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Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ET_B receptor, NADPH oxidase and caveolin-1

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1 Endothelin-1 (ET-1), an endothelium-derived vasoactive peptide, participates in the regulation of endothelial function through mechanisms that are not fully elucidated. This study examined the impact of ET-1 on oxidative stress, apoptosis and cell proliferation in human umbilical vein endothelial cells (HUVEC). HUVECs were challenged for 24 h with ET-1 (10 pM–10 nM) in the absence or presence of the ET_B receptor antagonist BQ788 (1 μM) or the NADPH oxidase inhibitor apocynin (1 μM). Reactive oxygen species (ROS) were detected using chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate. Apoptosis was evaluated with 4′,6′-diamidino-2′-phenylindoladihydrochloride staining and by the caspase-3 assay. Cell proliferation was measured by a colorimetric assay. Expression of NADPH oxidase, Akt, pAkt, Bcl-2, Bax, IκB, caveolin-1 and eNOS was evaluated by Western blot analysis.

2 ET-1 significantly enhanced ROS generation and cell proliferation following 24-h incubation, both of which were prevented by BQ788 or apocynin, consistent with the ability of ET-1 to directly upregulate NADPH oxidase. ET-1 itself did not affect apoptosis but attenuated homocysteine-induced apoptosis through an ET_B receptor-mediated mechanism. Western blot analysis indicated that ET-1 alleviated homocysteine (Hcy)-induced apoptosis, likely acting by antagonizing the Hcy-induced decreases in Akt, pAkt, pAkt-to-Akt, Bcl-2-to-Bax ratios and increases in Bax and caveolin-1 expression. Furthermore, ET-1 downregulated expression of caveolin-1 and eNOS, which was attenuated by BQ788 or apocynin.

3 In summary, our results suggest that ET-1 affects oxidative stress, proliferation and apoptosis possibly through ET_B, NADPH oxidase, Akt, Bax and caveolin-1-mediated mechanisms.
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Abbreviations: Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide; CM-H_2DCFDA, 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DAPI, 4′,6′-diamidino-2′-phenylindoladihydrochloride; ECGS, endothelial cell growth supplement; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; Hcy, homocysteine; HUVEC, human umbilical vein endothelial cells; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAP kinase: mitogen-activated protein kinase|NADPH, nicotinamide adenine dinucleotide phosphate; NFκB, nuclear transcription factor κB; NO, nitric oxide; pAkt, phosphorylated Akt; PKB, protein kinase B; ROS, reactive oxygen species

Introduction

Endothelin-1 (ET-1), a 21-amino-acid polypeptide produced by vascular endothelial cells, possesses potent vasoactive activity and has been implicated in the pathophysiology of many cardiovascular diseases such as hypertension, atherosclerosis and hypercholesterolemia (Barton *et al*., 1998; Best *et al*., 1999; Schiffrin, 2001). ET-1 has been shown to exert its biological effects through binding to specific G protein-coupled membrane receptors, namely ET_A and ET_B subtypes. ET_A receptor is located mainly on vascular smooth muscle cells and transmits the signal for vasoconstriction and proliferation, whereas the ET_B receptor is present predominantly on endothelial cells and mediates vasorelaxation through nitric oxide (NO) and prostacyclin (Schiffrin, 2001). The balance between vasoconstriction and vasorelaxation or ET_A and ET_B receptors is the most important factor in determining the regional blood flow and blood pressure regulatory effects of ET-1 (Schiffrin, 2001). Production of ET-1 has been found to be elevated under a number of hypertensive states such as salt-sensitive hypertension (Schiffrin, 2001). In addition to its vasoactive effects, ET-1 also affects cell proliferation, tissue remodeling and cell survival

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in various cell types including vascular smooth muscle and endothelial cells (Ziche *et al*., 1995; Salani *et al*., 2000; Schiffrin, 2001). ET-1 has been demonstrated to enhance vascular superoxide (O_2^-) production and to promote cell proliferation through induction of reactive oxygen species (ROS) (Wedgwood *et al*., 2001; Li *et al*., 2003a, b). However, the precise role of ET-1 as a modulator of apoptosis, the genetically programmed cell death process, remains controversial. ET-1 has been reported to be an antiapoptotic factor in various cell types including endothelial and vascular smooth muscle cells (Shichiri *et al*., 1997; 2000; Del Bufalo *et al*., 2002). On the contrary, the proapoptotic effect of ET-1 has been documented in human melanoma cells (Okazawa *et al*., 1998). Nevertheless, the signaling pathways responsible for the anti- vs proapoptotic properties of ET-1 have not been elucidated.

Signaling components of the ET-1 cascade such as the ET-1 receptor, extracellular signal-regulated kinase (ERK) and mitogen-activated protein (MAP) kinase molecules have been found to colocalize with caveolin-1 in caveolae (Chun *et al*., 1994; Peiro *et al*., 2000; Hua *et al*., 2003). Caveolin-1 is the major component of caveolae, the flask-shaped membrane invaginations participating in multiple cellular processes such as vesicular transport, cholesterol homeostasis and perhaps, most importantly, signal transduction (Okamoto *et al*., 1998). Caveolin-1 is known to suppress growth and cell proliferation (Engelman *et al*., 1997; Kifor *et al*., 2003). Increased caveolin-1 expression is associated with both macrophage apoptosis (Gargalovic & Dory, 2003) and antiapoptosis in certain prostate cancer-derived cell types (Timme *et al*., 2000). It is postulated that regulation of proliferation or cell death by caveolin-1 may be cell specific (Gargalovic & Dory, 2003). However, whether caveolin-1 is involved in ET-1-induced cell proliferation and antiapoptosis processes remains unknown. The aim of the present study was to examine the causal link among ET-1-induced responses on ROS generation, apoptosis and cell proliferation in human umbilical vein endothelial cells (HUVEC). We also examined expression of protein kinase B (PKB)/Akt, phosphorylated Akt (pAkt), anti- and proapoptotic enzymes Bcl-2 and Bax, respectively, the nuclear transcription factor κB (NFκB) inhibitory subunit IκB, caveolin-1 and its downstream signaling molecule eNOS following 24-h treatment of ET-1.

Methods

HUVEC cell culture

HUVECs were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in Kaight's F12K medium with 2 mM L-glutamine, 1.5 g l^{-1} sodium bicarbonate, 0.1 mg ml^{-1} heparin, 0.03–0.05 mg ml^{-1} endothelial cell growth supplement (ECGS), 10% fetal bovine serum, 100 μg ml^{-1} streptomycin and 100 μl ml^{-1} penicillin (ATCC, Manassas, VA, U.S.A.) at 37°C with 5% CO_2. HUVECs (10^5 cells ml^{-1}) were cultured for 0, 8, 16 and 24 h with ET-1 (10 pM–10 nM, endogenous ET-1 = 20–100 pM) for the time-dependent response of ET-1. For subsequent studies, confluent HUVECs were treated with ET-1 (10 pM–10 nM) for 24 h in the absence or presence of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin (1 μM), ET_B antagonist BQ788 (1 μM) or homocysteine (Hcy; 1 mM) serving as a proapoptosis control. Hcy or hyperhomocysteinemia is an integral component of many cardiovascular, neurodegenerative and alcoholic diseases. Hcy may unleash inflammatory mediators including NFκB, IL-1β and IL-6, promote generation of superoxide anion (O_2^-) leading to oxidative stress and endoplasmic reticulum stress *en route* to ultimate cell injury such as apoptosis and inflammation (Ji & Kaplowitz, 2004).

