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Endothelin-1 induces VCAM-1 expression-mediated inflammation via receptor tyrosine kinases and Elk/p300 in human tracheal smooth muscle cells

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Lin CC, Lin WN, Hou WC, Hsiao LD, Yang CM. Endothelin-1 induces VCAM-1 expression-mediated inflammation via receptor tyrosine kinases and Elk/p300 in human tracheal smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 309: L211–L225, 2015. First published June 12, 2015; doi:10.1152/ajplung.00232.2014.—The elevated level of endothelin-1 (ET-1) has been detected in the bronchoalveolar lavage of patients with severe asthma, acute lung injury, acute respiratory distress syndrome, and sepsis. ET-1 may affect vessel tone together with lung physiology and pathology. Vascular cell adhesion molecule-1 (VCAM-1) is one kind of adhesion molecules participating in the process of polymorphonuclear leukocyte transmigration and regulating the occurrence and amplification of tissue inflammation. However, the molecular mechanisms underlying ET-1-mediated expression of VCAM-1 on human tracheal smooth muscle cells (HTSMCs) were largely unknown. Here we reported that ET-1 stimulated expression of VCAM-1 gene on HTSMCs, which was blocked by pretreatment with the inhibitors of ET receptors, Src, matrix metalloproteinasases (MMPs), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), phosphatidylinositol 3-kinase (PI3K), AKT, MEK1/2, and p300, suggesting the participation of these signaling components in ET-1-regulated HTSMC responses. Furthermore, transfection with small-interfering RNA (siRNA) of Src, AKT, p42 mitogen-activated protein kinase (MAPK), or p300 downregulated the respective proteins and significantly attenuated ET-1-induced VCAM-1 expression. ET-1 also stimulated phosphorylation of Src, EGFR, PDGFR, AKT, p42/p44 MAPK, and Elk-1 and acetylation of histone H4 on HTSMCs. Immunoprecipitation assay showed the association between Elk-1 and p300 in the nucleus. Adhesion assay revealed that the adhesion of THP-1 to HTSMCs challenged with ET-1 was increased, which was attenuated by the inhibitors of ET receptors, Src, MMPs, EGFR, PDGFR, PI3K, AKT, p42/p44 MAPK, and p300. Taken together, these data suggested that ET-1 promotes occurrence and amplification of pathology-related airway inflammation via enhancing VCAM-1 expression in an ET receptor/Src/MMP/EGFR, PDGFR/PI3K/AKT/p42/p44 MAPK/Elk-1/p300 pathway in HTSMCs.

human tracheal smooth muscle cells; vascular cell adhesion molecule-1

Asthma, a chronic inflammatory disease, is characterized by airway smooth muscle contraction, hypersecretion of mucus, epithelial damage, thickening of the extracellular matrix, increased hyperresponsiveness via airway smooth muscle hyperplasia/hypertrophy, and inflammation within the airway (12). In addition to TNF-α, IL-1β, and lipopolysaccharide, endothelins (ETs) had been reported as promoters of airway hyperresponsiveness and inflammation in various animal models (15, 35). ETs are 21-amino-acid peptides functioning originally as pressure regulators. There are three types of ETs in humans, ET-1, ET-2, and ET-3, which are encoded by chromosomes 6, 1, and 20, respectively (36). Once released, ETs may exert their function via activation of ET receptors, type A (ET_A), type B1 (ET_B1), and type B2 (ET_B2) (32). ETs mediated vasoconstriction via binding to ET_A and ET_B2 receptors but caused production of nitric oxide from endothelial cells, resulting in vasodilation through activation of ET_B1 receptor. Thus, expression of ETs should be tightly regulated to maintain proper vessel pressure. Accumulating evidence suggested that dysregulation of ET expression is related to various pulmonary pathologies. It is found that, in addition to pulmonary hypertension, ET-1 level in bronchoalveolar lavage is significantly increased in the patients with severe asthma, acute lung injury, acute respiratory distress syndrome, and sepsis (14). The levels of ET-1 in the pulmonary system are particularly high compared with those of other tissues (16). The origin of ET-1 in the pulmonary system includes airway epithelial cells, macrophages, vascular endothelial cells, vascular smooth muscle cells, and fibroblasts (33). In addition to regulating vessel tone, ET-1 may also affect lung physiology and pulmonary pathology. It is found that ET-1 contributes to antigen-induced airway responses in sheep allergic to Ascaris suum (30). Aerosolized BQ-123 (ET_A antagonist) significantly blocked carbachol-induced airway hyperresponsiveness in ET-1-challenged sheep (30).

Recruitment and infiltration of polymorphonuclear leukocytes at sites of inflammation are regulated by expression of cell adhesion molecules in the pathogenesis of airway disease, including asthma (38). Vascular cell adhesion molecule-1 (VCAM-1), belonging to the immunoglobulin superfamily, is one kind of adhesion molecule participating in the process of polymorphonuclear leukocyte transmigration and regulating the occurrence and amplification of tissue inflammation. Up-regulation of VCAM-1 is found on human tracheal smooth muscle cells (HTSMCs) in response to TNF-α, IL-1β, or lipopolysaccharide (25, 28). VCAM-1 on the cell surface of...
HTSMCs is pivotal for interacting with a variety of inflammatory cells relevant for various pulmonary pathogenesis such as asthma (39). The main ligand of VCAM-1 is αβ1-integrin. After binding with αβ1 expressed on leukocytes, VCAM-1/αβ1 interactions critically regulate leukocyte adhesion and spreading, thus facilitating the extravascular migration of leukocytes across the endothelium in extravascular tissues (6). The effects of ET-1 on mediating vessel constriction had been widely studied; however, little is known about the actions of ET-1 on pulmonary resident cells. In addition to regulating the vessel system, whether ET-1 facilitated occurrence or amplification of pulmonary inflammation and led to pathologies via VCAM-1 expression is largely unknown. Thus, this study would mainly focus on the effects of ET-1 in mediating VCAM-1 expression on HTSMCs and the related mechanisms.

