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Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells: role of ET_A receptors and platelet-activating factor

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1 The potent coronary vasoconstrictor, endothelin-1 (ET-1) may also regulate neutrophil traffic into tissues. The aim of the present study was to characterize the endothelin receptors responsible and to investigate the underlying mechanisms.

2 ET-1 (1 nM – 1 μM) markedly enhanced attachment of human neutrophils to lipopolysaccharide-, and to a lesser extent, to ET-1-activated human coronary artery endothelial cells (HCAEC). This can partially be blocked by monoclonal antibodies against E-selectin, L-selectin or CD18, whereas combination of the three antibodies inhibited adhesion by ~83%. Increases in neutrophil adhesion evoked by ET-1 were also blocked by the platelet-activating factor (PAF) antagonists, BN 52021 (50 μM) and WEB 2086 (10 μM).

3 ET-1 downregulated the expression of L-selectin and upregulated expression of CD11b/CD18 and CD45 on the neutrophil surface and induced gelatinase release with EC_{50} values of ~2 nM. These actions of ET-1 were almost completely prevented by the ET_A receptor antagonist FR 139317 (1 μM) and the ET_A/ET_B receptor antagonist bosentan (10 μM), whereas the ET_B receptor antagonist BQ 788 (1 μM) had no effect. ET-1 slightly increased the expression of E-selectin and ICAM-1 on HCAEC, that was prevented by BQ 788, but not by FR 139317.

4 Receptor binding studies indicated the presence of ET_B receptors (K_D: 40 pM) on phosphoramidon-treated HCAEC and the predominant expression of ET_A receptors (K_D: 38 pM) on neutrophils.

5 These results indicate that promotion by ET-1 of neutrophil adhesion to HCAEC is predominantly mediated through activation of ET_A receptors on neutrophils and subsequent generation of PAF.

Keywords: Neutrophil granulocytes; endothelin-1; ET_A and ET_B receptors; adhesion molecules; selectins; integrins; ICAM-1; neutrophil-endothelial adhesion; platelet-activating factor; human coronary artery endothelial cells

Abbreviations: ET-1, endothelin-1; HBSS, Hanks' balanced salt solution; HCAEC, human coronary artery endothelial cells; LPS, lipopolysaccharide; mAb, monoclonal antibody; PAF, platelet-activating factor; PBS, phosphate buffered saline

Introduction

Endothelin-1 (ET-1), a potent constrictor of coronary arteries both *in vitro* and *in vivo* (reviewed by Rubanyi & Polokoff, 1994) has recently been recognized as one of the key mediators of myocardial ischaemia (Lüscher, 1991). Elevated tissue expression and plasma levels of ET-1 were found to occur in pathological conditions such as myocardial ischaemia (Miyauchi *et al*., 1989; Watanabe *et al*., 1991) and atherosclerosis (Lerman *et al*., 1991), which are also characterized by increased adhesiveness of leukocytes to endothelial cells (Lefer & Lefer, 1993).

Recent studies from several laboratories, including ours have suggested that ET-1 may be an important autocrine/paracrine modulator of neutrophil functions. ET-1 causes a selective neutrophil leukocytopenia in guinea-pigs (Filep *et al*., 1995b) and induces neutrophil accumulation in the isolated perfused heart (López-Farré *et al*., 1993), lung (Helset *et al*., 1996; Khimenko *et al*., 1996) and kidney (Espinosa *et al*., 1996). Neutrophils challenged with ET-1 migrate from the venous lumen into the tissue matrix of the human umbilical cord, and induce a massive tissue destruction (Halim *et al*., 1995). ET-1 promotes neutrophil aggregation (Gómez-Garré *et al*., 1992; López-Farré *et al*., 1995), stimulates surface expression of CD11b/CD18 on human neutrophils and augments their adhesion to cultured bovine endothelial cells (López-Farré *et al*., 1993). No data, however, are available on the effects of ET-1 on the expression of L-selectin (CD62L), or the ET receptor subtype(s) and the underlying mechanism(s) that mediate neutrophil-endothelial adhesion. To date two distinct mammalian ET receptor subtypes have been cloned, ET_A (which is highly selective for ET-1) and ET_B (non-isopeptide selective) (Arai *et al*., 1990; Sakurai *et al*., 1990) and pharmacological evidence suggest the existence of a third receptor subtype (termed non-ET_A/ET_B) on mammalian cells (Harrison *et al*., 1992). Previous studies reported that ET_A receptor blockade prevents ET-1-induced neutrophil leukocytopenia (Filep *et al*., 1995b) and neutrophil accumulation in rat perfused lung (Khimenko *et al*., 1996).

Accordingly, this study was undertaken to characterize the endothelin receptor subtypes which mediate the action of ET-1 on the surface expression of L-selectin and CD11b/CD18 on human neutrophils, expression of E-selectin and

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ICAM-1 on human coronary artery endothelial cells (HCAEC) and on adhesion of neutrophils to HCAEC by receptor binding assays and by using selective ET_A and ET_B receptor antagonists and agonists. Since multiple adhesion receptors are involved in neutrophil adhesion to activated endothelial cells (Spertini *et al*., 1991; Zouki *et al*., 1997), we assayed the contribution of L-selectin, E-selectin and CD18 to the attachment elicited by ET-1. In addition, we also investigated whether platelet-activating factor (PAF) mediates these actions of ET-1, because ET-1 stimulates PAF synthesis in neutrophils (Gómez-Garré *et al*., 1992) and PAF may function as a signal for neutrophils to bind to the endothelium (Zimmerman *et al*., 1990; Kuijpers *et al*., 1991).

Methods

Materials

ET-1 and IRL-1620 (Suc-[Glu^9,$Ala^{11,15}$]endothelin-1(18-21)) were synthesized in our laboratory by solid-phase method. The purity of peptides were greater than 98%, as analysed by high performance liquid chromatography. Bosentan (4-*tert*-butyl- N-[6-(2 hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′bi-pyrimidin-4-yl]-benzene-sulphonamide) was a gift from Dr M. Clozel (Hoffmann LaRoche Ltd. Basel, Switzerland), FR 139317 ((R)2-[(R)- 2 -[[1-(hexahydro-1H-azepinyl)]-carbonyl]-amino - 4 - methylpentanoyl]amino-3-[3-(1-methyl-1H-indoyl)]-propionyl]amino-3-(2-pyridyl) propionic acid) was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). BQ 788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) was obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). BN 52021 (ginkgolide B) was a gift from Dr P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France). WEB 2086 (3 - [4 - (2-chlorphenyl) - 9 - methyl - 6H -thienol[3,2-f][1,2,4]triazolo-[4,3-a] [1,4]-diazepine-2-yl]-1-(morpholinyl)-1-propanon) was a gift from Dr H. Heuer (Boehringer-Ingelheim KG, Ingelheim, Germany). Drugs were dissolved in 0.9% NaCl with the exception of BN 52021 which was dissolved in dimethylsulphoxide and was diluted further in with 0.9% NaCl as appropriate. Lipopolysaccharide (LPS, *E. coli*, serotype O111:B4) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphoramidon was purchased from Peptides International (Louisville, KY, U.S.A.).

