Salutary Effect of Kallistatin in Salt-Induced Renal Injury, Inflammation, and Fibrosis via Antioxidative Stress

Bo Shen, Makoto Hagiwara, Yu-Yu Yao, Lee Chao, Julie Chao

Abstract—An inverse relationship exists between kallistatin levels and salt-induced oxidative stress in Dahl-salt sensitive rats. We further investigated the role of kallistatin in inhibiting inflammation and fibrosis through antioxidative stress in Dahl-salt sensitive rats and cultured renal cells. High-salt intake in Dahl-salt sensitive rats induced elevation of thiobarbituric acid reactive substances (an indicator of lipid peroxidation), malondialdehyde levels, reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, and superoxide formation, whereas kallistatin gene delivery significantly reduced these oxidative stress parameters. Kallistatin treatment improved renal function and reduced kidney damage as evidenced by diminished proteinuria and serum urea nitrogen levels, glomerular sclerosis, tubular damage, and protein cast formation. Kallistatin significantly decreased interstitial monocyte-macrophage infiltration and the expression of tumor necrosis factor-α, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1. Kallistatin also reduced collagen fraction volume and the deposition and expression of collagen types I and III. Renal protection by kallistatin was associated with increased NO levels and endothelial NO synthase expression and decreased p38 mitogen-activated protein kinase, extracellular signal-regulated kinase phosphorylation, and transforming growth factor-β1 expression. Moreover, kallistatin attenuated tumor necrosis factor-α-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression via inhibition of reactive oxygen species formation and p38 mitogen-activated protein kinase and nuclear factor-κB activation in cultured proximal tubular cells. Kallistatin inhibited fibronectin and collagen expression by suppressing angiotensin II–induced reactive oxygen species generation and transforming growth factor-β1 expression in cultured mesangial cells. These combined findings reveal that kallistatin is a novel antioxidant, which prevents salt-induced kidney injury, inflammation, and fibrosis by inhibiting reactive oxygen species–induced proinflammatory cytokine and transforming growth factor-β1 expression. (Hypertension. 2008;51:1358-1365.)

Key Words: Dahl salt-sensitive rat ■ kallistatin ■ reactive oxygen species ■ inflammation ■ fibrosis

K allistatin is a plasma protein that belongs to the serine protease inhibitor family.1,2 Although most serine protease inhibitors are synthesized primarily in the liver, kallistatin is widely expressed in organs such as the kidney, heart, and blood vessel.3–6 Kallistatin is a negative acute-phase protein, because its expression in the liver is rapidly reduced after lipopolysaccharide-induced inflammation.7 Conversely, transgenic mice overexpressing human kallistatin are resistant to lipopolysaccharide-induced mortality.8 Local delivery of the kallistatin gene inhibited inflammatory responses and reduced joint swelling in a rat model of arthritis.9 Furthermore, our recent study showed that kallistatin gene transfer protected against acute myocardial ischemia-reperfusion injury by inhibition of cardiomyocyte apoptosis and inflammatory cell recruitment.10 Whether kallistatin plays a protective role against renal injury has not been investigated.

A strong correlation has been observed between oxidative stress and immune cell infiltration in salt-sensitive hypertension. In fact, oxidative stress is considered to be a major contributing factor in the development of renal injury, because it can stimulate the expression of proinflammatory and profibrotic molecules.11 Inflammation is crucial to the subsequent development of fibrosis, the final contributing factor to kidney failure. Dahl salt-sensitive (DSS) rats develop progressive and sclerotic renal lesions after salt loading, making them a popular model of human salt-sensitive hypertension.12,13 High-salt loading in DSS rats increases inflammatory cell infiltration, glomerular enlargement, and extracellular matrix protein accumulation in association with increased oxidative stress in the kidney.14 In this study, we investigated the mechanisms of kallistatin in inflammatory cell accumulation and renal fibrosis during the progression of renal injury in DSS rats after salt loading, as well as in cultured renal proximal tubular and mesangial cells. Our data demonstrate that kallistatin has a novel role as an antioxidant in the protection against renal injury by inhibiting salt-induced oxidative stress.
inflammatory cell recruitment and renal fibrosis in vivo and in cultured renal cells.

