Role of Reactive Oxygen Species in TGF-β1–Induced Mitogen-Activated Protein Kinase Activation and Epithelial-Mesenchymal Transition in Renal Tubular Epithelial Cells

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Epithelial-mesenchymal transition (EMT) plays an important role in renal tubulointerstitial fibrosis and TGF-β1 is the key inducer of EMT. Phosphorylation of Smad proteins and/or mitogen-activated protein kinases (MAPK) is required for TGF-β1–induced EMT. Because reactive oxygen species (ROS) are involved in TGF-β1 signaling and are upstream signaling molecules to MAPK, this study examined the role of ROS in TGF-β1–induced MAPK activation and EMT in rat proximal tubular epithelial cells. Growth-arrested and synchronized NRK-52E cells were stimulated with TGF-β1 (0.2 to 20 ng/ml) or H2O2 (1 to 500 μM) in the presence or absence of antioxidants (N-acetylcycteine or catalase), inhibitors of NADPH oxidase (diphenyleneiodonium and apocynin), mitochondrial electron transfer chain subunit I (rotenone), and MAPK (PD 98059, an MEK [MAP kinase/ERK kinase] inhibitor, or p38 MAPK inhibitor) for up to 96 h. TGF-β1 increased dichlorofluorescein-sensitive cellular ROS, phosphorylated Smad 2, p38 MAPK, extracellular signal-regulated kinases (ERK)1/2, α-smooth muscle actin (α-SMA) expression, and fibronectin secretion and decreased E-cadherin expression. Antioxidants effectively inhibited TGF-β1–induced cellular ROS, phosphorylation of Smad 2, p38 MAPK, and ERK, and EMT. H2O2 reproduced all of the effects of TGF-β1 with the exception of Smad 2 phosphorylation. Chemical inhibition of ERK but not p38 MAPK inhibited TGF-β1–induced Smad 2 phosphorylation, and both MAPK inhibitors inhibited TGF-β1– and H2O2-induced EMT. Diphenyleneiodonium, apocynin, and rotenone also significantly inhibited TGF-β1–induced ROS. Thus, this data suggest that ROS play an important role in TGF-β1–induced EMT primarily through activation of MAPK and subsequently through ERK-directed activation of Smad pathway in proximal tubular epithelial cells.


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Reactive oxygen species (ROS) have been shown to mediate TGF-β1–induced cellular responses in various cells (26–32). We (33) recently demonstrated that TGF-β1 upregulates plasminogen activator inhibitor-1 expression in glomerular mesangial cells through ROS. ROS are known to activate MAPK (34). However, it is not known whether ROS are involved in EMT. We therefore examined whether TGF-β1–induced EMT is mediated by ROS-MAPK pathway. For this purpose, we measured cellular ROS after stimulation with TGF-β1, examined the effect of antioxidants on TGF-β1–induced MAPK activation and EMT, and finally compared the effect of H2O2 with that of TGF-β1. We also examined the source and type of TGF-β1–induced cellular ROS and the cross-talk between MAPK and Smad 2.

Materials and Methods

All chemicals and tissue culture plates, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO) and Becton Dickinson Labware (Lincoln Park, NJ).

Cell Culture and Treatment

Rat proximal tubular epithelial cell line NRK52E was purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (Life Technologies BRL, Gaithersburg, MD) that contained 5% FBS (Life Technologies BRL), 100 U/ml penicillin, 100 µg/ml streptomycin, 44 mM NaHCO3, and 14 mM HEPES.

Cells were cultured at a density of 8 × 104 cells/well in six-well culture plates. Near confluent NRK-52E cells were incubated with serum-free media for 24 h to arrest and synchronize the cell growth. After this time period, the media were changed to fresh serum-free media that contained various concentrations of recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) or H2O2. In some experiments, cells were pretreated with antioxidants (5 mM N-acetylcysteine [NAC] and 500 U/ml catalase), NADPH oxidase inhibitors (100 nM diphenyleneiodonium [DPI] and 100 µM apocynin), mitochondrial electron transfer chain subunit I inhibitor (rotenone 1 µM), 5 µM p38 MAPK inhibitor (2-[1-[4-chlorophenyl]-4-[4-fluorophenyl]-5-pyridine-4-yl-1,2-dihydropyrazol-3–1; Calbiochem, San Diego, CA), or 50 µM PD98059, a known MEK (MAP kinase/ERK kinase) inhibitor (Calbiochem) for 1 h before the addition of TGF-β1 or H2O2. The effective concentrations of antioxidants, NADPH oxidase inhibitors, and rotenone were decided on the basis of our previous data (33,35) and MAPK inhibitors from our preliminary study. Cells were processed for measurement of cellular ROS and expression of E-cadherin, α-SMA, ERK, p38 MAPK, and Smad 2 at the end of incubation period as described below.