ET_A and ET_B receptors are seven-transmembrane G protein-coupled membrane proteins (24). By recruiting different Gα proteins, both receptors could activate various downstream protein kinases and mediate various physiological or pathological events. It is found that ET-1 activates the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) signaling pathway in many cell types, including cardiomyocytes, fibroblasts, glomerular mesangial cells, and vascular smooth muscle cells (21). Moreover, the roles of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway in ET-1-mediated Ca2+ influx or vessel contraction have also been proven in carotid artery and basilar artery (1). However, whether p42/p44 MAPK, PI3K, or AKT participated in ET-1-mediated VCAM-1 expression on HTSMCs still needs to be studied. In addition, several G protein-coupled receptors (GPCRs) are found to transactivate receptor tyrosine kinases (RTK) and nonreceptor tyrosine kinases such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and Src (11, 13). However, whether ET-1-activated ET receptors also affected cell functions via transactivation of EGFR or PDGFR is still largely unknown. Thus, the role of p42/p44 MAPK, PI3K, AKT, EGFR, or PDGFR in mediating ET-1-induced VCAM-1 expression on HTSMCs will be investigated.

We proposed that ET-1 contributed to airway inflammation and pulmonary pathology via upregulation of VCAM-1, thus facilitating the migration and adhesion of leukocytes in the pulmonary system. We found that ET-1 increased expression of VCAM-1 on HTSMCs via Src/matrix metalloproteinase (MMP)-dependent transactivation of the receptor tyrosine kinase/PI3K/AKT/p42/p44 MAPK pathway, leading to activation and interaction of Elk-1 and p300 and acetylation of histone H4. Upregulation of VCAM-1 increased adhesion between HTSMCs and leukocytes, facilitating the occurrence and amplification of tissue inflammation.

MATERIALS AND METHODS

Materials. DMEM/F-12 medium, FBS, and TRIZOL were purchased from Invitrogen (Carlsbad, CA). Hybond C membrane and the enhanced chemiluminescence (ECL) Western blotting detection reagents according to the instructions of the manufacturer. The transfection complex was diluted in 400 μl DMEM/F-12 medium and added directly to the cells. The medium was replaced with serum-free DMEM/F-12 after 24 h. Cells were analyzed at 72 h after transfection by Western blot. The transfection efficiency (~60%) was determined by transfection with green fluorescent protein (GFP).

Total RNA extraction and RT-PCR analysis. Total RNA was isolated from HTSMCs in 10-cm culture dishes with Trizol according to the manufacturer’s protocol. RNA concentration was spectrophotometrically determined at 260 nm. First-strand cDNA synthesis was

BQ-78, GPA_A, GPA_A, U-0126, PP1, AG-1478, AG-1296, GM-6001, MMP2/9, LY-294002, SH-5, wortmannin, and GR-334 were from Biomol (Plymouth Meeting, PA). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL). SDS-PAGE supplies were from MDBio (Taipei, Taiwan). Enzymes and other chemicals were from Sigma (St. Louis, MO).

Cell culture. Human HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and grown in DMEM/F-12 containing 10% FBS and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin, and 250 mg/ml fungizone) at 37°C in a humidified 5% CO2 atmosphere. When the cultures reached confluence, cells were treated with 0.05% trypsin/0.53 mM EDTA for 5 min at 37°C. The cell suspension was diluted with DMEM/F-12 containing 10% FBS to a concentration of 2 × 10^6 cells/ml. The cell suspension was plated on 12-well culture plates and 10-cm culture dishes for the measurement of protein expression and mRNA accumulation, respectively. Culture medium was changed after 24 h and then every 3 days.

To characterize the isolated and cultured HTSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by an indirect immunofluorescent staining using an antibody to smooth muscle actin or desmin. Over 95% of the cell preparation was found to be composed of tracheal smooth muscle cells.

Preparation of cell extracts and Western blot analysis. HTSMCs were shifted to serum-free DMEM/F-12 medium for 24 h. HTSMCs were incubated with different concentrations of ET-1 at 37°C for the indicated time periods. When inhibitors were used, they were added 1 h before the application of ET-1. After incubation, the cells were rapidly washed with ice-cold PBS, scraped, and collected by centrifugation at 1,000 g for 10 min. The collected cells were lysed with ice-cold lysis buffer containing: 25 mM Tris·HCl, pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium vanadate, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 0.5% Nonidet P-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 45,000 g for 1 h at 4°C to yield the whole cell extract. The protein concentration was determined by the BCA reagents according to the instructions of the manufacturer. Samples from these supernatant fractions (30 μg protein) were denatured and subjected to SDS-PAGE using a 12% running gel. Proteins were transferred to the nitrocellulose membrane, and the membrane was incubated successively at room temperature with 5% BSA in 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.4 (TTBS), for 1 h. Membranes were incubated overnight at 4°C with a primary antibody such as VCAM-1 or GAPDH used at a dilution of 1:1,000 in TTBS. Membranes were washed with TTBS three times for 10 min each and incubated with a 1:2,000 dilution of anti-rabbit or anti-mouse horseradish peroxidase antibody for 1 h. The membrane was washed extensively with TTBS. The immunoreactive bands were detected by ECL reagents.

