



## OPEN ASK-1 activation exacerbates kidney dysfunction via increment of glomerular permeability and accelerates cellular aging in diabetic kidney disease model mice

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Diabetic kidney disease (DKD) is a major disease characterized by early albuminuria and heightened risk of renal deterioration. Increased reactive oxygen species (ROS) production, especially in glomeruli, plays an important role in the progression of DKD. ROS also cause activation of Apoptosis signal-regulating kinase 1 (ASK-1), which is implicated in various organ injuries. However, the detailed mechanisms remain unclear. This study investigates ASK-1 activation in advanced DKD and its underlying mechanisms using GS442172, an ASK-1 inhibitor. In the DKD mouse model, activation of ASK-1 was observed. Although inhibition of ASK-1 activation improved hyperpermeability in glomerular endothelial cells. ASK-1 inhibition significantly reduced glomerular injury and albuminuria, while also attenuating tubular damage and interstitial fibrosis. RNA-seq analysis revealed an aging phenotype associated with ASK-1 activation in DKD. In vitro experiments demonstrated ASK-1 activation-induced cellular senescence in tubular cells via redox signaling. These results suggested that the critical role of ASK-1 activation in DKD pathogenesis, implicating glomerular injury, tubular damage, and cellular senescence. ASK-1 inhibitors are promising therapeutic strategies to mitigate the progression of DKD.

Chronic kidney disease (CKD) is a global public health burden, with a high prevalence and high risk of progression to end-stage renal disease (ESRD), cardiovascular disease (CVD), and premature death. In particular, DKD is an important disease for which there is still no adequate treatment to control its progression. DKD is characterized by the presence of albuminuria from an early stage. The albuminuria has been shown to be a strong risk factor for progression to renal failure<sup>1</sup>.

In DKD, elevated reactive oxygen species (ROS) production in the glomerulus occurs early in disease. ROS production sources include increased NADPH oxidase activity<sup>2</sup> and free-radical species derived from dysfunctional mitochondria<sup>3</sup>. Normal cellular function is maintained by appropriate ROS production<sup>4</sup>. However, excessive ROS production has been shown to cause progressive renal dysfunction<sup>5</sup>. Increased NADPH oxidase activity in glomerular endothelial cells promotes the onset of albuminuria<sup>6</sup>. Thus, ROS increases play an important role in the onset and progression of DKD.

Apoptosis signal-regulating kinase 1 (ASK-1) is a ubiquitously-expressed, apical MAPK that is activated by increased oxidative stress<sup>7</sup>. ASK-1 is normally bound and repressed by thiol-containing antioxidant proteins, including thioredoxins in the cytosol and mitochondria<sup>7</sup>. In settings of elevated oxidative stress, thioredoxin undergoes oxidation and dissociation from ASK-1, leading to autophosphorylation of ASK-1 at threonine 845 (ASK-T845) and resulting in ASK-1 activity<sup>7</sup>. ASK-1 activation has been reported to be involved in the progression of organ injury by promoting apoptosis, inflammation, and fibrosis<sup>8,9</sup>. ROS-mediated ASK-1 activation has been shown to be important in the transition to heart failure after myocardial infarction<sup>10</sup>. In glomeruli of DKD also, ASK-1 is known to be activated by ROS<sup>11</sup>. It has also been shown that ER stress in glomerular epithelial cells activates ASK-1, which is involved in the disruption of the coagulation wall, and it has

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been reported that ASK-1 has a certain role in glomerular pathogenesis<sup>11</sup>. These preclinical data indicate that ASK-1 activation is responsible for the development of renal damage in diabetic kidney disease. However, the detailed mechanism is unknown.

On the other hand, the importance of tubular and interstitial lesions as well as glomerular lesions in the development of renal damage has been reported. Excess albumin filtered from the glomerulus is reabsorbed in the tubules. Free fatty acid bound to albumin has been shown to induce mitochondrial stress due to excessive  $\beta$ -oxidation during tubular metabolism. This also results in an increase in mitochondria-derived ROS, leading to the secretion of inflammatory cytokines and increased inflammation<sup>12,13</sup>. This is an important pathway for renal injury. Thus, increased reactive oxygen species have been shown to be an important driver in tubular injury as well. However, the significance of ASK-1 activation in tubular injury is unknown.

Therefore, it is possible that the damaging consequences of oxidative stress in diabetic kidney disease progresses occurs as a result of ASK-1 activation. In this study, we evaluated the significance of ASK-1 activation in advanced diabetic kidney disease and provide insight into the mechanism of renal damage progression using GS-444217, an ASK-1 inhibitor, which has been shown previously to protect against kidney fibrosis and dysfunction in rodent models of kidney disease<sup>14,15</sup>.

## Results

### ASK-1-p38 pathway activation in DKD model mice

Physiological characteristics at sacrifice are shown in Table 1. Trends in body weight and blood glucose are also shown in the Supplementary Table. We first evaluated ASK-1 activation in a DKD model by western blotting of phosphorylation of p38, a downstream marker of ASK-1 activity. Compared to db/<sup>misty</sup> as control mice, eNOS-KO db/db mice showed increased phosphorylation of p38 (Fig. 1-A,B), which was abolished by the ASK-1 inhibitor GS-444217. There is a report that increased staining intensity was observed in p-p38 immunostaining in eNOS-KO/dbdb glomeruli<sup>15</sup>. In addition, immunohistochemistry revealed that ASK-1 was expressed in tubular cells (Supplementary Fig. 1). These results indicate that ASK-1 activation in the kidney occurs in diabetic kidney disease and is inhibited by GS-444217.

### ASK-1 inhibition reduced glomerular injury in DKD model mice

The tissues of mice aged 10 weeks before ASK-1 inhibitor administration, the eNOS-KO db/db mice showed marked glomerular sclerosis compared to the db/<sup>misty</sup> mice (Supplementary Fig. 2A–C). PAS staining after 8 weeks of drug administration are also shown in Fig. 2-A,B and Supplementary Fig. 3. eNOS-KO db/db showed prominent glomerulosclerosis, which were significantly suppressed by ASK-1 inhibition. At 8 weeks on administration, there was a marked increase in albuminuria in the eNOS-KO db/db group, but a significant decrease in the ASK-1 inhibitor group (Fig. 2-C). For further detail morphological evaluation, we observed the capillary wall by Transmission Electron Microscope (TEM). eNOS-KO db/db mice exhibited a disappearance of endothelial cell fenestrae, indicating morphological damage to the endothelial cells (Fig. 2-D, Supplementary Fig. 4). Foot process effacement was observed in the glomerular epithelial cells. In the group treated with ASK-1 inhibitor, some foot process fusion remained, but endothelial cell swelling was markedly improved. These results indicate that ASK-1 is involved in glomerular capillary wall injury, especially endothelial injury, in DKD.

