TNF-α induces MMP-9 expression via activation of Src/EGFR, PDGFR/PI3K/Akt cascade and promotion of NF-κB/p300 binding in human tracheal smooth muscle cells

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domain of p85, which permits docking of PI3K to the plasma membrane and allows allosteric modifications that increase its catalytic activity (10). MMP-9 expression and promoter activity are also regulated by Src and transactivation of the epidermal growth factor receptor (EGFR) in tumor cells or vascular smooth muscle cells (8, 46). Similar to EGFR transactivation, platelet-derived growth factor receptor (PDGFR) is also activated in human bronchial epithelial cells stimulated by lysophosphatidic acid (15). Hence, phosphorylation of PI3K/Akt by Src transactivation may be important for MMP-9 induction during airway diseases. Furthermore, TNF-α-stimulated PI3K/Akt and Src activation may lead to NF-κB activation (16, 35). Analysis of the MMP-9 promoter activity has shown to contain an essential proximal AP-1 element and an upstream NF-κB binding site (42). In addition, NF-κB-dependent MMP-9 gene transcription requires the presence of histone acetyltransferase (HAT) coactivators in PMA-stimulated cells (33). HATs such as p300 and CBP are phosphoproteins, which can be regulated by protein kinases, such as MAPKs (1), calcium/calmodulin-dependent protein kinase (CaMK) IV (21), and Akt (18). p300 and CREB binding protein (CBP) act as protein bridges, thereby connecting different transcriptional activators via protein-protein interactions to the basal transcriptional machinery, such as transcription factor IIB (TFIIB) and TATA-binding protein, as well as the RNA polymerase II complex (11). They also function as a scaffolding protein upon which to build a multicomponent transcriptional regulatory complex (8). Raised activity of intrinsic HAT may cause remodeling of chromatin structure by acetylation of the NH2 terminus of core nucleosomal histones. Phosphorylation of p300 at Ser1834 by Akt translocation into nucleus is critical for chromatin remodeling and gene transcription (18). Thus, activation of Akt may regulate MMP-9 gene transcription by phosphorylating HAT coactivators stimulated by TNF-α.

In the present study, we investigated whether TNF-α-stimulated Akt phosphorylation mediated through transactivation of Src and growth factor receptors. Phosphorylated Akt was translocated into nucleus and may eventually stimulate p300 activity, assemble transcription factors NF-κB and histone (H3), and recruit the transcriptional machinery to the MMP-9 promoter in human tracheal smooth muscle cells (HTSMCs).

MATERIALS AND METHODS

Materials. DMEM/F-12 medium, FBS, and TRIZol were obtained from Invitrogen (Carlsbad, CA). Hybond C membrane, ECL Western blotting detection system and Hyperfilms were obtained from Amer sham Biosciences (Buckinghamshire, UK). Recombinant human TNF-α was from R&D Systems (Minneapolis, MN). Monoclonal anti-MMP-9 Ab was obtained from NeoMarkers (Fremont, CA, USA). PhosphoPlus Src, EGFR, PDGFR, and Akt Ab kits were obtained from Cell Signaling (Beverly, MA). IκB-α and NF-κB (p65) Ab were obtained from Santa Cruz (Santa Cruz, CA). PPI, AG1296, AG1478, LY294002, wortmannin, curcumin, and helenalin were obtained from Biomol (Plymouth Meeting, PA). GAPDH was obtained from Biogenesis (Boumemouth, UK). Bicinchoninic acid protein assay kit was from Sigma (St. Louis, MO).

Cell culture. HTSMCs were isolated from human trachea and cultured as previously described (30). When the cultures reached confluence, cells were treated with 0.05% (wt/vol) 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 2×10^5 cells/mL. The cell suspension was plated onto (1 mL/well) 12-well culture plates and (10 mL/dish) 10-cm culture dishes for the measurement of protein expression and mRNA accumulation, respectively. Experiments were performed with cells from passages 3 to 8.

MMP gelatin zymography. HTSMCs were plated onto 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. Growth-arrested cells were incubated with different concentrations of TNF-α at 37°C for the indicated times. When inhibitors were used, they were added 1 h before the application of TNF-α. After treatment, the culture medium was collected and centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris. The supernatant was mixed with 5× nonreducing sample buffer (4:1, v/v) and electrophoresed on 12% SDS-PAGE containing 1% gelatin as a protease substrate. Following electrophoresis, gels were washed in 3% Triton X-100 for 1 h to remove SDS and then incubated for 40 h at 37°C in developing buffer (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM CaCl2, and 0.2% Brij 35) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue for 1 h followed by destaining. Mixed human MMP-2 and MMP-9 were used as positive controls. Gelatinolytic activity was manifested as horizontal white bands on a blue background.

MMP-9 activity. Detection of MMP activity with an EnzChek Gelatinase/Collagenase Assay kit was conducted according to the directions of the manufacturer (Molecular Probes). Fifty microliters of conditioned medium was added per well with 130 μl of substrate buffer (50 mM Tris·HCl, 5 mM CaCl2, pH 7.4) and 20 μl of fluorescence-labeled gelatin substrate (1 mg/ml). Samples were incubated for 24 h at 25°C in the dark. Samples were then slowly shaken for 1 min, and MMP activity was measured as an increase in fluorescence (excitation 495 nm, emission 515 nm), by a fluorometer Fusion (Packard Bioscience, Meriden, CT).

