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Upregulation of mitochondrial Nox4 mediates TGF-β-induced apoptosis in cultured mouse podocytes

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1Department of Physiology and Institute of Lifestyle Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea; 2Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea; and 3Department of Internal Medicine, Soonchunhyang University Cheonan Hospital, Cheonan, Korea

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DAS R, Xu S, Quan X, Nguyen TT, Kong ID, Chung CH, Lee EY, Cha SK, Park KS. Upregulation of mitochondrial Nox4 mediates TGF-β-induced apoptosis in cultured mouse podocytes. Am J Physiol Renal Physiol 306: F155–F167, 2014. First published November 20, 2013; doi:10.1152/ajprenal.00438.2013.—Injury to podocytes leads to the onset of chronic renal diseases characterized by proteinuria. Elevated transforming growth factor (TGF)-β in kidney tissue is associated with podocyte damage that ultimately results in apoptosis and detachment. We investigated the proapoptotic mechanism of TGF-β in immortalized mouse podocytes. Exogenous TGF-β1-induced podocyte apoptosis through caspase-3 activation, which was related to elevated ROS levels generated by selective upregulation of NADPH oxidase 4 (Nox4). In mouse podocytes, Nox4 was predominantly localized to mitochondria, and Nox4 upregulation by TGF-β1 markedly depolarized mitochondrial membrane potential. TGF-β1-induced ROS production and caspase activation were mitigated by an antioxidant, the Nox inhibitor diphenyleneiodonium, or small interfering RNA for Nox4. A TGF-β receptor I blocker, SB-431542, completely reversed the changes triggered by TGF-β1. Knockdown of either Smad2 or Smad3 prevented the increase of Nox4 expression, ROS generation, loss of mitochondrial membrane potential, and caspase-3 activation by TGF-β1. These results suggest that TGF-β1-induced mitochondrial Nox4 upregulation via the TGF-β receptor-Smad2/3 pathway is responsible for ROS production, mitochondrial dysfunction, and apoptosis, which may at least in part contribute to the development and progression of proteinuric glomerular diseases such as diabetic nephropathy.

podocyte; transforming growth factor-β; NADPH oxidase 4; mitochondria; apoptosis

PODOCYTES, the terminally differentiated and highly specialized visceral epithelial cells residing on the glomerular basement membrane, play an important role in glomerular filtration. Evidence shows that podocyte injury and loss leads to proteinuria, one of the early symptoms of chronic glomerular diseases (22, 37, 48). Structural changes in podocyte injury related to proteinuria include foot process effacement, hypertrophy, a reduction in slit diaphragm-associated proteins, and cell depletion from the glomerular basement membrane. Well-known mediators of podocyte damage are high glucose (14, 15), transforming growth factor (TGF)-β (38), ANG II, and advanced glycation end products (16, 55). In particular, TGF-β plays a critical pathogenic role in chronic glomerular lesions in diabetic nephropathy and progressive glomerulosclerosis (6). Accumulation of TGF-β has been documented in injured kidneys of experimental animals and in all types of human chronic kidney diseases (6). Furthermore, marked proteinuria with podocyte effacement has been induced in an adenosine-mediated TGF-β-overexpressing animal model (17). Exogenous TGF-β1 elicits remodeling of the renal basement membrane by altering the expression of matrix proteins such as collagen type IV (24) or fibronectin (21), causes epithelial to mesenchymal transdifferentiation (21, 32, 42), and induces apoptosis (38, 49, 53). Podocyte death induced by proapoptotic concentration of TGF-β1 has been classified into Smad-dependent and -independent pathways (46). The Smad-dependent pathway involves inhibition of NF-κB through Smad7 upregulation (38); on the other hand, the Smad-independent pathway is caused by the cleavage of caspases as a result of p38 MAPK activation (27, 49).

NADPH oxidase (Nox) is a major inducer of oxidative stress in different cell types. Seven different isoforms comprise the Nox family (Nox1–Nox5) and two dual oxidases (Duox) (12), among which Nox1, Nox2, and Nox4 have been reported to be expressed in the kidney cortex (3). The most abundant isoform of Nox in renal tissue is Nox4, also known as Renox (41). Nox4 in podocytes plays multiple roles in the presence of different stimuli. Insulin signaling, which accelerates Nox4-mediated surface expression of transient receptor potential cation channel 6 (TRPC6), is essential for normal podocyte function (28), whereas glucose-stimulated Nox4 activation induces apoptosis in cultured mouse podocytes as well as in diabetic mouse models (14, 15). Several lines of evidence have shown that TGF-β-induced superoxide production via Nox4 plays a crucial role in fibrosis and/or apoptosis of lung (10, 25), liver (9), and kidney tissue (5). Until now, however, activation of Nox4 and ROS generation induced by TGF-β has not been documented in podocytes.

Early studies have suggested the localization of Nox2 protein at the plasma membrane and in phagocytic granules (18). Interestingly, Nox4 in cardiac myocytes is primarily expressed in mitochondria, and upregulation of Nox4 induced by hypertrophic stimuli elicits mitochondrial dysfunction and cardiac failure (2). In breast or ovarian tumor cells, mitochondrial Nox4 contributes to oncogenesis (19). In vascular endothelial cells, however, Nox4 is expressed in the endoplasmic reticu-
lum (ER) and plays a specific role in redox-mediated ER signaling (47, 50). In this study, we observed from immortalized mouse podocyte cultures that Nox4 is predominantly expressed in mitochondria. Application of TGF-β1 increases the expression and activity of Nox4 in mitochondria, which burdens the oxidative stress, leading to mitochondrial depolarization and apoptosis in podocytes. These results are consistent with a previous report (44) showing that TGF-β suppresses mitochondrial respiratory chain activity, which is critical for maintaining metabolism and energy homeostasis in podocytes (1). We also demonstrated that TGF-β-induced caspase activation is the consequence of Smad2/3-mediated Nox4 upregulation, which is independent of Smad7 or MAPK. Inhibition of Nox4 or Smad2/3 ameliorates TGF-β-induced oxidative stress and apoptosis in cultured mouse podocytes.