### Expression of glycocalyx and VE-cadherin on capillary wall via ASK-1 activation

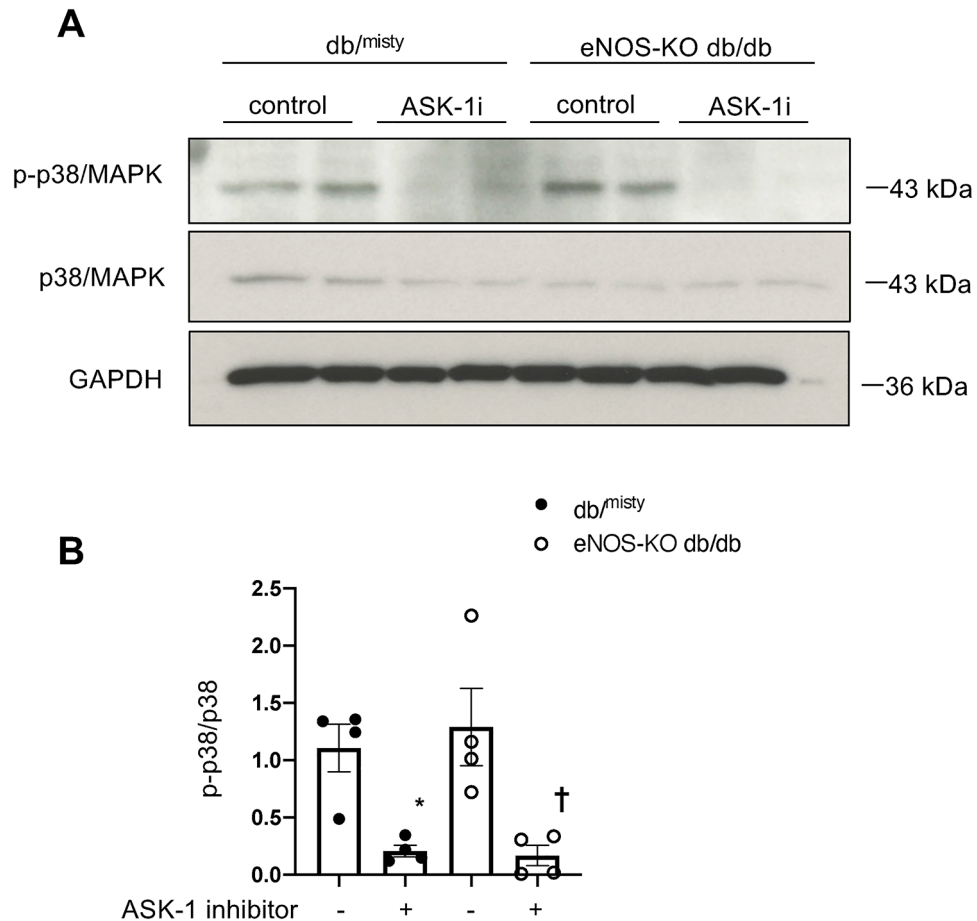
Glycocalyx and VE-cadherin, which are important for permeability control, were evaluated. VE-cadherin in endothelial cells was decreased in eNOS-KO db/db and was preserved in the ASK-1 inhibitor treated group (Fig. 3-A,C). Endothelial glycocalyx, which is important in regulating glomerular capillary wall permeability, was evaluated by lectin staining. Normal glomeruli show smooth lectin staining along the capillary wall, whereas eNOS-KO db/db shows a discontinuity of the lectin staining (Fig. 3-B,D) and WGA staining (Supplementary Fig. 5). To further investigate the molecular mechanism, we performed in vitro experiments using glomerular endothelial cells. Phosphorylation of VE-cadherin was observed after stimulation with H<sub>2</sub>O<sub>2</sub>, but was inhibited by ASK-1 inhibitors (Fig. 3-E,F). These results indicate that increased ROS in glomerular endothelial cells promote VE-cadherin degradation via ASK-1 activation (Fig. 3-G).

### ASK-1 activation induces tubular damage and interstitial fibrosis in DKD

Masson staining was used to evaluate interstitial fibrosis (Fig. 4-A). In eNOS-KO db/db, tubular cell collapse and fibrosis were observed, whereas in the ASK-1 inhibitor-treated group, the fibrosis was improved. The expression of fibrosis-related genes ( $\alpha$ SMA, Collagen1, Fibronectin1 and TGF $\beta$ ) was determined by quantitative PCR (Fig. 4-B). eNOS-KO db/db showed significantly elevated expression of these genes. ASK-1 inhibitor does not

	db/ <sup>misty</sup> control	db/ <sup>misty</sup> ASK-1 inhibitor	eNOS-KO db/db control	eNOS-KO db/db ASK-1 inhibitor
BW (g)	28.6 ± 1.3	22.7 ± 0.3	50.0 ± 1.4*	36.9 ± 1.7*†
BS (mg/dL)	148 ± 10	111 ± 9	368 ± 58*	380 ± 41*
BP (mmHg)	106 ± 1	98 ± 4	141 ± 13	137 ± 21
s-Cr (mg/dL)	0.10 ± 0.006	0.14 ± 0.005	0.10 ± 0.007	0.09 ± 0.007
BUN (mg/dL)	41.0 ± 3.0	32.0 ± 2.0	43.8 ± 2.9	35.3 ± 1.5†

**Table 1.** Average  $\pm$  SEM. BW Body weight, BS Blood sugar, BP Blood pressure. \*P < 0.05 vs. db/<sup>misty</sup> control. †P < 0.05 vs. eNOS-KO/db/db control.



**Fig. 1.** Expression levels of ASK-1-related genes increased in DKD model mice. These data were obtained from whole kidney tissue from 5 mice in each group. **(A)** Representative western blotting experiments and analysis data for phospho-p38/MAPK (Thr180/Tyr182), total-p38/MAPK and GAPDH. Original blots are presented in Supplementary Fig. 8-A,B. **(B)** phospho-p38/MAPK protein level expressed as fold change. Data are mean  $\pm$  SEM. \* $P < 0.05$  vs versus db/misty control. † $P < 0.05$  vs versus eNOS-KO/db/db control.

