Oxidative Modification of Nuclear Mitogen-activated Protein Kinase Phosphatase 1 Is Involved in Transforming Growth Factor β1-induced Expression of Plasminogen Activator Inhibitor 1 in Fibroblasts*

Received for publication, February 6, 2010, and in revised form, March 8, 2010 Published, JBC Papers in Press, March 12, 2010, DOI 10.1074/jbc.M110.111732

Rui-Ming Liu, Jinah Choi, Jian-He Wu, Kimberly A. Gaston Pravia, Karen M. Lewis, Jeffrey D. Brand, N. S. Reyes Mochel, David M. Krzywanski, J. David Lambeth, James S. Hagood, Henry Jay Forman, Victor J. Thannickal, and Edward M. Postlethwait

From the Department of Environmental Health Sciences, School of Public Health, and the Departments of Pathology, Pediatrics, and Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294, the Department of Natural Sciences, University of California, Merced, California 95343, and the Department of Pathology, Emory University, Atlanta, Georgia 30322

Transforming growth factor β (TGF-β) stimulates reactive oxygen species (ROS) production in various cell types, which mediates many of the effects of TGF-β. The molecular mechanisms whereby TGF-β increases ROS production and ROS modulate the signaling processes of TGF-β, however, remain poorly defined. In this study, we show that TGF-β1 stimulates NADPH oxidase 4 (Nox4) expression and ROS generation in the nucleus of murine embryo fibroblasts (NIH3T3 cells). This is associated with an increase in protein thiol modification and inactivation of MAPK phosphatase 1 (MKP-1), a nuclear phosphatase. Furthermore, knockdown of MKP-1 using small interfering RNA enhances TGF-β1-induced phosphorylation of JNK and p38 as well as the expression of plasminogen activator inhibitor 1 (PAI-1), a TGF-β-responsive gene involved in the pathogenesis of many diseases. Knockdown of Nox4 with Nox4 small interfering RNA, on the other hand, reduces TGF-β1-stimulated ROS production, p38 phosphorylation, and PAI-1 expression. TGF-β also increased the nuclear level of Nox4 protein as well as PAI-1 expression in human lung fibroblasts (CCL-210 cells), suggesting that TGF-β may induce PAI-1 expression by a similar mechanism in human lung fibroblasts. In summary, in this study we have identified nuclear MAPK phosphatase MKP-1 as a novel molecular target of ROS in TGF-β signaling pathways. Our data suggest that increased generation of ROS by Nox4 mediates TGF-β1-induced PAI-1 gene expression at least in part through oxidative modification and inhibition of MKP-1 leading to a sustained activation of JNK and p38 MAPKs.

Transforming growth factor β (TGF-β), a multi-functional protein, plays an essential role in the development of fibrosis under various pathological conditions involved in almost all organ systems. Emerging evidence indicates that TGF-β stimulates the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in different types of cells, which mediate many of the effects of TGF-β. Nonetheless, the molecular mechanisms whereby TGF-β stimulates ROS/RNS production and how ROS/RNS modulate TGF-β signaling remain poorly defined. NADPH oxidases (Noxs) are a family of heme-containing proteins that transfer electrons from NAD(P)H to molecular O2 to form ROS. Although their biological functions are still largely unknown, emerging evidence indicates that Nox family enzymes are key contributors to ROS generation in both phagocytic and nonphagocytic cells. Seven members of the Nox/Dual oxidase (Duox, which has both peroxidase homology domain and oxidase homology domain) family have been identified: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2. Different Nox/Duox proteins are expressed in different types of cells or tissues and induced by different stimuli. Interestingly, it has been reported that TGF-β specifically increases the expression of Nox4 and ROS production in myriad cell types including smooth muscle cells (1–3), hepatocytes (4–7), and fibroblasts (8, 9), which mediates the induction of smooth muscle cell proliferation, hepatocyte apoptosis, and myofibroblast differentiation by TGF-β. Nevertheless, how Nox4 induction/ROS production mediates the effects of TGF-β and the critical molecular target(s) of ROS in TGF-β signaling pathways remains unclear.