MATERIALS AND METHODS

Cell culture. An immortalized mouse podocyte cell line was a kind gift from Prof. Peter Mundel (Harvard Medical School, Charlestown, MA), and this line was cultured as previously described (15). Cells were grown as a monolayer on petri dishes coated with collagen type I (catalog no. A10483-01, Life Technologies, Grand Island, NY). Cells were grown in low-glucose DMEM (catalog no. 11885-084, GIBCO, Carlsbad, CA) supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at two different temperatures that either promoted proliferation or differentiation. At 33°C, cells are allowed to proliferate (permissive condition) in the presence of 20% FBS and allowed to differentiate (nonpermissive condition). Podocytes were thermoshifted to 37°C in the absence of interferon-γ for 14 days. Before the application of TGF-β1 (catalog no. 240-B, R&D Systems), cells were maintained in a serum-deprived condition (0.25% FBS) for 24 h.

Immunocytochemistry. At 37°C without interferon-γ, podocytes on 18-mm coverslips were grown for 14 days. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at 37°C. Next, cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min and blocked with 1% BSA in PBS for 30 min. Incubation with specific antibodies for Nox4 (5 µg/ml, catalog no. ab60940, Abcam, Cambridge, UK), mitochondrial complex IV subunit I (Cox I; 5 µg/ml, catalog no. 459600, Invitrogen, Camarillo, CA), and synaptopodin (1:100 dilution, catalog no. 03-65194, Invitrogen, Eugene, OR) for 1 h at room temperature in a dark room. Cells were washed and counterstained with 1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI) for 5 min and mounted on a glass slide. Fluorescence images were obtained using a laser scanning confocal microscope (TCS SPE, Leica Microsystems, Wetzlar, Germany).

Quantitative real-time PCR. Total RNA was isolated from cultured podocytes using the RNeasy kit (catalog no. 74134, Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 µg of total RNA with a reverse transcription kit (Applied Bioscience, Foster City, CA) using oligo-dt in a reaction volume of 20 µl according to the manufacturer’s protocol. Real-time PCR was performed to measure mRNA levels of synaptopodin, Nox1, Nox2, Nox4, cytochrome P-450 (CYP)4A, Smad2, Smad3, and Smad7 using sequence-specific primers (Table 1). GAPDH and β-actin were used as reference controls. For the analysis of each gene expression, experiments were conducted in triplicate in a real-time PCR system (7900HT, Applied Bioscience) using SYBR Green PCR Master Mix (catalog no. 204143, Qiagen). Data were analyzed following the ∆∆Ct method (where Ct is threshold cycle) (33).

Detection of condensed and apoptotic nuclei. Cells were grown on collagen-coated coverslips for 14 days. After 48 h of TGF-β1 treatment, cells were carefully washed three times with PBS and fixed using 4% paraformaldehyde in PBS for 15 min at 37°C. Cells were stained with DAPI (1 µg/ml, 5 min) for nuclear staining. Condensed and apoptotic nuclei were counted under an epi-fluorescence microscope (excitation at 358 nm and emission at 461 nm) and expressed as a percentage of total DAPI-stained nuclei.

TUNEL assay. Podocytes were seeded on a four-chambered glass slide (catalog no. 354114, BD Falcon, Bedford, MA) and allowed to differentiate for 14 days. Cells were treated with 0.25% FBS containing media before the application of TGF-β1 and inhibitors. TUNEL was performed using an ApopTag Fluorescein In Situ Apoptosis Detection Kit as per the manufacturer’s instructions (catalog no. S7110, Millipore, Billerica, MA).

Western blot analysis. For total protein extracted from cultured podocytes, cells were washed with ice-cold PBS and lysed with cold RIPA buffer (Thermo Scientific, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and supernatants were collected and used for Western blot analysis. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL), and equal amount of proteins were loaded for SDS-PAGE and electrophoresis before the application of TGF-β1 and inhibitors. TUNEL was performed using an ApopTag Fluorescein In Situ Apoptosis Detection Kit as per the manufacturer’s instructions (catalog no. S7110, Millipore, Billerica, MA).

Table 1. Sequence-specific primer pairs used for real-time PCR

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Nox, NADPH oxidase; CYP, cytochrome P-450.

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alog nos. 9523 and 9520, Cell Signaling Technology) were prepared in 0.1% Tris-buffered saline and Tween 20 (TBST) containing 5% BSA. β-Actin (1:5,000 dilution, catalog no. ab6276, Abcam) was used as a loading control. Horseradish peroxidase-conjugated secondary antibody against either mouse or rabbit IgG (catalog nos. 31450 and 31460, Thermo Scientific) was incubated for 1 h at room temperature. Bands were visualized with a UVP Biospectrum-600 imaging system using enhanced chemiluminescence solution (Luminata Forte, Millipore).

**Isolation of mitochondria.** To isolate mitochondria, podocytes were grown on 100-mm dishes for 14 days. Cells were harvested by trypsinization and fractionated into mitochondrial and cytosolic fractions using a commercial mitochondria isolation kit (catalog no. ab110171, Abcam) according to the manufacturer’s instructions.