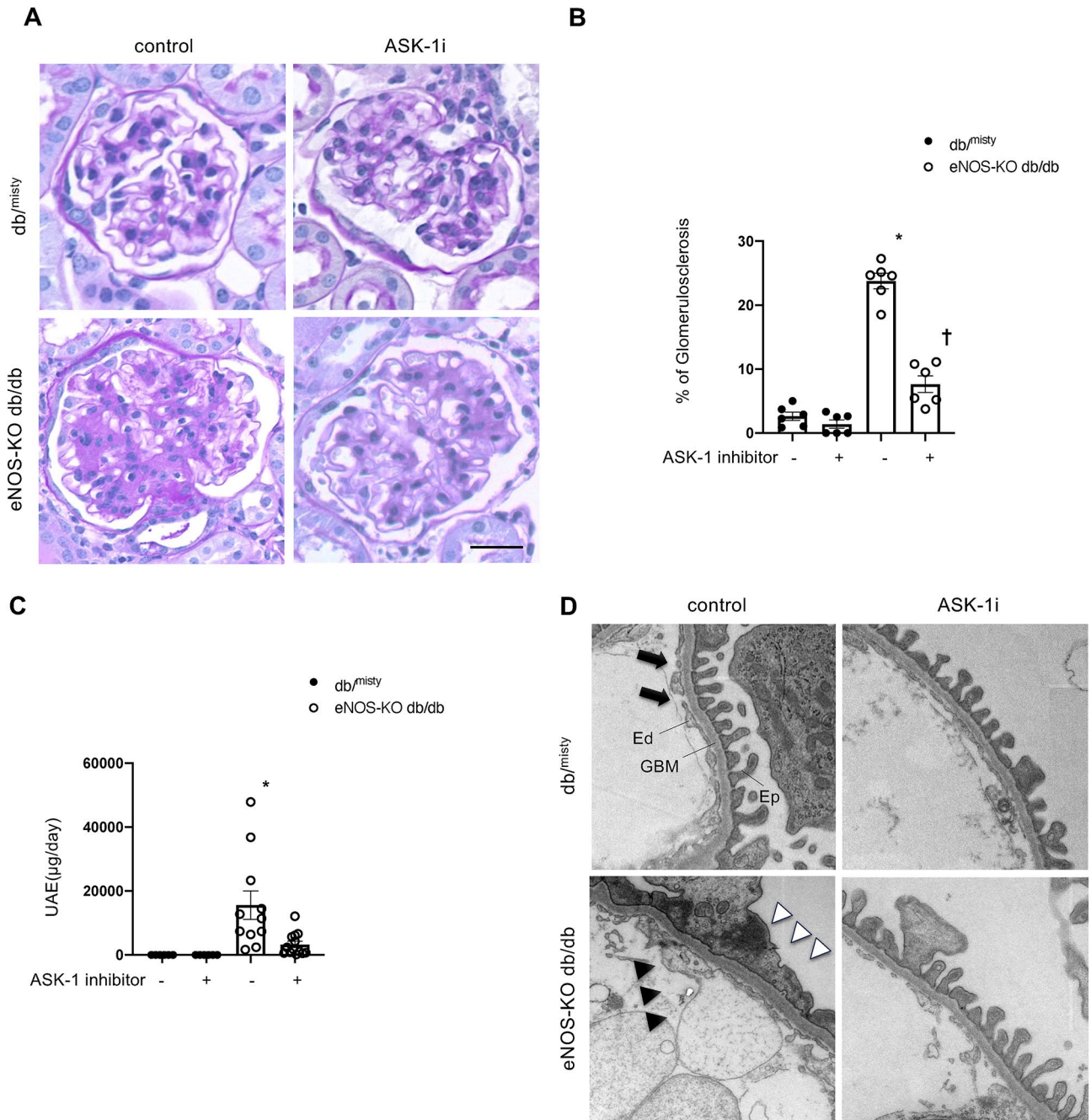
affect  $\alpha$ SMA gene expression compared with eNOS-KO db/db group. However, the expression of other fibrosis-related genes was significantly decreased with ASK-1 inhibitors. In addition, KIM-1 staining and urinary KIM-1 excretion were evaluated as markers of tubular damage (Fig. 4-C,D). The eNOS-KO db/db group showed a marked increase in the number of KIM-1 positive tubular cells and urinary KIM-1 excretion levels. ASK-1 inhibitor treatment suppressed these proximal tubular cell damage.

### RNA-seq data revealed ASK-1 activation leads to aging phenotype in DKD

We focused on clusters that are upregulated in eNOS-KO db/db compared to db/misty and are further downregulated by ASK-1 inhibitor (Fig. 5-A cluster 2). The results of the GO analysis are presented in the Supplementary Fig. 6. In particular, Fig. 5-B shows the results of Go analysis of cluster2, where most of the significant changes in molecular function were genes related to cytoskeleton and fibrosis. On the other hand, aging was observed in the GO term of the biological process. These results suggest that cellular senescence might be caused by ASK-1 activation depending on the pathology in DKD.

