〈Regular Article〉

The deficient of eNOS-NO pathway exacerbates kidney dysfunction via inflammasome activation in diabetic kidney disease

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ABSTRACT **Background**: Diabetic kidney disease (DKD) is a major cause of end-stage kidney disease. We have reported that inflammasome activation is involved in the pathogenesis of kidney disease and that the disruption of the endothelial nitric oxide synthase (eNOS)-nitric oxide (NO) pathway promotes inflammasome activation and renal interstitial inflammation in hypertensive and aging kidney models. Endothelial dysfunction, which is involved in the pathogenesis of renal injury, may regulate inflammasome activation. However, it is not known whether endothelial dysfunction promotes the progression of DKD via inflammasome activation.

Methods: Apoptosis-associated speck-like protein (ASC) is one of the components constituting the inflammasome. We crossed eNOS-deficient mice (eNOSKO) with ASC-deficient mice to create eNOS-ASC-double-deficient mice (eNOS-ASC-DKO). C57BL / 6 (WT), eNOSKO and eNOS-ASC-DKO were administered streptozotocin (STZ) to induce diabetes. Urine storage and blood pressure measurements were taken in these groups. Eight weeks after the onset of diabetes, they were sacrificed and examined. Tissue damage was examined by PAS staining, Masson staining, and tissue immunohistochemistry.

Results: The exacerbation of glomerular lesions were observed in eNOSKO-STZ mice. Kidney injury molecule-1 (KIM-1) positive impaired proximal tubules increased as well. The interstitial fibrosis was significantly increased. These changes were rescued by ASC deficiency. In addition, macrophage infiltrated into the glomeruli in eNOS-deficient mice. Interestingly, these

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inflammatory cell infiltrations were suppressed in eNOS-ASC-DKO-STZ mice.

Conclusions: This study used a DKD model to demonstrate the impact of endothelial dysfunction on chronic inflammation. The results indicate that controlling inflammasome activation may inhibit the progression of kidney disease.

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Key words：Endothelial cell, ASC-deficient mice, Chronic inflammation, Diabetes mellitus

INTRODUCTION

Chronic kidney disease (CKD) is a significant global public health concern and is linked to increased mortality. Among various kidney diseases, diabetic kidney disease (DKD) is particularly critical as it is the primary cause of dialysis initiation. Endothelial dysfunction plays a crucial role in the initiation and progression of DKD. NAD(P)H oxidase activation in endothelial cells has been identified as an early trigger for albuminuria[1].

Furthermore, it is well-established that endothelial nitric oxide synthase (eNOS)-deficient mice experience an accelerated progression of renal damage after the onset of diabetes due to an impaired eNOS-NO pathway[2].

Chronic inflammation, however, is known to be a common pathway for kidney injury; it has been reported that TLR4 signal is activated in glomeruli in DKD[3]. More recently, inflammasome activation has also been reported to be important. Inflammasomes are generated as an innate immune response to either exogenous pathogens or endogenous danger signals. Inflammasome is a multiprotein complex composed of pattern recognition receptors (PRRs), apoptosis-associated speck-like protein (ASC), and pro-caspase-1[4]. In particular, NLRP3, one of the PRRs, has been reported to be associated with various diseases such as autoimmune disorders, neurodegenerative diseases, and metabolic disorders. Activated NLRP3 inflammasomes activate caspase-1, which matures proinflammatory cytokines such as IL-1β and IL-18[5]. The significance of inflammasome activation has also been reported in renal diseases, especially DKD. In KK-Ay mice, the suppression of NLRP3 inflammasome activation prevents the progression of renal damage[6].

Moreover, we have reported that disruption of the eNOS-NO pathway promotes renal interstitial inflammation by inflammasome activation in hypertensive and aging renal models[7, 8]. These facts suggest that endothelial dysfunction involved in the development of renal injury may regulate inflammasome activation.

However, its role in DKD is unknown. In this study, we examined the relationship between endothelial dysfunction and inflammasome activation in DKD using eNOS- and ASC-deficient mice.

