Role of Tissue Kallikrein in Prevention and Recovery of Gentamicin-Induced Renal Injury

Grant Bledsoe,*,† Bo Shen,† Yu-Yu Yao,† Makato Hagiwara,† Brandon Mizell,* Michael Teuton,* Daniel Grass,* Lee Chao,† and Julie Chao†

*Department of Biology, Charleston Southern University, 9200 University Boulevard, Charleston, South Carolina 29406; and †Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, 171 Ashley Avenue, South Carolina 29425

Received November 14, 2007; accepted December 20, 2007

Gentamicin is an aminoglycoside antibiotic that induces severe nephrotoxicity and acute renal failure. In the current project, we investigated the protective effects of tissue kallikrein (TK) protein administration (1 μg/h via osmotic minipumps) on kidney damage, apoptosis, and inflammation both during and after a 10-day regimen of gentamicin (80 mg/kg body weight/day sc) in Sprague-Dawley rats. TK infusion during gentamicin treatment significantly attenuated drug-induced renal dysfunction, cortical damage, and apoptosis. Moreover, TK reduced inflammatory cell accumulation in conjunction with diminished superoxide production and decreased expression of tumor necrosis factor-α, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1. The protective effects of TK were blocked by coinfusion of icatibant (1.3 μg/h), indicating a kinin B2 receptor-mediated signaling event. After cessation of gentamicin treatment, TK infusion for 2 weeks completely restored kidney histology and morphology comparable to that of saline-treated animals. Furthermore, TK reduced gentamicin-induced renal dysfunction and fibrosis as evidenced by decreased myofibroblast and collagen accumulation in the kidney. In vitro, gentamicin increased the number of apoptotic cells and caspase-3 activity, but decreased phosphorylation of the prosurvival kinase Akt, in immortalized rat proximal tubular cells; addition of TK and bradykinin phosphorylation of the prosurvival kinase Akt, in immortalized rat proximal tubular cells; addition of TK and bradykinin

Key Words: aminoglycoside; apoptosis; fibrosis; inflammation; kidney.

Gentamicin is an aminoglycoside antibiotic commonly used in the treatment of gram-negative bacterial infections. However, 30% of patients treated with gentamicin for more than 7 days show signs of nephrotoxicity (Mathew, 1992). In addition, 10–15% of all cases of acute renal failure are attributed to gentamicin administration (Erdem et al., 2000). Although the majority of aminoglycosides is excreted in the urine after drug administration, approximately 5–10% of the dose accumulates in the renal cortex and remains there long after discontinuation of the drug (Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004). It is believed that the polycationic aminoglycoside gentamicin is taken up into proximal tubular cells of the nephron by binding to negatively charged phospholipids on the brush border and is then quickly transferred to the transmembrane protein megalin (Mingeot-Leclercq and Tulkens, 1999). After internalization via endocytosis, the aminoglycoside is transported to the lysosome and tightly binds to acidic phospholipids in the lipid bilayer, causing reduced phospholipase activity and a buildup of phospholipids. Accumulation of phospholipids, known as lysosomal phospholipidosis, results in tubular necrosis. Other factors that contribute to the pathogenesis of gentamicin nephrotoxicity include hydroxyradical formation, Na⁺-K⁺-ATPase inhibition, mitochondrial injury, and renin-angiotensin system activation (Ali, 1995).

Acute renal failure, due to ischemia or nephrotoxicant exposure, may lead to death or sublethal injury of proximal tubular cells. It is believed that the surviving cells repolarize and/or dedifferentiate, proliferate, migrate to denuded areas, differentiate, and restore nephron structure and function (Nony and Schnellmann, 2003). This process regenerates the tubular epithelium and leads to the restoration of overall renal function. Indeed, three decades ago, Houghton et al. (1976) noted that 4 weeks after a 10-day regimen of gentamicin, rat kidneys were visually comparable to controls, except for a few areas of interstitial fibrosis and tubular atrophy. More recently, it was shown that, despite recovery of renal function 30 days after cessation of gentamicin treatment, rats had increased macrophage infiltration, myofibroblast accumulation, and extracellular matrix (ECM) accretion in the kidney (Geleilete et al., 2002). The inflammatory and fibrogenic responses to gentamicin were associated with increases in transforming growth factor (TGF)-β, endothelin, and angiotensin II levels, implying their involvement in the progression of tubulointerstitial
nephritis. It has also been observed that gentamicin administration can induce apoptosis of cultured proximal tubular cells (El Mouadden et al., 2000). Furthermore, formation of reactive oxygen species (ROS), such as superoxide, has been shown to increase upon gentamicin treatment (Cuzzocrea et al., 2002; Sha and Schacht, 1999). This elevation of ROS would stimulate the activation or expression of proinflammatory and proapoptotic mediators, including NF-κB, leukocyte adhesion molecules, mitogen-activated protein kinases (MAPKs) and TGF-β1 (Diamond et al., 1994; Finkel, 1998; Pueyo et al., 2000; Tang et al., 1994), contributing to kidney damage induced by gentamicin. Thus, steps to prevent or reverse aminoglycoside-induced kidney injury would have significant clinical value.

