British Journal of Pharmacology (2008) 153, 1420–1431
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Activation of ET_B receptors regulates the abundance of ET-1 mRNA in vascular endothelial cells

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Background and purpose: The factors that influence the cellular levels of endothelin-1 (ET-1) include transcription, mRNA localization, stability and translation, post-translational maturation of preproET-1 and degradation of ET-1. We investigated the regulation of ET-1 mRNA abundance by extracellular ET-1 in porcine aortic endothelial cells (PAECs).
Experimental approach: Passsage one cultures of PAECs were incubated in starving medium in the presence or absence of ET-1 and antagonists or pharmacological inhibitors. PreproET-1 mRNA, *endothelin-1* promoter activity, Erk and p38 MAPK activation were determined.
Key results: Exogenous ET-1 reduced cellular ET-1 mRNA content: a reduction of 10 000-fold was observed after 4 h. ET-1 simultaneously reduced the stability of ET-1 mRNA and increased the loading of RNA Polymerase II at the *endothelin-1* promoter. In the absence of exogenous ET-1, the ET_B-selective antagonist, BQ788, increased ET-1 mRNA. An ET_A-selective antagonist had no effect. ET-1 mRNA returned to control levels within 24 h. Whereas activation of p38 MAPK induced by ET-1 peaked at 30 min and returned to control levels within 90 min, Erk1/2 remained active after 4 h of stimulation. Inhibition of p38 MAPK prevented the ET-1-induced decrease in ET-1 mRNA. In contrast, Erk1/2 inhibition increased ET-1 mRNA. Similarly, inhibition of receptor internalization increased ET-1 mRNA in the presence or absence of exogenous ET-1.
Conclusions and implications: These results suggest that extracellular ET-1 regulates the abundance of ET-1 mRNA in PAECs, in an ET_B receptor-dependent manner, by modulating both mRNA stability and transcription via mechanisms involving receptor endocytosis and both ERK and p38 MAPK pathways.
British Journal of Pharmacology (2008) **153,** 1420–1431; doi:10.1038/bjp.2008.25; published online 18 February 2008

Keywords: endothelin-1; vascular endothelial cells; autocrine regulation; mRNA stability; promoter activity; ET_B receptors

Abbreviations: ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; MAPK, mitogen-activated protein kinase; MK-2, MAPK-activated protein kinase 2; MKK6, MAPK kinase 6; PAECs, porcine aortic endothelial cells; S6c, sarafotoxin 6c; TBS, Tris-buffered saline

Introduction

Endothelium-derived endothelin-1 (ET-1) promotes vasoconstriction in response to acetylcholine in human coronary arteries (Thorin, 2001) and augments myogenic responses in rabbit mesenteric arteries (Nguyen *et al*., 1999). The mechanisms underlying the regulation of ET-1 expression in the vascular endothelium are not well established. Endothelial production of ET-1 is downregulated by sustained shear stress (Busse and Fleming, 2003), and nitric oxide (NO) plays a fundamental role in this pathway (Kedzierski and Yanagisawa, 2001; Ohkita *et al*., 2002). Aside from NO and compounds that increase cGMP levels, most agents increase ET-1 production. For example, angiotensin II, oxidized low-density lipoprotein, insulin, thrombin, transforming growth factor β, tumour necrosis factor-α, verotoxin and vascular endothelial growth factor directly increase endothelial ET-1 mRNA expression (Kedzierski and Yanagisawa, 2001; Mawji and Marsden, 2003).

Endothelin receptor subtype B (ET_B), the only ET-1 receptor subtype expressed in aortic endothelial cells, are generally thought to be responsible for the clearance of circulating ET-1 (Russell and Davenport, 1999; Dupuis, 2001; Sanchez *et al*., 2002). Experimental data suggest that ET_B receptors are expressed on the luminal side of the endothelium: i.v. administration of a pharmacological concentration of ET-1 induces a transient decrease in blood pressure

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Received 31 August 2007; revised 10 December 2007; accepted 11 January 2008; published online 18 February 2008

followed by a sustained hypertension (Gratton *et al*., 2000). The initial response, which is ET_B receptor dependent and NO mediated (Gratton *et al*., 1997), is not seen if big-ET-1, the precursor peptide of ET-1, is substituted for ET-1. In such cases, only the sustained hypertensive phase is observed (Gratton *et al*., 2000). This latter observation strongly suggests that big-ET-1 is cleaved at the abluminal side of the endothelium, leading to direct stimulation of endothelin receptor subtype A (ET_A) located on the smooth muscle (Russell and Davenport, 1999). In support of these data, Wagner *et al*. (1992) demonstrated that the release of ET-1 by endothelial cells was abluminal. Blockade of ET_B receptors increases circulating levels of ET-1 (Dupuis, 2001), which increases blood pressure by over-stimulating smooth muscle ET_A receptors (Gratton *et al*., 1997). Hence, whereas ET-1 is released towards the vascular wall, luminal endothelial ET_B receptors serve to clear circulating ET-1.

A previous study, employing northern analysis, demonstrated that exogenous ET-1 reduced the steady-state level of ET-1 mRNA in primary cultures of porcine aortic endothelial cells (PAECs) (Sanchez *et al*., 2002). As ET_B receptors serve to clear circulating ET-1 and ET-1 synthesis is subject to regulation, ET_B receptors may also play a role in sensing the extracellular concentration of ET-1 and regulating the biosynthesis of ET-1 in the vascular endothelium accordingly. In this study, we tested the hypothesis that ET-1-dependent stimulation of ET_B receptors regulates the abundance of ET-1 mRNA. Our results show that, in passage one cultures of PAECs, a cascade of events initiated by ET-1-dependent ET_B receptor activation leads to a transient reduction in levels of ET-1 mRNA. This regulatory mechanism, which required internalization of ET_B receptors and activation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, lead to reduced stability of ET-1 mRNA and enhanced RNA polymerase II binding to the ET-1 proximal promoter region.

Materials and methods

Isolation of endothelial cells

Porcine aortic endothelial cells were isolated by enzymatic digestion using type II collagenase as previously described (Thorin *et al*., 1994). Cells were passaged only once and all data collected were obtained using passage one cultures. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10% calf serum, penicillin/streptomycin and glutamine. When the cells reached 90% confluence, the culture medium was replaced by a Dulbecco's modified Eagle's medium 'starving' medium containing antibiotics and glutamine, but no serum. After 16 h, the starving medium was replaced and the cells were employed in the different experimental protocols described below.

Stimulation of vascular endothelial cells for ET-1 mRNA quantification

Cells were stimulated in starving medium for 10, 30 min, 1, 4 or 24 h by incubating in the presence of various combinations of ET-1 (0.1 μM), sarafotoxin 6c (S6c) (1.0 μM), BQ788 (1 μM), BQ123 (1 μM), SB202190 (1 μM), PD98059 (1 μM) or dansylcadaverine (50 μM), as indicated. To terminate the incubation, culture flasks were put on ice, the medium was aspirated, cells were washed with phosphate-buffered saline and the cells were subsequently removed by scraping directly into lysis buffer. Non-viable cells detach and were removed during aspiration of the medium. Total RNA was isolated using RNeasy Mini kits (Qiagen Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions.