MATERIAL AND METHODS

Animal

The experimental protocols followed in the current study were approved by the Ethics Review Committee for Animal Experimentation at Kawasaki Medical School, Kurashiki, Japan (20-076; 9 July 2020). C57BL / 6 (WT) and eNOS KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). ASC KO mice were kindly provided by Takahashi M (Jichi Medical University, Shimotsuke, Japan)[9]. Subsequently, eNOS-ASC-DKO mice were generated by intercrossing eNOS KO and ASC KO mice[8]. The mice were housed in a temperature- and humidity-controlled room with a 12-hour light–dark cycle and were fed standard laboratory animal chow with free access to tap

water. All mice in each group (n = 3-6, male) were euthanized at 16-18 weeks of age, and blood and kidney tissue were collected. At the beginning of the experiment (8 weeks old), the mice weighed 19-35 g. Blood samples were collected by inserting a 26-gauge needle into the right ventricle of the mouse. Kidneys were harvested from the body after perfusion with phosphate-buffered saline (PBS) (20 mL) from the left ventricle.

Physiological and Biochemical Measurements

Body weights were recorded, and blood pressures and pulse rates were measured using the tail-cuff method with an automatic sphygmomanometer (BP98A; Softron Co., Ltd., Tokyo, Japan). Blood glucose was measured by using Glutestmint (Sanwa Chemistry, Aichi, Japan). The 24 h urine samples were collected on the day before euthanasia. Urinary albumin levels were determined by ELISA using the primary monoclonal antibody of mouse albumin (A90-134A, Bethyl Laboratories, TX, USA). Urinary creatinine was measured using commercially available LabAssay Creatinine (Wako Pure Chemical Industries, Osaka, Japan). Serum creatinine and urea nitrogen levels were measured at a central clinical laboratory (SRL, Inc., Tokyo, Japan).

Histologic Analysis and Immunohistochemistry

Right kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin for histologic analysis. Tissue sections were deparaffinized and stained with periodic acid-Schiff (PAS) (2 μm thick) and Masson trichrome (Masson) staining (4 μm thick)[7]. Immunochemical staining was conducted on the deparaffinized kidney sections (4 μm thick) by heating them in a microwave at 500 W for 15 min for antigen retrieval, followed by incubating them overnight with an antibody against kidney injury molecule-1 (KIM-1) (AF1817, R&D Systems, Minneapolis, MN, USA), an alpha smooth muscle Actin (ab5694, Abcam, Cambridge, UK), Collagen IV (ab6586, Abcam, Cambridge, UK) and F4 / 80 (MCA497GA; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary antibody was detected using the Histofine Simple Stain MAX-PO kit (Nichirei Corporation, Tokyo, Japan) and 3, 3' -diaminobenzidine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All images were obtained with an all-in-one fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan). The fibrotic areas (stained blue with Masson's trichrome) and KIM-1 and Collagen IV positive areas were automatically calculated using a hybrid cell count application in the BZ-X Analyzer software (BZ-Ⅱ Analyzer, KEYENCE, Osaka, Japan). These results were expressed as a percentage of area in the scanned interstitium. The α-SMA and F4 / 80 positive areas were measured similarly. These results were expressed as a percentage of area in glomeruli.

Immunoblot Analysis

SDS-PAGE was performed (20-30 μg protein / lane) with antibodies against KIM-1 (AF1817, R&D Systems, Minneapolis, MN, USA) and glyceraldehyde phosphate dehydrogenase (1E6D9, proteintech, Rosemont, Illinois, USA). Signals were detected using Amersham ECL Western Blotting Detection Reagents (Cytiva, Marlborough, MA, USA). The relative optical densities of the bands were quantified using Image J software version 1.53t.

Statistical Analyses

All values are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Parameters were evaluated using one-way ANOVA analysis of variance for comparison of multiple means. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Assessment of glomerular damage

Physiological characteristics at 8 weeks after STZ administration are shown in Table 1. The mice developed diabetes after streptozotocin (STZ) administration, and WT-STZ, eNOSKO-STZ, and eNOS-ASC-DKO-STZ all showed significantly increased blood glucose levels compared to WT. There were no significant differences in blood glucose levels between WT-STZ, eNOSKO-STZ and eNOS-ASC-DKO-STZ.

