British Journal of Pharmacology (2007) 152, 313–322
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

The peptidase inhibitor CGS-26303 increases endothelin converting enzyme-1 expression in endothelial cells through accumulation of big endothelin-1

V Raoch[1,3], P Martinez-Miguel[1,3], I Arribas-Gomez[1], M Rodriguez-Puyol[2], D Rodriguez-Puyol[1] and S Lopez-Ongil[1]

[1]*Hospital Universitario Principe de Asturias, Research Unit and Nephrology section, Madrid, Spain and* [2]*Department of Physiology, Alcala University, Alcala de Henares, Madrid, Spain*

Background and purpose: CGS-26303 inhibits endothelin converting enzyme (ECE)-1 more specifically than phosphoramidon. We have studied the effect of CGS-26303 on ECE-1 expression in bovine aortic endothelial cells.
Methods: ECE-1 activity and big endothelin (ET)-1 levels were measured by ELISA, ECE-1 expression using western and northern blot and promoter activity using transfection assays.
Key results: ECE-1 activity was completely inhibited by CGS-26303 25 μM and phosphoramidon 100 μM. CGS-26303 and phosphoramidon, though not thiorphan, a neutral endopeptidase (NEP) inhibitor, stimulated ECE-1 expression in cells (maximal effect at 16 h, 25 μM). Cycloheximide abolished that effect. CGS-26303 induced ECE-1 mRNA expression and ECE-1 promoter activity. CGS-35066, a selective ECE-1 inhibitor, mimicked the effects of CGS-26303, suggesting that the effect was specific to ECE-1 inhibition. Big ET-1 accumulated in the cells and in the supernatants after CGS-26303 treatment. Neither exogenously added ET-1 nor the blockade of their receptors with bosentan modified ECE-1 protein. When big ET-1 was added to cells, significant increases in ECE-1 protein content and ECE-1 promoter activity were found. Bosentan did not block those effects. CGS-26303 did not modify prepro-ET-1 expression. CGS-26303 and big ET-1 induced the same effects in human endothelial cells, at lower doses.
Conclusions: These results suggest that the accumulation of big ET-1 is responsible for the effects of CGS-26303 on ECE-1 and they did not depend on NEP blockade. Changes in ECE-1 protein after the administration of CGS-26303 could lead to a decreased response in long-term treatments.
British Journal of Pharmacology (2007) **152**, 313–322; doi:10.1038/sj.bjp.0707398; published online 23 July 2007

Keywords: endothelial cells; endothelin-1; big ET-1; endothelin converting enzyme-1; ECE-1 regulation; CGS-26303; CGS-35066

Abbreviations: BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; NEP, neutral endopeptidase

Introduction

Endothelin-1 (ET-1), the most powerful endothelium-derived vasoconstrictor known, is generated from the posttranslational processing of a precursor protein, prepro-ET-1 to a peptide precursor, big ET-1, which is further processed to biologically active ET-1 by a specific phosphoramidon-sensitive metalloprotease called endothelin converting enzyme (ECE) (Xu *et al*., 1994). Two ECEs, labelled ECE-1 and ECE-2, are transcribed from different genes, and have been cloned and functionally characterized (Xu *et al*., 1994; Emoto and Yanagisawa, 1995). Both enzymes are type II membrane proteins and belong to the family of zinc metalloproteases related to neutral endopeptidase (NEP). The regulation of ECE gene expression in cultured endothelial cells has still to be fully described. It has been reported that ECE is up-regulated by phorbol ester through the transcriptional factor Ets-1 (Orzechowski *et al*., 1998), hydrogen peroxide through the transcriptional factor STAT 3 (Lopez-Ongil *et al*., 2002), thrombin through Erk1/2 (Eto *et al*., 2001), glucose (Keynan *et al*., 2004), oxidized LDL

Correspondence: Dr S Lopez-Ongil, Research Unit, 5°D, Fundacion para la Investigacion Biomedica, Hospital Universitario Principe de Asturias, Ctra. Alcala-Meco s/n, 28805 Alcala de Henares, Madrid, Spain.
E-mail: slopez.hupa@salud.madrid.org
[3]These authors contributed equally to this work.
Received 29 March 2007; revised 25 May 2007; accepted 19 June 2007; published online 23 July 2007

(Niemann *et al*., 2005) and vascular endothelial growth factor (Matsuura *et al*., 1997). On the other hand, it is downregulated by shear stress (Morawietz *et al*., 2000; Masatsugu *et al*., 2003), angiotensin II (Lopez-Ongil *et al*., 2005) and ET-1 (Naomi *et al*., 1998), and superoxide anion inhibits ECE-1 activity (Lopez-Ongil *et al*., 2000).

Since ET-1 is believed to play a critical role in different pathological settings (Lüscher and Barton, 2000; Aharinejad *et al*., 2005; Attina *et al*., 2005), the regulation of ECE synthesis or ECE activity could prove to be very helpful in controlling a number of cardiovascular or renal diseases. Phosphoramidon, the first reported ECE inhibitor, blocks the biological actions of big ET-1 both *in vitro* and *in vivo* (McMahon *et al*., 1991; Turner *et al*., 2001). However, it seems to be a rather nonspecific compound and it has been reported to increase the intracellular expression of ECE-1a and 1b (Isaka *et al*., 2003), an effect that could lead to a decreased response for long-term drug treatments. For these reasons, new ECE inhibitors are being developed and one of these, CGS-26303, has been shown to inhibit ECE-1 with an IC_{50} of 410 nM. *In vivo*, CGS-26303 was able to inhibit the pressor response to big ET-1 by 50% (Jeng *et al*., 2000). Long-term treatment with CGS-26303 decreases both preload and afterload, increases cardiac output, and diminishes left ventricular hypertrophy, dilatation and cardiac fibrosis (Mulder *et al*., 2004). The effect of CGS-26303 cannot be completely explained by its anti-ECE activity, as this drug also inhibits NEP (Jeng, 1997), subsequently increasing vasoactive peptides such as atrial natriuretic peptide (van der Zander *et al*., 1999; Horio *et al*., 2000).

In contrast to previous studies with phosphoramidon, the effect of CGS-26303 on ECE-1 expression has not been yet analysed. Changes in ECE-1 expression after the administration of CGS-26303 could determine an increased or decreased therapeutic response in long-term treatments. Present experiments were designed to analyse the effect of CGS-26303 on the cell content of ECE-1. Because a significantly increased expression of this protein was detected after treatment of cells with CGS-26303, the mechanisms responsible for these changes were explored.

Methods

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine thoracic aortas, using the previously described methods (Marsden *et al*., 1990). The cells were maintained in Rosewell Park Memorial Institute 1640 (RPMI 1640) supplemented with 15% calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in an atmosphere of 95% air and 5% CO_2. The human endothelial cell line (EA.hy926) was grown in DMEM supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in an atmosphere of 95% air and 5% CO_2. Experiments were routinely performed on confluent monolayers between passages 2–5, that were made quiescent by serum deprivation. The cellular toxicity of CGS-26303 and CGS-35066 were excluded in every experimental condition using the trypan blue dye exclusion method and measuring lactic dehydrogenase (LDH) activity.