Stimulation of vascular endothelial cells and preparation of lysates for enzymatic assays

Cells were stimulated in starving medium for 10, 30 min, 1, 4 or 24 h by addition of ET-1 (0.1 μM), S6c (1.0 μM), BQ788 (1 μM), SB202190 (1 μM), PD98059 (1 μM) and/or dansylcadaverine (50 μM), as indicated in figure legends. To terminate the incubation, culture flasks were placed on ice, the cells were washed twice with ice-cold Tris-buffered saline (TBS) (25 mM Tris-HCl, 150 mM NaCl, pH 7.5), drained and lysed by scraping in the presence of 1.0 ml of ice-cold lysis buffer comprising 50 mM Tris-HCl (pH 7.5 at 5 °C), 20 mM β-glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 10 mM benzamidine, 0.5 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} leupeptin, 5 mM dithiothreitol, 1 μM microcystin LR and 1% (v/v) Triton X-100 (reduced). Lysates were transferred to 1.5 ml microcentrifuge tubes, extracted by mixing for 15 min at 5 °C using a clinical rotator, centrifuged at 13 000 *g* and 5 °C for 15 min and the soluble fractions were retained.

Determination of phospho-ERK1/2 MAPKs

Phospho-ERK1/2 was determined in lysates using anti-phosphoprotein-specific antisera following the procedure described by the manufacturer. Briefly, 100 μg of each lysate was resolved on 10% (w/v) acrylamide mini-gels. Following SDS-polyacrylamide gel electrophoresis, samples were transferred at 100 V and 5 °C for 90 min onto 0.2 μm reinforced nitrocellulose membranes in a buffer comprising 25 mM Tris base, 192 mM glycine and 5% (v/v) methanol. Membranes were blocked for 2 h in a solution of 5% (w/v) skimmed milk powder (Carnation) in TBS containing 0.05% (v/v) Tween-20 (TBST). Membranes were incubated with primary antibodies, diluted 1:1000 with 1% (w/v) BSA plus 0.04% (w/v) sodium azide in TBST, for 16 h at 5 °C. After washing with TBST (3 × 10 min), membranes were re-blocked for 10 min with TBST containing 5% (w/v) skimmed milk powder and then incubated in the presence of horseradish peroxidase-labelled anti-rabbit immunoglobulin (Ig), diluted 1:20 000 in blocking buffer, for 2 h at room temperature. Immune complexes were detected by the ECL western blotting detection method (Renaissance Plus; NEN Life Sciences, Boston, MA, USA) according to the manufacturer's instructions and visualized using Kodak BioMax ML film. Immunoreactive bands were digitized and quantified using Quantity One software (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Exposure times were chosen to keep within the linear range of the detection system. To re-probe for total ERK

immunoreactivity, membranes were stripped by incubating twice for 15 min at room temperature in 0.2 M NaOH, with constant mixing, followed by a brief rinse with TBST (Suck and Krupinska, 1996; Wang *et al*., 1999). Membranes were subsequently blocked and incubated with primary antibodies as described above. Control experiments determined that this stripping method effectively removed the previous primary and secondary antibodies.

ERK immune complex assays were performed as described previously (Chevalier and Allen, 2000a) with modifications. ERK was precipitated from cell lysates (100 μg) using ERK-CT antisera (0.5 μg). To reduce nonspecific activity, immune complexes were washed once with 0.5 M NaI. Finally, the kinase reaction contained 5 μg of purified myelin basic protein.

Determination of MK-2 activity

Activation of the p38 MAPK cascade was assessed by measuring the activity of MAPK-activated protein kinase 2 (MK-2), a protein kinase that is phosphorylated and activated by p38 MAPKs, as described previously (Chevalier and Allen, 2000b). Lysates (100 μg) were incubated in the presence of antibodies to MK-2 (0.5 μg), precoupled to protein A/G Sepharose, at 5 °C for 16 h. Supernatants were removed and the pellets were washed twice with 1 ml of lysis buffer and twice with 50 mM Tris-HCl (pH 7.4 at 5 °C), 150 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, 1 mM benzamidine, 1 μg ml^{-1} leupeptin, 1 mM Na_3VO_4 and 0.1% (v/v) β-mercaptoethanol. MK-2 activity was assayed using recombinant canine hsp27 (Stokoe *et al*., 1992) as a substrate. The assay was for 60 min at 30 °C in a final volume of 30 μl in the presence of 50 mM Tris-HCl (pH 7.5 at 30 °C), 13 mM β-glycerophosphate, 1 μg hsp27, 10 mM $MgCl_2$, 1.3 mM EDTA, 2 mM EGTA, 1.0 mM Na_3VO_4, 10 μM [γ-^{32}P]ATP (33 Ci mmol^{-1}), 1 μM cAMP-dependent protein kinase inhibitor peptide, 10 μg ml^{-1} leupeptin and 10 mM dithiothreitol. Reactions were initiated by the addition of assay media and terminated by the addition of 10 μl of 4 × Laemmli sample buffer for a final volume of 40 μl. Samples were heated to 70 °C for 90 s and then 35 μl was applied to 10–20% (w/v) acrylamide-gradient SDS-polyacrylamide gel electrophoresis gels. Gels were stained in 45% (v/v) denatured ethanol, 10% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250 and diffusion destained in 20% (v/v) denatured ethanol containing 5% (v/v) acetic acid. Destained gels were dried between two sheets of cellophane (BioDesign Inc.) and exposed to Kodak BioMax MR film at −80 °C in cassettes fitted with Kodak TranScreen-HE intensifying screens. Following autoradiography, gels were exposed to molecular imaging screens for 16 h and ^{32}P incorporation into hsp27 was digitized and quantified by phosphor imaging (Bio-Rad GS 525 Molecular Analyzer) and expressed as pixel density × mm^2 × 10^2.

Competitive reverse transcriptase-PCR

Competitive reverse transcriptase (RT)-PCR, conducted to quantify ET-1 mRNA, was performed as described previously (Wang *et al*., 1998). An RNA mimic, used as an internal standard, had gene-specific primer pairs (forward and reverse) for the ET-1 mRNA at both ends and a 460-bp fragment of rabbit cardiac α-actin in the middle. For reverse transcription, a series of serial 10-fold dilutions of the RNA mimic were prepared, combined with 1 μg of sample RNA extracted from control or treated endothelial cells and denatured at 65 °C for 15 min. RT reactions (20 μl) were performed in a reaction mixture containing 1 × reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 1 mM of dNTPs, 3.2 μg of random primers, 5 mM dithiothreitol, 50 U RNase inhibitor and 200 U of M-MLV RT. The reaction mixtures were incubated at 42 °C for 60 min, for first-strand cDNA synthesis, and then heated to 99 °C for 5 min to inactivate the RT. PCR amplification was performed using oligonucleotide primers specific for porcine ET-1 mRNA (sense: 5′-ATGGATTATTTCCCCATGATTATCG-3′, nucleotides 75–99; antisense: 5′-TCAGTGTGTTCGGTTATGGGTCA-3′, nucleotides 690–714). These two primers define a specific 640-bp fragment within the preproET-1 mRNA. For each PCR reaction, 5 μl of first-strand cDNA template and 0.5 μM of each primer were combined in 20 μl of reaction mixture (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs and 2.5 U of Taq polymerase). Reactions were started with an incubation at 94 °C for 3 min (initial melt); then 30 cycles of the following sequential steps: 94 °C for 30 s (melt); 54 °C for 30 s (anneal) and 72 °C for 30 s (extension). A final incubation was performed at 72 °C for 5 min (final extension). The products of each PCR reaction were separated on 2% agarose gels, stained with ethidium bromide and visualized under u.v. light. Ethidium bromide fluorescence was digitized using a NightHawk imaging system, equipped with a charge-coupled device camera, and analysed using Quantity One software (Bio-Rad Laboratories). The mass of the amplified mimic and preproET-1 mRNA fragment DNA was calculated using the DNA mass ladder (1-Kb ladder, Stratagene, La Jolla, CA, USA, 1 μg loaded) as a mass standard.

