with those cells cultured in a phosphoramidon-free medium (Fig. 5a,c,e) in which there was no such localized accumulation of ECE immunoreactivity. Confocal images taken in a single, superficial plane of the cell confirmed the presence of cell-surface ECE in both treated and untreated cells (Fig. 5c,d) with additional perinuclear staining in phosphoramidon-treated cells (Fig. 5f). Intracellular staining for ECE (Fig. 6a) was located in a similar compartment to wheat germ agglutinin, a lectin marker for the Golgi (Virtanen et al., 1980) (Fig. 6b), and this was confirmed by double immunostaining (data not shown). When HUVEC were treated with 100 or 200 μM thiorphan and immunostained with the ECE antibody, a similar but less dramatic increase in ECE expression was revealed (data not shown). In a parallel experiment, HUVEC were incubated with 100 or 200 μM phosphoramidon or thiorphan and were immunostained for AP-N. In this case no change in intensity or redistribution of AP-N was observed, and the immunofluorescence remained similar to that shown in Fig. 3a.

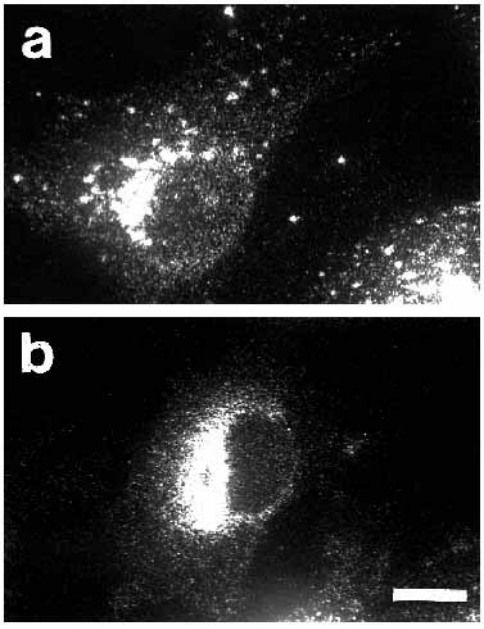

Fig. 6. Confocal microscopy of EA.hy926 cells stained for ECE-1 and wheat germ agglutinin. Cells were fixed with a methanol/acetone 1:1 (v/v) mixture. (a) Cells immunostained with AEC32-236 to ECE-1, followed by anti-mouse FITC; (b) cells stained with wheat germ agglutinin conjugated to TRIC. (a and b) Composite images formed from micrographs taken in several focal planes. The intense immunofluorescence for ECE-1 (a) colocalizes with the lectin Golgi staining in (b). Bar, 10 μm (a and b).

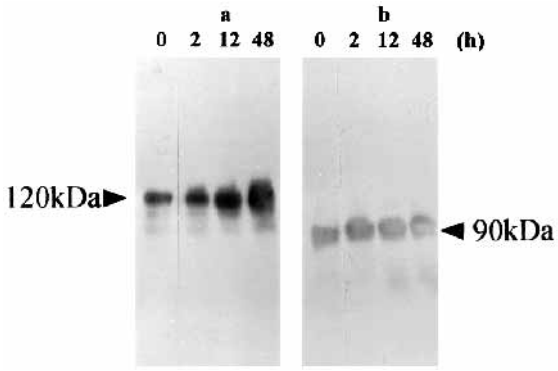

Fig. 7. Immunoblots for ECE and E-24.11 from phosphoramidon-treated EA.hy926 cells. EA.hy926 cells were grown without phosphoramidon for 48 hours (not shown) or with 100 μM phosphoramidon for times indicated. Cell membranes were solubilized with 1% Triton X-100 and samples of 10 μg protein subjected to SDS-PAGE and immunoblotting with antibodies AEC32-236 to ECE (a) and RP161 to E-24.11 (b).