### Redox status induced cellular senescence via ASK-1 activation in tubular cells

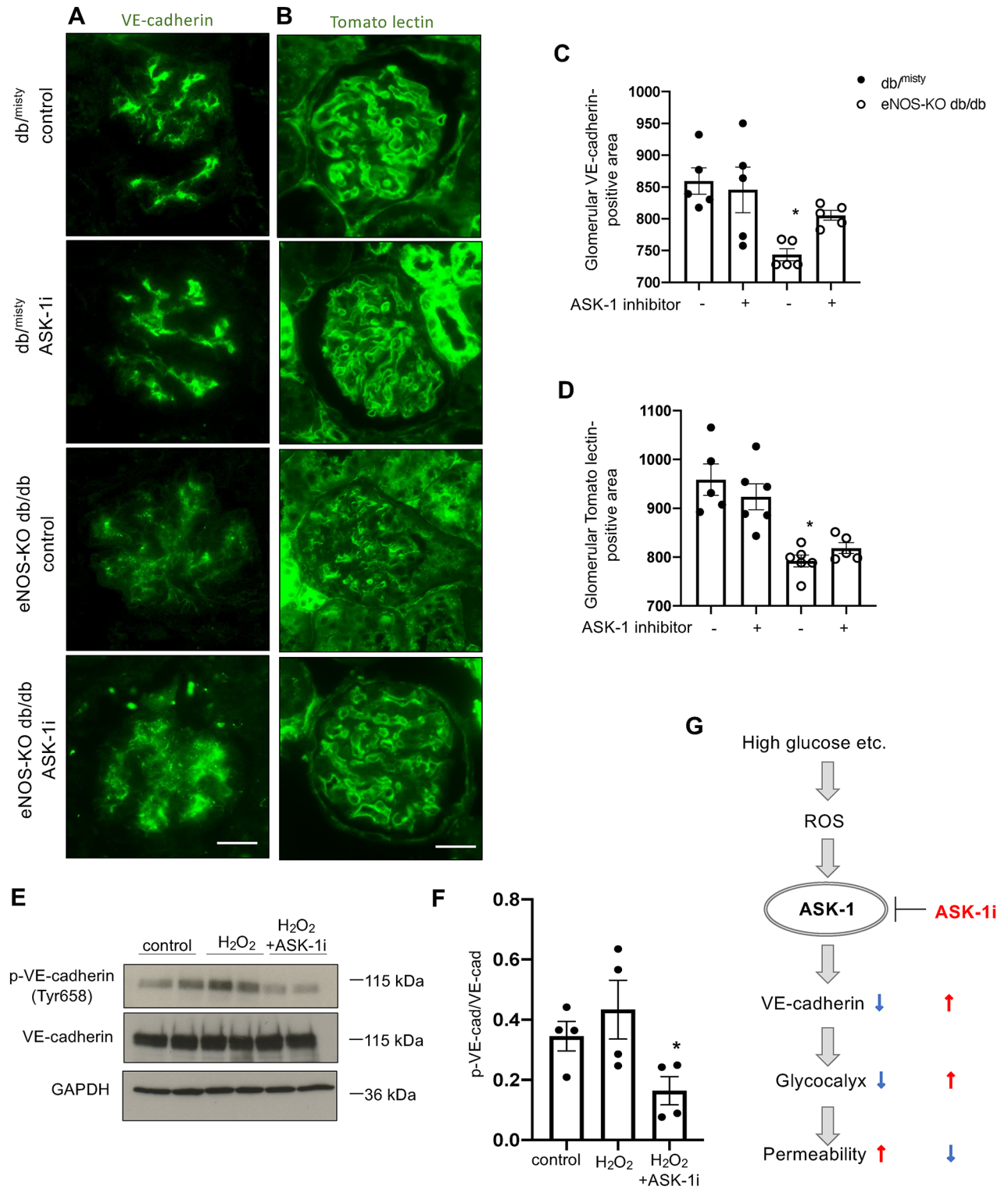
To investigate the direct involvement of ASK-1 activation and cellular senescence, we performed in vitro experiments using proximal tubular cells. SA- $\beta$ -GAL (senescence-associated beta-galactosidase) staining was used to evaluate cellular senescence. The SA- $\beta$ -GAL activity in tubular cells were increased by incubation with BSA + FFA (Fig. 6-A). This increase in activity was suppressed by administration of ASK-1 inhibitors. The p21 signaling pathway, a marker of aging, was evaluated by western blotting. Expression of p21 in Primary human proximal tubule epithelial cells (hPTECs; Lonza, Basel, Switzerland) were upregulated by the addition of BSA + FFA, but was suppressed by ASK-1 inhibitor treatment ( $P = 0.0983$  H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + ASK-1inhibitor) (Fig. 6-B,C). In vivo, eNOS-KO db/db mice showed higher p53 protein expression than db/misty mice, which was suppressed by ASK-1inhibitor treatment. Moreover, p21 protein levels, a transcriptional target of p53, was suppressed after treatment with ASK-1inhibitor (Fig. 6-D, Supplementary Fig. 7). The expression of genes associated with SASP (Senescence-associated secretory phenotype) (IL-1 $\beta$ , IL-18 and F4/80) was measured



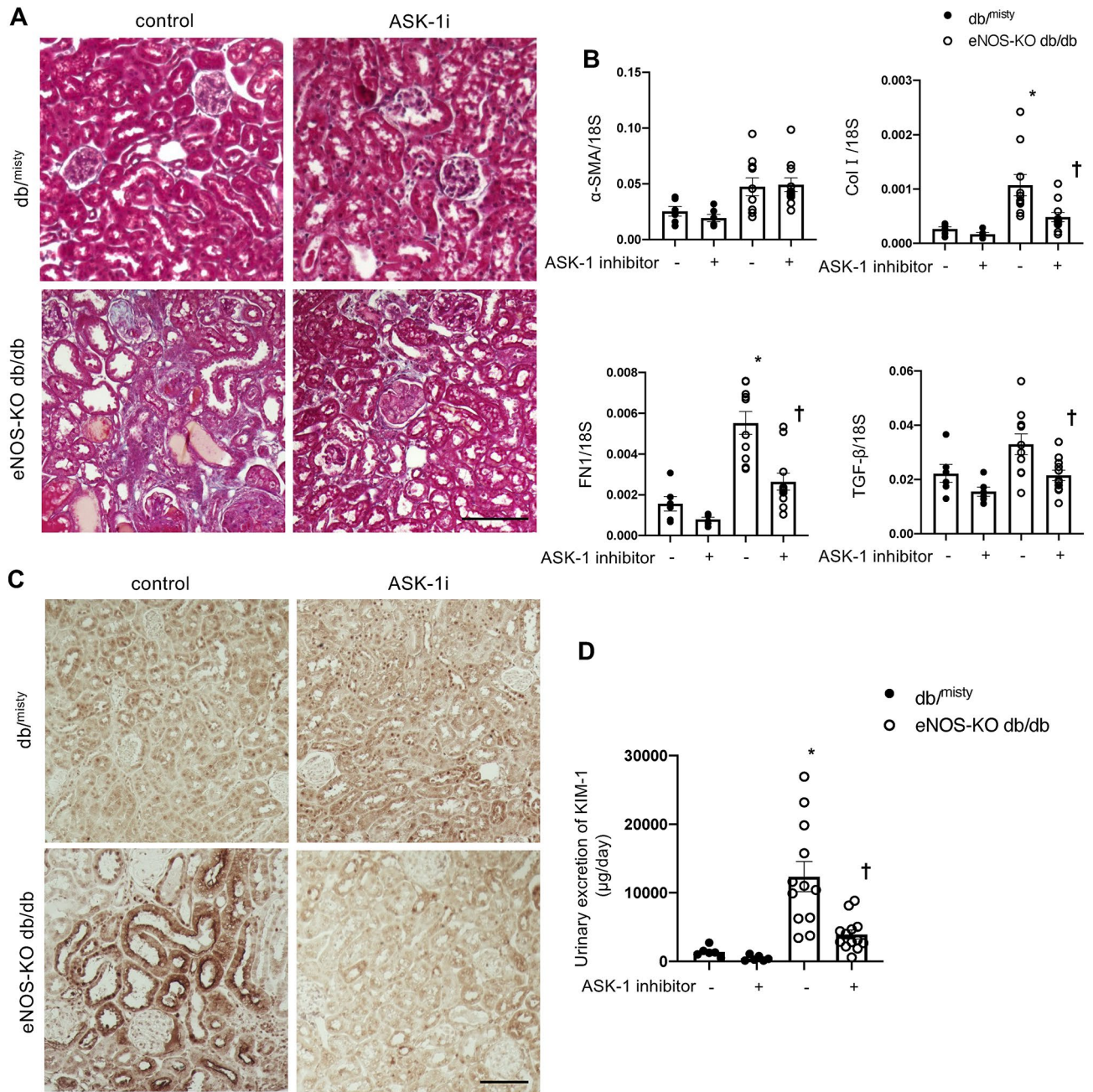
**Fig. 2.** ASK-1 inhibition reduced renal function and glomerular damage. **(A)** PAS staining of glomeruli (bar = 20 μm). **(B)** Percentage of Glomerulosclerosis (GS) score. data are mean ± SEM. \*P < 0.05 versus db/misty control. †P < 0.05 versus eNOS-KO db/db control. **(C)** Measurement of Urinary albumin excretion per day (n = 6–13). Data are expressed as means ± SEM. \*P < 0.05 vs versus db/misty control. **(D)** The cross-sectional transmission electron micrograph of glomerular capillary. Black arrow indicates endothelial fenestrae. Black arrowhead indicates the partial occlusion and disappearance of fenestrae. White arrowhead indicates swollen epithelial cells. *Ep* epithelial cells, *Ed* endothelial cells, *GBM* glomerular basement membrane.