Quantitative real-time PCR

For quantification of porcine ET-1 mRNA (NM_213882.1), the following primer set was designed (+495 to +555 relative to conserved (human) transcription start site): 5′-TGGACATCATCTGGGTCAACACT-3′ and 5′-GAAGGGCTTC CAAGTCCGTATG-3′. Relative fold change of ET-1 mRNA was determined by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) using an exogenous, *in vitro* transcribed, capped, polyadenylated luciferase RNA (0.25 ng) spiked into the cellular lysate to control for the efficiency of the RNA extraction procedure and first-strand cDNA synthesis steps. The utility of an *in vitro* synthesized reference RNA for real-time RT-PCR data normalization has previously been described (Gilsbach *et al*., 2006).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP Assay Kit (Millipore, Billerica, MA, USA, 17–295) as previously described (Fish *et al*., 2005). Briefly, ~0.5 × 10^6 cells were used per immunoprecipitation.

Crosslinking of proteins to DNA was performed by the addition of formaldehyde directly to the culture medium (final concentration, 1%) and incubation at 37 °C for 10 min. Crosslinked DNA was sheared on ice to chromatin fragments (200–400 bp) using a Vibra-Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA) equipped with a 3 mm tip at 30% maximum power (5×10 s pulses interrupted by 4×10 s pauses). Nonspecific background was reduced by pre-clearing with 80 μl of protein A agarose/salmon sperm DNA (50% slurry) for 2 h at 4 °C with agitation. Immunoprecipitations were performed in parallel overnight at 4 °C with rotation using 5 μg of anti-RNA polymerase II antibody, 5 μg of normal rabbit IgG, or a no antibody control, respectively. After immunoprecipitation, immune complexes were collected with 60 μl of protein A agarose/salmon sperm DNA (50% slurry) for 2 h at 4 °C with agitation. To recover the immunoprecipitated DNA fragments, pelleted beads were washed, crosslinks were reversed and a proteinase K digestion was performed prior to phenol/chloroform extraction. Recovered DNA was resuspended in a final volume of 40 μl of water prior to quantification by real-time PCR. An input control was collected immediately prior to immunoprecipitation.

Quantification by real-time PCR was performed on 2 μl of immunoprecipitated DNA using an Applied Biosystems Prism 7900HT Sequence Detection System and SYBR Green universal PCR master mix (Applied Biosystems, Foster City, CA, USA). Triplicate measurements were performed for each sample in a 10 μl final reaction volume with the following cycling parameters: 95 °C for 10 min, followed by two-step PCR for 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time PCR primers were designed with Oligo primer analysis software (v6.7; Molecular Biology Insights, Cascade, CO, USA) to computationally assess duplex and hairpin formation, as well as optimal primer internal stability. A BLAST search against the non-redundant database (http://www.ncbi.nlm.nih.gov/BLAST/) and melting curve analysis were used to confirm PCR product specificity. Optimal primer concentrations, that is, those that produced maximum product yield without formation of nonspecific products, were determined empirically using a standard primer matrix. For quantification of the porcine ET-1 proximal promoter immunoprecipitated by ChIP, characterization of the porcine ET-1 5′-regulatory region (AC092875.2) was first performed by multiple sequence alignment of the corresponding human, mouse, rat and cow nucleotide sequences using the Jalview alignment editor (Clamp *et al*., 2004). The following porcine ET-1 proximal promoter primer set was designed (−121 to +1 relative to conserved (human) transcription start site): 5′-GCCTGG TGGTGACTAATGACACA-3′ and 5′-TGACCTGGACAGCTC TCTCGG-3′. Anti-polymerase II immunoprecipitated target sequence copy numbers were determined by comparison of C_t values with a pig genomic DNA standard curve, correction for target sequences nonspecifically immunoprecipitated in control reactions (similar results were obtained with both normal rabbit IgG and no antibody controls) and normalization to diluted input controls, as previously described (Fish *et al*., 2005). Data represent the mean and s.e. ($n = 3$, biological replicates).

Miscellaneous methods

Protein concentrations were determined by the method of Bradford (1976) using γ-globulin as standard. The mass of each amplified mimic and ET-1 mRNA fragment DNA was calculated from their fluorescence intensity using the DNA mass ladder to generate a standard curve of mass versus fluorescence using a nonlinear curve-fitting program (GraphPad Prism version 4b for Mac) and extrapolating to zero. The half-life for ET-1 mRNA was determined by fitting to a single exponential function (GraphPad Prism).

Data analysis

Data are shown as the mean ± s.e. mean. Statistical analyses of real-time PCR cDNA and ChIP data were performed using a Student's *t*-test and analysis of variance (one-way ANOVA, Bonferroni correction), respectively. Otherwise, the significance of differences between groups was estimated by one- or two-way ANOVA followed by Bonferroni post-tests (GraphPad Prism version 4c for Mac). Differences were considered significant when $P < 0.05$.

Materials

[γ-^{32}P]ATP was from GE Healthcare (Baie d'Urfe, Quebec, Canada). Membrane grade (reduced) Triton X-100, leupeptin and phenylmethylsulphonyl fluoride were from Roche Molecular Biochemicals (Laval, Quebec, Canada). ET-1, S6c, BQ123 and BQ788 were from American Peptide Co., (Sunnyvale, CA, USA), Reagents for SDS-polyacrylamide gel electrophoresis and Bradford protein assays were from Bio-Rad Laboratories. Microcystin LR was from Calbiochem (Mississaga, Ontario, Canada). The specific p38 MAPK inhibitor, SB202190, was from Calbiochem. cAMP-dependent protein kinase inhibitor peptide (amino-acid sequence TTYADFIASGRTGRRNAIHD) was from the University of Calgary Peptide Synthesis Core Facility. Recombinant canine hsp27, cloned into the pET24a expression vector (Larsen *et al*., 1995), was a kind gift from Dr William Gerthoffer, Reno, NV. Myelin basic protein was purified from bovine brain as described previously (Chevalier and Allen, 2000a). Anti-ERK1-CT antisera, which recognizes both ERK1 and ERK2, was from Stressgen (Victoria, BC, Canada). Antisera for phospho-ERK1/2 (9101), specific for the dually phosphorylated form of the T-E-Y motif, and normal rabbit IgG (12-370) were from Millipore. Antibodies to MK-2 (sc-6621) and RNA polymerase II antibody (sc-899) were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Landrace male swine were obtained from JP Dion and Sons farm (Saint-Liboire, Quebec, Canada). All other reagents were of analytical grade or best grade available. NanoPure grade water was used throughout these studies.

Results

Effect of ET-1 on ET-1 mRNA levels

Using northern analysis it had been shown that exogenous ET-1 depressed steady-state levels of ET-1 mRNA in passage

one PAECs (Sanchez *et al*., 2002). Steady-state levels of ET-1 mRNA are a product of both transcription and mRNA degradation. In the present study, competitive RT-PCR was employed to quantify changes in ET-1 mRNA. ET-1 mRNA was present at 15.2 ± 0.5 fmol μg^{-1} total RNA in passage 1 PAECs. These cells secrete ET-1 in a constitutive manner (Sanchez *et al*., 2002) and BQ788 (1 μM), an ET_B-receptor selective antagonist, increased ET-1 mRNA with a maximum of 700 ± 18 fmol μg^{-1} detected after 4 h of exposure (Figure 1). PAECs do not express ET_A receptors and, consistent with this, BQ123 (1 μM), an ET_A receptor-selective antagonist had no effect on ET-1 mRNA levels (data not shown). A single application of ET-1 (0.1 μM) led to a profound decrease in ET-1 mRNA (Figures 1a and b), which declined to 2.36 ± 0.48 amol mg^{-1} total RNA 4 h after ET-1 administration and returned to baseline levels within 24 h. The IC_{50} for the suppression of ET-1 mRNA by exogenous ET-1 was 1.1 ± 0.9 nM ($n = 3$; data not shown), which is similar to the K_i measured previously in intact cardiac myoyctes (Allen *et al*., 2003). BQ788 (1 μM) did not significantly block the effect of 0.1 μM exogenous ET-1 (data not shown): this may result from differences in the dissociation of BQ788 versus ET-1 from ET_B receptors (Allen *et al*., 2003). The ET-1-mediated decrease in ET-1 mRNA abundance was also verified by real-time PCR (not shown). Furthermore, S6c (1.0 μM), a selective ET_B receptor agonist, mimicked the effects of ET-1 on ET-1 mRNA levels (Figure 2). The decrease in ET-1 mRNA was not a result of cell death, as dead cells detach and were washed away prior to lysis and RNA isolation.

