Endothelin suppresses cell migration via the JNK signaling pathway in a manner dependent upon Src kinase, Rac1, and Cdc42

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Abstract **Cell migration is a complex phenomenon that is stimulated by chemoattractive factors such as chemokines, a family of ligands for G protein-coupled receptors (GPCRs). In contrast, factors that suppress cell migration, and the mechanism of their action, remain largely unknown. In this study, we show that endothelin, a GPCR ligand, inhibits cell motility in a manner dependent upon signaling through the c-Jun N-terminal kinase (JNK) pathway. We further demonstrate that this effect is dependent upon Src kinase and small GTPases Rac1 and Cdc42. These findings provide new insight into GPCR-mediated regulation of cell migration.** © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cell motility; Endothelin; Src kinase; Rac1; Cdc42; c-Jun N-terminal kinase

1. Introduction

Cell migration is an important cellular process mediated by extracellular ligands that bind and activate cell surface receptors [1–5]. Such ligands include chemokines, which are chemoattractants that induce cell migration [1–4]. The cognate chemokine receptors comprise a subfamily of G protein-coupled receptors (GPCRs) characterized by a heptahelical seven-transmembrane structure [1–4].

In contrast to our understanding of the factors that induce cell migration, ligands that inhibit this process are largely unknown, with the exception of metastin and certain bioactive lipids [6–12]. Metastin is a bioactive peptide derived from a protein encoded by human melanoma metastasis suppressor gene, *KiSS-1*, and has been identified as a ligand of certain orphan GPCRs [6–9]. Bioactive lipids, such as sphingosine-1-phosphate and platelet-activating factor, have been shown to suppress cell motility and invasiveness, respectively, through GPCRs [10–12].

Growing evidence suggests that GPCRs stimulate a signaling pathway activating c-Jun N-terminal kinase (JNK), a subfamily of mitogen-activated protein kinases (MAPKs) [13–18]. We and other groups have shown that the GPCR-JNK signaling pathway involves tyrosine kinases and/or Rho family small GTPases [13–18]. However, the physiological significance of the signaling pathway remains still unclear.

The endothelin receptor is a widely distributed GPCR that has been implicated in pleiotropic functions [19,20]. Genetic studies of the endothelin/endothelin receptor system suggest an involvement in cell migration [19,20], although the detailed function of endothelin in cell migration remains largely unknown. In this study, we show that endothelin inhibits motility of human epithelial-like 293 cells. We also demonstrate that endothelin-induced inhibition of cell motility is mediated by the JNK signaling pathway. Furthermore, we investigate the signaling mechanism controlling inhibition of cell motility by endothelin.

2. Materials and methods

2.1. Materials

The following materials were employed in this study: anti-JNK (C-17), anti-RhoA (26C4), and anti-Src (SRC2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-Rac1 (102) and anti-Cdc42 (44) from BD Biosciences (San Jose, CA, USA); anti-phosphorylated (pY^{418}) Src (44–660) from BIOSOURCE International (Nivelles, Belgium); anti-FLAG epitope (M2) from Sigma-Aldrich Co. (St. Louis, MO, USA); anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase from Amersham Biosciences (Buckinghamshire, UK); PP1 and PP2 from Biomol (Plymouth Meeting, PA, USA); *Clostridium difficile* toxin B from Calbiochem-Novabiochem Co. (San Diego, CA, USA); endothelin-1 from Sigma-Aldrich Co.

2.2. Plasmids

The mammalian expression plasmids encoding FLAG-tagged MKK4K95R (dominant-inhibitory form of MKK4), constitutively active (CA)-MEKK1, Rac1G12V (constitutively active form of Rac1), and Cdc42G12V (constitutively active form of Cdc42) were constructed as described previously [14–18,21]. The pUSE-CA-Src plasmid was purchased from Upstate, Inc. (Lake Placid, NY, USA). The *Escherichia coli* expression plasmids encoding the RhoA-binding domain (RBD) of mDia1 and the Rac1- and Cdc42-binding domain (CRIB) of αPak were constructed as described previously [17,22,23]. The pGEX2T-c-Jun (amino acids 1–221) plasmid was generously provided by M. Karin (University of California, San Diego, CA, USA).