TGF-β signaling through the Smad pathway has been well described. In addition to the Smad pathway, there is increasing evidence that other pathways including mitogen-activated pro-

* This work was supported, in whole or in part, by National Institutes of Health Grants ES011831 and HL088141 (to J. C.). This work was also supported by funds from the American Lung Association (to R.-M. L.) and start-up funds from University of California at Merced (to J. C.).

1 To whom correspondence should be addressed: Dept. of Environmental Health Sciences, School of Public Health, University of Alabama at Birmingham, Birmingham, Al 35294. Tel.: 205-934-7028; Fax: 205-975-6341; E-mail: rliu@uab.edu.

2 The abbreviations used are: TGF-β, transforming growth factor β; ROS, reactive oxygen species; RNS, reactive nitrogen species; Nox, NADPH oxidase; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; PAI, plasminogen activator inhibitor; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; Hsp, heat shock protein; TBP, TATA-binding protein; BIAM, N-(biotinyl)-N’-(iodoacetyl)-ethylenediamine; ELISA, enzyme-linked immunosorbent assay.
tein kinases (MAPKs) are also important in TGF-β signaling (10–12). Numerous studies have shown that TGF-β increases ROS/RNS production, which activates MAPK pathways in various cell types. The redox-sensitive target(s) in TGF-β-MAPK signaling pathways, however, remain to be identified. MAPKs are activated by phosphorylation of threonine and tyrosine residues by MAPK kinases, which, in turn, are phosphorylated and activated by MAPK kinase kinases. Activated MAPKs, on the other hand, can be inactivated by dephosphorylation of threonine residues, tyrosine residues, or both, catalyzed by serine/threonine-specific MAPK phosphatase (MKPs), tyrosine-specific MKPs, and dual-specific MKPs, respectively. Although it is still debatable whether ROS/RNS can directly activate MAPKs, emerging evidence suggests that many of MAPK phosphatases, especially dual-specific MKPs, which contain a redox-sensitive cysteine motif in their active sites, are sensitive to oxidative inactivation by ROS/RNS (13). Whether inactivation of MKPs by ROS/RNS contributes to the sustained activation of MAPKs and increased expression of TGF-β-responsive genes remains to be determined.

In previous studies, we have shown that TGF-β1 stimulates ROS production in murine embryo fibroblasts (NIH3T3 cells), which leads to the activation of JNK and p38 MAPKs and induction of plasminogen activator inhibitor 1 (PAI-1), a protease inhibitor critical in many pathologic conditions including fibrosis (14–17). In this study, we show that TGF-β1 increases the expression and activity of Nox4 in the nucleus of NIH3T3 cells, which is associated with increased nuclear ROS production, thiol modifications, and inhibition of a nuclear phosphatase MKP-1. Nuclear Nox4 protein is also increased in TGF-β1-treated human lung fibroblasts. Using siRNAs, we further show that decreasing MKP-1 expression enhances TGF-β1-induced JNK/p38 phosphorylation and PAI-1 expression, whereas knocking down Nox4 expression reduces TGF-β1-stimulated ROS production, p38 phosphorylation, and PAI-1 expression. The data presented in this study reveal a novel mechanism whereby ROS modulate TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Mouse embryonic fibroblast NIH3T3 cells, obtained from the ATCC (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) as described (18). CCL-210 cells, normal human lung fibroblasts from ATCC, were cultured in ATCC-formulated Eagle’s minimum essential medium (catalogue number 30-003) supplemented with 10% fetal bovine serum. The cells were serum-starved for 6 h before treatment with 1 ng/ml TGF-β1 in the serum-free medium for various periods of time as indicated.

Separation of Cytosolic and Nuclear Fractions—The cytosolic and nuclear fractions were separated as described before (16). Briefly, after treatment, the cells were resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 0.5% Igepal, 2 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 1.0 μg/ml aprotinin) and incubated on ice for 10 min. After centrifugation at 14,000 × g for 1 min at 4 °C, supernatant (cytosol) and the pellets (nuclear fraction) were collected. Cross-contamination of the cytosolic and nuclear fractions was monitored by Western analyses of cytosol marker heat shock protein 90 (Hsp-90) and nuclear marker TATA-binding protein (TBP).