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**Fig. 1.** Transforming growth factor (TGF-β1)-induced podocyte apoptosis through ROS generation. **A:** immunofluorescence detection of fragmented and condensed nuclei (indicated by white arrow) after nuclear staining using 4′,6′-diamidino-2-phenylindole (DAPI). **B:** histogram showing percentage of apoptotic cells per DAPI-stained nuclei. **C:** representative Western blots of cleaved caspase-3 with different concentrations of TGF-β1. **D:** representative images of TUNEL-positive nuclei in the control group and TGF-β1-treated group (indicated by white arrows). **E:** representative images of ROS measurements in podocytes loaded with 2,7-dichlorofluorescein (DCF) dye. **F:** histogram analysis of the results shown in **E** for the comparison of average DCF intensities among different groups. **G:** Western blots showing the effect of N-acetylcysteine (NAC) on TGF-β1-induced caspase-3 cleavage. **H:** histogram showing podocyte cell death detected by TUNEL assays in the presence and/or absence of TGF-β1, Z-VAD (OMe)-FMK (zVAD), and NAC. All values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001.
Measurement of ROS. Intracellular ROS generation was detected by loading cultured podocytes with chloromethyl-7-di chloride fluorescein diacetate (CM-H2DCF-DA) (catalog no. C6827, Molecular Probes). In the presence of intracellular ROS, this dye is rapidly oxidized to 2,7-dichlorofluorescein (DCF), which is highly fluorescent. In brief, cultured podocytes, with or without TGF-β1, and with the inhibitors mentioned, were treated with 5 μM CM-H2DCF-DA for 20 min at 37°C and washed out using Krebs-Ringer bicarbonate (KRB) solution (135 mM NaCl, 3.6 mM KCl, 2 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, and 10 mM HEPES; pH 7.4). Fluorescence images (excitation/emission: 490/535 nm) were obtained using an inverted microscopy (IX81, Olympus, Tokyo, Japan) with a confocal spinning disk (CSU10, Yokogawa Electric, Tokyo, Japan), and intensity was analyzed using Metamorph 6.1 software (Molecular Devices, Sunnyvale, CA).

Detection of mitochondrial membrane potential. Mitochondrial membrane potential (∆Ψm) in podocytes was measured using the lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; catalog no. T3168, Molecular Probes). JC-1 monomers enter into mitochondria based on ∆Ψm and form J-aggregates inside the mitochondria, where they transmit red fluorescence (excitation/emission: 540/590 nm), in contrast to monomers that remain outside mitochondria, which transmit green fluorescence (excitation/emission: 490/535 nm). The ratio of red-to-green fluorescence was used as an indicator of ∆Ψm. For this experiment, cells were grown on 96-well microplates (catalog no. 3603, Corning, Corning, NY). Cells were washed twice with warm KRB solution after being loaded with JC-1 (300 nM) for 30 min, and fluorescence intensities were read by a fluorescence microplate reader (Flexstation II, Molecular Devices) as previously described (36).

Nox activity measurement. Total Nox activity was measured as previously described (15) with minor modifications. Podocytes were washed twice with warm PBS and scraped from the dishes with PBS containing protease inhibitor after centrifugation to 2,000 rpm for 3 min at 4°C. Cell pellets were dissolved in lysis buffer (20 mM KH2PO4, 1 mM EGTA, and protease inhibitor cocktail; pH 7.0). Cell suspensions were homogenized with 100 strokes in a Dounce homogenizer, and homogenates (50 μm) were added to 50 mM phosphate buffer (pH 7.0) including 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. To check mitochondrial Nox activity, 30 μg mitochondria (equivalent to mitochondrial protein) was considered. The reaction generated photon particles that were detected by a luminometer (Synergy 2, BioTek Instruments, Winooski, VT) every 20 s for 10 min continuously, and this was expressed as relative light units. The blank buffer reading was subtracted from each test reading, and superoxide generation was expressed as relative light units per minute per milligram of protein.

Small interfering RNA transfection. SiGENOME Smartpool small interfering (si)RNA duplexes for Nox4, Smad2, and Smad3 were purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO). siRNAs for Smad7 and nontargeting sequences (control siRNA) were synthesized from Bioneer (Daejeon, Korea). The sequences for Smad7 and nontargeting siRNA were 5′-GAGGCTGTGTTGCTGT-GAA-3′ and 5′-CCUACGCCACCAUUUCGU-3′, respectively. Podocytes were transfected with 50 nM siRNA mixed with DharmaFECT 1 siRNA transfection reagent (catalog no. T-2001-03, Thermo Fisher Scientific). Knockdown of genes was assessed at 48 h by determining mRNA levels using quantitative PCR. To assess protein levels, Western blot analysis was performed at 60 h after siRNA transfection. Functional assays for siRNA-mediated gene silencing were performed at 72 h after siRNA treatment.

Infection of adenovirus and transfection of plasmids. Doxycycline-inducible adenovirus encoding mitochondria-targeted cyan fluorescence protein (mitoCFP) was constructed using the Adeno-X Tet-On expression system (Clontech Laboratories, Mountain View, CA). Adenoviruses encoding mitoCFP were applied together with adenovirus expressing the reverse tetracycline transactivator for 90–120 min at 37°C, as previously described (36), and used after 48 h for experiments. D1ER plasmid was transfected into podocytes using FuGENE HD Transfection Reagent (catalog no. E231A, Promega, Madison, WI) according to the manufacturer’s instructions. Experiments were conducted 48 h after transfection. Fluorescence images for mitoCFP and D1ER (excitation/emission: 440/490 nm) were obtained using a laser scanning confocal microscope (TCS SPE, Leica Microsystems).

Statistical analysis. Experimental values are presented as means ± SE; n is the number of independent experiments. Statistical comparisons were made by Student’s t-test or one-way ANOVA, and P values of <0.05 were considered to be significant.