Assay of Cellular Reactive Oxygen Species

Dichlorofluorescin (DCF)-sensitive cellular ROS in NRK52E cells were measured by a FACS (Becton Dickinson, Mountain View, CA) as described previously (33). In brief, cells in various time points after stimulation with serum-free TGF-β1 or H2O2 were washed with serum-free and phenol red-free DMEM and loaded with 5 µM 5-(anilino)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR). After incubation for 20 min in the dark, the cells were washed with PBS, detached, and resuspended in 1 ml of PBS. Cellular ROS in 10,000 cells as a result of the oxidation of 2',7'-dihydrofluorescein was measured (excitation, 488 nm; emission, 515 to 540 nm).

Cellular superoxide anion and peroxinitrite were measured using dihydroethidine (36) and dihydrorhodamine 123 (DHR) (37), respectively. Dihydroethidine is cell permeable and reacts with O2•− to form ethidium, which in turn interacts with DNA, providing fluorescence at an excitation at 520 nm and an emission at 610 nm. DHR forms the fluorescence product rhodamine 123 upon oxidation by peroxides and notably ONOO−. Fluorescence was detected with emission at 530 nm and excitation at 485 nm and normalized to total cell protein and expressed as fold of control.

Western Blot Analysis

At the end of incubation, cells were washed with PBS and lysed in 150 µl of lysis buffer of 20 mM Tris-HCl (pH 7.9), 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 1% Triton-X that contained protease inhibitors for 10 min at 4°C. Conditioned media and cell lysates were centrifuged at 13,000 rpm at 4°C for 15 min to pellet cell debris, and the concentration of cellular protein was determined using Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Samples with equal concentrations of cellular protein (20 µg) were mixed with a 5× sample buffer that contained 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol, and 0.2% 2-mercaptoethanol and was heated at 95°C for 10 min and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris buffer (0.025 M Tris, HCl, 0.192 M glycine, and 20% methanol). The membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBS-Tween 20. Membranes were incubated at 4°C overnight with mouse mAb to E-cadherin (Becton Dickinson Labware; 1:2000), mouse anti–α-SMA mAb (clone 1A4; 1:2000), rabbit polyclonal anti-phosphorylated and total ERK1/2 antibody (Cell Signaling, Beverly, MA; 1:1000), or rabbit polyclonal anti-phosphorylated and total p38 MAPK antibody (CellSignaling; 1:1000) or anti-phosphorylated Smad 2 antibody (Calbiochem, San Diego, CA; 1:1000). After extensive washing in TBS-Tween 20, the membranes then were incubated with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. After washing, the membranes were incubated with enhanced chemiluminescence system (ECL) detection kit (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically, normalized by β-actin (for E-cadherin, α-SMA, and phosphorylated Smad 2), total ERK1/2 (1:2000, for phosphorylated ERK), or total p38 MAPK (1:2000, for phosphorylated p38 MAPK) and compared with controls. For fibronectin secreted into the media, 5% polyacrylamide gel and peroxidase-conjugated rabbit anti-human fibronectin (DAKO A/S, Glostrup, Denmark; 1:2000) were used.

Statistical Analyses

All results are expressed as mean ± SE with n as the number of experiments. ANOVA was used to assess the differences between multiple groups. When the F statistic was significant, the mean values obtained from each group then were compared by Fisher least significant difference method. P < 0.05 was used as the criterion for a statistically significant difference.

Results

Effect of TGF-β1 on E-Cadherin and α-SMA Expression

TGF-β1 decreased E-cadherin expression and increased α-SMA expression in a dose- (Figure 1, A and B) and a time-dependent manner (Figure 1, C and D) in NRK-52E cells. Statistically significant changes were observed at 10 ng/ml and from day 2 after TGF-β1.
Effect of TGF-β1 on p38 MAPK, ERK, and Smad 2 Activation

TGF-β1 at 10 ng/ml significantly increased phosphorylated p38 MAPK (Figure 2A) and ERK1/2 (Figure 2B) from 15 min, and this increase remained significant up to 2 h. TGF-β1–induced Smad 2 phosphorylation was evident from 5 min under our experimental condition (Figure 2C).

