Suppression of Par-4 Protects Human Renal Proximal Tubule Cells from Apoptosis Induced by Oxidative Stress

Bin Sun \textsuperscript{a} Chao Lu \textsuperscript{b} Guo-Ping Zhou \textsuperscript{b} Chang-Ying Xing \textsuperscript{a}

\textsuperscript{a}Division of Nephrology, Department of Internal Medicine, and \textsuperscript{b}Division of Paediatrics, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

**Key Words**
Apoptosis \cdot Human renal proximal tubule cells \cdot Oxidative stress \cdot PI3K/Akt signal transduction \cdot Prostate apoptosis response factor-4 \cdot siRNA experiments

**Abstract**

\textbf{Background:} Oxidative stress is an important inducer of cell apoptosis and plays a key role in the development of renal inflammation. The prostate apoptosis response factor-4 (Par-4) gene was originally identified in prostate cells undergoing apoptosis. Subsequently, Par-4 was found to possess potent pro-apoptotic activity in various cellular systems. However, it remains unclear whether Par-4 is involved in oxidant injury of renal tubular epithelial cells. **Aims:** To determine the role of Par-4 in renal proximal tubular cell apoptosis induced by oxidative stress. **Methods:** Par-4 gene expression was silenced by small interfering RNA. Renal proximal tubular cells were then exposed to hydrogen peroxide and the effect of Par-4 silencing on apoptosis and expression of phosphorylated Akt and vascular endothelial growth factor was determined. **Results:** Hydrogen peroxide induced apoptosis and increased Par-4 expression in human renal proximal tubular epithelial cells. Par-4 silencing significantly protected renal proximal tubular cells from apoptosis via activating the PI3K/Akt signaling pathway as Akt phosphorylation was enhanced. Par-4 silencing also ameliorated the downregulation of vascular endothelial growth factor expression induced by oxidative stress. **Conclusion:** Par-4 gene silencing resulted in PI3K/Akt signaling-dependent inhibition of renal proximal tubular cell apoptosis following oxidative stress.

**Introduction**

Oxidative stress plays an important role in the pathophysiology of various forms of renal disease. The resulting reactive oxygen species (ROS) have been shown to mediate renal injury in toxic, metabolic, ischemic and inflammatory renal diseases [1–3]. The proximal tubules are a primary target of injury in various renal diseases potentially mediated by oxidant injury [4–9]. For example, ischemia-reperfusion injury, a common cause of acute renal failure, is characterized by the excessive production of ROS and consequent tubular cell apoptosis and necrosis [10–12]. Despite much experimental and clinical study, the mechanisms involved in oxidative stress-induced apoptosis are largely unexplored. The identification of novel regulatory mediators of oxidative stress-induced apoptosis may lead to new therapies for protecting renal proximal tubular cells against oxidative stress.
Prostate apoptosis response factor-4 (Par-4) was initially identified in human prostate cell lines. Subsequently, Par-4 was found to possess potent pro-apoptotic activity in various cellular systems in response to numerous stimuli. The Par-4 gene maps to chromosome 12q21, a region frequently deleted in malignant tumors, and encodes a 38-kDa protein. Par-4 contains a leucine zipper domain in the carboxyterminal region that interacts with a variety of proteins, including the atypical protein kinases (aPKCs), PKCζ, and PKCl/I. Par-4 protein localizes predominantly to the nucleus but is also present in the cytoplasm. However, nuclear entry of Par-4 was essential for its pro-apoptotic activity [13–17]. Recently, Lee et al. [18] demonstrated that the ectopic expression of Par-4 sensitized human renal cancer cells to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). However, it remains unclear whether Par-4 is involved in the apoptosis of renal tubular epithelial cells induced by oxidative injury.

