

## Endothelin-1 pathway in human alveolar epithelial cell line A549 and human umbilical vein endothelial cells

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**KEY WORDS** endothelin-1; secretion; endothelin receptors

### ABSTRACT

**AIM:** This study was designed to characterize the endothelin pathway in an immortalized human adenocarcinoma-derived alveolar epithelial cell line (A549) and human umbilical vein endothelial cell line (HUVEC).

**METHODS:** The release of ET-1 and big-ET-1 was measured in the incubation medium of both cell lines. The expression of mRNAs coding for the endothelin isoforms (hppET-1, -2, -3), the endothelin converting enzymes (hECE-1a, b, c, and d) and the hET<sub>A</sub> and hET<sub>B</sub> receptors was investigated using RT-PCR. The expression of ECE-1 mRNA in various human tissues and in A549 cells was investigated by Northern blot analysis and the subcellular localization of ECE-1 in A549 cells was investigated by immunoblotting using a polyclonal antibody. **RESULTS:** Under control conditions, HUVEC release both ET-1 and big-ET-1 (ratio 5 to 1) while in A549 cells the big-ET-1 levels were below the threshold of detection. The release of these two peptides was minimally affected by various inhibitors of peptidases. However, in both cell lines phosphoramidon produced a concentration-dependent inhibition of ET-1 release and an enhanced accumulation of big-ET-1. Both HUVEC and A549 cells express the mRNAs for ppET-1, ET-A, and ET-B receptor subtypes and ECE-1 (isoforms ECE-1b, c and/or d). In addition, in HUVEC the mRNAs for ppET-2 and for the isoform ECE-1a were also detected. In A549 cells, ECE-1 had a preferential subcellular localization in the membrane fraction but was not detected in

the cytosol. **CONCLUSION:** Both A549 and HUVEC produce and release endothelin-1 through a specific enzymatic pathway, whether or not ECE-1 is the only enzyme involved remains to be determined. A549 might be used as a screening assay for drug discovery such as for inhibitors of endothelin-1 release.

### INTRODUCTION

Endothelins are a family of potent vasoactive 21-amino-acid peptides consisting of three peptides coded by three different genes; endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). The biosynthesis of ET-1 involves first the cleavage of preproendothelin-1 (pp-ET-1) to proendothelin-1 (big-ET-1) and then the cleavage of big-ET-1 by specific metalloproteases called endothelin converting enzyme (s) (ECE) to generate the bioactive peptide<sup>(1,2)</sup>. At least two different enzymes could be involved in big-ET-1 processing, ECE-1 and ECE-2<sup>(3)</sup>. In humans, ECE-1 appears to be the predominant converting enzyme<sup>(4)</sup>. Four isoforms of this endopeptidase; ECE-1a, ECE-1b, ECE-1c, and ECE-1d are generated from the same gene by differential mRNA splicing<sup>(5,6)</sup>. ECE(s) are membrane bound proteases with structural analogy with neutral-endopeptidase 24-11 and are sensitive to the metalloprotease inhibitor phosphoramidon<sup>(7)</sup>. However, non ECE-1 proteases could also be involved in the production of ET-1 as this peptide is still detected in tissue from mice subjected to targeted disruption of the endothelin converting enzyme-1 gene<sup>(8)</sup>. ET-1 can activate at least two subtypes of receptors ET<sub>A</sub> and ET<sub>B</sub><sup>(2)</sup>.

ET-1 is a potent vasoconstrictor that may play a role in the terminal stages of various cardiovascular diseases such as heart failure or coronary vasospasm<sup>(2)</sup> and possibly in the maintenance of basal vascular tone in humans<sup>(9)</sup>. Besides their endothelial origin, endothelins can be also generated in the respiratory tract and they could also be involved in pulmonary diseases such as

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Received 1999-12-22

Accepted 2000-03-10

asthma, lung fibrosis and pulmonary hypertension<sup>[10]</sup>. However, it is not yet clear whether or not the endothelin synthesis involves the same pathway (s) in vascular endothelial and airway epithelial cells.

The present experiments were designed to compare the endothelin pathway in an immortalized human umbilical vein endothelial cell line (HUVEC) and in a human adenocarcinoma-derived alveolar epithelial cell line (A549).