Effect of TGF-β1 on Cellular ROS Generation: Source and Type of ROS

TGF-β1 at 10 ng/ml significantly increased DCF-sensitive cellular ROS in NRK-52E cells from 5 to 30 min (Figure 3A). Cellular ROS at 10 min after TGF-β1 was two-fold higher than that of basal. Cellular ROS returned to basal level at 4 h after TGF-β1. There was a slight but statistically significant increase

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**Figure 1.** Effect of TGF-β1 on E-cadherin and α-smooth muscle actin (α-SMA) expression. After incubation of quiescent NRK-52E cells with increasing concentrations of TGF-β1 and at different time points, aliquots of cell lysate that contained the same concentrations of proteins were electrophoresed under reducing condition and Western blots were performed as described in the text. (A) Dose response of E-cadherin expression. (B) Dose response of α-SMA expression. (C) Time response of E-cadherin expression. (D) Time response of α-SMA expression. The top panel shows a representative Western blot, and the bottom panel represents relative change as mean ± SEM of four experiments. *P < 0.05 versus control.

**Figure 2.** (A) Effect of TGF-β1 on p38 mitogen-activated protein kinase (MAPK), (B) extracellular signal-regulated kinase (ERK), and (C) Smad 2 activation. After incubation of quiescent NRK-52E cells for different time periods, aliquots of cell lysate were subjected to Western blot analysis. The top panel shows a representative Western blot, and the bottom panel represents relative increase as mean ± SEM of five experiments. *P < 0.05 versus control.
in dihydrorhodamine-sensitive ROS (data not shown). There was, however, no significant increase in dihydroethidine-sensitive ROS (data not shown). Inhibitors of NADPH oxidase (100 nM DPI and 100 μM apocynin), an inhibitor of mitochondrial electron transfer chain subunit I (rotenone 1 μM), and antioxidants (5 mM NAC and 500 U/ml catalase) all significantly reduced TGF-β1-induced DCF-sensitive cellular ROS (Figure 3B).

Effect of Antioxidants on TGF-β1–Induced p38 MAPK, ERK, and Smad 2 Activation

Treatment with antioxidants NAC (5 mM) or catalase (500 U/ml) effectively suppressed TGF-β1-induced phosphorylation of p38 MAPK (Figure 4A), ERK1/2 (Figure 4B), and Smad 2 (Figure 4C). Neither NAC nor catalase affected basal phosphorylation of p38 MAPK, ERK1/2, and Smad2 (data not shown).

Effect of Antioxidants and MAPK Inhibitors on TGF-β1–Induced EMT

TGF-β1–induced downregulation of E-cadherin was effectively prevented by pretreatment with antioxidants NAC (5 mM) or catalase (500 U/ml; Figure 5A). Both NAC and catalase effectively inhibited TGF-β1–induced upregulation of α-SMA expression (Figure 5B). Pretreatment with 50 μM PD 98059, an MEK inhibitor, or 5 μM p38 MAPK inhibitor effectively prevented TGF-β1–induced changes in E-cadherin (Figure 6A) and α-SMA expression (Figure 6B).

Effect of NADPH Oxidase Inhibitors on TGF-β1–Induced Fibronectin Secretion

As shown in Figure 7, NADPH oxidase inhibitors DPI and apocynin significantly reduced TGF-β1–induced fibronectin secretion by NRK-52E cells.

Figure 3. (A) Effect of TGF-β1 on dichlorofluorescein (DCF)-sensitive cellular reactive oxygen species (ROS) and (B) effects of antioxidants. After incubation of quiescent NRK-52E cells for different time periods or in different experimental conditions, DCF-sensitive cellular ROS were measured as described in the text. Antioxidants including NAC, catalase, DPI, apocynin, and rotenone were administered 1 h before the addition of TGF-β1, and cellular ROS were measured at 15 min after the addition of TGF-β1. 0, time 0 or culture media without TGF-β1; Apo, apocynin 100 μM; DPI, diphenyleniodonium 100 nM; Rot, rotenone 1 μM; NAC, N-acetylcysteine 5 mM; Cat, catalase 500 U/ml. Values are expressed as mean ± SEM of six experiments. *P < 0.05 versus time 0; +P < 0.05 versus TGF-β1 control.