The serine/threonine kinase Akt is a critical component of the intracellular signaling pathway that supports cell survival and inhibits apoptosis [19–21]. The non-phosphorylated form of Akt is virtually inactive, and phosphorylation at Ser-473 generates active pAKT [19–22]. Vascular endothelial growth factor (VEGF) is a critical mediator of angiogenesis [20, 21] and plays a role in the pathogenesis of diabetic retinopathy, nephropathy, and vascular disease [23, 24]. VEGF is both an autocrine and paracrine factor and VEGF expression is increased in the kidneys of patients with type 1 [25] and type 2 diabetes [26]. In this context, increased VEGF expression is involved in glomerular and tubular hypertrophy, proteinuria and glomerular hyperfiltration [23, 24]. Activation of the PI3K/Akt pathway increases transcriptional activation of the VEGF promoter via the transcription factor Sp1 [27]. However, little is known about the effect of oxidative stress on the regulation of VEGF expression in human renal tubular epithelial cells.

In the present study, we provide evidence that the silencing of Par-4 gene expression promotes PI3K/Akt signal transduction and protects renal proximal tubular cells from apoptosis induced by oxidative stress.

Materials and Methods

Chemicals and Reagents

Fetal bovine serum and Dulbecco’s modified Eagle’s medium were obtained from GibcoBRL (Gaithersburg, Md., USA). Ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, Mo., USA). Caspase-3 assay kits and the caspase-3 inhibitor were purchased from Calbiochem (Cambridge, Mass., USA). Antibodies against Par-4, Akt, phosphorylated Akt (Ser-473) and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Lipofectamine was purchased from Invitrogen Life Technologies Inc. (Carlsbad, Calif., USA).

Cell Culture

Commercially available human proximal tubular epithelial cells prepared from human kidney biopsies were purchased from Cambrex Biosciences (East Rutherford, N.J., USA). Renal proximal tubular epithelial cells were grown in a renal epithelial growth medium as recommended by the manufacturer. HK-2 cells, a human renal proximal tubular epithelial cell line, were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Subconfluent cells were washed once and made quiescent by incubation in serum- and supplement-free medium for 24 or 48 h prior to experiments.

Preparation of Nuclear Extracts and Whole Cell Extracts

Cells were washed twice with ice-cold phosphate-buffered saline and collected after centrifugation at 300 g for 5 min. Cells were resuspended in ice-cold buffer containing 10 mM Hepes, pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 2 µg/ml pepstatin and kept on ice for 15 min. Cells were lysed in 0.1% Nonidet P-40 and vortexed for 10s, and the nuclear pellet was recovered after centrifugation at 13,000 g for 10 s at 4°C. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM Hepes, pH 7.8, 0.4 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 2 µg/ml pepstatin and incubated on ice for 20 min with shaking. Nuclear extracts were retained after centrifugation at 14,000 rpm for 5 min at 4°C, and the supernatant was separated into aliquots and stored at −80°C. Protein concentration was determined by the Bradford method (Bio-Rad). Whole cell extracts were prepared by sonication in Laemml sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 0.1% bromophenol blue).

Western Blot Analysis

Fifty micrograms of protein from either nuclear or whole cell extracts were loaded on precast SDS/Tris/glycine gels (Bio-Rad). After electrophoresis, proteins were transferred to nitrocellulose membranes for subsequent blotting with the appropriate primary antibody. Membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase for 1 h at room temperature. After washes in TBST (25 mM Tris, pH 8.0, 125 mM NaCl and 0.1% Tween-20), the blot was incubated in detection reagent (ECL Advance Western blotting detection kit) and exposed to a Hyperfilm ECL film (Pierce). Tubulin and nuclear protein Histone H1 served as loading controls.