Whole-blood incubation

Venous blood (anticoagulated with sodium heparin, 50 U ml^{-1}) was obtained from non-smoking healthy volunteers (male and female, 25–44 years old) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cells counts were between 4500 and 8500 cells per μl. Whole blood aliquots were transferred to polypropylene tubes, placed on a rotator, and preincubated with one of the following antagonists for 10 min at 37°C: the ET_A receptor selective antagonist FR 139317 (1 μM) (Aramori *et al*., 1993), the ET_A/ET_B antagonist bosentan (10 μM) (Clozel *et al*., 1994), the ET_B receptor selective antagonist BQ 788 (1 μM) (Ishikawa *et al*., 1994), or the PAF receptor antagonist BN 52021 (50 μM) (Braquet *et al*., 1985) or WEB 2081 (10 μM) (Casals-Stenzel *et al*., 1987), and then challenged with either ET-1 or the selective ET_B receptor agonist IRL 1620 (Takai *et al*., 1992) (both 1 pM – 1 μM) for 30 min at 37°C in 5% CO_2 in air.

Flow cytometry analysis

Direct immunofluorescence labelling of control or treated neutrophil granulocytes in whole blood were performed as described previously (Filep *et al*., 1997). Leukocytes were stained with saturating concentration of FITC-labelled anti-human L-selectin monoclonal antibody (mAb) DREG-56 (IgG_1, PharMingen, San Diego, CA, U.S.A.), R-phycoerythrin-conjugated mouse anti-human CD11b mAb Leu-185 (IgG_1, Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) or PerCP-labelled anti-human CD45 mAb 2D1 (IgG_1, Becton Dickinson). Appropriately labelled, class-matched irrelevant mouse IgG_1 was used as a negative control for each staining. Single-colour immunofluorescence staining was analysed by a FACScan Flow Cytometer (Becton Dickinson) with Lysis II software.

Isolation and treatment of neutrophil granulocytes

Neutrophil granulocytes were isolated from peripheral blood by centrifugation through Ficoll-Hypaque gradients (Pharmacia Diagnostics AB, Uppsala, Sweden), sedimentation through dextran (3%, w v^{-1}), and hypotonic lysis of erythrocytes. The resultant cell preparation contained >97% neutrophils. Neutrophils (5×10^6 cells ml^{-1}) were suspended in a modified Hanks' balanced salt solution (HBSS) consisting of (mM): NaCl 145, K_2PO_4 10, $CaCl_2$ 1.4, $MgCl_2$ 1.2, glucose 10, and 250 μg ml^{-1} human serum albumin, pH 7.4, preincubated with one of the antagonists for 10 min and then challenged with ET-1 or IRL 1620 for 30 min at 37°C. Then the cells were pelleted, and the supernatants were collected for further analysis.

Measurement of granule enzyme release

Lysozyme, β-glucuronidase and gelatinase were assayed as described previously (Filep *et al*., 1997; Zouki *et al*., 1997). Enzyme release was determined as the percentage of total enzyme units released from neutrophils treated with 0.1% Triton X-100.

Culture of endothelial cells

Normal human coronary artery endothelial cells obtained from Clonetics Corp. (San Diego, CA, U.S.A.) were cultured as described (Zouki *et al*., 1997). HCAEC (passages 4–6) seeded into 24-well or 96-well microplates and grown to confluence were used in the experiments.

Neutrophil-endothelial cell adhesion assay

The adhesion assay was performed as described previously (Zouki *et al*., 1997). In brief, monolayers of HCAEC in 96-well microplates were stimulated with LPS (1 μg ml^{-1}) or ET-1 (100 nM) for 6 h at 37°C in a 5% CO_2 atmosphere. The wells were then washed three times, and 2×10^5 ^{51}Cr-labelled neutrophils in 100 μl were added. Radiolabelled neutrophils were preincubated for 10 min with one of the antagonists at the concentration indicated. In another set of experiments, LPS or ET-1-activated HCAEC were incubated for 15 min with the function blocking anti-E-selectin mAb ENA-2 (IgG_1, purified $F(ab')_2$ fragments; Monosan, Uden, The Netherlands) at 10 μg ml^{-1} (Leeuwenberg *et al*., 1990)

or the irrelevant mAb MOPC-21 (IgG_1, PharMingen) before addition of neutrophils. The mAb reactive with E-selectin was also added back to the neutrophil suspensions so that mAb ENA-2 was present throughout the assay. Radiolabelled neutrophils were incubated with the anti-L-selectin mAb DREG-56 (IgG_1, PharMingen) at 20 μg ml^{-1} (Kishimoto *et al*., 1991) or the anti-CD18 mAb L130 (IgG_1, Becton Dickinson) at 10 μg ml^{-1} (Zouki *et al*., 1997) for 15 min at 37°C before addition to HCAEC. After incubation of endothelial cells plus neutrophils in the absence or presence of ET-1 (100 nM) for 30 min at 37°C on an orbital shaker at 90 r.p.m., loosely adherent or unattached neutrophils were washed three times, and the endothelial monolayer plus the adherent neutrophils were lysed in 200 μl of 0.1 M NaOH. The number of adhered neutrophils in each experiment was estimated from the radioactivity of a control sample. Treatment of HCAEC with any of the antibody used in these studies did not affect the integrity of viable endothelial monolayers.

Expression of E-selectin and ICAM-1

Monolayers of HCAEC in 24-well microplates were incubated for 4 h at 37°C in a 5% CO_2 atmosphere with LPS (1 μg ml^{-1}) or ET-1 (100 nM) in the absence or presence of ET receptor antagonists. The cells were then detached by exposure to EDTA (0.01%) in PBS for 10 min at 37°C followed by gentle trituration. The cells were resuspended in ice-cold NaCl solution (150 mM) containing 0.02% sodium azide, incubated with saturating concentrations of FITC-labelled mouse anti-human E-selectin mAb 1.2B6 (Serotec, Kidlington, England) and R-phycoerythrin-conjugated mouse anti-human ICAM-1 mAb HA58 (PharMingen) for 30 min at 4°C, washed and fixed in formaldehyde (3.9% in PBS). Appropriately labelled, class-matched irrelevant mouse IgG_1 was used as a negative control