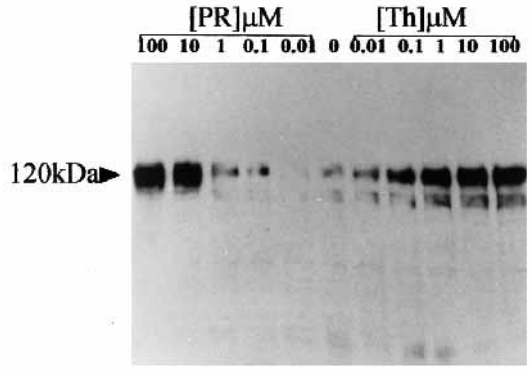

Fig. 8. Immunoblots of total cellular proteins from phosphoramidon- or thiorphan-treated transformed rat lung endothelial cells. TRLEC-03 cells were untreated, or treated with either phosphoramidon or thiorphan, for 48 hours at the concentrations indicated. Cells were solubilized with SDS-PAGE sample buffer containing 1% β-mercaptoethanol, and aliquots were subjected to immunoblotting using mAb AEC27-121.

The effect of phosphoramidon and thiorphan was also examined by immunoblotting. The increase in ECE levels induced by inhibitor varied considerably between cell lines when quantified by densitometry (Scanmaster 3, Howtek, USA). In the case of HUVEC the increase was assessed to be <2-fold, 2.5-fold for EA.hy926 (Fig. 7a), but was approx 10-fold for TRLEC-03 cells (Fig. 8). The increased immunoreactivity was paralleled by a similar increase in enzyme activity. The amount of E-24.11, however, remained constant as shown by immunoblotting (Fig. 7b) and by HPLC assay (data not shown). The increase in ECE levels induced by phosphoramidon or thiorphan showed a dose-dependent effect with maximum increase occurring above 10 μM of the inhibitors (Fig. 8) Incubation of EA.hy926 cells for 48 hours in the presence of some other peptidase inhibitors (amastatin, enalaprilat, pepstatin A, 10-30 μM) caused no increase in ECE levels indicating the specificity of the effect. To assess whether endothelin levels themselves affect the concentration of ECE-1 protein through a feedback action, the effects of incubation of cells in the presence of 1 μM ET-1, ET-2 or ET-3 were examined both in the presence and absence of PR. In neither case did ETs affect the levels of ECE assessed by immunoblotting (data not shown).

Phosphoramidon treatment does not affect ECE-1 mRNA levels

The effect of phosphoramidon pre-treatment of cells on the level of ECE-1 mRNA was examined in TRLEC-03 cells by reverse transcription-PCR. Under conditions which give rise to a 10-fold increase in ECE protein immunoreactivity, no change in the level of ECE-1 mRNA was observed (Fig. 9).

E-24.11 does not hydrolyse ECE protein

A sample of purified ECE (500 ng) was incubated with 1 μg of E-24.11 in a total volume of 100 μl 0.1% Triton in 0.1 M Tris/HCl, pH 7.4 (v/v), at 37°C. Samples, 20 μl, were taken at intervals over 24 hours. Dissociation buffer was added and the sample boiled for 5 minutes at 100°C and immediately frozen for storage at –20°C until required for immunoblotting. Phosphoramidon (10 μM final concentration) was included in some incubations. No phosphoramidon-sensitive degradation of ECE protein could be detected even after 24 hours (data not shown).

DISCUSSION

Since the discovery of ET by Yanagisawa (1988) all four

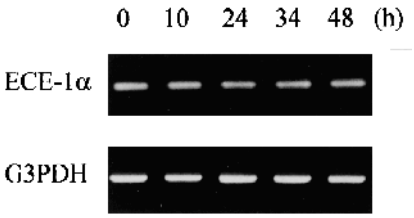

Fig. 9. Effect of phosphoramidon on ECE-1 mRNA levels. Single-stranded cDNAs were reverse-transcribed from the total RNA of TRLEC-03 cells which had been treated with 100 μM phosphoramidon for indicated time. Each cDNA was subjected to ECE-1α specific PCR (ECE-1α) or G3PDH specific PCR (G3PDH). The amplified products were subjected to electrophoresis on agarose gels and were visualized by staining with ethidium bromide.

