Albumin impairs renal tubular tight junctions via targeting the NLRP3 inflammasome

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Proteinuria is an independent causative factor leading to renal tubulointerstitial injury (2, 5). The pathological phenomena include tubular epithelial cell apoptosis (16), cellular phenotypic transition (21), tubulointerstitial inflammation (9, 13), oxidative stress (13), and others. In our previous study, we showed that albumin induced renal tubular cell apoptosis and phenotypic changes through NLRP3 [neuronal apoptosis inhibitory protein, major histocompatibility complex class 2 transcription activator, incompatibility locus protein from Podospora anserina, and telomerase-associated protein (NACHT); leucine-rich repeat (LRR); and pyrin domain (PYD) domains-containing protein 3] inflammasome activation (20).

The NLRP3 inflammasome, as one of the best characterized inflammasomes, is composed of NLRP3, apoptosis-associated speck-like protein containing a COOH-terminal caspase recruitment domain, and caspase-1, which is activated by many danger signals, e.g., oxidative stress, potassium efflux, and monosodium urate crystals (17, 19). The activation of the NLRP3 inflammasome triggers the maturation of interleukin (IL)-1β and IL-18 and promotes the release of active inflammatory cytokines, which ultimately contributes to the pathogenesis of many diseases (11, 14, 17).

In the present study, using mPTCs, we pretreated mPTCs with NLRP3 siRNA (siNLRP3) and found that NLRP3 knockout knocked down the expression of ZO-1 and claudin-1 induced by albumin. Similarly, in albumin-overloaded wild-type mice, both ZO-1 and claudin-1 were downregulated at the protein and mRNA levels in parallel with the impaired formation of the tight junctions on transmission electron microscopy and the abnormal renal tubular morphology on periodic acid-Schiff staining, which contrasted with the stimulation of NLRP3 in the renal tubules. In contrast, NLRP3 knockout (NLRP3−/−) mice preserved normal ZO-1 and claudin-1 expression as well as largely normal tight junctions and tubular morphology. More importantly, deletion of the NLRP3 pathway downstream component caspase-1 similarly blocked the albumin overload-induced downregulation of ZO-1 and claudin-1. Taken together, these findings demonstrated an important role of the albumin-NLRP3 inflammasome axis in mediating the impairment of renal tubular tight junctions and integrity.

MATERIALS AND METHODS

Animal studies. NLRP3-knockout (NLRP3−/−) and caspase-1 knockout (caspase-1−/−) mice on a C57BL/6J-129 background (Jackson Laboratory, Sacramento, CA) were used to examine the roles of NLRP3 and caspase-1 in albumin overload-induced renal tubular injury. In particular, heterozygous littermates were bred to generate homozygous knockout mice and littermate wild-type (WT) controls. Eight-week-old male mice were fed a high-protein diet (12% casein) and maintained on a 12-h light/dark cycle in a temperature-controlled (19 –21°C) room, fed a
standard rodent diet, and allowed free access to drinking water. At the termination of the experiments, the mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine/atropine solution. Plasma and kidney samples were then immediately frozen in liquid nitrogen and stored at −80°C until use. The study protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Nanjing Medical University, China.

**Cell culture studies.** Mouse proximal tubular cells (mPTCs), an immortalized cell line, were grown in serum-free keratinocyte medium supplemented with bovine pituitary extract and epidermal growth factor (Wisent, Saint-Jean-Baptiste, Quebec, Canada). The cells were specifically grown at 37°C with 5% CO2 and subcultured at 50–80% confluence using 0.25% trypsin-0.02% EDTA (Invitrogen, Carlsbad, CA). Dilapidated albumin was used to stimulate the mPTCs because albumin-bound lipids and fatty acids contribute to the pro-apoptotic effects of albumin on tubular cells.

**siNLRP3 transfection.** The mPTCs were cultivated to 50–60% confluence in culture medium containing no penicillin or streptomycin. NLRP3 siRNA (siNLRP3) was synthesized by GenePharma (Shanghai, China). The cells were then transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In particular, the cells were transfected with 500 nM siNLRP3 or control siRNA 24 h before albumin treatment. Scramble siRNA was used as the control siRNA (GenePharma).