Western blot analysis of immunoreactive ECE-1

BAEC were grown to confluence and then homogenized in 1 ml of RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.2 mM orthosodium vanadate. The homogenates were centrifuged at 10 000 *g* for 30 min. The protein concentration in each supernatant was determined with the Bio-Rad Protein Assay Kit. Proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis 6% (30 μg protein per lane) and transferred onto nitrocellulose membranes using the Universal buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol, 0.05% SDS). The nitrocellulose membranes were blocked with 5% (w/v) non-fat dried milk in phosphate-buffered saline (PBS) 1 h at room temperature, and then incubated with 10 μg ml^{-1} of the monoclonal antibody against bovine ECE-1 (Dr Kohei Shimada). After being washed in tween tris buffered saline (TTBS) (20 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20), the blots were incubated with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The blots were then reblotted with a monoclonal anti–tubulin antibody in order to normalize ECE-1 levels. The immunoreactive bands were visualized with the supersignal detection system.

RNA isolation and northern analysis

Total cellular RNA was isolated from the BAEC with the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). For northern analysis, 10 μg per lane of total RNA were subjected to electrophoresis in 1% agarose gels containing 0.66 M formaldehyde, transferred to Hybond nylon membranes, UV crosslinked and hybridized with labelled specific bovine probes (Lopez-Ongil *et al*., 2005) in hybridization solution (50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0.5% SDS and 100 μg herring sperm DNA) at 42°C. The blots were washed at final stringency conditions of 50°C, 0.2 × SSC, 0.5% SDS for 30 min, and exposed on UV-X film for 24–48 h using intensifying screens at −80°C. The filters were stripped by boiling in 0.1% SDS solution and reprobed with a ^{32}P-labelled 18 S cDNA (5.8 kb fragment digested by *Eco*RI). The densitometric analysis of the film was performed with an image scanner using the public domain software package National Institutes of Health Image 1.55 (Bethesda, MD, USA). Levels of ECE-1 and prepro-ET-1 were normalized using 18 S expressions in the same lane and expressed in relative densitometric units with respect to the control values.

Measurement of ET-1 synthesis and ECE activity

Supernatants of confluent monolayers of BAEC were collected, lyophilized and stored at −70°C until assay. ET-1 was measured by enzyme-linked immunosorbent assay

(ELISA) according to the kit instructions. Membrane proteins of confluent monolayers of BAEC were homogenized in 1 ml of homogenization buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM PMSF, 20 μM leupeptin, 20 μM aprotinin) using a Polytron homogenizer (Lopez-Ongil *et al*., 2000). The homogenates were centrifuged at 100 000 *g* for 45 min. The resultant membranes were washed three times by resuspension in homogenization buffer and recentrifuged after homogenization using a teflon/glass homogenizer. The protein concentration in each supernatant was determined using the Bio-Rad Protein Assay kit. Then 30 μg of this homogenate was incubated with bovine big ET-1 (100 ng) for 4 h at 37°C, in 250 μl of a reaction mixture containing 50 mM Tris-HCl buffer pH 7 (Ohnaka *et al*., 1990). The reaction was stopped by adding 600 μl of cold ethanol (−20°C). After centrifugation at 10 000 *g* for 10 min, supernatants were lyophilized. The dry residues were reconstituted with assay buffer, and ET-1 production in each sample was measured using ELISA.

Measurement of big endothelin-1 in cells and incubation media
Confluent monolayers of BAEC were treated with different ECE-1/NEP inhibitors for 16 h at 37°C. After this period, supernatants were collected and stored at −20°C. To assess whether big ET-1 was really able to cross the cell membrane, intracellular big ET-1 was measured in cells after adding CGS-26303 and exogenous big ET-1 for 16 h at 37°C. Afterwards, cells were sonicated in order to release the intracellular big ET-1. A commercial ELISA measured big ET-1 using a 96-well microtitre plate reader. To generate a standard curve for big ET-1, serial dilutions of big ET-1 stock ranging from 0.625–10 fmol ml^{-1} were used. A 4PL algorithm curve was automatically fitted to the standard and unknown values interpolated from the standard curves.

Transfection of BAEC with promoter/reporter constructs
We used the polymerase chain reaction (PCR) of HeLa cell genomic DNA to create the human ECE-1 α gene promoter with the Advantage Genomic PCR kit. Promoter was subcloned in the *Xho*I–*Hin*d III site of the pGL3 vector, upstream from a luciferase reporter gene. The plasmid was then grown in *Escherichia coli* DH5α and purified with Qiagen columns.

BAEC were grown in RPMI 1640 supplemented with 15% calf serum and antibiotics, and the cells were maintained in 5% CO_2 and plated approximately 24 h before transfection at a density of 60–80% of confluence in six-well plates with promoter/luciferase constructs. Transfections were performed by mixing 0.1 $\mu g\,\mu l^{-1}$ of plasmid DNA (pGL3-ECE-1) with 1 ng μl^{-1} of plasmid control from *Renilla* luciferase (pRL-SV40 vector) and 4 $\mu g\,ml^{-1}$ of Lipofectamine. The cells were washed with PBS 24 h after transfection, re-fed with RPMI 1640 and serum, and CGS-26303, big ET-1, or CGS-35066 was added at different times. The cells were harvested and assayed for luciferase activity using the Dual Luciferase Reporter Assay System. Luciferase activity was expressed as relative light units of plasmid DNA per milligram protein for each well.

Statistical analysis
Unless otherwise indicated, data are expressed as mean value ± s.e. mean obtained in at least three independent experiments, and are usually expressed as a percentage of the control values. Since the number of data in each distribution was never greater than 10, non-parametric statistics, in particular the Wilcoxon (two groups) or Friedman (more than two groups) tests, were selected to compare the paired results with the different experimental groups. The level of statistically significant difference was defined as $P<0.05$.

Materials
Bovine big ET-1, bovine ET-1, phosphoramidon, thiorphan, cycloheximide, actinomycin D, Triton X-100, PMSF, monoclonal anti–tubulin antibody, cell culture media RPMI 1640, calf serum, trypsin-EDTA (0.02%) and penicillin/streptomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). ET-1 (1–31) was purchased from Peptides International (Louisville, Kentuchy, USA). Dual Luciferase Reporter Assay System, pGL3 vector and pRL-SV40 vector were purchased from Innogenetics (Walkersville, MD, USA). Lipofectamine reagent was purchased from GIBCO-Invitrogen (Barcelona, Spain). Acrylamide–bisacrylamide was purchased from Hispanlab-Pronadisa (Madrid, Spain). MXB film was purchased from Kodak (Rochester, NY, USA). The supersignal detection system, secondary horseradish peroxidase-conjugated goat anti-mouse IgG, and NucleoBond PC 500 EF kits were purchased from Pierce (Rockford, USA). Protein markers, Bio-Rad protein assay kits, nitrocellulose membranes, plates and electrophoresis equipment were purchased from Bio-Rad Laboratories (Richmond, CA, USA). The endothelin-1 ELISA system, α-[^{32}P]dCTP and Hybond nylon membranes were from GE Healthcare (Buckinghamshire, UK). The Advantage Genomic PCR kit was purchased from Clontech Lab (Palo Alto, CA, USA). Qiagen columns were purchased from IZASA (GmbH, Hamburg, Germany). The big ET-1 ELISA system was purchased from BIOMEDICA (GmbH, Vienna, Austria).

Results

Effects of CGS-26303 on ECE-1
The ability of CGS-26303 to inhibit ECE activity was tested in cell extracts. When CGS-26303 was used at concentrations over 10 μM, the inhibition of the enzyme was maximal and comparable to that induced by 100 μM phosphoramidon (Figure 1a). In contrast, CGS-26303 induced a significant increase in ECE-1 protein content after 16 h, an effect that was also observed with phosphoramidon, but not with thiorphan, a specific NEP inhibitor (Figure 1b). Cells incubated with 25 μM CGS-26303 showed a time-dependent increase of ECE-1 protein content that was detectable at 4 h of treatment and maximal at 16 h (Figure 2a). A dose–response curve confirmed that the maximal effect of CGS-26303 on ECE-1 protein content took place at 25 μM, and was observable at concentrations between 5 and 50 μM (Figure 2b).