RESULTS

TGF-β1 induces podocyte apoptosis through ROS generation. We demonstrated the expression of synaptopodin by immuno-cytochemistry using a synaptopodin-specific monoclonal antibody, which is used as a specific marker for podocyte differentiation (43). Podocytes at 37°C but not at 33°C with inter-
feron-γ showed a distinct synaptopodin staining pattern that colocalized with actin fibers (data not shown). The transcriptional level of synaptopodin was analyzed by real-time PCR from podocytes cultured in the nonpermissive condition (37°C) at days 1, 3, 7, and 14. The synaptopodin mRNA level gradually increased from day 1 to day 14 in culture, whereas it was not detected from podocytes maintained in the permissive condition (33°C). Using differentiated podocytes, we performed a dose-dependent experiment where cells were treated with different concentration of TGF-β1. Apoptotic cells were estimated using nuclear staining with DAPI. Western blot analysis for cleaved caspase-3, and TUNEL assay. Compared with the control group, TGF-β1 stimulation greatly increased the proportion of condensed and fragmented nuclei and TUNEL-positive nuclei (indicated by the white arrows in Fig. 1, A and D). Numbers of apoptotic nuclei as assessed by DAPI staining showed that 5 ng/ml TGF-β1 induced the maximum apoptotic response (18.1 ± 0.9%, n = 5) compared with 10 ng/ml TGF-β1 (15.5 ± 0.7%, n = 6) in our culture condition (Fig. 1B). TGF-β1 also increased activation of caspase-3 maximally at 5 ng/ml (Fig. 1C). Thus, all subsequent experiments were carried out using 5 ng/ml TGF-β1 unless otherwise mentioned. To check whether cleaved caspase-3 activation induced podocyte cell death by TGF-β1 treatment, we performed the TUNEL assay in the presence of the pan caspase inhibitor Z-VAD (OMe)-FMK (z-VAD) (Millipore). Z-VAD (OMe)-FMK treatment began 1 h before TGF-β1 treatment and reduced podocyte cell death by 65.15% (n = 3, P < 0.05; Fig. 1H). To investigate the mechanism of podocyte apoptosis, we measured the generation of ROS by TGF-β1. Cells exposed to TGF-β1 for 24 h significantly increased the DCF fluorescent intensity compared with the untreated group (Fig. 1, E and F). Preincubation with N-acetylcysteine (NAC) scavenged TGF-β1-mediated ROS generation by 60.7% (n = 3, P < 0.05; Fig. 1F). NAC also prevented TGF-β1-stimulated caspase-3 activation, as demonstrated by Western blot analysis (Fig. 1G), and reduced TUNEL-positive nuclei (67.78% reduction of the Δincrease, n = 3, P < 0.05; Fig. 1H).

**TGF-β1 elevates Nox4 expression and total Nox activity.** To identify the possible ROS-generating enzyme, semiquantitative PCR was carried out for Nox1, Nox2, Nox4, and CYP4A in control and TGF-β1-treated podocytes (Fig. 2A). In our study, expression of Nox1 was not detected in differentiated podocytes. TGF-β1 application reduced the mRNA levels of Nox2 and CYP4A, whereas only Nox4 mRNA was increased by TGF-β1. To confirm the semiquantitative PCR data, real-time PCR was performed for Nox2, Nox4, and CYP4A. There was a pronounced reduction in Nox2 (0.13 ± 0.09, n = 3) and CYP4A (0.03 ± 0.08, n = 3) expression, whereas the Nox4 mRNA level increased after TGF-β1 treatment (5.66 ± 0.33, 1.08 ± 0.32). Western blot analysis showed that Nox4 expression progressively increased with treatment and was highest at 1 ng/ml of TGF-β1. (Fig. 2B). To identify the possible ROS-generating enzyme, semiquantitative PCR was done for Nox1, Nox2, Nox4, and CYP4A. There was a pronounced reduction in Nox2 (0.13 ± 0.09, n = 3) and CYP4A (0.03 ± 0.08, n = 3) expression, whereas the Nox4 mRNA level increased after TGF-β1 treatment (5.66 ± 0.33, 1.08 ± 0.32).

**Fig. 3.** Nox4 is predominantly localized to mitochondria in mouse podocytes. A: mitochondria in podocytes labeled with mitochondria-targeted cyan fluorescence protein (mitoCFP; left), rabbit isotype control IgG (middle), and the merged image (right). C: podocytes infected with mitoCFP adenovirus (left), labeled with Nox4 antibody (middle), and the merged image (right). E: podocytes stained with endogenous mitochondrial protein mitochondrial complex IV subunit I (Cox1; left), Nox4 (middle), and the merged image (right). G: mouse podocytes transfected with D1ER plasmid (left), Nox4 (middle), and the merged image (right).

**A** and **D**: relative fluorescence intensity plots along the line in the merged images in A, C, E, and G, respectively. **I**: the Nox4 protein level was checked in mitochondrial and cytosolic fractions using Western blots. Antibodies against complex I and β-actin were used to check the purity of the mitochondrial and cytosolic fractions, respectively. **J**: Western blots showing the increase in the mitochondrial Nox4 protein level by TGF-β1 stimulation.
Expression of Nox4 was increased time dependently during 48 h of TGF-β1 incubation (0.46 ± 0.25, n = 4 at 6 h, 1.62 ± 1.11, n = 4 at 12 h, 5.66 ± 0.33, n = 10 at 24 h, and 9.45 ± 3.51, n = 4 at 48 h). Consistent with the elevation of Nox4 transcripts, the Nox4 protein level was also increased by TGF-β1 (1.68 ± 0.11, n = 3; Fig. 2, E and F). In addition, we observed that total Nox activity was also significantly augmented after TGF-β1 application (1.83 ± 0.12-fold increase, n = 3; Fig. 2G).