ELISA. The amount of secreted MMP-9 was determined in the culture medium of HTSMCs using a commercial ELISA kit (R&D Systems), following the instructions of manufacturer. Conditioned media (100 μl) were applied to each well for the ELISA; each sample was run in duplicate, and the final measurement was read out using a plate reader at 450 nm. The concentration of MMP-9 protein in each sample was determined according to the standards (recombinant proteins) accompanied with the kits. MMP-9 levels in these samples were located within the linear range of the standard curves.

siRNA, plasmids, and transfection. The plasmids encoding dominant negative mutants Src (K295M), p85, and Akt were kindly provided by Dr. C. C. Chen (Department of Pharmacology, National Taiwan University, Taipei, Taiwan) and Dr. R. D. Ye (Department of Pharmacology, University of Illinois at Chicago, Chicago, IL). All plasmids were prepared by using Qiagen plasmid DNA preparation kits. ON-TARGETplus siRNA of Akt (sense sequence: 5′-CAUCAACAC-CAACCGUACCAAUU-3′; anti-sense: 5′-P-UUUGUGAGGUGGUUAGUUGU-3′) was from Dharmacon Research (Lafayette, CO).

HTSMCs cells were plated at 3×10^5 cells/ml in six-well culture plates for 24 h. Cells were transfected with 1 μg of dominant-negative mutants of Src, p85, Akt, and siRNA Akt for each well using DNA-Metafectene and incubated at 37°C for 24 h. Two ml of DMEM/F-12 medium containing 10% FBS were added and incubated for an additional 24 h. The cells were washed twice with PBS and maintained in serum-free DMEM/F-12 for 24 h. Further, TNF-α was added for the indicated time. The transfection efficiency (approximately 60%) was determined by transfection with EGFP.

Measurement of MMP-9 luciferase activity. A 710-bp (−720 to −11) segment from the S′-promoter region of the MMP-9 gene was cloned as described (37). Briefly, a 0.71-kb segment at the S′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (accession no. D10051): 5′-ACATTGTCGCGGACGCTCTGAAAG-3′ (forward/SacI) and 5′-AGGCGCTTGCCAGAAGCTTAGTG-3′ (reverse/HindIII). The pGL3-basic vector, containing a polyadenylation signal upstream...
from the luciferase gene, was used to construct the expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the SacI/HindIII site of the pGL3-basic vector. The PCR products (pGL3-MMP-9WT) were confirmed by their size, as determined by electrophoresis and by DNA sequencing. Additionally, the introduction of a double-point mutation into the NF-κB-site to generate pGL3-MMP-9mNF-κB was performed, using the following (forward) primer: 5′-CTGCGGAAGACAGGCCGTTGCCCCAGTGGAATTCCC-3′ (626 to 591) (7). The mutant was generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

MMP-9-luc plasmid was transient transfected into HTSMCs using Genejumper Transfection Reagent (Stratagene, La Jolla, CA). Briefly, plasmids (1.8 μg) and β-gal (0.2 μg) were formulated with Genejumper transfection according to the manufacturer’s instruction. The transfection complex was diluted into 900 μl of DMEM/F-12 medium and added directly to the cells. The medium was replaced with complete basal essential growth medium after 5 h. 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, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system. Firefly luciferase activities were standardized for β-galactosidase activity.

Preparation of cell extracts and Western blot analysis. HTSMCs were plated in 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. Growth-arrested cells were incubated with different concentrations of TNF-α at 37°C for the indicated time referenced in Fig 1. The cell lysates were collected and subjected to 10% (wt/vol) SDS-PAGE, as previously described (46). Proteins were transferred to nitrocellulose membranes, which were incubated successively at room temperature with 5% (wt/vol) BSA in TTBS [(50 mM Tris HCl, pH 8.1, 150 mM NaCl, 0.05% (wt/vol) Tween 20, pH 7.4)] for 1 h. Membranes were incubated overnight at 4°C with the PhosphoPlus Src, PYK2, EGFR, PDGFR, Akt, MPP-9, and GAPDH Ab used at a dilution of 1:1,000 in Tween-Tris buffer saline (TTBS). Membranes were washed with TTBS for four times for 5 min each, incubated with a 1:2,000 dilution of anti-goat or anti-mouse horseradish peroxidase Ab for 1 h. Following each incubation, the membranes were extensively washed with TTBS. The immunoreactive bands detected by enhanced chemiluminescence (ECL) reagents were developed by Hyperfilm-ECL.