To test the possibility that extracellular ET-1 altered the stability of ET-1 mRNA in PAECs, the effect of extracellular

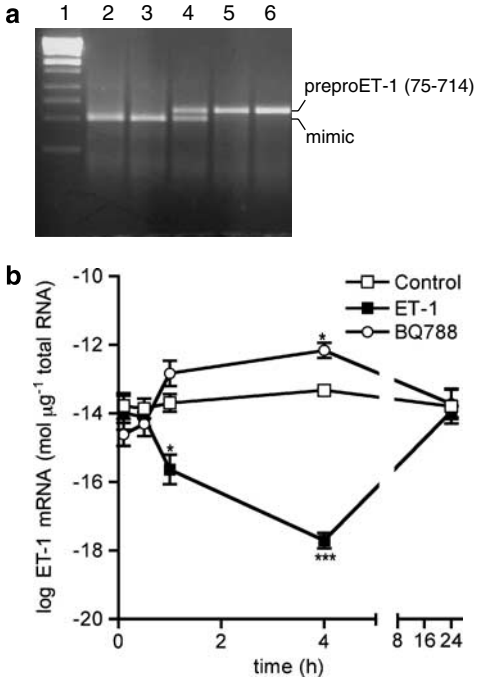

Figure 1 Exogenous endothelin-1 (ET-1) decreases ET-1 mRNA levels. Total mRNA was isolated from porcine aortic endothelial cells (PAECs) and ET-1 mRNA quantified by competitive reverse transcriptase (RT)-PCR. (**a**) Total RNA (1 μg) was combined with 20 ng (lane 2), 2 ng (lane 3), 200 pg (lane 4), 20 pg (lane 5) or 2 pg (lane 6) of RNA mimic. RT-PCR reactions were performed as described under 'Materials and methods'. The products of RT-PCR were separated on 2% agarose gels, visualized using u.v. light plus ethidium bromide, emitted fluorescence was digitized using a NightHawk imaging system and analysed using Quantity One software (Bio-Rad Laboratories). DNA mass ladder is shown in lane 1. Bands corresponding to the amplified mimic and preproET-1 (75–714) are indicated. (**b**) PAECs were incubated for 16 h in serum-free media and then in the absence (control) or presence of 0.1 μM ET-1 or 1.0 μM BQ788 for the indicated times. The values shown are the means (± s.e.mean) of four separate experiments employing four different preparations of endothelial cells. $^*P<0.05$ versus control; $^{***}P<0.001$ versus control (one-way ANOVA, Bonferroni post-tests).

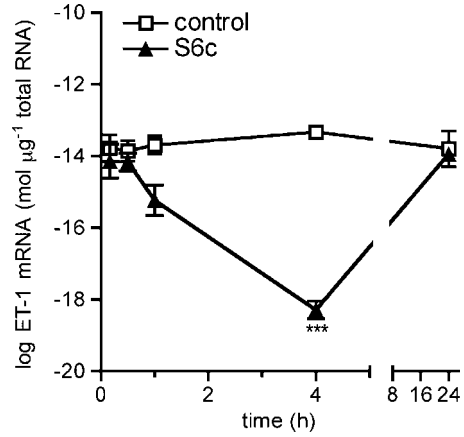

Figure 2 Endothelin receptor subtype B (ET_B) receptor-selective agonist S6c decreases endothelin-1 (ET-1) mRNA levels. Porcine aortic endothelial cells (PAECs) were incubated for 16 h in serum-free media and then in the absence (control) or presence of 1 μM sarafotoxin 6c (S6c) for the indicated times. The values shown are the means (± s.e.mean) of four separate experiments employing four different preparations of endothelial cells. $^{***}P<0.001$ versus control (one-way ANOVA, Bonferroni post-tests).

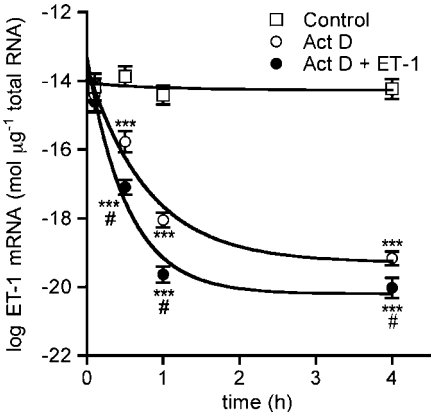

Figure 3 Exogenous endothelin-1 (ET-1) increases the degradation of ET-1 mRNA. Serum-starved porcine aortic endothelial cells (PAECs) were incubated in media (control), or in the presence of 50 μg/ml actinomycin D alone (Act D) or supplemented with 0.1 μM ET-1 (Act D + ET-1) for the indicated times. Total mRNA was isolated and preproET-1 mRNA was quantified as described in the legend of Figure 1. The values shown are the means (± s.e.mean) of four separate experiments employing four different preparations of endothelial cells. $^{\#}P<0.05$ versus actinomycin D. $^{***}P<0.001$ versus control (one-way ANOVA, Bonferroni post-tests).

ET-1 was examined following actinomycin D-induced transcriptional arrest. ET-1 mRNA is labile (Inoue *et al*., 1989) and, hence, in the presence of actinomycin D, the ET-1 mRNA content decreased rapidly (Figure 3) with an observed half-life of 33 min. Both the rate and magnitude of this decrease were enhanced when PAECs were incubated in the presence of actinomycin D plus ET-1, shortening the observed half-life of ET-1 mRNA to 22 min. However, actinomycin D affected cell viability, reflecting the stress such treatment imposed upon the cells, and thus the half-life