Intracellular fluorescence measurement of ROS

The membrane-permeable probe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, Eugene, OR, U.S.A.) enters the cells and produces a fluorescent signal after intracellular oxidation by ROS such as H_2O_2. Intracellular oxidant stress was monitored by changes in fluorescence intensity resulting from intracellular probe oxidation according to our previously described method (Privratsky *et al*., 2003). Following 24-h ET-1 (10 pM–10 nM) treatment with or without apocynin or BQ788, HUVECs were loaded with 10 μM CM-H_2DCFDA for 30 min at 37°C and washed with PBS buffer. Cells were sampled randomly using an Olympus BX-51 microscope equipped with an Olympus MagnaFire™ SP digital camera and ImagePro image analysis software (Media Cybernetics, Silver Spring, MD, U.S.A.). Fluorescence was calibrated with InSpeck microspheres (Molecular Probes). More than 500 cells per treatment group were evaluated using the grid-crossing method for cell selection in more than five visual fields per experiment (4–6 experiments).

Apoptosis with 4′,6′-diamidino-2′-phenylindoladihydrochloride (DAPI) staining

We used a DNA-specific fluorochrome staining technique to assess the effect of ET-1 on apoptosis (Li *et al*., 2004). Typical apoptotic changes comprise condensation of chromatin, its presence along the periphery of the nucleus and segmentation of the nucleus. The rate of apoptosis was determined as the percentage of apoptotic nuclei (nuclei with condensed and/or segmented chromatin were counted as apoptotic nuclei) per visual field. In brief, HUVECs growing on glass coverslips were rinsed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, then washed with PBS. DAPI (1 μg ml^{-1}) was added to the fixed cells before being examined by fluorescence microscopy. More than 500 cells per treatment group were evaluated randomly in more than five visual fields per experiment (4–6 experiments).

Apoptosis with caspase-3 assay

Caspase-3 is an enzyme activated during induction of apoptosis. The caspase-3 activity was determined according to the published method (Li *et al*., 2004). Briefly, 1 ml of PBS was added to a flask containing ET-1 (10 pM–10 nM)-treated HUVECs (with or without 1 μM apocynin, 1 μM BQ788 or 1 mM Hcy), monolayer HUVECs were scraped and collected in a microfuge tube. Cells were pelleted by centrifugation at 10,000 × *g* at 4°C for 10 min. The supernatant was discarded, and the cells were lysed in 100 μl of

ice-cold cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1% NP40). The assay for caspase-3 activity was carried out in a 96-well plate. Each well contained 20 μl of cell lysate, 70 μl of assay buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT and 1 mM EDTA) and 10 μl of caspase-3 colorimetric substrate *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) (Sigma Chemicals, St Louis, MO, U.S.A.). The 96-well plate was incubated at 37°C for 2 h, during which time caspase in the sample was allowed to cleave the chromophore pNA from the substrate molecule. Absorbance readings were obtained at 405 nm with the caspase-3 activity being directly proportional to the colorimetric reaction. Protein content was determined using the Bradford (1976) method.

Cell proliferation assay

Cell proliferation was assessed with a colorimetric cell proliferation kit (Oncogene, San Diego, CA, U.S.A.), which measures the increased activity of cellular mitochondrial dehydrogenases that can cleave the tetrazolium dye WST-1 to formazan. The formazan formation is then quantified by measuring the change in absorbance at 450 nm in a microplate reader. The activity of mitochondrial dehydrogenases is proportional to cell number. In brief, HUVECs were seeded onto 96-well plates and incubated in 150 μl culture medium at 37°C in a CO_2 tissue culture incubator for 24 h containing ET-1 (1 nM) with or without apocynin (1 μM) or BQ788 (1 μM). After 24 h incubation, 10 μl of the WST-1 labeling mixture was added and further incubated at 37°C for 2.5 h. The absorbance was read at 450 nm using a Spectra Max 190 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.) (Li *et al*., 2004).

Western blot analysis of NADPH oxidase $p47^{phox}$, Akt, pAkt, Bcl-2, Bax, caveolin-1 and eNOS

HUVECs were collected and sonicated in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and protease inhibitor cocktail. Equal amounts of protein lysates (50 μg per lane) were separated on 15% (caveolin-1, $p47^{phox}$, Bcl-2, Bax, β-actin, IκB and phospho-IκB) or 7% (Akt, pAkt and eNOS) SDS–polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes (0.2 μM). The membranes were blocked in 5% (w v^{-1}) nonfat milk in TBS-T buffer, and then incubated with anti-Cav-1 (1 : 1000), anti-$p47^{phox}$ (1 : 1000), anti-Bax (1 : 500), anti-Bcl-2 (1 : 500), anti-Akt (1 : 1000), anti-phospho-Akt (1 : 1000), anti-IκB (1 : 1000), anti-phospho-IκB (1 : 1000), anti-eNOS (1 : 1000) and β-actin (1 : 5000) antibodies at 4°C overnight. Anti-caveolin-1 polyclonal antibody was purchased from Sigma. Anti-$p47^{phox}$ monoclonal antibody was kindly provided by Dr Mark T. Quinn from Montana State University (Bozeman, MT, U.S.A.). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). After incubation with the primary antibody, blots were incubated with either anti-mouse or anti-rabbit IgG HRP-linked antibodies at a dilution of 1 : 5000 for 120 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI, U.S.A.). The intensity of bands was measured with a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software (Privratsky *et al*., 2003). For all Western blot analysis experiments, β-actin was used as an internal loading control.

Data analysis

For each experimental series, data are presented as mean ± s.e.m. Statistical significance ($P<0.05$) for each variable was estimated by two-way analysis of variance (ANOVA) or *t*-test, where appropriate. A Dunnett's test was used for *post hoc* analysis when required.

Results

Effect of ET-1 on oxidative stress: involvement of ET_B receptor and NADPH oxidase

Figure 1 demonstrated the time-dependent response of ET-1 (10 pM–10 nM) on ROS generation. While ET-1 failed to elicit any effect on ROS generation within 18 h of incubation, ET-1 (10 pM–10 nM) significantly promoted ROS generation after 24 h of incubation. Longer incubation time (up to 48 h) of ET-1 did not elicit any further enhancement of ROS generation (data not shown). Therefore, 24 h was used for all subsequent ET-1 incubation experiments. Figure 2 depicted that 24 h incubation of ET-1-induced enhancement of ROS reached a plateau phase between 100 pM and 10 nM. Interestingly, both the ET_B receptor antagonist BQ788 (1 μM) and the NADPH oxidase inhibitor apocynin (1 μM) completely prevented ET-1-induced elevation in ROS generation (Figure 2), suggesting a likely involvement of ET_B receptor and NADPH oxidase in ET-1-induced ROS generation. Neither apocynin nor BQ788 affected ROS generation itself (Figure 2). The ET_A receptor antagonist BQ123 (1 μM) did not affect ET-1-induced ROS generation (data not shown), supporting the notion that the ET_A receptor subtype does not exist in HUVECs (Duerrschmidt *et al*., 2000).