## MATERIALS AND METHODS

**Cell culture** HUVEC and A549, were obtained from the Institut Pasteur<sup>[11]</sup> and ECACC (ref: 86012804), respectively. The cells were grown in minimum essential medium (MEM, Gibco BRL, Cergy Pontoise, France) supplemented with 10 % fetal calf serum (FCS), 0.01 mol/L HEPES (Gibco BRL), glutamine 2 mmol/L (Gibco BRL) and antibiotics (Penicillin 100 kU/L and streptomycin 100 kG/L, Gibco BRL). Cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> and cultured for five days. Before the experiments the supplemented medium was washed out and the medium was renewed without the serum. At the end of the incubation period (6 to 18 h), aliquots of medium were collected and conserved at -20 °C. The number of cells per well was estimated using either a Malassez cell or fluorimetry (after incubation with propidium iodide 60 mg/L, Triton 0.05 % and edetic acid 0.01 mol/L for 18 h). Cell viability was estimated by Trypan blue exclusion.

**Preparation of mRNA** The cells ( $8 \times 10^5$  cells) were washed with Earle's balanced salt solution (EBSS; Gibco BRL) and collected. Total RNA was extracted from the cells with RNA-B solution (Quantum Biotechnologies, Montreuil-sous-bois, France). Poly adenylated mRNAs were purified by chromatography on an oligo (dT) column (QuickPrep Micro mRNA Purification kit, Pharmacia Biotech, Uppsala, Sweden).

**Reverse transcription (RT)-polymerase chain reaction (PCR)** Poly adenylated mRNA were reverse transcribed from oligo dT12-18 in a final volume of 15  $\mu$ L using the "First Strand cDNA synthesis kit" method (Pharmacia Biotech). PCR reactions were carried out in buffer containing Tris-HCl 10 mmol/L (pH 8.3), MgCl<sub>2</sub> 2.5 mmol/L, deoxynucleotide triphosphate 0.2 mmol/L, 5  $\mu$ L of RT-mix, 0.5  $\mu$ mol/L of each primer and 2 units of Taq polymerase (Pharmacia Biotech), in a final volume of 100  $\mu$ L and using a cycling program of 94 °C for 1 min 30'; 60 °C (hECE-1a) or 62 °C (hECE-1b,

hppET-1) or 65 °C (hppET-2,3 and hET<sub>A,B,R</sub>) for 2 min and 72 °C for 2 min. After 30 to 40 cycles, PCR products were analysed by electrophoresis on 2 % agarose gel stained by ethidium bromide. The positions of forward sense and reverse anti-sense primers used (Genset, France) were respectively 35-56 and 605-623 for hppET-1<sup>[12]</sup>, 1-21 and 510-530 for hppET-2<sup>[13]</sup>, 24-45 and 647-669<sup>[14]</sup>, 515-537 and 1261-1284 for hET<sub>A</sub> receptor<sup>[15]</sup>, 680-703 and 1510-1535 for hET<sub>B</sub> receptor<sup>[16]</sup>. The primers used to amplify hECE-1a and hECE-1b fragments were localized in the common part of the sequence for the reverse anti-sense primer (position 741-763)<sup>[17]</sup> and in the variable part of the sequence for the forward sense primers (positions 1-20 and 52-71 respectively for hECE-1a and hECE-1b)<sup>[17,18]</sup>. The 5' primer used to amplify the mRNA of the ECE-1b isoform also amplifies the mRNA for the ECE-1c and d isoforms<sup>[5,6]</sup>. The length of the amplified cDNA for the ECE-1c and d isoforms were identical to that of ECE-1b. The specificity of PCR products was established by predicted size fragments obtained by several restriction enzymes digestions. The mRNA not reverse-transcribed was used as negative control.

**Dosage of ET-1 and big-ET-1** Cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> and cultured for five days in 48 wells plates. At d 5 the cells numbers per well was  $4 \times 10^4$  and  $7 \times 10^5$  for HUVEC and A549 respectively. Aliquots of the incubation medium were analysed by enzyme linked immunosorbent assay (ELISA) to measure ET-1 and big-ET-1 release (Amersham kit, Les Ulis, France). The specificity of the ELISA measurement was verified with ET-1, ET-2, ET-3, big-ET-1 (1-38), big-ET-1 C-terminal fragment (22-38), ET-1 carboxy-terminal fragment (11-21) and a fragment which includes the cleavage site (19-26). The ELISA-kit for Big-ET-1 is specific while the ELISA-kit for ET-1 recognizes both ET-1 and ET-2.

