NADPH Oxidase-derived ROS Contributes to Upregulation of TRPC6 Expression in Puromycin Aminonucleoside-induced Podocyte Injury

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Key Words

Oxidative stress • Podocyte injury • TRPC6 • Focal segmental glomerulosclerosis

Abstract

Recent studies have demonstrated upregulation of transient receptor potential cation channel 6 (TRPC6) contributes to podocyte injury in acquired forms of proteinuric kidney diseases, such as focal segmental glomerulosclerosis (FSGS). However, under these pathophysiological conditions, the mechanisms of regulation of TRPC6 expression and activity remain unknown. The present study tested the hypothesis that NADPH oxidase-mediated redox signaling importantly participates in the development of podocyte injury by regulation of TRPC6 expression and activity. Injection of puromycin aminonucleoside (PAN) to rats produced severe proteinuria and mimics the lesions of FSGS. Podocyte effacement, NADPH oxidase subunit NOX4 expression, enzyme activity and TRPC6 expression were significantly increased in glomeruli from PAN nephrosis rats. Inhibition of NADPH oxidase activity by apocynin ameliorated proteinuria and podocyte effacement and reduced TRPC6 expression. In *in vitro* study, PAN significantly

increased NOX4 and TRPC6 expression levels in cultured podocytes. This increased TRPC6 expression was attenuated by apocynin or siRNA-NOX4. Our results provide direct evidence for the first time that NADPH oxidase-derived reactive oxygen species (ROS) is one of critical components of a signal transduction pathway that links PAN nephrosis to TRPC6-mediated Ca^{2+} signaling.

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Introduction

Podocytes are specialized cells with a complex cellular morphology that reside in the kidney glomerulus and cover the outer surface of the filtering capillaries. They function as glomerular basement membrane (GBM) turnover, maintenance of the glomerular filtration barrier and regulation of glomerular filtration. Podocyte injury is now considered as a hallmark of glomerular disease, which is generally displayed by the rearrangement of the podocyte slit diaphragm and the actin cytoskeleton. Several podocyte-associated proteins such as nephrin, podocin, α -actinin 4 (ACTN4), CD2-associated protein (CD2AP) and transient receptor potential cation channel 6 (TRPC6) have emerged to provide critical insight into

the pathogenesis of hereditary nephrotic syndromes [1-4]. Mutations in these genes lead to glomerular injury and the progression to end stage renal disease (ESRD). TRPC6 is the latest discovery of podocyte associated proteins. Mutation of TRPC6 that causes hereditary FSGS [5, 6] raises questions regarding the pathophysiological role of this channel in podocytes. In addition, elevated expression levels of TRPC6 are detected in acquired glomerular diseases, such as focal segmental glomerulosclerosis (FSGS), minimal-change disease (MCD) and membranous glomerulonephritis (MGN), suggesting that TRPC6 may also play a pivotal role in nongenetic forms of glomerular disease [7]. However, under these conditions, the mechanisms by which TRPC6 expression and activity are up-regulated and consequently resulting in glomerular injury remain unknown.

It is well known that reactive oxygen species (ROS) decisively contributes to cellular signaling, affecting almost all aspects of cellular functions including gene expression, proliferation, migration and apoptosis [8]. Although multiple enzymes contribute to oxidative stress in different tissues or cells, it is general accepted that NADPH oxidase is a major source of ROS in many non-phagocytic cells [9]. It has been demonstrated the contributing role of NADPH oxidase-derived ROS on the regulation of ion channels, such as K⁺ channels [10], plasma membrane Ca²⁺ channels [11]. This regulation of ion channels may occur either directly or through ROS-sensitive signaling systems [12]. However, it keeps unknown whether TRPC6 is a redox-sensitive channel and a potential molecular target of NADPH oxidase-derived ROS for the regulation of cellular events. Considering the role of NADPH oxidase on the regulation of ion channels and renal function, the present study was designed to test the hypothesis that NADPH oxidase-mediated redox signaling importantly participates in the development of podocyte injury by regulation of TRPC6 expression and activity.

As we known, FSGS is a major cause of idiopathic steroid-resistant nephrotic syndrome (NS) and ESRD [13]. Injection of puromycin aminonucleoside (PAN) to rats produces severe proteinuria and mimics the lesions of FSGS. In particular, PAN specifically injures podocytes, leading to foot process effacement, actin cytoskeleton disorganization and abnormal distribution of slit diaphragm proteins [14]. Therefore, in the present study, we utilized PAN-induced podocyte injury as an experimental FSGS animal model to explore related mechanisms. We also treated these rats with an NADPH oxidase inhibitor, apocynin, to observe the changes of TRPC6 expression, beneficial actions on PAN-induced podocyte injury.