**Quantitative real-time RT-PCR.** Total RNA from mPTCs and kidney cortex was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and TRIzol reagent (Invitrogen). Oligonucleotides (NLRP3: forward, 5'-GTGGTGACCCCTCTGTGAGGT-3', reverse, 5'-TCTTCTGGAGCGGTCTTCA A-3'; ZO-1: forward, 5'-AGCCGGTCACCCCTTGTT-3', reverse, 5'-AGGGCCTGGA TGGTTCA-3'; claudin-1: forward, 5'-TATCCCCAAGCAAACCTTCTC-3', reverse, 5'-AGCGCTCCCTCCTCTTGCT-3'; GAPDH: forward, 5'-GTCTTCATACCATGAGGA-3', reverse, 5'-CATGGATGACCTTGCCAG-3') were designed using Primer3 software (available at http://frodo.wi.mit.edu/) and synthesized by Invitrogen. qRT-PCR was then used to detect the mRNA expression of the target genes.

**Fig. 1.** Albumin time dependently downregulated zonula occludens 1 (ZO-1) and claudin-1 expression in mouse proximal tubular cells (mPTCs). A: Western blotting analysis of ZO-1 and claudin-1 in mPTCs following 10 mg/ml albumin treatment in a time-course (0–48 h) study. B: densitometric analysis of ZO-1 expression following 10 mg/ml albumin administration. C: densitometric analysis of claudin-1 expression following albumin administration. D and E: qRT-PCR analysis of ZO-1 (D) and claudin-1 (E) mRNA expression levels in mPTCs following a 10 mg/ml albumin treatment in a time-course (0–24 h) study. The values represent means ± SD (n = 6 per group). *P < 0.01 vs. control.
Reverse transcription was performed using the Promega Reverse Transcription System according to the manufacturer’s protocol (Madison, WI). Real-time PCR amplification was performed using the ABI 7500 real-time PCR detection system using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative mitochondrial DNA copy number was normalized to the 18S rRNA level encoded by the nuclear DNA, and mRNA levels were normalized to GAPDH as a control and calculated using the comparative cycle threshold (ΔΔCt) method.

Western blotting. The mPTCs and kidney cortex were lysed using a protein lysis buffer containing 50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 200 mM sodium fluoride, and 4 mM sodium orthovanadate as a protease inhibitor (pH 7.5). Immunoblotting was then performed using primary antibodies against ZO-1 (1:500), claudin-1 (1:250), NLRP3 (1:500), and β-actin (1:1,000), followed by the addition of horseradish peroxidase-labeled secondary antibodies. The blots were visualized using the Amersham Enhanced Chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, UK). Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, CA).

Kidney histological analysis. Harvested tissues from mice were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned transversely. Kidney sections (3 μm) were stained with periodic acid–Schiff (PAS).

Immunostaining. Kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections of each specimen were cut at a thickness of 3 μm (Cryostat 2800 Frigocut-E; Leica Microsystems, Wetzlar, Germany), and a standard protocol, using xylene and graded ethanol, was employed to deparaffinize and rehydrate the tissue. These sections were washed with PBS and treated with blocking buffer containing 50 mM NH₄Cl, 2% BSA, and 0.05% saponin in PBS for 20 min at room temperature. The sections were then incubated overnight at 4°C with anti-NLRP3 rabbit polyclonal antibody at 2–5 μg/ml. After being washed with PBS, the secondary antibody was applied, and the signals were visualized using an avidin/biotin complex kit (Santa Cruz Biotechnology, Santa Cruz, CA).

Fig. 2. Albumin dose dependently downregulated ZO-1 and claudin-1 expression in mPTCs. A: Western blotting analysis of ZO-1 and claudin-1 after treatment with different doses of albumin (0–20 mg/ml) for 48 h. B: densitometric analysis of ZO-1 expression following albumin administration. C: densitometric analysis of claudin-1 expression following albumin administration. D and E: qRT-PCR analysis of ZO-1 (D) and claudin-1 (E) mRNA expression levels in mPTCs after treatment with different doses of albumin (0–20 mg/ml) for 24 h. The values represent means ± SD (n = 6 per group). *P < 0.01 vs. control.
Transmission electron microscopy. Fresh kidney tissues were fixed in 1.25% glutaraldehyde/0.1 M phosphate buffer and postfixed in 1% OsO4/0.1 M phosphate buffer. Ultrathin sections (60 nm) were then cut on a microtome, placed on copper grids, stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEM-1010; JEOL, Tokyo, Japan).

Analysis of urinary albumin excretion. Urinary albumin excretion was determined using enzyme-linked immunosorbent assay kits (Exocell, Philadelphia, PA) according to the manufacturer’s instructions.

Statistical analysis. All of the data are presented as means ± SD. The statistical analysis was performed using ANOVA followed by Bonferroni’s test with SPSS 13 statistical software. P < 0.05 was considered significant.