Chromatin immunoprecipitation assay. To detect the in vitro association of nuclear proteins with human MMP-9 promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as previously described (39) with some modifications. HTSMCs in 100-mm dishes were grown to confluence and serum starved for 24 h. After treatment with TNF-α, protein-DNA complexes were fixed by 1% formaldehyde in PBS. The fixed cells were washed and lysed in SDS-lysis buffer (1% SDS, 5 mM EDTA, 1 mM PMSF, 50 mM Tris-HCl, pH 8.1), and sonicated on ice until the DNA size became ~200–1,000 base pairs. The samples were centrifuged, and the soluble chromatin was pre cleared by incubation with sheared salmon sperm DNA-proteinagarose A slurry (Upstate) for 30 min at 4°C with rotation. After centrifugation at 800 rpm for 1 min, one portion of the pre cleared supernatant was used as DNA input control, and the remains were subdivided into aliquots and then incubated with a nonimmune rabbit immunoglobulin G (IgG; Upstate), IgG Ab to NF-κB p65 (were from Santa Cruz), anti-phospho-Akt Ab (sc-585; Santa Cruz), or anti-acetylated histone H3 (06–599; Upstate), respectively, overnight at 4°C. The immunoprecipitated complexes of Ab-protein-DNA were collected by using the above protein A beads, and washed successively with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt buffer (same as the low-salt buffer but with 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and Trit-EDTA (pH 8.0), and then eluted with elution buffer (1% SDS, 100 mM NaHCO3). The cross-linking of protein-DNA complexes was reversed by incubation with 5 M NaCl at 65°C for 4 h, and DNA was digested with 10 μg of proteinase K (Sigma)/ml for 1 h at 45°C. The DNA was then extracted with phenol-chloroform, and the purified DNA pellet was precipitated with isopropanol. After washing, the DNA pellet was resuspended in H2O and subjected to PCR amplification with the forward (5′-TGGCTCTTTCAGCTGCTTCTGAAAG-3′) and reverse (5′-ACTCCAGGCTCTGCTCCTCAGTCA-3′), which were specifically designed from the MMP-9 promoter region (−657 to −484) (43). PCR products were analyzed on ethidium bromide-stained agarose gels.

Analysis of data. Concentration-effect curves were fitted and EC50 values were estimated using the GraphPad Prism Program (GraphPad, San Diego, CA). Data were expressed as the means ± SE and were analyzed with a one-way ANOVA at a P < 0.05 level of significance.
RT-PCR, using the primer specific for MMP-9 and \( \text{TNF-\alpha} \)-induced MMP-9 mRNA expression, the cells were incubated with 30 ng/ml TNF-\( \alpha \). Further confirmation of the up-regulation of MMP-9 protein by TNF-\( \alpha \), the conditioned medium was precipitated with trichloroacetic acid and centrifuged at 3,000 g for 30 min. The pellet was suspended in 100-\( \mu \)l sample buffer, run on 10\% SDS-PAGE, and blotted with an anti-MMP-9 Ab. As shown in Fig. 1B, the amount of MMP-9 protein was increased by incubation with TNF-\( \alpha \) in a timedependent manner similar to those of zymographic analysis. Moreover, analysis of RT-PCR showed that TNF-\( \alpha \)-induced MMP-9 mRNA expression in a time-dependent manner (Fig. 1C). There was a significant increase between 10 and 30 min and reached a maximal increase within 1 h.

**Inhibition of Src/EGFR-PDGFR/P13K/Akt blocks TNF-\( \alpha \)-induced MMP-9 expression.** To determine whether Src/EGFR, PDGFR/P13K/Akt and NF-\( \kappa \)B were involved in TNF-\( \alpha \)-induced MMP-9 expression, their respective inhibitors, PP1, AG1478, AG1296, LY294002, and helenalin, were used. As shown in Fig. 2A, preincubation of HTSMCs with these inhibitors for 1 h significantly attenuated TNF-\( \alpha \)-induced MMP-9 mRNA expression. The involvement of Src, P13K, and Akt in TNF-\( \alpha \)-induced responses was further confirmed by transfection with dominant-negative mutants of c-Src (K295M), p85, and Akt (K179A), which significantly decreased TNF-\( \alpha \)-induced MMP-9 mRNA expression, as revealed by zymographic analysis (Fig. 2B). These results suggested that TNF-\( \alpha \)-induced MMP-9 gene expression may be mediated through Src, EGFR, PDGFR, P13K/Akt, and NF-\( \kappa \)B in HTSMCs.