Figure 4. Effect of antioxidants on TGF-β1–induced (A) p38 MAPK, (B) ERK, and (C) Smad 2 activation. After incubation of quiescent NRK-52E cells in different experimental conditions, aliquots of cell lysate were subjected to Western blot analysis. NAC or Cat was administered 1 h before the addition of 10 ng/ml TGF-β1. Cells were treated with TGF-β1 for 1 h. The top panel shows a representative Western blot, and the bottom panel represents relative increase as mean ± SEM of four experiments. *P < 0.05 versus control; +P < 0.05 versus TGF-β1 without antioxidants.
Effect of H$_2$O$_2$ on MAPK, EMT, and Smad 2 Activation

H$_2$O$_2$ at 100 µM significantly increased p38 MAPK (Figure 8A) and ERK1/2 phosphorylation (Figure 8B) from 5 min to 2 h after the addition of H$_2$O$_2$. H$_2$O$_2$ decreased E-cadherin expression and increased α-SMA expression in a dose-dependent manner at day 4. H$_2$O$_2$ at concentrations of 100 µM and above significantly decreased E-cadherin expression (Figure 8C), and H$_2$O$_2$ at concentrations of 50 µM and above significantly increased α-SMA expression (Figure 8D) compared with control. H$_2$O$_2$ at 100 µM did not increase Smad 2 phosphorylation up to 120 min (Figure 9A). H$_2$O$_2$ up to 500 µM did not affect Smad 2 phosphorylation at 30 min (data not shown).

Effect of MAPK Inhibitors on H$_2$O$_2$-Induced EMT and TGF-β1-Induced Smad 2 Phosphorylation

Decreased E-cadherin expression by H$_2$O$_2$ was recovered to basal by p38 MAPK inhibitor or by an MEK inhibitor (Figure 9B). Both p38 MAPK inhibitor and MEK inhibitor effectively inhibited H$_2$O$_2$-induced α-SMA expression without significant effect on basal level (Figure 9C).

TGF-β1–induced Smad2 phosphorylation was effectively inhibited by PD98059 but not by p38 MAPK inhibitor at doses that inhibited the effect of TGF-β1 on E-cadherin and α-SMA (Figure 10). p38 MAPK inhibitor up to 10 µM (twice the dose that inhibited the effect of TGF-β1) did not inhibit TGF-β1-induced Smad 2 phosphorylation.
Signaling Pathways in TGF-β1–Induced EMT in NRK-52E Cells

Figure 11 summarizes the involvement of ROS in TGF-β1–induced EMT through both MAPK and Smad pathways.

Discussion

Activation of Smad pathway and/or MAPK pathway is required in TGF-β1–induced EMT (10). Although TGF-β1 is known to signal through ROS (26–33), it is not known whether ROS are involved in TGF-β1–induced renal tubular EMT. We hypothesized that ROS may play a role in TGF-β1–induced EMT in renal tubular epithelial cells through activation of Smad or MAPK pathway.

We confirmed in this study that TGF-β1 activates Smad 2, p38 MAPK, and ERK1/2 and decreases E-cadherin and increases α-SMA expression and fibronectin secretion by NRK-52E cells. We also confirmed that TGF-β1 increases DCF-sensitive cellular ROS. Inhibition of NADPH oxidase and mitochondrial electron transfer chain subunit I as well as antioxidants significantly reduced, to similar extent, TGF-β1–induced generation of cellular ROS, suggesting that NADPH oxidase and mitochondrial metabolism are important sources of TGF-β1–induced cellular ROS. We previously demonstrated that TGF-β1 upregulates NADPH oxidase subunit mRNA expression in LLC-PK1, another proximal tubular epithelial cell line (34). The effect of rotenone in this study is different from previous studies that demonstrated total inhibitory effect of DPI but not rotenone on TGF-β1–induced ROS in rat hepatocyte (38) and human lung fibroblast (39). A slight but statistically significant increase in DHR-sensitive ROS (peroxinitrite) was observed after TGF-β1 but not in dihydroethidine-sensitive ROS (superoxide anion; data not shown). This may be interpreted as a result of rapid conversion of superoxide anion to H2O2 by superoxide dismutase and even more rapid binding of superoxide to NO forming peroxinitrite.