classes of proteinases have been suggested as physiological endothelin-converting enzymes. Most of these have turned out to be irrelevant activities and the current consensus is that an endothelial, phosphoramidon-sensitive metallopeptidase resembling the plasma membrane ectoenzyme E-24.11 is the responsible enzyme (see Turner and Murphy, 1996, for review). The processing of big ET-1 to ET-1 which involves highly selective hydrolysis of a Trp-Val bond in the precursor is unique as a prohormone processing event. The location of this event in the constitutive secretory pathway and the precise localization of ECE is controversial, current evidence supporting both intracellular (Gui et al., 1993; Xu et al., 1994) and post-secretory processing (Takahashi et al., 1995). The recent molecular cloning of ECE (Ikura et al., 1994; Schmidt et al., 1994; Shimada et al., 1994, 1995a; Xu et al., 1994; Emoto and Yanagisawa, 1995) and the development of monoclonal antibodies to the enzyme (Shimada et al., 1994, 1995a) now allow these questions to be addressed more precisely.

Here we present both biochemical and immunological data with the aim of establishing the subcellular location of ECE. The results show a mainly cell-surface location but with an unexpected upregulation and redistribution of ECE to an intracellular compartment following treatment of cells with low concentrations of metallopeptidase inhibitors. The transformed human endothelial cell line, EA.hy926, has been particularly useful for studies of endothelin biology since it exhibits sustained expression of many differentiated functions of the endothelium (Edgell et al., 1983), expresses ET-1 mRNA and has been shown to secrete big ET-1 and ET-1 into the culture medium in a phosphoramidon-sensitive fashion (Saijonmaa et al., 1991). Phosphoramidon-sensitive ECE activity has also been partially characterized in this cell line (Waxman et al., 1994; Corder et al., 1995) although its immunohistochemical localization has not been addressed. Here we have established the presence of ECE-1 mRNA and protein in the EA.hy926 cells by in situ hybridization analysis and immunohistochemistry. Where possible we have compared results obtained from the EA.hy926 cells with a transformed rat endothelial cell line, TRLEC-03, and with the more physiologically relevant non-transformed HUVEC and porcine aortic endothelial cells.

Magnetic immunoseparation procedures demonstrate the presence of ECE activity associated with a membrane fraction from EA.hy926 cells, co-localizing with the activities of AP-N and E-24.11, both well-established plasma membrane ectoenzymes (see e.g. Kenny et al., 1987). This is consistent with the predicted topology of ECE-1 and its homology with E-24.11 (Shimada et al., 1994; Xu et al., 1994; Ikura et al., 1994; Schmidt et al., 1994; Turner and Murphy, 1996) implying that it may act largely as an ectoenzyme in a post-secretory processing role, analogous to the generation of angiotensin II from angiotensin I by ACE (Ohnaka et al., 1991; Corder et al., 1995 and Takahashi et al., 1995).

Immunofluorescence staining has established a clustered appearance of ECE on the plasma membrane of human EA.hy926 cells, and other endothelial cells, which we have shown here to be distinct from the more homogeneous distribution of AP-N. The nature of the surface ECE clusters is unknown but they could correspond to caveolae which are abundant on both endothelial and smooth muscle cells. Caveolae are plasma membrane invaginations that can close off to form non-clathrin-coated vesicles (Rothberg et al., 1992; Anderson, 1993). They contain high concentrations of the coat protein, caveolin, and many GPI-anchored membrane proteins (Turner, 1994). ACE has been reported to be localized to caveolae in pig aortic endothelial cells in culture (Ryan et al., 1976) and the endothelin ET_A receptor also clusters in caveolae (Chun et al., 1994). Caveolar-associated proteins are typically insoluble in Triton X-100 as a result of the sphingolipid-rich domains in which they locate and this phenomenon is commonly used as an initial step in purification of caveolae (Chang et al., 1994; Lisanti et al., 1994). Here we have failed to detect an association of ECE with caveolae both by biochemical and immunocytochemical criteria. Whether specific anchor proteins aid ECE clustering is unknown but the essential role of rapsyn in promoting the clustering of synaptic nicotinic acetylcholine receptors (Gautam et al., 1995) may provide a useful analogy.