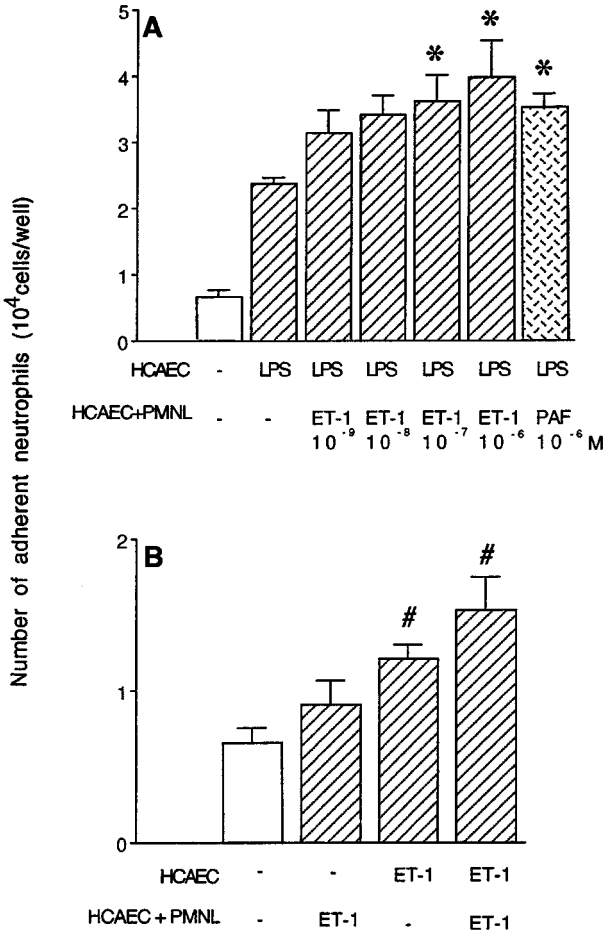

Figure 1 Endothelin-1 (ET-1) augments adhesion of neutrophils to coronary artery endothelial cells (HCAEC). Confluent HCAEC monolayers were cultured for 6 h with (A) 1 μg ml^{-1} lipopolysaccharide (LPS) or (B) 100 nM ET-1. Neutrophils (PMNL) together with ET-1 or PAF (1 μM) were then added and incubated with HCAEC for 30 min at 37°C (PMNL + HCAEC). Values are expressed as mean ± s.e.mean of three experiments in triplicate using neutrophils from different donors. *$P<0.05$ vs LPS; #$P<0.05$ vs unstimulated (open column).

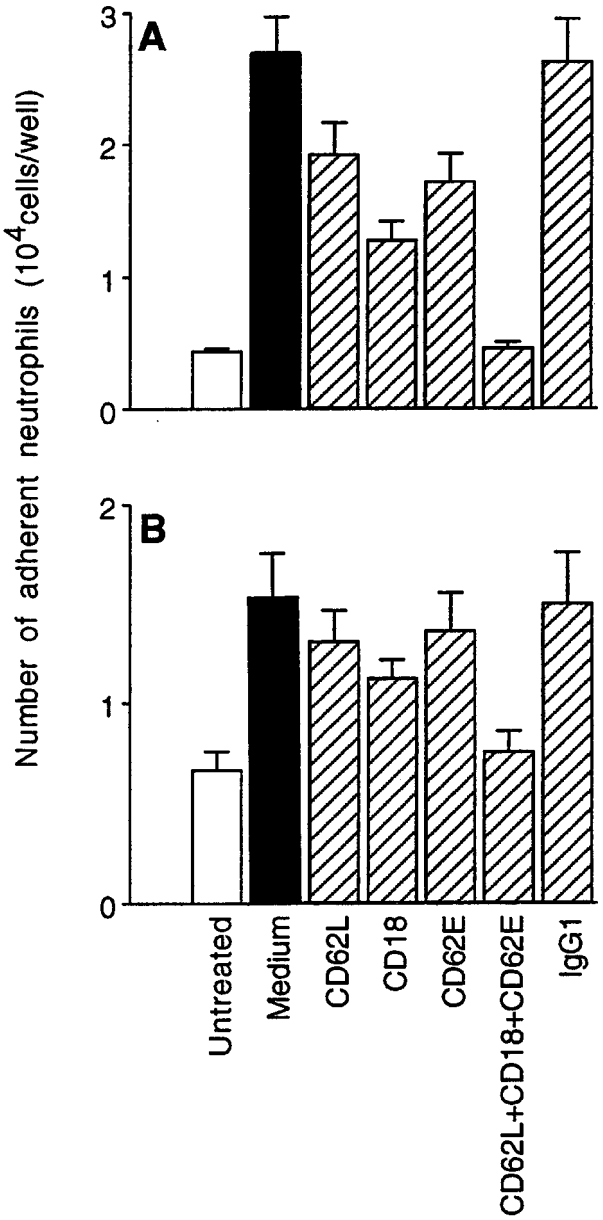

Figure 2 Inhibition of endothelin-1 (ET-1)-stimulated neutrophil attachment to coronary artery endothelial cells (HCAEC) by function-blocking monoclonal antibodies (mAbs) directed against E-selectin (CD62E) expressed on HCAEC, and against L-selectin (CD62L) and CD18 expressed by neutrophils. Radiolabelled neutrophils were added to (A) untreated or lipopolysaccharide (1 μg ml^{-1})-activated HCAEC or (B) ET-1 (100 nM)-activated HCAEC and incubated for 30 min at 37°C in the absence (medium) or presence of mAbs. The specificity of mAbs is indicated on the X-axis. The irrelevant mAb MOPC-21 (IgG_1) was used as a negative control. Results are expressed as mean ± s.e.mean of three experiments in triplicate using neutrophils from different donors.

for each staining. Immunofluorescence of HCAEC was then analysed with a FACScan flow cytometer.

Radioligand binding studies

Isolated neutrophil granulocytes (2×10^6 cells per ml) were resuspended in HBSS supplemented with 2 μM leupeptin, 1 μM pepstatin A and 0.1 mM phenylmethylsulphonyl fluoride. ET receptor antagonists BQ 788 and FR 139317 were added 10 min before addition of 92 pM ^{125}I-labelled ET-1 (specific activity: 2200 Ci mmol^{-1}, New England Nuclear, Cambridge, MA, U.S.A.). For competition studies, ET-1 was mixed with [^{125}I]-ET-1 before addition to cells. Binding assays were performed at 4°C for 60 min. Free and bound ligand were separated using a silicone oil centrifugation method (Filep & Földes-Filep, 1989). Pellets were solubilized with 0.5 ml 2% Triton X-100 and radioactivity was measured using a Wallac 1470 Wizard Automatic Gamma Counter (Turku, Finland). ET-1 binding to HCAEC was performed in accordance to the method of Fujitani *et al.* (1992). In brief, confluent monolayers of HCAEC in 24-well plates were cultured in the absence or presence of phosphoramidon (4 mM) for 2 days. Following extensive washing with HBSS, the cells were incubated in HBSS with [^{125}I]-ET-1 (50 pM) and the desired concentrations of unlabelled ET-1, BQ 788 and FR 139317. After incubation at 37°C for 60 min, which was sufficient to reach maximum binding, cells were washed with HBSS, harvested into 0.1 N NaOH and the cell associated radioactivity was counted. In all assays, non-specific binding was determined in the presence of 100 nM unlabelled ET-1. Specific binding was calculated as total binding minus nonspecific binding. Binding data were analysed with the EBDA-LIGAND software (Elsevier Biosoft Cambridge, U.K.) (Munson & Rodbard, 1980).

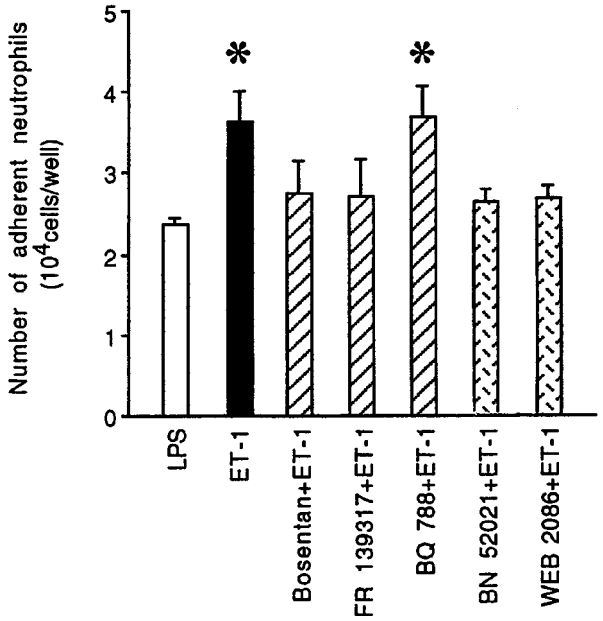

Figure 3 Inhibition of neutrophil adhesion to LPS-activated human coronary artery endothelial cells (HCAEC) by endothelin and platelet-activating factor receptor antagonists. Neutrophils preincubated with one of the antagonists for 10 min were cultured with lipopolysaccharide (LPS, 1 μg ml^{-1})-activated HCAEC in the presence of medium (LPS) or ET-1 (100 nM) for 30 min at 37°C. The following antagonists were used: bosentan (10 μM), FR 139317 (1 μM), BQ 788 (1 μM), BN 52021 (50 μM) and WEB 2086 (10 μM). Results are mean ± s.e.mean for three experiments performed in triplicate using neutrophils from different donors. *$P<0.05$ vs LPS.

