Tissue kallikrein attenuates salt-induced renal fibrosis by inhibition of oxidative stress

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Background. High salt intake induces hypertension, cardiac hypertrophy, and progressive renal damage. Progressive renal injury is the consequence of a process of destructive fibrosis. Using gene transfer approach, we have shown that the tissue kallikrein-kinin system (KKS) plays an important role in protection against renal injury in several hypertensive rat models. In this study, we further investigated the effect and potential mechanisms mediated by kallikrein on salt-induced renal fibrosis.

Methods. Adenovirus harboring the human tissue kallikrein gene was delivered intravenously into Dahl salt-sensitive (DSS) rats on a high salt diet for 4 weeks. Two weeks after gene delivery, the effect of kallikrein on renal fibrosis was examined by biochemical and histologic analysis.

Results. Kallikrein gene delivery resulted in reduced blood urea nitrogen (BUN), urinary protein and albumin levels in DSS rats on a high salt diet. Expression of recombinant human tissue kallikrein was detected in the sera and urine of rats injected with the kallikrein gene. Histologic investigation showed that kallikrein gene delivery significantly reduced glomerular and tubular fibrosis scores and collagen deposition, as well as renal cell proliferation, compared to rats on a high salt diet injected with control virus. Kallikrein gene transfer significantly decreased nitric oxide bioavailability and suppression of oxidative stress.

Conclusion. These results indicate that tissue kallikrein protects against renal fibrosis in hypertensive DSS rats through increased nitric oxide bioavailability and suppression of oxidative stress and TGF-β1 expression.

Renal fibrosis is the final common pathway to end-stage renal failure. Increased matrix protein synthesis and degradation of matrix protein all appear to increase the accumulation of extracellular matrix (ECM), which leads to glomerulosclerosis and tubulointerstitial scarring [1–3]. The extent and severity of interstitial and glomerular lesions are also known to reflect the deterioration of renal function [4–5]. Fibrogenic factors transforming growth factor-β (TGF-β), angiotensin II, and plasminogen activator inhibitor-1 (PAI-1) are the main contributors to renal fibrosis [2, 6]. TGF-β, a premier fibrosis-promoting molecule, exerts biologic effects on target cells via binding to specific cell membrane receptors and activation of intracellular signaling pathways that ultimately affect target cell gene transcription, including expression of matrix genes, genes of inhibitors of matrix-degrading enzymes, and matrix-binding receptors [2, 7, 8]. TGF-β1 synthesis and ECM gene expression are induced by reactive oxygen species (ROS) in cultured human mesangial cells [9]. ROS play an important role in the pathogenesis of acute and chronic renal diseases as characterized by varying degree of decreased renal function, increased proliferation of renal cells, and progressive accumulation of ECM proteins [10–13]. These findings suggest that oxidative stress may underlie the development and progression of renal fibrosis.

High salt intake in Dahl salt-sensitive (DSS) rats results in enhanced production of superoxide radicals within the arterioles and venules in addition to elevated levels of hydrogen peroxide in plasma [14]. A recent study showed that oxidative stress contributes to salt-sensitive hypertension in DSS rats and the accompanying renal damage [15]. Antioxidant treatment with vitamin E markedly reduced blood pressure and ameliorated renal injury in DSS rats on a high salt diet [16]. High salt intake in DSS rats also increases TGF-β1 transcription as well as latent TGF-β1 secretion from sclerotic glomeruli and intrarenal vascular walls [17]. These results indicate that increased expression of TGF-β1 induced by a high salt diet may be related to ECM accumulation in both thickened vascular walls and sclerotic glomeruli of hypertensive individuals.

Tissue kallikrein cleaves low-molecular-weight kininogen substrate to release kinin by limited proteolysis [18, 19]. By binding to endothelial bradykinin B2 receptors,
kinin stimulates release of nitric oxide, leading to increased local blood flow and smooth muscle relaxation [20]. In addition, nitric oxide suppresses the superoxide-generating activity of NADH/NADPH oxidase and the levels TGF-β1 and PAI-1 in vascular smooth muscle cells and mesangial cells [21–23]. Taken together, these findings indicate that increased nitric oxide production may play a protective role in reducing oxidative stress and fibrosis. It has been shown that renal and urinary kallikrein levels are reduced in DSS rats on a high salt diet (8% NaCl) as compared to DSS rats on a normal diet [24]. We previously demonstrated that a continuous supply of tissue kallikrein by adeno-virus-mediated gene transfer reduced blood pressure, improved renal function, and reduced renal injury in DSS rats on a high salt diet [25]. In the present study, we further investigated the effect of kallikrein on renal cell proliferation and fibrosis as well as the potential factors mediating kallikrein’s action in the antifibrogenic process.

