British Journal of Pharmacology (2002) 135, 1167–1174

www.nature.com/bjp

Extracellular signal-regulated kinase plays an essential role in endothelin-1-induced homotypic adhesion of human neutrophil granulocytes

[1]Levente József, [1]Tarek Khreiss, [2]Alain Fournier, [3]John S.D. Chan & *,[1]János G. Filep

[1]Research Center, Maisonneuve-Rosemont Hospital and Department of Medicine, University of Montréal, Montréal, Québec, Canada H1T 2M4; [2]INRS-Institut Armand-Frappier, Pointe-Claire, Québec, Canada H9R 1G6 and [3]CHUM-Hôtel-Dieu, Centre de Recherche, Pavillon Masson, University of Montréal, Montréal, Québec, Canada H2W 1T8

1 Endothelin-1 (ET-1) stimulates integrin-dependent adhesion of neutrophil granulocytes to endothelial cells, one of the early key events in acute inflammation. However, the signalling pathway(s) of ET-1-stimulated neutrophil adhesive responses has not been elucidated. Previous studies indicated that extracellular signal-regulated kinase (ERK) activation could mediate rapid responses of neutrophil granulocytes to various stimuli. In this study, we investigated the role of ERK signalling in human neutrophil granulocytes challenged with ET-1.

2 ET-1 rapidly down-regulated the expression of L-selectin and up-regulated the expression of CD11b/CD18 on the neutrophil surface. Concomitantly, ET-1 induced homotypic adhesion (aggregation) of neutrophils, that was blocked by a monoclonal antibody to CD18.

3 ET-1, through ET_A receptors, evoked activation of Ras and subsequent phosphorylation of Raf-1, mitogen-activated protein kinase kinase (MAPK/ERK kinase) and ERK 1/2. ERK activation by ET-1 was rapid, concordant with the kinetics of ET-1-stimulated neutrophil aggregation.

4 Neutrophil responses to ET-1 were markedly attenuated by the MAPK/ERK kinase inhibitor PD98059, whereas inhibitors of p38 MAPK, tyrosine kinases and phosphatidylinositol 3-kinase had no detectable effects. We have observed a tight correlation between neutrophil ERK activation and homotypic adhesion.

5 These data indicate an essential role for ERK in mediating ET-1-stimulated adhesive responses of human neutrophil granulocytes.

British Journal of Pharmacology (2002) **135,** 1167–1174

Keywords: Leukocytes; endothelin-1; ETA receptor; mitogen-activated protein kinase; extracellular signal-regulated kinase; adhesion molecules; integrins; neutrophil aggregation; inflammation

Abbreviations: ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FITC, fluorescein-isothiocyanate; mAb, monoclonal antibody, MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PAF, platelet-activating factor; PBS, phosphate buffered saline; RFU, relative fluorescence units

Introduction

Neutrophil activation and migration into tissues are critical events in the host response to infection and tissue injury (Malech & Gallin, 1987). Considerable evidence accumulated during the past decade indicates that the vasoactive peptide endothelin-1 (ET-1) (Yanagisawa *et al*., 1988) possesses potent pro-inflammatory actions (Filep & Hay, 1999) including autocrine/paracrine modulation of neutrophil functions. ET-1 down-regulates surface expression of L-selectin (Zouki *et al*., 1999), up-regulates expression of CD11b/CD18 on human neutrophils (López-Farré *et al*., 1993; Zouki *et al*., 1999), promotes neutrophil aggregation (homotypic adhesion) (Gómez-Garré *et al*., 1992; López-Farré *et al*., 1995), and β_2-integrin-dependent attachment of neutrophils to cultured human and bovine vascular endothelial cells (heterotypic cell adhesion) (Zouki *et al*., 1999; López-Farré *et al*., 1993). ET-1 causes a selective neutrophil leukocytopenia in guinea-pigs (Filep *et al*., 1995) and induces neutrophil accumulation in the isolated perfused heart (López-Farré *et al*., 1993), lung (Helset *et al*., 1996; Khimenko *et al*., 1996), kidney (Espinosa *et al*., 1996), and mesenterium (Boros *et al*., 1998) predominantly *via* activation of ET_A receptors (Filep *et al*., 1995; Khimenko *et al*., 1996; Boros *et al*., 1998). 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). Of interest, ET-1 alone fails to stimulate superoxide generation (Ishida *et al*., 1990), suggesting a rather selective degree of neutrophil stimulation instead of a massive activation as observed with neutrophil chemoattractants. While these studies clearly document ET-1 activation of neutrophils, the underlying molecular mechanisms that couple the ET-1 signal initiated at the cell membrane to the functional responses remain poorly understood.

The extracellular signal-regulated kinases 1/2 (ERK 1/2) are serine/threonine protein kinases that, in mitotic cells, are involved in the regulation of cell growth and differentiation (Cobb *et al*., 1994). ERK activation plays an essential role in ET-1-induced cardiomyocyte hypertrophy (Yue *et al*., 2000)

*Author for correspondence at: Research Center, Maisonneuve-Rosemont Hospital, 5415 boulevard de l'Assomption, Montréal, Québec, Canada H1T 2M4; E-mail: janos.g.filep@umontreal.ca

as well as in the anti-apoptotic action of ET-1 in vascular and prostatic smooth muscle cells (Wu-Wong *et al*., 2000; Shichiri *et al*., 2000). Circulating human neutrophils are post-mitotic and terminally differentiated. Studies of mitogen-activated protein kinases (MAPKs) in neutrophils have found an association between ERK stimulation by chemoattractants, arachidonic acid or peroxynitrite and neutrophil adhesive function (Thompson *et al*., 1993; Pillinger *et al*., 1996; Capodici *et al*., 1998; Zouki *et al*., 2001), but not superoxide generation (Yu *et al*., 1995). These studies also suggest that ERK activation is mediated, at least in part, by pathways similar to those initiated by protein tyrosine kinase receptors, i.e. through activation of Ras, Raf-1 and MAPK/ERK kinase (MEK) (Pillinger *et al*., 1996; Gardner *et al*., 1993; Worthen *et al*., 1994). Thus, ERK in neutrophils appears to have a specific, non-mitotic signalling function. In the present study, we tested the hypothesis that ET-1-stimulated neutrophil activation and adhesion are ERK-dependent. Our data indicate that ET-1 *via* ET_A receptors stimulates ERK, consistent with a role in rapid neutrophil responses. ET-1 stimulation of Erk was MEK dependent and was associated with activation of Ras and Raf-1. Finally, our data also demonstrate that the ERK pathway in neutrophils plays an essential role in the regulation of surface expression of adhesion molecules and adhesive responses of these cells to ET-1.