by quantitative PCR (Fig. 6-E). The lipopolysaccharide-mediated signaling pathway of RNA-seq bioprocess exhibited the same trend (Supplementary Fig. 6). These results indicate that elevated ROS in proximal tubules promotes cellular senescence via ASK-1 activation.

The results of this study show that in DKD, ASK-1 activation in glomerular endothelial cells causes increased permeability due to accelerated VE-cadherin degradation, resulting in increased albuminuria and tubular damage. ROS produced during this process also promote cellular senescence via ASK-1 activation and are implicated in the formation of tubulointerstitial lesions.



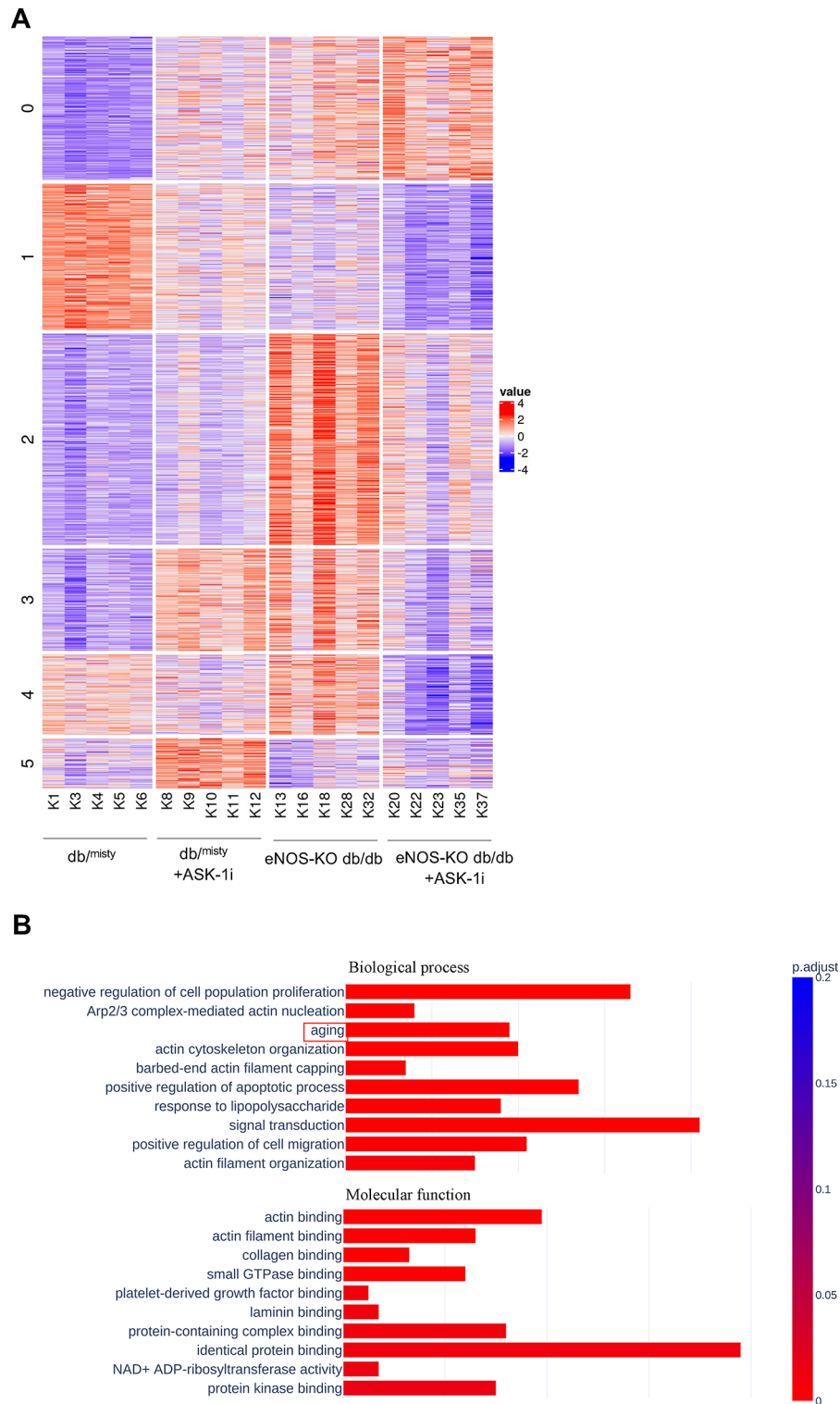
**Fig. 3.** Altered expression of glycans and VE-cadherin on capillary walls via ASK-1 activation. **(A)** immunofluorescence staining of VE-cadherin in kidney tissue. Staining (bar = 20µm). **(B)** Glomerular ESL was evaluated by tomato lectin staining (bar = 20µm). **(C)** Expression levels of VE-cadherin. **(D)** Expression levels of Tomato lectin. **(E)** Representative western blotting experiments and analysis data for phospho-VE-cadherin (Tyr658), total-VE-cadherin, and GAPDH. Original blots are presented in Supplementary Fig. 9-A-C. **(F)** phosphorylated-VE-cadherin protein level expressed as fold change. Data are expressed as means ± SEM. \*P < 0.05 versus H<sub>2</sub>O<sub>2</sub>. **(G)** Scheme showing ASK-1-mediated regulation of glomerular permeability.



