NLRP3 deletion protects against renal fibrosis and attenuates mitochondrial abnormality in mouse with 5/6 nephrectomy

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Gong W, Mao S, Yu J, Song J, Jia Z, Huang S, Zhang A. NLRP3 deletion protects against renal fibrosis and attenuates mitochondrial abnormality in mouse with 5/6 nephrectomy. Am J Physiol Renal Physiol 310: F1081–F1088, 2016. First published February 17, 2016; doi:10.1152/ajprenal.00534.2015.—Progressive fibrosis in chronic kidney disease (CKD) is the well-recognized cause leading to the progressive loss of renal function. Emerging evidence indicated a pathogenic role of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome in mediating kidney injury. However, the role of NLRP3 in the remnant kidney disease model is still undefined. The present study was undertaken to evaluate the function of NLRP3 in modulating renal fibrosis in a CKD model of 5/6 nephrectomy (5/6 Nx) and the potential involvement of mitochondrial dysfunction in the pathogenesis. Employing NLRP3+/− and NLRP3−/− mice with or without 5/6 Nx, we examined renal fibrotic response and mitochondrial function. Strikingly, tubulointerstitial fibrosis was remarkably attenuated in NLRP3−/− mice as evidenced by the blockade of extracellular matrix deposition. Meanwhile, renal tubular cells in NLRP3−/− mice maintained better mitochondrial morphology and higher mitochondrial DNA copy number, indicating an amelioration of mitochondrial abnormality. Moreover, NLRP3 deletion also blunted the severity of proteinuria and CKD-related hypertension. To further evaluate the direct role of NLRP3 in triggering fibrogenesis, mouse proximal tubular cells (PTCs) were subjected to transforming growth factor β1 (TGF-β1), and the cellular phenotypic changes were detected. As expected, TGF-β1-induced alterations of PTC phenotype were abolished by NLRP3 small interfering RNA, in line with a protection of mitochondrial function. Taken together, NLRP3 deletion protected against renal fibrosis in the 5/6 Nx disease model, possibly via inhibiting mitochondrial dysfunction.

chronic kidney disease; mitochondrial dysfunction; NLRP3 inflammasome

CHRONIC RENAL FAILURE (CRF) is a syndrome with multiple dysfunctions in kidney excretory capability, blood pressure regulation, and so on (5, 8). It is the common end-stage of many chronic kidney diseases (CKDs) with no effective therapy (10). An understanding of the underlying mechanisms leading to the CRF is important and urgent for effective clinical intervention. Recent studies indicated that the progression of chronic kidney injury to CRF appeared to be associated with persistent inflammation, which contributes to the tubulointerstitial fibrosis and loss of renal function (1, 2, 18).

An inflammatory response is usually considered to be the homeostatic response of the body to defend against infection (3). During this process, the pattern recognition receptor (PRR) plays a primary role to recognize the intra- and extracellular danger signals (17). NLRP3 is one member of the nucleotide-binding oligomerization domain-like receptor family belonging to PRRs. Its activation could oligomerize and recruit the adaptor protein apoptosis-associated specklike protein containing a caspase recruitment domain and the protease caspase-1 to form the protein complex termed “inflammasome” (15–17), which finally results in the cleavage of prointerleukin (pro-IL)-β and pro-IL-18 into the proinflammatory cytokines of IL-1β and IL-18 (25). Reports implicated that cytokines associated with NLRP3 inflammasome contributed to inflammation and fibrogenesis (4, 14). However, the exact role of NLRP3 in modulating renal fibrosis in a remnant kidney disease model [5/6 nephrectomy (5/6 Nx)] is still undefined.