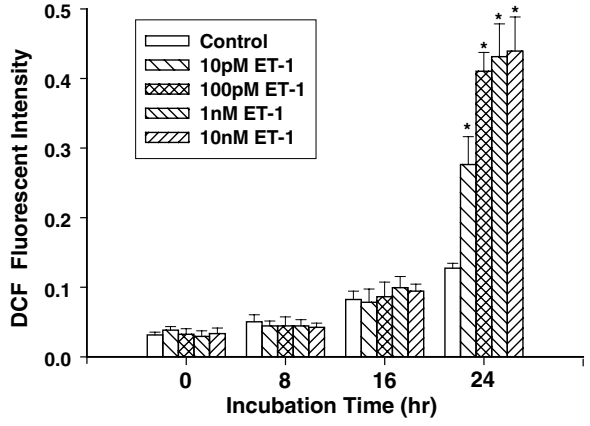

Figure 1 Time-dependent response of ET-1 (10 pM–10 nM) on intracellular ROS generation in HUVECs. ROS generation was detected using CM-H_2DCFDA. Mean ± s.e.m., $n=4$, $^*P<0.05$ vs respective control group.

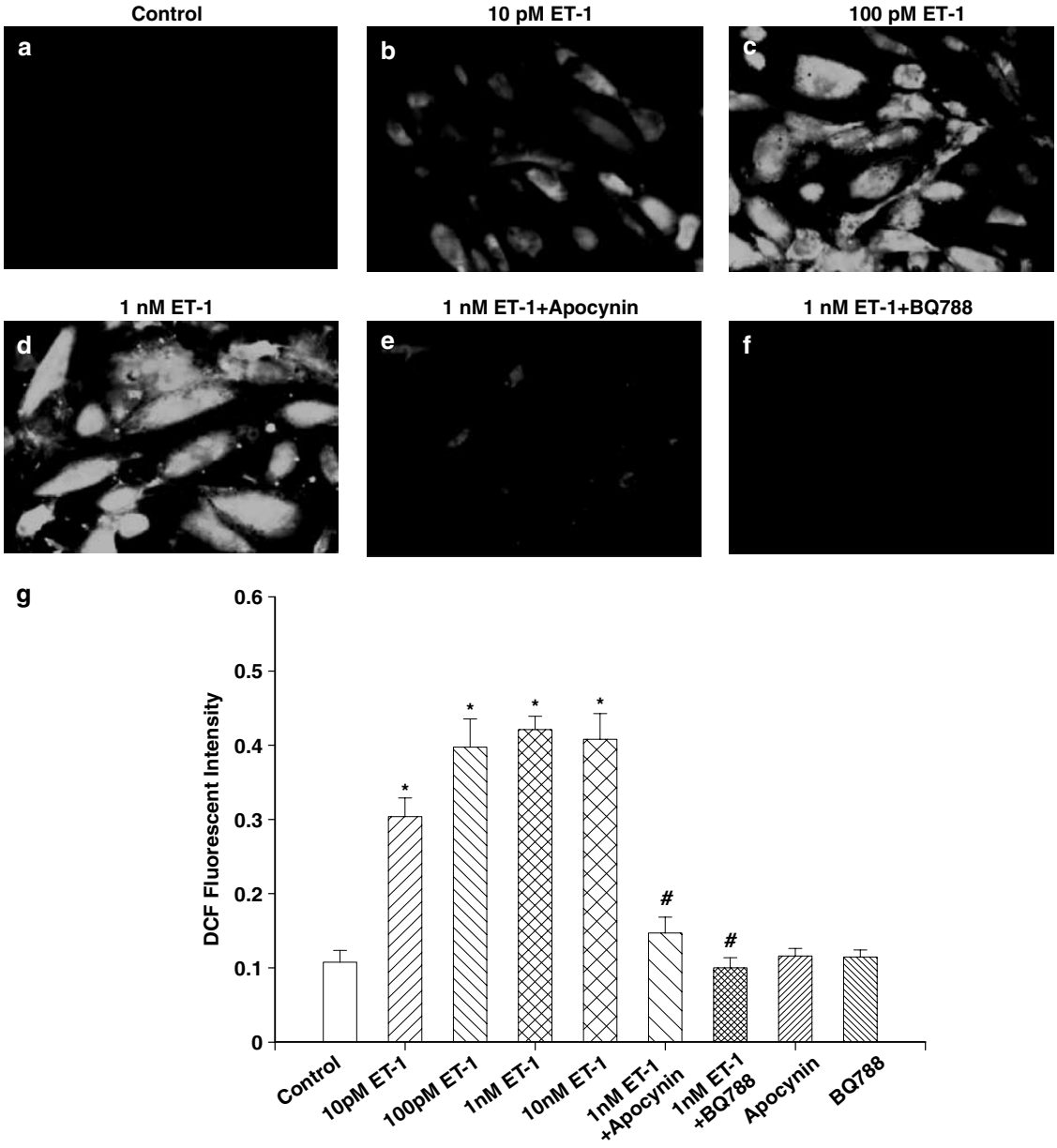

Figure 2 Effect of ET_B receptor antagonism and NADPH oxidase inhibition on ET-1-induced ROS generation. HUVECs were incubated with ET-1 (10 pM–10 nM) for 24 h in the absence or presence of the ET_B receptor antagonist BQ788 or the NADPH oxidase inhibitor apocynin before ROS was detected using CM-H_2DCFDA. (a) Control; (b) 10 pM ET-1; (c) 100 pM ET-1; (d) 1 nM ET-1; (e) 1 nM ET-1 + apocynin (1 μM); (f) 1 nM ET-1 + BQ788 (1 μM). (g) Summary of four independent experiments. Mean ± s.e.m., $n = 4$–6 experiments, $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs 1 nM ET-1.

Effect of ET-1 on apoptosis in HUVEC

Our results indicated that ET-1 alone (10 pM–10 nM) did not significantly affect apoptosis in HUVECs following a 24-h treatment (Figure 3j and k). As expected, Hcy (Hcy, 1 mM) elicited overt apoptotic cell death measured with DAPI staining and in the caspase-3 activity assay. While lower concentrations of ET-1 (10 and 100 pM) did not affect Hcy-induced apoptosis, higher levels of ET-1 (1 and 10 nM) significantly attenuated Hcy-induced apoptosis in HUVECs. The ET-1-induced antiapoptotic effect against Hcy was abolished by the ET_B receptor antagonist BQ788 (1 μM) (Figure 3a–i). BQ788 itself did not affect Hcy-induced apoptosis (data not shown).