Although the above studies imply a plasma membrane location for ECE, under certain conditions some ECE immunoreactivity is detectable in a perinuclear site co-localizing with wheat germ agglutinin-binding sites. The apparent ‘upregulation’ of ECE activity by the ECE inhibitor phosphoramidon was first briefly noted by Schmidt et al. (1994) who exploited this observation to aid ECE purification but the mechanism of this effect was not addressed. The degree of upregulation of ECE immunoreactivity by phosphoramidon as assessed here by Western blotting varies in different endothelial cell lines, with ECE induction in TRLEC-03 cells showing a more substantial increase than in EA.hy926 or HUVEC. Furthermore, under similar conditions, the levels of the closely related phosphoramidon-sensitive enzyme, E-24.11, do not increase. We also observed that the distribution of another ectopeptidase, AP-N, remained unaffected when treated with phosphoramidon. One interpretation of these data might be that inhibition of ECE by phosphoramidon leads to an intracellular accumulation of the intermediate big ET which, in turn, regulates ECE transcription or translation through a feedback mechanism. However, the failure to detect any increase in the amount of ECE-1α mRNA (this cell line does not express ECE-1β; Shimada et al., 1995b) argues against this explanation and suggests an inhibition of the degradation of ECE-1 protein and hence its intracellular accumulation. That the effect is seen both with phosphoramidon and thiorphan, as demonstrated here by immunoblotting and by immunocytochemistry in EA.hy926, TRLEC-03 and HUVEC cells, rules out an autocatalytic effect since ECE is generally regarded to

be insensitive to thiorphan (for review see Turner and Murphy, 1996). The presence of endothelins in the culture medium did not affect the increase in ECE levels indicating that endothelin concentration is not a contributory factor in modulating ECE levels. An alternative explanation that the phosphoramidon effect is at the level of post-translational processing or transport of ECE appears unlikely since the M_r of ECE as assessed by immunoblotting is unchanged after phosphoramidon treatment of cells and no change is seen in the levels of AP-N, another cell-surface peptidase following the same intracellular transport pathway,

The only known mammalian enzyme sensitive to both phosphoramidon and thiorphan is E-24.11. However, E-24.11 has a strict specificity for oligopeptides (Kenny et al., 1987) and incubation of E-24.11 with ECE produced no phosphoramidon-sensitive degradation of ECE. Overall these data would suggest the presence of a novel, intracellular phosphoramidon- and thiorphan-sensitive metalloprotease involved in the turnover of ECE, and perhaps some other proteins. This activity did not, however, appear to affect the levels of AP-N nor E-24.11. The extent of the increase in ECE levels was dependent on cell type, with a particularly marked effect in TRLEC-03 cells. This cell-line should therefore prove a valuable model system to characterize this novel metalloprotease and examine its potential role in intracellular protein turnover. Its sensitivity to phosphoramidon suggests it may represent a new member of the mammalian E-24.11/ECE family. The selective up-regulation of ECE by metallopeptidase inhibitors, especially phosphoramidon, has considerable therapeutic implications. The potential application of ECE inhibitors in cardiovascular conditions may be compromised if they simultaneously increase the levels of ECE within endothelial cells.

