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# Activation of the Nlrp3 inflammasome by mitochondrial reactive oxygen species: A novel mechanism of albumin-induced tubulointerstitial inflammation

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ABSTRACT

Albuminuria is not only an important marker of chronic kidney disease but also a crucial contributor to tubulointerstitial inflammation (TIF). In this study, we determined whether activation of the Nlrp3 inflammasome is involved in albuminuria induced-TIF and the underlying mechanisms of inflammasome activation by mitochondrial reactive oxygen species (mROS). We established an albumin-overload induced rat nephropathy model characterised by albuminuria, renal infiltration of inflammatory cells, tubular dilation and atrophy. The renal expression levels of the Nlrp3 inflammasome, IL-1β and IL-18 were significantly increased in this animal model. In vitro, albumin time- and dose-dependently increased the expression levels of the Nlrp3 inflammasome, IL-1β and IL18. Moreover, the silencing of the Nlrp3 gene or the use of the caspase-1 inhibitor Z-VAD-fmk significantly attenuated the albumin-induced increase in IL-1β and IL-18 expression in HK2 cells. In addition, mROS generation was elevated by albumin stimulation, whereas the ROS scavenger N-acetyl-L-cysteine (NAC) inhibited Nlrp3 expression and the release of IL-1β and IL-18. In kidney biopsy specimens obtained from patients with IgA nephropathy, Nlrp3 expression was localised to the proximal tubular epithelial cells, and this result is closely correlated with the extent of proteinuria and TIF. In summary, this study demonstrates that albuminuria may serve as an endogenous danger-associated molecular pattern (DAMP) that stimulates TIF via the mROS-mediated activation of the cytoplasmic Nlrp3 inflammasome.



## 1. Introduction

Tubulointerstitial inflammation plays a central role in the loss of renal function in chronic renal disease, but the exact mechanism remains largely unknown (Lee and Kalluri, 2010). A number of studies have demonstrated that albuminuria is not only a well-recognised hallmark of kidney disease but also a pathogenic factor involved in the development of proteinuric nephropathy, which is characterised by alterations in proximal tubular epithelial cell (PTEC) death and inflammatory cytokine production (Abbate et al., 2006; Baines and Brunskill, 2011; Eddy and Giachelli, 1995; Li et al., 2010). Albuminuria stimulates proximal tubular cells to synthesise chemokines (MCP-1 and RANTES) that recruit monocytes and T cells and contributes to the release of cytokines that attract neutrophils and fibrosis-promoting molecules (e.g., endothelin, angiotensin II, and TGF-α) through phospholipase C, MAPK, or NF-κB signal activation (Drumm et al., 2002; Gomez-Garre et al., 2001; Gorriz and Martinez-Castelao, 2012; Han et al., 2005; Liu et al., 2009; Takase et al., 2008; Wang et al., 1999). These studies raised a concern about the pathogenic role of proteinuria in the progression of chronic renal disease. However, the exact molecular mechanisms that regulate inflammation by albuminuria have not been fully elucidated.

More recent studies have focused on innate immune-sensing receptors and their role in inflammation and disease processes. The Nlrp3 inflammasome is a cytoplasmic multiprotein that contains the Nod-like receptor and ASC adaptor (Lorenz et al., 2014)

*Abbreviations:* TIF, tubulointerstitial inflammation; NLR, nucleotide-binding domain leucine-rich repeat-containing (protein/family); ASC, apoptosis-associated speck-like protein containing a CARD; siRNA, small interfering RNA; NAC, N-acetyl-L-cysteine; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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and triggers the activation of caspase-1, IL-1β and IL-18 to engage innate immune defences and execute inflammatory responses (Latz et al., 2013; Schroder and Tschopp, 2010; Shi et al., 2012). Previous studies have confirmed that the inflammasome is activated by a variety of stimuli, including pathogens associated with virtually every type of pathogen-associated molecular pattern (PAMP). It is also triggered by several danger signals, including pore-forming toxins from bacteria, extracellular ATP (Mariathasan et al., 2006), ROS (Martinon et al., 2009; Zhou et al., 2010), monosodium urate crystals (Martinon et al., 2006), nucleic acids (Muruve et al., 2008), extracellular matrix components, including hyaluronan (Yamasaki et al., 2009), biglycans (Babelova et al., 2009), and environmental microparticles, such as asbestos and silica (Dostert et al., 2008; Martinon, 2012). The activation of the Nlrp3 inflammasome requires a two-step signal. Initially, the transcription and translation of immature pro-IL-1β (pro-IL-18) mRNA is induced via NF-κB activation by a primary signal, which may be derived from TLR, TNFR or IL-1R signalling. A secondary signal then activates the inflammasome and cleaves mature IL-1β/IL-18 released by inflammasome stimuli (Gross et al., 2011; Lorenz et al., 2014).