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney *U*-test. P values <0.05 were considered significant for all tests.

Results

Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells

Only a few neutrophils were able to bind to unstimulated HCAEC. Neutrophil adherence was enhanced 3.6 fold by activation of HCAEC with LPS (Figure 1A). ET-1 produced further, concentration-dependent increases in the number of adhering neutrophils onto LPS-activated HCAEC (Figure 1A). ET-1 did not enhance neutrophil adhesion to unstimulated HCAEC. However, when neutrophils were added to HCAEC cultured with ET-1 (100 nM) for 6 h, on average 1.8 fold more neutrophils adhered to stimulated than to unstimulated HCAEC (Figure 1B). The number of adhering neutrophils was further enhanced when the adhesion assay was performed in the presence of ET-1

Table 1 E-selectin and ICAM-1 expression on human coronary artery endothelial cells challenged with ET-1 and LPS

	E-selectin		*ICAM-1*	
	MFI	*Positive cells* (%)	*MFI*	*Positive cells* (%)
Control	1.7 ± 0.2	4.1 ± 0.2	28.5 ± 6.1	57.9 ± 5.3
LPS, 1 μg ml^{-1}	43.9 ± 7.1**	81.0 ± 2.1**	364.0 ± 18.4**	96.6 ± 0.7*
ET-1, 10^{-9} M	3.1 ± 0.3	5.0 ± 0.7	31.7 ± 8.2	52.8 ± 6.1
ET-1, 10^{-8} M	4.3 ± 0.6*	7.2 ± 0.5	44.5 ± 7.5*	63.3 ± 2.5
ET-1, 10^{-7} M	5.0 ± 0.5*	8.0 ± 0.6*	68.5 ± 4.8*	70.0 ± 2.7*
ET-1, 10^{-6} M	4.7 ± 0.7*	7.3 ± 0.5*	64.4 ± 2.5*	70.7 ± 2.8*
BQ 788 + ET-1, 10^{-7} M	2.4 ± 0.1#	7.2 ± 0.4	37.0 ± 4.3#	68.7 ± 1.9
FR 139317 + ET-1, 10^{-7} M	4.7 ± 0.4	7.6 ± 0.4	68.8 ± 4.8	67.9 ± 2.1

Monolayers of HCAEC were cultured with LPS or ET-1 for 4 h at 37°C in the absence or presence of BQ 788 (1 μM) or FR 139317 (1 μM). E-selectin and ICAM-1 expression was assessed by flow cytometry following detachment and staining of the cells with fluorescent dye-labelled monoclonal antibodies. Antibody staining is expressed as mean fluorescence intensity (MFI) after subtracting nonspecific immunostaining. Positive cells represent the percentage of the total cells which stained positive for the indicated adhesion molecule. Values are expressed as mean ± s.e.mean of 3–4 independent measurements. *$P<0.05$; **$P<0.01$ vs control (i.e. cells cultured in medium only); #$P<0.05$ vs ET-1, 10^{-7} M.

(1.17 ± 0.05 vs $1.53 \pm 0.22 \times 10^4$ adherent neutrophils per well, $n=3$, $P<0.05$) (Figure 1B). These results indicate that ET-1 by itself is capable of enhancing neutrophil adhesion to HCAEC.

Since multiple receptors are involved in neutrophil adhesion to LPS-stimulated HCAEC even under nonstatic conditions (Spertini *et al*., 1991; Zouki *et al*., 1997), we assayed the contribution of L-selectin, CD18 integrins and E-selectin to the ET-1-stimulated binding interaction. A significant proportion of neutrophil-HCAEC attachment was blocked by mAb binding to E-selectin ($36 \pm 2\%$, $n=3$), L-selectin ($29 \pm 2\%$) and CD18 ($45 \pm 5\%$) (Figure 2A). The combination of these mAbs inhibited the neutrophil adhesion by $\sim 83\%$ (Figure 2A). ET-1-stimulated neutrophil adhesion to ET-1-activated HCAEC was blocked by mAb binding to E-selectin ($25 \pm 3\%$, $n=3$), L-selectin ($28 \pm 2\%$) and CD18 ($39 \pm 2\%$) (Figure 2B). The combination of these three mAbs inhibited the neutrophil adhesion by $\sim 70\%$ (Figure 2B).

Inhibition of neutrophil-endothelial attachment by ET_A receptor and platelet-activating factor receptor antagonists

Preincubation of neutrophils with the selective ET_A receptor antagonist, FR 139317 (1 μM) or with the ET_A/ET_B receptor antagonist, bosentan (10 μM) markedly attenuated the ET-1-stimulated neutrophil adherence to LPS-activated HCAEC (Figure 3). No significant difference could be detected between the degree of inhibition with FR 139317 and bosentan. Furthermore, the ET_B receptor selective antagonist BQ 788 (1 μM) had no detectable effect on the number of adhering neutrophils (Figure 3), further indicating ET_A receptors as the relevant ET receptor subtype responsible for the adhesion enhancing action of ET-1. Since ET-1 induces the synthesis and release of PAF from neutrophils (Gómez-Garré *et al*., 1992), we next investigated the role of PAF in mediating ET-1-induced neutrophil adherence. The PAF receptor antagonists BN 52021 (50 μM) and WEB 2086 (10 μM) significantly attenuated ET-1-stimulated neutrophil adherence (Figure 3). The degree of inhibition observed with BN 52021 or WEB 2086 did not differ from those seen with FR 139317 or bosentan. The vehicle of these antagonists had no effect on the adhesion (data not shown).