METHODS

Animals

DSS rats (male, 4 weeks old) (Sprague-Dawley Harlan, Indianapolis, IN, USA) were used in this study. Rats were divided into two groups. The first group was fed standard rat chow (0.4% NaCl) and the other group was fed a high salt diet (4% NaCl) (Harlan Teklad, Madison, WI, USA). All rats had free access to water. All 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, Bethesda, MD, USA).

Preparation of replication-deficient adenovirus vector Ad.CMV-CHK-4F2

Human tissue kallikrein plasmid cDNA (cHK) along with cytomegalovirus (CMV) promoter was inserted at XhoI and BglII site of Kan-pShut plasmid. Subsequently, the 4F2 enhancer and bovine growth hormone poly (A) sequence were inserted at XhoI site in the Kan-pShut vector. The Kan-pShut plasmid was modified from pHM4 plasmid (kindly provided by Dr. Mark A. Kay, Stanford University Medical Center, Stanford, CA, USA) and engineered by insertion of a kanamycin-resistant gene expression unit. The expression cassettes of human tissue kallikrein cDNA along with the kanamycin-resistant gene were released with I-CeuI and PI-SceI and inserted at the same sites of pAdHM4 plasmid, a backbone vector of E1/E3-deleted adenovirus. The expression cassettes of human tissue kallikrein cDNA along with the E1/E3-deleted adenoviral backbone DNA were released with PacI and transected into human embryonic kidney (HEK) 293 cells. The E1/E3-deleted adenovirus, Ad.CMV-cHK-4F2, was generated and amplified through six passages and purified as previously described [26].

Intravenous delivery of adeno-viral vectors

DSS rats fed a high salt diet containing 4% NaCl for 4 weeks were randomly divided into two groups and intravenously injected through the tail-vein with either Ad.CMV-cHK-4F2 (N = 7) or control virus encoding the luciferase gene Ad.CMV-Luc (N = 6) at a dosage of 1.6 × 1010 pfu (plaque formation unit) per rat.

Urine and blood collection

Twenty-four–hour urine was collected from rats in metabolic cages at 7 days post-gene delivery. In order to eliminate contamination of urine samples, animals received only water during the 24-hour collection period. Urine was collected and centrifuged at 1,000 × g to remove particles. The supernatant of urine was used for further analysis. Rat serum was collected at various time points after gene delivery.

Assays for blood urea nitrogen (BUN), human tissue kallikrein, urinary protein, albumin, nitric oxide, and cyclic guanosine monophosphate (cGMP)

BUN levels were determined using a modified urease-indophenol method [27]. Human tissue kallikrein levels in rat urine and sera were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described [28]. Radioimmunoassays of urinary cGMP and albumin were examined at 7 days post-gene transfer according to the published protocols [29, 30]. Total urinary protein levels were measured by Lowry’s method [31]. Nitric oxide levels in urine samples collected at 7 days after gene delivery were measured by a fluorometric assay for nitrite/nitrate [32].

Tissue preparation

Rats were anaesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally) and perfused with saline through left ventricle. Kidneys were removed, cleaned, blotted, and weighed. A slice of kidney section was preserved in zinc formalin (10% zinc dichromate, 4% formaldehyde, and 0.9% NaCl) and embedded in paraffin. Sections 4 μm in thickness were prepared for histochi-chemical and immunohistochemical studies.