Methods

For an expanded Methods section, please see http://hyper.ahajournals.org.

Preparation of Replication-Deficient Adenoviral Vectors Containing Human Kallistatin and Purification of Recombinant Kallistatin

Adenoviral vectors carrying the human kallistatin cDNA under the control of the cytomegalovirus enhancer-promoter (Ad.HKS) or the adenoviral vector alone (Ad.Null) were constructed and prepared as described previously.10 Expression and purification of recombinant human kallistatin were performed as described previously.15

Animal Treatments

All of the procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences). Four-week–old, male DSS rats (Sprague-Dawley Harlan) were used. Rats were fed either a normal-salt (0.4% NaCl) or high-salt (4% NaCl) diet for 2 weeks, and those on the high-salt diet were fed a high-salt diet for 6 weeks (2.70±0.21 versus 1.77±0.01 μmol/L; n=6 to 8; P<0.01) with a concomitant decrease in serum kallistatin levels (103.6±6.0 versus 167.5±4.2 μg/mL; n=6 to 8; P<0.01). Kallistatin levels in the kidney were also reduced in DSS rats fed a high-salt diet compared with normal-salt diet (149.3±8.2 versus 272.4±5.3 ng/mg of protein; n=6 to 8; P<0.01). These results indicate that oxidative stress is inversely correlated with kallistatin levels under pathological conditions.

Renal Kallistatin Expression After Gene Delivery Improves Renal Function

Immunoreactive human kallistatin was expressed in glomerular and tubular cells after kallistatin gene delivery. No specific staining was found in the kidney with control adenovirus injection (data not shown). Human kallistatin levels in renal extracts after gene delivery were 82.9±18.1 ng/mg of protein (n=3) but were not detectable in the rats receiving Ad.Null. Kallistatin gene delivery significantly reduced circulating TBARS levels and improved renal function (Table 1). High-salt loading for 6 weeks resulted in a dramatic increase in urinary protein excretion, and kallistatin administration significantly decreased urinary protein levels. Moreover, kallistatin treatment completely reversed salt-induced elevation of serum urea nitrogen levels and creatinine clearance. Blood pressure markedly increased after salt loading, and kallistatin administration resulted in a slight but significant reduction of blood pressure (Table 1). However, blood pressure was still significantly higher in the kallistatin group compared with DSS rats on a normal-salt diet. DSS rats

<table>
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<th>HS + Ad.HKS, Mean±SEM</th>
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<tr>
<td>KW/BW, g/100 g of BW</td>
<td>0.59±0.02*</td>
<td>0.93±0.04</td>
<td>0.80±0.02†</td>
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<td>BP, mm Hg</td>
<td>138.7±3.5*</td>
<td>217.8±1.9</td>
<td>198.2±2.6†</td>
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<td>TBARS, μmol/L</td>
<td>1.77±0.01*</td>
<td>2.70±0.21</td>
<td>2.03±0.11*</td>
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<td>Urinary protein, mg/d/100 g of BW</td>
<td>66.3±4.4*</td>
<td>155.8±8.2</td>
<td>125.3±7.8†</td>
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<td>Serum urea nitrogen, mg/mL</td>
<td>0.55±0.03*</td>
<td>0.80±0.10</td>
<td>0.57±0.06*</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>1.15±0.04*</td>
<td>0.51±0.09</td>
<td>1.07±0.09†</td>
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<td>Glomerular sclerotic score</td>
<td>0*</td>
<td>2.84±0.44</td>
<td>1.49±0.15†</td>
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<td>Arterio-arteriolar sclerotic score</td>
<td>0.33±0.10*</td>
<td>2.42±0.28</td>
<td>1.8±0.12†</td>
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<td>Tubulointerstitial injury score</td>
<td>0*</td>
<td>3.29±0.18</td>
<td>2.33±0.21†</td>
</tr>
</tbody>
</table>

NS indicates normal salt; HS, high salt; BW, body weight; KW, kidney weight; BP, blood pressure. n=7. *P<0.05 vs HS + Ad.Null; †P<0.05 vs NS.
on a normal-salt diet were normotensive throughout the experimental period.