Endothelin-1 induces E-selectin and ICAM-1 expression via ET_B receptors

The expression of adhesion molecules by treated and untreated HCAEC was quantitated by flow cytometry analysis. Under our experimental conditions, approximately 60 and 4% of untreated HCAEC expressed ICAM-1 and E-selectin, respectively (Table 1). Treatment of HCAEC for

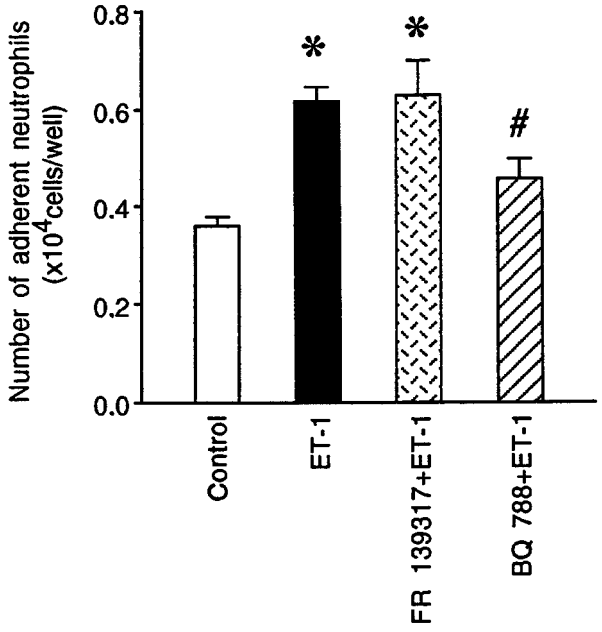

Figure 4 Effects of endothelin receptor antagonists on adhesion of neutrophils to human coronary artery endothelial cells (HCAEC) cultured with ET-1. HCAEC were left unstimulated (control) or activated with ET-1 (100 nM) for 6 h in the absence (ET-1) or presence of the ET_A-selective antagonist FR 139317 (1 μM) or the ET_B-selective antagonist BQ 788 (1 μM). Radiolabelled unstimulated neutrophils were then added and incubated with HCAEC for 30 min at 37°C. Values are expressed as mean ± s.e.mean of three experiments in triplicate using neutrophils from different donors. $*P<0.05$ vs control: $\#P<0.05$ vs ET-1.

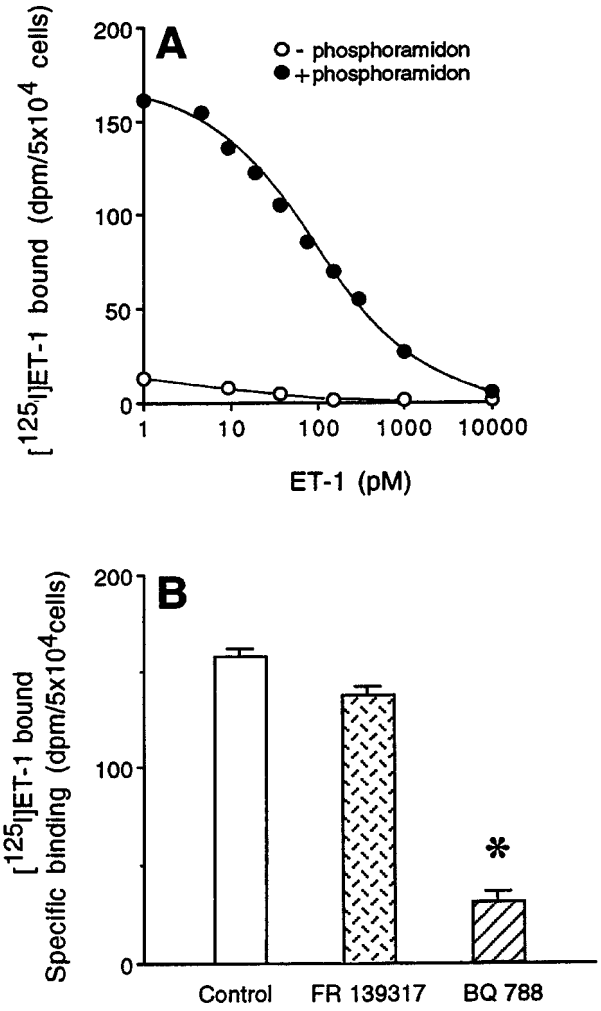

Figure 5 Endothelin receptor expression by human coronary artery endothelial cells (HCAEC). (A) Competitive binding of 50 pM [^{125}I]-ET-1 to HCAEC cultured in the absence (open circles) and presence of phosphoramidon (closed circles). Phosphoramidon was added at a concentration of 4 mM for 48 h. (B) Specific binding of 90 pM [^{125}I]-ET-1 to phosphoramidon (4 mM for 48 h)-treated HCAEC in the absence (control) and presence of FR 139317 (100 nM) or BQ 788 (100 nM). Nonspecific binding was determined in the presence of 100 nM ET-1. Results are expressed as mean ± s.e.mean for three experiments performed in duplicate. $*P<0.05$ vs control.

4 h with ET-1 increased the overall expression of E-selectin and ICAM-1 in a concentration-dependent fashion, apparent maximum changes were observed at 10^{-7} M (Table 1). Increasing the concentration of ET-1 did not further enhance adhesion molecule expression. ET-1 treatment also increased the percentage of cells expressing these adhesion molecules (Table 1). As a positive control, LPS treatment evoked approximately 26 and 13 fold increases in E-selectin and ICAM-1 expression, respectively, and 81 and 97% of cells stained positive for these adhesion molecules, respectively (Table 1). The ET-1 (10^{-7} M)-induced increases in E-selectin and ICAM-1 expression were effectively inhibited by BQ 788 (1 μM), whereas FR 139317 (1 μM) had no detectable effects (Table 1). Consistently, the increases in the number of adherent neutrophils following activation of HCAEC with ET-1 were prevented by treatment with BQ 788, but not by FR 139317 (Figure 4).

Endothelin-1 binding to human coronary artery endothelial cells

Receptor binding studies were performed to characterize the ET receptors expressed on HCAEC. The specific binding of [^{125}I]-ET-1 to untreated HCAEC was too low to allow competition experiments (Figure 5). Treatment of the cells with phosphoramidon for 2 days increased the maximal specific binding by 12 fold (Figure 5A). Previous studies have shown that phosphoramidon does not effect the affinity of ET-1 (Fujitani *et al*., 1992; Clozel *et al*., 1993). Unlabelled ET-1 displaced specific [^{125}I]-ET-1 binding with an IC_{50} value of 90 pM (Figure 5A). The estimated dissociation constant (K_D) and maximum binding (B_{max}) were 40 pM and 0.06 fmol per 5×10^4 cells, respectively. Specific binding of [^{125}I]-ET-1 was markedly reduced in the presence of BQ 788, whereas ET_A receptor blockade with FR 139317 was without effect (Figure 5B), indicating that HCAEC predominantly express ET_B receptors.