2.3. Recombinant proteins

Recombinant GST-tagged mDia1RBD, αPakCRIB, and c-Jun proteins were purified using *E. coli* BL21(DE3) pLysS, as described previously [17].

2.4. Cell culture and transfection

Maintenance of 293 cells and transient transfection (2 μg of DNA

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per 6 cm dish) using Lipofectamine Plus (Invitrogen Co., Carlsbad, CA, USA) were performed as described previously [17,18]. The medium was replaced 24 h after transfection, and cells were starved in serum-free medium for 24 h.

2.5. Cell migration assay

Cell migration was measured using a 24-well Boyden chamber (Becton Dickinson Labware, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Briefly, upper wells with polyethylene terephthalate filters (8 μm pore size) were coated with 10 μg/ml extracellular matrix E-C-L (Upstate). Serum-starved cells (2×10^6 cells in 500 μl of Dulbecco's modified Eagle's medium (DMEM) per well) were loaded into upper wells, which were immediately plated on a chamber containing 165 nM endothelin (750 μl of DMEM per well). After incubation at 37°C for 3 h, upper filters were stained with a Diff-Quick staining kit (International Reagents Co., Kobe, Japan), according to the manufacturer's protocol. Cells that had not

migrated were wiped away from the inner surface of the upper wells. Using an optical microscope, the number of stained, migrated cells was counted in at least three independent experiments.

2.6. JNK assay

Unless otherwise indicated, serum-starved cells were stimulated with or without 100 nM endothelin for 5 min, and then lysed in lysis buffer A (20 mM HEPES–NaOH (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% NP-40). Endogenous JNK was immunoprecipitated with an anti-JNK antibody, and the activity of JNK was measured as the [^{32}P]-radioactivity incorporated into recombinant c-Jun, as described previously [14–18]. To estimate the amount of JNK in the immunoprecipitates, we performed immunoblot analysis. The band intensity of JNK in the immunoblot was semi-quantified using NIH Image 1.61. Levels of radioactivity incorporated into c-Jun
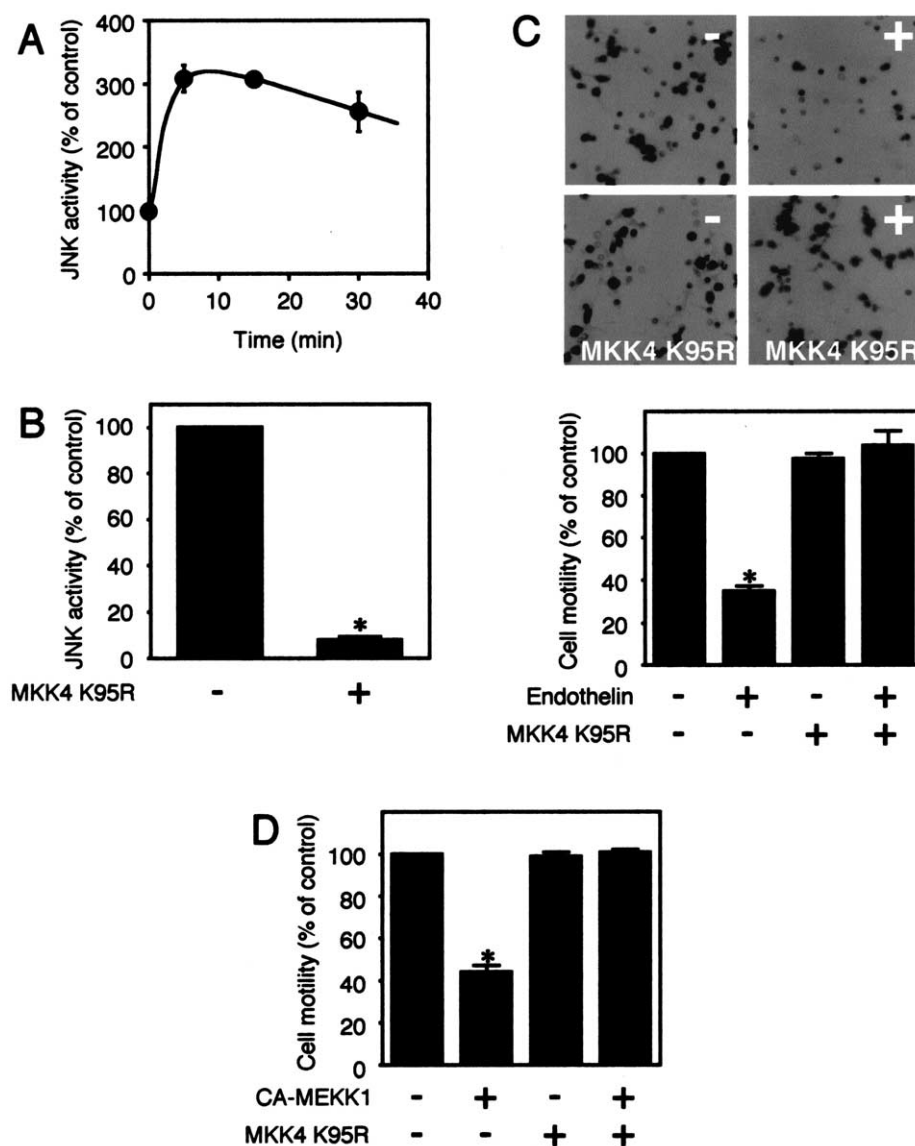