A part of the kidney was homogenized in guanidine isothiocyanate solution and stored at −80°C for total RNA extraction. A piece of renal cortex was snap-frozen in liquid nitrogen and stored in the −80°C for biochemical assays.
Western blot analysis

Frozen renal cortex was homogenized by sonicator (The Vir Tis Company, Inc., Gardiner, NY, USA) in radioimmunoprecipitation buffer (RIPA) [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L disodium ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L egtazic acid (EGTA), 1.0% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate] with protease inhibitors (Protease Inhibitor Cocktail) Sigma Chemical Co., St. Louis, MO, USA). Samples were centrifuged at 15,000 × g for 30 minutes at 4°C, and the supernatant was collected. Total protein of each sample was determined by Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Samples containing 150 μg of total protein were boiled in SDS sample loading buffer for 5 minutes, resolved in 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto a nitrocellulose membrane. Following incubation in blocking buffer TBST (5% nonfat dry milk in Tris-buffered saline, pH 7.6, and 0.1% Tween 20), the membrane was prehybridized for at least 2 hours at 42°C overnight in hybridization solution (5 × standard saline citrate (SSC), 5 × Denhardt’s solution, 0.1 mg/mL of salmon sperm DNA, 0.1% SDS, and 50% formamide). Murine TGF-β1 cDNA fragment, 974 bp, was released from mTGF-β1-A plasmid DNA (kindly provided by Dr. Moses, Vanderbilt University Medical Center, Nashville, TN, USA) by SmaI digestion. DNA fragment was electrophoresed and purified by Qiaex II gel extraction kit (Qiagen Inc., Valencia, CA, USA). cDNA probes for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with 32P-deoxyctydine triphosphate (dCTP) by random primer method and the membranes were hybridized at 42°C overnight [17]. Autoradiography was performed by the standard method. The densitometric value of each mRNA was corrected against that of GAPDH. The ratio of each mRNA/GAPDH RNA was averaged in each group.

Detection of superoxide by ferricytochrome c reduction assay

Superoxide production in renal extracts was measured as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c at 550 nm according to modified previous protocol [33, 34]. Samples were mixed with cytochrome c (final concentration 1 mg/mL), with or without SOD (final concentration 510 U/mL). The absorbance was read at 550 nm against a distilled-water blank. Reduction of cytochrome c in the presence of SOD was subtracted from values without SOD. The difference in absorbance between comparable wells with or without SOD was converted to equivalent O2− production by using molar extinction coefficient for cytochrome c (21.0 × 103 cm−1 mol−1).

Determination of NADH/NADPH oxidase activity

NADH/NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescent detection of superoxide in a luminometer (TD-20/20) (Turner Designs, Sunnyvale, CA, USA) according to the previously published protocol [34]. Briefly, reaction buffer contained 50 mmol/L phosphate buffer (pH 7.5), 0.01 mmol/L EDTA, 250 μmol/L lucigenin as electron acceptor, and 2 mmol/L NADH or NADPH. The reaction was started by addition of kidney homogenates (100 to 300 μg protein). Luminescence was measured as the rate of photon counts per mg protein following subtraction of the counts obtained from a buffer blank.

Histochemical investigation

Four micrometer thick sections were cut and stained with sirius red [35] and then analyzed microscopically and morphometrically in a single- or double-blinded way. To analyze semiquantitatively for glomerular matrix accumulation, 20 glomeruli from each animal were selected randomly, and scoring of relative area of mesangial matrix was as follows: 0, little or no detectable mesangial matrix; 1+, detectable mesangial matrix of about one fourth of glomerular area; 2+, detectable mesangial matrix of one fourth to one half of glomerular area; 3+, detectable mesangial matrix of one half to three fourths of glomerular area; and 4+, detectable mesangial matrix of three fourths to total of glomerular area. The number of glomeruli showing a lesion of 0, 1+, 2+, 3+, or 4+ was set as n0, n1, n2, n3, or n4, respectively, and correspondingly. Twenty glomeruli were examined independently and then
fibrosis score was obtained by the following formula: 
\[ \left( \frac{0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4}{20} \right) \times 100. \]  
Tubulointerstitial fibrosis was scored semiquantitatively using a previously published method [36]. Cortical tubulointerstitial fields were examined under 40× objective with a 2.5×2.5 mm² ocular grid. A minimum of 10 fields was assessed in each section. The following semiquantitative score was used: 0, normal interstitium and tubules; 1+, less than 10% of fibrosis with minimal interstitial thickening between tubules; 2+, 10% to 25% fibrosis with moderate interstitial thickening between the tubules; and 3+, 26% to 50% fibrosis with severe interstitial thickening between the tubules. Final tubular fibrosis score in each individual was averaged from 10 different fields.