Transfection of siRNAs for Src, AKT, p85α, p110, p42, and p300. SMARTpool RNA duplexes corresponding to human Src, AKT, p85α, p110, p42, p300, and Scrambled no. 2 siRNA were from Dharmacon Research (Lafayette, CO). HTSMCs (passage 4 or 5) were cultured on 12-well plates. At 70–80% confluence, the media were replaced with serum-free DMEM/F-12 after 24 h. Cells were analyzed at 72 h after transfection by Western blot.
performed with 2 µg of total RNA using random hexamers as primers in a final volume of 20 µl (5 µg/µl random hexamers, 1 mM dNTPs, 2 U/µl RNasin, and 10 U/µl Moloney murine leukemia virus reverse transcriptase). The reaction was carried out at 37°C for 60 min. cDNAs encoding β-actin and VCAM-1 were amplified from 3 to 5 µl of the cDNA reaction mixture using specific gene primers. Oligonucleotide primers for β-actin and VCAM-1 were as follows: β-actin: 5'-TGAAGGCGTCAACCCACTGTCGCTA-3' (sense), 5'-GCTAAGAGCTTTGCGTGAGATG-3' (antisense); VCAM-1: 5'-GGAGAATTTGACGTTACAGTGACAGGCTCC-3' (sense), 5'-CAAGTTACATATCCAGGA-3' (antisense).

The amplification profile includes 1 cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, and extension at 72°C for 1 min and then 1 cycle of final extension at 72°C for 5 min. The expression of β-actin was used as an internal control.

**Measurement of VCAM-1 luciferase activity.** For construction of the VCAM-1-luc plasmid, a region spanning −1,716 to −119 bp (kindly provided by Dr. W. C. Aird, Department of Molecular Medicine, Beth Israel Deaconess Medical Center, Boston, MA) was cloned into pGL3-basic vector (Promega, Madison, WI). VCAM-1-luc plasmid was transiently transfected into HTSMCs using GeneFamer transfection reagent. Briefly, VCAM-1-luc plasmid and β-galactosidase (β-gal) were formulated with GeneJammer transfection reagent. The transfection complex was diluted into 900 µl of DMEM/F-12 medium and then added directly to the cells for 6 h. The medium was replaced with DMEM/F-12 containing 10% FBS for 24 h. Next, the cells were starved for 24 h in serum-free DMEM/F-12 medium. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation at 1,000 revolutions/min for 2 min, aliquots of the supernatants were tested for luciferase activity using a luciferase assay system (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were standardized for β-gal activity.

**Commmunoprecipitation assay.** Cell lysates containing 1 µg of protein were incubated with 2 µg of anti-p300 antibody at 4°C for 24 h, and then 10 µl of 50% protein A-agarose beads were added and mixed for 24 h at 4°C. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100. Laemmli buffer was added and then subjected to electrophoresis on 12% SDS-PAGE. Western blot analysis was performed using antibodies against p300 and Elk-1.

**Immunofluorescence staining.** HTSMCs were plated on six-well culture plates with cover slips. Cells were further cultured in serum-free DMEM/F-12 medium for 24 h, and then 10 µl of 50% protein A-agarose beads were added and mixed for 24 h at 4°C. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100. Fifty microliters of Laemmli buffer were added and then subjected to electrophoresis on 12% SDS-PAGE. Western blot analysis was performed using antibodies against p300 and Elk-1.

**RESULTS**

**ET-1 induced VCAM-1 gene expression.** To investigate the effect of ET-1 on VCAM-1 expression, HTSMCs were incubated with various concentrations of ET-1 for the indicated time periods. The amount of de novo synthesis of VCAM-1 protein was determined using Western blot analysis. As shown in Fig. 1A, ET-1 induced VCAM-1 expression in a time-dependent manner with maximum expression at 16 h and then slightly declined at 24 h. The maximal VCAM-1 expression was achieved with 30 nM ET-1 at 16 h. The blot was stripped and reprobed with an anti-GAPDH antibody to demonstrate an equivalent amount of GAPDH expression.

To further examine whether the effect of ET-1 on VCAM-1 expression occurred at the level of transcription, VCAM-1 mRNA expression was determined by RT-PCR. As shown in Fig. 1B, ET-1 induced VCAM-1 mRNA accumulation in a time-dependent manner. The expression of VCAM-1 mRNA occurred within 2 h and achieved maximum within 6 h. To determine whether ET-1 upregulated VCAM-1 promoter activity, VCAM-1 promoter plasmid was cloned and transfected into HTSMCs. ET-1 stimulated activation of VCAM-1 promoter in a time-dependent manner with a maximal response within 4–6 h (Fig. 1C). Thus, ET-1 mediated VCAM-1 protein expression via upregulation of VCAM-1 promoter activity and mRNA expression in HTSMCs.

**Participation of ET receptors and G proteins in ET-1-induced VCAM-1 expression.** It is reported that ET-1 exerts its function via activation of GPCRs (9). First, we showed that both ETA and ETB receptors expressed on HTSMCs (Fig. 2A). To address the roles of different subtypes of ET receptors or G proteins in ET-1-induced VCAM-1 expression, HTSMCs were
pretreated with the inhibitor of ETA (BQ-123), ETB (BQ-788), G\textsubscript{i/o} or G\textsubscript{i} (GPA2), or G\textsubscript{i} (GPA2A) for 1 h before exposure to ET-1 for 16 h. As shown in Fig. 2A, ET-1-induced expression of VCAM-1 was significantly attenuated by pretreatment with BQ-123, BQ-788, GPA2, or GPA2A, suggesting the participation of G\textsubscript{i/o} or G\textsubscript{i}-coupled ETA and ETB receptors in ET-1-mediated VCAM-1 expression. Similarly, ET-1-stimulated VCAM-1 promoter activity and mRNA expression are also inhibited by pretreatment with these inhibitors (Fig. 2A, B and C). These results suggested that ET-1 induced VCAM-1 gene expression via both G protein-coupled ETA and ETB receptors in HTSMCs.