**Kallistatin Reduces Salt-Induced Renal Injury and Inflammatory Response**

The morphology of renal injury induced by high-salt intake was evaluated by Periodic acid-Schiff staining. Kidneys of DSS rats on a normal-salt diet had normal structure. However, DSS rats on a high-salt diet for 6 weeks exhibited tubular dilatation, glomerular sclerosis, and extensive protein cast formation. Kallistatin gene transfer in DSS rats attenuated tubular damage and also resulted in fewer protein casts and sclerotic glomeruli compared with rats in the Ad.Null group. Quantitative analysis indicated that kallistatin significantly reduced glomerular sclerotic, arterio-arteriolar sclerotic, and tubulointerstitial injury scores (Table 1). Accumulation of monocytes/macrophages was determined by immunohistochemical staining against ED-1. DSS rats fed a normal-salt diet had a small number of ED-1–positive cells. In contrast, significant accumulation of monocytes/macrophages was observed in DSS rats a high-salt diet for 6 weeks (Figure 1A). Kallistatin gene delivery significantly inhibited salt-induced monocytes/macrophages accumulation in the kidney (Figure 1A and 1B). Kallistatin significantly reduced salt-induced protein and gene expression of TNF-α, intercellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1; Figure 1C through 1E).

**Kallistatin Attenuates Salt-Induced Renal Fibrosis and Collagen Expression**

Kidney sections were stained with Sirius red for the determination of total collagen in DSS rats (Figure 2A). Rats fed a normal-salt diet exhibited a small amount of collagen in the interstitial space and glomeruli. Although high-salt loading increased the accumulation of collagen in the interstitium and in glomeruli, kallistatin gene transfer attenuated collagen deposition (Figure 2B). Immunohistochemical staining of collagen types I and III indicated that kallistatin gene transfer reduced salt-induced collagen expression in the interstitial space and periglomeruli (Figure 2A). Furthermore, kallistatin significantly inhibited collagen types I and III mRNA levels in the kidney compared with the high-salt group (Figure 2C and 2D).

**Kallistatin Restores Renal Endothelial NO Synthase Expression and Nitrogen Oxide Levels and Reduces Oxidative Stress**

Kallistatin gene transfer significantly increased urinary nitrogen oxide levels and reduced oxidative stress compared with DSS rats on a high-salt diet (Table 2). Kallistatin administration also significantly restored endothelial NO synthase expression in high-salt–loaded DSS rats (see the online data supplement). Renal MDA levels were increased after the high-salt diet compared with the normal diet but were lowered by kallistatin gene delivery. Moreover, a high-salt diet induced a significant upregulation of the expression of NADPH oxidase subunit-p47phox expression (see the online data supplement).
data supplement) and elevated NADPH oxidase activity compared with DSS rats on a normal diet. Kallistatin gene delivery significantly reversed these increases. Superoxide formation paralleled NADPH oxidase activity. Superoxide levels were elevated in the Ad.Null group above those in the normal-salt group. Kallistatin gene transfer significantly lowered salt-induced superoxide formation. These results indicate that kallistatin increases NO production and suppresses oxidative stress in salt-loaded DSS rats.

Kallistatin Inhibits p38MAPK and Extracellular Signal-Regulated Kinase Activation and TGF-β1 Expression

Western blot analysis showed that kallistatin gene delivery markedly reduced salt-induced phosphorylation of p38MAPK and extracellular signal-regulated kinase (Figure 3A). Renal TGF-β1 protein levels, determined by ELISA, were dramatically elevated in the high-salt group compared with the normal-salt group and were diminished in DSS rats injected with Ad.HKS (Figure 3B). Similarly, real-time PCR showed that kallistatin reduced salt-induced TGF-β1 mRNA levels (Figure 3C).