Methods

Isolation and activation of neutrophil granulocytes

Neutrophils were isolated from the peripheral blood obtained from non-smoking healthy volunteers (male and female, aged 23–49 years) by centrifugation through Ficoll-Hypaque gradients (Pharmacia Diagnostics AB, Uppsala, Sweden), sedimentation through dextran (3% w/v) and hypotonic lysis of red blood cells (Zouki *et al*., 1997). The resultant cell preparations contained >97% neutrophils. Isolated neutrophils were resuspended in a modified Hanks' balanced salt solution ((mM): NaCl 145; K_2PO_4 10; $CaCl_2$ 1.4; $MgCl_2$ 1.2; glucose 5 and bovine serum albumin 250 μg ml^{-1}, pH 7.4) and used within 30 min of preparation. Neutrophils were preincubated with one of the following antagonists for 10 min at 37°C: the ET_A receptor selective antagonist FR 139317 (1 μM, Fujisawa Pharmaceuticals Co., Osaka, Japan) (Aramori *et al*., 1993), the ET_B receptor selective antagonist BQ 788 (10 μM, Novabiochem Corp., San Diego, CA, U.S.A.) (Ishikawa *et al*., 1994), the MAPK kinase inhibitor PD 98059 (100 μM, Calbiochem, San Diego, CA, U.S.A.) (Dudley *et al*., 1995), the phosphatidylinositol 3-kinase inhibitor wortmannin (2 μM, Sigma Chemicals, St. Louis, MO, U.S.A.), the tyrosine kinase inhibitor genistein (100 μM, Sigma Chemicals) or the p38 MAPK inhibitor SB 203580 (1 μM, Calbiochem) (Cuenda *et al*., 1995) and then challenged with ET-1 for the indicated times at 37°C.

Flow cytometry analysis

Direct immunofluorescence labelling of control or treated neutrophil granulocytes was performed as described previously (Zouki *et al*., 1997). Leukocytes were stained with a 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-labelled anti-human CD18 mAb (IgG_1, BD Biosciences, Mountain View, CA, U.S.A.) or FITC-labelled anti-human CD11a mAb G-25.2 (IgG_1, BD Biosciences). Appropriately labelled, class-matched irrelevant mouse IgG_1 was used as a negative control for each staining. Single or double-colour immunofluorescence staining was analysed by a FACScan Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) with Lysis II software. The results are presented as relative fluorescence units (RFU): $RFU = (FU_{experimental} - FU_{isotype}) \times 100/(FU_{control} - FU_{isotype})$, where $FU_{experimental}$ and $FU_{control}$ are the L-selectin and CD11b mean fluorescence intensity of treated cells and cells cultured in medium only, respectively, and $FU_{isotype}$ is the mean fluorescence intensity of class-matched irrelevant antibody.

MAPK phosphorylation and activity assays

Neutrophils were lysed in ice-cold lysis buffer (mM): Tris 20; EGTA 1; Na_3VO_4 2; NaF 25; 0.5% Triton X-100; PMSF 2; aprotinin 40 μg ml^{-1} and 10 μg ml^{-1} each of chymostatin, leupeptin and pepstatin A, pH 7.4) for 15 min and centrifuged at 4°C for 10 min at $14.000 \times g$. Western blot analysis of phosphorylated MEK and ERK1/2 (p44/42 MAPK) was performed as in (Zouki *et al*., 2001) using the Phospho Plus MEK 1/2 and ERK1/2 MAP kinase antibody kits (New England Biolabs, Beverly, MA, U.S.A.). ERK1/2 activity was assayed following immunoprecipitation of ERK1/2 from the cell lysates with an immobilized anti-phospho-p44/42 MAP kinase antibody using Elk-1 fusion protein, a specific target for ERK 1/2 (Marais *et al*., 1993).

Raf-1 kinase assay

Raf-1 kinase activity was determined by a modification of the method of Gardner *et al*. (1994). In brief, Raf-1 was immunoprecipitated with an anti-Raf-1 antiserum (C-12, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), antigen-antibody complexes were then isolated by protein A-Sepharose CL-4B, and Raf-1 activity was measured using the Raf-1 Kinase Cascade Assay kit (Upstate Biotechnology, Lake Placid, NY, U.S.A.) in accordance with the manufacturer's protocol.

Ras activation assay

Activated $p21^{Ras}$ (Ras-GTP) from neutrophil lysates was affinity precipitated using GST-Ras binding domain of Raf-1 (residues 1–149) fusion protein conjugated to agarose (Upstate Biotechnology) as described previously (Zouki *et al*., 2001). The beads were washed extensively and boiled in reducing sample buffer. The eluted proteins were resolved on a 10% SDS-acrylamide gel, transferred to a PVDF membrane, probed with a mouse anti-Ras mAb (clone RAS10, Upstate Biotechnology) and visualized using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (BioRad, Mississauga, ON, Canada) and a chemiluminescence detection system.

Measurement of superoxide production and gelatinase release

Superoxide production was determined by measuring superoxide dismutase-inhibitable reduction of ferricytochrome c (Filep & Földes-Filep, 1989). Gelatinolytic activity was determined using [^{3}H]-acetylated gelatin as a substrate following activation of the latent gelatinase by 1.7 mM phenylmercuric acetate, and gelatinase release was expressed as the percentage of total enzyme units released from neutrophils treated with 0.1% Triton X-100 (Zouki *et al*., 1997).

Neutrophil homotypic adhesion

Neutrophil aggregation was measured on-line as the increase in light transmittance through a stirred suspension of neutrophils (10^7 cells ml^{-1}) in a platelet aggregometer (BioData, Hatboro, PA, U.S.A.) and quantitated as the area under the aggregation curve for the first 2 min after stimulation (Capodici *et al*., 1998). In some experiments, neutrophils were pretreated with a function-blocking anti-human CD18 mAb L130 (IgG_1, 10 μg ml^{-1}, BD Biosciences) (Zouki *et al*., 1997) or with an irrelevant mAb MOPC-21 for 10 min before addition to ET-1.