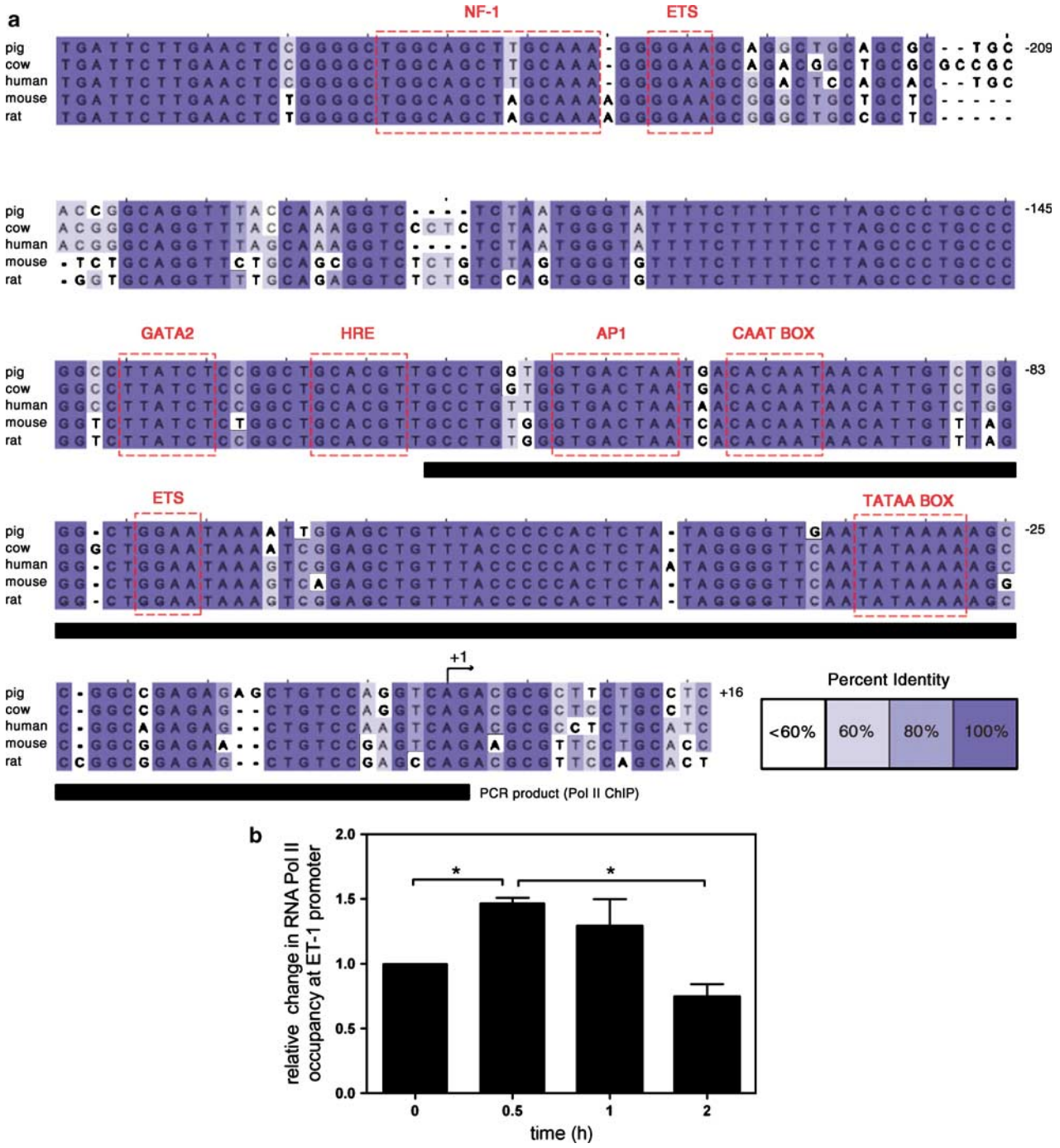

Figure 4 Exogenous endothelin-1 (ET-1) paradoxically increases the transcription of the ET-1 gene. Chromatin immunoprecipitation (ChIP) with anti-RNA polymerase II antibody was used to assess transcription of the ET-1 gene. (**a**) To characterize the pig ET-1 proximal promoter, a multiple sequence alignment of pig, cow, human, mouse and rat ET-1 5′-regulatory regions was performed using the Jalview alignment editor. Alignment gaps are indicated by dashes. Percent sequence identity across species is highlighted by shading. Conserved, functionally relevant *cis*-regulatory elements are outlined by boxes. The PCR product amplified by pig ET-1 ChIP primers (−121 to +1) is identified by a solid black line. Numbering is relative to the conserved, human transcription start site. (**b**) Porcine aortic endothelial cells (PAECs) were serum starved for 16 h prior to treatment with 0.1 μM ET-1 for the indicated times. The relative change in RNA polymerase II occupancy at the ET-1 promoter was determined after correction for nonspecific binding using a normal rabbit immunoglobulin (Ig) G control immunoprecipitation and normalization to input DNA. Means (± s.e.mean; $n=3$, biological replicates) are shown. *$P<0.05$, (one-way ANOVA, Bonferroni post-tests).

for ET-1 mRNA observed in the presence of actinomycin D may differ from the true value. Thus, binding of ET-1 to ET_B receptors resulted in reduced levels of ET-1 mRNA and this decrease in message levels involved a reduction in mRNA stability.

Extracellular ET-1 reduced ET-1 mRNA levels; however, this effect was transient and mRNA abundance returned to control levels within 24 h. This suggests extracellular ET-1 may also regulate *endothelin-1* (*Edn1*) promoter activity. RNA polymerase II ChIP (RNA Pol ChIP) assays revealed that ET-1 increased the loading of RNA polymerase II (Figure 4b) onto the *Edn1* proximal promoter region (Figure 4a). Thus, extracellular ET-1 altered *Edn1* transcription as well as mRNA stability.

ET-1-dependent activation of p38 and ERK1/2 MAPK pathways

The ERK and p38 MAPK pathways have been implicated in regulating gene expression (Edmunds and Mahadevan, 2004) and mRNA stability (Kotlyarov and Gaestel, 2002; Numahata *et al*., 2003; Chrestensen *et al*., 2004). Hence, we sought to determine if activation of ET_B receptors induced activation of either the ERK or p38 MAPK pathway with a time course similar to the reduction in ET-1 mRNA levels. In these studies, activation of the ERK1/2 MAPK cascade was assessed by determining changes in the level of ERK1/2 phosphorylation using a commercially available phospho-specific antibody. ERK activity was confirmed by immune complex assay wherein ERK1/2 was immunoprecipitated with a specific antibody and myelin basic protein was employed as phosphate acceptor. Activation of the p38 MAPK cascade was determined by measuring the activity of MK-2, which is phosphorylated and activated by p38 MAPKs and hence reflects activation of the p38 cascade (Chevalier and Allen, 2000b). Addition of exogenous ET-1 (0.1 μM) or S6c (1 μM) to PAECs increased the activity of both the p38 and ERK MAPK pathways, but activation of the signalling cascades demonstrated significantly different time courses. Activation of MK-2 by ET-1 (Figure 5a) or S6c (Figure 5b) was maximal at 30 min and had returned to control levels within 4 h. BQ788, which had no intrinsic effect upon MK-2 activity (data not shown), prevented its activation in response to exogenous ET-1 or S6c. This contrasts with the kinetics observed for activation of the ERK cascade: phospho-ERK levels, both p42 (ERK2) and p44 (ERK1), rose within 10–30 min of ET-1 (Figures 6a and b) or S6c (Figures 6c and d) administration and remained elevated 4 h after the initial application of ET-1. Immune complex assays revealed that changes in ERK activity paralleled those in ERK phosphorylation (data not shown). Furthermore, whereas BQ788 inhibited ET-1- or S6c-dependent activation of ERK1/2, BQ788 alone also increased ERK phosphorylation, albeit to a lesser extent than ET-1 or S6c.

ERK and p38 MAPK cascades are involved in regulating the levels of ET-1 mRNA

The ability of ET-1 to decrease the stability of ET-1 mRNA was blocked by SB202190 (1 μM), an inhibitor of p38 MAPK activity (Figure 7). In contrast, PD98059 (1 μM), an inhibitor of ERK1/2 (p42/44) activation, not only prevented the decrease in ET-1 mRNA levels but also induced an increase