Small Interfering RNA-Based (siRNA) Experiments

A siRNA strategy was employed to silence Par-4 expression in renal proximal tubular cells. The experiments were performed as described previously [17]. Par-4 and scrambled control siRNAs were generated using the procedure of siSTRIKE™ U6 Hairpin Cloning Systems (Promega). The Par-4 siRNA had the following
Plasmids. Cells were then exposed to hydrogen peroxide (H$_2$O$_2$) transfected with the indicated concentrations of Par-4 siRNA

Results

H$_2$O$_2$ Induced Apoptosis and Increased Par-4 Protein Expression in Human Renal Proximal Tubular Epithelial Cells

We employed H$_2$O$_2$ to initiate intracellular oxidative stress, as H$_2$O$_2$ is involved in renal disease and is readily permeable to the plasma membrane [28]. A treatment dose of 0.75 mM H$_2$O$_2$ was chosen according to preliminary studies (data not shown) and cell apoptosis was monitored over a period of up to 24 h after H$_2$O$_2$ treatment. Obvious morphological changes were observed in renal proximal tubular cells within 2 h with cell shrinkage (the morphological hallmark of apoptosis) developing afterwards. Flow cytometric analysis using FITC-labeled annexin V to bind phosphatidylserine was employed to assess the level of apoptosis whilst cell viability was assessed by staining with fluorescent PI that binds DNA. Treatment with 0.75 mM H$_2$O$_2$ induced significant time-dependent apoptosis of renal proximal tubular cells starting 6 h after H$_2$O$_2$ treatment (fig. 1).

Western blotting was performed to determine the involvement of Par-4 in the regulation of H$_2$O$_2$-induced apoptosis in renal proximal tubule cells. As the pro-apoptotic function of Par-4 has been attributed to its nuclear translocation, Western blotting was performed on nuclear extracts. H$_2$O$_2$ induced a time-dependent increase in Par-4 expression in renal proximal tubular cells compared to control with similar results obtained in HK-2 cells (fig. 2a).

Since the activation of caspase-3 has been implicated as a common downstream effector of diverse apoptotic pathways [29], we examined caspase-3 activation in renal proximal tubule cells exposed to H$_2$O$_2$. Treatment with H$_2$O$_2$ significantly increased caspase-3 activation in both human primary renal proximal tubular epithelial cells and HK-2 cells (fig. 2b).

These data indicate that H$_2$O$_2$ induced apoptosis in human renal proximal tubular epithelial cells and that this was associated with increased Par-4 protein expression.

Par-4 Suppression Protects Renal Proximal Tubular Cells from H$_2$O$_2$-Induced Apoptosis via Activation of the PI3K/Akt Signaling Pathway

To investigate whether Par-4 suppression may protect renal proximal tubular cells from oxidative stress-induced apoptosis, we performed siRNA experiments. Renal proximal tubular cells were transfected with either Par-4-specific siRNA, control scrambled siRNA, or no

Suppression of Par-4 Protects Human RPTCs from Apoptosis

Flow Cytometric Analysis of Apoptosis

Renal proximal tubular cells were grown to the exponential phase, seeded at a density of 2 × 10$^5$ cells per 60-mm dish, and transfected with the indicated concentrations of Par-4 siRNA plasmids. Cells were then exposed to hydrogen peroxide (H$_2$O$_2$) or control medium for various periods of time. The cells were collected by trypsinization and washed in PBS. After incubation with 5 μl of Annexin-V FITC and 10 μl of PI (50 μg/ml) at room temperature for 15 min in the dark, cells were analyzed by flow cytometry using a FACS Caliber. The percentage of cells undergoing apoptosis was calculated and the result presented relative to the levels of apoptosis in controls.

Measurement of Caspase-3 Activity

Caspase-3 activity was measured by a caspase-3 fluorometric protease assay kit (MBL) following the manufacturer’s instructions. The fluorogenic synthetic peptide DEVD-7-amino-4-trifluoromethylcoumarin (AFC) was used as a substrate for caspase-3 and the fluorescence of the released AFC was measured with an excitation wavelength of 360 nm and an emission wavelength of 530 nm.