Moreover, our *in vitro* studies further demonstrated that NADPH oxidase-produced ROS contributes to TRPC6 expression in PAN-treated podocytes and enhanced NOX4 subunit expression is associated with PAN-induced NADPH oxidase activity. Our results provide direct evidence that NADPH oxidase-derived ROS is one of critical components of a signal transduction pathway that links puromycin aminonucleoside nephrosis to TRPC6-mediated Ca²⁺ signaling.

Materials and Methods

Animals

Male Wistar rats (Laboratory Animals Center of Shandong University, China) were given free access to standard laboratory chow. Previous studies have shown that apocynin had no effect on glomerular structure and function in normal rats [15]. Therefore, in the following mechanism studies, the rats were randomly divided into three groups: the controls, the PAN nephrosis and PAN nephrosis with NADPH oxidase inhibitor apocynin treatment group. The rats of the PAN nephrosis group were administered 80 mg/kg body weight of PAN dissolved in 1 ml of 0.9% NaCl (saline) via the tail vein to induce nephrosis. Rats that received an injection of normal bovine serum (5 ml/kg body wt) served as controls. For the prevention group, apocynin (15 mg/kg/day in drinking water) was administrated 2 days before PAN injection and continued throughout the experiment period of 14 days. All protocols were approved by Institutional Animal Care and Use Committee of Shandong University. During recording days, a 24-hour urine samples were collected. Urinary albumin excretion was measured using a rat albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). Simultaneously, rats were killed under ketamine anesthesia. Kidney tissues were processed for isolation of glomeruli, light microscopy, immunofluorescence staining, and electron microscopy. Glomeruli were prepared by a graded or series sieving as described previously [16].

Morphological examinations

The fixed kidneys were paraffin-embedded, and sections were prepared and stained with periodic acid-Schiff stain. Glomerular injury index was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion as described previously [16]. To examine the ultrastructural changes in glomeruli, the renal cortical tissue was fixed for electron microscopic examinations. Electron microscopic sample handling and detection were performed by Ultrastructure Laboratory of Shandong University School of Medicine.

Immunofluorescence Staining

Immunofluorescence staining was performed as described previously [17]. Rabbit anti-TRPC6 1: 100 (abcam, Cambridge, MA, USA) was used in this study.

Fig. 1. The effects of NADPH oxidase inhibitor apocynin on glomerular injury in PAN nephrosis rats. **A:** Urinary albumin excretion in 3 different groups of rats as indicated by the control, the PAN nephrosis and NADPH oxidase inhibitor apocynin treatment groups. **B:** Photomicrographs (original magnification x250) showing typical glomerular structure and summarized glomerular damage index (GDI) by semiquantitation of scores in 3 different groups. **C:** Apocynin ameliorated podocyte foot process effacement induced by PAN via transmission electron microscopy observation. * $P < 0.05$ compared with control; # $P < 0.05$ compared with the values obtained from PAN nephrosis rats ($n = 6$).

Cell culture

A clonal cell line of conditionally-immortalized murine podocytes was the generous gift from Peter Mundel, Department of Medicine, Mount Sinai School of Medicine, New York, USA. Briefly, cells were cultured under growth-permissive conditions on collagen type I-coated plastic dishes (BD Bioscience, Bedford, MA, USA) at 33 °C in RPMI 1640 (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen), 20 U/ml mouse recombinant interferon- γ (Sigma, St. Louis, MO, USA), and 100 U/ml penicillin plus 0.1 mg/ml streptomycin (Sigma). To induce differentiation, podocytes were maintained in nonpermissive conditions at 37 °C without interferon- γ for 7 days and used for the experiments [3]. Apocynin (100 μ M) was added to the media before 2 h for PAN treatment.

RNA interference

Small interference RNAs (siRNAs) were synthesized by QIAGEN. The DNA target sequence for siRNA-NOX4 is 5'-CAG TGA ACT ATA GTG AAC ATT TCCT-3'. The scrambled small RNA (AAT TCT CGA ACT GTC ACG T) has been confirmed as non-silencing double stranded RNA and was used as control in the present study. In these experiments, siRNA transfection was performed according to the manufacturer's instruction in Bio-Red siLentFect™ Lipid transfection kit.