Western Blot Analysis—Cell lysates, cytosol, or nuclear fractions equivalent to 20–50 μg of protein were resolved on 10% SDS-PAGE gels. The separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane and blocked with 5% nonfat dry milk. The membranes were probed at 4 °C overnight with antibodies to Nox4 (1:400), phospho-p38 (1:500), p38 (1:1000), phospho-JNK (1:500), JNK (1:1000), Hsp-90 (1:1000), or TBP (1:500) and then with the corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. The protein bands were visualized by chemiluminescence using the ECL detection system (Amersham Biosciences). The Nox4 antibody was generated in Dr. David Lambeth’s laboratory. All other antibodies, except for PAI-1 (Molecular Innovations, Southfield, MI), Hsp-90 (BD), and TBP (Abcam), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunocytochemical Staining to Detect Nox4—Cells, cultured on coverslips inside 24-well plates, were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then permeabilized in 0.2% Triton X-100 for 15 min and blocked with 2% bovine serum albumin at room temperature for 30 min, followed by incubation with the anti-Nox4 antibody at 4 °C overnight. After washing and blocking, the cells were then incubated with the corresponding secondary antibody plus Oregon Green® 488 phallidin (Molecular Probes) (to stain nuclei) at room temperature for 1 h. The coverslips were mounted with Vectashield mounting medium, and multiple images from each slide were captured using a Nikon TE2000E-2 epifluorescent microscope or a confocal microscopy and analyzed using Image Pro Plus version 5.1.2 (Media Cybernetics).

Determination of NADPH Oxidase Activity—The cytosolic and nuclear fractions were separated as described above, and the nuclei were resuspended in an intracellular-like buffer containing 140 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 1 mM EGTA, 0.193 mM CaCl₂, and protease inhibitors. Superoxide production was measured using cytochrome c as an electron acceptor as described before (19). Briefly, 20–50 μg of protein was incubated with 200 μl of intracellular-like buffer plus 100 μM ATP and 100 μM NADPH with or without diphenyldiamonium at 37 °C for 15 min. The reaction was started by adding 100 μM of cytochrome c and monitored at 550 nm for 30 min. Diphenyldiamonium inhibitable reduction of cytochrome c was used to calculate the NADPH oxidase activity, and the results were normalized by protein concentration.

Measurement of Intracellular ROS/RNS—Intracellular ROS/RNS levels were determined using a redox-sensitive fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (Sigma) by epifluorescence or confocal microscopy as we have described before (16). For mitochondrial ROS/RNS detection, the cells were incubated with 5 μM dihydrorhodamine (Molecular Probes) in serum-free medium at 37 °C for 30 min after treatment and then stained with Hoescht. The intensity of fluorescent staining was quantified using the imaging software. The
average fluorescence intensity from four independent samples for each treatment is presented.

Determination of Thioli Modifications in MKP-1 Protein—After treatment, the cells were lysed with a buffer containing 0.5% (v/v) Triton X-100, 1 mM EDTA, 1 mM diethylentriaminepentaacetic acid (an iron chelating agent), protease inhibitors, and 100 μM N-(biotinoyl)-N’-(iodoacetyl)-ethylendiamine (BIAM), which labels reduced protein thiols. Unreacted BIAM was removed by centrifugation through Microcon-10 (Amicon, Beverly, MA). MKP-1 was then pulled down using anti-MKP-1 antibody and protein A/G PLUS-agarose beads (Santa Cruz) was removed by centrifugation. After incubation, the beads were washed four times with cold lysis buffer, and biotinylated proteins were eluted by incubating beads with 40 μl of release solution (2% SDS, 30 mM biotin, 50 mM phosphate, 100 mM NaCl, 6 M urea, 2 M thiourea, pH 12) at 96 °C for 15 min. After brief centrifugation, the supernatants were used for Western analysis of MKP-1 using anti-MKP-1 antibody.