**Immunoblotting** Confluent monolayers of A549 cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested in the presence of edetic acid 5 mmol/L and collected by centrifugation ( $1000 \times g$  at 4 °C). The cells were resuspended in ten volumes of buffer A [Tris-HCl 10 mmol/L pH 7.5, edetic acid 1 mmol/L, sucrose 250 mmol/L supplemented with Complete<sup>TM</sup> protease inhibitor cocktail (Boehringer)] and lysed using a polytron homogenizer (Brinkman). The lysate was centrifuged at  $14\,000 \times g$  at 4 °C for 30 min. The resulting pellet was resuspended in buffer A and cen-

trifuged in the same condition. Membrane fraction (pellet) was resuspended in PBS buffer supplemented with KCl 5, MgCl<sub>2</sub> 25, sucrose 250, and edetic acid 1 mmol/L. The protein concentration of the membrane fraction and of the cytosol (first supernatant) was measured using a BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as standard. Membrane and cytosolic proteins of A549 cells were separated by SDS-polyacrylamide (7 % w/v) gel electrophoresis<sup>[23]</sup> and electrotransferred to nitrocellulose filters. The blot was first incubated for two hours at 25 °C in blocking PBS containing 5 % (w/v) BSA and 0.2 % (v/v) Tween 20 (buffer B), then overnight at 4 °C with anti-carboxy-terminal peptide (YRCPPGSPMNPCHKCE) of human ECE-1 rabbit polyclonal antibody (diluted at 1/3000) in antibody PBS containing 2 % (w/v) BSA, 0.5 % (w/v) ovalbumin and 0.1 % (v/v) Tween 20 (buffer C). This antibody allows the detection of the four ECE-1 subtypes (ECE-1a, b, c and d) as they possess the same carboxy-terminal region. Immunoblots were washed three times with buffer B and incubated for one hour at 25 °C in buffer C with polyclonal horseradish peroxidase conjugated goat anti-mouse antibody (Promega) diluted at 1/3500. After three washes, peroxidase activity was revealed by ECL-reagent and exposure to Hyperfilm in accordance with the manufacturer's instructions (Amersham).

**Northern blot analysis** Northern blot membranes containing mRNA (2 µg/lane) from various human tissues were purchased from Clontech. The mRNA (2 µg) extracted from A549 cells (Clontech) was subjected to formaldehyde/agarose gel (1 % w/v) electrophoresis and blotted on hybond N<sup>+</sup> filter (Amersham). The membranes were prehybridized for two hours at 42 °C in 5 × Standard Saline Citrate, 5 × Denhardt's solution, sheared salmon sperm DNA 100 mg/L, 50 % v/v formamide and 0.1 % w/v SDS. This was followed by overnight hybridization at 42 °C in the same buffer containing 10<sup>9</sup> cpm/L of random priming labeled probe (specific activity 2 × 10<sup>9</sup> cpm/µg DNA). The probe was a 1.35 kb fragment DNA generated by RT-PCR corresponding to the coding region (position 52 to 1400) of human ECE-1 mRNA<sup>[17]</sup> that does not discriminate between the 4 isoforms of ECE-1 (ECE-1a, b, c, or d). The membranes were washed twice with 2 × Standard Saline Citrate, 0.1 % SDS at room temperature and twice for 30 min. at high stringency 0.1 × Standard Saline Citrate, 0.1 % SDS at 65 °C. The blots were exposed with Kodak X-AR-5 film and intensifying screens for 18 h at -80 °C. The integrity of the mRNA was

confirmed by the presence of intact β-actin transcript.

**Pharmacological reagents** Pepstatin, phosphoramidon and actinonin were purchased from Sigma (France); thiorphan, ET-1, ET-2, ET-3, big-ET-1 (1-38), C-terminal fragment (22-38) from Bachem (Voisins le Bx, France); captopril from Squibb. ET-1 (11-21) from Neosystem (France) and big ET-1-(19-26) from Novabiochem (France). The compounds were dissolved in Earle's balanced salt solution except for thiorphan and actinonin which were dissolved in dimethylsulfoxide (maximal concentration 0.4 %).

**Statistical analysis** Results of the experiments are expressed as mean ± SEM. Student's *t* test for unpaired observations was used for the statistical analysis. Differences were considered to be significant when *P* was less than 0.05.

## RESULTS

**Release of ET-1 and big-ET-1** After six hours in a culture medium deprived of serum, ET-1 was released by both HUVEC and A549 cells. HUVEC produced significantly more ET-1 per 10<sup>5</sup> cells than A549 [79.7 ± 13.6 and 11.4 ± 2.4 pmol · L<sup>-1</sup>/10<sup>5</sup> cells, respectively (*n* = 11)]. Big-ET-1 could be measured in the cultured medium of HUVEC (17.8 ± 1.1 pmol · L<sup>-1</sup>/10<sup>5</sup> cells; *n* = 7) but was below the threshold of detection for A549.