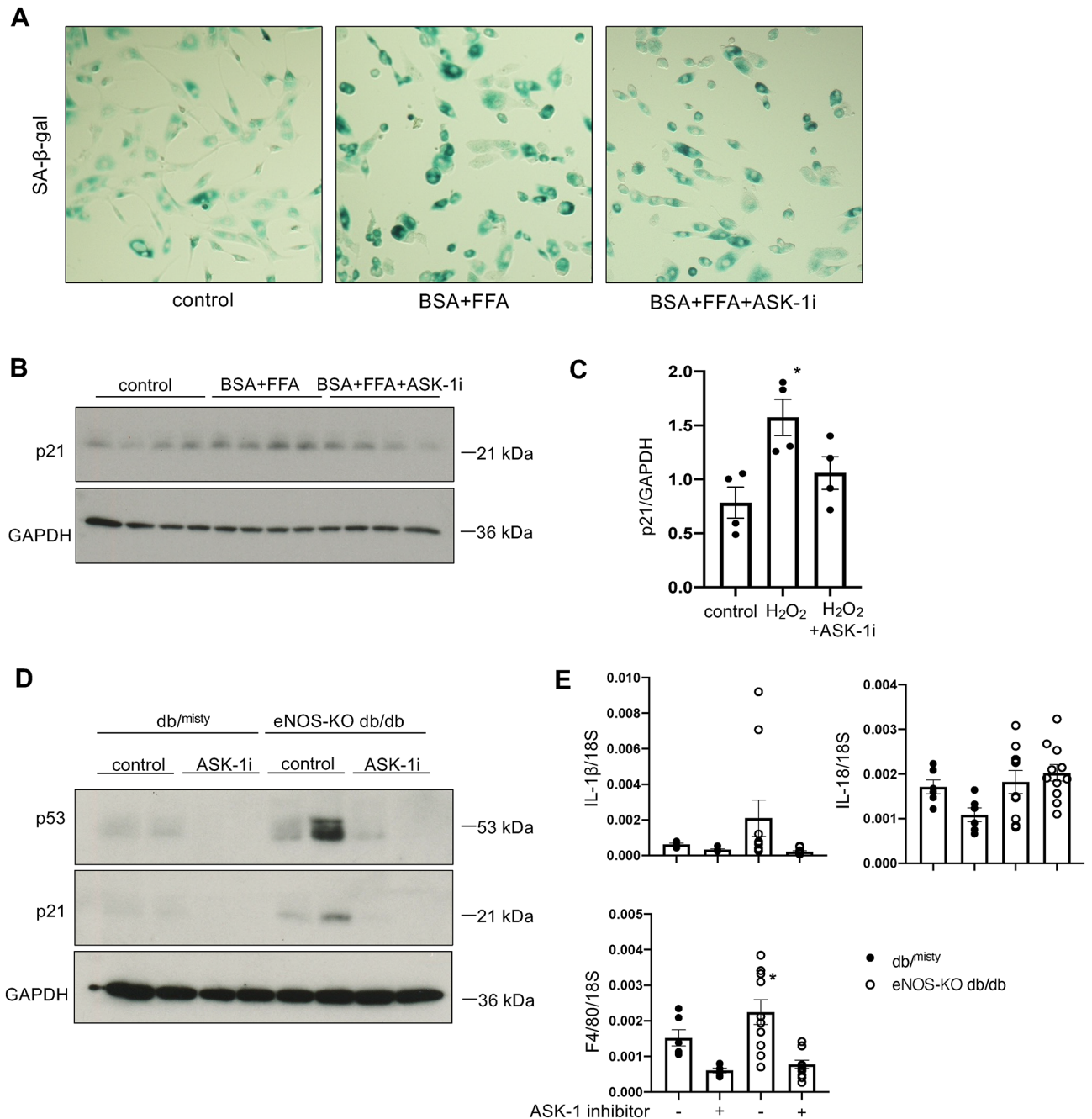
**Fig. 4.** ASK-1 activation induce tubular damage and interstitial fibrosis in DKD. (A) Masson trichrome staining (bar = 100 µm). (B) Expression of fibrogenic gene (*alpha smooth muscle actin* [α-SMA], *Collagen type I* [Col I], *Fibronectin 1* [FN 1] and *Transforming growth factor-β* [TGF-β]) were assessed with quantitative reverse transcription PCR (qRT-PCR). (C) Immunohistochemical staining for KIM-1 (bar = 100µm). (D) ELISA quantifying kidney expression of KIM-1. Data are expressed as means ± SEM. \*P < 0.05 versus db/misty; †P < 0.05 versus eNOS-KO db/db. db/misty control; db/misty ASK-1 inhibitor; eNOS-KO db/db control; eNOS-KO db/db ASK-1 inhibitor. The number of mice n = 6–11.