MKP-1 Activity Analysis—The assays were performed in 96-well plates as described previously (20). Briefly, after immunoprecipitation of MKP-1 from 200 μg of cell lysates with anti-MKP-1 antibody, the phosphatase activities in the immunoprecipitates were determined using a specific phosphorylated substrate 6,8-difluoro-4-methylumbelliferyl phosphate (Molecular Probes) with a fluorescence plate reader following the protocol provided by the manufacturer. The activities were calculated based on standard curves generated using 6,8-difluoro-7-hydroxy-4-methylcoumarin.

Determination of JNK-directed Phosphate Activity—After treatment, endogenous JNK (phosphorylated and nonphosphorylated) was depleted by immunoprecipitation using anti-JNK monoclonal antibody. JNK-directed phosphatase activity was determined using exogenous active JNK (pJNK; from Upstate). Briefly, JNK-depleted cell lysates from control and TGF-β-treated cells were incubated with equal amount of exogenous pJNK at 30 °C for 30 min. The remaining undeveloped exogenous pJNK was determined by Western analysis. Reaction mixtures containing only exogenous pJNK or only cell lysate were used as positive and negative controls, respectively. The membrane was also probed with β-actin and JNK to show that equal amounts of sample protein and equal amounts of exogenous pJNK were used between reactions.

siRNA Knockdown of MKP-1 or Nox4—The cells, cultured in 24-well plates (for immunocytocchemical staining analysis) or 6-well plates (for Western analysis) overnight, were transfected with 100 nM predesigned mouse MKP-1 siRNA or 100 nM Nox4 siRNA (Dharmacon Inc., Chicago, IL) using DharmaFECT transfection reagent (Thermo Scientific) according to the manufacturers’ protocols. 30 h (for Nox4 siRNA) or 24 h (for MKP-1 siRNA) after transfection, the cells were serum-starved for 6 h and then treated with TGF-β1 in serum-free medium for different periods of time as indicated in the figure legends.

Total PAI-1 ELISA—Total PAI-1 protein content in the culture medium was determined by ELISA as we have described previously (16). Briefly, the plates were coated with antihuman PAI-1 monoclonal antibody (catalogue number MA31C9-1005; Molecular Innovations) at 4 °C overnight. After blocking and washing, samples or PAI-1 standards were added to the wells and incubated at 25 °C for 30 min.
anti-human PAI-1 antibody (catalogue number ASHPAI-GF; Molecular Innovations) and horseradish peroxidase-conjugated anti-rabbit antibody were used to detect captured PAI-1 by TMB ready-to-use substrate (Sigma). The results were calculated based on the standard curve run under the same conditions.

Statistical Analysis—The data are presented as the means ± S.E. and were evaluated by one-way analysis of variance. Statistical significance was determined post-hoc by Tukey’s test wherein \( p < 0.05 \) was considered significant.

RESULTS

TGF-β1 Increases Nuclear Nox4 Expression and Activity in NIH3T3 Cells—Although it has been well documented that TGF-β increases ROS production in various cell types, the mechanism and precise location(s) of ROS production remain unclear. TGF-β has been shown to increase Nox4 expression in different types of cells. To determine whether TGF-β1 increases ROS production in NIH3T3 cells as we have shown previously (16, 18) by inducing Nox4, the amount and location of Nox4 protein was determined by immunocytochemical staining and by Western analysis after cell fractionation. Immunocytochemical staining showed that Nox4 protein was located primarily in the nucleus of unstimulated NIH3T3 cells, and its expression increased after treating cells with TGF-β1 for 1 h (Fig. 1A). Subcellular fractionation-Western analyses further confirmed that Nox4 was mainly expressed in the nucleus of NIH3T3 cells and that its expression increased by 2.8- and 4.5-fold after the cells were treated with TGF-β1 for 2 and 18 h, respectively (Fig. 1B). To confirm the increase in nuclear Nox4 expression, NADPH oxidase activity was determined in nuclear compartment by measuring diphenylidonium inhibitable reduction of cytochrome c in the presence of NADPH. The results showed that although a slight increase in NADPH oxidase activity could be observed in the cytosol, TGF-β1 treatment led to a significant increase in NADPH oxidase activity in the nucleus (211 ± 31.8%, \( p < 0.05 \)) (Fig. 1C).