Effect of ET-1 on cell proliferation in HUVEC via *an ET_B-NADPH oxidase-dependent pathway*

Figure 4a shows that ET-1 (1 nM) significantly promoted cell proliferation of HUVECs after 24-h incubation, which was completely prevented by the NADPH oxidase inhibitor apocynin (1 μM) and the ET_B receptor antagonist BQ788 (1 μM), suggesting that ET_B receptor and/or NADPH oxidase likely mediate the proliferative response of ET-1. Neither apocynin nor BQ788 alone affected cell proliferation (Figure 4). To confirm the involvement of NADPH oxidase in ET-1-induced proliferation and ROS generation in HUVECs, Western blot analysis was performed and the results indicated that ET-1 (1 nM) significantly

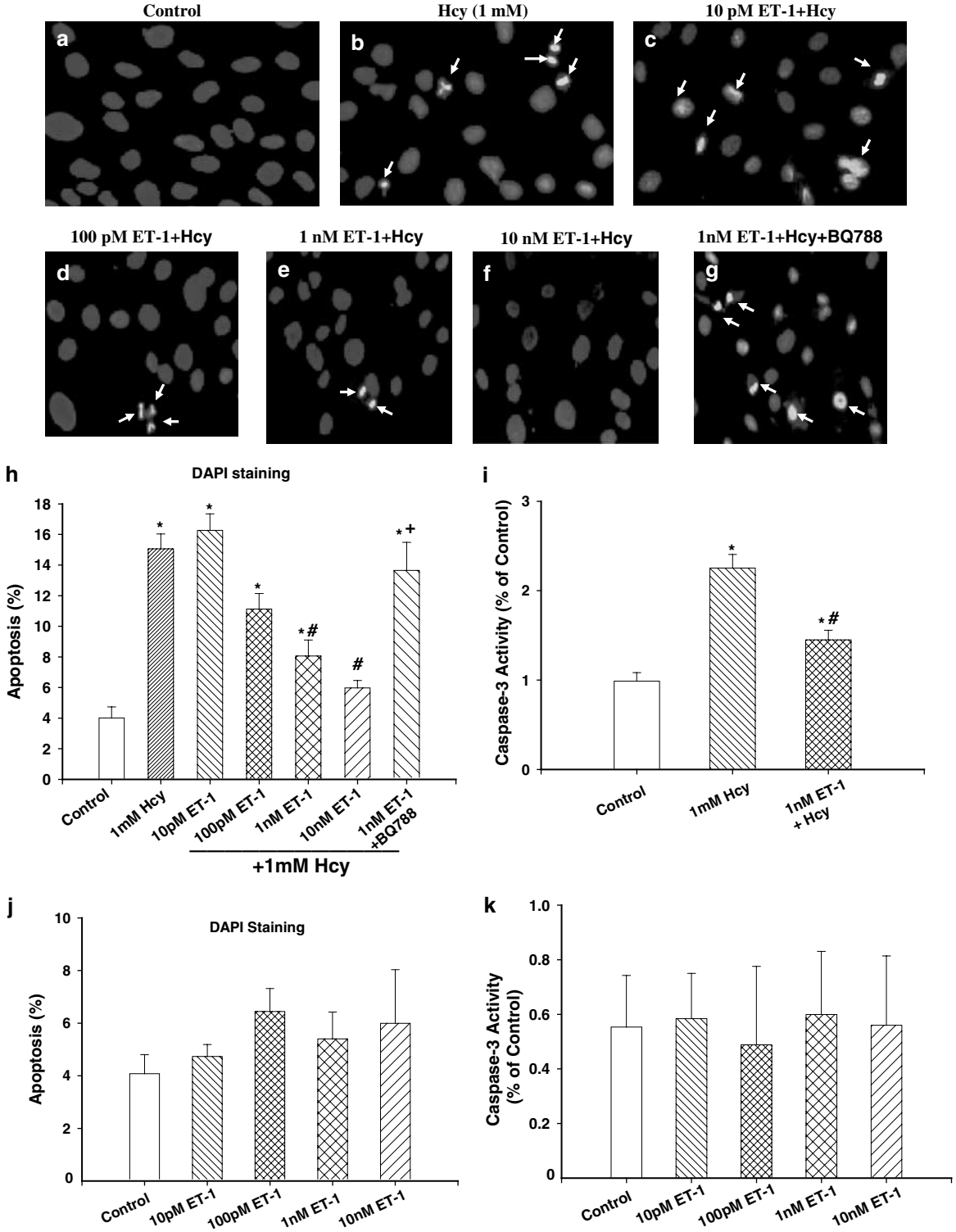

Figure 3 Effect of ET-1 on basal and Hcy-induced apoptosis. HUVECs were incubated with 1 mM Hcy for 24 h in the absence or presence of ET-1 (10 pM–10 nM) or BQ788 (1 μM). (a) Control; (b) Hcy; (c) Hcy + 10 pM ET-1; (d) Hcy + 100 pM ET-1; (e) Hcy + 1 nM ET-1; (f) Hcy + 10 nM ET-1; (g) Hcy + 1 nM ET-1 + BQ788. (h) Summary of 4–6 independent DAPI apoptotic assays. (i) Effect of ET-1 on Hcy (1 mM)-induced change in caspase-3 activity. (j) Effect of ET-1 on basal apoptosis (DAPI staining); (k) Effect of ET-1 on caspase-3 activity. Mean ± s.e.m., $n = 4$–6 experiments, $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs 1 mM Hcy; $^{+}P < 0.05$ vs Hcy + 1 nM ET-1.

upregulated protein expression of the $p47^{phox}$ subunit of NADPH oxidase after 24-h incubation. Protein expression of β-actin confirmed equal loading between ET-1-treated and untreated groups (Figure 4b). This result validated direct activation of NADPH oxidase by ET-1, which was consistent with the inhibitory effect of the NADPH oxidase inhibitor apocynin on ET-1-induced ROS generation and cell proliferation.

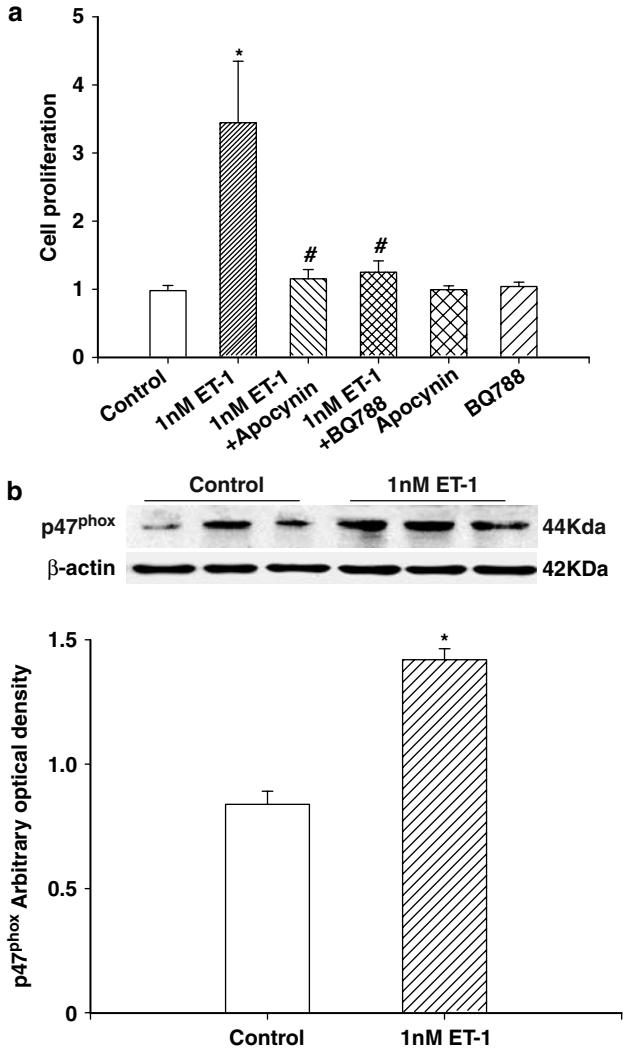

Figure 4 (a) Effect of ET-1 on proliferation in HUVEC. HUVECs were incubated with ET-1 (1 nM) for 24 h with or without apocynin and BQ788. (b) Effect of ET-1 (1 nM) on the $p47^{phox}$ subunit of NADPH oxidase in HUVECs after 24 h incubation using immunoblot technique. Inset shows representative blot of immunostaining using anti-$p47^{phox}$ and anti-β-actin (as loading control) antibodies. Mean ± s.e.m., $n = 4$–6 experiments, $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs 1 nM ET-1.