Statistical analysis

All results are presented as mean$\pm$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

ET-1-induced changes in adhesion molecule expression on human neutrophils and inhibition by an ET_A receptor antagonist and PD98059

Incubation of neutrophils with ET-1 down-regulated the expression of L-selectin and up-regulated the expression of CD11b in a concentration-dependent fashion (Figure 1), with an apparent EC_{50} concentration of about 9 nM. Similar increases were detected in CD18 expression (data not shown). The maximum changes that could be achieved with ET-1 were similar to those evoked by 1 μM PAF (46$\pm$2% and 57$\pm$4% decreases in L-selectin expression by ET-1 and PAF, respectively; $n=6$, $P<0.05$; 56$\pm$4% *vs* 73$\pm$7% increases in CD11b expression by ET-1 and PAF, $P<0.05$).

Next we investigated whether ET-1 could affect the affinity of β_2 integrins using the mAb G-25.2, an antibody that recognizes the β-propeller domain in CD11a/CD18 (LFA-1) (McDowall *et al*., 1998). Conformational changes and association of the I domain with the β-propeller domain are thought to be required for the formation of a higher affinity form of CD11a/CD18 and CD11b/CD18 (Mac-1) (McDowall *et al*., 1998; Springer, 1997). Incubation of neutrophils with ET-1 did not induce a detectable increase in staining with mAb G-25.2 (Figure 2a). As a positive

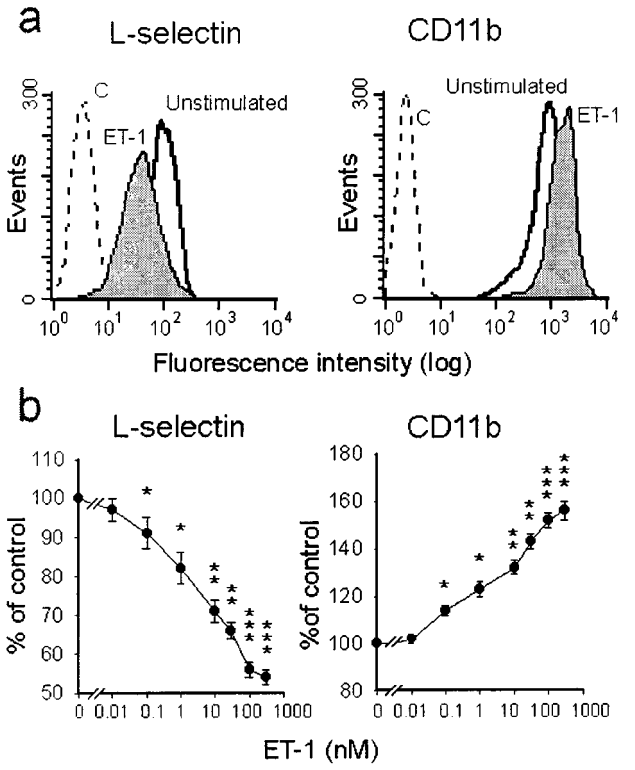

Figure 1 Effects of ET-1 on surface expression of L-selectin and CD11b on human neutrophils. Isolated neutrophils were challenged with ET-1 for 30 min at 37°C. (a) Representative immunostaining of neutrophils challenged with 30 nM ET-1. In each histogram is also displayed the negative control of immunostaining with class-matched irrelevant antibodies (C). Shown is a representative of six experiments using neutrophils from different blood donors. (b) Concentration-dependent effects of ET-1. Fluorescence intensity is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils incubated in medium only. Results are the mean$\pm$s.e.mean. for six experiments using neutrophils from different donors. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with control.

control, neutrophils were incubated with Mg^{2+} and EGTA, which induces the formation of a higher affinity form of CD11a/CD18 without inducing clustering (McDowall *et al*., 1998). As shown in Figure 2b, such treatment results in increased mAb G-25.2 immunostaining.

Neither the ET_A receptor selective antagonist FR139317 nor the ET_B receptor selective antagonist BQ788 on their own affected expression of adhesion molecules on resting neutrophils. FR 139317 markedly attenuated ET-1 (100 nM)-induced changes in L-selectin and CD11b expression (37$\pm$3% and 5$\pm$4% decreases in L-selectin expression, and 56$\pm$4% and 9$\pm$2% increases in CD11b expression in response to ET-1 in the absence and presence of FR 139317, respectively, $n=5$, both $P<0.01$), whereas BQ 788 had no detectable effects (34$\pm$7% decreases in L-selectin expression and 54$\pm$6% increases in CD11b expression in response to ET-1 in the presence of BQ 788, $n=5$, both $P>0.1$ compared with ET-1). The MAPK kinase (MEK) inhibitor PD98059 effectively prevented ET-1-induced up-regulation of CD11b/CD18 expression, whereas it was less effective in reversing down-regulation of L-selectin expression (Figure 3). The phosphatidylinositol 3-kinase inhibitor wortmannin, the tyrosine kinase inhibitor genistein or the

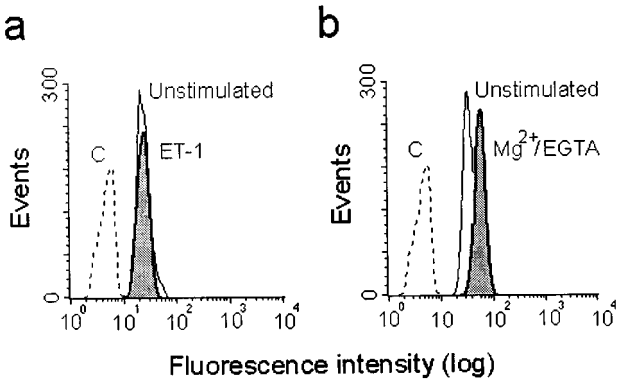

Figure 2 Effect of ET-1 on the expression of CD11a/CD18 (LFA-1) epitope expressed by the β-propeller domain and associated with higher binding affinity of CD11a/CD18 on neutrophils. Mean fluorescence of mAb G-25.2 on neutrophils challenged with (a) 100 nM ET-1 or (b) Mg^{2+} (1 mM) and EGTA (2 mM) for 30 min at 37°C. The dotted lines represent control mAb G-25.2 binding at 4°C. Shown is a representative of three experiments.

selective p38 MAP kinase inhibitor SB 203580 did not affect significantly ET-1-induced changes in adhesion molecule expression, in spite of the fact that both wortmannin and genistein by themselves induced downregulation of L-selectin expression (Figure 3b).