TGF-β1 Increases Nuclear and Cytosolic ROS Levels in NIH3T3 Cells—To determine the sites where ROS/RNS are produced in TGF-β1-treated NIH3T3 cells, redox-sensitive fluorescent dyes 2′,7′-dichlorodihydrofluorescein diacetate (DCF), which can easily pass cell membranes, and dihydrorhodamine, which is specifically concentrated in mitochondria, were used to monitor ROS/RNS signals in TGF-β1-treated NIH3T3 cells using epifluorescence and confocal microscopy. The results showed that the 2′,7′-dichlorodihydrofluorescein diacetate signal was significantly increased in both nucleus and cytosol in NIH3T3 cells after 1 h of TGF-β1 treatment, suggesting an increase in both nuclear and cytosolic ROS/RNS levels (Fig. 2A). The increase in the 2′,7′-dichlorodihydrofluorescein diacetate signal lasted for at least 5 h (data not shown). Interestingly, although an increase in cytosolic ROS/RNS could be observed, we did not find any significant increase in mitochondrial ROS/RNS with TGF-β1 treatment (Fig. 2B). These data suggest that TGF-β1 treatment increases ROS/RNS levels in NIH3T3 cells mainly in the nucleus and possibly cytosolic compartment(s) other than the mitochondria. These data further suggest that increased expression and activity of Nox4 may underlie the increase in ROS/RNS production in TGF-β1-treated NIH3T3 cells.

TGF-β1 Inhibits MKP-1 Activity and Increases Protein Thiolytic Modification of MKP-1 without Altering Its Protein Level in NIH3T3 Cells—Although it has been well documented that ROS mediate the activation of MAPKs by TGF-β, the molecular mechanism remains poorly defined. MKPs contain a redox-sensitive cysteine residue in their active site and therefore are sensitive to the redox status of the environment. To explore whether ROS mediate the activation of MAPKs by TGF-β1 through inactivation of MKPs, we monitored the protein thiol modifications and the activity of MKP-1, a dual specific nuclear MAPK phosphatase, in TGF-β1-treated NIH3T3 cells. Two techniques were used to demonstrate MKP-1 protein thiol modifications: 1) after labeling unmodified protein thiol residues with BIAM, MKP-1 protein was immunoprecipitated with MKP-1 antibody, followed by Western analysis of BIAM-labeled MKP-1 using horseradish peroxidase-conjugated streptavidin; and 2) after labeling, BIAM-labeled proteins were pulled down with streptavidin resin, followed by Western analysis of BIAM-labeled MKP-1 using MKP-1 antibody. The results...
showed that treatment of NIH3T3 cells with TGF-β1 for 1 h induced thiol modifications in MKP-1 protein, resulting in a decrease in BIAM labeling/signals (Fig. 3, A and B). Importantly, we showed that, associated with increased thiol modifications, TGF-β1 treatment for 1 h decreased the MKP-1 activity by half (Fig. 3C). These results suggest that inactivation of MAPK phosphatases such as MKP-1 by ROS may underlie the sustained activation of JNK and p38 in these fibroblasts.

**TGF-β1 Inhibits the Activity of JNK-directed Phosphatase in NIH3T3 Cells**—To further demonstrate that TGF-β1 treatment leads to inactivation of MKPs, we measured the activity of JNK-directed phosphatase, which selectively dephosphorylate pJNK, in TGF-β1-treated NIH3T3 cells. The results showed that treatment of NIH3T3 cells with TGF-β1 for 30 or 60 min significantly reduced the activity of JNK-directed phosphatases in our cell model (Fig. 4). These data confirm an inhibition of the MKP activity by TGF-β1 treatment in our cell model and further suggest that inhibition of MKP activity may be responsible for the sustained activation of MAPKs by TGF-β1 as shown previously.