Western blot analysis

Western blotting was performed as described previously [18]. Primary antibodies anti-TRPC6, anti-NOX4 (1:1000 dilution, abcam, Cambridge, MA, USA), anti-p47^{phox}, and anti-NOX2 (gp91^{phox}) (1:1000 dilution, BD Transduction Laboratories, Lexington, KY, USA) and secondly antibodies horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG (1:6000 dilution, ProteinTech Group, Chicago, IL, USA) were used this studies. To document the loading controls, the membrane was reprobred with a primary antibody against housekeeping protein β -actin.

RNA extraction and real time RT-PCR

Total RNA was isolated from glomeruli or podocytes using TRIzol reagent (GIBCO, Life Technologies, Carlsbad, CA, USA) according to the protocol described by the manufacturer. The mRNA levels for target genes were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA). The mRNA level of target genes was normalized to the 18S rRNA. The specific primers for NOX4 based on the core sequence of rat NOX4 (Accession number NM_053524): GAG CAA CAA ACC TGT CAC CAT (forward) and GCT TTG GTT TCA GTA GGA CAC A (reverse); NOX2 (Accession number NM_024160): TGT GGC TGT GAT AAG CAG GA (forward) and TGT GGC TGT GAT AAG CAG GA (reverse); TRPC6 (Accession number NM_053559): TCC CAC TAA CAT CAC CAC CT (forward) and CCC AGG AAA ATG GTG AAG GA (reverse).

Measurements of NADPH oxidase activity: NADPH oxidase activity by measurement of O_2^- production was determined in isolated glomeruli or podocyte cell homogenates. Fluorescence spectrometry for O_2^- production was performed by using a modified DHE fluorescent spectrometric assay as described previously [19].

Statistics

Data are expressed as means $\pm$ SE. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Inhibition of NADPH oxidase activity ameliorated PAN-induced podocyte injury

As shown in Figure 1A, urinary albumin excretion was significantly increased in PAN-treated rats.

Fig. 2. NADPH oxidase activity and subunit expressions in glomeruli from different groups of rats. A: NADPH oxidase activity in the glomeruli from different groups of rats. B: Immunoblot for NOX2 (gp91^{phox}), NOX4 and p47^{phox} in the homogenates from glomeruli of these rats. C: Summarized data showing changes in NOX2, NOX4 and p47^{phox}. * $P < 0.05$ compared with control; # $P < 0.05$ compared with the values obtained from PAN nephrosis rats ($n=6$).

However, apocynin markedly reduced urinary albumin excretion in PAN nephrosis rats. Morphological analysis showed the overall histological changes of glomeruli were not obviously at 14 days after PAN injection (Figure 1B). We further examined the integrity of podocyte foot processes by transmission electron microscopy. As shown in Figure 1C, foot processes were fused or effaced in PAN nephrosis rats. In apocynin-treated rats, apocynin ameliorated podocyte foot process effacement, although effaced processes were noted in some areas.

Fig. 3. Effects of apocynin on TRPC6 expression in glomeruli from PAN nephrosis rats. A: Relative quantitation of mRNA levels in TRPC6 by real-time RT-PCR analysis in glomeruli from different groups. B: Representative Western blot gel document (upper panel) and summarized data for protein levels of TRPC6 in glomeruli. C: Representative immunofluorescent micrographs for TRPC6 in glomeruli from three different groups. * $P < 0.05$ compared with control; # $P < 0.05$ compared with the values obtained from PAN nephrosis rats ($n=6$).

PAN induced NADPH oxidase activity and expression in glomeruli isolated from PAN nephrosis rats

The activity of NADPH oxidase was determined by fluorescence spectrometric assay. Summarized data showed that NADPH oxidase activity was increased by 2.9-fold in isolated glomeruli of PAN nephrosis rats compared with that of control rats. Administration of apocynin markedly attenuated PAN-induced NADPH oxidase activity by over 60% (Figure 2A). To explore the

Fig. 4. Effect of PAN on NADPH oxidase activity (A) and NOX4 mRNA levels at different time treatments. *P < 0.05 compared with control (n=6).

mechanism by which PAN-induced NADPH oxidase activity in PAN-treated rats, we examined expression levels of NADPH oxidase subunits in glomeruli. The representative functional subunits NOX2 and NOX4, two major isoforms of NOX in the kidney, and one crucial cytosolic subunit p47^{phox}, were detected. As shown in Figure 2B and 2C, NOX4 expression was significantly increased in glomeruli of PAN-treated rats. NOX2 level was shown an increase tendency, although no statistics significance. p47^{phox} expression level had no change, suggesting that NOX4 may mainly contribute to increased NADPH oxidase activity.