Effect of ET-1 on Akt, Bcl-2, Bax, IκB, caveolin-1 and eNOS protein expression

To examine the potential signaling pathways involved in the ET-1-induced antiapoptotic and proliferative response, several relevant anti-/proapoptotic signals were examined in Hcy-treated HUVECs using Western blot analysis following 24-h ET-1 incubation. Figure 5 revealed that Hcy (1 mM) significantly depressed protein levels of total Akt, pAkt and the ratio of pAkt-to-total Akt, all of which may be antagonized by co-incubation with ET-1 (1 nM). ET-1 itself significantly enhanced pAkt and pAkt-to-total ratio. Neither Hcy nor ET-1 significantly affected the Bcl-2 expression following a 24-h incubation. However, Hcy (1 mM) significantly enhanced expression of the proapoptotic gene *Bax* and reduced the Bcl-2-to-Bax ratio, both of which were reversed by ET-1 (1 nM) (Figure 6). ET-1 itself did not significantly affect expression of

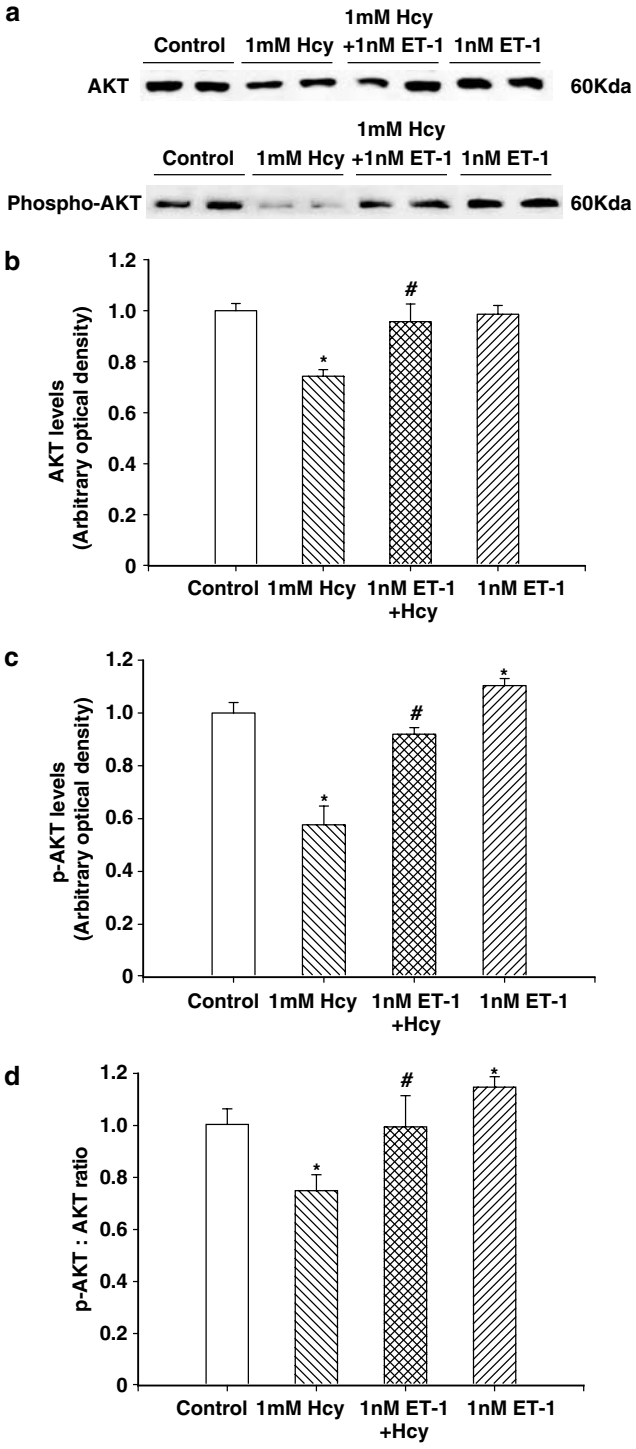

Figure 5 Effect of ET-1 (1 nM) on Hcy-induced response of Akt and pAkt in HUVEC. HUVECs were incubated with Hcy (1 mM) in the absence or presence of ET-1 (1 nM) for 24 h. (a) Representative blots showing immunostaining with anti-Akt or anti-pAkt antibodies in HUVECs; (b, c) Akt and pAkt protein expression after 24-h incubation Hcy (1 mM) with or without ET-1 (1 nM); (d) pAkt-to-Akt ratio. Mean ± s.e.m., $n = 4$–6 experiments, $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs Hcy (1 mM).

Bcl-2 or Bax (data not shown). While neither Hcy (1 mM) nor ET-1 (1 nM) affected total protein expression of the NFκB inhibitory subunit IκB, they both stimulated phosphorylation of IκB (which removes the inhibition of IκB on NFκB). ET-1

did not affect Hcy-induced phosphorylation of IκB (Figure 7). Hcy (1 mM) significantly enhanced the protein expression of caveolin-1, which may be antagonized by ET-1 (1 nM). ET-1 induced downregulation of caveolin-1 expression, which was abolished by apocynin or BQ788, suggesting that both the ET_B receptor and NADPH oxidase are likely involved in ET-1-induced regulation of caveolin-1 (Figure 8). On the other hand, both Hcy (1 mM) and ET-1 (1 nM) significantly reduced eNOS protein expression. Hcy-induced decrease in eNOS was further amplified with the presence of ET-1. ET-1-induced decrease in eNOS expression was attenuated by apocynin and BQ788, suggesting that both the ET_B receptor and NADPH oxidase are likely involved, at least in part, in ET-1-induced regulation of eNOS (Figure 9). Protein expression of β-actin was examined in all Western blot analyses to ensure equal protein loading (data not shown).

Discussion

Our results indicated that ET-1 enhanced oxidative stress in HUVECs following 24-h incubation. The observation of enhanced ROS generation in response to ET-1 exposure is consistent with the finding that ET-1 is capable of promoting O_2^- generation, however, through an ET_A receptor-NADPH oxidase-mediated pathway in the vasculature (Li *et al*., 2003b). Recent evidence suggested that ROS, such as O_2^- and H_2O_2, may play a role in the proliferation of vascular smooth muscle cells (Wedgwood *et al*., 2001) and fibroblasts (Cheng *et al*., 2003). ROS may elicit a wide variety of biological/pathological responses, depending upon the cell type, the magnitude and the duration of exposure. It was suggested that low doses of ROS are mitogenic and promote cell proliferation, whereas intermediate doses may result in temporary or permanent growth arrest. Severe oxidative stress due to a high ROS environment usually leads to cell death *via* either apoptotic or necrotic mechanisms (Martindale & Holbrook, 2002).