To assess renal cell proliferation, kidney sections were stained for proliferating cell nuclear antigen (PCNA) (1:3000 dilution) (Sigma Chemical Co.) using Vectastain ABC kit (Vector laboratories, Inc., Burlingame, CA, USA). Renal tubular cells and interstitial cells with distinct and intense staining were counted and mean number of proliferating cells was determined by counting PCNA-positive cells in 10 fields per tissue section at a magnification of 200× with a 0.5×0.5 mm² ocular grid.

To assess TGF-β1 and collagen I immunostaining, the kidney sections were stained with rabbit polyclonal anti-TGF-β1 (1:200 dilution) (Santa Cruz Biotechnology) or mouse monoclonal anti-collagen I (1:800 dilution) (Sigma Chemical Co.) using Vectastain ABC kit. Collagen I density was assessed by evaluating low power (4× objective) fields of cross-sections in Adobe PhotoShop (version 5.0) to determine the percentage of immunohistochemical-positive staining to total tissue area.

Statistical analysis
Statistical significance was determined by one-way analysis of variance (ANOVA) with Fisher multiple comparison test. All data were expressed as mean ± SEM, and differences were considered significant at a value of \( P < 0.05 \).

RESULTS
Expression of human tissue kallikrein in DSS rats after kallikrein gene transfer
Following intravenous injection of adenovirus encoding human tissue kallikrein gene, recombinant human kallikrein levels in rat sera and urine were measured by an ELISA specific for human tissue kallikrein. Immunoreactive human tissue kallikrein levels in rat serum was 1068.6 ± 93.2 ng/mL (\( N = 7 \)) at day 4 and reduced to 118.7 ± 25.8 ng/mL at day 12 post-gene delivery. Human tissue kallikrein levels were also detected in rat urine at day 7 after gene transfer (1.6 ± 0.1 μg/100 g body weight/day, \( N = 7 \)). Recombinant human tissue kallikrein was not detectable in the sera or urine of control DSS rats with or without injection with control virus Ad.CMV-Luc.

Effect of kallikrein gene delivery on BUN, urinary protein, and albumin levels in DSS rats
Figure 1A shows that BUN levels in DSS rats on high salt diet and injected with Ad.CMV-Luc were 3.1-fold higher than in the control group fed a normal salt diet [1.53 ± 0.54 mg/mL (\( N = 6 \)) vs. 0.49 ± 0.03 mg/mL (\( N = 5 \)) \( P < 0.05 \)]. Kallikrein gene transfer significantly reduced salt-induced BUN levels as compared to the luciferase group at 14 days post-gene delivery [0.59 ± 0.12 mg/dL (\( N = 7 \)) vs. 1.53 ± 0.54 mg/mL (\( N = 6 \)) \( P < 0.05 \)]. At 7 days post-gene delivery, high salt diet increased urinary total protein levels compared to DSS rats on a normal salt diet [63.2 ± 6.3 mg/day/100 g body weight (\( N = 6 \)) vs. 47.9 ± 3.2 mg/day/100 g body weight (\( N = 5 \)) \( P < 0.05 \)] (Fig. 1B), whereas kallikrein gene transfer significantly reduced urinary protein levels compared to the luciferase group [43.9 ± 3.5 mg/day/100 g body weight (\( N = 7 \)) vs. 63.2 ± 6.3 mg/day/100 g body weight (\( N = 6 \)) \( P < 0.01 \)] (Fig. 1B). Urinary albumin levels corroborate with these results, as a similar reduction of albumin levels were observed in the kallikrein group compared to the luciferase group [19.7 ± 1.7 mg/day/100 g body weight (\( N = 7 \)) vs. 33.3 ± 4.0 mg/day/100 g body weight (\( N = 6 \)) \( P < 0.01 \)] (Fig. 1C).

Effect of kallikrein gene transfer on fibrosis scores and collagen type I immunostaining
Renal fibrosis was analyzed semiquantitatively by staining kidney section with sirius red, which stained cytoplasm yellow and collagen red [35, 37]. Only a small amount of fibrillar collagen was present in renal tubular interstitium of control rats on a normal diet (Fig. 2A). Six weeks of high salt intake resulted in a large area of collagen deposition in the glomerular and interstitial areas (Fig. 2A). However, kallikrein gene transfer led to a reduction of glomerular collagen accumulation and interstitial fibrosis as compared to kidneys in rats injected with control adenovirus (Fig. 2A). Semiquantitative analysis of glomerular fibrosis and tubular fibrosis showed that kallikrein gene delivery markedly reduced salt-induced glomerular fibrosis scores [1.7 ± 0.1 (\( N = 7 \)) vs. 2.5 ± 0.2 (\( N = 6 \)) \( P < 0.01 \)] and tubular fibrosis scores [1.6 ± 0.2 (\( N = 7 \)) vs. 2.5 ± 0.3 (\( N = 5 \)) \( P < 0.01 \)], as compared to the control luciferase group (Fig. 2B).