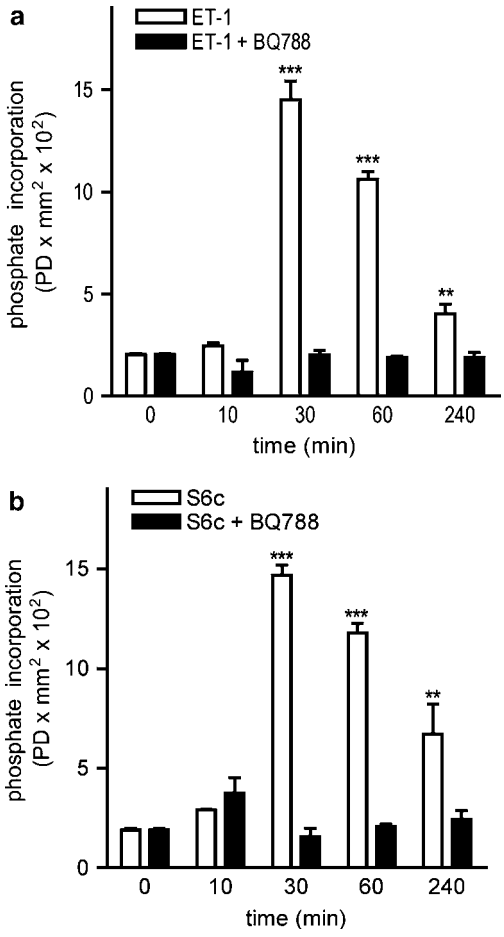

Figure 5 Endothelin-1 (ET-1) and sarafotoxin 6c (S6c) activate the p38 MAPK pathway. Serum-starved porcine aortic endothelial cells (PAECs) were incubated with (**a**) 0.1 μM ET-1 in the absence (ET-1) or presence of 1.0 μM BQ788 (ET-1 + BQ788) or (**b**) 1.0 μM S6c in the absence (S6c) or presence of 1.0 μM BQ788 (S6c + BQ788) for the indicated times. Activation of the p38 MAPK pathway was determined by measuring the activity of MK-2, which is phosphorylated and activated by p38 MAPKs, by immune complex assay. MK-2-mediated hsp27 phosphorylation was determined as described in Materials and methods. The values shown, expressed as pixel density (PD) × mm^2 × 10^2, are the means (± s.e.mean) of three separate experiments employing three different preparations of endothelial cells. ***$P<0.001$ versus BQ788; **$P<0.01$ versus BQ788 (two-way ANOVA, Bonferroni post-tests).

over control levels (Figure 7), an effect similar to that produced by BQ788 (Figure 1). In the absence of exogenously added ET-1, PD98059 had no effect on the level of ET-1 mRNA (Figure 7). SB202190 alone produced a small decrease in ET-1 mRNA to levels similar to those detected in the presence of ET-1 plus SB202190. The concentrations of PD98059 and SB101290 employed herein were sufficient to block ET-1-mediated activation of ERK and MK-2, respectively (data not shown).

Effect of ET_B receptor internalization on ET-1 mRNA

Pharmacological inhibition of the internalization of ET_B receptors with dansylcadaverine (50 μM; Sanchez *et al*., 2002) increased ET-1 mRNA in both the presence and absence of exogenously added ET-1 (Figure 8), mimicking the effects of

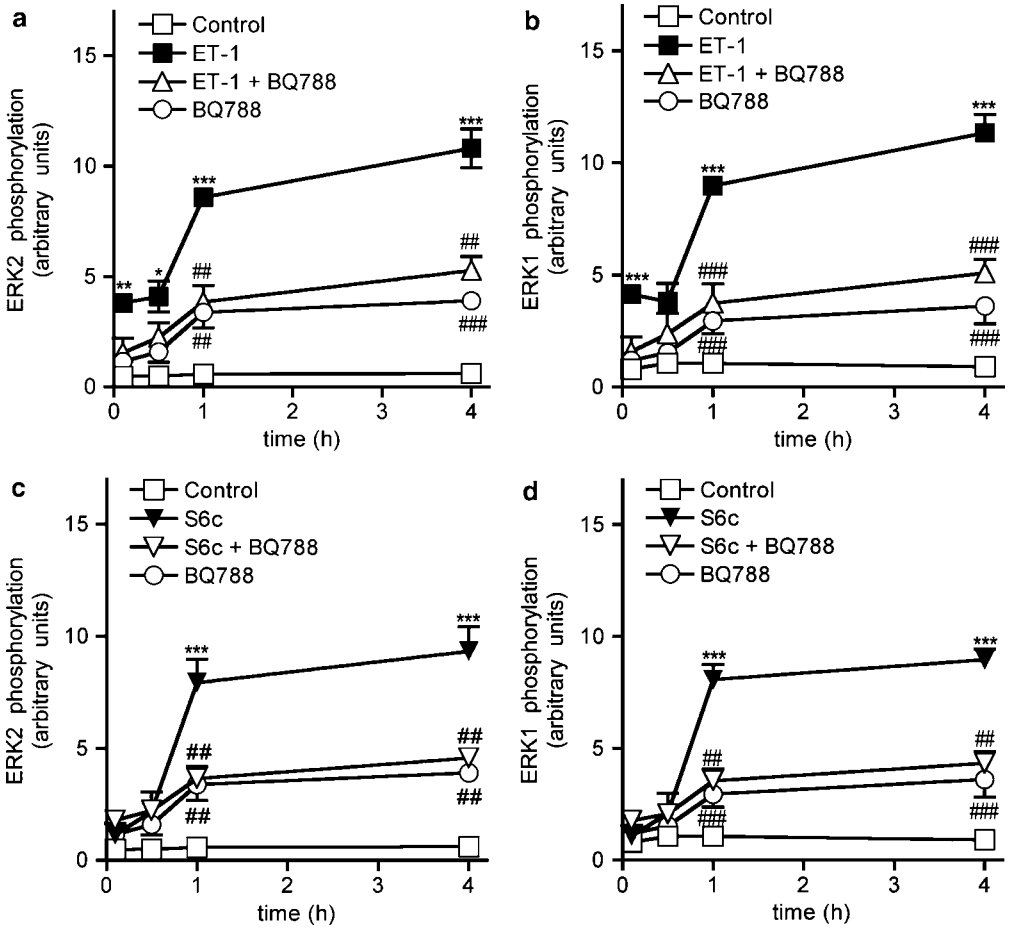

Figure 6 Endothelin-1 (ET-1) and sarafotoxin 6c (S6c) activate the extracellular signal-regulated kinase (ERK) MAPK pathway. (**a**, **b**) Serum-starved porcine aortic endothelial cells (PAECs) were incubated with media alone (control), or containing 0.1 μM ET-1, 1 μM BQ788 or 0.1 μM ET-1 plus 1 μM BQ788 for the indicated times. (**c**, **d**) Serum-starved PAECs were incubated with media alone (control), or containing 1.0 μM S6c, 1.0 μM BQ788 or S6c plus BQ788 for the indicated times. Activation of ERK2 (p42; **a**, **c**) and ERK1 (p44; **b**, **d**) was subsequently determined in cell lysates by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using phospho-specific antibodies as described under Materials and methods. The values shown are the means (±s.e.mean) of three separate experiments employing three different cell preparations. $^{*}P<0.05$ versus control; $^{**}P<0.01$ versus control; $^{***}P<0.001$ versus control; $^{\#}P<0.05$ versus ET-1 (**a**, **b**) or S6c (**c**, **d**); $^{\#\#}P<0.01$ versus ET-1 (**a**, **b**) or S6c (**c**, **d**); $^{\#\#\#}P<0.001$ versus ET-1 (**a**, **b**) or S6c (**c**, **d**) (one-way ANOVA, Bonferroni post-tests).

BQ788 (Figure 2). Under the conditions employed in these studies, dansylcadaverine inhibited the internalization of a fluorescently labelled ET_B receptor ligand, rhodamine-ET-3 (not shown). The dansylcadaverine-induced upregulation of ET-1 mRNA was prevented upon inhibition of p38 MAPK (SB202190, 1 μM) (Figure 8). In contrast, inhibiting the activation of ERK1/2 (PD98059, 1 μM) had no effect. However, it is noteworthy here that, whereas PD98059 increased ET-1 mRNA levels in the presence of ET-1 (Figure 7), in the presence of dansylcadaverine plus ET-1, PD98059 did not produce any additional increase in mRNA effect over that observed in the presence of ET-1 plus dansylcadaverine. Thus, internalization of activated ET_B receptors initiates or participates in the regulation of the abundance of ET-1 mRNA in PAECs.