**Involvement of MMPs and Src in ETA-stimulated VCAM-1 expression.** Studies reported that GPCR may transactivate RTK via extracellular activation of MMPs or intracellular phosphorylation of Src (3, 10, 13). To differentiate whether MMPs or Src participated in ET-1-mediated VCAM-1 expression, HTSMCs were pretreated with conventional MMP inhibitor (GM-6001), specific MMP2/9 inhibitor, or Src inhibitor (PP1). Data in Fig. 3, A and B, showed that ET-1-induced VCAM-1 expression was significantly attenuated by pretreatment with the inhibitor of MMP or Src, suggesting that MMPs, especially MMP2 and MMP9, and Src may participate in the regulation of VCAM-1 expression. To confirm the role of Src, transfection with Src siRNA significantly reduced Src expression and attenuated ET-1-induced VCAM-1 expression (Fig. 3C). Similarly, pretreatment of HTSMCs with GM-6001, MMP2/9 inhibitor, or PP1 also reduced ET-1-induced VCAM-1 promoter activity and mRNA expression (Fig. 3D and E). Moreover, we found that ET-1 directly stimulated phosphorylation of Src in a time-dependent manner with maximal response within 5 min, which was reduced by pretreatment with the inhibitor of Src (PP1), ETA receptor (BQ-123), ETB receptor (BQ-788), G\textsubscript{i} protein (GPA2A), or G\textsubscript{i} protein (GPA2) (Fig. 3F). Taken together, these data suggested that both MMPs and Src may participate in ET-1-induced VCAM-1 expression in HTSMCs.

**Transactivation of receptor tyrosine kinases in VCAM-1 expression induced by ET-1.** To investigate whether activation of RTK such as EGFR or PDGFR was involved in ET-1-mediated VCAM-1 upregulation, specific inhibitors against EGFR (AG-1478) and PDGFR (AG-1296) were used. As shown in Fig. 4A, pretreatment with AG-1468 or AG-1296 attenuated ET-1-induced VCAM-1 protein expression in a concentration-dependent manner. Similarly, ET-1-stimulated VCAM-1 promoter activity (Fig. 4B) and mRNA expression (Fig. 4C) were also attenuated by AG-1478 and AG-1296. These results suggested that EGFR and PDGFR are involved in ET-1-induced VCAM-1 gene expression. To determine ET-1-mediated VCAM-1 expression mediated through phosphorylation of EGFR or PDGFR, we found that ET-1 stimulated phosphorylation of EGFR and PDGFR within 5–30 min, which were attenuated by pretreatment with the inhibitors of ETA (BQ-123), ETB (BQ-788), G\textsubscript{i} protein (GPA2), G\textsubscript{i} protein (GPA2A), MMP inhibitors (GM-6001 and MMP2/9), EGFR (AG-1478), or PDGFR (AG-1296) (Fig. 4D, E and F). Collectively, these results suggested that ET-1 transactivated PDGFR/EGFR through ET receptor (ETA and ETB)-dependent activation of c-Src/MMP and thus led to an increase of VCAM-1 gene expression in HTSMCs.

**Involvement of PI3K-AKT in ET-1-induced VCAM-1 expression.** AKT is widely known as a downstream component of PI3K that has been shown to be activated via a c-Src/RTK transactivation pathway (2). To further detect whether activation of PI3K/AKT was required for ET-1-induced VCAM-1 expression, the inhibitors of PI3K (wortmannin, LY-294002) and AKT (SH-5) were used. The results showed that pretreat-
ment with wortmannin, LY-294002, or SH-5 significantly inhibited the ET-1-mediated increase of VCAM-1 protein expression (Fig. 5A). To confirm the role of PI3K and AKT in this response, HTSMCs were transiently transfected with siRNA against AKT, p85 α (the regulatory subunit of PI3K), or p110 (the catalytic subunit of PI3K) and then incubated with 30 nM ET-1. We found that siRNA against AKT reduced AKT expression and attenuated ET-1-induced VCAM-1 expression (Fig. 5B). Similarly, siRNA against p85 α or p110 reduced protein expression of PI3K subunits together with abolishment of ET-1-stimulated VCAM-1 expression in HTSMCs (Fig. 5B). Moreover, VCAM-1 promoter activity and mRNA expression were also attenuated by inhibitor of PI3K or AKT pre-treatment (Fig. 5, C and D). To examine whether ET-1 stimulated AKT phosphorylation leading to VCAM-1 expression, we found that ET-1 stimulated AKT phosphorylation as early as 3 min, reached maximum at 5 min, and then declined (Fig. 5E). The phosphorylation of AKT was attenuated by pretreatment with the inhibitor of ETA (BQ-123), ETB (BQ-788), Gα protein (GPA2 A), Gα protein (GPA2), EGFR (AG-1478), PDGFR (AG-1296), MMP (GM-6001 and MMP2/9), Src (PP1), PI3K (LY-294002 and wortmannin), or AKT (SH-5) (Fig. 5E). Thus, these results indicated that ET-1-stimulated phosphorylation of AKT was mediated through ETA/ETB receptor-dependent activation of the Src/MMP/RTK/PI3K pathway in HTSMCs.

Fig. 2. Involvement of ET receptors and G proteins in ET-1-induced VCAM-1 expression. A: expression of ETA and ETB receptors in HTSMCs was determined by RT-PCR. HTSMCs were pretreated with various concentrations of BQ-123, BQ-788, GPA2 A, and GPA2 A for 1 h and then incubated with 30 nM ET-1 for 16 h. Expression of VCAM-1 was determined. GAPDH was used as an internal control. B: cells were transiently transfected with VCAM-1-luc reporter gene, pretreated with 10 nM of BQ-123, BQ-788, GPA2 A, and GPA2 A and then incubated with 30 nM ET-1 for 6 h. Promoter activity was determined and normalized to β-galactosidase activity. C: cells were pretreated with BQ-123, BQ-788, GPA2 A, and GPA2 A and then incubated with 30 nM ET-1 for 6 h. VCAM-1 mRNA was determined by RT-PCR. Data are expressed as means ± SE of 5 independent experiments (n = 5). *P < 0.05 and #P < 0.01 compared with cells exposed to vehicle alone.
Involvement of p42/p44 MAPK in ET-1-induced VCAM-1 expression. It is found that EGF activates p42/p44 MAPK phosphorylation in an AKT-dependent manner (20, 34). However, the PI3K inhibitor LY-294004 failed to inhibit PDGF-stimulated phosphorylation of p42/p44 MAPK in oligodendrocytes (19). To examine whether ET-1 mediated VCAM-1 expression via PI3K/AKT-dependent activation of p42/p44 MAPK, MEK1/2 inhibitor (U-0126) and p42 siRNA were used. We found that ET-1-induced VCAM-1 expression was significantly attenuated by U-0126 or p42 siRNA transfection (Fig. 6A), suggesting that ET-1-induced VCAM-1 expression is mediated through p42/p44 MAPK. Similarly, ET-1-induced VCAM-1 promoter activity and mRNA expression were also reduced by U-0126 (Fig. 6, B and C). To elucidate the rela-