RESULTS

Albumin reduced ZO-1 and claudin-1 expression levels in mPTCs. Albumin is known to contribute to renal tubular epithelial cell injury. In the present study, we examined whether albumin could affect the expression of the TJ proteins ZO-1 and claudin-1 at the protein and mRNA levels. As shown in Fig. 1, albumin at a dose of 10 mg/ml time dependently downregulated ZO-1 and claudin-1 at both the mRNA and protein levels during a 24-h observation. In a separate study, both ZO-1 and claudin-1 were reduced in a dose-dependent manner (0–20 mg/ml) (Fig. 2, A–E). These results suggested that albumin is a direct regulator of TJ proteins.

Knockdown of NLRP3 by siRNA restored albumin-induced downregulation of ZO-1 and claudin-1 in mPTCs. Application of NLRP3 siRNA significantly reduced the expression of

Fig. 3. NLRP3 [neuronal apoptosis inhibitor protein, major histocompatibility complex class 2 transcription activator, incompatibility locus protein from Podospora anserina, and telomerase-associated protein (NACHT); leucine-rich repeat (LRR); and pyrin domain (PYD) domains-containing protein 3] siRNA transfection in mPTCs. Cells were transfected with NLRP3 siRNA (siNLRP3) (500 nM) or scrambled siRNA (Vehi, 500 nM) for 24 h, and untreated cells were used as the control. A: Western blotting analysis of NLRP3 protein expression. B: densitometric analysis of NLRP3 Western blots following siNLRP3 transfection. C: qRT-PCR analysis of NLRP3 mRNA expression. The values represent means ± SD (n = 6 per group). *P < 0.01 vs. control (Cntl).

Fig. 4. Albumin-induced downregulation of ZO-1 and claudin-1 was diminished by NLRP3 siRNA treatment in mPTCs. Confluent mPTCs were transfected with siNLRP3 for 48 h and then incubated with albumin (10 mg/ml) for an additional 48 h. A: Western blotting analysis of ZO-1 and claudin-1 expression levels in mPTCs after treatment with 10 mg/ml albumin. B: densitometric analysis of ZO-1 protein expression. C: densitometric analysis of claudin-1 protein expression. The values represent means ± SD (n = 6 per group). *P < 0.01 vs. control. #P < 0.01 vs. albumin-treated mPTCs.
NLRP3 in mPTCs at both the RNA and protein levels (Fig. 3, A–C). Interestingly, blockade of NLRP3 by siRNA normalized the downregulation of ZO-1 and claudin-1 following albumin treatment, as determined by Western blotting (Fig. 4, A–C). These results suggested that albumin reduced TJ proteins via NLRP3 in vitro.

The NLRP3 inflammasome mediated the effects of albumin on TJ protein expression. We next investigated the in vivo roles of albuminuria and NLRP3 in TJ protein regulation in kidneys. Following albumin overload, the urinary albumin excretion was markedly elevated by 3.4-fold in WT mice and 2.9-fold in NLRP3−/− mice, respectively, with no significant difference between genotypes (Fig. 5A). As expected, albumin overload remarkably reduced the ZO-1 and claudin-1 expression levels at the protein and mRNA levels in kidney, which was entirely blocked by the systemic deletion of NLRP3 (Fig. 5, B–F).

The effects of NLRP3 deletion on kidney morphology and tubular TJ formation. Twelve days of albumin overload markedly stimulated NLRP3 protein expression in renal tubular cells (Fig. 6A). On PAS staining, albumin overload resulted in renal tubular dilation, epithelial cell flattening, and the loss of the brush border (Fig. 6B). In contrast, systemic NLRP3 deletion strikingly improved these morphological alterations (Fig. 6B). Importantly, this impairment was strikingly ameliorated in NLRP3−/− mice (Fig. 6C). These in vivo results provided further evidence of the important role of NLRP3 in main-

![Figure 5](http://ajprenal.physiology.org/)

Fig. 5. Downregulation of renal ZO-1 and claudin-1 expression following 12 days of albumin overload was blocked in NLRP3 knockout (NLRP3−/−) mice. A: 24-h urinary albumin excretion following albumin overload. B: Western blotting analysis of ZO-1 and claudin-1 expression in wild-type (WT) and NLRP3−/− mice following albumin overload. C: densitometric analysis of ZO-1 protein expression. D: densitometric analysis of claudin-1 protein expression. E and F: qRT-PCR analysis of the ZO-1 (E) and claudin-1 (F) mRNA expression levels in WT and NLRP3−/− mice following albumin overload. The values represent means ± SD (n = 8). *P < 0.01 vs. control. #P < 0.01 vs. albumin-overloaded WT mice.
containing normal TJ and tubular integrity under albuminuric conditions.