ET-1 activates ERK in human neutrophils

MEK and ERK were rapidly phosphorylated in neutrophils challenged with ET-1 (Figure 4). The maximal phosphorylation was observed at 2–5 min after exposure, and the effects of ET-1 were concentration-dependent (Figure 4). ERK activity was measured as the ability of neutrophil lysates to phosphorylate Elk-1, a specific target for ERK (Marais *et al*., 1993). ERK activation by ET-1 was rapid (peak at around 2 min), concordant with the kinetics of ET-1-stimulated neutrophil homotypic aggregation (Figure 5a). Maximal activation of ERK by ET-1 was observed at concentrations between 30 and 100 nM ($EC_{50}=12$ nM), again concordant with its effect on neutrophil aggregation (Figure 5b). Furthermore, ET-1 (100 nM)-induced increases in ERK activity (872±52% of control) was markedly attenuated in the presence of 1 μM FR 139317 (ERK activity: 164±12% of control, $n=4$, $P<0.01$), whereas BQ 788 (10 μM) had no significant effect (ERK activity: 815±47% of control, $n=4$, $P>0.1$).

ET-1 stimulation of neutrophil ERK activity and aggregation is MEK dependent

To provide further evidence that ET-1 stimulation of neutrophil ERK is mediated via Raf-1 and MEK, we studied whether PD98059, a specific inhibitor that binds to MEK and prevents its phosphorylation and activation by Raf-1 and other kinases (Dudley *et al*., 1995; Alessi *et al*., 1995), has an effect on neutrophil responses. Preincubation of neutrophils with PD98059 (100 μM) resulted in 83±2%, 85±8% and 73±11% inhibition of ET-1-stimulated ERK activity, upregulation of CD11b/CD18 expression and aggregation, respectively (Figures 3 and 5c).

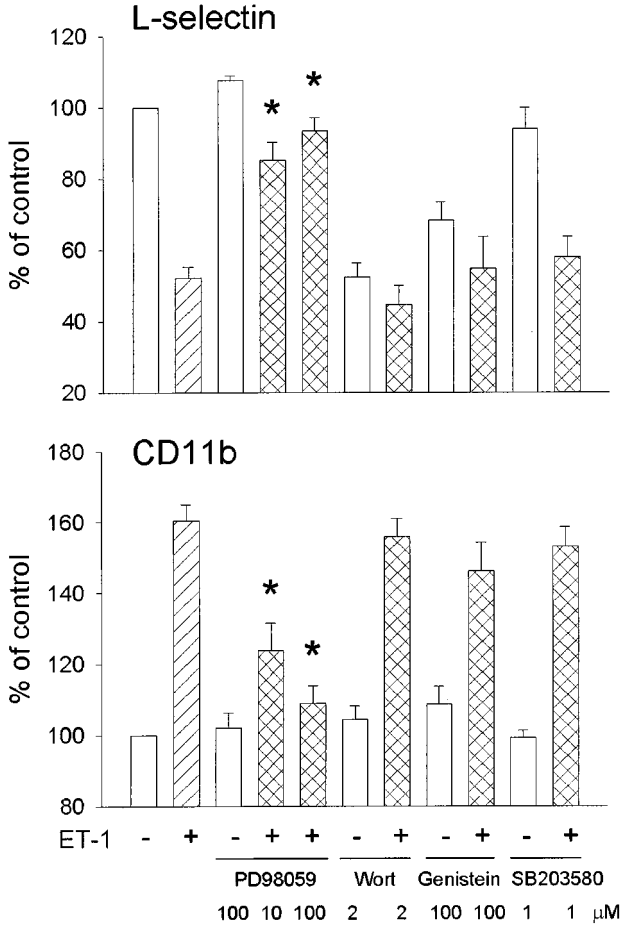

Figure 3 Effects of protein kinase inhibitors on neutrophil expression of L-selectin and CD11b. Neutrophils were preincubated with the MAPK kinase inhibitor PD98059, the phosphatidylinositol 3-kinase inhibitor wortmannin, the tyrosine kinase inhibitor genistein or the p38 MAPK inhibitor SB 203580 for 20 min at 37°C, and then challenged with ET-1 (100 nM) for 30 min. Adhesion molecule expression is presented as the percentage of control. Mean fluorescence intensity for control samples, L-selectin: 78±9; CD11b: 580±38. Values are the mean±s.e.mean of four independent experiments. *$P<0.05$ compared with ET-1 alone (hatched columns).

To test whether ET-1 stimulates Raf-1 in neutrophils, we immunoprecipitated Raf-1 from lysates of unstimulated and ET-1-stimulated neutrophils and assayed Raf-1 activity using a MEK/ERK-1/myelin basic protein cascade system. ET-1 activated Raf-1 kinase in a concentration-dependent manner (Figure 6a). Furthermore, ET-1 also evoked a concentration and time-dependent association of Ras with the GST-Ras binding domain of Raf-1 (Figure 6b,c), indicating Ras activation.

ET-1-induced neutrophil homotypic adhesion is β_2 integrin dependent

Neutrophil aggregation in response to 100 nM ET-1 was blocked with an anti-human CD18 mAb (88±6% versus 26±6% maximum aggregation, respectively, $n=4$, $P<0.05$), whereas the irrelevant mAb MOPC-21 was without effect (100±3% maximum aggregation, $n=4$, $P>0.1$).

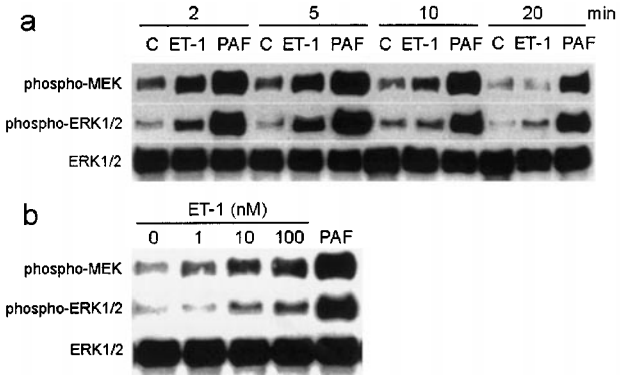

Figure 4 ET-1 induces time-, and concentration-dependent phosphorylation of MEK and ERK in human neutrophils. Neutrophils were challenged with ET-1 (100 nM) for the indicated time periods (a) or with various concentrations of ET-1 for 2 min (b). The effects of PAF (1 μM) are shown for comparisons. Results are representative of four experiments.

ET-1 induces gelatinase release, but not superoxide production

To assess further neutrophil activation by ET-1, we studied superoxide production and gelatinase release. ET-1 did not induce detectable superoxide production (unstimulated neutrophils produced 0.2 ± 0.2 nmol superoxide per 10^7 cells per min vs 0.3 ± 0.2 nmol superoxide per 10^7 cells per min in response to 100 nM ET-1, $n = 5$, $P > 0.1$), whereas it evoked a concentration-dependent release of gelatinase that was prevented by PD98059 (Figure 7).