**RESULTS**

**TNF-\( \alpha \) induces MMP-9 activity, protein, and mRNA expression in HTSMCs.** To determine the effect of TNF-\( \alpha \) on MMP-9 expression, HTSMCs were treated with 30 ng/ml TNF-\( \alpha \) for the time indicated in Fig. 1. The enzyme activity of MMP-9 in conditioned media was determined using gelatin zymography. In the present study, TNF-\( \alpha \)-induced MMP-9 expression in a time- and concentration-dependent manner. For time course study, various concentrations of TNF-\( \alpha \) (0.15, 1.5, and 15 ng/ml) also used to induce MMP-9 expression (data not shown). As shown in Fig. 1A, media from control HTSMCs displayed proteolytic activity at 92 and 72 kDa, corresponding to pro-MMP-9 and pro-MMP-2, respectively. MMP-9 expression was induced by 30 ng/ml TNF-\( \alpha \) in a time-dependent manner. There was a significant increase between 10 and 30 min. A maximal increase was achieved within 1 h and then decreased to the basal level within 24 h. In contrast, MMP-2 expression was not changed by TNF-\( \alpha \) during the period of observation. Thus, in the following experiments, HTSMCs were incubated with 30 ng/ml TNF-\( \alpha \) for 1 h. To further confirm the up-regulation of MMP-9 protein by TNF-\( \alpha \), the conditioned medium was precipitated with trichloroacetic acid and centrifuged at 3,000 g for 30 min. The pellet was suspended in 100-\( \mu \)l sample buffer, run on 10\% SDS-PAGE, and blotted with an anti-MMP-9 Ab. As shown in Fig. 1B, the amount of MMP-9 protein was increased by incubation with TNF-\( \alpha \) in a timedependent manner similar to those of zymographic analysis. Moreover, analysis of RT-PCR showed that TNF-\( \alpha \)-induced MMP-9 mRNA expression in a time-dependent manner (Fig. 1C). There was a significant increase between 10 and 30 min and reached a maximal increase within 1 h.

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TNF-α stimulates phosphorylation of protein tyrosine kinases in HTSMCs. To examine whether TNF-α stimulated the phosphorylation of protein tyrosine kinases, HTSMCs were incubated with TNF-α (30 ng/ml) for the indicated time shown in Fig. 3. As shown in Fig. 3A, TNF-α stimulated tyrosine phosphorylation of proteins including ~190, ~170, ~116, and ~60 kDa proteins, which may be identical to PDGFR (190 kDa), EGFR (170 kDa), proline-rich tyrosine kinase 2 (PYK2; 116 kDa), and c-Src tyrosine kinase (60 kDa), respectively. The blot was stripped and reprobed with an anti-GAPDH Ab to demonstrate equivalent amount of GAPDH expression.

Next, to ensure the phosphorylated components were Src, PDGFR, and EGFR, the membranes were reprobed with their respective antibodies. As shown in Fig. 3B, treatment of HTSMCs with 30 ng/ml TNF-α stimulated phosphorylation of c-Src, EGFR, and PDGFR from 5 to 10 min, respectively. TNF-α-induced phosphorylation of c-Src and EGFR, and PDGFR was inhibited by PP1, PP1 and AG1478, as well as PP1 and AG1296, respectively, but not by LY294002 (a PI3K inhibitor) (data not shown). Transfection with a dominant-negative mutant of c-Src (K295M) can also block phosphorylation of EGFR and PDGFR stimulated by TNF-α. These results suggested that TNF-α-stimulated transactivation of growth factor receptors may be mediated through Src phosphorylation leading to MMP-9 expression in HTSMCs.

Effect of Src kinase inhibitor on the induction of MMP-9 activity by TNF-α in HTSMCs. TNF-α-induced MMP-9 expression has been shown to be mediated through a Src-dependent pathway in human keratinocytic tumor cells (9). Thus, we further examined whether Src was involved in TNF-α-induced MMP-9 expression in HTSMCs. Pretreatment of cells with a Src inhibitor (PP1) for 1 h before exposure to TNF-α for 1 h attenuated MMP-9 expression in a concentration-dependent manner revealed by zymographic analysis (Fig. 4A). At a concentration of 10 μM PP1 almost completely blocked TNF-α-induced MMP-9 protein expression.

Next, to confirm Src phosphorylation linked to MMP-9 expression, cells were pretreated with 10 μM PP1 for 1 h and then stimulated with 30 ng/ml of TNF-α for the indicated time. The cell lysates were immunoprecipitated with anti-c-Src antibody. The immunoprecipitates were subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and then blotted using an antiserum reactive with antiphospho-Src, EGFR, and PDGFR Ab. Membranes were stripped and reprobed with anti-GAPDH Ab as an internal control. Results are presented from one representative of three independent experiments.
was not mediated through EGFR, PDGFR, and PI3K. Src may be an upstream component of growth factor receptor transactivation pathway. These results showed that c-Src phosphorylation was required for TNF-α-induced MMP-9 expression.

**TNF-α induces MMP-9 expression mediated through EGFR transactivation.** MMP-9 expression has been shown to be mediated through Src/EGFR transactivation induced by arsenite (9). Hence, to further investigate whether EGFR activation was involved in TNF-α-induced MMP-9 expression, EGFR inhibitor (AG1478) was used. Pretreatment with AG1478 significantly attenuated TNF-α-induced MMP-9 expression in a concentration-dependent manner, as revealed by zymographic analysis (Fig. 5A). At a concentration of 10 μM, AG1478 almost completely blocked TNF-α-induced MMP-9 protein expression. Because Src-dependent EGFR phosphorylation was shown to be involved in this response, coimmunoprecipitation of c-Src and EGFR was performed to examine whether c-Src directly regulated phosphorylation of EGFR. Cells were treated with 30 ng/ml of TNF-α for the indicated times. The cell lysates were immunoprecipitated with anti-c-Src Ab. The immunoprecipitates were subjected to 10% SDS-PAGE, transferred to membranes, and blotted with anti-phospho-EGFR, c-Src, or GAPDH Ab. As shown in Fig. 5B, TNF-α stimulated EGFR phosphorylation in a time-dependent manner, which was blocked by PP1 and AG1478. These results indicated that c-Src can directly associate with EGFR and phosphorylate their tyrosine residues by TNF-α stimulation. These results suggested that transactivation of EGFR through Src phosphorylation was involved in TNF-α-induced MMP-9 expression in HTSMCs.