**Knockdown of MKP-1 Enhances TGF-β1-induced JNK/p38 Phosphorylation and PAI-1 Expression in NIH3T3 Cells**—To further elucidate the role of MKP-1 in MAPK activation and PAI-1 induction by TGF-β1 in our cell model, the MKP-1 expression was knocked down using siRNA techniques. Nontargeting siRNA was used as a negative control. The results showed that although knocking down MKP-1 expression by 70% alone had no significant effect on JNK/p38 activation or PAI-1 expression, it significantly enhanced TGF-β1-induced JNK (by 60 ± 1.4%) and p38 (by 185 ± 30%) phosphorylation as well as PAI-1 expression (by 162 ± 28%) compared with TGF-β-treated, nontargeting siRNA-transfected cells (Fig. 5). The results further suggest that inactivation of MKP-1 is responsible for the sustained activation of JNK/p38 and the induction of PAI-1 by TGF-β1 in these fibroblasts.

**Down-regulation of Nox4 Gene Expression with Nox4 siRNA Attenuates TGF-β1-induced ROS Production, p38 Phosphorylation, and PAI-1 Expression in NIH3T3 Cells**—To delineate the role of Nox4 in TGF-β1-induced ROS production, MAPK activation, and PAI-1 expression in our cell model, Nox4 siRNA was used to specifically knock down Nox4 protein expression. The cells were then treated with TGF-β1 for 2 or 15 h. ROS production, p38 phosphorylation, and PAI-1 expression were monitored as described above. The results showed that transfecting cells with Nox4 siRNA completely blocked TGF-β1-induced Nox4 expression at both the 2- and 15-h time points, revealed by immunocytochemical staining and Western analyses (Fig. 6, A and B). Associated with the down-regulation of Nox4 expression, TGF-β-stimulated ROS signal was dramatically reduced (Fig. 6C), compared with nontargeting siRNA-transfected cells. Most importantly, we showed that TGF-β1-induced phosphorylation of p38 MAPK was reduced by 61 ± 3.7 and 46 ± 5.6%, whereas PAI-1 expression was reduced by 66 ± 1.2 and 51 ± 3.8%, respectively, at 2 and 15 h after TGF-β treatment in Nox4 siRNA-transfected cells as compared with nontargeting siRNA-transfected controls (Fig. 6B). These results strongly suggest that Nox4 induction/activation contributes importantly to TGF-β1-induced ROS production, JNK/p38 activation, and PAI-1 expression in fibroblasts.
TGF-β, Nox4, ROS, MKP-1, and PAI-1 Induction

TGF-β Increases the Nuclear Nox4 Protein Level and Induces PAI-1 Expression in Human Lung Fibroblasts—To further test whether the increase in the nuclear Nox4 expression upon TGF-β stimulation occurs in other fibroblasts, such as human fibroblasts, we examined whether TGF-β induced Nox4 expression in CCL-210 cells, which are normal human lung fibroblasts. Nuclear fractionation-Western and the immunocytochemical staining analyses showed that although Nox4 was mainly found in the cytosol in these cells, treating cells with 1 ng/ml TGF-β for 20 h significantly increases nuclear Nox4 protein levels with no significant effect on the amount of cytosolic Nox4 protein (Fig. 7, A and B). Associated with increased nuclear Nox4 expression, PAI-1 protein level in the cultured medium was increased by 13.8-fold (measured by ELISA) as shown in Fig. 7C. The results suggest that TGF-β may induce PAI-1 expression in human lung fibroblasts by a similar mechanism as shown in NIH3T3 cells.

DISCUSSION

Studies from our lab and others have shown that TGF-β1 increases ROS production in various cell types, which mediates the induction of many of TGF-β-responsive genes including PAI-1 through MAPK pathways. The molecular mechanism underlying TGF-β stimulation of ROS production and the redox-sensitive targets in TGF-β-MAPKs signaling pathways, however, remained unclear. In this study, we showed that TGF-β1 increased Nox4 expression/activity mainly in the nucleus of murine embryo fibroblasts (NIH3T3 cells) and human lung fibroblasts (CCL-210 cells). Such an increase in the nuclear Nox4 expression/activity was associated with increased ROS production as well as thiol modifications and inhibition of nuclear MAPK phosphatase MKP-1 in NIH3T3 cells. Knocking down MKP-1 with MKP-1 siRNA enhanced TGF-β1-induced phosphorylation of JNK and p38 as well as the expression of PAI-1, whereas knocking down Nox4 with Nox4 siRNA suppressed TGF-β1-induced ROS production, p38 phosphorylation, and PAI-1 expression. The results presented in this study reveal a novel redox-sensitive target in TGF-β1 signaling pathways and suggest that oxidative modifications/inactivation of MAPK phosphatases may underlie TGF-β1-induced sustained activation of MAPKs and thereby the expression of many TGF-β-responsive genes such as PAI-1.