Fig. 1. Endothelin inhibits cell motility through the JNK signaling pathway. A: 293 cells were treated with 100 nM endothelin for the indicated times. The activity of endogenous JNK was measured by the amount of [^{32}P]-radioactivity incorporated into recombinant c-Jun. B: Cells were transiently transfected with the plasmid encoding mock or MKK4K95R. 48 h posttransfection, JNK activity was measured 5 min after the addition of 100 nM endothelin. C: Cells were transfected with the plasmid encoding mock or MKK4K95R. 48 h posttransfection, cell motility was measured using a Boyden chamber. After incubation at 37°C for 3 h with or without endothelin (100 nM, final concentration), cells attached to the filters were stained and analyzed under a microscope (upper photos). The number of stained, migrated cells was also counted (lower figure). D: Cells were transfected with the plasmid encoding mock, CA-MEKK1, or MKK4K95R. Cell motility was measured using a Boyden chamber at 37°C for 3 h.

were normalized to the amount of immunoprecipitated JNK as estimated from these band intensities.

2.7. Pull-down assays of Rho family small GTPases

Serum-starved cells were stimulated with or without 100 nM endothelin for 5 min, and then lysed in lysis buffer B (50 mM HEPES–NaOH (pH 7.5), 20 mM MgCl$_2$, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 µg/ml leupeptin, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 10 mM NaF, and 0.5% NP-40). To detect endogenous GTP-bound Rho family small GTPases in the cell lysate, we performed pull-down assays using recombinant GST-tagged mDia1RBD (for RhoA) and αPakCRIB (for Rac1 and Cdc42) as described previously [17,22,23]. Cell lysates were subjected to immunoblot analysis to estimate the amount of small GTPases. Representative results from three separate experiments are shown in Fig. 3.

2.8. Statistical analysis

Statistical analysis was performed using SAS StatView 5.0. Values shown represent the mean ± S.E.M. from at least three separate experiments. Student's t-test was carried out for intergroup comparisons (*, $P < 0.01$).

3. Results and discussion

3.1. Endothelin inhibits cell motility through the JNK pathway

We previously showed that in 293 cells transfected transiently with the plasmids encoding cDNAs of adrenergic and muscarinic acetylcholine receptors, receptor stimulation activates JNK through the JNK kinase MKK4 [14, 17,18]. We first examined whether endothelin treatment activates JNK in 293 cells, which express the endothelin receptor natively [24]. We have detected that endothelin type A and type B receptors express and the type A receptor mainly expresses in 293 cells, using the method of reverse transcriptase-polymerase chain reaction (data not shown). This result is consistent with the result of Howard et al. [24]. As shown in Fig. 1A, endothelin treatment of cells activated JNK in a time-dependent manner.
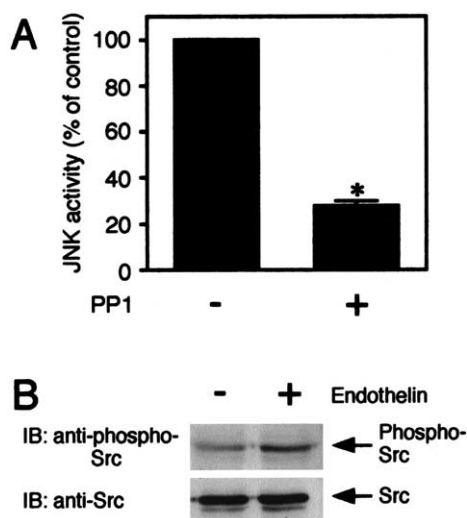