ET-1 has been shown to rescue cells from apoptosis induced by various apoptotic stimuli including paclitaxel, NO, serum deprivation and c-Myc (Shichiri *et al*., 1998; 2000; Del Bufalo *et al*., 2002). Consistently, our results showed that ET-1 possesses an antiapoptotic effect against Hcy through an ET_B-mediated mechanism. Our study revealed that the antiapoptotic mechanisms of ET-1 against Hcy-induced apoptosis may be mediated through its antagonism against Hcy-induced decrease in pAkt : Akt ratio, increase in Bax expression and a decrease in Bcl-2 : Bax ratio. These proapoptotic (Bax) and antiapoptotic (Akt, Bcl-2) molecules appear to display tissue and cell specificity in response to ET-1 exposure. For example, ET-1 was shown to induce expression of Bcl-2 in cardiac myocytes *via* a cyclosporin A-dependent manner without affecting Bax expression (Kakita *et al*., 2001). No effect was observed for ET-1 on Bcl-2 or Bax expression in vascular smooth muscle cells (Diep *et al*., 2000). ET-1 has been reported to stimulate Akt phosphorylation, which was abolished by the ET_B receptor antagonist BQ788 (Del Bufalo *et al*., 2002). Akt phosphorylation was demonstrated to occur within minutes of ET-1 receptor binding, peaked at 30 min and subsequently declined, consistent with the notion of rapid signaling and 'prolonged desensitization' of ET-1 signal pathways (Liu *et al*., 2003). Interestingly, our study revealed significantly elevated Akt phosphorylation following 24-h incubation of ET-1,

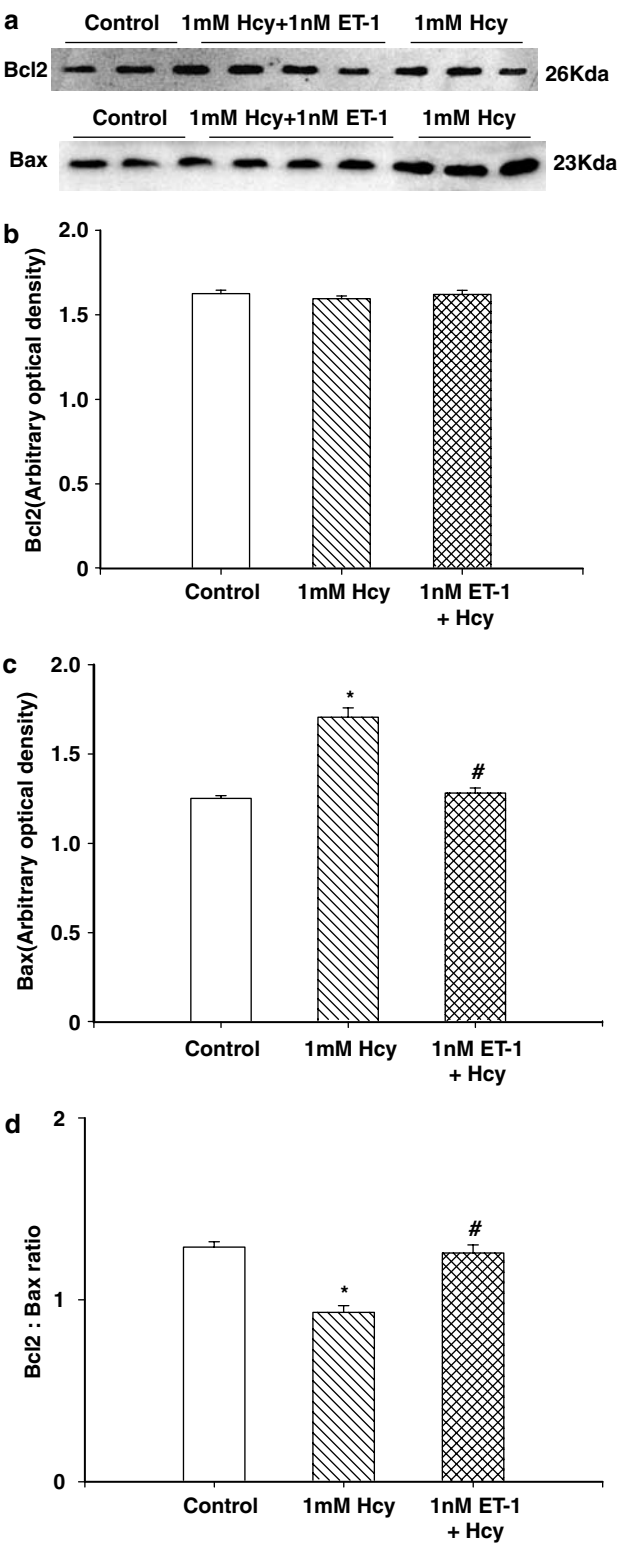

Figure 6 Effect of ET-1 (1 nM) on Hcy-induced response of Bcl-2 and Bax in HUVEC. HUVECs were incubated with Hcy (1 mM) in the absence or presence of ET-1 (1 nM) for 24 h. (a) Representative blots showing immunostaining with anti-Bcl-2 or anti-Bax antibodies in HUVECs; (b, c) Bcl-2 and Bax protein expression after 24-h incubation Hcy (1 mM) with or without ET-1 (1 nM); (d) Bcl-2-to-Bax ratio. Mean ± s.e.m., $n = 4$–6 experiments, $^*P < 0.05$ vs control, $^{\#}P < 0.05$ vs Hcy (1 mM).