Blockade of enhanced TRPC6 expression in glomeruli of PAN nephrosis rats by apocynin treatment

To examine whether regulation of TRPC6 expression is associated with NADPH oxidase-mediated redox signaling pathway in PAN nephrosis rats, real time RT-PCR and Western blot analyses were performed and the results are summarized in Figure 3A and 3B, TRPC6 mRNA and protein levels in glomeruli from PAN nephrosis rats were increased by 188 and 110%, respectively, Apocynin significantly attenuated PAN-induced increases. Kidneys were further examined by immunofluorescence using a primary antibody against TRPC6 as shown in Figure 3C. In the kidneys of control rats, TRPC6 staining was detected as a dotted, linear-like pattern. In PAN

Fig. 5. Efficiency of siRNA-NOX4 on the silencing of NOX4 expression in podocytes. A: Quantitative RT-PCR analysis of NOX4 mRNA levels in control, scrambled RNA and siRNA-NOX4 transfected podocytes. B: Representative Western blot gel documents of NOX4 expression in the homogenate from transfected podocytes. C: Summarized data showing relative NOX4 protein levels in control, scrambled RNA- and siRNA-NOX4 transfected podocytes after normalized to β-actin. * P<0.05 vs. control (n=5).

nephrosis rats, TRPC6 staining was much stronger and clots were appeared. Inhibition of NADPH oxidase activity reduced PAN-induced TRPC6 expression.

Upregulation of NOX4 contributed to TRPC6 expression in PAN-treated podocyte in vitro

To further test whether the regulation of TRPC6 expression is associated with NADPH oxidase activity, we used PAN to treat conditionally-immortalized murine podocytes. It was found that PAN increased NOX4 mRNA level, NADPH oxidase activity in a time dependent manner under 100 µg/ml of PAN treatment (Figure 4). We also found that PAN-induced TRPC6 expression was inhibited by apocynin (data not shown). To further investigate the role of NOX4 on the regulation

Fig. 6. Effects of siRNA-NOX4 on the NADPH oxidase activity, NOX4 and TRPC6 expressions in PAN-treated podocytes. **A:** Summarized data depicting NADPH oxidase activity in siRNA-NOX4 transfected podocytes with PAN treatments. **B:** Representative Western blot gel documents of NOX4 and TRPC6 in the homogenate from transfected podocytes. **C:** Summarized data showing relative NOX4 and TRPC6 protein levels. * $P < 0.05$ vs. control. # $P < 0.05$ vs. vehicle of PAN treatment ($n=5$).

of TRPC6 in PAN-treated podocytes, NOX4 gene silencing was used in this study because of no specific inhibitor available for NOX4. By real-time RT-PCR and Western blot analysis, NOX4 mRNA and protein expression were decreased by 73% and 52 % in siRNA-NOX4 transfected podocytes, respectively. This siRNA-NOX4 had no effect on the NOX2 expression level, indicating the specificity of siRNA to NOX4 (Figure 5). Therefore, we determined the effect of siRNA-NOX4 on NADPH oxidase activity. It was found that PAN significantly increased NADPH oxidase activity, which was markedly blocked by siRNA-NOX4 (Figure 6). Finally, we examined the effect of siRNA-NOX4 on the

TRPC6 expression levels, it was found that siRNA-NOX4 significantly blocked PAN-induced TRPC6 expression, suggesting the contributing role of NOX4 on the regulation of TRPC6.

Discussion

There is considerable evidence that the kidney is vulnerable to oxidative damage [20]. Although the origin of increased ROS generation in renal diseases is multifactorial, NADPH oxidase is now known as a major source for ROS in the kidney. In the kidney, NADPH oxidase can be detected in fibroblasts, endothelial cells, mesangial cells, tubular cells, and podocytes [21]. Increasing experimental evidence indicates that NADPH oxidase-produced O_2^- is involved in the pathogenesis or development of many kidney diseases. A number of reports have indirectly linked PAN-induced nephrosis with oxidant injury of podocytes [22, 23]. However, the role of NADPH oxidase-derived ROS in podocyte injury still keeps unclear. The present study demonstrated *in vivo* that NADPH oxidase activity significantly increased in glomeruli from PAN nephrosis rats. NADPH oxidase inhibitor apocynin decreased urinary protein excretion and ameliorated podocyte injury in PAN nephrosis rats. These results suggest that the PAN-induced injury to podocytes resulting in experimental nephrotic syndrome is mediated by NADPH oxidase-derived ROS.