To analyse the molecular pathways involved in the CGS-26303-dependent ECE-1 upregulation, three kinds of

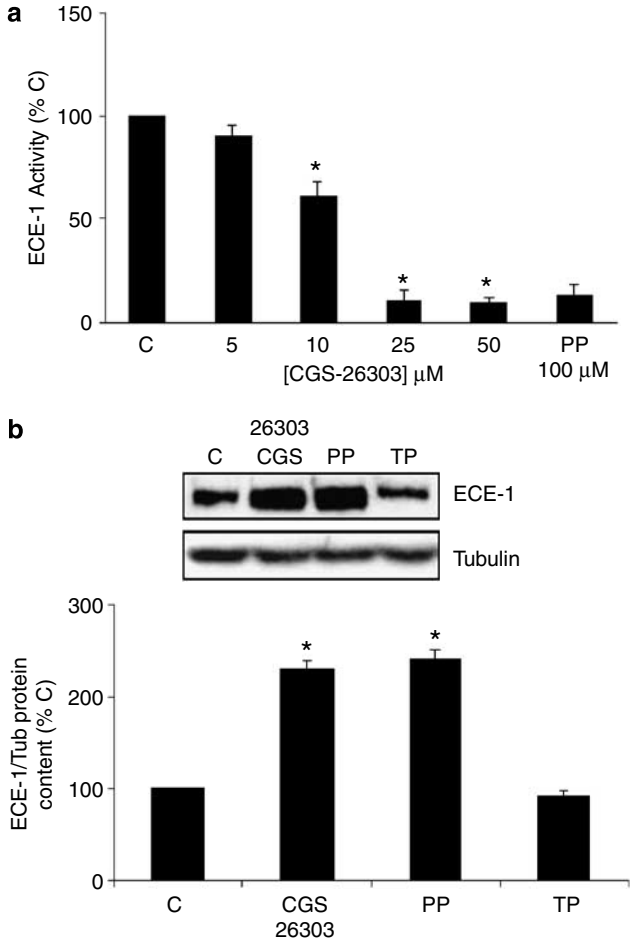

Figure 1 Effects of CGS-26303 on ECE-1 activity and ECE-1 protein content. (**a**) Cell extracts from BAEC were incubated with different concentrations of CGS-26303 for 6 h and ECE-1 activity was measured. 100 μM phosphoramidon (PP) was used as an internal control. Results are the mean value ± s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). (**b**) BAEC were incubated for 16 h with 25 μM CGS-26303, 100 μM PP, and 10 μM thiorphan (TP). A representative western blot is shown in the upper part of the panel, whereas in the lower part, the densitometric analysis of three independent experiments is shown (mean value ± s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PP, phosphoramidon; TP, thiorphan.

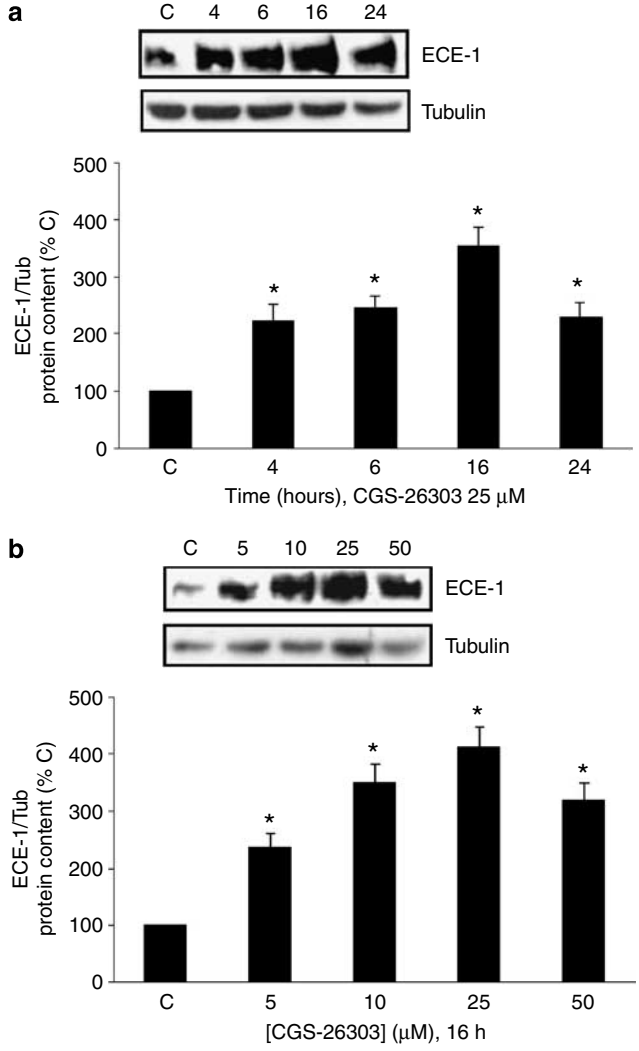

Figure 2 Effect of CGS-26303 on ECE-1 protein content. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**), or with different concentrations of CGS-26303 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part, the densitometric analysis of five independent experiments is shown (mean value ± s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1.

experiments were performed. First, the importance of *de novo* synthesis of proteins was tested using cycloheximide. As shown in Figure 3a, incubation with cycloheximide abolished the stimulation of ECE-1 induced by CGS-26303. The effects of CGS-26303 on ECE-1 mRNA content in BAEC were then considered. A statistically significant, dose–response induction of ECE-1 mRNA was elicited with CGS-26303 treatment (Figure 3b). This mRNA increase was not due to mRNA stabilization, as ECE-1 mRNA expression levels were comparable in cells treated with and without CGS-26303, when mRNA synthesis was blocked with actinomycin D (Figure 3c). Finally, the drug induced a time- and dose-dependent induction of ECE-1a promoter activity, with a similar pattern to the one observed in the mRNA ECE-1 expression (Figure 4).

To confirm the specific effect of CGS-26303 on ECE-1 upregulation, various experiments with a more selective ECE-1 inhibitor, CGS-35066, were performed. A time- (Figure 5a) and dose-dependent (Figure 5b) increase in ECE-1 protein content was also observed, appearing after 4 h of cell incubation and reaching its peak at 24 h (Figure 5a). The stimulation was maximal at 5 μM CGS-35066 (Figure 5b). Increased ECE-1 promoter activity was also observed in BAEC incubated with CGS-35066, as also happened with CGS-26303, but its maximal effect appeared at lower concentrations (Figure 5c).