**TNF-α induces MMP-9 expression mediated through PDGFR transactivation.** It has been reported that GPCR ligands such as ANG II transactivate not only EGFR but also PDGFR (14). We investigated whether PDGFR transactivation was also involved in TNF-α-induced MMP-9 expression. Pretreatment of HTSMCs with a PDGFR inhibitor (AG1296) for 1 h before exposure to TNF-α for 1 h caused an attenuation of MMP-9 expression in a concentration-dependent manner, as determined by zymographic analysis (Fig. 6A). At a concentration of 10 μM, AG1296 almost completely blocked TNF-α-induced proMMP-9 protein expression. To examine whether c-Src directly regulated phosphorylation of PDGFR, cells were...
treated with 30 ng/ml of TNF-α for the indicated times. Cell lysates were immunoprecipitated with anti-c-Src Ab; the immunoprecipitates were subjected to 10% SDS-PAGE, transferred to membranes, and blotted with anti-phospho-PDGFR, Src, or GAPDH Ab. As shown in Fig. 6A, TNF-α stimulated PDGFR phosphorylation in a time-dependent manner, which was blocked by PP1 and AG1296. These results indicated that c-Src can directly associate with PDGFR and phosphorylate their tyrosine residues by TNF-α stimulation. These results demonstrated that transactivation of PDGFR through Src phosphorylation was involved in TNF-α-induced MMP-9 expression in HTSMCs.

TNF-α stimulates Akt phosphorylation via Src/EGFR-PDGFR leading to MMP-9 expression. We have shown that TNF-α-induced MMP-9 expression was inhibited by the dominant-negative mutants of p85 and Akt. Here, we presented the evidence that PI3K/Akt activation was involved in TNF-α-induced MMP-9 expression. Pretreatment of HTSMCs with PI3K inhibitors LY294002 and wortmannin for 1 h before exposure to TNF-α for 1 h caused an attenuation of MMP-9 expression in a concentration-dependent manner, as revealed by zymographic analysis (Fig. 7A). Akt has been shown to be a downstream component of Src/EGFR transactivation pathway (6). Furthermore, TNF-α-induced Akt phosphorylation was determined by Western blot analysis using a specific antiserum for active and phosphorylated Akt. As shown in Fig. 7B, TNF-α induced Akt phosphorylation in a time-dependent manner. There was a significant increase in 3–5 min and a maximal response was obtained within 10–30 min. To confirm whether Akt activation was downstream of Src/EGFR pathway, cells were pretreated with PP1, AG1478, AG1296, or LY294002 for 1 h, and then treated with 30 ng/ml of TNF-α for 30 min. As shown in Figs. 7, C–F, Akt phosphorylation was inhibited by PP1, AG1478, AG1296, or LY294002, indicating the requirement of Src and growth factor receptor transactivation for the Akt phosphorylation and, finally, MMP-9 expression.

TNF-α induces MMP-9 expression mediated through NF-κB. Promoter of MMP-9 has been shown to consist of an upstream NF-κB binding site (32). We investigated whether NF-κB activation was involved in TNF-α-induced MMP-9 expression. Pretreatment of HTSMCs with a selective NF-κB inhibitor helenalin for 1 h before exposure to TNF-α for 1 h caused an attenuation of MMP-9 expression in a concentration-dependent manner determined by zymographic analysis (Fig. 8A). At the highest concentration used, helenalin almost completely inhibited MMP-9 protein expression induced by TNF-α in HTSMCs. In our previous study, TNF-α has been shown to stimulate translocation of NF-κB (p65) into nucleus in HTSMCs (29). Furthermore, NF-κB activation by TNF-α may be mediated through Akt phosphorylation (12, 13). To examine this possibility, the nuclear fraction was used to determine NF-κB translocation by Western blot analysis using anti-NF-κB (p65) Ab. Activation of NF-κB was assessed following TNF-α stimulation in the presence of PI3K inhibitors either LY294002 or wortmannin. As shown in Fig. 8B, LY294002 or wortmannin failed to block NF-κB translocation, as determined by Western blot analysis. Correspondingly, the images of immunofluorescence staining showed that TNF-α-induced NF-κB translocation was also not blocked by LY294002 or wortmannin (Fig. 8C). These results implied that TNF-α-regulated MMP-9 expression was independently mediated through NF-κB and PI3K/Akt.