In both cell lines, thiorphan (10 µmol/L), captopril (1 µmol/L), pepstatin (100 µmol/L), actinonin (100 µmol) and a combination of these four inhibitors were administered throughout the incubation period. These inhibitors produced none or very minor changes in the release of both ET-1 and big-ET-1 by both cell lines (data not shown). In HUVEC, thiorphan produced a modest but significant increase in the release of big-ET-1 (24.7 ± 1.1 pmol · L<sup>-1</sup>/10<sup>5</sup> cells; *n* = 7).

Incubation with phosphoramidon (0.01 to 1 mmol/L) for 6 h induced a concentration dependent inhibition of ET-1 concentration and a parallel increase in big-ET-1 concentration in both cell lines (Fig 1-2). Thiorphan (10 µmol/L) did not significantly affect the effects of phosphoramidon (Fig 1-2). The inhibitory effect of phosphoramidon (1 mmol/L) was still observed after an 18-h incubation period. The inhibitor did not significantly affect cells viability or the ELISA measurements (data not shown).

**Expression of human endothelin isoforms (hppET-1, -2, -3), endothelin converting enzyme**

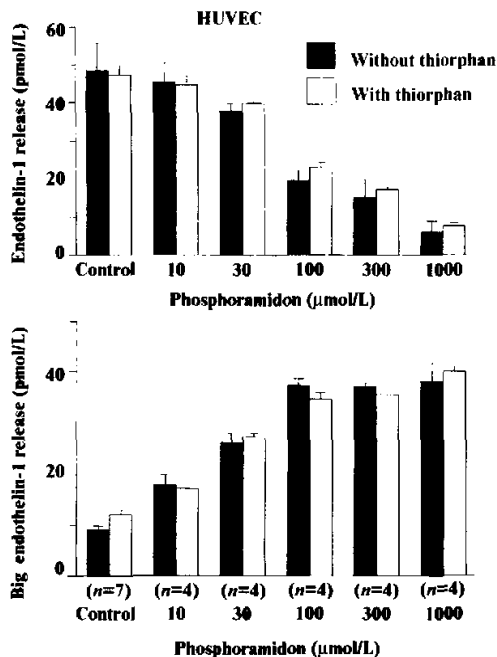


Fig 1. Effect of phosphoramidon on the endothelin and big-endothelin release by HUVEC. Top panel: endothelin release in the absence ( $n = 4 - 11$ ) or presence ( $n = 3 - 6$ ) of thiorphan ( $10 \mu\text{mol/L}$ ). Lower panel: Big endothelin release in the absence or presence of thiorphan ( $10 \mu\text{mol/L}$ ). Data are shown as means  $\pm$  SEM. The number of HUVEC cells per well was approximately  $5 \times 10^4$ . Control experiments were performed in the presence of  $\text{Me}_2\text{SO}$  ( $0.004\%$ ).

isoforms (hECE-1a, b, c, and d) and hET<sub>A,B</sub> receptors mRNA. The presence of PCR products with the expected size corresponding to mRNA encoding for hpp-ET-1 (593 bp) were detected both in HUVEC and A549 (Fig 3). The hpp-ET-2 mRNA was present only in HUVEC since the corresponding 530 bp cDNA was not amplified in A549 cells (Fig 3). PCR products specific for hppET-3 mRNA were undetectable both in HUVEC and A549 cells, even after 40 polymerase chain reaction cycles (Fig 3). In contrast, with human placenta mRNA the amplification of the expected 655 bp hpp-ET-3 cDNA was observed with the hppET-3 specific primers (data not shown).

A 726 bp cDNA corresponding to hECE-1b, c, and d was amplified from both HUVEC and A549 (Fig 3). The 732 bp cDNA encoding hECE-1a was amplified from HUVEC cells mRNAs but not from A549 cells mRNAs (Fig 3). The cDNAs corresponding to hET<sub>A</sub> and hET<sub>B</sub>