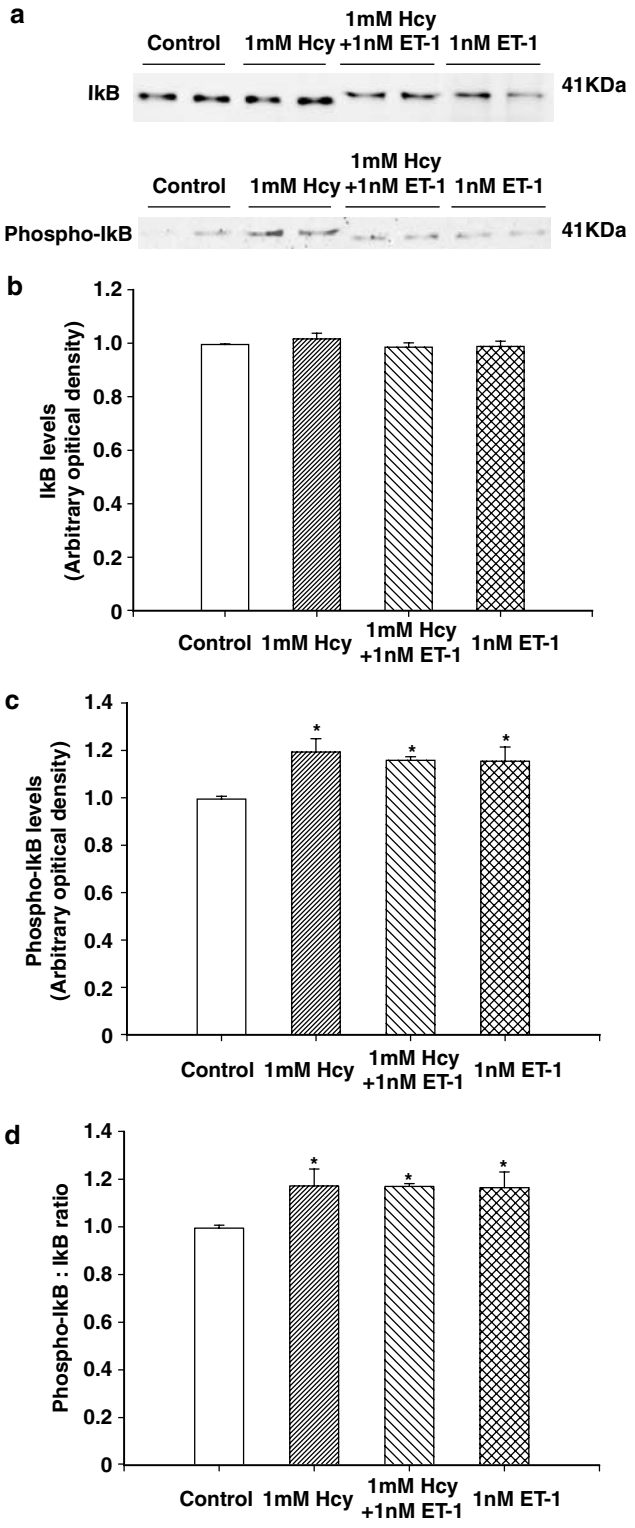

Figure 7 Effect of ET-1 (1 nM) on Hcy-induced response of total and phosphorylated IκB in HUVEC. HUVECs were incubated with Hcy (1 mM) in the absence or presence of ET-1 (1 nM) for 24 h. (a) Representative blots showing immunostaining with anti-IκB or anti-phospho-IκB antibodies in HUVECs; (b, c) IκB and phospho-IκB protein expression after 24-h incubation Hcy (1 mM) with or without ET-1 (1 nM); (d) phospho-IκB-to-IκB ratio. Mean$\pm$s.e.m., $n=4$–6 experiments, $^{*}P<0.05$ vs control.

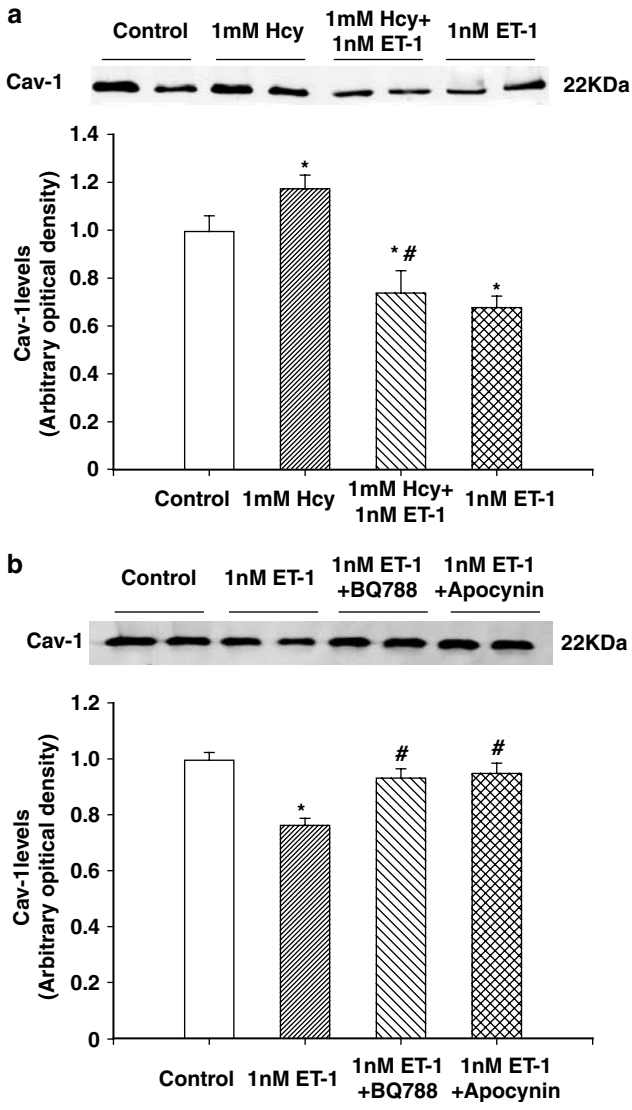

Figure 8 (a) Effect of ET-1 (1 nM) on Hcy-induced response of caveolin-1 (Cav-1) expression in HUVEC. HUVECs were incubated with Hcy (1 mM) in the absence or presence of ET-1 (1 nM) for 24 h. (b) Effect of NADPH oxidase and ET_B receptor inhibition on ET-1-induced decrease in Cav-1 protein expression in HUVEC. HUVECs were incubated with ET-1 (1 nM) for 24 h with or without apocynin (1 μM) and BQ788 (1 μM). Insets show representative immunostaining using anti-Cav-1 antibody. Mean$\pm$s.e.m., $n=4$–6 experiments, $^{*}P<0.05$ vs control; $^{\#}P<0.05$ vs ET-1 (1 nM).

suggesting that 24 h incubation of ET-1 may not lead to desensitized Akt phosphorylation and subsequently insulin resistance, which occurs for chronic ET-1 treatment *in vivo* (Wilkes *et al*., 2003). Although NFκB has been implicated in ET_A receptor activation-induced cell proliferation and inhibition of apoptosis (Mangelus *et al*., 2001b), data from our present study do not favor a role of NFκB in ET-1-induced antiapoptotic effect against Hcy since ET-1 and Hcy both triggered phosphorylation of the NFκB inhibitory subunit IκB (Figure 7). ET-1 has also been shown to activate a cascade of signaling molecules including ERK1/2, p38 MAP kinase and c-Jun N-terminal kinase (JNK) (Cheng *et al*., 2003), which may play a role in ET-1-mediated protection against apoptosis (Martindale & Holbrook, 2002). Further study is warranted to