Table 2. Effect of Kallistatin Gene Delivery on Urinary NO Content and Oxidative Stress

<table>
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<th>HS+Ad.HKS, Mean±SEM</th>
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<td>Urinary nitrogen oxide levels, μmol/d/100 g of BW</td>
<td>1.65±0.03*</td>
<td>0.86±0.13</td>
<td>1.38±0.03*</td>
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<td>Renal MDA levels, μmol/mg of protein</td>
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<td>0.69±0.03</td>
<td>0.55±0.04†</td>
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<tr>
<td>NADPH oxidase activity, rlu/min/mg of protein</td>
<td>0.27±0.02*</td>
<td>0.37±0.03</td>
<td>0.30±0.01*</td>
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<tr>
<td>Superoxide formation, nmol/min/mg of protein</td>
<td>0.30±0.07*</td>
<td>0.99±0.13</td>
<td>0.40±0.07*</td>
</tr>
</tbody>
</table>

NS indicates normal salt; HS, high salt; MDA, malondialdehyde; rlu, relative light units. n=7.

*P<0.05 vs HS+Ad.Null; †P<0.05 vs NS.

Kallistatin Inhibits TNF-α–Induced ROS Formation, p38MAPK, and IκBα Phosphorylation and ICAM-1 and VCAM-1 Expression in Proximal Tubular Cells

Kallistatin significantly inhibited TNF-α–induced ROS formation in cultured proximal tubular cells in situ as detected by elevated intensity of 2′,7′-dichlorofluorescein diacetate fluorescence (Figure 4A and 4B). Moreover, kallistatin reduced p38MAPK and IκBα phosphorylation in a dose-dependent manner (Figure 4C). Inhibition of p38MAPK activation by kallistatin led to inhibition of TNF-α–induced ICAM-1 and VCAM-1 expression.
VCAM-1 expression. TNF-α–induced proinflammatory mediator expression was mediated by p38MAPK activation, as the effect of TNF-α was blocked by SB202190, a p38MAPK inhibitor (see the online data supplement). Taken together, kallistatin, through inhibition of ROS formation, attenuated TNF-α–induced inflammatory cytokine expression by suppressing p38MAPK and nuclear factor κB (NF-κB) activation.

Kallistatin Inhibits Angiotensin II–Induced ROS Formation, TGF-β1, Fibronectin, and Collagen Expression in Mesangial Cells

Angiotensin (Ang) II treatment significantly increased ROS formation in cultured mesangial cells (Figure 5A). Pretreatment with kallistatin or apocynin, an NADPH oxidase inhibitor, significantly blocked Ang II–induced ROS production (Figure 5A and 5B). Kallistatin and apocynin also abrogated Ang II–stimulated TGF-β1 protein and mRNA levels (Figure 5C and 5D). Furthermore, kallistatin abolished TGF-β1–induced fibronectin and collagen I expression in mesangial cells (see the online data supplement). These results indicate that kallistatin inhibited TGF-β1 expression through suppression of NADPH oxidase activity and ROS formation.

Discussion

This is the first study to demonstrate that kallistatin levels are inversely correlated with oxidative stress and that kallistatin...
supplementation by gene delivery reduced oxidative stress in the serum and kidney. The antioxidative activity of kallistatin inhibited inflammation and fibrosis in DSS rats and on cultured proximal tubular and mesangial cells. The antiinflammatory effect of kallistatin observed in this study is consistent with our previous reports that kallistatin gene transfer reduces inflammatory cell recruitment and TNF-α levels in animal models of rat arthritis and myocardial ischemia-reperfusion.9,10 Our results showed that kallistatin inhibited proinflammatory cytokine and TGF-β1 expression through suppression of NADPH oxidase activity, ROS formation, and, thus, p38MAPK and NF-κB activation in cultured renal cells. The results obtained from the in vivo and cell culture studies provide strong evidence that kallistatin, as a potent antioxidant, protects against salt-induced renal injury.