Caspase-1 deletion restored albumin overload-induced downregulation of TJ proteins in vivo. For further validation of the role of NLRP3 inflammasome activation in mediating the effects of albuminuria on TJ protein regulation by caspase-1, a downstream molecule of NLRP3 (8, 17), knockout (caspase-1\(^{-/-}\)) mice were subjected to albumin overload for 12 days. Following albumin overload, the urinary albumin excretion was markedly enhanced by 3.1-fold in WT mice and 2.7-fold in caspase-1\(^{-/-}\) mice, respectively, with no significant difference between genotypes (Fig. 7A). As expected, the downregulation of ZO-1 and claudin-1 was completely blocked in caspase-1\(^{-/-}\) animals (Fig. 7, B–E). These data strongly suggested that albumin targets the NLRP3-caspase-1 pathway to impair renal tubular TJs and their integrity.

**DISCUSSION**

To date, proteinuria has been recognized as, not only a common feature of chronic kidney disease (CKD), but also as a direct causative factor in promoting renal injury (2, 10). However, the mechanism underlying proteinuria-induced renal injury is not fully understood. Additionally, there is no specific target for treating proteinuria-associated kidney injury in the clinic. In the present study, we evaluated the effects of albumin on TJ protein regulation both in vitro in renal epithelial cells and in vivo in animal kidneys. The apical junctional complex encompassing the TJ and adherens junction plays a pivotal role in the control of the epithelial barrier function (3). TJs prevent the passage of molecules and ions through the space between the plasma membranes of adjacent cells and also help to maintain the polarity of cells (18). The major types of proteins forming the TJs include ZO proteins, claudins, and occludins. In confluent mPTCs, a pathological dose of albumin (10 mg/ml) strikingly downregulated ZO-1 and claudin-1 expression in a time- and dose-dependent manner, demonstrating the direct role of albumin in downregulating TJ proteins. Our previous study demonstrated that albumin could positively activate the NLRP3 inflammasome, which contributed to albumin-induced apoptosis and phenotypic transition in mPTCs (20). In the present study, knockdown of the NLRP3 using an siRNA strategy entirely abolished the effects of albumin on TJ protein downregulation, suggesting a key role for the NLRP3 inflammasome in mediating albumin-induced TJ impairment in this pathological process.

Albumin overload is a widely used model to study the pathological effects of albumin on renal injury (1, 6, 15). Although this model cannot entirely mimic the disease status of patients with proteinuria in the clinic, it might be a cleaner model for studying the effect of albuminuria on kidney injury because of the exclusion of nonproteinuria insults, such as diabetes, hypertension, and lipid disorders. Similarly to previous in vitro studies, albumin overload for 12 days strikingly downregulated the expression of the TJ proteins ZO-1 and claudin-1 at the mRNA and protein levels. Systemic deletion of NLRP3 in mice completely abolished this downregulation. In
addition to TJ protein changes, TJ formation was also impaired as determined by TEM. In line with the TJ impairment, albumin overload also led to tubular morphological damage that paralleled the stimulation of NLRP3. Importantly, such abnormalities were markedly attenuated by NLRP3 invalidation. All of these in vivo data further suggested that albuminuria could impair renal tubular integrity via targeting the NLRP3 inflammasome.

Caspase-1 is a key component of the NLRP3 inflammasome (8), and it is a unique caspase that activates proinflammatory cytokines including IL-1β and IL-18 (17). With the use of caspase-1<sup>−/−</sup> mice, the albumin overload-induced downregulation of ZO-1 and claudin-1 was also restored, which mimicked the same phenotype observed in NLRP3<sup>−/−</sup> mice. At the same time, the urinary excretion of IL-1β and IL-18 was remarkably blunted in both NLRP3<sup>−/−</sup> and caspase-1<sup>−/−</sup> mice following albumin overload (data not shown).

In summary, the present study suggested that albumin targeted the NLRP3 inflammasome to impair the renal tubular TJs and tubular integrity. Impairment of renal tubular integrity could increase their permeability to urinary components, including the urinary proteins into the tubular interstitium, further promoting the inflammatory and fibrotic response. Currently, there are few specific therapies to prevent CKD progression. Our findings characterized a key role for the NLRP3 inflammasome in mediating albuminuria-related renal tubular injury, shedding new light on its pathogenesis and establishing a therapeutic target for CKDs, particularly for proteinuric kidney diseases.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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