Recent studies have demonstrated a unique role for the Ca^{2+} -permeable ion channel protein TRPC6 as a regulator of glomerular ultrafiltration [24]. In both genetic and acquired forms of proteinuric kidney disease, dysregulation of podocyte TRPC6 plays a very important role under pathogenic conditions. Although physiologically, TRPC activation is triggered by activation of the phospholipase C pathway following activation of G-protein-coupled receptors or receptor tyrosine kinases [25, 26], the molecular mechanisms of enhanced TRPC6 expression and activity under pathophysiological processes remain poorly understood. It has been demonstrated that ROS can regulate intracellular [27] and plasma membrane [12, 28] ion channels. Given the role of ROS on the regulation of plasma membrane ion channels and gene expression, the major purpose of this study was to investigate whether TRPC6 is a redox-sensitive channel and a potential molecular target of NADPH oxidase-derived ROS for regulation of cellular events. Therefore, we detected the effect of NADPH oxidase-derived ROS on the regulation of TRPC6 expression. In *in vivo* study,

we found that the regulation of TRPC6 expression is associated with NADPH oxidase activity, indicating the contributing role of NADPH oxidase on the regulation of TRPC6 in PAN nephrosis rats. While the present study did not attempt to address how NADPH oxidase-derived ROS induce TRPC6 expression, there is abundant evidence for the regulation of gene expression by NADPH oxidase-dependent ROS including TNF- α [29], angiotensin II, monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1 [30]. Most studies have indicated that transcriptional upregulation is the major cause for mRNA upregulation in response to ROS. In particular, it has been reported that the activation of JNK and STAT3 is required for upregulation of TRPC6 induced by endothelin-1 (ET-1) in pulmonary vascular smooth muscle cells [31]. Nishida M et al. further demonstrated that G $_{\alpha 12/13}$ -NADPH oxidase-derived ROS-JNK pathway participates in the transcriptional activation of the TRPC6 gene in response to endothelin-1 [32]. Therefore, it is possible that this signaling pathway is also involved in the PAN-induced TRPC6 expression. Further studies will be performed to address this issue.

To explore the mechanisms by which PAN activates NADPH oxidase in PAN nephrosis rats, we then examined expression levels of major NADPH oxidase subunits NOX2, NOX4 and p47^{phox}. Western blot analysis showed that PAN significantly increased NOX4 expression and had no significant effect on NOX2 and p47^{phox} expression, although an increase tendency was observed in NOX2 expression. These results suggest that NOX4 may play a major role on the regulation of NADPH oxidase activity. NOX4 was originally identified as an NADPH oxidase homolog highly expressed in the kidney [33]. Upregulation of NOX4 has been reported in response to endoplasmic reticulum stress, hypoxia and ischemia, TGF- β 1 and TNF- α stimulation [34-37]. Some studies have reported that NOX4 does not require cytosolic subunits for its activity [38, 39], indicating that NOX4 might be a constitutively active enzyme. In the present study, we further performed gene silencing by siRNA to inhibit NOX4 expression and addressed the role of NOX4 in the regulation of NADPH oxidase activity induced by PAN in podocytes. Our results indicate that NOX4 importantly participates in PAN-induced increase in NADPH oxidase activity and

consequent upregulation of TRPC6 expression. It is suggested that NOX4 may serve as an early target molecule by PAN to increase TRPC6 expression and thereby activate the mechanistic cascade of podocyte injury such as Ca²⁺ signaling in foot processes for podocyte structure and function.

It should be noted that, as discussed above, NADPH oxidase-derived ROS can increase TRPC6 expression. On the other hand, Ca²⁺ signaling also contributes to the regulation of NADPH oxidase activity [12]. Therefore, it is possible that Ca²⁺ signaling mediated by TRPC6 channel further enhances NADPH oxidase activity, whereby superoxide is produced more and more, thereby producing a feedforward regulation, resulting in amplification of NADPH oxidase-TRPC6 signaling in PAN-induced podocyte injury. Therefore, NADPH oxidase-produced ROS and TRPC6-mediated Ca²⁺ signaling interactions may constitute a redox signaling network and thereby mediate the actions of PAN in podocyte injury. Further studies are needed to address this issue.

In summary, the present study addressed the role of NADPH oxidase-TRPC6 signaling in the development of PAN-induced podocyte injury. These findings for the first time demonstrate that NADPH oxidase-derived ROS is one of critical components of a signal transduction pathway that links PAN-induced podocyte injury to TRPC6-mediated Ca²⁺ signaling. These suggest a new pathogenic pathway contributing to glomerular injury in FSGS, which may provide an effective treatment for acquired forms of proteinuric kidney disease by NADPH oxidase-TRPC6 signaling blockade.

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