Inhibition of Src/EGFR/PDGFR/PI3K/Akt blocks TNF-α-induced MMP-9 promoter activity. This regulation of MMP-9 gene transcription through Src/EGFR/PDGFR/PI3K/Akt and NF-κB pathways induced by TNF-α was further confirmed by gene luciferase activity assay. MMP-9 luciferase reporter gene was transfected into HTSMCs and then stimulated with TNF-α for 30 ng/ml. Data in Fig. 9A showed that TNF-α stimulated MMP-9-luciferase activity within 30 min, peaked at 60 min, and sustained for over 2 h. Moreover, TNF-α-induced MMP-9 promoter activation was inhibited by selective inhibitors including PP1, AG1296, AG1478, LY294002, and helenalin for Src, EGFR, PDGFR, PI3K, and NF-κB, respectively (Fig. 9B). To further confirm the functional role of NF-κB transcription factor in TNF-α-mediated MMP-9 promoter induction, point-mutated MMP-9 promoter construct was used to test this induction by TNF-α. As shown in Fig. 9C, TNF-α-stimulated MMP-9 luciferase activity was totally lost in HTSMCs transfected with mutated NF-κB promoter, indicating that the NF-κB binding site is required for MMP-9 promoter activation by TNF-α. These results indicated the involvement of Src/
EGFR, PDGFR/PI3K/Akt, and NF-κB pathways in TNF-α-induced MMP-9 gene transcription.

Nuclear Akt and NF-κB p65 interact with acetyl-histone H3 and p300 in response to TNF-α. As NF-κB-dependent MMP-9 transcription has been demonstrated to require the presence of p300/CBP coactivators in PMA-stimulated cells (11), we aimed to study the possibility of NF-κB p65-p300 association in TNF-α-stimulated HTSMCs regulated by PI3K/Akt cascade. As shown in Fig. 10, A and B, immunofluorescence staining and Western blot analysis demonstrated that TNF-α stimulated nuclear translocation and phosphorylation of Akt, which was attenuated by LY294002. Furthermore, we presented the evidence that Akt or NF-κB p65 interacted with p300 and histone (H3) in vivo, immunoprecipitation of nuclear lysates with p300 or acetyl-H3 Ab, followed by immunoblot analysis using anti-Akt or anti-p65 Ab, was performed. As shown in Fig. 10C, p300 and histone (H3) formed a complex with Akt and NF-κB p65 after TNF-α stimulation, which was blocked by LY294002 and curcumin (a novel specific inhibitor of p300). TNF-α-induced MMP-9 expression was also atten-
uated by curcumin at a concentration of 0.1 μM, as determined by zymographic analysis (Fig. 10D), suggesting that p300 may involve in MMP-9 promoter regulation. To further investigate the roles of these transcription factors and the MMP-9 promoter regulatory elements in TNF-α-induced MMP-9 transcription, in vivo association of these transcription factors with the MMP-9 promoter was evaluated by the ChIP assay. To determine whether the transcription factors could specifically associate with the MMP-9 promoter, PCR amplifications were conducted on an equal amount of immunoprecipitated DNA, followed by 45 cycles of PCR with the specific primer pairs encompassing position 657 to 484 region of the human MMP-9 promoter, which contains NF-κB binding sites (Fig. 10E). After TNF-α treatment, an enrichment of p65-, p300-, and histone H3-associated MMP-9 promoter DNA appeared at 30 min and sustained up to 2 h, compared with the nonimmune IgG immunoprecipitated control. Association of p300, histone H3, and NF-κB with the MMP-9 promoter was blocked by pretreatment with LY294002 (Fig. 10E). The p300 acetylation activity was revealed by Western blot analysis using antiacetylated histone H3. As shown in Fig. 10F, TNF-α-induced histone H3 acetylation within 30 and 60 min, this effect is impaired by transfection with siRNA of Akt. These data indicated that NF-κB (p65), p300, and histone H3 were involved in TNF-α-induced MMP-9 transcription and regulated by PI3K/Akt-dependent pathway in HTSMCs.

Determination of TNF-α-stimulated MMP-9 protein activity and expression using fluorescein-substrate-based assay and ELISA. To determine the effect of TNF-α on MMP-9 activity, HTSMCs were treated with 30 ng/ml TNF-α for the indicated time. The enzyme activity of MMP-9 in conditioned media was determined using EnzChek Gelatinase/Collagenase Assay kit. As shown in Fig. 11A, the addition of 30 ng/ml TNF-α for 1 h caused a 30% increase in dye-quenched (DQ) gelatin cleavage, over the basal value. Thrombin (0.1 U/ml) also caused a 10% increase in MMP-9 activity under similar conditions. Next, to further confirm zymographic data, the secreted amount of MMP-9 was determined by ELISA. As shown in Fig. 11B, TNF-α induced a significant increase MMP-9 expression within 1 h (2.3-fold over the basal level, ~750 pg/ml), which was inhibited by pretreatment with PP1, AG1296, AG1478, LY294002, and helenalin.
DISCUSSION