Immunohistochemical and biochemical evidence has now unequivocally localized ECE-1 mainly to the plasma membrane and, in particular circumstances, its redistribution to a perinuclear organelle, probably the Golgi, in a number of different endothelial cell types. In view of the existence of isoforms of ECE, further study, including at the ultrastructural level, is necessary to evaluate their individual and co-operative involvement in the cellular and post-secretory processing of endothelin precursors.

A.J.T. and K.B. thank the British Heart Foundation and the Medical Research Council (UK) for financial support. We thank Mr L. J. Murphy and Dr A. Cox for assistance with ECE assays and for cell culture respectively. We thank Dr S. Tsurufuji for the kind gift of TRLEC-03 cells and Dr R. Corder for the ET-1(16-21) antibody.

REFERENCES

Anderson, R. G. W. (1993). Potocytosis of small molecules and ions by caveolae. *Trends Cell Biol.* **3**, 69-71.

Barnes, K., Bourne, A., Cook, P. A., Turner, A. J. and Kenny, A. J. (1991). Membrane peptidases in the peripheral nervous system of the pig: their localisation by immunohistochemistry at light and electron microscopic levels. *Neuroscience* **44**, 245-261.

Barnes, K., Kenny, A. J. and Turner, A. J. (1994). Localization of aminopeptidase N and dipeptidyl peptidase IV in pig striatum and in neuronal and glial cell cultures. *Eur. J. Neurosci.* **6**, 531-537.

Chang, W.-J., Ying, Y.-S, Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambliel, H. A. De Gunzburg, J., Mumby, S. M., Gilman, A. G. and Anderson, R. G. W. (1994). Purification and characterisation of smooth muscle cell caveolae. *J. Cell Biol.* **126**, 127-138.

Chun, M., Liyanage, U. K., Lisanti, M. P. and Lodish, H. F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: Colocalisation of endothelin and its receptor with caveolin. *Proc. Nat. Acad. Sci. USA* **91**, 11728-11732.

Corder, R., Harrison, V. J., Khan, N., Änggård, E. E. and Vane, J. R. (1993). Effects of phosphoramidon in endothelial cell cultures on the endogenous synthesis of endothelin-1 and on conversion of exogenous big endothelin-1 to endothelin-1. *J. Cardiovasc. Pharmacol.* Suppl. **8**, S73-S76.

Corder, R., Khan, N. and Harrison, V. J. (1995). A simple method for isolating human endothelin converting enzyme (ECE-1) free from contamination by neutral endopeptidase 24. 11. *Biochem. Biophys. Res. Commun.* **207**, 355-362.

Corder, R. and Vane, J. R. (1995). Radioimmunoassay evidence that the pressor effect of big endothelin-1 is due to local conversion to endothelin-1. *Biochem. Pharmacol.* **49**, 375-380.

Edgell, C.-J. S., McDonald, C. C. and Graham, J. B. (1983). Permanent cell-line expressing human factor VIII-related antigen established by hybridization. *Proc. Nat. Acad. Sci. USA* **80**, 3734-3737.

Emoto, N. and Yanagisawa, M. (1995). Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J. Biol. Chem.* **270**, 15262-15268.

Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232-236.

Gee, N. S., Matsas, R. and Kenny, A. J. (1983). A monoclonal antibody to endopeptidase-24. 11. Its application in immunoadsorbent purification and immunofluorescent microscopy of kidney and intestine. *Biochem. J.* **214**, 377-386.

Gee, N. S. and Kenny, A. J. (1985). Dissection of renal brush border membranes with monoclonal antibodies. *Biochem. Soc. Trans.* **13**, 101-102.

George, F., Brisson, C., Poncelet, P., Laurent, J. C., Massot, O., Arnoux, D., Ambrosi, P., Klein-Soyer, C., Cazenave, J. P. and Sampol, J. (1992). Rapid isolation of human endothelial cells from whole blood using S-endo 1 monoclonal antibody coupled to immuno-magnetic beads: demonstration of endothelial injury after angioplasty. *Thromb. Haem.* **67**, 147-153.