We (40) and others (41) have previously demonstrated that H2O2 upregulates TGF-β1 mRNA and protein expression in glomerular mesangial cells and that antioxidants effectively inhibit high glucose–induced TGF-β1 expression in mesangial cells (42) and diabetic kidneys (43). These observations together with the finding of this study that TGF-β1 induces generation of ROS suggest that TGF-β1 might be a key mediator of renal tubular EMT.

Figure 8. Effect of H2O2 on activation of (A) p38 MAPK and (B) ERK1/2 and expression of (C) E-cadherin and (D) α-SMA. After incubation of quiescent NRK-52E cells for different time periods or at different concentrations of H2O2, aliquots of cell lysate were subjected to Western blot analysis. Time-response of MAPK activation (A and B) was performed with 100 μM H2O2 and dose-response for E-cadherin (C) and α-SMA (D) expression at day 4. The top panel shows a representative Western blot, and the bottom panel represents relative increase as mean ± SEM of four experiments. *P < 0.05 versus time 0 or control without H2O2.
of cellular ROS (H₂O₂) suggest that ROS amplify TGF-β1 signaling in renal cells.

In this study, we found that antioxidants effectively reversed TGF-β1–induced phosphorylation of Smad 2 and MAPK and the changes in E-cadherin and α-SMA expression. Antioxidants, NADPH oxidase inhibitors, and an inhibitor of mitochondrial electron transfer chain subunit I all reduced TGF-β1–induced DCF-sensitive cellular ROS, and NADPH oxidase inhibitors significantly reduced TGF-β1–induced fibronectin secretion by NRK-52E cells. Exogenous H₂O₂ increased phosphorylated MAPK and induced EMT, strongly suggesting the role of ROS-MAPK in EMT. H₂O₂ failed to induce phosphorylation of Smad 2. Inhibition of ERK, however, effectively inhibited TGF-β1–induced Smad 2 phosphorylation and H₂O₂-induced EMT, further suggesting the role of ROS-MAPK in EMT. A cross-talk between ERK and Smad has been known to exist in TGF-β1 signaling (10,25). Elevated ERK activity can enhance Smad 2 activity, and ERK inhibition reduces TGF-β1–stimulated Smad 2 phosphorylation (25). Our data are consistent with these earlier observations and provide a novel molecular signaling mechanism in which ROS-MAPK pathway mediates TGF-β1–induced EMT in renal proximal tubular epithelial cells. It seems that the effect of H₂O₂ on Smad phosphorylation varies in different cells. H₂O₂ increases Smad 2 phosphorylation in human diploid fibroblasts (44) but not in articular chondrocytes (45). In renal tubular epithelial cells, H₂O₂ failed to induce directly Smad 2 phosphorylation.
Smad have been implicated in TGF-β1-induced EMT (9,10,15,19), and our observations suggest that this could be, in part, through ROS-MAPK pathway. There is enough evidence that TGF-β signals through MAPK. A microarray-based screen of transcriptional profiles of TGF-β1-induced EMT in human keratinocytes revealed that ERK plays a role in TGF-β1-directed cell motility and disruption of adherens junctions (19). ERK activation is also required in TGF-β1-induced EMT in pancreatic cancer cells (20) and mammary epithelial cells (21). Activation of p38 MAPK is required in TGF-β1-induced EMT in mammary epithelial cells (22) and in migration and proliferation of healing corneal epithelium (23). In agreement with these observations, our data also suggest that both p38 MAPK and ERK are required in TGF-β1-induced EMT in NRK-52E cells. Both p38 MAPK inhibitor and MEK inhibitor effectively inhibited TGF-β1- and H2O2-induced EMT. Inhibition of ERK but not p38 MAPK blocked TGF-β1-induced Smad 2 phosphorylation. Our observation that antioxidants and ERK inhibitor (MEKi) prevented TGF-β1-induced Smad 2 phosphorylation although exogenous H2O2 failed to induce directly Smad 2 phosphorylation suggest that ROS-MAPK pathway is also involved in EMT through Smad pathway.

In conclusion, we demonstrated that ROS mediates TGF-β1-induced EMT in renal tubular epithelial cells directly through activation of MAPK and indirectly through ERK-directed Smad 2 phosphorylation and suggest that antioxidants and MAPK inhibitors may prevent EMT through both MAPK and Smad pathways and subsequent tubulointerstitial fibrosis.

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