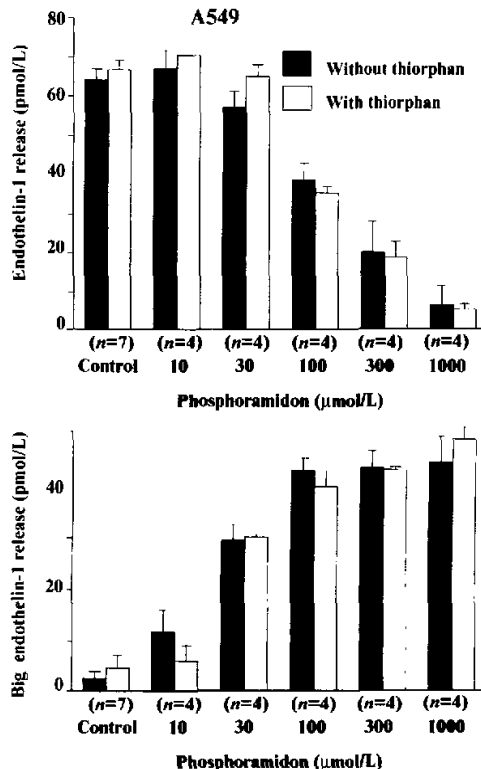


Fig 2. Effect of phosphoramidon on the endothelin and big-endothelin release by A549 cells. Top panel: endothelin release in the absence or presence of thiorphan ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ ). Lower panel: Big endothelin release in the absence or presence of thiorphan ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ ). Data are shown as means  $\pm$  SEM. The number of A549 cells per well was approximately  $8 \times 10^5$ . Control experiments were performed in the presence of  $\text{Me}_2\text{SO}$  ( $0.004\%$ ).

receptors (769 and 905 bp, respectively)<sup>[15,16]</sup> were amplified from both HUVEC and A549 cells mRNAs (Fig 3).

No signal was observed when reverse transcriptase was omitted from the first-strand cDNA conversion. This suggests that the PCR products observed were not due to any genomic DNA contaminating RNA.

**Northern blot analysis of ECE-1 mRNAs in A549 cells and in various human tissues** Two mRNAs of 1.3 and 4.8 kb, respectively were revealed (Fig 4). The mRNA of 4.8 kb was highly expressed in human lung, heart, placenta, pancreas and in A549 cells and very weakly expressed in brain, liver, kidney and skeletal muscle. The 1.3 kb mRNA was detected in each human tissue and cells studied with a preferential expression in placenta, heart and pancreas. Two additional

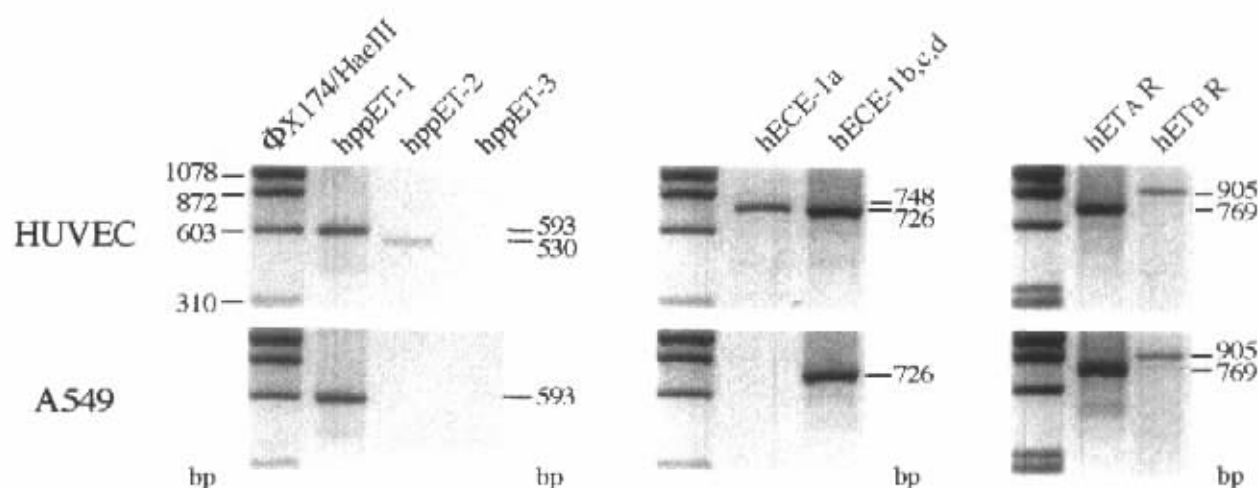


Fig 3. Expression of human mRNA coding to preproendothelins [hppET<sub>1,2,3</sub>], endothelin converting enzymes (hECE-1a, b, c, d) and endothelin receptors (hET<sub>A,B</sub>R) in HUVEC and A549 cells. The mRNA (250 ng) of HUVEC and A549 cells were reverse transcribed and amplified by 35 PCR cycles using specific primers. The PCR products were analyzed on 2% agarose gel stained by ethidium bromide. The length of the PCR fragments was estimated by molecular weight marker ( $\Phi$ X 174/Hae III) on the left and indicated in base pairs on the right.