A strong association between dysregulated inflammasome activity and certain human inflammatory diseases suggests the importance of this pathway in innate immune responses, such as gouty arthritis (Martinon et al., 2006), type 2 diabetes mellitus, atherosclerosis, and inflammatory bowel disease (Strowig et al., 2012). Previous studies have demonstrated that IL-18 and caspase-1, which are two key markers of Nlrp3 inflammasome activation, are expressed in the renal tubular epithelium of patients with chronic kidney disease, suggesting that the Nlrp3 inflammasome may play an important role in regulating inflammation in kidney diseases (Gauer et al., 2007; Lonnemann et al., 2003). However, the role of albuminuria in the activation of the tubular Nlrp3 inflammasome and its subsequent contribution to tubulointerstitial inflammation remains speculative. The purpose of this study was therefore to investigate whether albuminuria directly induces mROS generation to activate the Nlrp3 inflammasome pathway in tubular cells during the genesis of tubulointerstitial inflammation.

## 2. Materials and methods

### 2.1. Animal model

Protein-overload nephropathy (Eddy, 1989) was induced via intraperitoneal injection of bovine serum albumin (BSA) in male Wistar rats one week after right nephrectomy (initial weight 120–130 g, Academy of Military Medical Science, Animal Experiment Centre). The rats were fed standard rat chow ad libitum, given free access to water and randomly divided into two groups. In the albumin-overload group (AO, $n = 10$), the rats were administered a daily intraperitoneal injection of BSA (5.0 g/kg/d, fatty acid-free, low endotoxin, Roche). The rats in the control group ($n = 8$) received an intraperitoneal injection of an equivalent volume of saline at pH 7.4. BSA was dissolved in normal saline at a concentration of 33% (pH 7.4). The BSA injection was sustained for nine weeks. At the end of week 10, the animals were anesthetised with chloral hydrate and sacrificed. The animal care protocols and experimental protocol used in this study were approved by the Ethics Review Committees for Animal Experimentation of Southeast University.

### 2.2. Urine and blood measurements

The body weight was measured weekly. Samples were collected every 24 h from rats housed in metabolic cages for 0, 2, 5, 7, 9 and 10 weeks with access to drinking water only. The urinary protein and albumin excretion were measured using the Coomassie Blue method (Jiancheng, Nanjing) or an ELISA kit according to the manufacturer's instructions. The urinary N-acetyl-β-D-glycosaminidase (NAG) levels was measured using an ELISA kit (Jiancheng, Nanjing). Blood samples were collected on weeks 0, 2, 5, 7, 9 and 10 (at death) from the inner canthus or heart after sacrifice in the 10th week to assess the changes in biochemical parameters (Hitachi, Tokyo, Japan).

### 2.3. Renal histological preparation and assessment

The left kidneys were collected after perfusion with 50 ml of ice-cold normal saline. A portion of coronal tissue was fixed with 10% buffered formalin and then embedded in paraffin for staining with haematoxylin-eosin (HE), periodic acid-Schiff reagent (PAS), and immunohistochemistry. To assess the pathological scoring for tubular injury, the PAS-stained slides (3 μm-thick sections) were reviewed by two independent renal pathologists in a blinded manner. A tubular injury scoring system adapted from Vilaysane et al. (2010) was used. The percentage of cortical tubular necrosis was assigned a score as follows: 0 = normal, 1 = <20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, and 5 = >80%. The levels of tubulointerstitial inflammation in 20 randomly selected non-overlapping fields from the kidney of each rat or IgAN patient were scored at 400× magnification with periodic acid-Schiff staining. The scoring of interstitial inflammatory cell infiltration in each specimen was as follows: 0 = normal, 1 = <25% of the interstitium was affected, 2 = 25–50% of the interstitium was affected, and 3 = >50% of the interstitium was affected (Yu et al., 2010).

### 2.4. Immunohistochemistry staining

For analysis and localising the expression of the endocytic receptors, the components of the renal tubular Nlrp3 inflammasome were examined. Briefly, paraffin-embedded sections from the kidney cortex were incubated with primary antibodies to Nlrp3, caspase-1, ASC, IL-18 (Santa Cruz Biotechnology, USA) and IL-1β (Cell Signalling Technology, USA), and the sections from renal biopsies of patients with IgA nephropathy were incubated with primary antibodies to Nlrp3 and IL-18. The sections were then analysed using an appropriate immunohistochemical kit processing (Maxim, China) according to the manufacturer's instructions. An immunohistochemistry semiquantitative analysis was conducted using the Image Pro Plus image analysis system.

### 2.5. Electron microscopy analysis

For transmission electron microscopy (TEM) observations of ultrastructural changes in proximal tubular epithelial cells, the kidneys or collected HK-2 cell clusters were immersed in a fixative containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer. After fixation and dehydration with ethanol, the samples were embedded in Durcupan resin for ultra-thin sectioning and TEM examination in the VCU electron microscopy core facility.

### 2.6. Tubular epithelial cell isolation and mitochondrial extraction

The renal capsule was removed, and the cortex was dissected from the medulla. The cortex was then finely minced to 1–2 $mm^3$, washed with PBS, spun three times at 800 × g, placed in collagenase A (Sigma–Aldrich, St. Louis, MO), and incubated at 37 °C for 30 min with frequent shaking. After incubation, the digested mixture was differentially sieved through descending pore sizes (80, 100, and 200 mesh) and washed three times with fresh media. The sieved contents were then centrifuged, pelleted, and layered over 25 ml of 45% Percoll (Sigma–Aldrich, USA). After spinning the Percoll