Mechanisms involved in CGS-26303-induced upregulation of ECE-1

Considering the pharmacological activity of CGS-26303, there are two main mechanisms that could account for the

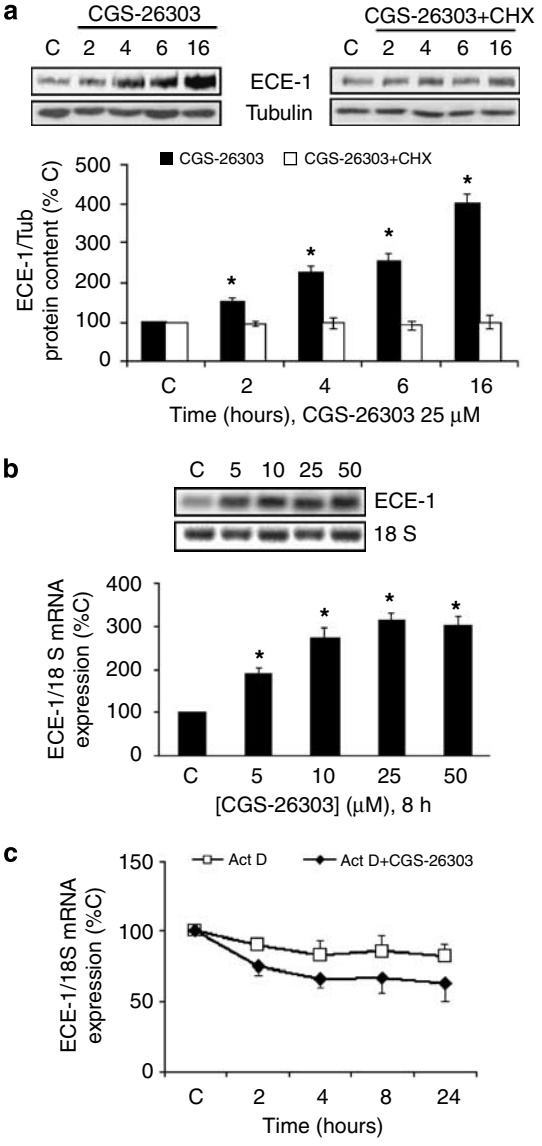

Figure 3 Mechanisms involved in the CGS-26303-dependent upregulation of ECE-1. Importance of protein synthesis, mRNA expression and mRNA stability. (**a**) BAEC were incubated with 25 μM CGS-26303 for different periods of time in basal conditions or in the presence of 1 μM cycloheximide (CHX). A representative western blot is shown above the densitometric analysis of three independent experiments (mean value $\pm$ s.e. mean). *$P<0.05$ vs control cells (C). (**b**) BAEC were incubated with different concentrations of CGS-26303 for 8 h. A representative northern blot is shown in the upper part of the panel, whereas in the lower part, the densitometric analysis of three independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control cells (C). (**c**) BAEC were incubated with actinomycin D (Act D) (10 μg ml^{-1}) with and without 25 μM CGS-26303, at different times. The mean values of mRNA levels in three independent experiments are shown (mean value $\pm$ s.e. mean). Act D alone; Act D + CGS-26303. Act D, actinomycin D; BAEC, bovine aortic endothelial cells; CHX, cycloheximide; ECE-1, endothelin converting enzyme-1.

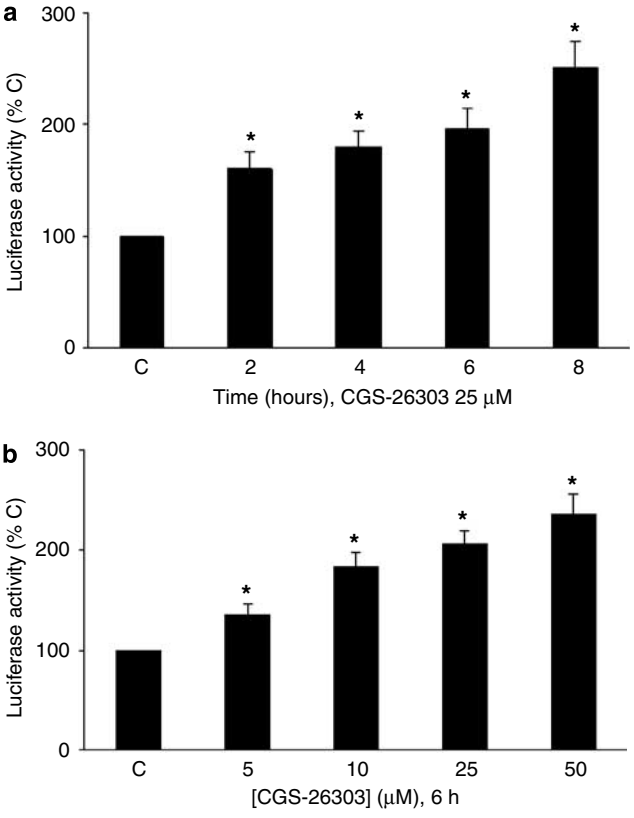

Figure 4 Mechanism involved in the CGS-26303-dependent upregulation of ECE-1. Importance of ECE-1 promoter activation. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**), or with different concentrations of CGS-26303 for 6 h (**b**). Results are the mean value $\pm$ s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with phorbol myristate acetate (PMA; 0.3 μM) for 6 h, which was used as a positive control, was 283 $\pm$ 38% ($n=4$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PMA, phorbol myristate acetate.

upregulation of ECE-1: the inhibition of the ET-1 synthetic pathway or the NEP blockade. As Figure 1b shows, NEP blockade was not the cause of this effect, since thiorphan was unable to induce any change in ECE-1 protein content. The hypothesis that lower levels of ET-1, or that the accumulation of big ET-1, could be involved in this activation, was then tested. Big ET-1 did accumulate in cell supernatants (Figure 6a) and extracts (Figure 6b) after CGS-26303 treatment. Neither exogenously added ET-1 nor the blockade of their receptors modified ECE-1 protein content (Figure 7a). Different ET-1 concentrations (5–50 nM) at different times were tested and no changes in this protein were found (results not shown). Similar results were observed with ET-1 (1–31) (Figure 7b). In contrast, cell treatment with big ET-1 induced a significantly increased amount of ECE-1 protein in cells (Figure 7c). This effect, as well as CGS-26303-dependent stimulation, continued in the presence of bosentan, the dual ET-1 receptor blocker. Moreover, the stimulation induced by big ET-1 showed a time- and dose-dependent pattern (Figure 8), appearing after 6 h of cell incubation with big ET-1 and reaching its maximum between 16 and 24 h (Figure 8a). The minimum dose of big ET-1 that elicited this stimulation was 10 nM (Figure 8b). As can be seen in Figure 9, these changes in ECE-1 protein content reflect the modulation of ECE-1a promoter activity by big ET-1. Exogenously added big ET-1 was able to cross the cell membrane, as