Measurement of VEGF Production in the Culture Supernatants

The VEGF levels of cell culture supernatant were determined using ELISA kits (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer’s instructions. Briefly, 200 μl of cell culture supernatants, controls or standards were added to wells previously coated with monoclonal anti-VEGF antibody. After 2 h of incubation, wells were washed and incubated with an enzyme-linked polyclonal anti-VEGF antibody. Following another wash, a substrate solution was added to wells with color development being proportional to the amount of bound VEGF. The plate was read on a Dynex plate reader with an absorbance of 450 nm. Results were calculated from the standard curve generated from several known VEGF concentrations.

Statistical Analysis

When appropriate, data were expressed as mean ± SE. Data were analyzed by factorial ANOVA and Fisher’s least significant difference test when appropriate. Statistical significance was accepted at p < 0.05.

Sense and antisense strands were annealed and ligated into the linearized psiSTRIKE Vector following the manufacturer’s directions. Sequence analysis of randomly picked transformed clones was used to confirm the sequence integrity of the Par-4 shRNA plasmids. Cells were transfected with siRNA or the indicated constructs using Lipofectamine 2000 (Invitrogen) in Opti-MEM I for 24 h, and then the medium was changed back to growth medium for additional incubation. Green fluorescent protein phMGEF vector was co-transfected to determine transfection efficiency by flow cytometry. Forty-eight hours after the transfection, total RNA was prepared using TRI Reagent according to the manufacturer’s instructions and used to perform real-time quantitative PCR analysis. The level of target RNA suppression in transfected cells was determined by normalizing for transfection efficiency.

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siRNA (mock transfection). Par-4 mRNA and protein expression was assessed by real-time quantitative PCR analysis and Western blotting respectively. Par-4-specific siRNA effectively reduced Par-4 mRNA and protein expression (fig. 3a). Control and Par-4-specific siRNA transfected renal proximal tubular cells were then exposed to 0.75 mM H₂O₂ for 24 h. Flow cytometric analysis indicated that the suppression of Par-4 expression significantly attenuated H₂O₂-induced apoptosis (fig. 3b).

Because it has been demonstrated that Par-4 deficiency leads to activation of the Akt pathway in vivo and in several cellular systems [18, 26, 27], we assessed the effects of Par-4 silencing on the PI3K/Akt signaling pathway in renal proximal tubular cells exposed to H₂O₂. Primary renal proximal tubular cells and HK-2 cells were treated with 0.75 mM H₂O₂ for between 5 min and 24 h at which point lysates were analyzed by Western blotting. H₂O₂ treatment increased Akt phosphorylation (Ser-473) that peaked at 10 min and declined to basal levels within 3 h (fig. 4a). We then sought to determine whether the anti-apoptotic effect of Par-4 suppression was related to sustained activation of the PI3K/AKT signaling pathway. Renal proximal tubular cells were transfected with Par-4-specific siRNA followed by treatment with the PI3K inhibitor LY294002 (20 μM) or medium alone for 1 h. Then, cells were exposed to 0.75 mM H₂O₂ for 10 min and Akt phosphorylation (Ser-473) detected with Western blotting. Transfection of cells with Par-4-specific siRNA resulted in increased Akt phosphorylation in H₂O₂-treated renal proximal tubular cells (fig. 4b). However, LY294002

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**Fig. 1.** H₂O₂ induces time-dependent apoptosis in human proximal tubule epithelial cells. Primary human proximal tubular epithelial cells were treated with H₂O₂ (0.75 mM) for 0 (control), 6, 12 or 24 h. Flow cytometric analysis was employed to assess the level of apoptosis. *p < 0.05 compared to control cells.
effectively blocked the upregulated expression of phosphorylated Akt induced by transfection with Par-4-specific siRNA (fig. 4b).

Collectively, these results demonstrate that the PI3K/Akt signaling pathway is a key mediator of the anti-apoptotic effects of Par-4-specific siRNA transfection of renal proximal tubular cells exposed to oxidative stress.