Discussion

In this study we examined the molecular mechanisms of ET-1 signalling in human neutrophils, observing a close association between ET-1-stimulated ERK activation and neutrophil adhesive responses.

Consistent with previous reports (Zouki *et al*., 1999; López-Farré *et al*., 1993), our data demonstrate that at nanomolar concentrations, ET-1 down-regulated L-selectin expression and up-regulated CD11b/CD18 expression on neutrophils and released gelatinase, reflecting neutrophil activation. 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, up-regulation of CD11b/CD18 can occur without degranulation of azurophil and specific granules. Indeed, ET-1 evoked a concentration-dependent release of gelatinase, whereas (up to a concentration of 300 nM) it did not induce release of β-glucuronidase and lysozyme (Zouki *et al*., 1999). Furthermore, ET-1 did not induce superoxide production (Ishida *et al*., 1990; and the present study). Thus, ET-1 may function as a rather selective neutrophil agonist, i.e. it is capable of enhancing adhesive properties of neutrophils without major increases in superoxide production or induction of degranulation of primary and secondary granules. The present observations that ET-1-induced changes in adhesion molecule expression and gelatinase release can be effectively attenuated by PD98059

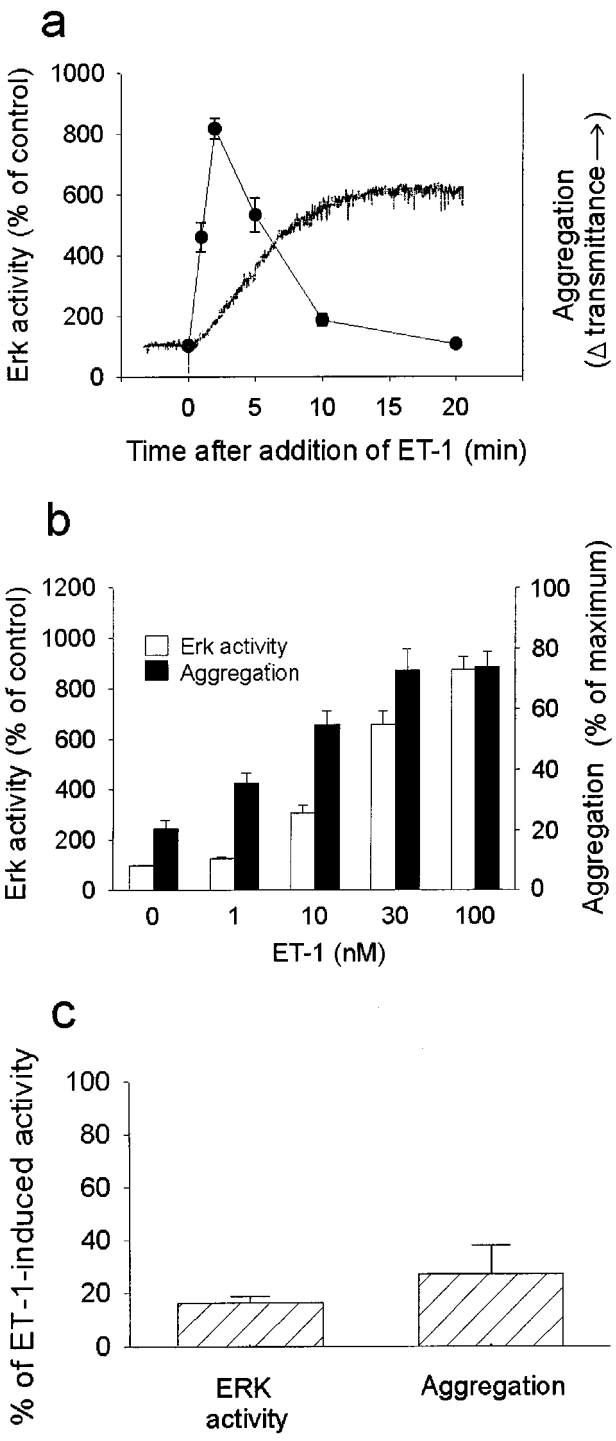

Figure 5 ET-1 stimulates neutrophil ERK activity. (a) Neutrophils were challenged with ET-1 (100 nM), then lysed at the indicated times, and ERK activity was assayed (thin line) or assayed on-line for homotypic aggregation (thick line). (b) Concentration-dependent activation of ERK and induction of neutrophil aggregation. Neutrophils were challenged with ET-1 for 3 min at 37°C, then either lysed and analysed for ERK activity or homotypic aggregation was monitored on-line for 10 min. Results shown are the mean ± s.e.mean or representative of four independent experiments. (c) ET-1 stimulation of human neutrophil granulocytes is MEK dependent. Neutrophils were incubated in the absence or presence of PD98059 (100 μM) for 10 min, challenged for 2 min with ET-1 (100 nM), lyzed and analysed for ERK activity or aggregation. Results shown are the mean ± s.e.mean of 4–5 experiments.

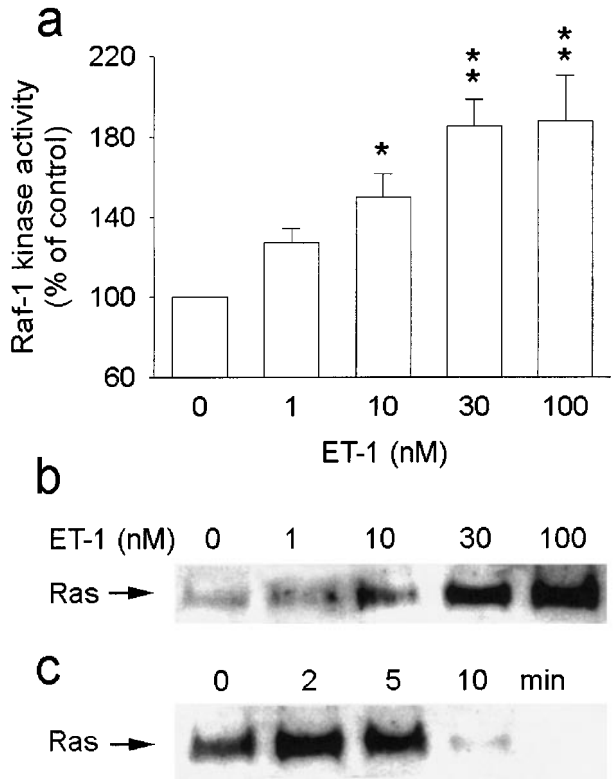

Figure 6 ET-1 induces activation of Ras and Raf-1 kinase. (a) Activation of Raf-1. Neutrophils were challenged with ET-1 for 2 min at 37°C, lysed, Raf-1 was immunoprecipitated, and Raf-1 kinase activity was measured as described in Methods. Results shown are the mean ± s.e.mean of four experiments. $*P<0.05$; $**P<0.01$ compared with unchallenged. Neutrophils were challenged (b) with various concentrations of ET-1 for 3 min or (c) with 100 nM ET-1 for the indicated times. GTP-bound active Ras was isolated from neutrophils by affinity precipitation with a GST-Ras binding domain fusion protein followed by immunoblot analysis with an anti-Ras antibody. Shown is a representative result, the experiments were repeated four times.