Collagen type I is the major type of interstitial collagen, which is not present in normal glomeruli [38]. In control kidney sections, type I collagen was localized primarily surrounding the blood vessels with light staining around Bowman’s capsule, identified by immunohistochemistry (Fig. 2C). In the kidney sections from rats on a high salt
diet and injected with the luciferase gene, extensive areas of type I collagen were observed in the renal interstitium (mostly around distal tubules) and periglomeruli. Occasional staining was also found in sclerotic glomeruli as well as surrounding glomeruli (Fig. 2C). In sections of the kidney from rats injected with the kallikrein gene, less interstitial collagen I was evident as compared to the luciferase group (Fig. 2C). Figure 2D shows semiquantitative results from collagen I staining. High salt diet markedly increased renal collagen I staining as compared to normal control [2.8 ± 0.4% (N = 6) vs. 1.2 ± 0.1% (N = 5) P < 0.01], whereas kallikrein gene delivery significantly reduced salt-induced collagen deposition [1.9 ± 0.1% (N = 7) vs. 2.8 ± 0.4% (N = 6) P < 0.05].

Effect of kallikrein gene transfer on renal cell proliferation

Renal cell proliferation was determined by PCNA immunohistochemistry. Figure 3 shows that, compared to rats on a normal diet, a high salt diet significantly increased renal cellular proliferation primarily in proximal tubular cells, although some staining was also seen in interstitial and glomerular cells [24 ± 3 cells/field (N = 6) vs. 9 ± 1 cells/field (N = 5) P < 0.01]. Kallikrein gene delivery markedly reduced renal tubular cell and interstitial cell proliferation as compared to the luciferase group [16 ± 1 cells/field (N = 7) vs. 24 ± 3 cells/field (N = 6) P < 0.05].

Effect of kallikrein gene transfer on urinary nitric oxide and cGMP levels

As shown in Figure 4A, high salt intake in DSS rats significantly reduced urinary (or renal) nitrite/nitrate levels compared to rats on a normal salt diet [1.1 ± 0.1 μmol/day/100 g body weight (N = 6) vs. 2.5 ± 0.2 μmol/day/100 g body weight (N = 5) P < 0.01]. Kallikrein gene transfer significantly increased nitrite/nitrate levels as compared to DSS rats on a high salt diet (1.6 ± 0.1 μmol/day/100 g body weight (N = 7) vs. 1.1 ± 0.1 μmol/day/100 g body weight (N = 6) P < 0.05). Similarly, urinary cGMP levels increased significantly after kallikrein gene delivery as compared to control rats receiving Ad.CMV-Luc [13.0 ± 0.9 nmol/day 100 g body weight (N = 7) vs. 8.0 ± 0.8 nmol/day/100 g body weight (N = 6) P < 0.05] (Fig. 4B).

Effect of kallikrein gene delivery on NADH/NADPH oxidase activity and superoxide generation

To determine the effect of kallikrein gene transfer on salt-induced oxidative stress, we measured
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Fig. 2. Evaluation of renal fibrosis in kidney cortex. (A) Representative histologic sections of kidney cortex. Kidney sections were stained with sirius red, which stained cytoplasm yellow and collagen red. Normal glomeruli and tubular interstitium are shown in the control group. Large areas of extracellular matrix appeared in the glomerular mesangial area and tubulointerstitium in the high salt Ad.CMV-Luc group. Glomerular and tubular lesions were obviously attenuated in the Ad.CMV-cHK group. (B) Degree of glomerular and tubular fibrosis. Values are presented as mean ± SEM (N = 5 to 7). Glomerular fibrosis and tubular fibrosis were scored as described in the Methods section. (C) Immunohistochemical staining for type I collagen in renal sections. Very light collagen I staining was observed in control group. Large areas of collagen I staining appeared in the interstitium of Ad.CMV-Luc. Some glomeruli also stained positively. Less collagen I positive areas were seen in the Ad.CMV-cHK. (D) Collagen I density. Values are expressed as mean ± SEM (N = 5 to 7).