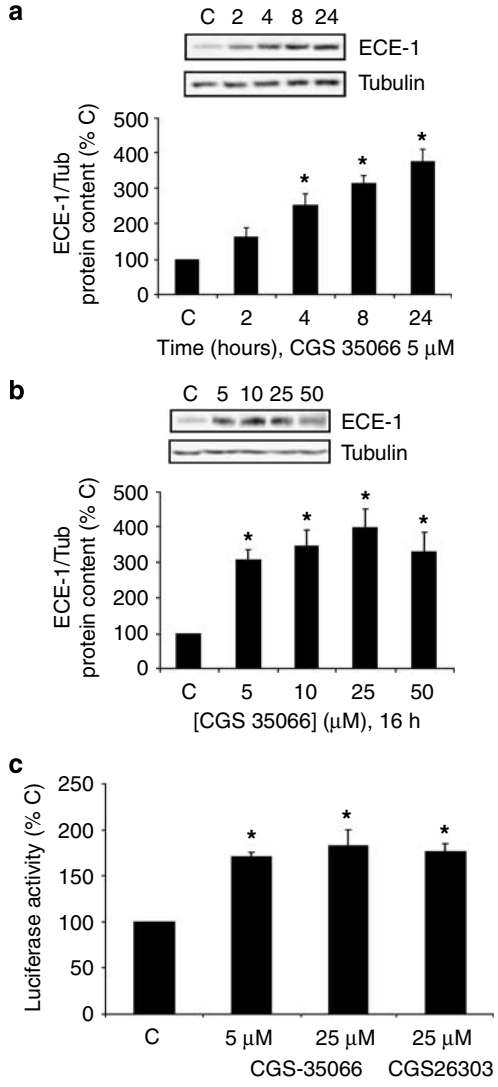

Figure 5 Effects of CGS-35066 on ECE-1 protein content and ECE-1 promoter activity. (**a**, **b**) BAEC were incubated for different periods of time with 5 μM CGS-35066 (**a**), or with different concentrations of CGS-35066 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of five independent experiments is shown (mean value$\pm$s.e. mean). *$P<0.05$ vs control values (C). (**c**) BAEC were incubated with two doses of CGS-35066 and with 25 μM of CGS-26303 for 6 h. Results are the mean value$\pm$s.e. mean of three independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with PMA (0.3 μM) for 6 h, which was used as a positive control, was 349$\pm$42% ($n=3$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PMA, phorbol myristate acetate.

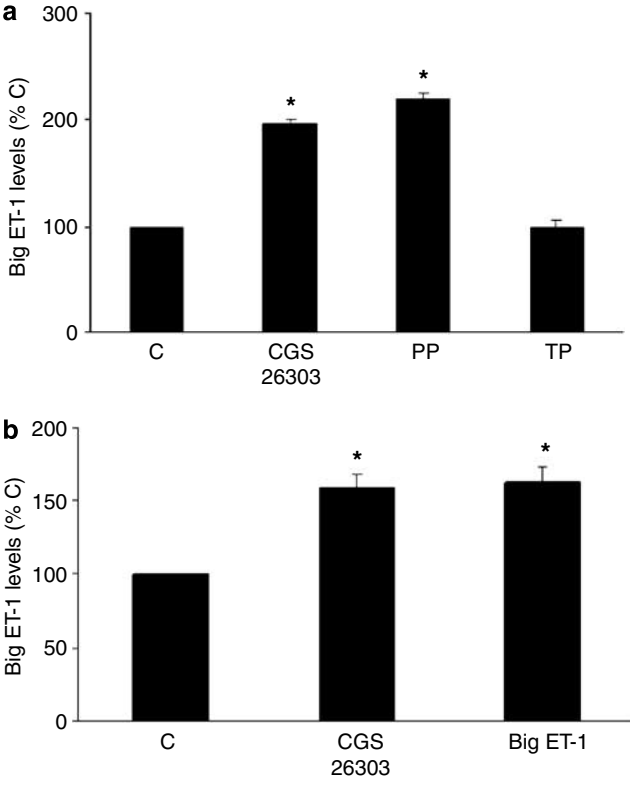

Figure 6 Effect of CGS-26303 on big ET-1 levels in cells and supernatants. (**a**) BAEC were incubated for 16 h with 25 μM CGS-26303, 100 μM phosphoramidon (PP) or 10 μM thiorphan (TP), and big ET-1 was measured in cell supernatants. Results are the mean value$\pm$s.e. mean of five independent experiments. *$P<0.05$ vs control values (C). Big ET-1 concentration was 29$\pm$3 fmol ml^{-1} in control cells. (**b**) BAEC were incubated for 16 h with 25 μM CGS-26303 and 25 nM big ET-1, and intracellular big ET-1 was measured in cells. *$P<0.05$ vs control values (C). Intracellular big ET-1 concentration was 25$\pm$4 fmol ml^{-1} mg^{-1} protein in control cells. BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; PP, phosphoramidon; TP, thiorphan.

increased concentrations of this protein were detected in cell extracts after big ET-1 incubation (Figure 6b).

The ECE-1 upregulation observed in endothelial cells treated with CGS-26303 may have relevant functional consequences. In fact, ET-1 concentration in cell supernatants after incubation for 6 h with 25 μM CGS-26303 decreased between 25 and 50% (mean inhibition 33$\pm$6%, $n=5$), a value significantly lower than the *in vitro* enzyme inhibition, which approached 100%. This dissociation may be explained by ECE-1 upregulation, since prepro-ET-1 expression was not modified by CGS-26303 treatment (Figure 10).

To study the effect of CGS-26303 on a different endothelial cell type, a human endothelial cell line (EA.hy926) was used. Cells were treated with 25 nM big ET-1, 25 μM CGS-26303 and 5 μM CGS-35066 for 16 h. Figure 11a shows the effect of these compounds on ECE-1 protein content. The stimulatory effect was similar for all of them. Moreover, lower doses of CGS-26303 were needed to induce ECE-1 protein content in human cells (Figure 11b).

Discussion

The present results clearly demonstrate that the cellular content of ECE-1 was increased by incubation with the dual ECE/NEP inhibitor CGS-26303, as well as with the more specific ECE-1 inhibitor CGS-35066, in both bovine and human endothelial cells. These inhibitors also define some of the primary mechanisms responsible for this stimulation. In short, in the presence of CGS-26303, a rapid increase in