Discussion and conclusions

ET-1 is a potent regulator of vascular tone in addition to having proliferative effects upon fibroblasts and vascular smooth muscle cells. Hence, regulation of ET-1 production and release, in addition to circulating levels, must be tightly controlled. Numerous factors regulate the cellular content of ET-1 mRNA, including sex steroid hormones (Wang *et al.*, 1997), transforming growth factor-β (Kurihara *et al.*, 1989), shear stress (Malek *et al.*, 1993), changes in the actin cytoskeleton (Malek *et al.*, 1997), hypoxia (Kourembanas *et al.*, 1991; Blanchard *et al.*, 1992), phorbol ester (Imai *et al.*, 1992), thrombin (Kitazumi and Tasaka, 1993), vasoconstrictors (Imai *et al.*, 1992), hypertension (Larivière *et al.*, 1995), NO (Blanchard *et al.*, 1992) and congestive heart failure (Øie *et al.*, 1997). In vascular smooth muscle cells, exogenous ET-1 induces ET-1 expression (Hahn *et al.*, 1990). As the ET_B receptors on vascular endothelial cells serve to clear circulating ET-1, these receptors are also in a key position to play a role in regulating local ET-1 biosynthesis. These cells do not express ET_A receptors. It was recently demonstrated that extracellular ET-1 reduces the cellular content of ET-1 mRNA in vascular endothelial cells (Sanchez *et al.*, 2002). However, nothing is known regarding the

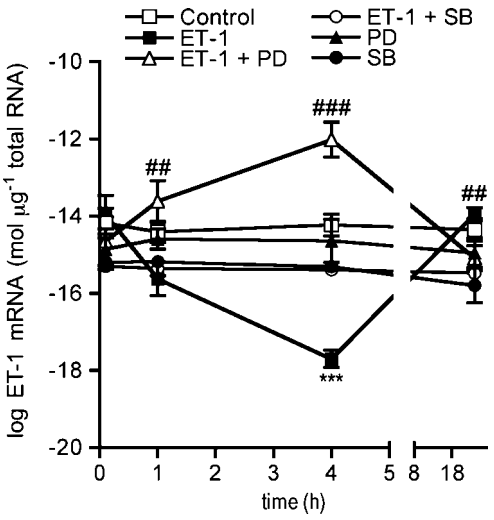

Figure 7 Activation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways is required for endothelin-1 (ET-1)-mediated decrease in ET-1 mRNA. Serum-starved endothelial cells were incubated for the indicated times in media alone (control), or supplemented with 0.1 μM ET-1, 1.0 μM SB202190 (SB) alone or in the presence of 0.1 μM ET-1, 1 μM PD98059 (PD) alone or in the presence of ET-1. Total mRNA was isolated and preproET-1 mRNA quantified as described in the legend of Figure 1. The values shown are the means (±s.e.mean) of four experiments employing four different preparations of endothelial cells. ***$P<0.001$ versus control; $^{\#\#}P<0.01$ versus ET-1; $^{\#\#\#}P<0.001$ versus ET-1 (one-way ANOVA, Bonferroni post-tests).

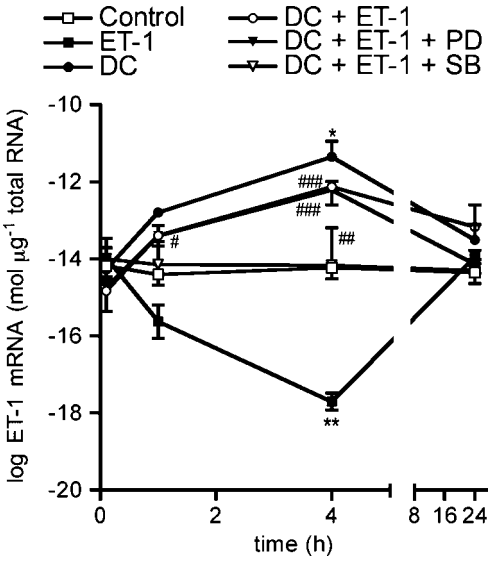

Figure 8 Internalization of endothelin-B receptor subtype B (ET_B) receptors is necessary for the endothelin-1 (ET-1)-mediated decrease in ET-1 mRNA. Serum-starved porcine aortic endothelial cells (PAECs) were incubated for the indicated times in media alone (control), 0.1 μM ET-1, 50 μM dansylcadaverine (DC) alone or in the presence of 0.1 μM ET-1, 0.1 μM ET-1 plus 1 μM PD98059 (PD), or 0.1 μM ET-1 plus 1 μM SB202190 (SB). Total mRNA was isolated and preproET-1 mRNA quantified as described in the legend of Figure 1. The values shown are the means (±s.e.mean) of four experiments employing four different preparations of endothelial cells. *$P<0.05$ versus media; **$P<0.01$ versus media; $^{\#}P<0.05$ versus ET-1; $^{\#\#}P<0.01$ versus ET-1; $^{\#\#\#}P<0.001$ versus ET-1 (one-way ANOVA, Bonferroni post-tests).

mechanism(s) whereby this regulation occurs. In this study, we present evidence that in vascular endothelial cells, ET-1 regulates the stability of ET-1 mRNA via a mechanism involving internalization of ET_B receptors and activation of both p38 and ERK MAPK cascades.

Activation of ET_B receptor signalling by the addition of exogenous ET-1 to the culture media led to a transient decrease in the cellular content of ET-1 mRNA (Figure 1). ET-1 mRNA is labile: human ET-1 mRNA has an intracellular half-life of 15 min (Inoue *et al*., 1989) and in porcine ECs, actinomycin D-mediated transcriptional arrest resulted in a rapid decrease in ET-1 mRNA. In the presence of actinomycin D, ET-1 further reduced ET-1 mRNA (Figure 3). However, ET-1 induced a transient increase in the association of RNA polymerase II with the ET-1 proximal promoter (Figure 4b). Thus, the modulation of cellular ET-1 mRNA content by extracellular ET-1 involves simultaneous changes in both transcription and mRNA stability. The magnitude of the reduction in cellular ET-1 mRNA content indicates that vascular endothelial cells are able to rapidly modulate their ET-1 synthesis in response to changes in the local concentration of circulating ET-1. Similarly, the lipopolysaccharide-induced increase in ET-1 mRNA in bovine aortic ECs results from a modest increase in transcription, but a two-fold increase in mRNA stability (Douthwaite *et al*., 2003). The 3′-untranslated region of human ET-1 mRNA contains two elements, termed DE-1 and DE-2, that constitutively destabilize ET-1 mRNA (Mawji *et al*., 2004a) and hence, they may play a key regulatory role in determining ET-1 mRNA stability.