Fig. 3. Involvement of matrix metalloproteinases (MMPs) and Src in ET-1-induced VCAM-1 expression. A and B: HTSMCs were pretreated with various concentrations of GM-6001, MMP2/9 inhibitor, or PP1 for 1 h and then incubated with 30 nM ET-1 for 16 h. Expression of VCAM-1 was determined. GAPDH was used as an internal control. C: HTSMCs were transfected with Src or scramble small-interfering RNA (siRNA) for 16 h and then incubated with 30 nM ET-1 for 16 h. The levels of VCAM-1 and Src proteins were determined by Western blot. D: cells were transiently transfected with VCAM-1-luc reporter gene, pretreated with PP1 (100 nM), GM-6001 (10 μM), or MMP2/9 inhibitor (10 nM) for 1 h, and then incubated with 30 nM ET-1 for 6 h. Promoter activity was determined and normalized to β-galactosidase activity. E: cells were pretreated with PP1 (100 nM), GM-6001 (10 μM), or MMP2/9 inhibitor (10 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h. VCAM-1 mRNA was determined by RT-PCR. To fit the construct of data layout, the data were rearranged from the same gel except nonrelated inhibitors and disclosed by the insertion of white spaces. F: HTSMCs were pretreated without or with PP1 (100 nM), BQ-123 (10 nM), BQ-788 (10 μM), GPA2A (10 nM), or GPA2 (10 μM) for 1 h and then incubated with 30 nM ET-1 for the indicated time intervals. Phosphorylation of Src was detected by Western blot. Data are expressed as means ± SE of 5 independent experiments (n = 5). #P < 0.01 compared with cells exposed to vehicle alone.
Fig. 4. Involvement of RTK in ET-1-induced VCAM-1 expression. A: HTSMCs were pretreated with various concentrations of AG-1478 or AG-1296 for 1 h and then incubated with 30 nM ET-1 for 16 h. Expression of VCAM-1 was determined. GAPDH was used as an internal control. B: VCAM-1-luc reporter gene transiently transfected cells were pretreated with AG-1478 (100 nM) or AG-1296 (100 nM) for 1 h and then stimulated with 30 nM of ET-1 for 6 h. Promoter activity was determined and normalized to β-galactosidase activity. C: cells were pretreated with AG-1478 (100 nM) or AG-1296 (100 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h; VCAM-1 mRNA was determined by RT-PCR. D and E: HTSMCs were pretreated with BQ-123 (10 nM), BQ-788 (10 μM), GPA2 (10 μM), GPA2A (10 nM), PP1 (100 nM), GM-6001 (10 μM), MMP2/9 inhibitor (10 nM), AG-1478 (100 nM), or AG-1296 (100 nM) for 1 h and then stimulated with 30 nM ET-1 for the indicated time intervals. Phosphorylation of epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) was detected by Western blot. Data are expressed as means ± SE of 5 independent experiments (n = 5). #P < 0.01 compared with cells exposed to vehicle alone.
L218

ET-1-INDUCED VCAM-1 EXPRESSION

A

[Image of immunoblot with densitometry units showing VCAM-1 and GAPDH levels with ET-1 and Wort concentrations.]

B

[Image of immunoblot with densitometry units showing Akt and p85α levels with ET-1 and Wort concentrations.]

C

[Graph showing VCAM-1 promoter activity with Inhibitor concentrations.]

D

[Graph showing VCAM-1 levels with ET-1 and Inhibitor concentrations.]

E

[Table showing fold of basal Akt and p-Akt levels with various inhibitors and ET-1 concentrations.]

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tionship between p42/p44 MAPK and PI3K/AKT in ET-1-mediated responses, HTSMCs were treated with 30 nM ET-1 for the indicated time periods. We found that ET-1 stimulate phosphorylation of p42/p44 MAPK, which was attenuated by pretreatment with U-0126 (Fig. 6D) and the inhibitor of ET\textsubscript{A}, ET\textsubscript{B} receptor, G\textsubscript{q} protein, G\textsubscript{i} protein, EGFR, PDGFR, MMP, Src, PI3K, or AKT (data not shown). These data indicated that ET-1-induced VCAM-1 expression was mediated through p42/p44 MAPK in a Src/MMP/RTK/PI3K/AKT-dependent manner in HTSMCs.