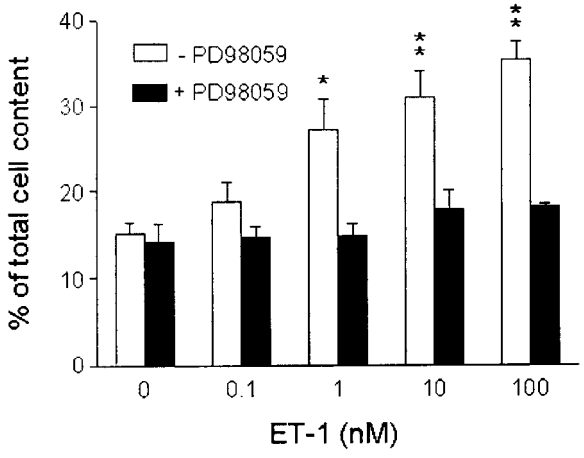

Figure 7 ET-1-induced gelatinase release is MEK dependent. Neutrophils were preincubated with PD 98059 (100 μM) for 20 min at 37°C, and then challenged with ET-1 for 30 min. Values are expressed as percentage of total cellular gelatinase activity released by neutrophils into the culture medium, and are means ± s.e.mean of four independent experiments. $*P<0.05$; $**P<0.01$ compared with control (unstimulated).

coupled with those of Yu *et al.* (1995), who have reported a dissociation between neutrophil ERK activation and superoxide generation, are consistent with this notion.

With respect to β_2 integrin expression, ET-1 did not affect mAb G-25.2 binding to the β-propeller domain of CD11a/CD18, whose expression is associated with the formation of a higher affinity CD11a/CD18 binding reaction (McDowall *et al.*, 1998; Springer, 1997). These results would suggest that ET-1 does not alter the affinity of CD11a/CD18, and probably CD11b/CD18, but rather induces clustering of β_2 integrins, thereby increasing the overall strength of binding. The ET-1-induced rapid changes in CD11b/CD18 expression do not support a role for *de novo* synthesis of neutrophil adhesion molecules, but rather suggest receptor translocation from intracellular stores in response to neutrophil activation (Arnaut, 1990). However, our results do not preclude the possibility that, in the presence of integrin ligands, ET-1 might affect a ligand-induced affinity increase secondary to integrin clustering.

Our previous pharmacological and receptor binding studies showed that human neutrophils predominantly express ET_A receptors (Zouki *et al.*, 1999). Scatchard analysis of ET-1 displacement of [^{125}I]-ET-1 binding to neutrophils revealed a single class of high affinity binding sites with a K_d value of 39 ± 9 pM (Zouki *et al.*, 1999). The actions of ET-1 on surface expression of L-selectin and CD11b/CD18, ERK activation and gelatinase release were significantly inhibited by the selective ET_A receptor antagonist FR 139317 (Aramori *et al.*, 1993), but not by the ET_B receptor antagonist BQ 788 (Ishikawa *et al.*, 1994). These findings clearly point to the ET_A receptor as being the relevant receptor subtype responsible for these actions of ET-1. The observations that ET-1 induces leukocyte adherence predominantly through an ET_A receptor-mediated mechanism in the venules of the intestinal circulation (Boros *et al.*, 1998), are consistent with an action of neutrophils, since endothelial cells are thought to express ET_B receptors. It should be noted that homotypic and heterotypic neutrophil adhesive responses may also be regulated *via* ET_B receptors expressed on endothelial or other cells. However, the role of ET_B receptors appears to be controversial, for both anti-adhesive (Murohara & Lefer, 1996) and pro-adhesive functions (McCarron *et al.*, 1993; Hayasaki *et al.*, 1996) have been reported. The observations that human neutrophils do not express ET_B receptors would argue against an autocrine regulatory function of these receptors in human neutrophils.

The present results indicate that ERK activation is required for ET-1 stimulation of neutrophil homotypic adhesion. ET-1 stimulated ERK activity and neutrophil aggregation with similar concentration-dependent responses and kinetics. The kinetics of ERK activation by ET-1 in neutrophils appears to be distinct from that observed in mitotic cells, rapidly peaking at 2–5 min for ET-1 in neutrophils compared to 5–10 min observed in mitotic cells (Hii *et al.*, 1994; Rao *et al.*, 1994). Furthermore, the specific MEK inhibitor PD98059 also inhibited ERK phosphorylation and neutrophil responses to ET-1. In this study, we used PD98059 to assess whether neutrophil responses to ET-1 are MEK dependent. As shown in Figure 5c, PD98059 at 100 μM effectively inhibited ERK phosphorylation. The short half-life (about 12 h) of human peripheral blood neutrophils precludes performing transient transfection with dominant-

negative mutants to confirm the role of ERK in neutrophil responses. The present observations are consistent with previous studies which have found a correlation between ERK activation and neutrophil aggregation evoked by chemoattractants (Pillinger *et al*., 1996) and arachidonic acid (Capodici *et al*., 1998). ERK phosphorylation in response to protein tyrosine kinase receptors and some G-protein-coupled receptors proceeds *via* Ras, Raf-1, and MEK (Pillinger *et al*., 1996; Gardner *et al*., 1993; Worthen *et al*., 1994). Our results suggest that ET-1 stimulation of ERK also involves this signalling pathway, since ET-1 (a) stimulated association of Ras with Raf-1; (b) increased Raf-1 kinase activity; and (c) resulted in phosphorylation of MEK. The observations that ET-1-induced changes in adhesion molecule expression, in particular the down-regulation of L-selectin expression, were never completely blocked by PD98059 suggest the involvement of other yet unidentified intracellular pathway(s) in ET-1 signalling in neutrophils. The lack of inhibitory effect of wortmannin, genistein and SB203580 would, however, argue against the involvement of phosphatidylinositol 3-kinase, tyrosine kinases and p38 MAPK.