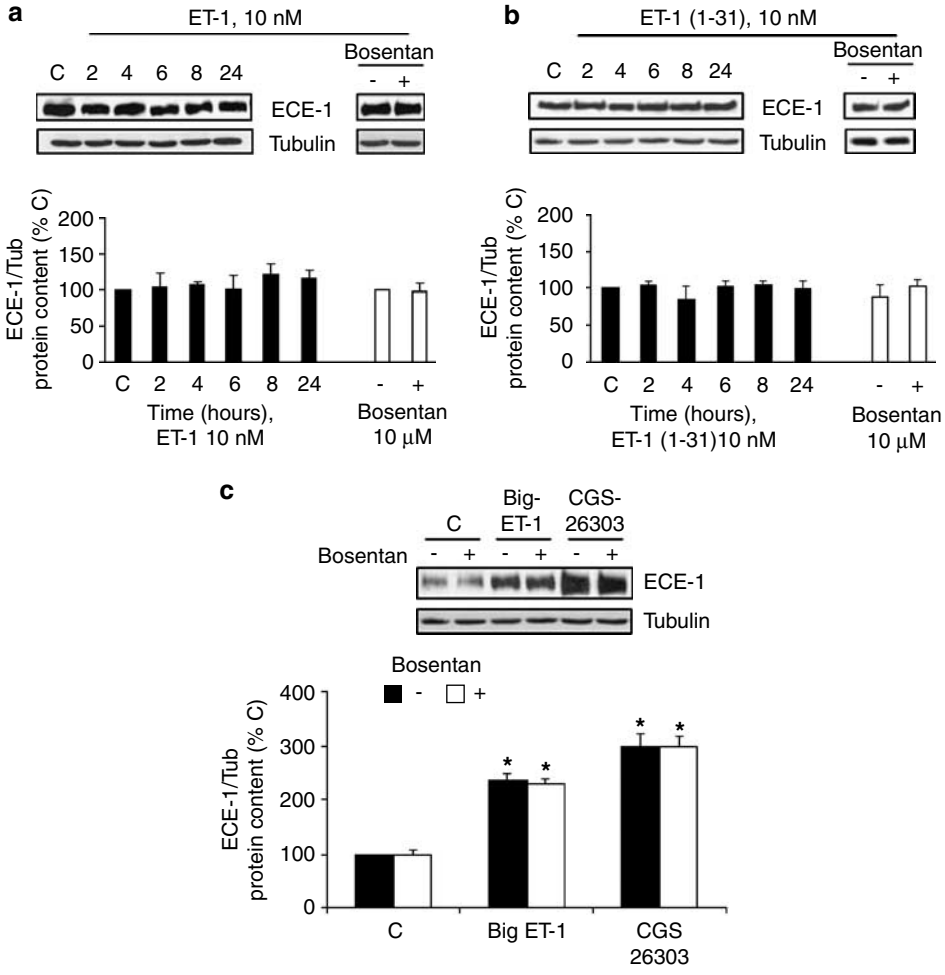

Figure 7 Role of ET-1, ET-1 (1–31) and big ET-1 in the CGS-26303-dependent ECE-1 upregulation. (**a**) BAEC were incubated for different periods of time with 10 nM ET-1 alone, or with 10 nM ET-1, in the absence or presence of 10 μM bosentan for 16 h. A representative western blot is shown in the upper part of the panel, whereas the densitometric analysis of four independent experiments is shown below (mean value $\pm$ s.e. mean). (**b**) BAEC were incubated for different periods of time with 10 nM ET-1 (1–31) alone or with 10 nM ET-1 (1–31) for 16 h, in the absence or presence of 10 μM bosentan. A representative western blot is shown in the upper part of the panel, whereas the densitometric analysis of three independent experiments is shown below (mean value $\pm$ s.e. mean). (C) BAEC were incubated for 16 h in control conditions (C), or with 25 nM big ET-1 or 25 μM CGS-26303 in the absence or presence of 10 μM bosentan. A representative western blot is shown above, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control values (**c**). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

ECE-1a promoter activity was detected, with a subsequent rise in ECE-1 mRNA steady-state levels. The translation of this mRNA determined the increase in protein content that was observed in the cells. Although alternative mechanisms such as protein or mRNA stabilization could also be proposed to explain the changes observed in ECE-1, our different experimental data, including the analysis of the steady-state mRNA levels, and the promoter activity, as well as the cycloheximide and actinomycin D studies, allow us to reasonably exclude these possibilities.

The consequences of this upregulation may be clinically relevant, since such a compensatory mechanism could induce a reduced response to enzyme inhibition after the chronic administration of an inhibitor drug. In fact, in our hands, CGS-26303 in a dose that almost completely inhibited ECE activity *in vitro* only partially decreased ET-1 synthesis in cultured cells. Although cultured cells and cellular extracts are not completely comparable when interpreting the results with drugs, these discrepancies ought to be considered when analysing the biological response to a particular treatment. Differences between the experimental approaches could also explain the apparent discrepancies between the dose-response curves shown in Figures 1 and 2. For instance, 5 μM CGS-26303 did not modify ECE-1 activity in cell extracts, but it did increase ECE-1 protein content in BAEC. In addition to differences in the types of measurements, it could be suggested that the balance between the moderate increase in ECE-1 and the presence of the inhibitor at this concentration might not lead to any changes in ECE-1 activity.

Considering the pharmacological activity of CGS-26303, at least three main mechanisms could account for the ECE-1 upregulation detected: reduced ET-1 synthesis, the accumulation of big ET-1 and the increased local concentration of

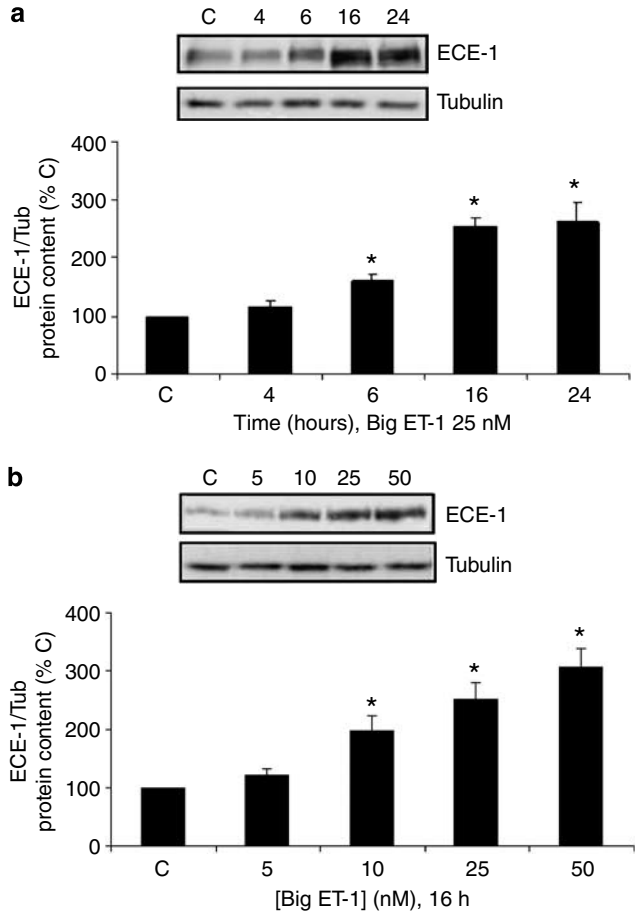

Figure 8 Effect of big ET-1 on ECE-1 protein content. BAEC were incubated for different periods of time with 25 nM big ET-1 (**a**) or with different doses of big ET-1 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value$\pm$s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

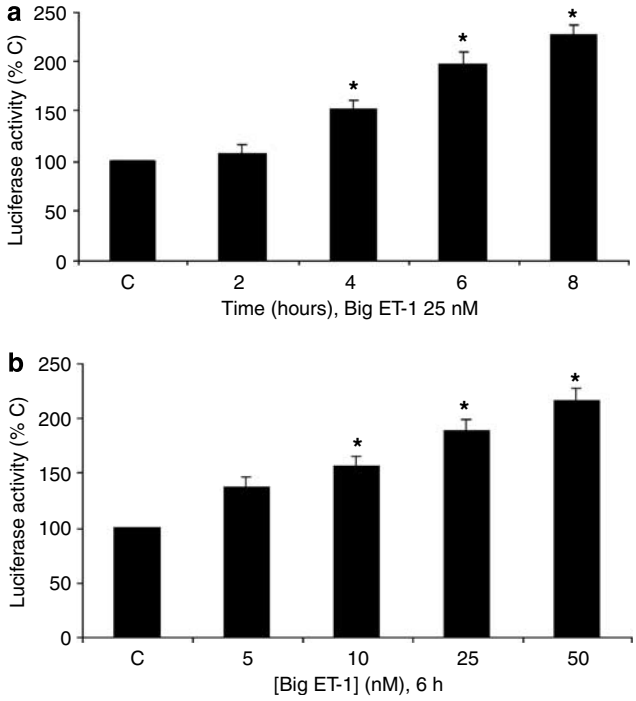

Figure 9 Effect of big ET-1 on ECE-1 promoter activity. BAEC were incubated for different periods of time with 25 nM big ET-1 (**a**) or with different doses of big ET-1 for 6 h (**b**). Results are the mean value$\pm$s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with PMA (0.3 μM) for 6 h, which was used as a positive control, was 272$\pm$25% ($n=4$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; PMA, phorbol myristate acetate.

peptides degraded by neutral endopeptidases. Reduced ET-1 synthesis, by activating a negative feedback loop, could increase ECE-1 expression. In fact, it is already known that ET-1 downregulated ECE-1 gene expression in cultured rat pulmonary endothelial cells (Naomi *et al.*, 1998). In our cells, however, the results obtained do not support this hypothesis; neither cell incubation with ET-1 at different doses nor the blockade of the ET-1 receptor with bosentan (10 μM) modified ECE protein content in endothelial cells. The differences between our results and those of Naomi *et al.* (1998) could be attributed to a difference in species or ECE-1 isoforms. Similar results were obtained with ET-1 (1–31). In contrast, when endothelial cells were incubated directly with big ET-1 at different doses, a significant increase in ECE-1 protein content, as well as in ECE-1a promoter activity, was detected, suggesting that the accumulation of this substrate was the main factor in the up-regulation of ECE-1. Neither the thiorphan experiments nor the results obtained with CGS-35066, a specific ECE blocker, support a role for NEP inhibition in the genesis of the observed effects.