Our results clearly demonstrate that the Akt plays a critical role in mediating the TNF-α-dependent production of MMP-9 in HTSMCs through the downstream activation of p300. Specifically, we showed first that the expressed and secreted MMP-9 molecule was obtained from the test culture medium of HTSMCs induced by TNF-α/H9251. This molecule was characterized by the following properties: it appeared as a latent form with a molecular mass of 92 kDa; it reacted with antibodies raised against anti-MMP-9 mAb; and it comigrated with the zymographic activities (Fig. 1A). Transfection with the dominant-negative mutants of c-Src (KM), p85, and Akt (KA) can also reduce both of MMP-9 activity and protein level (Fig. 2B). In addition to increased protein expression, TNF-α-induced an increased MMP-9 expression at the mRNA level in a time-dependent manner (Fig. 2A). Second, such expression of MMP-9 mRNA, promoter activation, and protein activity were blocked in parallel by the inhibition of phosphorylation of Src, EGFR, PDGFR, and PI3K by their specific inhibitors of PP1, AG1478, AG1296, and LY294002, respectively. Third, Akt
MMPs are capable of degrading all components of the ECM and play key roles in normal physiological and pathological processes, such as wound healing and airway remodeling (28). MMP-9 has been shown to be one of the most important MMPs in the airway tissue remodeling in asthmatic patients and is believed to result from chronic and/or short-term exposure to inflammatory stimuli (28). Increased evidence suggests that after injury, the airway smooth muscle phenotype may initiate a wound-healing process to restore its barrier integrity, bronchial hyperresponsiveness, and airway inflammation (3). In the present study, zymographic analysis showed that TNF-α-stimulated MMP-9 expression was increased in a time-dependent manner in HTSMCs (Fig. 1A). This short-term expression of MMP-9 by HTSMCs may lead to generation of chemotactic fragments from the degraded ECM, attracting inflammatory cells and facilitating the extravasation of inflammatory cells through the vascular endothelium, as well as their migration into the ECM and through the airway epithelium. These events can promote more immune cells to be recruited to the inflammatory sites. Induction of MMP-9 also resulted in the migration of several cell types, such as inflammatory leukocytes, macrophages, and human bronchial epithelial cells, through reconstitution of the basement membrane (28, 40). Although the levels of MMP-9 production from TSMCs are lower than those of inflammatory cells, the existence of these latent MMP-9 isoforms (92, 130, and 225 kDa) have been confirmed in TSMCs and neutrophils by Atkinson and Senior (4). Therefore, we strongly suggest that release of MMP-9 by TSMCs may be an important factor in the initiation of airway inflammatory responses.

Our data are consistent with a recent study demonstrating that activation of the PI3K/Akt is mediated through a Src-dependent transactivation pathway. In particular, Akt controls important functions in the pathogenesis, including tumorgenesis, angiogenesis, and airway inflammatory responses (23). It also plays a pivotal role in MMP-9 expression (9). In our previous study, Akt phosphorylation is required for bradykinin-induced MMP-9 in astrocytes (17). A recent study has shown that Akt is involved in the induction of MMP-9 in monocytes stimulated by LPS (32). We found that pretreatment with selective inhibitors of Src (PP1), EGFR (AG1478), PDGFR (AG1296), and PI3K (LY294002) decreased MMP-9 expression in both protein and mRNA level induced by TNF-α. Moreover, Akt phosphorylation was also attenuated by these inhibitors (Fig. 6, C and D). The Src kinase family has been shown to exert a necessary and sufficient role in the transactivation of ligands by GPCRs and growth factor receptors, which are involved in human airway smooth muscle cell proliferation and migration (25). Activation of the EGFR and PI3K/Akt by PGE2 is dependent on the activation of Src, since treatment with Src kinase inhibitors PP1 and PP2 also attenuates the phosphorylation of these signaling proteins (6). Here, as determined by immunoprecipitation, TNF-α was shown to stimulate Src activity and form a molecule complex with growth factor receptors such as EGFR and PDGFR (Figs. 3B, 4B, 5B, and 6B). Src phosphorylation and the complex association were inhibited by pretreatment with PP1, but not by AG1478 or AG1296. Therefore, Src may act as an upstream effector of the EGFR or PDGFR. These results suggested that Akt phosphorylation mediated through a Src-dependent transactivation pathway was required for MMP-9 expression in

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**Fig. 11. Quench fluorescent stopped assay to measure MMP activity and ELISA for total MMP-9.**

A: time dependence of TNF-α-increased MMP-9 activity, HTSMCs were cultured at 37°C for 24 h in RPMI serum-free medium without phenol red and then treated with 30 ng/ml TNF-α for various times. The conditioned media were collected and analyzed by EnzChek Gelatinase/Collagenase Assay kit. Standard relative fluorescence was normalized to unstimulated cells. B: cells were incubated in the absence or presence of PP1, AG1296, AG1478, LY294002, and helenalin for 1 h and then treated with 30 ng/ml TNF-α for 1 h. Conditioned media were collected and assayed using ELISA kits for human MMP-9.