Based on these observations, we suggest that ET-1 binds to neutrophil ET_A receptors, G-protein activity then initiates ERK phosphorylation *via* Ras, Raf-1 and MEK, as previously observed in other systems. Our results indicate that ERK activation is required for ET-1 up-regulation of CD11b/CD18 expression and consequent stimulation of neutrophil aggregation. The similar degree of inhibition of neutrophil aggregation observed with PD98059 and a function-blocking anti-CD18 mAb would lend further support to this notion. These results also indicate that the role of ET_A receptor-coupled ERK signalling pathway in rapid responses of terminally differentiated neutrophils may be limited to specific functions including adhesion and release of gelatinase from tertiary granules, and clearly differs from that in mitotic cells, such as cardiomyocytes (Yue *et al*., 2000). The observations that PD98059 produced similar inhibitory effects on up-regulation of surface expression of CD11b/CD18 and exocytosis of specific granules suggest that there is either a single signal for these changes or signalling for these events downstream from ERK activation is tightly linked.

In conclusion, the present study demonstrates that inhibition of the ERK signalling pathway blocked ET-1-induced upregulation of surface expression of CD11b/CD18 and neutrophil homotypic adhesion. Although we cannot exclude the involvement of other signalling pathways in neutrophil responses to ET-1, the present results strongly support the notion that ERK activation plays an essential role in the signalling mechanisms that lead to increased neutrophil adhesive responses.

This study was supported by grants from the Medical Research Council of Canada/Canadian Institutes of Health Research (MT-12573/MOP-12573 to JGF, and MT-13420 to JSD Chan and JG Filep).

References

ALESSI, D.R., CUENDA, A., COHEN, P., DUDLY, D.T. & SALTIEL, A.R. (1995). PD09859 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.,* **270,** 27489 – 27494.

ARAMORI, I., NIREI, H., SHOUBO, M., OGABE, K., NAKAMURA, K., KOJO, H., NOTSU, Y., ONO, T. & NAKANISHI, S. (1993). Subtype selectivity of a novel endothelin antagonist, FR 139317, for the two endothelin receptor in transfected Chinese hamster ovary cells. *Mol. Pharmacol.,* **43,** 127 – 131.

ARNAUT, M.A. (1990). Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood,* **75,** 1037 – 1050.

BOROS, M., MASSBERG, S., BARANY, L., OKADA, H. & MESSMER, K. (1998). Endothelin-1 induces leukocyte adhesion in submucosal venules of the rat small intestine. *Gastroenterology,* **114,** 103 – 114.

BORREGAARD, N., MILLER, L.J. & SPRINGER, T.A. (1987). Chemoattractant-regulated fusion of a novel, mobilizable intracellular compartment with the plasma membrane in human neutrophils. *Science,* **237,** 1204 – 1206.

CAPODICI, C., PILLINGER, M.H., HAN, G., PHILIPS, M.R. & WEISSMANN, G. (1998). Integrin-dependent homotypic adhesion of neutrophils. Arachidonic acid activates Raf-1/MEK/Erk via a lipoxygenase-dependent pathway. *J. Clin. Invest.,* **102,** 165 – 175.

COBB, M.H., HEPLER, J.R., CHENG, M. & ROBBINS, D. (1994). The mitogen-activated protein kinases, ERK1 and ERK2. *Sem. Cancer Biol.,* **5,** 261 – 268.

CUENDA, A., ROUSE, J., DOZA, Y.N., MEIER, R., COHEN, P., GALLAPHER, T.F., YOUNG, P.R. & LEE, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.,* **364,** 229 – 233.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7686 – 7689.

ESPINOSA, G., LÓPEZ-FARRÉ, A., CERNADAS, M.R., MANZARBEITIA, F., TAN, D., DIGIUNI, E., MOSQUERA, J.R., MONTÓN, M., MILLÁS, I, HERNANDO, L., CASADO, S. & CARAMELO, C. (1996). Role of endothelin in the pathophysiology of renal ischemia-reperfusion in normal rabbits. *Kidney Int.,* **50,** 776 – 782.

FILEP, J.G. & FÖLDES-FILEP, E. (1989). Effects of C-reactive protein on human neutrophil granulocytes challenged with N-formyl-methionyl-leucyl-phenylalanine and platelet-activating factor. *Life Sci.,* **44,** 517 – 524.

FILEP, J.G., FOURNIER, A., & FÖLDES-FILEP, E. (1995). Acute pro-inflammatory actions of endothelin-1 in the guinea-pig lung: involvement of ET_A and ET_B receptors. *Br. J. Pharmacol.,* **115,** 227 – 236.

FILEP, J.G. & HAY, D.W.P. (1999). Endothelin as a proinflammatory mediator. In *Pulmonary Actions of the Endothelins*. ed. Goldie, R.G. & Hay, D.W.P. pp. 177 – 196, Basel, Switzerland: Birkhauser Verlag.

GARDNER, A.M., LANGE-CARTER, C.A., VAILLANCOURT, R.R. & JOHNSON, G.L. (1994). Measuring activation of kinases in mitogen-activated protein kinase regulatory network. *Methods Enzymol.,* **238,** 258 – 270.

GARDNER, A.M., VAILLANCOURT, R.R. & JOHNSON, G.L. (1993). Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase by G protein and tyrosine kinase oncoproteins. *J. Biol. Chem.,* **268,** 17896 – 17901.

GÓMEZ-GARRÉ, D., GUERRA, M., GONZALEZ, E., LÓPEZ-FARRÉ, A., RIESCO, A. ESCANERO, J. & EGIDO, J. (1992). Aggregation of human polymorphonuclear leukocytes by endothelin: role of platelet-activating factor. *Eur. J. Pharmacol.,* **224,** 167 – 172.

HALIM, A., KANAYAMA, N., EL MARADNY, E., MAEHARA, K. & TERAO, T. (1995). Activated neutrophil by endothelin-1 caused tissue damage in human umbilical cord. *Thromb. Res.,* **77,** 321 – 327.

HAYASAKI, Y., NAKAJIMA, M., KITANO, Y., IWASAKI, T., SHIMAMURA, T. & IWAKI, K. (1996). ICAM-1 expression on cardiac myocytes and aortic endothelial cells via their specific endothelin receptor subtype. *Biochem. Biophys. Res. Commun.,* **229,** 817–824.