Fig. 2. Src kinase mediates endothelin-induced JNK activation. A: Cells were pretreated with or without 10 µM PP1 for 18 h. JNK activity was measured 5 min after the addition of 100 nM endothelin. B: The autophosphorylation of Src was assessed 5 min after the addition of 100 nM endothelin, using an antibody specific for phosphorylated (pY^{418}) Src. Levels of Src protein were measured by immunoblot analysis with an antibody that recognizes total Src protein.
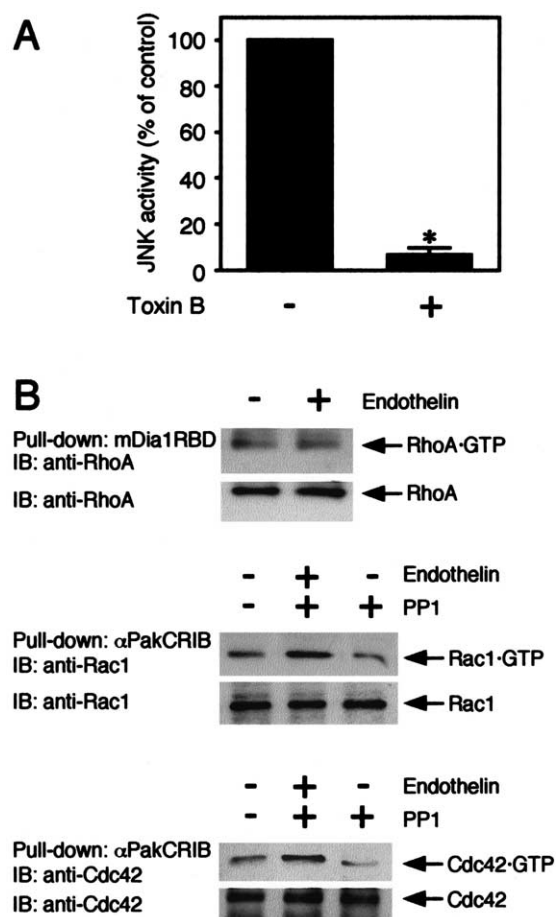

Fig. 3. Rac1 and Cdc42 mediate endothelin-induced JNK activation. A: Cells were pretreated with or without 2 ng/ml toxin B for 18 h. JNK activity was measured 5 min after the addition of 100 nM endothelin. B: Cells were pretreated with or without 10 µM PP1 for 18 h. The activity of Rho family small GTPases was measured 5 min after the addition of 100 nM endothelin by pull-down assay using recombinant mDia1RBD or αPakCRIB. Levels of Rho family small GTPases in the cell lysates were estimated by immunoblot analysis.

Next, we confirmed that MKK4K95R blocks endothelin-induced JNK activation. MKK4K95R is a kinase-deficient variant that inhibits transmission of signals from GPCRs to JNK [14,16–18]. As shown in Fig. 1B, transfection of the plasmid encoding MKK4K95R blocked JNK activation by endothelin. These results suggested that the JNK cascade was activated in 293 cells by endothelin acting through its cognate GPCR.