Several studies demonstrated that activated p42/p44 MAPK may translocate into the nucleus to modulate gene transcription (4, 5). To detect whether nuclear translocation of p42/p44 MAPK occurred in ET-1-stimulated HTSMCs, as shown in Fig. 6E, ET-1 mediated nuclear translocation of p42/p44 MAPK as early as 15 min and sustained over 60 min. To confirm the translocation of p42/p44 MAPK from the cytosol to the nucleus, the nuclear fraction was prepared from ET-1-stimulated HTSMCs and subjected to 10% SDS-PAGE. As shown in Fig. 6F, ET-1 stimulated nuclear translocation of p42/p44 MAPK, which was attenuated by pretreatment with ET\textsubscript{A} (BQ-123), ET\textsubscript{B} receptor (BQ-788), G\textsubscript{q} protein (GPA\textsubscript{2}A), G\textsubscript{i} protein (GPA\textsubscript{2A}), or p42/p44 MAPK (U-0126) inhibitor. These results suggested that ET-1-mediated nuclear translocation of p42/p44 MAPK is also mediated through a Src/MMP/RTK/PI3K/AKT-dependent way and leads to VCAM-1 expression in HTSMCs.

ET-1 mediated phosphorylation of Elk-1. Elk-1 is a member of the Ets family of transcription activators. It is an essential component in the serum response ternary complex and has been shown to regulate expression of immediate-early response genes (26). Activation of Elk-1 depends on the phosphorylation of Ser\textsuperscript{383} and Ser\textsuperscript{389} in the transcription activation domain by p42/p44 MAPK, which enhances DNA-binding affinity (16a). It has been shown that phosphorylation of Elk-1 by p42/p44 MAPK leads Elk-1 bound to MMP-9 promoter, thereby inducing transcription of MMP-9 by bradykinin (18). To evaluate whether Elk-1 participated in ET-1-mediated VCAM-1 expression, as shown in Fig. 7, ET-1 time-dependently stimulated phosphorylation of Elk-1 with a maximal response within 30 min, which was attenuated by pretreatment with BQ-123, BQ-788, GPA\textsubscript{2}A, GPA\textsubscript{2}, AG-1478, AG-1296, PP1, GM-6001, MMP2/9 inhibitor, wortmannin, AG-1478, AG-1296, wortmannin, LY-294002, SH-5, U-0126, or GR-343 (Fig. 8E). These results suggested that ET-1 stimulates phosphorylation of histone H4 via the ET receptor-activated RTK/PI3K/AKT/p42/p44 MAPK/Elk-1/p300 pathway. Taken together, these data suggested that ET-1-induced VCAM-1 expression is mediated by promoting Elk/p300 interaction and leading to an increase of histone H4 acetylation in an ET receptor/Src/RTK/PI3K/AKT/p42/p44 MAPK manner in HTSMCs.

ET-1 mediated VCAM-1 expression via activation of p300. It is found that phosphorylation on the Ser\textsuperscript{383} and Ser\textsuperscript{389} of Elk-1 by p42/p44 MAPK promotes Elk-1 interaction with p300, which changes the histone acetyltransferase activity in the Elk-1-associated complex that could play a critical role in chromatin remodeling and gene activation (26). To investigate whether p300 is also involved in ET-1-induced VCAM-1 expression via interacting with activated Elk-1, we first examine the interaction between Elk-1 and p300. HTSMCs were treated with 30 nM ET-1, and immunoprecipitation assay was performed using an anti-Elk-1 or anti-p300 antibody. We found that ET-1 stimulated interaction of Elk-1 and p300 (Fig. 8A), which was significantly attenuated by pretreatment with U-0126, suggesting that ET-1-induced Elk-1 and p300 interaction is mediated through p42/p44 MAPK. To confirm the role of p300 in ET-1-induced VCAM-1 expression, GR-343 (p300 inhibitor) or siRNA against p300 was used. We found that pretreatment with GR-343 significantly inhibited ET-1-induced VCAM-1 expression (Fig. 8B), which was confirmed by p300 siRNA that decreased the intracellular p300 level together with attenuation of VCAM-1 expression (Fig. 8C). Similarly, the ET-1-stimulated increase of VCAM-1 promoter activity and mRNA expression was inhibited by pretreatment with GR-343 (Fig. 8D), indicating that p300 associated with Elk-1 is involved in ET-1-induced VCAM-1 expression, promoter activity, and mRNA expression in HTSMCs.

p300 is an acetyltransferase and regulates lysine acetylation of histone. To evaluate the function of ET-1-activated p300 and the related signaling mechanisms, the level of histone H4 acetylation was detected. HTSMCs were pretreated with or without various inhibitors and then incubated with 30 nM ET-1 for the indicated time periods. The cell lysate was extracted and analyzed by Western blot using an anti-acetyl histone H4 antibody. As shown in Fig. 8E, ET-1 stimulated an increase of acetylated histone H4 that was attenuated by pretreatment with BQ-788, BQ-123, GPA\textsubscript{2}A, GPA\textsubscript{2}, PP1, GM-6001, MMP2/9 inhibitor, AG-1478, AG-1296, wortmannin, LY-294002, SH-5, U-0126, or GR-343 (Fig. 8E). These results suggested that ET-1 stimulates acetylation of histone H4 via the ET receptor-transactivated RTK/PI3K/AKT/p42/p44 MAPK/Elk-1/p300 pathway. Taken together, these data suggested that ET-1-induced VCAM-1 expression is mediated by promoting Elk/p300 interaction and leading to an increase of histone H4 acetylation in an ET receptor/Src/RTK/PI3K/AKT/p42/p44 MAPK manner in HTSMCs.