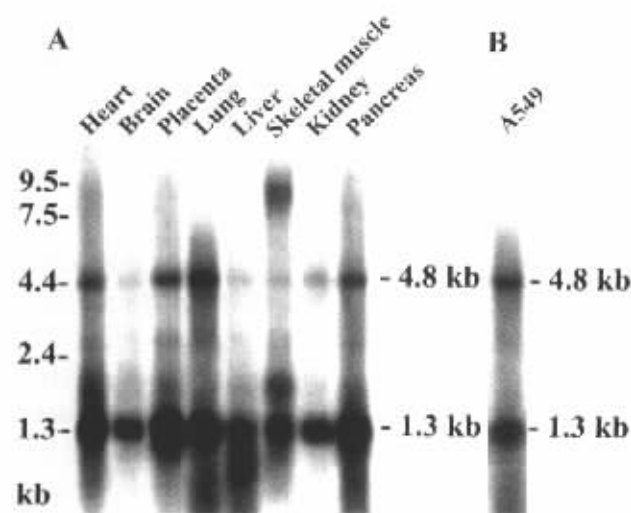


Fig 4. Northern Blot analysis of ECE-1 mRNAs. Expression of the ECE-1 mRNAs in various human tissues (A) and in A459 cells (B) was investigated by Northern blot analysis. Each lane contains 2  $\mu$ g of poly (A<sup>+</sup>) RNA. The filters were analysed with a probe corresponding to the coding region (position +52 to +1400) of human ECE-1 mRNA (non specific for the isoforms ECE-1a, b, c, or d; Yorimitsu *et al*, 1995). An 18 h exposure at -80 °C using intensifying screens of the autoradiograph is shown. The mobility of RNA size markers and ECE-1 mRNA were indicated on the left and on the right, respectively.

mRNA species of 1.6 and 9.5 kb were also detected in skeletal muscle (data not shown).

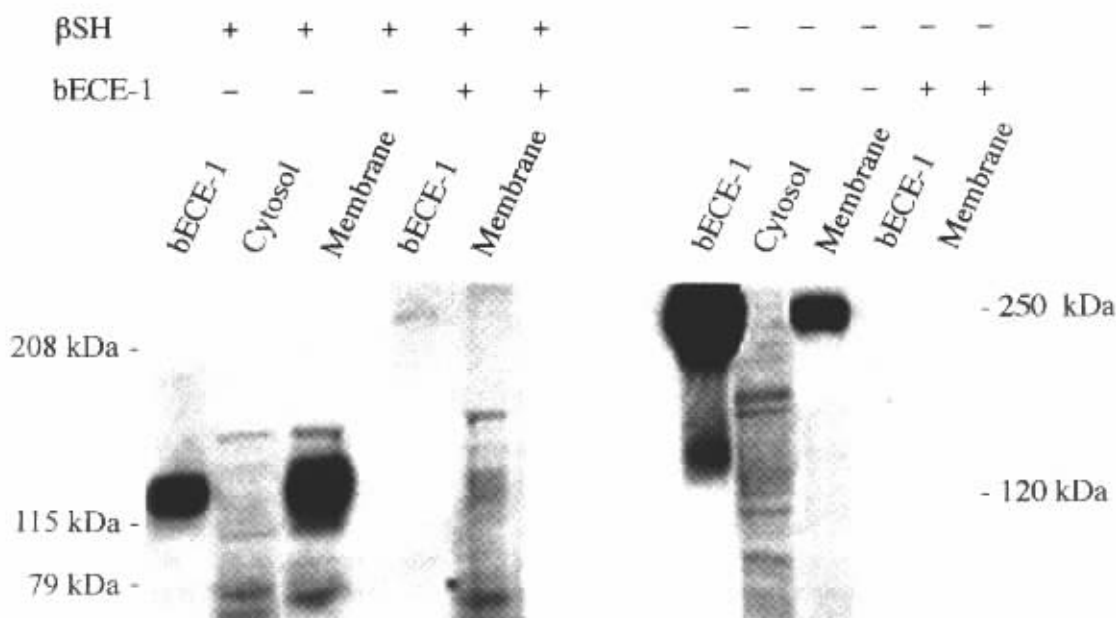
#### Immunoblot analysis of ECE-1 in A549 cells

ECE-1 was observed in the membrane fraction of A549 cells (Fig 5) but not in the cytosol. In the presence of  $\beta$ -mercaptoethanol, the antibody recognized a polypeptide of 120 kDa whereas in the absence of this reducing agent the antibody detected a polypeptide of 250 kDa (Fig 5). These results were also observed using partially purified bovine ECE-1 (Fig 5). Both the human ECE-1, expressed in A549 cells, and the purified bovine ECE-1 could not be detected when the antibody was first saturated with 5  $\mu$ g of purified bECE-1.