Par-4 Silencing Reduces the Downregulated Expression of VEGF Induced by Oxidative Stress

Previous studies had demonstrated that exogenous VEGF promoted the survival of renal tubular epithelial cells and that activation of PI3K/Akt could increase VEGF transcription [27, 30, 31]. Thus, we determined whether VEGF was involved in Par-4 modulation of primary renal proximal tubule cell apoptosis. Control primary renal proximal tubular cells and HK-2 cells expressed and secreted VEGF protein (fig. 5). Treatment with H2O2 for 24 h reduced secretion and expression of VEGF protein by both primary renal proximal tubular cells and HK-2 cells (fig. 5). Transfection with Par-4 siRNA resulted in significant inhibition of the H2O2-induced VEGF downregulation (fig. 5) with VEGF expression being comparable to control conditions. Furthermore, treatment of Par-4 siRNA-treated cells with the PI3K/Akt inhibitor LY294002 resulted in reduced VEGF expression compared to control Par-4 siRNA-treated cells. These data indicate that the effect of Par-4 silencing upon VEGF expression was at least partially dependent upon the PI3K/Akt signaling pathway (fig. 5).
Oxidative stress contributes to cell damage in a variety of kidney diseases. Renal ischemia/reperfusion injury is the most common cause of acute renal failure and is a major clinical problem with high morbidity and mortality. Renal ischemia/reperfusion injury produces abundant ROS that overwhelm the renal scavenging capacity resulting in ROS-mediated lipid peroxidation, DNA damage and protein dysfunction that lead to acute renal failure and acute tubular necrosis [1–5, 11, 12]. In this study, our results demonstrate that H$_2$O$_2$ induced apoptosis in human renal proximal tubular epithelial cells as shown by other investigators. For example, Iqbal et al. [32] showed that H$_2$O$_2$ could induce peroxidation of microsomal membrane lipids and DNA damage in human renal proximal tubular epithelial cells.

Although the pro-apoptotic protein Par-4 was initially identified as the product of a gene upregulated in prostate tumor cells undergoing apoptosis, there is growing evidence that Par-4 also regulates apoptosis in various cell lines [13–17]. We demonstrated that H$_2$O$_2$ induced a time-dependent increase of Par-4 expression in renal proximal tubular cells compared with control cells. Suppression of Par-4 expression using specific siRNA transfection greatly attenuated the level of apoptosis of renal proximal tubule cells exposed to H$_2$O$_2$ thereby implying that Par-4 was involved in oxidant injury of renal tubular epithelial cells. In view of previous work demonstrating that Par-4 expression enhanced activation of caspase family members in other cell lines [17], we evaluated caspase-3 activity and found that H$_2$O$_2$ significantly enhanced activation of caspase-3 in renal proximal tubular cells. These results indicate that Par-4 may play a critical role in renal ischemia/reperfusion injury.

**Fig. 3.** Silencing of Par-4 gene expression significantly reduces Par-4 protein expression and protects human proximal tubular epithelial cells from H$_2$O$_2$-induced apoptosis. **a** Primary proximal tubular epithelial cells were transfected with either Par-4-specific siRNA, control scrambled siRNA or no siRNA (mock transfection). Par-4-specific siRNA significantly reduced Par-4 mRNA expression levels (assessed by real-time quantitative PCR analysis) and Par-4 protein levels (assessed by Western blots of whole cell extracts). * p < 0.05 compared to control and mock-transfected cells. **b** Following transfection with either Par-4-specific siRNA, control scrambled siRNA or no siRNA (mock transfection), proximal tubular epithelial cells and HK-2 cells were treated with 0.75 mM H$_2$O$_2$ for 24 h. Flow cytometric analysis was employed to assess apoptosis. * p < 0.05 compared to control and mock-transfected primary tubular cells. * p < 0.05 compared to control and mock-transfected HK-2 cells. Results shown are representative of three independent experiments.
role in the H$_2$O$_2$-induced apoptosis of human renal proximal tubular epithelial cells.