phosphorylation was inhibited by the same inhibitors that blocked the expression of MMP-9 (Fig. 7, B and C), indicating the requirement of Src and transactivation of growth factor receptors for Akt phosphorylation. Besides the Akt pathway, we found that inhibition of NF-κB translocation by helenalin also attenuated MMP-9 gene expression (Fig. 8A). However, pretreatment with LY294002 and wortmannin did not inhibit NF-κB translocation into the nucleus (Figs. 8, B and C), suggesting that MMP-9 expression was independently regulated by phosphorylation of Akt and activation of NF-κB. Finally, we found that the phosphorylation of Akt was translocated into the nucleus, and enhanced activity of p300 may eventually lead to assembly of the NF-κB factor and may recruit basal transcriptional machinery to the MMP-9 promoter, as revealed by ChIP assays. Thus our results support the notion that the TNF-α specifically induces MMP-9 expression in HTSMCs, and the expression is sequentially mediated by the Src/EGFR, PDGFR/PI3K/Akt/p300 and NF-κB signaling pathways.
response to TNF-α in HTSMCs. EGFR can also be phosphorylated by HB-EGF (an EGF ligand), which has been known to be released from cell membrane after cleavage by MMP-9 and other proteases (A disintegrin and metalloprotease family). Pretreatment with GM6001 (an inhibitor of MMPs) and CRM197 (an inhibitor of HB-EGF) can also attenuate EGFR phosphorylation in response to TNF-α stimulation (data not shown). In addition, a recent study has demonstrated that CD21 activation triggers Cbl tyrosine phosphorylation within an early event, which requires c-Src kinase activity and then may interact with SH2 domain of p85 subunit in PI3K, in human B lymphoma cells (31). In T47D cells, activation of Akt requires Src and Cbl phosphorylation but not EGF (22). Actually, it has been reported that VCAM-1 ligation enhances PI3K activity mediated through Cbl-association in human ASM cells (27). It is possible that phosphorylation of Akt in response to TNF-α may directly mediate through either Cbl/PI3K activation or extra-transactivation by HB-EGF release, which needs to be investigated in detail in the future.

Nevertheless, activation of c-Src converges at IKKα/β and leads to activate NF-κB, via serine phosphorylation and degradation of IkBα, and finally initiates COX-2 or adhesion molecule expression (19, 20). NF-κB has been identified to regulate expression of many inflammatory genes, including MMP-9 (32, 33, 43, 50). Studies on the role of the PI3K-Akt pathway in NF-κB-dependent gene expression are controversial. Several studies have demonstrated that activation of NF-κB is mediated via Akt/protein kinase B (PKB) through IκB kinase (IKK)/IκB in a cell- and stimulus-specific manner (13, 32, 34, 38). Our results demonstrated that the PI3K-Akt pathway has no effect on NF-κB translocation in TNF-α-stimulated HTSMCs. On the basis of cell fractionation and immunofluorescence staining studies, although LY294002 and wortmannin were present, the NF-κB translocation into nucleus still occurred. However, both LY294002 and helenalin attenuated MMP-9 gene expression, suggesting that PI3K/Akt and NF-κB independently regulated MMP-9 expression in HTSMCs.

Interestingly, as shown in Fig. 8D, MMP-9 expression was significantly blocked by a novel specific inhibitor of p300, curcumin (5). Previous studies indicate that promoter of MMP-9 gene transcription, chromatin remodeling, and histone modification are regulated by p300/CBP (33). Two structurally and functionally related HATs, CBP and p300, play important roles in activation of gene transcription, as well as recruitment of other transcriptional factors. When proinflammatory transcription factors such as NF-κB are activated, they bind to specific recognition sequences in DNA and subsequently interact with coactivator molecules, such as CREB-binding protein (CBP), p300, and p300/CBP-associated factor. These coactivator molecules act as the molecular switches that control gene transcription, and all have intrinsic HAT activity. Enhanced activity of p300 or CBP has been shown to be stimulated by protein kinases such as Akt (18). Here, we propose that p300 may be involved in the initiation of MMP-9 expression at the transcription level. We found that p300 and histone (H3) formed a tight complex with Akt and p65 after TNF-α stimulation in the coimmunoprecipitation assay of nuclear lysates. The association of these complexes was blocked by pretreatment with LY294002 and curcumin. ChIP assay was also performed to examine the association of p300, histone (H3), and p65 on MMP-9 promoter region. On the basis of our results, an enrichment of NF-κB p65-, p300-, and histone H3-associated MMP-9 promoter (containing NF-κB binding sites) DNA appeared at 30 min and was sustained up to 2 h in HTSMCs stimulated by TNF-α. The direct link between MMP-9 promoter and NF-κB p65-, p300-, or histone H3 was blocked by LY294002. These data suggest that acetylation of histone H3 by p300 is dependent on Akt-phosphorylation and may induce chromatin remodeling, and, finally, enhance NF-κB binding to the MMP-9 promoter binding site.

On the basis of reported observations from the literature and our findings, a schematic pathway depicts a model for the roles of Src/EGFR-PDGFR/PI3K/Akt, NF-κB, and p300 activation associated with MMP-9 expression in HTSMCs exposed to TNF-α (Fig. 12). In conclusion, this study was the first to demonstrate that in HTSMCs, the mechanisms underlying TNF-α receptor—mediated through transactivation of Src/EGFR-PDGFR/PI3K/Akt and NF-κB as well as p300—were required for expression of MMP-9. The mechanisms by which TNF-α-induced MMP-9 expression in HTSMCs may be an important link in the pathogenesis of airway inflammatory diseases. Therefore, understanding the mechanisms underlying TNF-α-induced MMP-9 expression in HTSMCs is important to develop new therapeutic strategies.

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REFERENCES