#### DISCUSSION

The present findings confirm that the two cell lines of human origin, the HUVEC and the A549 produce endothelin-1 and its precursor big-ET-1<sup>[19,20]</sup> and that an enzymatic pathway specifically sensitive to phosphoramidon is required for the production of ET-1. In both HUVEC and A549 the mRNAs for the two endothelin receptor subtypes (ET<sub>A</sub> and ET<sub>B</sub>) are expressed.

The A549 cells expressed exclusively the mRNA for hpp-ET-1<sup>[12]</sup> while HUVEC expressed mRNAs for both hpp-ET-1 and hpp-ET-2<sup>[13]</sup>, the former was significantly more represented than the latter. The mRNA for hpp-ET-3 could not be detected in either cell line in contrast to previous observations in A549<sup>[17]</sup>. However, the expression reported in this earlier work was at a very low level. The present findings are unlikely to be attributed to technical difficulties as in experiments performed with



**Fig 5. Immunoblot analysis of ECE-1 in A549 cells.** Membrane (M) and cytosolic (C) proteins (60  $\mu$ g) or partially purified bovine ECE-1 (bECE-1, 2  $\mu$ g) were analysed by SDS-polyacrylamide (7 % v/v) gel electrophoresis in the presence or absence of  $\beta$ -mercaptoethanol ( $\beta$ SH). The proteins were then immunoblotted using anti-carboxy-terminal peptide of human ECE-1 polyclonal antibody either saturated or not with partially purified bovine ECE-1 (5  $\mu$ g). Detection of primary antibody binding was performed with a specific HRP-conjugated secondary antibody followed by ECL-directed exposure to Hyperfilm. The blots were calibrated with Rainbow molecular weight markers (Amersham) as indicated on the left. The estimated molecular weights of the ECE-1 are indicated on the right. A representative experiment, out of the three performed, is shown.

human placenta the expression of the mRNA for hpp-ET-3 was observed consistently<sup>[14]</sup>, thereby confirming that under the used experimental conditions HUVEC and A549 cells do not express the hpp-ET-3 isoform.

The ELISA-kit for ET-1 was relatively specific as it did not recognize big-ET-1 or the various peptidic fragments preceding, following or including the cleavage site (Trp<sup>21</sup>-Val<sup>22</sup>) for the endothelin-converting enzyme. However, the dosage kit could not discriminate ET-1 and ET-2. In A549 cells, the measurement of ET-1 release can be considered as accurate as only the mRNA for hpp-ET-1 is expressed in those cells. In contrast, in HUVEC, the measurement of ET-1 release can represent both ET-1 and ET-2 as these endothelial cells express mRNAs for both hpp-ET-1 and hpp-ET-2. However, the mRNA of the hpp-ET-2, seems poorly expressed when compared to the mRNAs for hpp-ET-1, suggesting that most of the endothelin measured by the ELISA-kit corresponds to the ET-1 isoform.

In both cell lines the conversion of big-ET-1 to ET-1 does not involve angiotensin converting enzyme, neutral endopeptidase, aspartic endopeptidase, or aminopeptidase

M as the release of ET-1 was not affected by the various specific inhibitors of these peptidases (captopril, thiorphan, pepstatin, and actinonin, respectively). These observations rule out a previous suggestion that endothelin converting enzyme could be aspartic endopeptidase active under acidic pH<sup>[21]</sup>. Big-ET-1 can be degraded into non-biologically active peptidic fragments by enzymes different than the endothelin converting enzyme, such as the neutral endopeptidase<sup>[22]</sup>. Indeed, in HUVEC an increase in big-ET-1 was observed in presence of thiorphan. However, as the effects of thiorphan are minimal, this pathway cannot be considered as preponderant under the present experimental conditions.

In HUVEC and in A549 cells, phosphoramidon produced a significant concentration-dependent inhibition of ET-1 release. The effect of phosphoramidon can be attributed to the inhibition of the converting enzyme because this compound produced a parallel accumulation of big-ET-1 in the culture medium and did not show any toxic effect. The effects of phosphoramidon are not influenced by the presence of thiorphan confirming that neutral endopeptidase is not involved in the conversion of

big-ET-1 to ET-1. The concentrations of phosphoramidon required to inhibit endothelin-1 release in HUVEC and A549 are similar. They are higher than the ones required to inhibit the activity of the isolated ECE enzyme but are consistent with the concentrations previously reported to inhibit ET-1 production by intact cells<sup>[2,23]</sup>. These observations suggest that in both HUVEC and A549 cells the same enzyme family is involved in the formation of ET-1 and that phosphoramidon must penetrate inside the cells in order to be active.