Systolic blood pressure was significantly higher in eNOSKO and eNOS-ASC-DKO compared to WT. eNOSKO and eNOS-ASC-DKO were not significantly different. Urinary albumin excretion was significantly increased in eNOSKO-STZ compared to WT-STZ (Fig. 1A). eNOSKO-STZ was evaluated for glomerular sclerosis lesions by PAS staining. Glomerulosclerosis was observed in eNOSKO-STZ compared to WT-STZ (Fig. 1B). To evaluate mesangial cell damage, α-SMA staining was performed. α-SMA positive area was increased in eNOSKO-STZ mice compared to WT-STZ. These glomerular damages observed in eNOSKO-STZ were ameliorated in eNOS-ASC-DKO-STZ (Fig. 1A-D).

Assessment of macrophage infiltration

To assess inflammation, F4 / 80 staining of kidney tissue was performed to evaluate macrophage infiltration. The positive area of F4 / 80 was increased in eNOSKO-STZ mice compared to WT-STZ. This macrophage infiltration observed with eNOSKO-STZ was improved with eNOS-ASC-DKO-STZ (Fig. 2A, B).

Assessment of tubulointerstitial fibrosis

The extent of fibrosis was evaluated by Masson staining and Collagen IV staining. In both cases, the area of fibrosis was increased in eNOSKO-STZ compared to WT-STZ (Fig. 3A-D). These tubulointerstitial fibroses seen in eNOSKO-STZ were improved in eNOS-ASC-DKO-STZ (Fig. 3A-D).

Assessment of proximal tubular damage

Tissue immunostaining and immunoblot of kidney tissues were performed using KIM-1 antibody as a marker of proximal tubular damage. eNOSKO-STZ increased KIM-1 positive areas compared to WT-STZ (Fig. 4A, B). These tubular defects observed in eNOSKO-STZ were ameliorated in eNOS-ASC-DKO-STZ (Fig. 4A, B). In immunoblot, eNOSKO-STZ showed a significant increase in the KIM-1 protein compared to WT-STZ (Fig. 4C, D).

DISCUSSION

The purpose of this study was to determine the relationship between endothelial dysfunction and inflammasome activation in DKD. The results showed an exacerbation of glomerular lesions in eNOSKO-STZ. KIM-1 positive impaired proximal tubules were similarly increased. In addition, interstitial fibrosis was exacerbated as well. These

Table 1. Physiological data

	Body weight (g)	Systolic blood pressure (mmHg)	Blood glucose (mg / dL)
WT	31.7 ± 0.8	106 ± 2	214 ± 21
WT-STZ	24.6 ± 1.6	98 ± 4	573 ± 39*
eNOSKO	28.5 ± 1.4	141 ± 4* †	–
eNOSKO-STZ	22.7 ± 0.4	134 ± 3* †	649 ± 81*
eNOS-ASC-DKO	32.7 ± 2.6	135 ± 3* †	–
eNOS-ASC-DKO-STZ	24.1 ± 0.9	126 ± 3* †	696 ± 59*

average ± SEM, *P < 0.05 vs. WT; † P < 0.05 vs. WT-STZ.