Besides inflammation, emerging evidence strongly suggested a pathogenic role of mitochondrial dysfunction in renal injuries (9, 13, 24). Mitochondria are the centers of energy metabolism and are vulnerable to various insults. Severe and global mitochondrial dysfunction leads to reduced ATP production, increased reactive oxygen species generation, and release of proapoptotic products such as mitochondrial DNA (mtDNA) and cytochrome c. Our recent report demonstrated that the activation of NLRP3 inflammasome deteriorated renal tubular mitochondrial dysfunction in proteinuric kidney disease (29). However, the role of NLRP3 inflammasome in modulating mitochondrial function in a CKD disease model of 5/6 Nx is unknown.

In this study, using NLRP3−/− mice and NLRP3 small interfering RNA (siRNA), we demonstrated that NLRP3 played a detrimental role in promoting a renal fibrotic response both in vivo and in vitro. Besides, NLRP3 inactivation inhibited mitochondrial dysfunction both in 5/6 Nx mice and transforming growth factor β1 (TGF-β1)-treated mouse proximal tubular cells (PTCs). The findings highly suggested that activation of NLRP3 inflammasome may trigger a renal fibrotic response via mitochondrial dysfunction. Targeting NLRP3 could be a novel therapeutic strategy for the treatment of CKDs.

MATERIALS AND METHODS

Reagents. DMEM/F12 and FBS were purchased from Gibco (Grand Island, NY). TGF-β1 and 2,7-dichlorofluorescein diacetate (DCFDA) were obtained from Sigma (St. Louis, MO). Antibodies of IL-1β, IL-18, collagen-I, collagen-III, vimentin, α-smooth muscle actin (α-SMA), E-cadherin, and β-actin were provided by Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was ordered from Cell Signaling Technology (Danvers, MA).

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Cell culture and siRNA experiments. Mouse PTCs were bought from Sciencell Research Laboratory (catalog No. M4100) and were cultured in the medium of DMEM/F12 supplemented with 10% FBS at 37°C with 5% CO₂ and subcultured at 70–80% confluence using 0.25% trypsin and 0.02% EDTA (Invitrogen, San Diego, CA). For in vitro experiments, cells were divided into four groups: control siRNA group, NLRP3 siRNA group, TGF-β1 group, and NLRP3 siRNA plus TGF-β1 group (NLRP3⁻/⁻ + TGF-β1). For details, siRNA for NLRP3 (GenePharma, Shanghai, China) was transfected into PTCs with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, the silencing effect of siRNA was detected by real-time quantitative PCR and Western blotting. Following siRNA transfection, cells were stimulated with TGF-β1 (10 ng/ml) for another 48 h to induce phenotypic alteration.

Animal studies. NLRP3⁻/⁻ mice on a C57BL/6J background (Jackson Laboratory, Sacramento, CA) were utilized to test the role of NLRP3 in the 5/6 Nx model. Mice were maintained on a 12:12-h light-dark cycle in a temperature-controlled (19–21°C) room, fed a (Jackson Laboratory, Sacramento, CA) diet, and given free access to water. The 5/6 Nx was performed in 16-wk-old NLRP3 wild-type (WT) and light-dark cycle in a temperature-controlled (19 –21°C) room, fed a diet, and given free access to water. The 5/6 Nx mice were anesthetized with an intraperitoneal injection of a ketamine-xylazine-atropine mixture, and the renal tissues were harvested and lysed using the 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). Immunofluorescence and immunohistochemical staining were performed as previously described (27).