At least two different enzymes could be involved in big-ET-1 processing, ECE-1 and ECE-2<sup>[3]</sup>. In humans, ECE-1 appears to be the predominant converting enzyme<sup>[4]</sup>. Four isoforms of this endopeptidase: ECE-1a, ECE-1b, ECE-1c, and ECE-1d are generated from the same gene by four promoters and differential mRNA splicing<sup>[5,6]</sup>. In both HUVEC and A549 cells the ECE-1 enzyme was detected. The ECE-1b, c or/and d isoforms were observed in both cells<sup>[17]</sup> while the ECE-1a isoform was present only in HUVEC<sup>[18]</sup>. The primers used in this study do not allow the discrimination between the ECE-1b, ECE-1c, and ECE-1d isoforms.

Northern blot analysis confirmed the expression of ECE-1 mRNA in A549 cells. The probe used in the present study can be hybridized with the mRNAs coding for the four ECE-1 subtypes (ECE-1a, b, c, and d). Two mRNAs of 4.8 and 1.3 kb were revealed. The 1.3 kb mRNA was detected in each human tissue and cells studied with a preferential expression in placenta, heart and pancreas. The size of the 1.3 kb mRNA is smaller than the coding region of ECE-1 mRNA (> 2 kb). This mRNA may correspond to an unknown alternative splicing of ECE gene or to a sequence sharing some homology with ECE-1 that may be hybridized by the probe. The mRNAs of 4.8 kb was highly expressed in A549 cells but also in human lung, heart, placenta, pancreas while it was very weakly expressed in brain, liver, kidney and skeletal muscle. The tissue distribution and the length of the mRNA 4.8 kb corresponds to the ECE-1 mRNA previously described<sup>[5,23]</sup>. The predominant sites for ET-1 mRNA expression are the lungs followed by the large intestines, but the mRNA expression has been detected in every organ examined so far<sup>[2]</sup>. These results may suggest a parallel tissular gene expression between ECE-1 and ET-1. However, in bovine aortic endothelial cells that express the mRNAs for both ppET-1 and ECE-1, the expression of ET-1 and ECE-1 are independently regulated<sup>[24]</sup>, suggesting that ECE-1 may not be the only enzyme involved in ET-1 production. Finally, the mR-

NA 1.6 and 9.5 kb observed exclusively in the skeletal muscle have not been identified at present.

In A549 cells, a polyclonal antibody against human ECE-1 has detected a polypeptide of 250 kDa. In the presence of the reducing agent  $\beta$ -mercaptoethanol, the antibody recognized a peptide of 120 kDa. The predicted molecular weight of ECE-1, is approximately 85 kDa but ECE-1 is heavily glycosylated and the apparent molecular weight under reducing conditions is 120 kDa. Furthermore, ECE-1 forms homodimer with interchain disulfid bonds leading to a 250 kDa peptide in non reducing conditions<sup>[23,25]</sup>. This data confirms the specificity of the observations performed in the present study as  $\beta$ -mercaptoethanol induces the monomerization of ECE-1. The polyclonal antibody against human ECE-1 did not discriminate between the four ECE-1 subtypes (ECE-1a, b, c, and d). ECE-1 was observed in the membrane fraction of A549 cells but not in the cytosol. This observation is consistent with previous observations demonstrating that ECE-1 is a membrane bound metalloprotease<sup>[3,23]</sup>. However, the subcellular location of these isoforms may differ<sup>[26,27]</sup>.

Non ECE-1 proteases could also be involved in the production of ET-1 as this peptide is still detected in tissues from mice subjected to targeted disruption of endothelin converting enzyme-1 gene<sup>[8]</sup>. ECE-2, a membrane-bound phosphoramidon sensitive metalloprotease<sup>[4]</sup> is also expressed in cultured human vascular endothelial cells and is also localized in intracellular secretory vesicle<sup>[28]</sup>. Whether or not this enzyme is present in A549 cells and the respective role of each enzyme in the production of ET-1 remains to be determined.

In conclusion, although the endothelial cells release ten times more endothelin-1 than the human alveolar epithelial cells A549, the latter cell line can be used to explore the enzymatic pathway involved in the production of endothelin-1. Furthermore, considering the ease in cultivating A549 and their rapid growth, this cell line might be used as a screening assay for drug discovery such as for inhibitors of endothelin-1 release.

**ABBREVIATIONS** Endothelin-1; ET-1; Big endothelin-1; big-ET-1; Prepro-Endothelin-1; pp-ET-1; Endothelin converting enzyme; ECE; human umbilical vein endothelial cells; HUVEC; Reverse transcription-polymerase chain reaction; RT-PCR.

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人肺泡上皮细胞株 A549 和脐静脉内皮细胞中的内皮素-1 通路

关键词 内皮素-1;分泌;内皮素受体

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