The physiological effects of the activation of ET_B receptors include vasodilation, inhibition of renal tubular reabsorption, increasing sympathetic nerve activity and development of the enteric nervous system. In the vascular endothelium, ET_B receptors are thought to act as clearance receptors for circulating ET-1 (Dupuis, 2001). Furthermore, the ET_B receptor-mediated clearance of ET-1 may serve to compartmentalize circulating ET-1 levels, and therefore its regulatory effects, as ET_B receptors were recently shown to prevent ET-1 secreted in the lungs or kidneys from spilling over into heart (Johnström *et al*., 2005). Consistent with the concept of the vascular endothelium playing a key role in ET-1 clearance and regulating local ET-1 levels, exogenous ET-1 is bound and internalized by cultured PAECs and reduces the cellular content of ET-1 mRNA (Sanchez *et al*., 2002). Dansylcadaverine, an inhibitor of clathrin-coated pit formation and the internalization of ET_B receptors (Sanchez *et al*., 2002), blocked the ET-1-mediated reduction in ET-1 mRNA, suggesting that internalization of ET_B receptors is required for this regulatory effect. In Chinese hamster ovary cells expressing a fusion protein comprising green fluorescent protein (GFP) and ET_B receptors (GFP-ET_B), GFP-ET_B receptors were observed to internalize constitutively, without a requirement for ligand binding and the internalized receptors were targeted to the lysosomes for degradation (Bremnes *et al*., 2000; Oksche *et al*., 2000). Although the passage one cultures of PAECs used in the present study were quiescent, due to their maintenance in serum-free culture medium, they continued to produce and release a limited quantity of endogenous ET-1 (Sanchez *et al*., 2002). Accordingly,

addition of the ET_B receptor antagonist BQ788, which blocks the interaction of exogenous or endogenous ET-1 with ET_B receptors, led to an increase in the amount of ET-1 mRNA. Similarly, BQ788 increases luciferase activity in bovine aortic endothelial cells transfected with an ET-1 promoter/luciferase reporter gene (Peled *et al*., 2006). Taken together, these results suggest that, in vascular endothelial cells, a negative feedback loop exists whereby internalization of an ET-1/ET_B receptor complex acutely reduces the steady-state levels of ET-1 mRNA. One could postulate that the ligand-bound and unoccupied forms of the ET_B receptor mediated this effect via different internalization pathways; however, GFP-ET_B receptors bound with cyanin3-conjugate ET-1 (cy3-ET-1) co-traffic from the cell surface to lysosomes, remaining as a complex within the cell for up to 4 h (Oksche *et al*., 2000). Hence, internalized ET-1/ET_B receptor complexes may evoke signalling events distinct from internalized, unoccupied ET_B receptors.

MAPKs phosphorylate and regulate the function of numerous transcription factors and both the ERK and p38 MAPK pathways have previously been shown to be activated in response to ET-1. Hence, to elucidate the signalling events involved in the regulation of ET-1 mRNA stability, the activities of p38 and ERK MAPK pathways were examined. First, ET_B receptor activation by exogenous ET-1 or S6c led to an increase in the activity of MK-2, a protein kinase that is phosphorylated and activated by p38 MAPKs. This activation occurred within minutes, was maximal after 30–40 min and had returned to near baseline within 4 h of stimulation (Figure 5). This contrasts with the activation of the ERK cascade where a slowly developing, late phase of activation commenced 30–60 min after ET-1 application and persisted for at least 3 h (Figure 6). Hence, both the p38 and ERK MAPK pathways were activated in PAECs in response to external ET-1 and the time courses of activation were comparable to the time course for the reduction in ET-1 mRNA evoked by exogenous ET-1. However, the relative roles of p38 and ERK signalling in regulating ET-1 promoter activity versus mRNA stability remain to be established.

The p38 MAPK pathway has been shown to regulate both mRNA stability and translation depending upon the presence of specific adenosine/uridine-rich elements within the 3′-untranslated region of mRNAs, the type of cell and the presence of specific mRNA-binding proteins. p38 MAPKs regulate tumour necrosis factor-α (Kotlyarov *et al*., 1999) and epithelial interleukin-8 expression (Yu *et al*., 2003) via translational control. In addition, the p38 MAPK pathway positively regulates the stability of several mRNAs, including interleukin-8 (Hoffmann *et al*., 2002), interleukin-6 (Winzen *et al*., 1999; Neininger *et al*., 2002), urokinase plasminogen activator (Han *et al*., 2002), cyclooxygenase-2 (Cheng and Harris, 2002), natural resistance associated macrophage protein-1 (Lafuse *et al*., 2002) and a multifunctional matrix protein, thrombospondin-1 (Okamoto *et al*., 2002). p38 MAPKs are involved in the increased vascular endothelial growth factor mRNA levels, but not the increased half-life, induced by oxidized low-density lipoprotein (Salomonsson *et al*., 2002). The ERK, but not the p38, MAPK pathway stabilizes granulocyte-macrophage colony-stimulating factor mRNA in tumour necrosis factor-α plus fibronectin-activated peripheral blood eosinophils (Esnault and Malter, 2002). Although, in general, these studies have employed pharmacological inhibitors of p38 MAPK (for example, SB203580 and SB202190) and implicate inhibitor-sensitive p38 isoforms α and β, other studies employing MK-2 knockout mice indicate that MK-2 plays an important role in the post-transcriptional regulation of gene expression (see Kotlyarov and Gaestel, 2002). Via actions at adenosine/uridine-rich elements within the 3′-untranslated region of mRNA, MK-2 has been shown to regulate mRNA translation and degradation (Neininger *et al*., 2002). In the present study, the ET-1-mediated decrease in ET-1 mRNA levels was blocked by the p38 MAPK inhibitor SB202190 (Figure 7) indicating that, in the case of the ET-1 transcript, p38 MAPK activation lead to decreased mRNA stability. Similarly, the amount of MAPK kinase 6 (MKK6) mRNA was recently shown to increase following treatment with the p38 inhibitor SB203580 or in $p38\alpha^{-/-}$ mice, indicating that p38α negatively regulates the stability of MKK6 mRNA (Ambrosino *et al*., 2003). Furthermore, the 3′-untranslated region of MKK6 mRNA differentially regulates the stability of a *lacZ* reporter gene in a p38α-dependent manner (Ambrosino *et al*., 2003). Thus, p38α activity suppresses the level, and therefore the activity, of one of its own upstream activators. With respect to the regulation of ET-1 mRNA, whereas blockade of p38 prevented an ET-1-mediated change in mRNA levels, blockade of the ERK pathway resulted in an increase in ET-1 mRNA levels (Figure 7) similar to that observed upon blockade of ET_B receptors with BQ788 (Figure 1b). In both cases, the effect of kinase blockade upon message level was dependent upon the addition of ET-1. Furthermore, the increase in ET-1 mRNA observed upon blocking receptor internalization was also prevented by SB202190 (Figure 8). Consistent with other mRNA species regulated by the p38 MAPK pathway, human ET-1 mRNA contains adenosine/uridine-rich elements within the 3′-untranslated region (Inoue *et al*., 1989) and the 3′-untranslated region of human ET-1 contains elements that enhance the lability of the mRNA in vascular endothelial cells (Mawji *et al*., 2004b).

In conclusion, in vascular endothelial cells, activation of ET_B receptors with exogenous ET-1 induced a transient decrease in the abundance of cellular ET-1 mRNA that involved concerted changes in both promoter activity and mRNA stability. This regulatory process required internalization of an ET_B receptor–ET-1 complex and activation of both p38 and ERK MAPK pathways. A negative regulatory role of p38 was recently demonstrated upon the stability of MKK6 mRNA. As other signalling systems, such as oxidative stress and angiotensin II, also activate the p38 and ERK MAPK cascades, experiments are currently underway to determine the impact of other signaling pathways on ET-1 mRNA in vascular endothelial cells.

Acknowledgements

This study was supported by grants from the Canadian Institutes of Health Research (MOP-64183 to BGA; MOP-14496 to ET) and the Fonds de la Recherche de l'Institut de Cardiologie de Montréal. BGA and ET were New Investigators

of the Heart and Stroke Foundation of Canada and currently hold Senior Scientist Awards from the Fonds de la Recherche en Santé du Québec (FRSQ). NF is the recipient of a bursary from the Fonds de la Recherche de l'Institut de Cardiologie de Montréal. We thank Dr Nathalie Thorin-Trescases for her assistance with isolation and culture of PAECs and Dr Maya Khairallah for her critical reading of the manuscript.

Conflict of interest

The authors state no conflict of interest.

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