Reverse transcription and quantitative real-time PCR (qRT-PCR). Total RNA was isolated from mouse proximal tubular cells (PTCs) or kidney cortex tissues following the manufacturer’s instructions. In brief, 1 μg of RNA was reverse transcribed in a 20-μl system using a Revert AId First-Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Subsequently, 1 μl complementary DNA was used as the template, and qRT-PCR was performed using SybrGreen PCR Master Mix (with Rox; Invitrogen) and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative mRNA expression levels of the target genes against that of GAPDH were calculated as 2⁻ΔΔCt according to the manufacturer’s specifications. qRT-PCR was used to detect mtDNA copy number as previously described (27). Relative amounts of mtDNA copy number were normalized to 18S ribosomal RNA levels encoded by the nuclear DNA. The sequences of primers were as follows: GAPDH: forward 5’-TCATGGATGACCTTGCCAG-3’ and reverse 5’-GTCTTCACTACCATGGAAGG-3’; NLRP3: forward 5’-TCTTCTGAGCGCTTCTCA-3’ and reverse 5’-GTGGTGACCCTCTGTGAAGT-3’; E-cadherin: forward 5’-CCTCTGTGAGGTGCCAAGCAGCAATACATCCT-3’ and reverse 5’-GGCAAGCAGCAATACATCCT-3’; vimentin: forward 5’-ACTACTGGCCAGCTTGAGA-3’ and reverse 5’-CCAATGAGATGGCTGGAA-3’; α-SMA: forward 5’-GACCTCTTCCACCACCTCTTC-3’ and reverse 5’-TTCCTCCTATTCTTTTGCT-3’; mtDNA: forward 5’-TTTTATCTGCGACTGAGTTATATCCTTG-3’ and reverse 5’-CTCTTCTGAGCGCTTCTCA-3’. Western blot analysis. Mouse PTCs or kidney cortex tissues were lysed using the 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). Immunofluorescence and immunohistochemical staining were performed as previously described (27).
nblotting was then performed using primary antibodies against IL-1β (1:1,000), IL-18 (1:1,000), E-cadherin (1:1,000), vimentin (1:500), α-SMA (1:500), collagen-I (1:1,000), collagen-III (1:1,000), and β-actin (1:1,000), followed by the addition of HRP-labeled secondary antibodies. Protein bands were visualized using enhanced chemiluminescence plus Western blot detection reagents (Millipore, Bedford, MA). Each blot was a representative of three independent experiments, and band intensity was measured using Image J software (NIH, Bethesda, MD).

Analysis of reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) in mouse PTCs. ROS production was measured by 2',7'-dichlorofluorescein diacetate as described previously (27), and the quantification of ROS detected by the fluorescence levels of 2',7'-dichlorofluorescein were measured using flow cytometry. MMP was determined using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide (JC-1; Molecular Probes) as described previously (29). The quantification of JC-1 fluorescence levels was analyzed by flow cytometry.

Fig. 2. Effect of NLRP3 deletion on renal fibrosis in 5/6 Nx mice. A: Masson staining of remnant kidneys of NLRP3 WT and knockout (KO) mice. B–D: mRNA levels of fibronectin (B), collagen-I (C), and collagen-III (D) in remnant kidneys of NLRP3 WT and KO mice as determined by qRT-PCR. Values are means ± SD (n = 5 in each group); *P < 0.05.
Transmission electron microscopy. To evaluate the mitochondrial morphology both in mouse PTC and renal cortex, cells and tissues were collected, respectively, and fixed in 1.25% glutaraldehyde-0.1 mol/l phosphate buffer. After postfixing in 1% OsO₄-0.1 mol/l phosphate buffer, ultrathin sections (60 nm) were cut on a microtome, placed on copper grids, stained with uranyl acetate and lead citrate, and examined in an electron microscope (JEOL JEM-1010; Tokyo, Japan) as previously described (29).

Statistical analysis. Each experiment was repeated at least three times, and all analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were expressed as means ± SD and were analyzed by one-way ANOVA followed by Bonferroni’s comparison test. *P < 0.05 was considered significant.