Although oxidative stress influences multiple anti- and pro-apoptotic signaling pathways, we focused on the Akt pathway in the light of previous reports that Par-4 deficiency activates the Akt pathway in vivo and in several cellular systems [18, 33, 34]. In our study, Akt existed predominantly in an inactivated non-phosphorylated form in control renal proximal tubule cells, but was transiently activated and phosphorylated under conditions of oxidative stress. Western blot analysis showed that H$_2$O$_2$ treatment increased Akt phosphorylation (Ser-473) that peaked at 10 min and subsequently declined to basal levels within 3 h. The decline of phosphorylated Akt might contribute to the molecular mechanisms responsible for H$_2$O$_2$-induced apoptosis in renal proximal tubular epithelial cells. Furthermore, our results showed that silencing of Par-4 increased Akt phosphorylation in renal proximal tubular cells treated with H$_2$O$_2$. This effect was inhibited by the PI3K inhibitor LY294002 (20 μM) or medium alone for 1 h. Cells were then exposed to 0.75 mM H$_2$O$_2$ for 10 min and Akt phosphorylation (Ser-473) detected by Western blotting. $^*$ $p < 0.05$ compared to non-transfected primary tubular epithelial cells not treated with H$_2$O$_2$ (first bar at left). $^{\#} p < 0.05$ compared to non-transfected HK-2 cells not treated with H$_2$O$_2$ (second bar at left). $^{\#} p < 0.05$ compared to primary tubular cells transfected with Par-4 siRNA and treated with H$_2$O$_2$. $^* p < 0.05$ compared to HK-2 cells transfected with Par-4 siRNA and treated with H$_2$O$_2$.

The Par-4 protein can interact with PKCζ via the leucine zipper domain in the COOH-terminal region and it has been reported that the Par-4/PKCζ cassette is an important regulator of Akt activation. This effect most likely secondary to the ability of PKCζ to directly phosphorylate Ser-124 of Akt with a subsequent impact on the phosphorylation of residue Ser-473 that is critical for regulating Akt activity and function [35].
Acknowledgments

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Fig. 5. Par-4 silencing ameliorates the downregulation of VEGF expression induced by H$_2$O$_2$. Following transfection with Par-4-specific siRNA, primary human proximal tubular epithelial cells and HK-2 cells were treated with the PI3K inhibitor LY294002 (20 μM) or medium alone for 1 h. Cells were then exposed to 0.75 mM H$_2$O$_2$ for 24 h. a Levels of secreted VEGF in the culture supernatant were determined using ELISA kits. b Cytosolic levels of VEGF protein were detected by Western blotting with tubulin used as loading control. *p<0.05 compared to control and mock-transfected primary tubular cells. *p<0.05 compared to control and mock-transfected HK-2 cells. *p<0.05 compared to primary tubular cells transfected with Par-4-specific siRNA. *p<0.05 compared to HK-2 cells transfected with Par-4-specific siRNA. Results shown are representative of three independent experiments.

In the present study, our results demonstrate that Par-4-specific siRNA transfection significantly ameliorated the downregulation of VEGF protein expression induced by H$_2$O$_2$ treatment of renal proximal tubule cells. Moreover, PI3K/Akt inhibitor LY294002 effectively reduced VEGF expression, indicating the regulatory effects of Par-4 suppression on VEGF expression was partially PI3K/Akt signaling pathway. Consistently, previous studies had demonstrated that VEGF promoted survival of renal epithelial cells and activation of PI3K/Akt could increase VEGF transcription [27, 30, 31].

In conclusion, our results suggested that Par-4 gene silencing protected renal proximal tubule cells from oxidative stress-induced apoptosis. The anti-apoptotic effect of Par-4 suppression is partially dependent on PI3K/Akt signal transduction, which ameliorated oxidative stress-downregulated expression of VEGF. Our findings may be useful to clarify the molecular mechanisms responsible for oxidative stress associated kidney disease, which benefits the therapy as well.
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