The Nox family has been increasingly recognized as important ROS producers not only for phagocytic but also for nonphagocytic cells. The expression of different Nox family members appears to be cell-, tissue-, and probably stimuli-specific and is associated with different cell compartments, although the prototypic NADPH oxidase Nox2 (also called gp91-PHOX) is mainly expressed on the plasma membrane. TGF-β has been shown to increase the expression of Nox4 in different types of cells, although the localization of Nox4 is still debatable. Using three kinds of affinity-purified anti-human Nox4 antibodies as well as immunocytochemistry and immunoelectron microscopy techniques, Kuroda et al. (28) showed that Nox4 was preferentially localized to the nucleus of human umbilical vein endothelial cells. A nuclear co-localization of Nox4 and p22^phox^ was also reported in human pulmonary artery endothelial cells, and the Nox4 protein level was shown to increase as early as 3 h after exposure of the cells to hypoxia (29). A recent study further shows that Nox4-dependent nuclear H₂O₂ production is responsible for the DNA oxidation in a murine endothelial cell model of hemangiomas (30). In addition to nuclear localization, Nox4 has been found to be associated with endoplasmic reticulum (2, 31–33) and perinuclear space (34). Interestingly, we showed in this study that Nox4 expression was increased predominantly in the nucleus of two types of fibroblasts upon TGF-β1 treatment, although it was expressed mainly in the cytosol of untreated human lung fibroblasts (CCL-210 cells) or in the nucleus of untreated NIH3T3 cell. Importantly, our data showed that the Nox4 protein level was increased 1 h after TGF-β1 treatment and that knocking down Nox4 with siRNA techniques dramatically reduced TGF-β1-stimulated ROS signal in NIH3T3 cells. These data suggest that Nox4 is an early response gene, probably regulated at both transcription and post-transcription levels, and that a major source of ROS in TGF-β1-stimulated fibroblast cells is Nox4. Nonetheless, whether TGF-β1 also induces other Nox family members in these fibroblast cells and the
**Mechanism underlying the induction of nuclear Nox4 expression by TGF-β1 remain to be explored.**

TGF-β1 increases ROS production in numerous types of cells such as endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts (1, 5, 8, 21–23); the underlying mechanism, however, remains obscure. Thannickal et al. (21, 24, 25) reported that TGF-β1 stimulated ROS production in human lung fibroblasts and bovine pulmonary artery endothelial cells through activation of cell-membrane associated oxidases, which led to an increased release of H2O2 to the extracellular space. Yoon et al. (22), on the other hand, reported that TGF-β1 induced prolonged mitochondrial ROS production in Mv1Lu cells, a mink lung epithelial cell line, by decreasing complex IV activity. Herrera et al. (26) also demonstrated an increased mitochondrial ROS production in TGF-β1-treated rat hepatocytes. Using different inhibitors, Albright et al.
**TGF-β1, Nox4, ROS, MKP-1, and PAI-1 Induction**

(27) showed, however, that both mitochondria and microsomes were involved in the ROS production in TGF-β-treated rat hepatocytes. In this study, we found that TGF-β1 treatment increased ROS signals in the nucleus and cytosol, although no significant change in ROS signals was observed in mitochondria. The reason for such differences in terms of the locations of ROS production between different types of cells in response to TGF-β is unclear at the moment, probably because of different enzymes expressed or different signaling pathways/receptors affected in different types of cells by TGF-β. Nonetheless, the differences in the locations of ROS generation may underlie the different responses of different types of cells to TGF-β. For example, mitochondrial ROS production has been shown to be associated with TGF-β-induced apoptosis in hepatocytes (26, 27). On the other hand, nuclear ROS production is associated with retinoblastoma protein phosphorylation and cell proliferation in TGF-β-treated human airway smooth muscle cells (2). In this study, we showed that TGF-β-induced nuclear ROS generation was associated with an inhibition of a nuclear MKP (MKP-1). Nevertheless, the molecular mechanism and biological significance associated with the site-specific production of ROS in different cells upon TGF-β treatment remain to be further investigated.