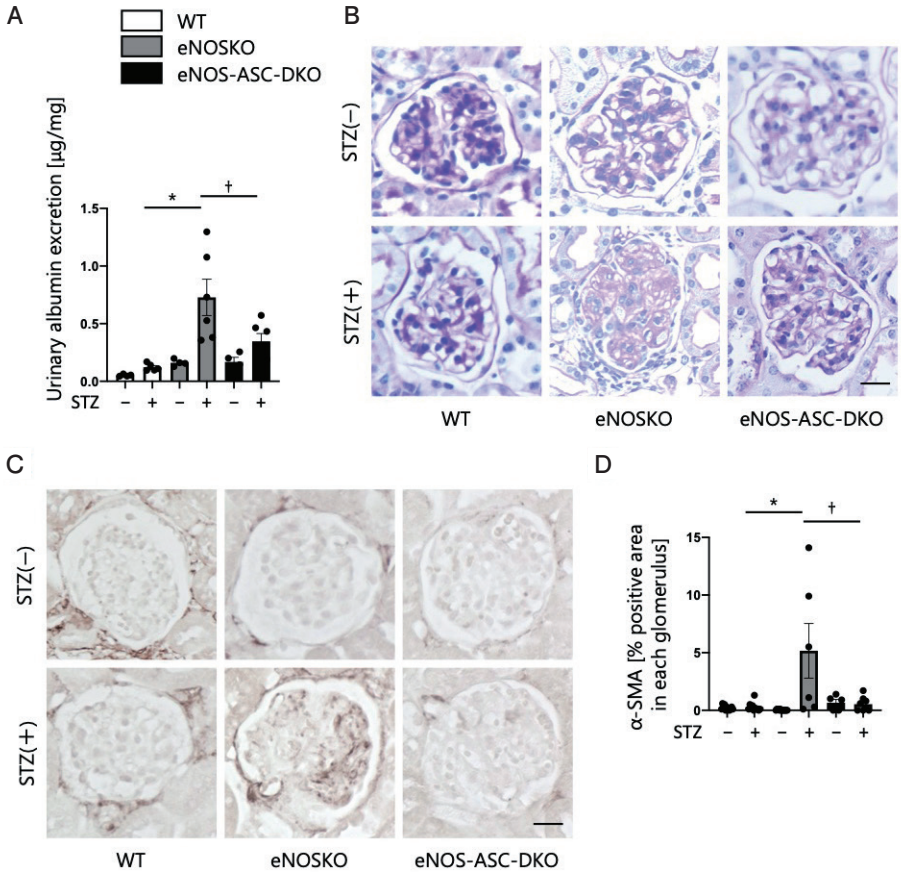

Fig. 1. Evaluation of kidney function and glomerular damage
(A) Measurement of U-Alb / U-Crn (n = 4～6). (B) PAS staining of glomeruli (bar = 20 μm). (C) Immunohistochemical staining for α-SMA (bar = 20 μm). (D) Percentage of α-SMA positive area in one glomerulus. Data are expressed as means ± SEM. * $P < 0.05$ versus WT-STZ; † $P < 0.05$ versus eNOSKO-STZ. Bar = 20 μm. WT, Wild Type; eNOSKO, eNOSKO mice; eNOS-ASC-DKO, eNOS-ASC-DKO mice; U-Alb / U-Crn, urinary albumin / urinary creatinine; PAS, periodic acid-schiff; α-SMA, alpha-smooth muscle actin.

changes were ameliorated by ASC deficiency. In addition, macrophage infiltration was observed in the glomeruli of eNOS-deficient mice. eNOS-ASC-DKO-STZ suppressed these inflammatory cell infiltrations. These results suggest that disruption the eNOS-NO pathway promotes DKD progression. The involvement of increased inflammasome activation was suggested as a possible mechanism.

One of the most important endothelial functions is the ability to generate bioavailable NO. It is known that decreased production of bioavailable NO occurs in various renal disorders, including DKD. We have reported that eNOS uncoupling is one of the mechanisms of eNOS dysfunction, especially in glomeruli[10]. It is not known, however, how reduced NO production may promote glomerular or interstitial lesions. Our studies have shown that NO suppresses chronic inflammation and fibrotic lesions of the tubulointerstitium. Meanwhile, ischemia may occur when NO levels are reduced. Since chronic ischemia is an important common pathway for interstitial fibrosis[11], disrupting the

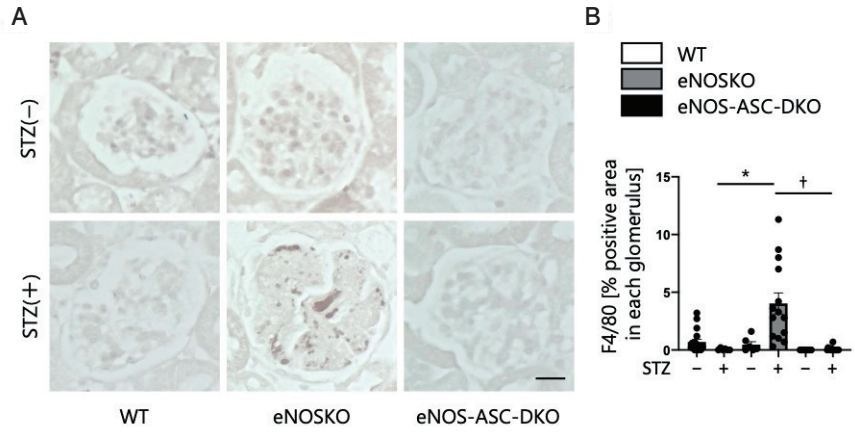

Fig. 2. Evaluation of macrophage infiltration into the glomerulus
(A) Immunohistochemical staining for F4 / 80 (bar = 20 μm). (B) Percentage of F4 / 80 positive area in one glomerulus. Data are expressed as means ± SEM. * P < 0.05 versus WT-STZ; † P < 0.05 versus eNOSKO-STZ. Bar = 20 μm. WT, Wild Type; eNOSKO, eNOSKO mice; eNOS-ASC-DKO, eNOS-ASC-DKO mice.