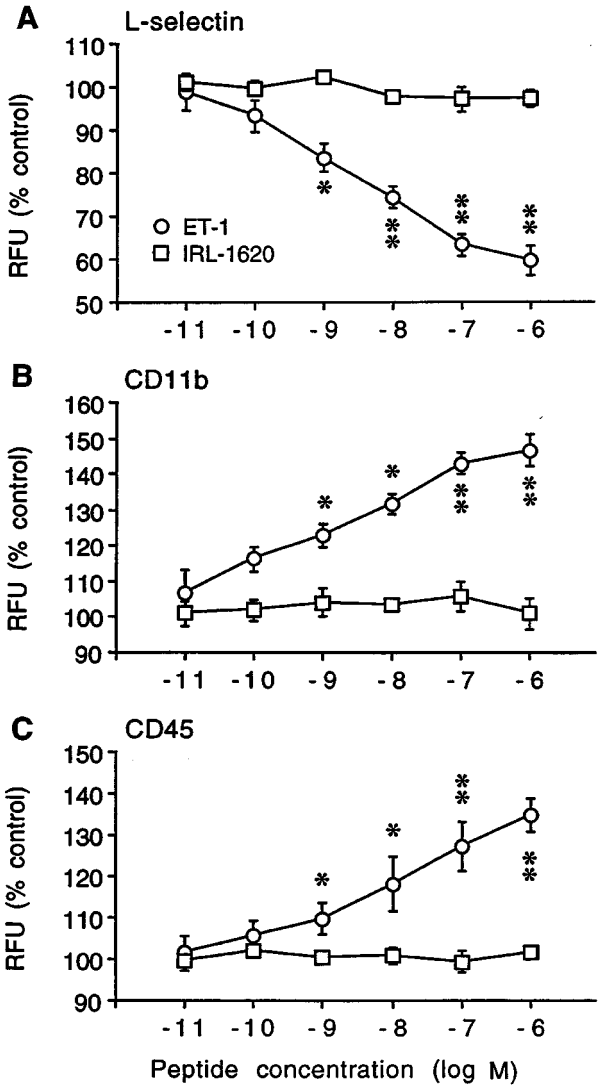

Figure 6 Concentration-dependent effect of endothelin-1 (ET-1) on surface expression of L-selectin (A), CD11b (B) and CD45 (C) on neutrophil granulocytes. Aliquots of whole blood were challenged with ET-1 or IRL-1620 for 30 min at 37°C. Fluorescence intensity (relative fluorescence unit, RFU) is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils cultured in medium only. Results are expressed as mean ± s.e.mean for 5–6 experiments using blood from different donors. *$P<0.05$; **$P<0.01$ vs control.

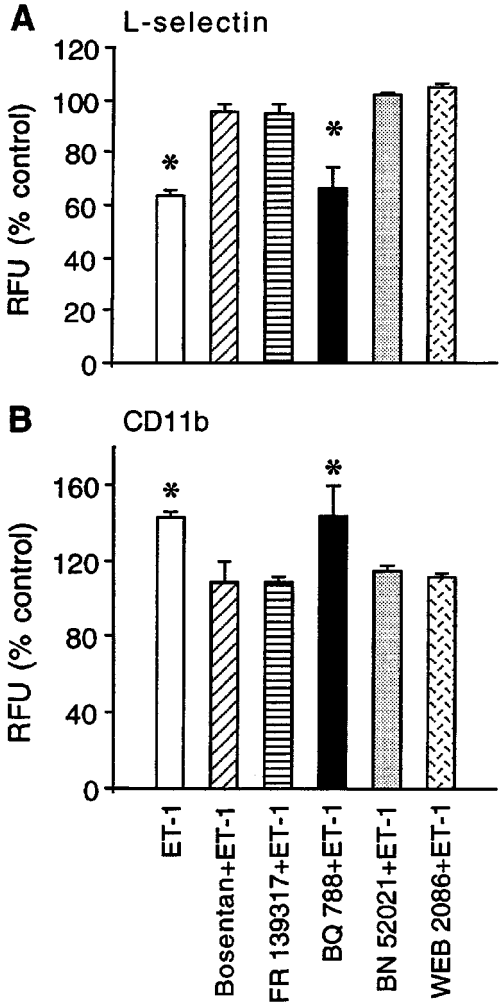

Figure 7 Inhibition of endothelin-1-induced downregulation of L-selectin (A) and upregulation of CD11b expression (B) by endothelin and platelet-activating factor receptor antagonists. Whole blood aliquots were preincubated with one of the antagonists for 10 min and then challenged with 100 nM ET-1 for 30 min at 37°C. Fluorescence intensity (relative fluorescence unit, RFU) is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils cultured in medium only. Results are expressed as mean ± s.e.mean for 5–6 experiments using blood from different donors. *$P<0.05$ vs control.

Table 2 Granule enzyme release from human neutrophils by endothelin-1 (ET-1)

	Enzyme release		
	Gelatinase	*β-Glucuronidase*	*Lysozyme*
Control	18.1±2.0	2.6±0.3	5.8±0.5
PAF, 1 μM	59.1±3.0*	2.7±0.5	6.4±0.7
ET-1, 1000 nM	37.0±1.7*	2.5±0.4	6.1±0.6
ET-1, 100 nM	31.6±1.9*	2.8±0.4	5.9±0.7
ET-1, 10 nM	27.7±2.4*	2.7±0.5	6.0±0.6
ET-1, 1 nM	19.8±1.8	2.5±0.4	5.9±0.4
Bosentan+ET-1, 100 nM	20.5±1.9	2.8±0.3	5.7±0.5
FR 139317+ET-1, 100 nM	21.4±2.3	2.9±0.3	5.9±0.7
BQ 788+ET-1, 100 nM	33.3±1.0*	3.0±0.4	6.3±0.8
BN 52021+ET-1, 100 nM	21.9±1.4	2.9±0.5	6.2±0.7
WEB 2086+ET-1, 100 nM	21.4±1.2	2.7±0.4	6.3±0.6

Values are expressed as percentage of total cellular enzyme activity released by neutrophils to the culture medium after incubation for 30 min at 37°C with ET-1 or platelet-activating factor (PAF) in the absence and presence of one of the following inhibitors: bosentan (10 μM), FR 139317 (1 μM), BQ 788 (1 μM), BN 52021 (50 μM) and WEB 2086 (10 μM). Values are means±s.e.mean of six independent experiments. *$P<0.05$ vs control (unstimulated neutrophils).

Endothelin-1 regulates expression of L-selectin and CD18 on neutrophils through activation of ET_A receptor and platelet-activating factor

Flow cytometry analysis of whole blood challenged with ET-1 showed that the neutrophil surface expression of L-selectin was downregulated (Figure 6A), whereas expression of CD11b was upregulated by ET-1 in a concentration-dependent fashion (Figure 6B) with an apparent EC_{50} concentration of ~2 nM. Similar increases were detected in CD18 expression (data not shown). No significant changes were detected with IRL-1620 over the concentration range studied (Figure 6A and B). The maximum changes that could be achieved with ET-1 were smaller than those evoked by 1 μM PAF (40±4 and 57±4% decreases in L-selectin expression by ET-1 and PAF, respectively; $n=6$, $P<0.05$; 47±4 vs 73±7% increase in CD11b expression by ET-1 and PAF, $P<0.05$). Furthermore, ET-1 also upregulated expression of CD45 (Figure 6C), indicating neutrophil activation.

None of the receptor antagonists used in this study on their own had an effect on expression of adhesion molecules on resting neutrophils. Both FR 139317 and bosentan markedly attenuated ET-1 (100 nM)-induced changes in L-selectin and CD11b expression, whereas BQ 788 had no detectable effects (Figure 7). The inhibitory actions of FR 139317 and bosentan did not differ significantly (Figure 7). Downregulation of L-selectin and upregulation of CD11b expression by ET-1 were also inhibited by the PAF receptor antagonists, BN 52021 and WEB 2086 (Figure 7).

Endothelin-1-induced granule enzyme release

To provide further evidence for neutrophil activation by ET-1, we studied degranulation of isolated neutrophils in response to ET-1. ET-1 did not induce release of β-glucuronidase (a marker for azurophil granules) or lysozyme (a marker for specific plus azurophil granules), whereas it evoked gelatinase release (a marker for tertiary granules) in a concentration-dependent fashion (Table 2). Similar changes were observed with PAF, albeit PAF released higher amounts of gelatinase than those observed with ET-1. The ET-1-induced gelatinase release was inhibited by FR 139317, bosentan, BN 52021 and WEB 2086, but not by BQ 788 (Table 2). IRL-1620 did not induce gelatinase release (data not shown). Lactate dehydrogenase release never exceeded 3% of the total cell content (data not shown), indicating that neutrophil integrity was not affected.