High-salt loading in DSS rats induced renal injury and blood pressure elevation in association with increased circulating ROS levels and reduced NO bioavailability. Kallistatin treatment protected against salt-induced renal dysfunction, as evidenced by reduced serum urea nitrogen and urinary protein levels. Although kallistatin administration partially lowered salt-induced blood pressure rise, it almost completely prevented renal injury. Moreover, kallistatin treatment restored endothelial NO synthase-nitrogen oxide levels and reduced salt-induced superoxide formation. Furthermore, previous studies showed that hypertensive treatment with amloidipine and hydralazine did not prevent glomerulosclerosis and proteinuria in DSS rats despite the reversal of systemic high blood pressure to the normal level.18,19 In addition, treatments with antioxidant or antiinflammatory agents are capable of reducing arterial pressure, as well as improving renal function in salt-sensitive hypertension.20,21 Therefore, we can conclude that the renoprotective effects of kallistatin are not primarily attributed to its blood pressure–lowering ability. A modest reduction in blood pressure after kallistatin treatment may be related to a combination of NO-mediated vasodilation and amelioration of ROS generation.

Oxidative stress is a major contributing factor in inducing renal injury, because it can stimulate the expression of proinflammatory and profibrotic molecules.12 High-salt intake has been shown to increase renal NADPH activity, urinary H₂O₂, 8-isoprostane, and thromboxane B₂ excretion.22 NO, a potent antioxidant, is capable of inhibiting neutrophil superoxide anion production via a direct action on the membrane components of NADPH oxidase and the assembly of reduced nicotinamide-adenine dinucleotide/NADPH oxidase subunits.23 In DSS rats fed a high-salt diet, NO production is impaired because of a significant reduction in NO synthase activity.24 Consistent with previous findings, significantly increased serum TBARS levels and renal ROS formation, as well as decreased endothelial NO synthase expression, were observed in DSS rats after high-salt loading. Kallistatin administration increased nitrogen oxide levels partly by restoring endothelial NO synthase expression and also effectively decreased NADPH oxidase activity and superoxide production in the kidney. Increased NO formation and lowered oxidative stress after kallistatin treatment are crucial in renal protection for suppressing inflammation and fibrosis.

It is well known that inflammation and oxidative stress are intrinsically interrelated. In fact, ROS can trigger an inflamma-
ory response through activation of the TNF-α pathway.25 ROS activates p38MAPK and the transcription factor NF-κB, which leads to proinflammatory cytokine release and inflammatory cell accumulation in the kidney.26,27 Sustained inflammatory responses may contribute to the progressive renal injury.28 Moreover, inflammation of renal cells in culture is associated with increased oxidative stress.29 We observed that kallistatin gene delivery effectively blocked high-salt–induced inflammatory cell infiltration into the interstitial tissues and ameliorated hypertensive-induced renal damage.30 In agreement with the in vivo study, we found that recombinant kallistatin inhibited TNF-α–induced ROS formation, IkBα degradation (an inhibitor of NF-κB activation), and inflammatory cytokine expression in cultured proximal tubular cells. These results indicate that kallistatin exerts antifibrotic effects via suppression of oxidative stress and NF-κB activation.

The development of fibrosis, the final contributing factor to kidney failure, is preceded by oxidative stress and inflammation. Oxidative stress has been shown to promote inflammation and to increase the release of active TGF-β1 via activation of mitogen-activated protein kinase pathways.31,32 Activated TGF-β1 participates in the development of renal failure in controlling extracellular matrix deposition and remodeling by stimulating collagen and fibronectin synthesis.33,34 Despite the positive-feedback loop between TGF-β1 and NO under physiological conditions, the inhibitory effect of NO on TGF-β1 production was found to be reduced after salt intake in DSS rats.35,36 In this study, we showed that kallistatin administration restored NO levels and significantly prevented salt-induced TGF-β1 expression. Recombinant kallistatin also reduced Ang II–induced ROS generation and TGF-β1 secretion and expression in cultured mesangial cells. Ang II–induced effects were abrogated by apocynin, an inhibitor of NADPH oxidase. Moreover, kallistatin inhibited TGF-β1–induced collagen and fibronectin gene expression in mesangial cells. These findings indicated that the antifibrotic effect of kallistatin is mediated by inhibition of oxidative stress and TGF-β expression.