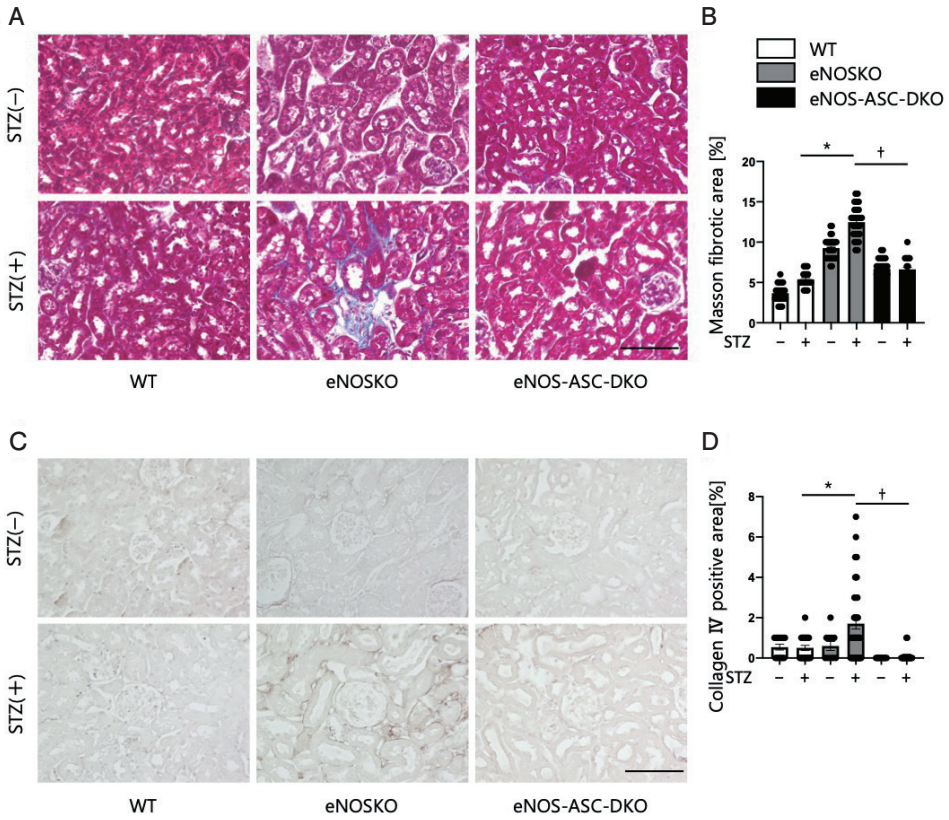

Fig. 3. Evaluation of tubulointerstitial fibrosis
(A) Masson trichrome staining. (B) Percentage of aniline blue-positive area in Masson trichrome staining. (C) Immunohistochemical staining for Collagen Ⅳ. (D) Percentage of Collagen Ⅳ positive area. Data are expressed as means ± SEM. * P < 0.05 versus WT-STZ; † P < 0.05 versus eNOSKO-STZ. Bar = 100 μm. WT, Wild Type; eNOSKO, eNOSKO mice; eNOS-ASC-DKO, eNOS-ASC-DKO mice.