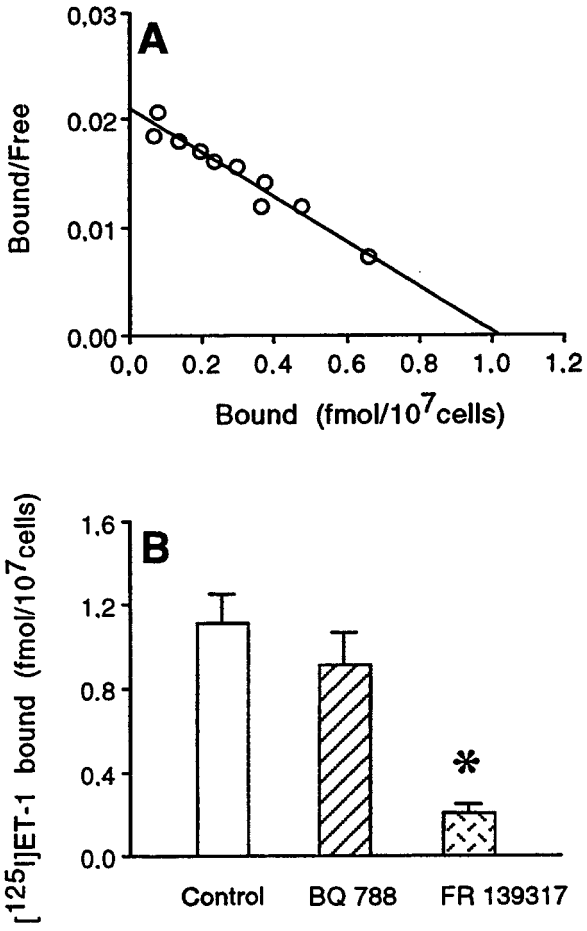

Figure 8 Endothelin receptor expression by human neutrophils. (A) Scatchard analysis of the specific binding of [^{125}I]-ET-1 to neutrophils. Specific binding was calculated as the difference between total binding and binding in the presence of 100 nM ET-1 (non-specific binding). This is a representative result for six experiments. (B) Specific binding of [^{125}I]-ET-1 to neutrophils was evaluated in the absence (control) and presence of the ET_A-selective antagonist FR 139317 (100 nM) or the ET_B-selective antagonist BQ 788 (100 nM). Nonspecific binding was determined in the presence of 100 nM ET-1 and represent approximately 10–15% of total binding. Values are mean±s.e.mean for seven experiments. *$P<0.05$ vs control.

Endothelin-1 binding to neutrophil granulocytes

Scatchard analysis of ET-1 displacement of [^{125}I]-ET-1 binding to neutrophils indicated a single class of high-affinity binding sites with a K_D value of 38±5 pM and a maximum binding of 1.03±0.08 fmol per 10^7 cells ($n=6$, Figure 8A). Hill coefficients calculated with the LIGAND program were 0.97±0.03 ($n=6$). Specific binding of [^{125}I]-ET-1 was reduced markedly in the presence of the ET_A-selective antagonist FR 139317. In contrast, the ET_B-selective antagonist BQ 788 did not inhibit [^{125}I]-ET-1 binding (Figure 8B). These data

indicate predominant expression of ET_A receptors on human neutrophils.

Discussion

The novel finding of the present study relevant to the role of ET-1 in regulating leukocyte-endothelial interaction is that ET-1 promotes neutrophil adhesion to HCAEC predominantly through activation of ET_A receptors and subsequent release of PAF. This adhesion enhancing action can be attributed to the effects of ET-1 on neutrophils, and, to a lesser extent, to effects on the endothelium and involves multiple adhesion molecules, L-selectin, E-selectin and CD18 integrins.

Our study showed that ET-1 markedly enhances neutrophil adhesion to HCAEC stimulated with either LPS or ET-1, whereas it slightly increased neutrophil adherence to non-activated HCAEC and to feline coronary artery segments (Murohara & Lefer, 1996). López-Farré *et al*. (1993) reported that 30 min of incubation of bovine endothelial cells alone with ET-1 had no stimulatory effect on neutrophil adhesion. In our study, culture of HCAEC alone with ET-1 for 4–6 h resulted in increases in the expression of the endothelial adhesion molecules, E-selectin and ICAM-1, and significantly increased the number of adhering neutrophils. Therefore, it is conceivable that, like with other stimuli, such as LPS, induction by ET-1 of expression of ICAM-1 and E-selectin may require a long exposure time. However, ET-1 is a less potent activator of HCAEC than LPS, as evidenced by analysis of E-selectin and ICAM-1 expression and adhesion assays. Since in the adhesion assays, neutrophils were incubated with activated HCAEC in the presence of ET-1 for 30 min, stimulation of neutrophil adhesion by ET-1 can primarily be attributed to the effects of this peptide on neutrophils. No adhesion experiments were performed with neutrophils alone preincubated with ET-1 since ET-1 may induce neutrophil aggregation (Gómez-Garré *et al*., 1992; López-Farré *et al*., 1995), therefore making interpretation of the results difficult.

Leukocyte-endothelial cell interaction involves a complex interplay among adhesion molecules (Butcher, 1991; Springer, 1994). Indeed, the experiments with function-blocking mAbs revealed the involvement of CD18 integrins, E-selectin and L-selectin in mediating ET-1-induced adhesion of neutrophils to activated HCAEC. However, the relative contribution of these adhesion molecules varied when HCAEC was stimulated with LPS or ET-1. Thus, neutrophil adhesion to ET-1-stimulated HCAEC was only slightly inhibited by the anti-E-selectin mAb. This is consistent with the observation that 4 h incubation of HCAEC with ET-1 increased E-selectin expression only in 8% of cells compared to 71% cells positive for ICAM-1, the counterreceptor for CD18 integrins. Increases in both the intensity of staining for adhesion molecules and the percentage of HCAEC expressing E-selectin and ICAM-1 indicate that in addition to upregulating the expression on cells already expressing E-selectin and ICAM-1, ET-1 also induced expression of these adhesion molecules on previously negative cells. The findings that ET-1 increases E-selectin expression on about 20% of human brain microvascular endothelial cells (McCarron *et al*., 1993) suggest the existence of regional differences in the ET-1 induction of adhesion molecule expression. The marked difference in the degree of inhibition of neutrophil adhesion with anti-CD18 mAb to HCAEC (40–50% inhibition) and to bovine endothelial cells (80–90% inhibition) (López-Farré *et al*., 1993), most likely indicates species differences in the role of CD18 integrins in adhesion.

Consistent with previous results with human umbilical vein endothelial cells (Fujitani *et al*., 1992; Clozel *et al*., 1993), we also found that ET receptors on HCAEC became detectable by binding assay only after pretreatment of the cells with phosphoramidon, an inhibitor of ET-1 converting enzyme (Ikegawa *et al*., 1991). Thus, it is plausible to assume that autocrine production of ET-1 by HCAEC decreases, either by binding or by downregulation, the number of ET-1 binding sites. Although the limited availability of HCAEC did not allow us to perform binding studies with the ET isopeptides, our results indicate the presence of a single class of high affinity ET receptor on HCAEC. The selective inhibition of [^{125}I]-ET-1 binding in the presence of BQ 788, but not FR 139317, is consistent with constitutive expression of ET_B receptors on HCAEC. Furthermore, the ET-1 induced expression of E-selectin and ICAM-1 was mediated through ET_B receptor activation, for this was also selectively blocked by BQ 788.