Adhesion of THP-1 on HTSMCs challenged with ET-1. Previous studies indicated that upregulation of VCAM-1 increases the adhesion between leukocytes and cells (27). To determine the functional activity of VCAM-1 expressed on HTSMCs by ET-1, the binding activity of the HTSMC monolayer was assessed by coculture with BCECF-loaded THP-1. As shown in Fig. 9, A and B, adhesion of THP-1 to HTSMCs challenged with ET-1 was enhanced, which was attenuated by pretreatment with BQ-788, BQ-123, GPA\textsubscript{2}A, GPA\textsubscript{2}, PP1, GM-6001, MMP2/9 inhibitor, AG-1478, AG-
Fig. 6. Nuclear translocation of p42/p44 mitogen-activated protein kinase (MAPK) in ET-1-induced VCAM-1 expression. A: HTSMCs were pretreated with various concentrations of U-0126 for 1 h and then incubated with 30 nM ET-1 for 16 h. Expression of VCAM-1 was determined. GAPDH was used as an internal control. HTSMCs were transfected with p42 or scramble siRNA for 24 h and then incubated with 30 nM ET-1 for 16 h. The levels of VCAM-1 and p42 were determined by Western blot using an anti-VCAM-1 or anti-p42 antibody (Ab). GAPDH was used as an internal control. B: VCAM-1-luc reporter gene transiently transfected cells were pretreated with U-0126 (100 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h. Promoter activity was determined and normalized to β-galactosidase activity. C: cells were pretreated with U-0126 (100 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h. VCAM-1 mRNA was determined by RT-PCR. D: cells were pretreated with 100 nM of U-0126 for 1 h and then incubated with 30 nM of ET-1 for the indicated time intervals. Phosphorylation of p42/p44 MAPK was detected by Western blot.

E: cells were stimulated with 30 nM ET-1 for the indicated time intervals. Immunofluorescence assays were performed. Scale bar was 20 μm. F: HTSMCs were pretreated without or with BQ-123 (10 nM), BQ-788 (10 μM), GPA2 (10 μM), GPA2A (10 nM), or U-0126 (100 nM) for 1 h and then stimulated with 30 nM ET-1 for the indicated time intervals or 15 min. The nuclear fractions were subjected to 12% SDS-PAGE and blotted using an anti-phospho-p42/p44 MAPK or anti-lamin A (as a control) Ab. To fit the construct of data layout, the data were rearranged from the same gel except nonrelated inhibitors and disclosed by the insertion of white spaces rearranged from the original capture. Data are expressed as means ± SE of 5 independent experiments (n = 5). *P < 0.05 and #P < 0.01 compared with cells exposed to vehicle alone.
These data revealed that adhesion of THP-1 on HTSMCs challenged with ET-1 is mediated via an ET receptor-transactivated Src/RTK/PI3K/AKT/p42/p44 MAPK/Elk-1/p300 pathway in HTSMCs.

**DISCUSSION**

Pathological vascular remodeling, resulting from endothelial dysfunction, is believed to be the main factor of pulmonary hypertension. It is found that circulating plasma levels of endothelial cell-derived vasoactive mediators, ET-1, are raised in pulmonary hypertension patients, and ET-1 is also increased in their pulmonary tissues (8). In other circumstances, such as severe asthma, acute lung injury, acute respiratory distress syndrome, and sepsis, increased ET-1 level in serum and bronchoalveolar lavage is also reported in patients (14). Once released, the possibilities of ET-1 to affect surrounding cells and tissues were largely increased. Thus, we made an attempt to investigate the effects of ET-1 on airway resident cells, HTSMCs, and to reveal the possible mechanisms underlying ET-1-mediated airway inflammation. Our results showed that ET-1-stimulated promoter activity and mRNA and protein expression of VCAM-1 were attenuated by the inhibitors of ET receptors, G proteins, MMPs, Src, EGFR, PDGFR, PI3K, AKT, p42/p44 MAPK, and p300 or by transfection with the respective siRNAs. Moreover, ET-1 enhanced phosphorylation of Src, EGFR, PDGFR, AKT, p42/p44 MAPK, and Elk-1, which were attenuated by pretreatment with their respective inhibitors. Thus, activation of ET receptors/Src/RTK/PI3K/AKT/p42/p44 MAPK cascade resulted in Elk-1 phosphorylation and an increase of VCAM-1 expression in ET-1-stimulated HTSMCs. We also found that activated Elk-1 associated with p300 enhanced acetylation of histone H4 and mediated promoter activation, mRNA expression, and protein translation of VCAM-1. Increase in VCAM-1 expression led to enhancement of adhesion between monocytes and HTSMCs challenged with ET-1. Thus, ET-1 may increase the risk of airway inflammation via promoting VCAM-1 expression on HTSMCs.

Fig. 7. ET-1 stimulated phosphorylation of Elk-1 in HTSMCs. Cells were pretreated with BQ-123 (10 nM), BQ-788 (10 μM), GPA2 (10 μM), GPA2A (10 nM), AG-1478 (100 nM), AG-1296 (100 nM), GM-6001 (10 μM), MMP2/9 inhibitor (10 nM), PP1 (100 nM), LY-294002 (300 nM), wortmannin (300 nM), or SH-5 (100 nM) for 1 h and then incubated with 30 nM ET-1 for the indicated time intervals. Phosphorylation of Elk-1 was detected by Western blot. *P < 0.05 and #P < 0.01 compared with cells exposed to ET-1 alone.
Fig. 8. ET-1 induced VCAM-1 expression via activation of p300. A: HTSMCs were pre-treated with 100 nM U-0126 for 1 h and then incubated with 30 nM ET-1 for another 1 h. The cell lysates were subjected to immunoprecipitation using an anti-Elk-1 or anti-p300 Ab, and then the immunoprecipitates were analyzed by Western blot using an anti-Elk-1 or anti-p300 Ab. B: HTSMCs were pre-treated with various concentrations of GR-343 for 1 h and then incubated with 30 nM ET-1 for 16 h. Expression of VCAM-1 was determined. GAPDH was used as an internal control. C: HTSMCs were transfected with p300 or scramble siRNA for 24 h and then incubated with 30 nM ET-1 for 16 h. The levels of VCAM-1 and p300 were determined by Western blot using an anti-VCAM-1 and anti-p300 Ab. GAPDH was used as an internal control. D: top, VCAM-1-luc reporter gene transiently transfected cells were pretreated with GR-343 (10 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h. Promoter activity was determined and normalized with β-galactosidase activity. Bottom, cells were pre-treated with GR-343 (10 nM) for 1 h and then incubated with 30 nM ET-1 for 6 h. VCAM-1 mRNA was determined by RT-PCR. E: cells were pretreated without or with BQ-123 (10 nM), BQ-788 (10 μM), GPA2 (10 μM), GPA2A (10 nM), AG-1478 (100 nM), AG-1296 (100 nM), GM-6001 (10 μM), MMP2/9 inhibitor (10 nM), PP1 (100 nM), LY-294002 (300 nM), wortmannin (300 nM), SH-5 (100 nM), U-0126 (100 nM), or GR-343 (10 nM) for 1 h and then incubated with 30 nM ET-1 for the indicated time intervals. Acetylation of histone H4 was detected by Western blot with anti-acetyl-histone H4 Ab. β-Actin was used as an internal control. *P < 0.05 and #P < 0.01 compared with cells exposed to ET-1-alone.
ET-1 is a dominant mediator in the pathogenesis of pulmonary hypertension. However, once released by pulmonary resident cells such as airway epithelial cells, macrophages, or fibroblasts (33), ET-1, other than as vasoconstrictor, may have diverse effects on pulmonary resident cells. ET-1 may promote hypertrophy and inhibit apoptosis in HTSMCs and thus induces increased smooth muscle mass in asthma (29). In addition, several studies also reported that ET-1 stimulates cell proliferation and contributes to airway remodeling in pig bronchial smooth muscle cells or rat airway smooth muscle cells (23, 40). Here we found that ET-1 exerted as a proinflammatory factor. Our results indicated that ET-1 mediated expression of VCAM-1 protein on HTSMCs via enhancing promoter activity and mRNA transcription. Thus, ET-1 may contribute to occurrence or amplification of inflammation via VCAM-1 expression.