RESULTS

NLRP3 deletion alleviated the renal fibrosis in 5/6 Nx mice. First, we examined the regulation of NLRP3 and its downstream inflammatory cytokines IL-1β and IL-18 in remnant kidneys of 5/6 Nx mice. As expected, NLRP3 mRNA expression was elevated 3.2-fold in remnant kidneys in line with the remarkable induction of IL-1β and IL-18 (Fig. 1, A–D). In contrast, NLRP3 deletion entirely abolished the induction of IL-1β and IL-18 in remnant kidneys (Fig. 1, B–D). Then we tested the role of NLRP3 in renal fibrosis in the 5/6 Nx model using NLRP3 mutant mice. Masson staining showed significant deposition of extracellular matrix in kidneys of WT mice with 5/6 Nx. Compared with WT 5/6 Nx mice, NLRP3 deletion significantly reduced tubulointerstitial fibrosis (Fig. 2A). Consistently, the mRNA levels of the components of extracellular matrix including fibronectin, collagen-I, and collagen-III in remnant kidneys were remarkably increased in WT mice, which was strikingly blunted in mice with NLRP3 invalidation (Fig. 2, B–D). With the use of Western blotting, we further confirmed the regulation of collagen-I and collagen-III at protein levels (Fig. 3, A–C). These results strongly suggested that NLRP3 played a detrimental role in mediating renal fibrosis in vivo.

NLRP3 deletion attenuated mitochondrial abnormality in remnant kidney. Previous studies showed that mitochondrial dysfunction occurred in the proximal tubular epithelial cells following albumin challenge, and such a mitochondrial abnormality was triggered by the NLRP3 inflammasome (29). Here we tested the mitochondrial status in the remnant kidneys and examined the effect of NLRP3 deletion on mitochondria in this particular CKD model. As shown in Fig. 4, A and B, 5/6 Nx strikingly altered mitochondrial morphology and reduced mtDNA copy number, indicating a severe mitochondrial abnormality in this model. Importantly, such abnormalities were significantly attenuated by deletion of NLRP3.

NLRP3 deletion improved proteinuria and CKD-related hypertension in 5/6 Nx mice. Proteinuria and high blood pressure are also common features of CKDs and contribute to the progression of kidney injury. Therefore, we examined the two indices in NLRP3 WT and knockout (KO) mice with or without 5/6 Nx. As shown in Fig. 5, A and B, there was no significant difference in systolic blood pressure and urine albumin output between genotypes in sham controls. However, 5/6 Nx-caused proteinuria and hypertension in WT mice were remarkably blunted in NLRP3-deficient mice. These data indicated that NLRP3 plays a role in modulating proteinuria and blood pressure in CKD.

Fig. 3. Effect of NLRP3 deletion on the protein levels of extracellular matrix in remnant kidneys of 5/6 Nx mice. A: representative images of Western blots of collagen I and collagen III. B and C: densitometric analysis of Western blots of collagen I and collagen III. Values are means ± SD (n = 5 in each group); *P < 0.05.
NLRP3 deficiency attenuated TGF-β1-induced phenotypic alteration in mouse PTCs. To further explore the effect of NLRP3 on renal fibrotic response, TGF-β1 was applied to PTCs with or without NLRP3 deficiency. By using a siRNA strategy (Fig. 6, A and B), we found that NLRP3 siRNA markedly blocked TGF-β1-induced downregulation of E-cadherin and induction of vimentin and α-SMA (Fig. 6, C–F), suggesting an antifibrotic potential of NLRP3 invalidation in vitro.

NLRP3 deficiency inhibited mitochondrial dysfunction in TGF-β1-treated mouse PTCs. To investigate the effect of NLRP3 on mitochondrial dysfunction in vitro, we examined mtDNA copy number, MMP, and ROS production. As shown in Fig. 7, A–C, TGF-β1 induced ROS production and reduced MMP and mtDNA copy number in PTCs. Significantly, NLRP3 siRNA strikingly rescued these parameters, demonstrating an important role of NLRP3 in mediating mitochondrial dysfunction.

DISCUSSION

During the process of CKD progression, fibrosis is one of the key factors leading to the progressive loss of renal function. Although a number of studies have been performed to reveal the mask of renal fibrosis, an effective therapy by specifically targeting the fibrotic response is still absent. Therefore, more studies are still required for exploring the mechanisms mediating renal fibrogenesis. Here we found that NLRP3 deletion improved renal fibrosis in 5/6 Nx mice, suggesting that NLRP3 contributed to fibrosis in CKD.