The mechanism by which big ET-1 modulates ECE-1 synthesis was not extensively explored; however, some indirect data allows hypotheses to be made about this mechanism. The effect of big ET-1 was not blocked by high concentrations of bosentan, suggesting that it is not dependent on the synthesis of new ET-1 or the interaction of this molecule with ET-1 receptors. Moreover, the big ET-1-dependent upregulation of ECE-1 was first observed after 6 h of incubation, whereas upregulation induced by CGS-26303 was already clear after 4 h of incubation. This would suggest that the exogenous big ET-1 must be transferred to the intracellular compartment in order to elicit its actions. At this point, results concerning the direct measurement of big ET-1 concentrations in cells and supernatants must be considered. These experiments were performed for two reasons. First, it was necessary to confirm the ability of CGS-26303 to induce big ET-1 accumulation. Big ET-1 concentration was significantly higher in cell extracts and supernatants after incubation with CGS-26303, probably reflecting the accumulation that takes place in the intracellular compartments after ECE-1 inhibition and the subsequent release in the culture media. Second, to demonstrate that exogenously added big ET-1 moves through the cell membrane and into the intracellular compartment.

There are no previous references to the possible regulatory role of big ET-1. The possibility that big ET-1 elicits specific actions has remained almost completely unexplored. Salvati

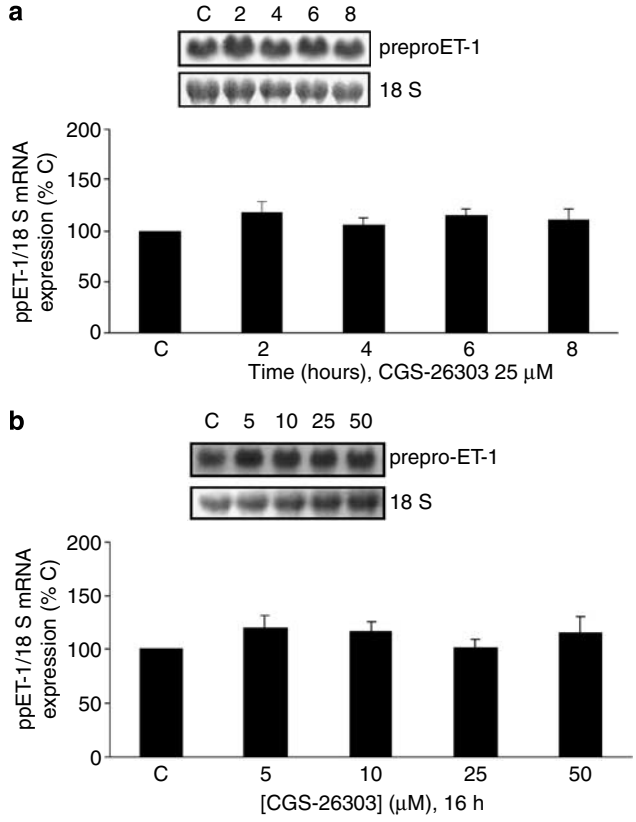

Figure 10 Effect of CGS-26303 on prepro-ET-1 (ppET-1) mRNA expression. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**) or with different concentrations of CGS-26303 for 16 h (**b**). A representative northern blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). BAEC, bovine aortic endothelial cells; ppET-1, prepro-ET-1.

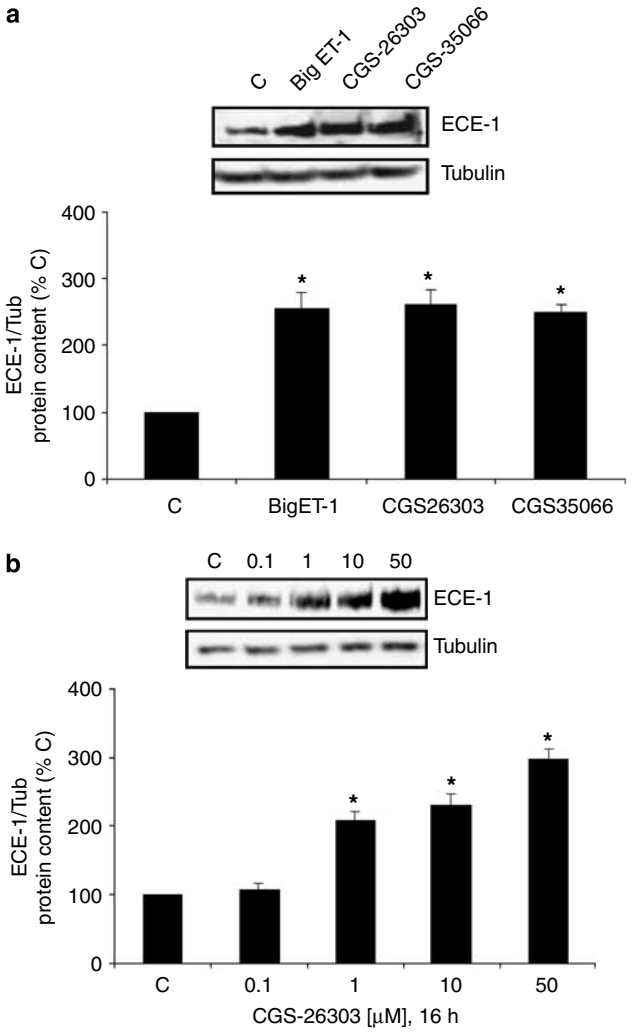

Figure 11 Effects of CGS-26303, big ET-1 and CGS-35066 on ECE-1 protein content in human endothelial cells (EA.hy926). (**a**) EA.hy926 were incubated with different compounds, 25 nM big ET-1, 25 μM CGS-26303 and 5 μM CGS-35066 for 16 h. (**b**) EA.hy926 were incubated with different doses of CGS-26303 for 16 h. A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control values (C). ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

et al. (1992) proposed a direct vasoconstrictor effect of big ET-1 on renal vascular resistance in the rat kidneys. Tirapelli *et al*. (2006) demonstrated that rabbit aortas generate ET-1 (1–31) from exogenously administered big ET-1 when ECE and NEP are inhibited by phosphoramidon. A chymase-like enzyme is probably involved in this process and synthesis of the 31-amino-acid peptide could explain the effects attributed to big ET-1. However, our results did not support that. Even so, no studies have analysed the possibility that big ET-1 acts directly in the intracellular compartment and that it regulates ECE-1 protein content.

With respect to ECE-1 upregulation by CGS-26303, previous studies have reported similar results. Thus, phosphoramidon, the first known ECE inhibitor, induced an increase in the expression levels of ECE-1a and ECE-1b, though not ECE-1c, in CHO-K1 cells (Isaka *et al*., 2003). On the other hand, the pharmacological inhibition of the angiotensin converting enzyme by lisinopril or captopril also induced the expression of this enzyme in porcine pulmonary artery endothelial cells (King and Oparil, 1992). However, these previous reports did not explore the mechanisms responsible for the effects observed. Our results stress the importance of the accumulation of substrate, in this case big ET-1, in the upregulation of the protein. Moreover, they point to a transcriptional mechanism, without changes in mRNA stability, as being primarily responsible for increased ECE-1 protein content after cell treatment with CGS-26303 or big ET-1.