Nox4 is predominantly localized to mitochondria in podocytes. A recent study (30) has shown that Nox4 is localized to mitochondria and that Nox4-induced ROS production is responsible for mitochondrial dysfunction. In other cell types, however, Nox4 is present in the ER, where it plays an important role in cellular signaling (50). We examined the intracellular distribution of Nox4 protein in podocytes by demonstrating the colocalization with other proteins expressed in mitochondria or the ER. To confirm the colocalization, the relative intensities of the two fluorescent signals were plotted along a line drawn on the merged images. Colocalization of the two proteins was determined to be where the peaks of the two different intensities overlapped. mitoCFP was visualized in differentiated podocytes by an adenovirus-mediated overexpression system (Fig. 3, A and C). Immunostaining of Nox4 using rabbit polyclonal antibody clearly colocalized with mitoCFP, as indicated by the presence of multiple yellow puncta in the merged image (Fig. 3, C and D). Endogenous mitochondrial protein Cox1 labeled with mouse monoclonal Cox1 antibody colocalized with Nox4 (Fig. 3, E and F). In the presence of rabbit isotype control IgG antibody, however, no signal was detected, indicating the specificity of Nox4 antibody (Fig. 3, A and B). We also considered the possibility that Nox4 might be localized in the ER of podocytes. To address that question, the ER was labeled by transfection of D1ER fluorescent protein, which is specifically targeted to the luminal side of the ER. As shown in Fig. 3, G and H, D1ER fluorescence did not colocalize with Nox4, implying that Nox4 was primarily localized to mitochondria, not the ER. In a different approach, podocytes were fractionated into mitochondrial and cytosolic fractions, and Nox4 protein levels were checked using Western blot analysis. Antibodies against complex I and β-actin were used to check the purity of the mitochondrial and cytosolic fractions, respectively. Compared with the cytosolic fraction, the maximum amount of Nox4 was detected in the mitochondrial fraction (Fig. 3I). The protein level of Nox4 in the mitochondrial fraction was increased by TGF-β1 (64.5%, n = 3; Fig. 3J), which was consistent with changes of the total Nox4 protein level.

TGF-β1-induced oxidative stress and apoptosis are Nox4 dependent. To confirm the role of Nox4 in TGF-β1-induced apoptosis, we examined the effects of the Nox inhibitor diphenyleneiodonium (DPI) and siRNA for Nox4. Preincubation with DPI for 1 h successfully attenuated the TGF-β1-mediated cytosolic ROS increase (87.3% inhibition of the Δincrease, n = 3; Fig. 4A). We measured Nox activity from the mitochondrial fraction of podocytes and found that DPI markedly reduced basal Nox activities (86.5 ± 7.6% inhibition, n = 3; Fig. 4B). Moreover, the increased mitochondrial Nox activities mediated by TGF-β1 were completely abolished by DPI pretreatment (n = 3; Fig. 4B). Mitochondrial ROS production is known to depolarize ΔΨm, which may further deteriorate mitochondrial function and induce apoptosis (2). DPI rescued the TGF-β1-induced loss of ΔΨm (75.0% recovery, n = 5, P < 0.01; Fig. 4C). The antioxidant NAC also ameliorated mitochondrial depolarization mediated by TGF-β1 (37.7% recovery, n = 3, P < 0.01; data not shown). As a consequence, pretreatment with DPI blocked the activation of caspase-3 by TGF-β1 (7.73 ± 1.42-fold by TGF-β1, 7.6% inhibition, n = 3, P < 0.01; Fig. 4D and E). To identify the specific action of Nox4, siRNA for Nox4 was transfected into podocytes. The mRNA level of Nox4 in Nox4 siRNA-treated cells was reduced by 73.5 ± 8.9% compared with control siRNA (Fig. 5A). Knockdown of the Nox4 protein level by Nox4 siRNA was also confirmed by Western
blot analysis (55.3 ± 9.3% reduction, n = 3; Fig. 5, B and C). Specific knockdown of Nox4 in podocytes prevented the TGF-β1-mediated ROS increase (84.0% reduction of the ΔInc, n = 3, P < 0.05; Fig. 5D), ΔΨm depolarization (54% recovery, n = 4, P < 0.05), and caspase-3 activation (Fig. 5E) and significantly decreased TUNEL-positive nuclei (68.93% reduction of the ΔInc, n = 3, P < 0.05; Fig. 5F).

TGF-β receptor I-Smad2/3 signaling cascades mediate TGF-β1-induced apoptosis via Nox4 upregulation. Phosphorylation of TGF-β receptor I is an early signaling event after the binding of ligands to TGF-β receptor II. TGF-β1-mediated Nox4 upregulation was completely blocked by SB-431542, a potent TGF-β1 inhibitor (4.86 ± 0.65-fold increase by TGF-β1 only compared with 1.01 ± 0.06-fold by TGF-β1 with SB-431542, n = 4, P < 0.001; Fig. 6A). Treatment with SB-431542 also abrogated TGF-β1-stimulated ROS generation (n = 5; Fig. 6B) and prevented mitochondrial membrane depolarization (n = 5; Fig. 6C) and caspase-3 activation in the presence of TGF-β1 (Fig. 6D).

TGF-β1 is known to activate both Smad-dependent and independent apoptotic pathways in podocytes (46). We observed that both Smad2 and Smad3 phosphorylation were detected within 10 min after TGF-β1 stimulation, which reached a peak at 30 min and gradually decreased over time (Fig. 7A). To determine the involvement of Smads in the regulation of the expression of Nox4, Smad2 and Smad3 were separately knocked down using specific siRNA, and the transcriptional levels of Smad2 and Smad3 were successfully reduced by 73.34 ± 1.2% and 77.0 ± 4.58%, respectively (Fig. 7B). Knockdowns of Smad2 and Smad3 were also evaluated at the protein level, which showed 57.49 ± 2.72% and 63.63 ± 12.54% reductions, respectively (Fig. 7C). TGF-β1-induced Nox4 upregulation was clearly prevented by siRNA for either Smad2 or Smad3 (Fig. 7D). Knockdown of Smad2 and Smad3 showed >100% (n = 3) and 72.4% (n = 4) reductions of the ROS increase (Fig. 7E) and 76.2% (n = 3) and 74.2% (n = 3) recovery of the ΔΨm loss induced by TGF-β1 (Fig. 7F), respectively. Both Smad2 siRNA and Smad3 siRNA indepen-
dently reduced TGF-β1-induced podocyte apoptosis, as demonstrated by caspase-3 activation (7.35 ± 2.89-fold by control siRNA with TGF-β1 compared with 2.12 ± 1.23-fold by Smad3 siRNA with TGF-β1, n = 3; Fig. 7G) and TUNEL-positive nuclei (66.53% and 72.74% reduction of the increase by Smad2 siRNA and Smad3 siRNA, respectively, n = 3; Fig. 7H).