NADH/NADPH oxidase activities in renal extracts using luminescence assay. High salt diet in DSS rats caused marked increases in NADH and NADPH oxidase activities [NADH 1.2 ± 0.3 relative light units/mg protein/min (N = 6) vs. 0.17 ± 0.04 relative light units/mg protein/min (N = 5) P < 0.01; NADPH 2.6 ± 0.5 relative light units/mg protein/min (N = 6) vs. 0.7 ± 0.1 relative light units/mg protein/min (N = 5) P < 0.01] (Fig. 5A and B), whereas kallikrein gene significantly reduced salt-induced NADH and NADPH oxidase activities [NADH 0.3 ± 0.04 relative light units/mg protein/min (N = 7) vs.1.2 ± 0.3 relative light units/mg protein/min (N = 6) P < 0.01; NADPH 1.1 ± 0.2 relative light units/mg protein/min (N = 7) vs. 2.6 ± 0.5 relative light units/mg protein/min (N = 6) P < 0.01] (Fig. 5A and B). NADH/NADPH oxidase is the primary source of superoxide in the kidney [39]. Consistent with these results, high salt intake significantly induced renal superoxide formation compared to control rats on normal salt diet [6.3 ± 1.5 nmol/mg protein/min (N = 6) vs. 2.3 ± 0.9 nmol/mg protein/min (N = 5) P < 0.05] (Fig. 5C). Kallikrein gene transfer markedly reduced salt-induced superoxide formation as compared
to the luciferase group [2.6 ± 0.6 nmol/mg protein/min (N = 7)] vs. 6.3 ± 1.5 nmol/mg protein/min (N = 6) P < 0.05] (Fig. 5C).

**Effect of kallikrein gene transfer on TGF-β1 expression and localization**

TGF-β1 mRNA levels in the kidney were analyzed by Northern blot analysis using GAPDH mRNA as loading control (Fig. 6A). Densitometric analysis indicated that kallikrein gene delivery significantly reduced salt-induced ratio of TGF-β1/GAPDH mRNA as compared to the control luciferase group. Figure 6B shows Western blot analysis for TGF-β1 protein using β-actin protein as loading control. Densitometric analysis showed that high salt diet increased TGF-β1/β-actin protein about 15-fold, whereas kallikrein gene transfer significantly reduced TGF-β1/β-actin protein to only 8.2-fold compared to the rats on a normal salt diet.

TGF-β1 immunohistochemistry showed that TGF-β1 protein was primarily localized in the proximal tubules and present in the protein casts as well as in glomerular compartments. Immunohistochemical staining of TGF-β1 protein in the kidney showed that very few positively stained proximal tubules were observed in the control group (Fig. 6C). Large areas of intense staining were present in swollen proximal tubules found in the luciferase group. TGF-β1 immunoreactivity was found in podocytes and mesangial cells of damaged glomeruli in the luciferase group (Fig. 6C). However, fewer TGF-β1-positive proximal tubules and glomeruli were found in the rats receiving the kallikrein gene (Fig. 6C).

**DISCUSSION**

This is the first study to demonstrate that tissue kallikrein protects against salt-induced renal function impairment and renal fibrosis. High salt diet in DSS rats causes renal dysfunction and renal injury as evidenced by elevation in BUN and proteinuria levels, which are indicators of renal dysfunction. Kallikrein gene transfer significantly reduced salt-induced renal cell proliferation, ECM protein and collagen deposition in glomeruli and tubular interstitium, resulting in attenuation of glomerular and tubular interstitial fibrosis. Salt loading in DSS rats markedly reduced nitric oxide levels compared to rats on normal diet, whereas kallikrein gene transfer significantly increased nitric oxide levels. It has been well documented that nitric oxide production is impaired in DSS rats fed a high salt diet [40, 41], implying that nitric oxide bioavailability plays an important role in the risk for cardiovascular and renal complications. Thus, the ability of kallikrein gene transfer to up-regulate nitric oxide formation is significant. Our results showed that DSS rats on high salt intake do not have a significant reduction in urinary cGMP levels compared to DSS rats receiving a low salt diet. Most likely, this is due to other factors that may contribute to cGMP production besides nitric oxide, such as atrial natriuretic peptide. Increased nitric oxide levels were in conjunction with reduced NADH/NADPH oxidase and superoxide anion formation. Oxidative stress may underlie the pathogenesis resulting in acute and chronic renal diseases, as characterized by decreased renal function and induced proliferation of renal cells [11, 12, 39]. Our results indicate that reduced nitric oxide bioavailability and increased oxidative stress may be a contributing factor in salt-induced renal injury and fibrosis in DSS rats.