To examine the effect of endothelin on cell motility, we carried out the assay using Boyden chamber [5–7,10,11]. As shown in Fig. 1C, endothelin treatment of cells significantly inhibited cell motility in this assay. As far as we know, this is the first report of inhibition of cell motility by endothelin. Transfection of the plasmid encoding MKK4K95R blocked endothelin-induced inhibition of cell motility (Fig. 1C), suggesting that the JNK signaling cascade mediated the effect of endothelin on cell motility. Constitutively active (CA)-MEKK1 is an isolated kinase domain of JNK kinase kinase MEKK1 and acts as an activator of the JNK cascade [21]. Transfection of the plasmid encoding CA-MEKK1 suppressed
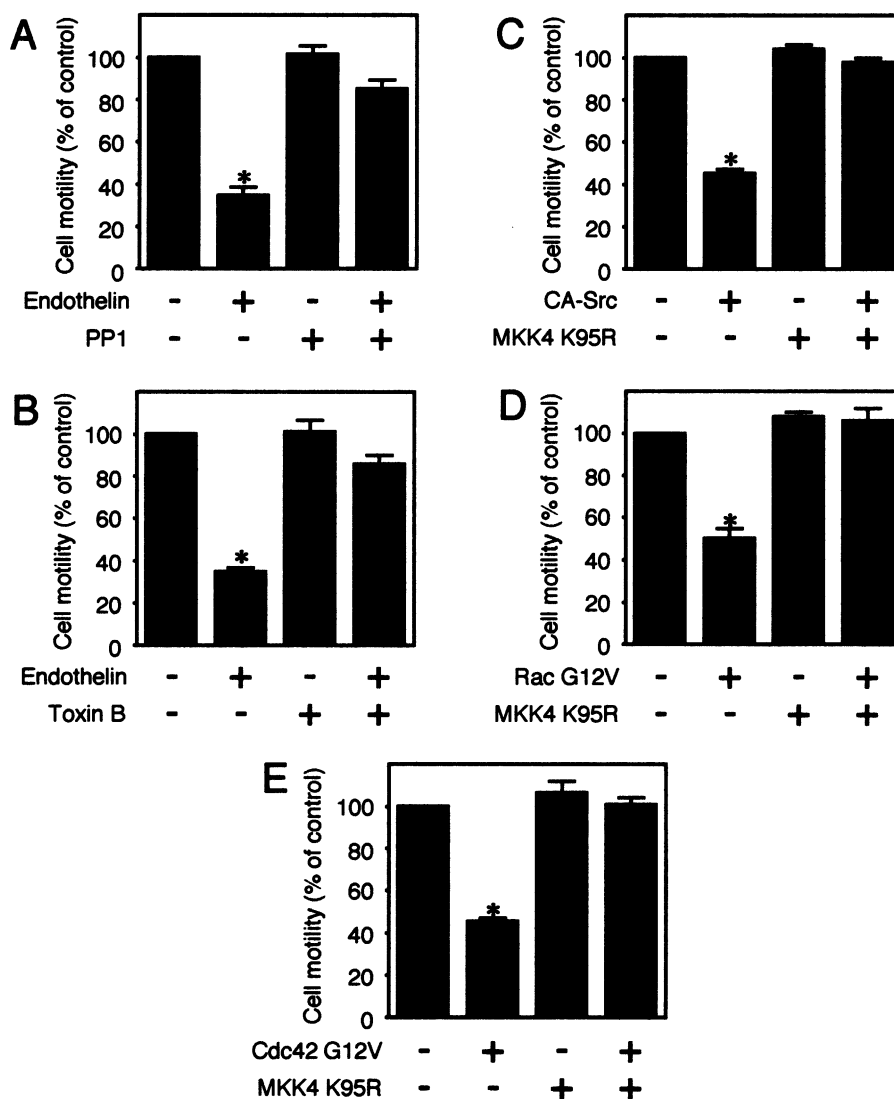
Fig. 4. Endothelin-induced inhibition of cell motility requires the JNK pathway and is dependent upon Src kinase, Rac1, and Cdc42. A: Cells were pretreated with or without 10 µM PP1 for 18 h, and then treated with or without endothelin (100 nM, final concentration) at 37°C for 3 h. B: Cells were pretreated with or without 2 ng/ml toxin B for 18 h, and treated with or without endothelin (100 nM, final concentration) at 37°C for 3 h. C: Cells were transfected with the plasmid encoding mock, CA-Src, or MKK4K95R. D: Cells were transfected with the plasmid encoding mock, Rac1G12V, or MKK4K95R. E: Cells were transfected with or without the plasmid encoding mock, Cdc42G12V, or MKK4K95R. Cell motility was measured using a Boyden chamber.

cell motility and this effect was blocked by co-transfection of MKK4K95R (Fig. 1D). These results further supported the idea that endothelin inhibits cell motility through the JNK signaling cascade.