### Discussion

This study investigated the role of ASK-1 activation in the eNOS-KO db/db mouse model of DKD, and build on previous research<sup>15</sup>. Herein, we demonstrate that ASK-1 activation was observed in eNOS-KO db/db mice compared to db/misty mice as assessed by phospho-p38, a downstream marker of ASK-1 activity. Inhibition of ASK-1 with the ASK-1-selective inhibitor, GS-444217, significantly reduced p-p38 and reduced glomerular injury, as evidenced by decreased glomerulosclerosis and albuminuria. ASK-1 inhibition also reduced endothelial injury in glomerular capillary walls and reduced disruption of glycocalyx and VE-cadherin expression. Moreover, ASK-1 inhibition reduced tubular damage and interstitial fibrosis, as shown by histological and molecular markers. Bulk kidney RNA-seq data indicated that the eNOS-KO db/db mouse model results in a senescence



**Fig. 5.** RNA-seq data revealed ASK-1 activation leads to aging phenotype in DKD. **(A)** Z-scores of TPM values were calculated and heat maps were created. **(B)** GO analysis for cluster 2 was shown in bar graphs. Horizontal axis: number of genes. Vertical axis: GO term. Color: adjusted p-value. GO analysis for all clusters is shown in the Supplementary Fig. 6. **(B)** GO analysis for cluster 2 was shown in bar graphs. Horizontal axis: number of genes. Vertical axis: GO term. Color: adjusted p-value. GO analysis for all clusters is shown in the Supplementary Fig. 6.



**Fig. 6.** Redox status induced cellular senescence via ASK-1 activation in tubular cells. (A) Representative images of SA-β-gal-stained hPTECs. (B) Immunoblot analysis quantifying cell expression of p21 and GAPDH. Original blots are presented in Supplementary Fig. 10-A,B. (C) p21 protein level expressed as fold change. Data are expressed as means ± SEM. \*P < 0.05 versus control. (D) In vivo, representative western blotting experiments for p53, p21 and GAPDH. These marker protein level expressed as fold change in Supplementary Fig. 7. Original blots are presented in Supplementary Fig. 10-C. (E) Expression of SASP related gene (interleukin-1β[IL-1β], interleukin-18[IL-18], F4/80) were assessed with quantitative reverse transcription PCR (qRT-PCR). Data are expressed as means ± SEM. \*P < 0.05 versus eNOS-KO db/db control.

gene signature, which was reversed by ASK-1 inhibition. Mechanistic studies performed in vitro demonstrated that ASK-1 activation induced cellular senescence in tubular cells via redox signaling. Overall, these findings suggest that ASK-1 activation plays a crucial role in DKD pathogenesis by promoting glomerular injury, tubular damage, and cellular senescence.

ASK-1 has been shown to be involved in the regulation of vascular permeability as well as cell death. In a stroke model, ASK-1 activation has been shown to exacerbate brain edema<sup>16</sup>. ASK-1 is also involved in regulating the expression of VE-cadherin, a tight junction that is important in the regulation of vascular permeability. It



has been shown that VE-cadherin is degraded by ASK-1 activation in tumor lesions. This increases vascular permeability, which is inhibited by ASK-1 inhibitors<sup>17</sup>. Interestingly, it has recently been reported that decreased VE-cadherin expression causes decreased glycocalyx expression in diabetic kidney disease<sup>18</sup>. The results of the present study indicate that ASK-1 activation is involved in hyperpermeability in diabetic kidney disease via direct VE-cadherin degradation in glomerular endothelial cells.

Free fatty acid (FFA) that is bound to albumin has been shown to induce tubular damage. Excess FFAs induce mitochondrial damage and promote the production of mitochondria-derived reactive oxygen species (ROS)<sup>19</sup>. Improving mitochondrial damage in proximal tubular cells in DKD is one therapeutic strategy. We also demonstrated previously, that selective estrogen receptor modulators manifest nephroprotective effects through suppression of mitochondrial damage<sup>12</sup>. Mitochondrial dysfunction is known to induce apoptosis, a Caspase 3-dependent cell death<sup>20</sup>. It is now known that ASK-1 activity is downstream of mitochondrial ROS in kidney disease, resulting in apoptosis<sup>21</sup>. The results of our study also indicate that mitochondrial dysfunction and ROS in proximal tubular cells promotes interstitial lesions in DKD via ASK-1 activation.

There is a close association between ROS induction and cellular senescence. An increase in ROS through NADPH oxidase activity, for example, causes cellular senescence via DNA damage<sup>22</sup>. Interestingly, the use of NADPH oxidase inhibitors can also prevent aging<sup>23</sup>. Mitochondria-derived ROS have also been shown to be involved in cellular senescence<sup>24</sup>. The senescent cells promote organ damage by producing SASP and inducing inflammation. On the other hand, SASP-related genes have been shown to move through various pathways. CCAAT/enhancer binding protein- $\beta$  (C/EBP- $\beta$ ), nuclear factor kappa-light chain enhancer (NF- $\kappa$ B) of activated B cells, H2A histone family member X ( $\gamma$ H2AX), ATM, macroH2A1 histone mutant, Janus kinase 1/2 (JAK1/2), p38 and mitogen-activated protein kinase (MAPK) are also involved in SASP<sup>25</sup>. The present results indicate that MAPKs, especially ASK-1 activation, are involved in cellular senescence in tubular cells.

In summary, ASK-1 activation was shown to be involved in the pathogenesis of DKD. In glomerular lesions, ASK-1 activation is involved in the regulation of the permeability of the glomerular wall. In tubular damage, ASK-1 activation was found to promote aging. These are direct pathways in which ASK-1 inhibitors may potentially provide therapeutic intervention.

## Methods

### Animals

The experimental protocols (no. 23-069) were approved by the Animal Research Committee of Kawasaki Medical School and were based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). Additionally, this study was reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (<https://arriveguidelines.org>). Experiments in eNOS-KO db/db mice were performed as described previously<sup>26</sup>. db/<sup>misty</sup>, eNOS-KO db/db were randomly divided into two groups. Each genotype was randomly divided into two groups, one continuing on a control diet from the age of 10 weeks and a feed mixed with ASK-1 inhibitor (0.3% GS-444217; provided by Gilead Sciences Inc, db/<sup>misty</sup> control, db/<sup>misty</sup> + ASK-1inhibitor, eNOS-KO db/db control and eNOS-KO db/db + ASK-1inhibitor, n = 6–11 mice in each group). The mice were housed in a temperature- and humidity-controlled room with a 14:10 h light–dark cycle and were fed standard laboratory animal chow with free access to tap water. The pentobarbital (200 mg/kg) used for euthanising the mice.