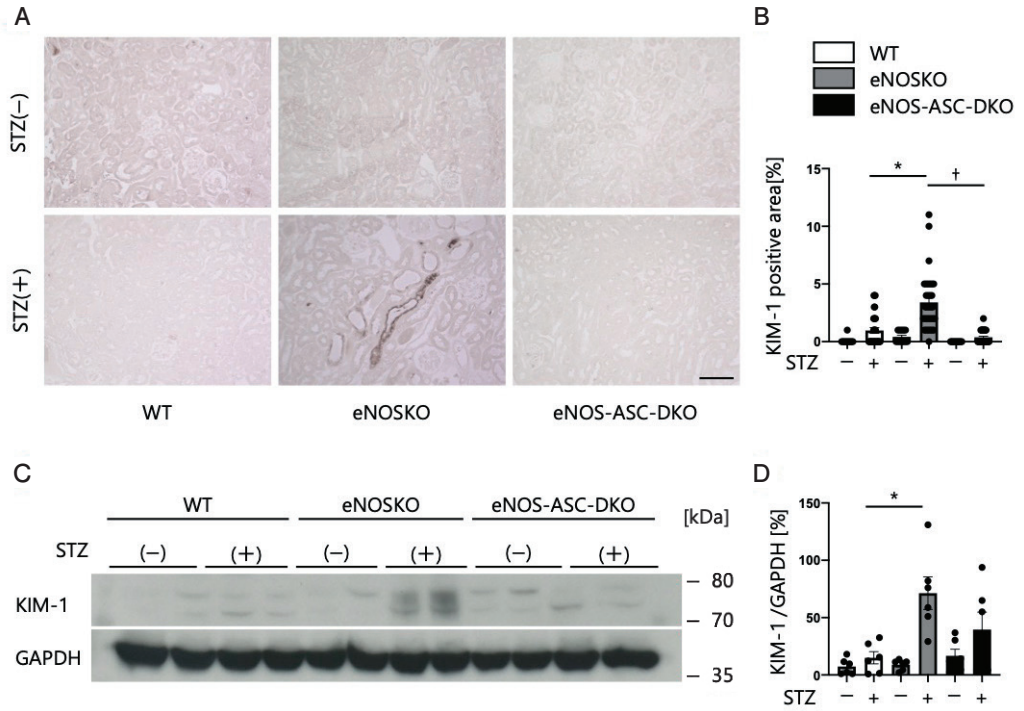

Fig. 4. Evaluation of KIM-1 level expression
(A) Immunohistochemical staining for KIM-1. (B) Percentage of KIM-1 positive area. (C) Immunoblot analysis quantifying kidney expression of KIM-1 (n = 6). (D) KIM-1 protein level expressed as fold change (n = 6). Data are expressed as means ± SEM. * P < 0.05 versus WT-STZ; † P < 0.05 versus eNOSKO-STZ. Bar = 100 μm. WT, Wild Type; eNOSKO, eNOSKO mice; eNOS-ASC-DKO, eNOS-ASC-DKO mice; KIM-1, kidney injury molecule 1.

eNOS-NO pathway may have promoted interstitial fibrosis via ischemia. The present study suggested that ASC deficiency could be improved interstitial fibrotic lesions, indicating a potential link to chronic inflammation, partially attributed to inflammasome activation. (Fig. 2, 3).

It is still unclear in which cells inflammasome activation occurs in DKD. Inflammasome activation in glomerular epithelial cells has been reported to be important in DKD[12, 13]. It also cannot be excluded in DKD, as inflammasome activation of the collecting ducts has been reported to be important in other models[14]. Recently, it has also been found that infiltration of bone marrow-derived cells is prominent in glomerular lesions in diabetes[15]. Thus, depending on the pathological model and stage of the disease, which cells are important for inflammasome activation may be variable. In our study, F4 / 80-positive cells were also infiltrating, suggesting macrophage involvement (Fig. 2). However, our study used general ASC-deficient mice, and thus we cannot comment on this point.

Various types of inflammasome exist; it has been reported that NLRP3 inflammasome inhibitors could be a potential treatment for DKD[16, 17]. NLRP3 inflammasome is activated by a two-hit theory. In general, the TLR4 signal is important as the first hit and is subsequently activated by various danger signals[18]. Furthermore, AIM2 inflammasome has been found to be involved in inflammation in renal disease[19]. In this study, we used ASC deficiency, as ASC is an essential protein for different types of inflammasome activation. Therefore, our results do not allow us to identify which type of inflammasome

activation is important. In the future, we would like to prove this using drug intervention and other methods.

This study demonstrates the impact of endothelial dysfunction on chronic inflammation using the DKD model. We found that regulating inflammasome activation may inhibit the progression of renal disease.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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