Neither Smad7 nor MAPK signaling participate in TGF-β1-induced Nox4 upregulation. In podocytes, Smad7 has been demonstrated to behave as a proapoptotic factor that is induced by TGF-β1 (38). We measured the transcriptional level of Smad7 using quantitative PCR, which peaked (8-fold increase) at 1 h after TGF-β1 treatment. To identify the role of Smad7 in TGF-β1-induced Nox4 upregulation, we applied siRNA for Smad7, which successfully knocked down the mRNA level of Smad7 (0.25 ± 0.08, n = 3; Fig. 8A). However, TGF-β1-induced Nox4 upregulation (6.64 ± 0.73-fold, n = 3) was not attenuated but, in fact, was increased by Smad7 siRNA (10.45 ± 2.79-fold, n = 2; Fig. 8B). Knockdown with Smad7 siRNA also did not prevent the mitochondrial depolarization elicited by TGF-β1 (n = 3; Fig. 8C).

As a Smad-independent pathway, activation of p38 MAPK or ERK has been known to mediate TGF-β-induced apoptosis in podocytes (38, 53). Phosphorylation of p38 MAPK became evident at 30 min after TGF-β1 application and started decreasing after 6 h, although it was detected until 24 h after treatment (Fig. 8D). TGF-β1-induced p38 MAPK phosphorylation was not blocked by the Nox inhibitor DPI (Fig. 8E). In addition, SB-202190, a p38 MAPK inhibitor, had no effect on Nox4 upregulation and mitochondrial depolarization induced by TGF-β1 (Fig. 8, F and G). SB-202190 also did not alleviate cytosolic ROS production by TGF-β1 (data not shown), which is consistent with it being a result of Nox4 upregulation. Upon TGF-β1 stimulation, phosphorylation of ERK1/2 MAPK also reached a peak at 30 min, when it began to slowly return to normal levels (data not shown). To clarify the signaling role of ERK1/2 phosphorylation on Nox4 upregulation, we investigated the effect of U-0126 (a MEK1/2 inhibitor, thereby inhibiting ERK1/2 activation) on TGF-β1-triggered Nox4 upregulation. However, the increase in the Nox4 mRNA level caused by TGF-β1 treatment was not blocked by U-0126 (data not shown), which indicates that neither p38 MAPK nor ERK1/2 signaling triggered by TGF-β1 were involved in Nox4-mediated apoptosis in mouse podocytes.

DISCUSSION

In the present study, we demonstrated a novel molecular pathway in podocytes that involves TGF-β-induced oxidative stress and apoptosis through the upregulation of mitochondrial Nox4. We proved that the TGF-β receptor-Smad2/3 pathway exclusively mediates the transcriptional regulation of Nox4 by TGF-β1, which is not affected by Smad-independent signaling, as in the case with MAPK activation. These results are also quite different from previous reports (38, 46) in podocytes that argued for a Smad-dependent apoptotic pathway that is caspase independent and primarily mediated by Smad7.
In an effort to screen possible ROS-generating enzymes, mRNA levels of CYP4A, Nox1, Nox2, and Nox4 were determined by real-time PCR. These enzymes have been previously shown to increase ROS levels in podocytes under various conditions related to diabetes (15). Of note, Nox1 mRNA expression was not detected in our culture condition. CYP4A, one of the family members of CYP, produces 20-HETE through arachidonic acid metabolism. Eid et al. (15) has shown that high glucose increases 20-HETE production, which subsequently upregulates Nox1 and Nox4 expression and triggers ROS-induced podocyte apoptosis. Activation of mammalian target of rapamycin also increases Nox4 activity (13), whereas ROS-induced podocyte apoptosis. The role of Smad2- and Smad3-mediated signaling in Nox4 upregulation has been known that mitochondrial ROS precipitate in collapse of the mitochondrial transmembrane potential preceding cytochrome c release and caspase activation (20, 31, 56). ROS produced by mitochondrial Nox4 have been shown to induce mitochondrial dysfunction and apoptosis in cardiac myocytes (2).

To clarify the role of mitochondrial Nox4 in TGF-β1-induced podocyte apoptosis, we used the Nox inhibitor DPI and Nox4 siRNA. The flavoprotein inhibitor DPI causes inhibition by reversing TGF-β1-stimulated ROS production, mitochondrial Nox4 in podocyte is fully functional as TGF-β1 treatment increased a substantial degree of Nox activity in the mitochondrial fraction, which was almost completely suppressed by a Nox inhibitor (Fig. 4B). It has been known that mitochondrial ROS precipitate in collapse of the mitochondrial transmembrane potential preceding cytochrome c release and caspase activation (20, 31, 56). ROS produced by mitochondrial Nox4 have been shown to induce mitochondrial dysfunction and apoptosis in cardiac myocytes (2).