**Perspectives**

Oxidative stress is a major contributing factor in the development of renal injury by the stimulation of proinflammatory and profibrotic molecule expression. This study demonstrates an inverse relationship of kallistatin with oxidative stress parameters and identifies kallistatin as a novel antioxidant in preventing salt-induced renal injury, inflammation, and fibrosis in DSS rats, as well as in cultured proximal tubular and mesangial cells. Inhibition of ROS formation by kallistatin leads to lower proinflammatory cytokine and profibrotic mediator expression and, thus, protection against oxidative kidney damage. Our study reveals that kallistatin, as a potent antioxidant, may have therapeutic potential for the treatment of oxidative organ failure.

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**Disclosures**

None.

**References**


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Salutary Effect of Kallistatin in Salt-Induced Renal Injury, Inflammation and Fibrosis via Anti-Oxidative Stress

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Abstract words: 248
Figure numbers: 7

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Methods

Blood Pressure Measurement

Systolic blood pressure was measured with DASYlab 5.5 software (Kent Scientific Corporation) by the tail-cuff method. Unanesthetized rats were placed in a plastic holder resting on a warm pad maintained at 37°C during the measurements. Average readings were taken for each animal after the animals had become acclimated to the environment.

Assays for TBARS, Malondialdehyde (MDA), Blood Urea Nitrogen (BUN) and Urinary Protein Levels

Serum lipid peroxidation, an indicator of oxidative stress, was determined by measuring TBARS levels at 535 nm using MDA standards (0 to 3 µM) (Sigma). MDA levels in renal extracts were measured in a similar fashion with cytosolic protein from renal extracts. BUN levels were determined using a modified urease-indophenol method. Protein levels in urine were measured by Bio-Rad DC Protein Assay (Bio-Rad Laboratories).

Morphological and Histological Investigations

Four-µm-thick sections were obtained from each sample. Sections were stained with periodic acid-Schiff (PAS) for morphometric analysis and with Sirius red to determine the extent of fibrosis. Renal glomerulosclerosis, arterio-arteriolar sclerosis, and tubulointerstitial damage were scored as described previously. Immunohistochemistry
was performed using the Vectastain Universal Elite ABC Kit (Vector Laboratories), following the supplied instructions. Kidney sections were incubated at 4°C overnight with antibodies against the monocyte/macrophage marker ED-1 (1:100; Chemicon Inc.), collagen type I (1:100; Sigma) and collagen type III (1:400; Sigma). Quantification of monocytes/macrophages was determined by counting ED-1 positive cells in 10 fields at 400x magnification.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Human kallistatin levels in the serum were determined using an ELISA specific for human kallistatin as described previously. Renal TGF-β1 levels were determined using ELISA kit (R & D Systems) and following the supplied instructions.

**Western Blot Analysis**

Renal tissues were homogenized in lysis buffer containing 1:100 protease inhibitor cocktail (Sigma) and centrifuged at 15,000 x g at 4°C for 30 min. After centrifugation, the cytosolic fraction protein concentration was measured Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories). Western blot analysis of tissue extracts was performed for p38MAPK, phospho-p38MAPK, ERK and phospho-ERK (Cell Signaling Technology Inc). Cell culture lysates also underwent western blot for p38MAPK, phospho-p38MAPK, IκBα and phospho-IκBα (Cell Signaling Technology Inc).