MKP-1 is a nuclear dual specificity MAPK phosphatase with specificity toward JNK and p38 in most cells (35–39). Like other dual specific MKPs, MKP-1 bears a redox-sensitive cysteine motif in its active site and therefore is sensitive to ROS. In this study, we show for the first time that TGF-β1 treatment increases thiol modifications of MKP-1 and inhibits its activity but has no significant effect on the amount of MKP-1 protein in NIH3T3 cells. Furthermore, we show that knocking down MKP-1 expression using MKP-1 siRNA enhances TGF-β1-induced phosphorylation of JNK and p38 MAPKs and the expression of the PAI-1 gene. These data support the notion that the MKPs are the target of ROS and that the oxidative inactivation of MKP1 may contribute to TGF-β1-induced sustained activation of MAPKs and thus the expression of PAI-1 gene (Fig. 8).

It should be mentioned that TGF-β has been shown to induce MKP-1 gene expression, which leads to inactivation of JNK or p38 MAPK and therefore the suppression of inflammatory cytokine production in macrophages and other immune response cells (40–42). Such induction of MKP-1 has been proposed to be one of the mechanisms responsible for the suppression of inflammatory response by TGF-β. In this study, we found, however, that TGF-β treatment has no effect on the MKP-1 protein level but significantly decreases MKP-1 activity in NIH3T3 cells. Differences between our results and others may be due to the differences in the cells used because it has been well documented that different types of cells bear different receptors and signaling pathways and respond differently to TGF-β. Nonetheless, although no data are available in the literature showing that TGF-β inhibits MKP-1 activity, ROS generated by other stimuli have been reported to inactivate MKP-1. Hou et al. (43) showed that treatment of pancreatic β-cells with low glucose induced ROS production, JNK and p38 phosphorylation, and MKP-1 oxidation, whereas ROS scavengers prevented MKP-1 oxidation and JNK activation. Interestingly,

**FIGURE 8. Potential source of ROS and mechanism whereby ROS mediate TGF-β induction of PAI-1 in NIH3T3 cells. TGF-β increases ROS production by inducing nuclear Nox4 expression and stimulating other ROS generation mechanisms. ROS inactivate MKP-1, which leads to sustained activation of JNK and p38 and thus the induction of PAI-1 expression.**

Mishra *et al.* (44) reported that exposure of newborn piglets to hypoxia increased MKP-1 and MKP-3 protein levels but decreased their activities in the neuronal nucleus. Treatment of animals with neuronal nitric-oxide synthase inhibitor prevented hypoxia-induced changes in MKP-1 and MKP-3, suggesting that hypoxia decreases MKP-1 and MKP-3 activities by causing oxidative modifications of MKP-1 and MKP-3 proteins rather than by decreasing the expression/production of these MKPs (44).

**CONCLUSION**

TGF-β increases ROS production, and ROS modulates TGF-β signaling processes; the molecular mechanism underlying TGF-β stimulation of ROS production and whereby ROS modulate TGF-β signaling, however, was poorly defined. The results from this study reveal a novel molecular target of ROS in TGF-β signaling pathway. Because TGF-β plays a vital role in the pathogenesis of many diseases and ROS mediate many of the effects of TGF-β, identifying redox-sensitive targets in TGF-β signaling pathways will not only help us to understand the mechanism whereby TGF-β exerts its biological and/or pathogenic effects but also aid in the development of treatments for the diseases in which TGF-β plays a pivotal role, such as fibrosis.

**ACKNOWLEDGMENT**—We thank Dr. Carol Ballinger for critical review of the manuscript.

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