ET-1 activated cells via binding to its receptors, ET\(_A\) or ET\(_B\). Both ET\(_A\) and ET\(_B\) are able to differently couple to pertussis toxin-sensitive (G\(_\alpha\)) and -insensitive (G\(_{q/11}\)) families of G proteins (22). Here we confirmed that both ET\(_A\) and ET\(_B\) receptors were expressed on HTSMCs. These two receptors coupled to G\(_q\) and G\(_i\) proteins were implicated in the ET-1-induced VCAM-1 expression, which were attenuated by BQ-123, BQ-788, GPA\(_2\), and GPA\(_2\)A, suggesting that ET-1-mediated proinflammatory effects are mediated through ET\(_A\) or ET\(_B\) receptor-coupled G\(_q\) or G\(_i\) protein in HTSMCs. These results were similar to the effects of ET-1 on rat mesenteric small arteries that VCAM-1 expression is mediated through activation of ET\(_B\) (31) and the finding that pretreatment with ET\(_A\) receptor antagonist, BQ-610, prevents the stimulatory effect of ET-1 on SOCS-3 gene expression in 3T3-L1 adipocytes (7).

It has been indicated that GPCRs may activate downstream signaling such as MAPK via transactivation of RTK in several cell types. Here we found that inhibition of ET\(_A\), ET\(_B\), G\(_q\), or G\(_i\) by BQ-123, BQ-788, GPA\(_2\), and GPA\(_2\)A significantly attenuated ET-1-stimulated phosphorylation of EGFR and PDGFR. Moreover, pretreatment with PP1, GM-6001, or MMP2/9 inhibitor blocked ET-1-mediated EGFR and PDGFR phosphorylation. ET-1-induced VCAM-1 expression was also attenuated by the inhibitor of Src, MMP, EGFR, or PDGFR in HTSMCs. These results were consistent with the finding of GPCR transducing signaling via activation of RTK (11) and to the action of ET-1 on COX-2 expression via c-Src-dependent transactivation of EGFR in brain microvascular endothelial cells (17). Thus, our results revealed that, in HTSMCs, ET-1-stimulated activation of p42/p44 MAPK was mediated by ET\(_A\)/ET\(_B\) receptor/Src/MMP/EGFR and PDGFR, leading to VCAM-1 expression.
It is found that MAPK phosphorylation may lead to activation of Elk-1 and p300 (26). Here we reported that ET-1 may activate Elk-1 via activation of p42/p44 MAPK in an ET receptor-transactivated EGFR/PDGFR manner. We found that activated p42/p44 MAPK translocated into cell nucleus and resulted in phosphorylation of Elk-1 in HTSMCs. Furthermore, immunoprecipitation assay revealed the physical association between Elk-1 and p300 in ET1-challenged HTSMCs. p300 is a transcriptional activator and promotes the acetylation of histone H4. Here, we also confirmed that ET-1 stimulated an increase of histone H4 acetylation via an ET receptor/Src/MMP/EGFR, PDGFR/Pi3K/Akt/p42/p44 MAPK/p300 manner. This finding was consistent with the results obtained with human K562 cells indicating that heat shock leads to the expression of heme-regulated inhibitor via Elk-1 and p300 (37).

On the basis of the literature and our findings, an ET-1 signaling model was established revealing that ET-1 stimulated EGFR/PDGFR activation through Src and MMP and led to phosphorylation of AKT and p42/p44 MAPK. Furthermore, nuclear translocation of activated p42/p44 MAPK promoted the association of Elk-1 and p300, which upregulated VCAM-1 promoter activity and mRNA and protein expression in HTSMCs. Increased expression of VCAM-1 enhanced the adhesion of THP-1 to HTSMCs challenged with ET-1, thus promoting the occurrence and amplification of airway inflammation. These findings suggested that ET-1-induced VCAM-1 expression via ET receptors may be mediated through trans-activation of the Src/MMP/EGFR/PDGFR/Pi3K/Akt/p42/p44 MAPK pathway in HTSMCs. Understanding the mechanisms underlying ET-1-mediated pulmonary inflammation may provide opportunities of therapeutic strategies for ET-1-induced lung injuries.

REFERENCES