The present study showed that ET-1 at concentrations two order of magnitude lower than those used in the study of López-Farré *et al*. (1993) is capable of upregulating CD11b expression on neutrophils. Furthermore, at nanomolar concentrations, ET-1 downregulated L-selectin expression, upregulated CD45 expression on neutrophils and released gelatinase from tertiary granules, reflecting neutrophil activation. However, ET-1 up to a concentration of 1 μM did not induce β-glucuronidase and lysozyme release. Because the most readily mobilizable store of CD11b/CD18 is in a granule distinct from the classic azurophil and secondary granules Borregaard *et al*., 1987), but may be associated with tertiary granules, upregulation of CD18 can occur without degranulation of azurophil and specific granules. Furthermore, ET-1 (over the range of 1 pM–1 μM) does not induce respiratory burst and superoxide production (Ishida *et al*., 1990). A possible interpretation of these apparently discordant observations is that ET-1 may function as a quite selective neutrophil agonist, i.e. it is capable of enhancing adhesive properties of neutrophils without major increases in respiratory burst and superoxide production.

The actions of ET-1 on neutrophil-HCAEC adhesion and neutrophil expression of L-selectin and CD11b, and gelatinase release were significantly inhibited by the selective ET_A receptor antagonist FR 139317, but not by the ET_B receptor antagonist BQ 788, and were not mimicked by selective ET_B receptor stimulation with IRL-1620. These findings clearly point to the ET_A receptor as being the relevant receptor subtype responsible for these actions of ET-1. Indeed, receptor binding studies revealed the presence of a single class of high affinity ET-1 binding site on neutrophils. The observations that ET-1 binding was inhibited in the presence of FR 139317, whereas it was insensitive to BQ 788 are consistent with predominant expression of ET_A receptors by human neutrophils. Previous studies on experimental animals also showed that ET_A receptor activation augments leukocyte trafficking into tissues (Filep *et al*., 1995b; Khimenko *et al*., 1996). Further insight into the characteristics of the ET-1 effect was obtained by studying the involvement of PAF. Previous studies have suggested that the effects of ET-1 on microvascular permeability (Filep *et al*., 1991), renal mesangial cell contractility (López-Farré *et al*., 1991) and homotypic aggregation of human neutrophils (Gómez-Garré *et al*., 1992; López-Farré *et al*., 1995) are related to an increase in PAF synthesis by those cells. Indeed, ET-1 stimulates both the synthesis and release of PAF from human neutrophils (Gómez-Garré *et al*., 1992) and rat brain microvessels (Catalán

et al., 1996). The ET-1-induced augmentation of neutrophil adhesion to HCAEC can be blocked by BN 52021 and WEB 2086, two potent and specific PAF receptor antagonists (Braquet *et al*., 1985; Casals-Stenzel *et al*., 1987) to a similar extent as observed with ET_A receptor blockade. Moreover, exogenous PAF produced similar changes as those observed with ET-1. Therefore, it appears that PAF plays a pivotal role in mediating the proadhesive action of ET-1. These findings provide further support to the notion that PAF, an autocrine/paracrine regulator of neutrophil activation, also functions as a signal for neutrophils to bind to the endothelium (Zimmerman *et al*., 1990; Kuijpers *et al*., 1991).

Recent studies showed that ET-1 may also exert an antiadhesive action. Thus, short-term (10 min) incubation of feline coronary artery segments with ET-1 was found to inhibit adhesion of neutrophils to thrombin-stimulated endothelium (Murohara & Lefer, 1996). This is a result of inhibition of P-selectin-mediated leukocyte-endothelial interaction by nitric oxide released upon activation of endothelial ET_B receptors (Murohara & Lefer, 1996). In our neutrophil-HCAEC binding assay, P-selectin-dependent adhesion was not studied, since P-selectin expression occurs within 10–20 min after application of inflammatory stimuli and is sustained for about 60 min (Weyrich *et al*., 1995). Autocrine production of nitric oxide by human monocytes triggered by ET_B receptor activation has also been reported to reduce monocyte adhesion to the endothelium (King *et al*., 1997). Based on these and the present results, we propose a model in which ET-1 exerts a dual action on neutrophil adhesion; a transient antiadhesive action, which is mediated through ET_B receptor-coupled NO production and a proadhesive action which is predominantly mediated through ET_A receptor activation-induced changes in surface expression of adhesion molecules on neutrophils. In addition, ET-1 could also upregulate expression of E-selectin and ICAM-1 by HCAEC through the activation of ET_B receptors, as discussed above. These may also lead to an increase in the number of adherent neutrophils. However, only a small portion of neutrophil attachment could be attributed to ET_B-receptor-mediated adhesion under our assay conditions. By contrast, activation of the ET_B receptor on rat aortic endothelial cells markedly enhanced neutrophil attachment (Hayasaki *et al*., 1996). It is possible that short-term and long-term exposure of endothelial cells to ET-1 might effect endothelial adhesiveness differently, although the underlying mechanisms remain to be investigated.

The ET-1 concentrations required to evoke significant increases in neutrophil-endothelial adhesion were higher than those detected in the venous plasma or coronary sinus (1–23 fmol ml^{-1}) of patients with acute ischaemic coronary artery diseases (Yasuda *et al*., 1990; Matsuyama *et al*., 1991; Ray *et al*., 1992). However, local ET-1 levels might be even higher than those detected in the plasma. Neutrophils themselves can also influence local levels of ET-1, either by increasing (i.e. enhanced conversion of big ET-1 to ET-1) or decreasing (increased degradation of ET-1) the amount of bioactive ET-1, depending on their state of activation (Caramelo *et al*., 1997). Our observation that ET-1 at concentrations as low as 0.1–1 nM, is capable of inducing changes in expression of adhesion molecules on neutrophils would suggest that local ET-1 concentrations might be sufficiently high to promote neutrophil adhesion. The close association of elevated plasma ET-1 levels and increased neutrophil infiltration in vascular lesions in skin biopsies from patients with mixed connective tissue disease (Filep *et al*., 1995a) would lend further support to this notion.

In conclusion, the present study documents the existence of ET_A receptors on neutrophils and the expression of ET_B receptors on HCAEC. Our results indicate that ET_A receptor-coupled PAF production and release by human neutrophils may provide a novel mechanism for autocrine or paracrine regulation of adhesion, resulting in augmented neutrophil adherence to HCAEC. These data taken together with rapid release of ET-1 from the sites of vascular injury (see Rubanyi & Polokoff, 1994), and the role of PAF in strengthening neutrophil adhesion to the endothelium (Zimmerman *et al*., 1990; Kuijpers *et al*., 1991) may provide important mechanisms for regulation of neutrophil-endothelial interactions at sites of vascular injury. Endothelin antagonists may therefore be of therapeutical value to reduce neutrophil accumulation and consequently tissue damage in myocardial diseases associated with elevated ET-1 production.

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