**Measurement of NOx Levels, NADPH Oxidase Activity and Superoxide Formation**
Nitric oxide levels in urine samples collected at 3 weeks after gene delivery were measured by a fluorometric assay for nitrite/nitrate. NADPH oxidase activity in the renal extracts was measured in the presence of NADPH substrate (100 μM) and lucigenin (75 μM) by chemiluminescence assay. Light emission levels were expressed as relative light units (rlu) per min per mg of protein. Superoxide production was measured using a ferricytochrome c reduction assay according to a modified previous protocol.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from kidney and cultured cells using Trizol reagent (Invitrogen). cDNA was transcribed from 2 μg of RNA using a high cDNA archive kit (Applied Biosystems) following the manufacturer’s instructions. The qRT-PCR reaction was carried out using the Gene Expression Assay and running on 7300 real-time PCR system (Applied Biosystems, Foster, CA). Quantification was determined by Relative Quantification Software (Applied Biosystems).

**Proximal Tubular Cell Culture**

Immortalized rat proximal tubular cells, a generous gift from Dr. Julie Ingelfinger from Harvard Medical School, were cultured as previously described. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) at 34°C in a humidified 5% CO₂ atmosphere. Cells were cultured in 12-well plates until they achieved 80% confluence, then the culture medium was replaced with serum-free DMEM for 16 hour. Cells were then incubated with TNF-α (10 ng/ml) in the absence or presence of
kallistatin (0.2-0.4 µM) or SB202190 (a p38MAPK inhibitor, 5 µM) for 15 min for
western blot analysis or 24 hour for qRT-PCR.

**Glomerular Mesangial Cell Culture**

Rat glomerular mesangial cells were purchased from American Type Culture Collection
(ATCC). The cells were cultured in DMEM with 0.4 mg/ml G418 at 37°C in a
humidified 5% CO₂ atmosphere. Cells were cultured in 12-well plates until they
achieved 80% confluence, after which the culture medium was replaced with serum-free
DMEM for 16 hour. Cells were then incubated with angiotensin (Ang) II (50 nM) or
TGF-β1 (5 ng/ml) in the absence or presence of kallistatin (0.2 and 0.4 µM) or apocynin
(a NAPDH oxidase inhibitor, 500 µM) for 24 hour. The medium was then collected and
centrifuged, and the supernatant was frozen at -80°C for subsequent assay of TGF-β1.
qRT-PCR was performed to determine TGF-β1, fibronectin and collagen expression.

**Detection of Reactive Oxygen Species (ROS) Formation**

Intracellular production of ROS was measured by using 2',7'-dichlorofluorescein
diacetate (DCF-DA; Molecular Probes).¹³ Rat renal cells were grown in 6 well plates.
When the cells reached 80% confluence, they were incubated for 30 min with 50 pM
DCF-DA diluted in DMEM medium. The cells were then pretreated with human
kallistatin or apocynin (500 µM) for 30 min before addition of Ang II for 1 hour or
TNF-α for 15 min. After incubation the cells were washed twice with PBS and imaged
using fluorescence microscope. To measure ROS levels, the cells were seeded to a 96-
well plate, and were treated as described above. Relative fluorescence was measured
using a fluorescence plate reader Victor3™ (Perkin Elmer Life Science) at excitation and emission wavelengths of 485 and 528 nm, respectively.

Reference:


Figures:

**Figure S1:** Effect of kallistatin gene delivery on renal eNOS and NADPH subunit p47phox expression. (A) Real-time PCR analysis of mRNA levels for renal eNOS. (B) Real-time PCR analysis of mRNA levels for renal p47phox. Values are expressed as mean ± SEM, n=7.
Figure S2: Effect of kallistatin on renal cells. Kallistatin treatment inhibited TNF-α-induced ICAM-1 and VCAM-1 expression in cultured proximal tubular cells. (A) ICAM-1 and (B) VCAM-1 levels were measured by real-time PCR and normalized with GAPDH. SB: SB 202190, a p38MAPK inhibitor. Kallistatin treatment inhibited TGF-β1 induced fibronectin and collage I expression in cultured rat mesangial cells. (C) fibronectin and (D) collagen I levels were measured by real-time PCR and normalized with GAPDH. Values are expressed as means ± SEM, n=3.