Oxidative stress has been shown to contribute to saltsensitive hypertension in DSS rats and the accompanying renal damage [15]. We showed that kallikrein gene transfer significantly restored nitric oxide levels, which was accompanied by reduced oxidative stress and renal fibrosis.
injury in hypertensive DSS rats after salt loading. Nitric oxide induces oxidation of sulphydryl groups in the cysteine residue of membrane and cytosolic components of NADH/NADPH oxidase and inhibits the assembly process of NADH/NADPH oxidase [21]. Therefore, inhibition of NADH/NADPH oxidase activities by nitric oxide leads to reduced ROS formation. In addition, nitric oxide can scavenge superoxide anions and thus reduce oxidative stress [42].

Stimulated ROS initiate a number of signaling cascades leading to renal cell proliferation and progressive accumulation of ECM protein via activation of transcription and production of TGF-β1 [13, 43, 44]. TGF-β plays a key role in matrix synthesis and degradation in several renal diseases [45, 46]. In agreement with a previous report that TGF-β1 expression is induced in kidney of DSS rats on a high salt diet [17], we demonstrated that high salt intake led to increases in renal TGF-β1 mRNA and protein levels. Immunohistochemical staining revealed that TGF-β1 protein was predominately localized in the damaged renal proximal tubules and glomeruli. This local generation of TGF-β1 could directly stimulate fibroblast proliferation and ECM protein accumulation resulting in both interstitial and glomerular fibrosis. This assumption is supported by a previous study showing stimulation of cortical fibroblast proliferation and collagen synthesis in vitro after addition of conditioned media isolated from cultured proximal tubule cells, which was blocked by TGF-β neutralizing antibody [47]. Kallikrein gene delivery inhibited salt-induced TGF-β1 expression and immunoreactive TGF-β levels as well as collagen accumulation in the kidney. In mesangial cells cocultured with glomerular endothelial cells, nitric oxide has been shown to reduce TGF-β1 mRNA as well as TGF-β1 and collagen synthesis, partially by inhibiting protein kinase C activity [48]. Therefore, the beneficial effect of kallikrein on salt-induced renal fibrosis may be attributed to increased nitric oxide bioavailability. However, even though nitric oxide and TGF-β1 levels were significantly reversed after kallikrein gene delivery, they were not normalized to control levels. This may indicate that additional mechanisms are involved in kallikrein’s protective effect against salt-induced renal fibrosis. Furthermore, since NADH/NADPH oxidase activity and superoxide levels were completely abolished by kallikrein gene delivery, this would suggest that ROS may play a role in regulating nitric oxide and TGF-β1 levels as well as other factors that contribute to renal fibrosis.

Exposure of kidney to high pressure leads to secretion of vasoactive substances, cytokines, and growth factors such as platelet-derived growth factor, basic fibroblast growth factor, and TGF-β [49]. TGF-β and/or other cytokines are able to directly or indirectly stimulate the proliferation of tubular and interstitial cells and contribute to the production of ECM proteins [49–52]. Using immunohistochemical staining for PCNA, a marker of cell proliferation [53], we observed that diffuse renal tubular and interstitial positive cells were associated with renal fibrosis in response to salt loading, whereas proliferating cells were significantly reduced in the rats injected with the kallikrein gene. Suppression of proliferating renal cells may account for reduction of collagen deposition in the kidneys. The inhibitory effect of kallikrein on renal cell proliferation may be, in part, due to suppression of a TGF-β–mediated signaling event.