### Physiological and biochemical measurement

Physiological parameters were measured just before the mice were killed at 18 weeks of age. Blood samples were obtained using a 21-gauge needle inserted into the right atrium of mice that fasted for 24 h. 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 before the feed change, 4 weeks after the feed change, and 8 weeks after the feed change before euthanasia. Urinary albumin levels were determined by ELISA using the primary monoclonal antibody of mouse albumin (A90-134A, Bethyl Laboratories, TX, USA). Urinary KIM-1 levels were determined by ELISA using the primary monoclonal antibody of mouse albumin (A90-134A, Bethyl Laboratories, TX, USA). Serum creatinine and urea nitrogen levels were measured at a central clinical laboratory (SRL, Inc., Tokyo, Japan).

### Histological analysis and immunohistochemistry

Right kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. Paraffin-embedded tissue was sectioned at 4  $\mu$ m thickness and frozen tissue at 10  $\mu$ m. Tissue sections were deparaffinized and stained with PAS and Masson staining. Three nephrologists semiquantitatively analysed PAS stained sections in a blind fashion. One point was counted if the sclerosis involved the entire glomerulus. Deparaffinized kidney sections were heated in a microwave at 500 W, 15 min for antigen retrieval and then incubated overnight with antibody against KIM-1 (AF1817, R&D Systems, Minneapolis, MN, USA). The primary antibody was detected using the Histofine Simple Stain MAX PO kit (Nichirei Corporation, Tokyo, Japan) and 3,3'-diaminobenzidine (Sigma-Aldrich). All images were obtained with an all-in-one fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan).

Frozen tissue sections were incubated overnight with antibody against VE-cadherin (ab33168, abcam, Cambridge, UK); secondary antibody was FITC (F0205, Dako, Glostrup, DNK).

The glomerular glycocalyx, which is a main component of endothelial surface layer (ESL), was evaluated by staining paraffin-embedded tissue with tomato lectin (L0401, SIGMA, Kanagawa, Japan).

### Western blot analysis

SDS-PAGE was performed (20–30 µg protein/lane) with antibodies against phospho-p38/MAPK (Thr180/Tyr182) (9211S, Cell signaling, Danvers, USA), total-p38/MAPK (9211, Cell signaling, Danvers, USA), phospho-VE-cadherin (Tyr658) (44-1144G, Invitrogen, Waltham, USA), total-VE-cadherin (ab33168, abcam, Cambridge, UK) 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.

### RNA extraction and real-time quantitative PCR

Total RNA extraction and real-time quantitative RT-PCR were performed, as previously described<sup>27</sup>. Briefly, total RNA was isolated from the kidneys using TRIzol (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by digestion with DNase (Sigma-Aldrich). cDNA was synthesized from total RNA (1 µg) using Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific Inc.) with oligo (dT)12–18 as a primer (Thermo Fisher Scientific Inc.). Reverse transcription was performed for 50 min at 37°C according to the manufacturer's protocol (Thermo Fisher Scientific Inc.). The primers and probes for TaqMan analysis were designed using sequence information from GenBank (National Institutes of Health)<sup>28</sup> and the Primer3 online software (<http://frodo.wi.mit.edu/primer3/>; accessed July 1, 2015). The primer and probe sequences are listed in Table 2. TaKaRa Premix Ex Taq (Takara Bio Inc., Otsu, Japan), with a final reaction volume of 20 µL, was used for the TaqMan probe-based RT-PCR reaction, which was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.). Plasmid cDNA of each gene was used to prepare absolute standards. The mRNA expression levels of each gene were normalized to those of the housekeeping 18S ribosomal RNA gene.

### RNA-sequencing

RNA-sequencing was performed by using mRNA from whole kidney. Samples were processed and analyzed by Novaseq (Gene Nex, Beijing, China). Gene ontology (GO) enrichment analysis was performed using goatools<sup>29</sup> and GOplot<sup>30</sup>. These data analysis was performed by Rhelixa Inc., Tokyo 104-0042, Japan.

### Cell culture

Primary human glomerular endothelial cells (hGEC; cell systems, Seattle, USA) were used for the in vitro assays. After starvation, subconfluent cells (0.5% fetal bovine serum for 24 h) were stimulated with 200 µM H<sub>2</sub>O<sub>2</sub> for 1 h. ASK-1 inhibitor (10µM) was added to the culture medium 30 min before stimulation with H<sub>2</sub>O<sub>2</sub>.

hPTECs were cultured in a renal epithelial basal medium (Lonza) supplemented with 5% fetal bovine serum. After starvation, subconfluent cells (0.5% fetal bovine serum for 24 h) were stimulated with 0.5% BSA (MilliporeSigma) binding 450 mM palmitic acid (BSA + FFA) for 6 h. ASK-1 inhibitor (10µM) was added to the culture medium 30 min before stimulation with BSA + FFA.

### Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining in hPTECs were performed using a Senescence β-Galactosidase Staining Kit (#9860; Cell Signaling), following the manufacturer's instructions.

### Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism7 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was evaluated using one-way ANOVA with Tukey–Kramer post hoc tests for comparisons among multiple groups. P values less than 0.05 were considered statistically significant.

Gene	ID	NAME	Sequence
aSMA	NM_007392	Forward primer	caggcattgctgacaggat
		Reverse primer	gttctggaggggcaatgat
		Probe	ctcgaccaccagcaccatgaaga
COL1a1	NM_007742	Forward primer	tgtcgcgatgacgtgcaat
		Reverse primer	ttgggtccctcgactctac
		Probe	actggactgtcccaacccccaaag
FN1	NM_010233	Forward primer	atgatgaggtgacacgtgtgt
		Reverse primer	tgacgcttgggaactgt
		Probe	tcgtggagaatgggcatgca
TGFβ	NM_011577	Forward primer	acctgcaagaccatgacatg
		Reverse primer	cgagccttagttggacaggat
		Probe	aagcgcacgaagccatcctg
GAPDH	NM_008084	Forward primer	aatgacccttcattgacctc
		Reverse primer	gtcttctgggtggcagtgat

**Table 2.** The primer and probe sequences for qRT-PCR.

## Data availability

The datasets generated and analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, GSE275379.

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Study design: E.K., H.N. N.K.; Interpretation of data and statistical analysis: E.K. M.T., R.T., A.H., I.T., R.U., Y.W.; Drafting of the manuscript: E.K., H.N., S.K.; Revision of the submitted manuscript: E.K. H.N. S.I., H.K., K.K.,

T.S., N.K. All authors performed the experiments in this study and designed research plan.

### **Additional information**

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