Greenhough, K., Walkden, B. J., Murphy, L. J., McLaren, H. E., Barnes, K. and Turner, A. J. (1994). Membrane peptidase activity of a human endothelial cell line (EA. hy926). *Biochem. Soc. Trans.* **22**, 454S.

Gui, G., Xu, D., Emoto, N. and Yanagisawa, M. (1993). Intracellular localisation of membrane-bound endothelin-converting enzyme from rat lung. *J. Cardiovasc. Pharmacol.* **22** (suppl. **8**), S53-56.

Harrison, V. J., Corder, R., Ånggard, E. E. and Vane, J. R. (1993). Evidence for vesicles that transport endothelin-1 in bovine aortic endothelial cells. *J. Cardiovasc. Pharmacol.* **22** (suppl. **8**), S57-S60.

Harrison, V. J., Barnes, K., Turner, A. J., Wood, E., Corder, R. and Vane, J. R. (1995). Identification of endothelin 1 and big endothelin 1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proc. Nat. Acad. Sci. USA* **92**, 6344-6348.

Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. and Morimoto, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the secretion of endothelin-1 from cultured endothelial cells by inhibiting a big endothelin-1 converting enzyme. *Biochem. Biophys. Res. Commun.* **171**, 669-675.

Ikura, T., Sawamura, T., Shiraki, T., Hosokawa, H., Kido, T., Hoshikawa, H., Shimada, K., Tanzawa, K., Kobayashi, S., Miwa, S. and Masaki, T. (1994). cDNA cloning and expression of bovine endothelin converting enzyme. *Biochem. Biophys. Res. Commun.* **203**, 1417-1422.

Kenny, A. J., Stephenson, S. L. and Turner, A. J. (1987). Cell surface peptidases. In *Mammalian ectoenzymes* (ed. A. J. Kenny and A. J. Turner), pp. 169-210, Elsevier, Amsterdam.

Lisanti, M. P., Scherer, P. E. Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y. H., Cook, R. F. and Sargiacomo, M. (1994). Characterisation of caveolin-rich domains isolated from an endothelial rich source: implications for human disease. *J. Cell Biol.* **126**, 111-126.

Jackson, C. J., Garbutt, P. K., Nesson, B. and Schreiber, L. (1990). Binding of human endothelin to *Ulex europeans* 1-coated Dynabeads: application to the isolation of microvascular endothelin. *J. Cell Sci.* **96**, 257-262.

Matsas, R., Fulcher, I. S., Kenny, A. J. and Turner, A. J. (1983). Substance P and [Leu] enkephalin are hydrolysed by an enzyme in pig caudate synaptic membranes that is identical to the endopeptidase of kidney microvilli. *Proc. Nat. Acad. Sci. USA* **80**, 3111-3115.

Matsas, R., Stephenson, S. L., Hryszko, J., Kenny, A. J. and Turner, A. J. (1985). The metabolism of neuropeptides: phase separation of synaptic membrane preparations with Triton X-114 reveals the presence of aminopeptidase-N. *Biochem. J.* **231**, 445-449.

Matsumura, Y., Hisaki, K., Takaoka, M. and Morimoto, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.* **185**, 103-106.

McMahon, E. G., Palomo, M. A., Moore, W. M., McDonald, J. F. and Stern, M. K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin-1 (1-39) in vivo and conversion of big endothelin-1 (1-39) to endothelin-1 (1-21) *in vitro*. *Proc. Nat. Acad. Sci. USA* **88**, 703-707.

Ohnaka, K., Takayanagi, R., Ohashi, M. and Nawata, H. (1991). Conversion of big endothelin isopeptides to mature endothelin isopeptides by cultured bovine endothelial cells. *J. Cardiovasc. Pharmacol.* **17** (suppl. **7**), S17-19.

Opgenorth, T. J., Wu-Wong, J. R. and Shiozaki, K. (1992). Endothelin-converting enzymes. *FASEB J.* **6**, 2653-2659.