Several pieces of evidence suggest that Nox4 is expressed in the ER (50), mitochondria (2, 4), and plasma membrane (28), which might be cell type dependent. Block et al. (4) observed the presence of Nox4 in mitochondria of mesangial cells and podocytes. To our knowledge, the role of mitochondrial Nox4 in podocytes has not been examined in detail. Our colocalization analysis, which used mitoCFP adenosine targeted to mitochondria and the endogenous mitochondrial marker protein Cox1 of the mitochondrial electron transport chain complex IV, showed that Nox4 is localized to mitochondria. Our western blot data also supported similar findings that revealed the presence of the majority of Nox4 protein in the mitochondrial fraction (Fig. 3F). Mitochondrial Nox4 in podocyte is functional as TGF-β1 treatment increased a substantial increase of Nox activity in the mitochondrial fraction, which was almost completely suppressed by a Nox inhibitor (Fig. 4B). It has been known that mitochondrial ROS precipitate in collapse of the mitochondrial transmembrane potential preceding cytochrome c release and caspase activation (20, 31, 56). ROS produced by mitochondrial Nox4 have been shown to induce mitochondrial dysfunction and apoptosis in cardiac myocytes (2).
cells (52). Poldip2 is another regulatory cytosolic subunit for Nox4 and regulates its activity in vascular smooth muscle cells (34). Interestingly, mRNA levels of none of these Nox4 sub-units were upregulated in TGF-β1-treated podocytes (data not shown), which suggests that an increase in Nox4 protein is sufficient for ROS-mediated podocyte apoptosis. Similar to the effects of DPI, knockdown of Nox4 using siRNA protected podocytes from TGF-β1-mediated ROS generation and activation of caspase-3 (Fig. 5). Thus, pharmacological and genetic inhibition clearly demonstrate the importance of Nox4 upregulation in podocyte apoptosis upon TGF-β1 stimulation.

Even though DPI markedly reduce Nox-induced superoxide production in the mitochondrial fraction (Fig. 4B), it produced only a small reduction of DCF intensity in control cells (Fig. 4A). A similar effect of DPI has been reported by Parinandi et al. (35), who showed that DPI completely inhibited Nox activity in human lung endothelial cells, whereas the intracellular ROS level measured by DCF-DA was not significantly altered under the same condition. It is conceivable that the necessity of maintaining a basal ROS level is indispensable for cell survival, which may be compensated by other ROS-producing systems in the absence of Nox-derived ROS. Needless to say, apart from Noxs, other ROS-generating enzymes exist in cells, including xanthine oxidase, proteins of the mitochondrial electron transport chain, cyclooxygenase, and CYP. Our observations show that inhibition of Nox activity by DPI in the absence of TGF-β1 has a very minimal effect on ∆Ψm. This is consistent with the finding that the total ROS level was not greatly affected by eliminating basal Nox activities.

A previous report (44) has shown that TGF-β1 induces mitochondrial dysfunction in podocytes by reducing citrate synthase activity and mitochondrial complex IV and V activity and by increasing total NADH content. Simultaneously, it has been observed that Nox4 in the mitochondrial fraction can use NADH more efficiently than NADPH to produce superoxide (2). Here, we also speculate that elevated NADH by TGF-β1 treatment would be a better substrate for superoxide production by Nox4 in mitochondria. Oxidative stress in mitochondria can trigger leakage of electrons from mitochondria and accelerate further oxidative stress, causing “ROS-induced ROS release.” This vicious cycle may thereby promote mitochondrial depolarization, oxidation modification of mitochondrial permeability transition pore components, release of cytochrome c, activation of caspase-3, and apoptosis (23, 56).

The multifunctional cytokine TGF-β acts through its binding with the membrane-bound TGF-β receptor. Upon ligand stimulation, TGF-β receptor types I and II form heteromeric...
complexes and phosphorylate Smads through the canonical pathway (46, 49). Phosphorylated Smad2 and Smad3 bind to Smad4, and this complex translocates from the cytosol to nucleus to act as a transcription factor. In addition to the Smad-dependent pathway, TGF-β activates TGF-β-activated kinase 1, phosphorylation of which activates p38 MAPK, ERK1/2, and JNK by the Smad-independent pathway (29). Interestingly, a TGF-β receptor type I kinase inhibitor, SB-431542, does not block Smad-independent TGF-β-activated kinase 1 activation but completely abrogates Smad2/3 phosphorylation (29). In our study, SB-431542 completely reverses TGF-β1-induced ΔΨm (Fig. 6), further emphasizing the importance of Smad during TGF-β1-induced apoptosis. It is noteworthy that the Smad2/3 double-knockout podocytes are resistant to TGF-β1-induced cell death (51). However, the molecular mechanism seems unclear, as Smad3−/− podocyte clones show reduced p38 MAPK activation (49) even though p38 MAPK-induced apoptosis is Smad independent (38).

Previous studies have shown that the Smad3-Nox4 axis is involved in renal myofibroblast activation (5) and the epithelial to mesenchymal transition and migration of breast epithelial cells (7). On the other hand, Smad2 phosphorylation is responsible for Nox4-mediated epithelial cell death during the development of lung fibrosis (10). Moreover, evidence shows that both Smad2 and Smad3 can also regulate the expression of a single gene, for example, STAT5 in mammary epithelial cells (11). In this study using siRNAs for Smad2 and Smad3, we clearly demonstrated that Smad2 and Smad3 are responsible for TGF-β1-induced Nox4 expression, ROS generation, mitochondrial membrane depolarization, and cleavage of caspase-3 (Fig. 7). Thus, contrary to previous findings (38, 40), this mode of apoptosis by Smads follows a caspase-dependent mechanism that is activated by Nox4-induced oxidative stress. Moreover, Carmona-Cuenca et al. (9) have shown that the rat Nox4 promoter region contains putative Smad-binding elements, implying direct regulation of Nox4 transcription by Smad2 and Smad3. We have also observed downregulation of Nox4 transcripts in Smad3 siRNA-treated cells in the presence of TGF-β1 (data not shown).