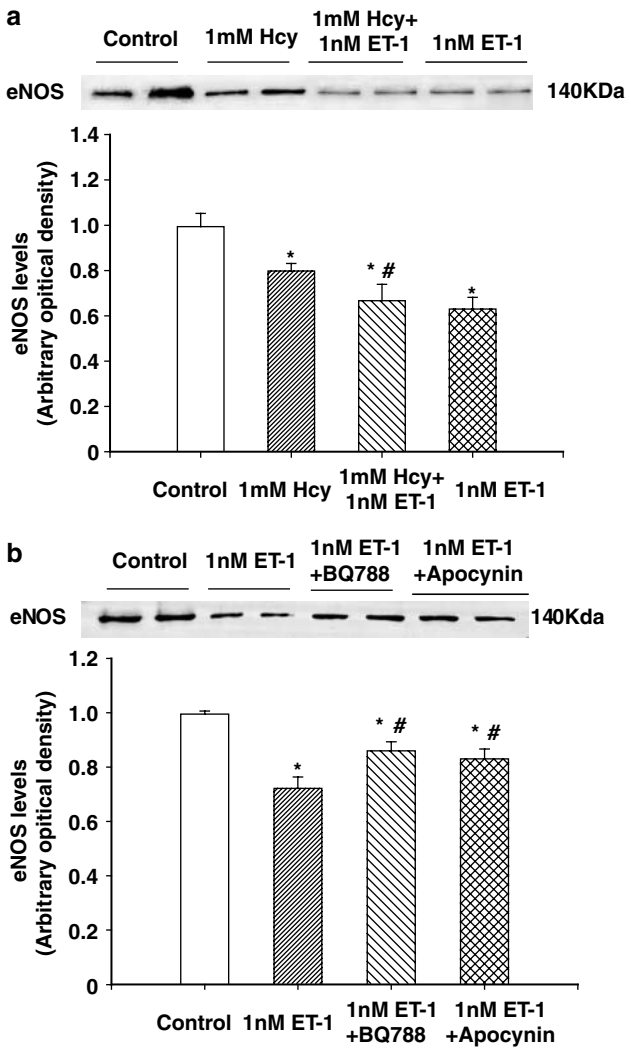

Figure 9 (a) Effect of ET-1 (1 nM) on Hcy-induced response of eNOS expression in HUVEC. HUVECs were incubated with Hcy (1 mM) in the absence or presence of ET-1 (1 nM) for 24 h. (b) Effect of NADPH oxidase and ET_B receptor inhibition on ET-1-induced decrease in eNOS protein expression in HUVEC. HUVECs were incubated with ET-1 (1 nM) for 24 h with or without apocynin (1 μM) and BQ788 (1 μM). Insets show representative immunostaining using anti-eNOS antibody. Mean $\pm$ s.e.m., $n = 4$–6 experiments, $^*P < 0.05$ vs control; $^{\#}P < 0.05$ vs ET-1 (1 nM).

elucidate the role of these signaling pathways in ET-1-elicited antiapoptotic effect.

One interesting finding is that ET-1-elicited antiapoptotic effect against Hcy is mirrored to some extent by alterations in the levels of caveolin-1 (Figure 8). Caveolin-1 organizes proteins in caveolae, thus enabling finely tuned regulation of physiological responses (Okamoto *et al*., 1998). As a suppressor of growth and cell proliferation (Engelman *et al*., 1997; Kifor *et al*., 2003), caveolin-1 level is associated with apoptosis (Gargalovic & Dory, 2003). Overexpression of caveolin-1 may sensitize cells to apoptotic stimuli, possibly *via* inhibition of PI-3 kinase and/or activation of caspase-3 (Zundel *et al*., 2000; Liu *et al*., 2001). This is consistent with our finding of similar changes of caspase-3 activity and caveolin-1 expression in response to ET-1 exposure. It is suggested that ET_B receptor forms a complex with caveolin-1 in cells in which these two proteins were co-expressed. The ET_B/caveolin-1 complex was formed efficiently only when ET_B was unoccupied or bound to an antagonist. ET-1 may dissociate this complex. In contrast, ET_A (although not present in HUVECs; Duerrschmidt *et al*., 2000) can bind to caveolin-1 regardless of ligand-binding status. Caveolin-1 utilizes its scaffolding domain (residues 82–101) and the C-terminal domain (residues 136–178) to bind to ET_B receptor (Yamaguchi *et al*., 2003). We speculate that ET-1 may compete with caveolin-1 for the ET_B receptor, and therefore disrupt the ET_B/caveolin-1 complex and associated apoptosis. Nevertheless, the precise relationship between caveolin-1 and apoptosis may be complicated since metastatic prostate cancer cells exhibit increased expression of caveolin-1 and reduced apoptosis (Nasu *et al*., 1998; Li *et al*., 2001).

NO participates in the regulation of ROS generation and apoptosis by both inducing and suppressing ROS generation and apoptosis depending upon the species or cell types. NO was shown to inhibit proliferation, induce ROS generation and apoptosis (Gordon *et al*., 2001; Del Bufalo *et al*., 2002). Results from our current study displayed that a 24-h treatment of Hcy or ET-1 reduced eNOS expression, indicating that the protection of ET-1 against Hcy-induced apoptosis is unlikely related to alteration of eNOS protein expression. However, ET-1 downregulated eNOS expression was mediated through an ET_B-NADPH oxidase-dependent mechanism, similar to its effect on ROS generation. Reduced eNOS expression in response to ET-1 exposure is likely to play a role in ET-1-induced ROS generation. ET-1 further decreased Hcy-induced eNOS protein expression (Figure 9). This may be attributed to ET-1-induced ROS accumulation since ROS itself can reduce eNOS expression and the association of eNOS with caveolin-1 (Peterson *et al*., 1999). Diminished eNOS protein expression and NOS activity have been reported following ET-1 and Hcy treatment (Li *et al*., 2002; Tognetti *et al*., 2003), suggesting the existence of a functional relationship between ET-1 and eNOS leading to ROS generation. It should be noted that ET-1 through ET_B may also stimulate NO production in sinusoidal endothelial cells as a result of Akt activation and subsequently eNOS phosphorylation (Del Bufalo *et al*., 2002). eNOS is an important substrate for Akt and may be activated by pAkt (Liu *et al*., 2003). The discrepant responses of ET-1 on eNOS and Akt phosphorylation are not fully understood, but may be related to the duration of ET-1 treatment.

Experimental limitations

Although our study indicated that ET-1 is implicated in the regulation of ROS, apoptosis and cell proliferation likely through an ET_B receptor/NADPH oxidase-mediated pathway(s) in HUVEC, these results did not fully address if enhanced cell proliferation may be responsible for discrepant responses of ROS generation and antiapoptosis against Hcy upon ET-1 exposure. An ample amount of evidence suggests that increased cell proliferation is usually accompanied with reduced apoptosis in response to extracellular stimuli including ET_A receptor activation (Mangelus *et al*., 2001). However, inhibition of cell proliferation is often associated with enhanced apoptosis (de Ruijter *et al*., 2004). Nevertheless, these complex events among ROS generation, cell proliferation and apoptosis in response to ET-1 exposure are not yet understood and warrant further work. In addition, we do not know if the cells being studied were in the quiescent or

proliferative state. Position of the cell cycle may be an important factor for apoptosis. Since ET-1 may activate the ERK1/2, p38 MAP kinase and JNK signaling molecules (Cheng *et al*., 2003), potential participation of these signaling pathways in ET-1-induced cell proliferation should not be ruled out at this time. Lastly, the Western blot technique used for signaling mechanisms of ET-1 in our study is somewhat semiquantitative and the degree of change in these proteins was relatively modest. It should not be considered the sole evidence of a mechanistic link, without confirmatory functional as well as specific gene overexpression or deletion data.

In summary, our results suggested that ET-1 may induce oxidative stress, proliferation and protect against Hcy-induced apoptosis in HUVEC through ET_B receptor/NADPH oxidase-dependent pathways. Our study indicated that antagonism against Hcy-induced decrease in Akt phosphorylation and Bcl-2/Bax ratio as well as downregulated caveolin-1 protein expression involving an ET_B/NADPH oxidase-dependent pathway may play a role in ET-1-mediated cellular effects (such as antiapoptosis) in HUVECs. Further study is underway to establish the definitive connection among ROS generation, cell proliferation and apoptosis, as well as role of caveolin-1 and eNOS in ET-1-induced regulation of cell survival.

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