Inflammation and fibrosis are featured events in the progress of CKD, regardless of the initial cause (6). Interstitial fibrosis is the common final outcome of progressive CKDs and results in irreversible renal damage. In general, renal fibrotic lesions aggravate the inflammatory response, and exacerbated inflammation further accelerates fibrosis and disease progression concomitantly (23, 26, 28). The NLRP3 inflammasome is an important contributor of inflammation via processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (25).

NLRP3, in line with its downstream inflammatory cytokines IL-1β and IL-18, was induced in remnant kidneys of NLRP3 WT mice but not KO mice, indicating a potential contribution of the NLRP3 inflammasome-mediated inflammatory response in 5/6 Nx-induced CRF. Our present study demonstrated that NLRP3−/− mice subjected to 5/6 Nx had less renal fibrosis, as evidenced by reduced expressions of fibronectin, collagen-I, and collagen-III. Consistently, NLRP3 deficiency by using a siRNA strategy attenuated TGF-β1-induced phenotypic alteration in mouse PTCs in vitro, demonstrating a direct role of NLRP3 in promoting fibrogenesis. Overall, these data highly
suggested a pathogenic role of NLRP3 in mediating renal fibrosis in CKD. However, a limitation of the current study is that we could not define the cell-specific role of NLRP3 in this disease model. In the future, experiments using cell-specific (inflammatory cells, renal epithelial cells, or renal fibroblast cells) NLRP3 KO mice may be required to address this question.

Mitochondrial dysfunction has been shown to be an early and key event leading to kidney damage (7, 22). Mitochondrial dysfunction could be induced by many insults including infection and noninfection factors via release of inflammatory cytokines and excessive ROS (11, 12, 19, 20). Our previous studies also suggested that NLRP3 inflammasome activation mediated albumin-induced renal tubular injury through impaired mitochondrial function (29). In this study, mitochondrial dysfunction occurred in remnant kidneys of 5/6 Nx mice and TGF-β1-treated mouse PTCs as evidenced by decreased mtDNA copy number and MMP and increased ROS. Transmission electron microscopy analysis also showed a significant abnormality of mitochondrial morphology in the PTCs of 5/6 Nx mice. NLRP3 deficiency led a remarkable amelioration of mitochondrial dysfunction in vivo and in vitro, which might be a potential mechanism by which NLRP3 invalidation blocked renal fibrosis. In theory, inflammation can cause mitochondrial damage leading to the mitochondria-originated oxidative stress and apoptosis, while mitochondrial dysfunction also contributes to the occurrence and promotion of inflammation, thus forming a positive-feedback loop between inflammation and mitochondrial dysfunction.

Besides the attenuation of renal fibrosis, NLRP3 deletion in mice also ameliorated the severity of proteinuria in 5/6 Nx mice. This interesting phenomenon could be due to the blockade of NLRP3-mediated inflammation and mitochondrial dysfunction. To fully investigate the role of NLRP3 in podocytes in modulating proteinuria, studies in podocyte-specific NLRP3 KO animals are needed in the future. Here, ameliorated severity of proteinuria levels could be involved in attenuating renal fibrosis to some extent. To understand blunted hypertension in NLRP3−/− mice with 5/6 Nx, more work using genetic strategies to specifically invalidate NLRP3 in vascular and kidney
cells is definitely required. In the present study, systemic deletion of NLRP3 did not change the contribution of NLRP3 in the vasculature, kidney, and central nervous system in regulating blood pressure in the 5/6 Nx CKD model.

In summary, these in vivo and in vitro data demonstrated that NLRP3 invalidation protected against renal fibrosis and improved mitochondrial function. The latter may serve as a possible mechanism in antagonizing fibrogenesis in remnant kidneys. This study not only provided evidence showing a pathogenic role for NLRP3 in CKDs but also offered us more confidence in treating renal fibrosis by targeting NLRP3.

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

AUTHOR CONTRIBUTIONS

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