Smad7 is one of the inhibitory Smads that negatively regulates Smad2 and Smad3 signaling in podocytes. It has also been shown to be upregulated in different glomerular diseases (40) and recognized as a proapoptotic factor induced by TGF-β1 (38). On the other hand, Smad6 expression is not enhanced by TGF-β stimulation in podocytes (40) and is not involved in negative regulation of Smad signaling in podocytes. These observations led us to investigate possible crosstalk between Smad7 and Nox4 and to determine if there is a functional link between these signaling molecules in the regulation of apoptotic signaling. Time kinetic mRNA analysis revealed that TGF-β1 stimulates Smad7 mRNA expression earlier than that of Nox4. Therefore, it is possible that Smad7 could positively or negatively control the transcription of Nox4. We expected either one of these two outcomes from siRNA-mediated Smad7 knockdown experiments. First, if Nox4 is a downstream target of Smad7, knockdown of Smad7 would reduce the expression and functional activity of Nox4. Second, if Nox4 is regulated by Smad2/Smad3, knockdown of Smad7 might increase the expression and activity of Nox4. As expected according to our hypothesis that Nox4 transcription is under the control of Smad2 and Smad3, knockdown of Smad7 unmasked Nox4 upregulation with TGF-β1 stimulation (Fig. 8B). This observation suggests that the apoptotic pathway mediated by TGF-β1-Smad2/3-Nox4 cascade is independent of Smad7.

Apart from canonical Smad signaling, TGF-β1 can trigger many other noncanonical pathways simultaneously, including phosphatidylinositol 3-kinase/Akt pathways and MAPKs (54). It has been previously reported that TGF-β1 phosphorylates p38 MAPK, which, in turn, increases the expression of proapoptotic Bax protein in podocytes (38). Bax translocation to mitochondria releases cytochrome c, which activates cleaved caspase-3, resulting in apoptosis. We observed that TGF-β1 activates p38 MAPK as early as 30 min and that the activation is sustained for 24 h. Because p38 MAPK activation could be caused by oxidative stress (8, 26, 41), we investigated the possibility of p38 MAPK activation by Nox4-induced ROS. Our results show that the Nox inhibitor DPI has no inhibitory effect on p38 MAPK phosphorylation (Fig. 8E). p38 MAPK is also a multifunctional protein kinase that regulates gene expression. In podocytes, the time kinetics of p38 MAPK activation by TGF-β1 revealed that its phosphorylation occurs earlier than the upregulation of Nox4 mRNA under the same stimulus. We checked the possibility of Nox4 upregulation by p38 MAPK. We found that p38 MAPK inhibition does not block TGF-β1-induced Nox4 upregulation, ROS generation (data not shown), and mitochondrial depolarization, indicating that p38 MAPK has no impact on Nox4-mediated ROS production in podocytes (Fig. 8, F and G). Yu et al. (53) have shown that the Smad3-ERK-NF-κB axis mediates TGF-β1-induced podocyte apoptosis through membrane translocation of TRPC6. They clearly demonstrated sequential activation of ERK1/2 after Smad3 activation, which could possibly involve ERK1/2-mediated Rel/p65 translocation to the nucleus. However, this process of ERK activation by Smad3 is not well understood due to the lack of inhibitory studies on Smad3. In contrast, TGF-β1 stimulates rapid ERK1/2 phosphorylation in a Smad-independent manner in mouse podocytes (39, 51). In this study, inhibition of ERK did not affect TGF-β1-stimulated apoptosis in podocytes. This observation suggests that the apoptotic pathway mediated by TGF-β1-Smad2/3-Nox4 cascade is independent of Smad7.

The proposed mechanism of TGF-β1-induced podocyte apoptosis is shown in Fig. 9.

![Fig. 9. Proposed mechanism of TGF-β1-induced podocyte apoptosis.](http://ajprenal.physiology.org/DownloadedFrom/10.1152/ajprenal.00438.2013)
Nox4 upregulation, indicating that Nox4-induced apoptosis may follow an ERK1/2-independent pathway.

Using a diabetic mouse model, Eid et al. (14, 15) demonstrated that podocytes undergo foot process effacement, glomerular basement membrane thickening, and proteinuria through Nox4-induced oxidative stress, while Susztak et al. (45) determined that high glucose-induced podocyte apoptosis is mediated by increased ROS. Although the magnitude of apoptotic podocytes was not very high in these cases, the evidence reflected statistically significant increases of apoptosis in diabetic animal models. In another study with albumin/TGF-β transgenic mice, Schiffer et al. (38) proved that TUNEL-positive apoptotic podocytes increased in TGF-β1 transgenic mice compared with wild-type mice and that 2-wk-old transgenic mice showed significantly higher rates of apoptosis compared with 5-wk-old transgenic mice. This observation indicates that age of the experimental animals should be considered when examining podocyte apoptosis in animal models. It is assumed that apoptotic podocytes might be lost with the age of disease progression and excreted out with urine, which may decrease the chance to detect apoptosis in vivo. Because of a limitation in our results using in vitro cultured podocytes, further evidence from in vivo study is required to strongly suggest the pathogenic mechanism in a diabetic glomerular injury.

Taken together, the present study demonstrates that the TGF-β receptor-Smad2/3-Nox4 axis is a pathophysiologic mechanism of TGF-β-induced podocyte apoptosis independent of Smad7 and p38 MAPK activation (Fig. 9). In particular, ROS generation through mitochondrial Nox4 might be a key inducer of mitochondrial dysfunction and apoptosis. Because TGF-β levels in kidney tissue are elevated in diabetic nephropathy and other chronic renal diseases, it is of critical importance to find possible targets of therapeutic or preventive treatment within the apoptotic cascade triggered by TGF-β1. The pathogenic consequence of Nox4-generated oxidative stress is not restricted to podocyte depletion but is also significant in causing defective glomerular basement membranes, foot process abnormalities, and fibrotic sclerosis, all of which play a significant role in the development and progression of chronic proteinuric glomerular diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


24. Iglesias-de la Cruz MC, Ziyadeh FN, Isomo N, Kouahou M, Han DC, Kalluri R, Mundel P, Chen S. Effects of high glucose and TGF-β1 on the
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