HELSET, E., LINDAL, S., OLSEN, R., HYKLEBUST, R. & JORGENSEN, L. (1996). Endothelin-1 causes sequential trapping of platelets and neutrophils in pulmonary microcirculation in rats. *Am. J. Physiol.,* **271,** L538–L546.

HII, C.S.T., FERRANTE, A., EDWARDS, Y.S., HUANG, Z.H., HARTFIELD, P.J., RATHJEN, D.A., POULOS, A. & MURRAY, A.W. (1994). Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C-dependent mechanism. *J. Biol. Chem.,* **270,** 4201–4204.

ISHIDA, K., TAKESHIGE, K. & MINAKAMI, S. (1990). Endothelin-1 enhances superoxide generation of human neutrophils stimulated by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine. *Biochem. Biophys. Res. Commun.,* **173,** 496–500.

ISHIKAWA, K., IHARA, M., FUKAMI, T., OZAKI, S., NAGASE, T., NISHIKIBE, M. & YANO, M. (1994). Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 4892–4896.

KHIMENKO, P.L., MOORE, T.M. & TAYLOR, A.E. (1996). Blocked ETA receptors prevent ischemia and reperfusion injury in rat lungs. *J. Appl. Physiol.,* **80,** 203–207.

LÓPEZ-FARRÉ, A., CARAMELO, C., ESTEBAN, A., ALBEROLA, ML., MILLÁS, I., MONTÓN, M. & CASADO, S. (1995). Effects of aspirin on platelet-neutrophil interactions: role of nitric oxide and endothelin-1. *Circulation,* **91,** 2080–2088.

LÓPEZ-FARRÉ, A., RIESCO, A., ESPINOSA, G., DIGIUNI, E., CERNADAS, M.R., ALVAREZ, V., MONTÓN, M., RIVAS, F., GALLEGO, M.J., EGIDO, J., CASADO, S. & CARAMELO, C. (1993). Effect of endothelin-1 on neutrophil adhesion to endothelial cells and perfused hearts. *Circulation,* **88,** 1166–1171.

MALECH, M.L. & GALLIN, J.I. (1987). Neutrophils in human diseases. *N. Engl. J. Med.,* **317,** 687–694.

MARAIS, R., WYNNE, J. & TREISMAN, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell,* **73,** 381–393.

McCARRON, R.M., WANG, L., STANIMIROVIC, D.B. & SPATZ, M. (1993). Endothelin induction of adhesion molecule expression on human brain microvascular endothelial cells. *Neurosci Lett.,* **156,** 31–34.

McDOWALL, A., LEITINGER, B., STANLEY, P., BATES, P.A., RADI, A.M. & HOGG, N. (1998). The I domain of integrin leukocyte function-associated antigen-1 is involved in a conformational change leading to high affinity binding to ligand intercellular adhesion molecule-1 (ICAM-1). *J. Biol. Chem.,* **273,** 27396–27403.

MUROHARA, T. & LEFER, A.M. (1996). Autocrine effects of endothelin-1 on leukocyte-endothelial interaction: Stimulation of endothelin B receptor subtype reduces endothelial adhesiveness via a nitric oxide-dependent mechanism. *Blood,* **88,** 3894–3900.

PILLINGER, M.H., FEOKTISTOV, A.S., CAPODICI, C., SOLITAR, B., LEVY, J., OEI, T.T. & PHILIPS, M. R. (1996). Mitogen-activated protein kinase in neutrophils and enucleate neutrophil cytoplasts: evidence for regulation of cell-cell adhesion. *J. Biol. Chem.,* **271,** 12049–12056.

RAO, G.N., BAAS, A.S., GLASGAW, W.C., ELING, T.E., RUNGE, M.S. & ALEXANDER, R.W. (1994). Activation of mitogen-activated protein kinases by arachidonic acid and its metabolites in vascular smooth muscle cells. *J. Biol. Chem.,* **269,** 32586–32591.

SHICHIRI, M., YOKOKURA, M., MARUMO, F. & HIRATA, Y. (2000). Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway. *Arterioscler. Thromb. Vasc. Biol.,* **20,** 989–997.

SPRINGER, T.A. (1997). Folding of the N-terminal, ligand binding region of integrin β-subunits into a β-propeller domain. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 65–72.

THOMPSON, H.L., SHIROO, M. & SAKLATVALA, J. (1993). The chemotactic factor N-formyl-methionyl-leucyl-phenylalanine activates microtubule-associated protein-2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leukocytes. *Biochem. J.,* **290,** 483–488.

WORTHEN, G.S., AVDI, N., BUHL, A.M., SUZUKI, N. & JOHNSON, G.L. (1994). FMLP activates Ras and Raf in human neutrophils. Potential role in activation of MAP kinase. *J. Clin. Invest.,* **94,** 815–823.

WU-WONG, J.R., CHIOU, W.J. & WANG, J. (2000). Extracellular signal-regulated kinases are involved in the antiapoptotic effect of endothelin-1. *J. Pharmacol. Exp. Ther.,* **293,** 514–521.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* **332,** 411–415.

YU, H., SUCHARD, S.J., NAIRN, R. & JOVE, R. (1995). Dissociation of mitogen-activated protein kinase activation from the oxidative burst in differentiated HL-60 cells and human neutrophils. *J. Biol. Chem.,* **270,** 15719–15724.

YUE T.-L., GU, J.-L., WANG, C., REITH, A.D., LEE, J.C., MIRABILE, R.C., KREUTZ, R., WANG, Y., MALEEFF, B., PARSONS, A.A. & OHLSTEIN, E.A. (2000). Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J. Biol. Chem.,* **275,** 37895–37901.

ZOUKI, C., BARON, C., FOURNIER, A. & FILEP, J.G. (1999). Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells: role of ET_A receptors and platelet-activating factor. *Br. J. Pharmacol.,* **127,** 969–979.

ZOUKI, C., BEAUCHAMP, M., BARON, C. & FILEP, J.G. (1997). Prevention of in vitro neutrophil adhesion to endothelial cells through shedding of L-selectin by C-reactive protein and peptides derived from C-reactive protein. *J. Clin. Invest.,* **100,** 522–529.

ZOUKI, C., ZHANG, S.L., CHAN, J.S.D. & FILEP, J.G. (2001). Peroxynitrite induces integrin-dependent adhesion of human neutrophils to endothelial cells via activation of the Raf-1/MEK/Erk pathway. *FASEB J.,* **15,** 25–27. (10.1096/fj.00-0521fje, published online November 9, 2000).

(Received October 8, 2001
Revised December 11, 2001
Accepted December 17, 2001)