In the present study, we showed that kallikrein gene delivery reduced BUN, proteinuria, NADH/NADPH oxidase activity, and superoxide formation to levels similar to DSS rats on a normal salt diet. These results suggest that kallikrein is capable of protection against salt-induced renal damage by suppression of oxidative stress. However, since salt-induced renal fibrosis causes

**Fig. 5. Determination of oxidative stress in renal extracts.** (A) NADH and (B) NADPH oxidase activity in renal extracts. Kallikrein gene delivery decreased salt-induced NADH/NADPH oxidase activities in the renal cortex collected two weeks after gene delivery. Values are expressed as mean ± SEM (N = 5 to 7). (C) Superoxide formation in the renal tissue extracts. Renal cortex was collected two weeks after kallikrein gene transfer. Kallikrein reduced salt-induced superoxide formation. Values are expressed as mean ± SEM (N = 5 to 7).
structural changes in the kidney, the reduction of oxidative stress measured at 2 weeks after gene delivery may reflect the time point at which the biochemical signaling is altered while the kidney is still undergoing morphologic and functional changes. This would imply that the reduction of oxidative stress we observed occurred before the changes in kidney morphology and function were complete.

Our previous study showed that after more than 5 weeks of high salt loading (or 11 days after virus injection), DSS rats injected with control virus had a systolic blood pressure value of 240 mm Hg, which was slightly reduced by 20 mm Hg in the DSS rats receiving the kallikrein gene [28]. It is likely that this decrease in blood pressure by kallikrein gene transfer may potentially contribute to the renal protective effects observed.
in the current study. However, the blood pressure of rats receiving the kallikrein gene was still 50 to 60 mm Hg higher than DSS rats on low salt [28]. Based on this data, renal protection mediated by kallikrein gene transfer appears to be only partially dependent on blood pressure change. In support of this notion, it has been shown that a long-term infusion of rat urinary kallikrein in DSS rats on high salt intake was able to attenuate renal injury without affecting systolic blood pressure [54]. Furthermore, infusion of the B₂ receptor antagonist HOE140 in salt-loaded DSS rats abolished kallikrein’s protective effect in the kidney, but had no effect on the time-dependent rise in blood pressure [55]. Taken together, these results suggest that kallikrein/kinin attenuates salt-induced kidney damage through mechanisms independent of blood pressure reduction.

Our previous studies indicate that there are no significant differences between the results from rats treated with the same experimental procedure with or without injection of adenovirus containing a reporter gene. For instance, Agata et al [56] demonstrated no significant differences of neointima formation in rat artery after balloon angioplasty with or without Ad.CMV-Luc gene transfer. In addition, Wolf et al [57] showed that in rats subjected to 5/6 renal mass reduction with or without receiving Ad.CMV-Luc gene transfer, no significant differences were observed in blood pressure, BUN, indices of renal and cardiac injury, or urinary kinin, nitric oxide, and cGMP levels. Furthermore, our previous study showed that the blood pressure did not differ between DSS rats fed 4% NaCl injected with or without control virus [58]. Overall, these findings provide evidences that the injection of control virus alone does not contribute to any additional effects in our animal models as compared to rats without injection. In addition, overexpression of human tissue kallikrein in transgenic mice may result in inflammatory response, as we observed changes in the cytotoarchitectures of several lymphatic organs [59]. However, we have shown that kallikrein gene transfer into genetically hypertensive rats does not cause an immune response, as determined by the absence of antibodies against human tissue kallikrein or its DNA in the sera after gene transfer [60, 61]. These results indicate that somatic delivery of the kallikrein gene does not elicit an immune response. It should also be noted that endogenous kallikrein is primarily located in the distal nephron of the kidney, whereas exogenous kallikrein after systemic gene transfer can be expressed at various sites of the kidney; secreted into the circulation; and excreted in the urine [25, 28]. Therefore, kinins released by exogenous kallikrein activity may act on regions of the kidney that are not restricted to the location of endogenous kallikrein. In this regard, it is possible that human tissue kallikrein transfer may affect areas that are not directly regulated by endogenous kallikrein.

**CONCLUSION**

The current study demonstrated that kallikrein gene delivery offers protection against salt-induced renal cell proliferation and fibrosis, due most likely to reduced oxidative stress and TGF-β1 expression. The beneficial effects of kallikrein on renal fibrosis may be mainly mediated via activation of nitric oxide/cGMP signaling pathways. The finding that kallikrein/kinin attenuates renal fibrosis provides significant insight into future therapeutic applications in treating salt-induced end-stage renal diseases.

**ACKNOWLEDGMENTS**

This work was supported by National Institutes of Health grants HL29397 and DK066350. We thank Dr. Jo Anne Simson for her assistance in the evaluation of kidney histology.

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