Acknowledgements

We would like to thank Dr Kohei Shimada (Biological Research Laboratories, Sankyo Co. Ltd., Tokyo, Japan) for providing monoclonal antibody against bovine ECE-1 (mAb AEC32-236) and Dr Arco Y Jeng (Novartis Pharmaceuticals Corporation, USA) for providing CGS-26303 and CGS-35066. We would also like to thank Dr Edgell S Cora-Jean (Pathology, University of North Carolina, USA) for providing

the human endothelial cell line, EA.hy926. Financial support for this paper was provided by the Fondo de Investigacion Sanitaria (FIS 01/3057) and the Fundacion Mutua Madrileña (CC-FMM05). SLO received funding from FIS 01/3057, MRP and DRP from the Ministerio de Educacion y Ciencia (SAF2004-07845) and VRM from CC-FMM05.

Conflict of interest

The authors state no conflict of interest.

References

Aharinejad S, Krenn K, Paulus P, Schafer R, Zuckermann A, Grimm M *et al*. (2005). Differential role of TGF-beta1/bFGF and ET-1 in graft fibrosis in heart failure patients. *Am J Transplant* **5**: 2185–2192.

Attina T, Camidge R, Newby DE, Webb DJ (2005). Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. *Heart* **91**: 825–831.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol chloroform extraction. *Anal Biochem* **162**: 156–159.

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

Eto M, Barandier C, Rathgeb L, Kozai T, Joch H, Yang Z *et al*. (2001). Thrombin suppresses endothelial nitric oxide synthase and upregulates endothelin-converting enzyme-1 expression by distinct pathways: role of Rho/ROCK and mitogen-activated protein kinase. *Circ Res* **89**: 583–590.

Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, Kangawa K (2000). Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension* **35**: 19–24.

Isaka D, Emoto N, Raharjo SB, Yokoyama M, Matsuo M (2003). The effects of phosphoramidon on the expression of human endothelin-converting enzyme-1 (ECE-1) isoforms. *J Cardiovasc Pharmacol* **42**: 136–141.

Jeng AJ (1997). Therapeutic potential of endothelin-converting enzyme inhibitors. *Exp Opin Ther Patents* **7**: 1283–1295.

Jeng AY, De Lombaert S, Beil ME, Bruseo CW, Savage P, Chou M *et al*. (2000). Design and synthesis of a potent and selective endothelin-converting enzyme inhibitor, CGS 35066. *J Cardiovasc Pharmacol* **36**: S36–S39.

Keynan S, Khamaisi M, Dahan R, Barnes K, Jackson CD, Turner AJ *et al*. (2004). Increased expression of endothelin-converting enzyme-1c isoform in response to high glucose levels in endothelial cells. *J Vasc Res* **41**: 131–140.

King SJ, Oparil S (1992). Converting-enzyme inhibitors increase converting-enzyme mRNA and activity in endothelial cells. *Am J Physiol* **263** (Cell Physiol 32): C743–C749.

Lopez-Ongil S, Diez-Marques ML, Griera M, Rodriguez-Puyol M, Rodriguez-Puyol D (2005). Crosstalk between mesangial and endothelial cells: angiotensin II down-regulates endothelin-converting enzyme 1. *Cell Physiol Biochem* **15**: 135–144.

Lopez-Ongil S, Saura M, Zaragoza C, Gonzalez-Santiago L, Rodriguez-Puyol M, Lowenstein CJ *et al*. (2002). Hydrogen peroxide regulation of bovine endothelin-converting enzyme-1. *Free Radic Biol Med* **32**: 406–413.

Lopez-Ongil S, Senchak V, Saura M, Zaragoza C, Ames M, Ballermann B *et al*. (2000). Superoxide regulation of endothelin-converting enzyme. *J Biol Chem* **275**: 26423–26427.

Lüscher TF, Barton M (2000). Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circulation* **102**: 2434–2440.

Marsden PA, Brock TA, Ballermann BJ (1990). Glomerular endothelial cells respond to calcium-mobilizing agonists with release of EDRF. *Am J Physiol* **258**: F1295–F1303.

Masatsugu K, Itoh H, Chun TH, Saito T, Yamashita J, Doi K *et al*. (2003). Shear stress attenuates endothelin and endothelin-converting enzyme expression through oxidative stress. *Regul Pept* **111**: 13–19.

Matsuura A, Kawashima S, Yamochi W, Hirata K, Yamaguchi T, Emoto N *et al*. (1997). Vascular endothelial growth factor increases endothelin-converting enzyme expression in vascular endothelial cells. *Biochem Biophys Res Commun* **235**: 713–716.

McMahon EG, Palomo MA, Moore WM, McDonald JF, Stern MK (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 Natl Acad Sci USA* **88**: 703–707.

Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B *et al*. (2000). Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol* **525**: 761–770.

Mulder P, Barbier S, Monteil C, Jeng AY, Henry JP, Renet S *et al*. (2004). Sustained improvement of cardiac function and prevention of cardiac remodeling after long-term dual ECE-NEP inhibition in rats with congestive heart failure. *J Cardiovasc Pharmacol* **43**: 489–494.

Naomi S, Iwaoka T, Disashi T, Inoue J, Kanesaka Y, Tokunaga H *et al*. (1998). Endothelin-1 inhibits endothelin-converting enzyme-1 expression in cultured rat pulmonary endothelial cells. *Circulation* **97**: 234–236.

Niemann B, Rohrbach S, Catar RA, Muller G, Barton M, Morawietz H (2005). Native and oxidized low-density lipoproteins stimulate endothelin-converting enzyme-1 expression in human endothelial cells. *Biochem Biophys Res Commun* **334**: 747–753.

Ohnaka K, Takayanagi R, Yamauchi T, Okazaki H, Ohashi M, Umeda F *et al*. (1990). Identification and characterization of endothelin-converting enzyme activity in cultured bovine endothelial cells. *Biochem Biophys Res Commun* **168**: 1128–1136.

Orzechowski HD, Gunther A, Menzel S, Funke-Kaiser H, Richter M, Bohnemeier H *et al*. (1998). Endothelial expression of endothelin-converting enzyme-1 beta mRNA is regulated by the transcription factor Ets-1. *J Cardiovasc Pharmacol* **31**: S55–S57.

Salvati P, Dho L, Calabresi M, Rosa B, Patrono C (1992). Evidence for a direct vasoconstrictor effect of bit ET-1 in the rat kidney. *Eur J Pharmacol* **221**: 267–273.

Tirapelli CR, Fecteau MH, Honore JC, Legros E, Gobeil F, D'Orleans-Juste P (2006). Enzymatic pathways involved in the generation of endothelin-1 (1–31) from exogenous big endothelin-1 in the rabbit aorta. *Br J Pharmacol* **148**: 527–535.

Turner AJ, Isaac RE, Coates D (2001). The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays* **23**: 261–269.

van der Zander K, Houben AJ, Kroon AA, de Leeuw PW (1999). Effects of brain natriuretic peptide on forearm vasculature: comparison with atrial natriuretic peptide. *Cardiovasc Res* **44**: 595–600.

Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D *et al*. (1994). ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **78**: 473–485.