Pollock, D. M. and Opgenorth, T. J. (1991). Evidence for metalloprotease involvement in the in vivo effects of big endothelin-1. *Am. J. Physiol.* **261**, R257-R263.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R. and Anderson R. G. W. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-682.

Ryan, J. W., Day, A. R., Schulz, D. R., Ryan, U. S., Chung, A., Marlborough D. I. and Dorer, F. E. (1976). Localisation of angiotensin converting enzyme (kininase II). I. Preparation of antibody hemeoctapeptide conjugates. *Tissue Cell* **8**, 111-124.

Saijonmaa, O., Nyman, T., Hohenthal, U. and Fyhrquist, F. (1991). Endothelin-1 is expressed and released by a human endothelial hybrid line (EA. hy926). *Biochem. Biophys. Res. Commun.* **181**, 529-536.

Sawamura, T., Kasuya, Y., Matsushita, Y., Suzuki, N., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1991). Phosphoramidon inhibits the intracellular conversion of big endothelin-1 to endothelin-1 in cultured endothelial cells. *Biochem. Biophys. Res. Commun.* **174**, 779-784.

Schmidt, M., Kröger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. and Hillen, H. (1994). Molecular characterisation of human and bovine endothelin converting enzyme (ECE-1). *FEBS Lett.* **356**, 238-243.

Shimada, K., Takahashi, M. and Tanzawa, K. (1994). Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J. Biol. Chem.* **269**, 18275-18278.

Shimada, K., Matsushita, Y., Wakabayashi, K., Takahashi, M., Matsubara, A., Iijima, Y. and Tanzawa, K. (1995a). Cloning and functional expression of human endothelin-converting enzyme cDNA. *Biochem. Biophys. Res. Commun.* **207**, 807-812.

Shimada, K., Takahashi, M., Ikeda, M. and Tanzawa, K. (1995b). Characterization of two isoforms of an endothelin-converting enzyme-1. *FEBS Lett.* **371**, 140-144.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* **150**, 76-85.

Takahashi, M., Matsushita, Y., Iijima, Y. and Tanzawa, K. (1993). Purification and characterization of endothelin-converting enzyme from rat lung. *J. Biol. Chem.* **268**, 21394-21398.

Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, M., Koike, H., Yamamoto, Y. and Tanzawa, K. (1995). Localization of rat endothelin-converting enzyme to vascular endothelial cells and some secretory cells. *Biochem. J.* **311**, 657-665.

Turner, A. J. (1994). PIG-tailed membrane proteins. *Essays Biochem.* **28**, 113-127.

Turner, A. J. and Murphy, L. J. (1996). Molecular pharmacology of endothelin converting-enzymes. *Biochem. Pharmacol.* **51**, 91-102.

Virtanen, I., Ekblom, P. and Laurila, P. (1980). Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* **85**, 429-434.

Waxman, L., Doshi, K. P., Gaul, S. L., Wang, S., Bednar, R. A. and Stern, A. M. (1994). Identification and characterisation of endothelin converting activity from EAHY 926 cells: Evidence for the physiologically relevant human enzyme. *Arch. Biochem. Biophys.* **308**, 240-253.

Williams, T. A., Barnes, K., Kenny, A. J., Turner, A. J. and Hooper, N. M. (1992). A comparison of the zinc contents and substrate specificities of the endothelial and testicular forms of porcine angiotensin converting enzyme and the preparation of isoenzyme-specific antisera. *Biochem. J.* **288**, 875-881.

Xu, D., Emoto, M., Giaid, A., Slaughter, C., Kaw, S., de Wit, D. and Yanagisawa, M. (1994). ECE-1: a membrane-bound metalloprotease that catalyses the proteolytic activation of big endothelin-1. *Cell* **78**, 473-485.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayishi, Y., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent, vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 441-415.

(Received 8 December 1995 - Accepted 5 February 1996)