3.2. JNK activation by endothelin is mediated by Src kinase

It has been reported that Src kinase mediates JNK activation induced by certain GPCRs [14,15,17]. Therefore, we examined the involvement of Src kinase in endothelin-induced JNK activation. As shown in Fig. 2A, pretreatment of cells with PP1, a specific inhibitor of Src kinase, inhibited endothelin-induced JNK activation. Similarly, JNK activation by endothelin was blocked by pretreatment of cells with PP2, another inhibitor of Src kinase (data not shown). We next determined whether endothelin treatment of cells results in activation of Src kinase, using an antibody specific for the

autophosphorylated Src. We detected activation of Src, as demonstrated by its autophosphorylation at Tyr[418], in response to endothelin treatment (Fig. 2B). These results suggested that Src kinase mediates activation of JNK by endothelin.

3.3. JNK activation by endothelin is mediated by Rac1 and Cdc42

GPCR-mediated activation of JNK has been shown to involve small GTPases of the Rho family [13–17]. Thus, we examined the involvement of these small GTPases in endothelin-induced JNK activation. *C. difficile* toxin B glycosylates Rho family small GTPases RhoA, Rac1, and Cdc42, and inhibits their cellular functions. Pretreatment of cells with toxin B suppressed endothelin-induced JNK activation (Fig. 3A). Next, we measured the intrinsic activities of Rho family small

GTPases by pull-down assays, using GST-tagged mDia1RBD and αPakCRIB. mDia1RBD specifically interacts with active GTP-bound RhoA [22], and αPakCRIB specifically associates with active GTP-bound Rac1 or Cdc42 [23]. αPakCRIB bound both Rac1 and Cdc42 from extracts prepared from endothelin-treated cells, but mDia1RBD did not bind RhoA, suggesting that endothelin activated Rac1 and Cdc42, but not RhoA (Fig. 3B). Furthermore, pretreatment of cells with PP1 inhibited endothelin-induced activation of Rac1 and Cdc42 (Fig. 3B). These results suggested that endothelin activates JNK through Rac1 and Cdc42, and that this effect is dependent upon active Src kinase.

3.4. Endothelin inhibits cell motility through the JNK pathway in a manner dependent upon Src kinase, Rac1, and Cdc42

We next investigated whether Src kinase, Rac1, and Cdc42 participate in endothelin-induced inhibition of cell motility. As shown in Fig. 4A, pretreatment of cells with PP1 suppressed endothelin-induced inhibition of cell motility. Similarly, pretreatment of cells with toxin B also reduced endothelin-induced inhibition of cell motility (Fig. 4B). These results suggested that active Src kinase and Rho family small GTPases are necessary for inhibition of cell motility by endothelin.

We further examined the effect of Src kinase, Rac1, and Cdc42 on cell motility through transfection studies making use of a constitutively active form of these signaling components. Transfection of constitutively active (CA)-Src inhibited cell motility (Fig. 4C). Co-transfection of CA-Src with MKK4K95R counteracted this effect, demonstrating that Src kinase inhibits cell motility in a manner dependent upon the JNK pathway (Fig. 4C). Similarly, transfection of Rac1G12V and Cdc42G12V, which are constitutively active forms of Rac1 and Cdc42, respectively, inhibited cell motility (Fig. 4D and E). Once again, this effect was blocked by co-transfection with MKK4K95R, demonstrating a dependence upon the JNK pathway (Fig. 4D and E). Taken together, these results suggested that endothelin inhibits cell motility through the JNK pathway in a manner dependent upon Src kinase, Rac1, and Cdc42.

Rac1 has been implicated in migration of cells such as fibroblasts and endothelial cells [25–28]. In addition, a dominant-inhibitory form of Cdc42 blocks the migration of macrophages [29]. These findings suggest that Rac1 and Cdc42 induce cell migration in certain types of cells. We have demonstrated the involvement of Rac1 and Cdc42 in endothelin-mediated inhibition of cell motility in 293 cells. The effect of Rac1 and Cdc42 on cell migration may vary, depending on the cell type.

In this study, we have shown that endothelin inhibits cell motility through activation of the JNK pathway in a manner dependent upon Src kinase, Rac1, and Cdc42. Further study is necessary to clarify how the endothelin receptor suppresses cell motility. Such studies should enhance our understanding of the general mechanism by which GPCRs regulate the JNK pathway and in turn cell migration.

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