Tissue kallikrein (TK) is a serine proteinase that releases the vasoactive kinin peptide from low-molecular weight kininogen (Bhoola et al., 1992). The binding of kinins to the constitutively expressed kinin B2 receptor causes an increase in intracellular Ca2+ levels which can enhance endothelial nitric oxide synthase activity and thus nitric oxide (NO) production. NO, in turn, can activate soluble guanylate cyclase, triggering cGMP formation. Through these mechanisms, kinin can elicit a broad range of biological responses, including vasodilation, blood pressure reduction, and vascular permeability. Rats treated with gentamicin have been reported to have dramatically reduced levels of urinary kallikrein compared to untreated control rats (Higa et al., 1985). This suggests that low amounts of TK may contribute to the pathogenesis of acute renal failure. However, kallikrein replacement by gene delivery was found to attenuate kidney damage in rats receiving gentamicin treatment (Bledsoe et al., 2006a; Murakami et al., 1998). In the present study, we examined the preventive and potential regenerative effects of TK administration by protein infusion both during and after gentamicin treatment.

MATERIALS AND METHODS

Animal treatment. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–220 g were used in this study. The rats were housed at a constant room temperature with a 12-h light/dark cycle and had free access to tap water and rat chow. 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). The protocol for our animal studies was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. This study was divided into two parts: (1) investigation of the preventive effect of TK infusion during 10 days of gentamicin treatment and (2) examination of the reparative/regenerative effect of TK on kidney recovery 2 weeks after cessation of the 10-day gentamicin regimen. On each day of the treatment, gentamicin (Sigma, St Louis, MO) was freshly prepared in sterile saline immediately before sc injection into rats. TK was purified from rat salivary glands according to the previously described procedures (Chao and Margolius, 1979).

For the preventive study, rats were randomly divided into four groups. Control rats received daily sc injections of saline. The second group received sc injections of gentamicin daily for 10 days at a dose of 80 mg/kg body weight (BW)/day. The third and fourth groups were injected with gentamicin along with infusion of TK (1 μg/h) via sc implantation of osmotic minipumps (Durect Corporation, Cupertino, CA), with or without confinon of icatibant (1.3 μg/h, Sigma), a kinin B2 receptor antagonist. On the 11th day, all rats were anesthetized by ip injection of pentobarbital (50 mg/kg BW).

For the reparative/regenerative study, rats either received daily sc injections of saline or gentamicin for 10 days at a dose of 80 mg/kg BW/day. On the 11th day, one group of gentamicin-treated rats was euthanized (Gent Day11), and another group was sacrificed 2 weeks later (Gent Day25). An additional group received TK infusion (1 μg/h), beginning the 11th day, for 2 weeks and were then euthanized (Gen/TK Day25).

Blood collection and assays. Blood was collected via tail vein withdrawal or cardiac puncture. After collection, blood samples were centrifuged at 1000 × g for 20 min, and sera were removed and stored at −20°C. Blood urea nitrogen (BUN) levels were measured using a modified urease-indophenol method (Zhang et al., 2004), and serum creatinine (sCr) levels were determined by a commercial kit (BioAssay Systems, Hayward, CA).

Blood pressure measurement. Systolic blood pressure was measured with DASYlab 5.5 software (Kent Scientific, Torrington, CT) 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 animal had become acclimated to the environment.

Morphological and histological investigations. At the time of sacrifice, kidneys were removed, fixed in 4% formaldehyde solution, dehydrated, and paraffin embedded. Kidney sections (4 μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or Sirius red for histological analyses. PAS stains carbohydrates and carbohydrate moieties of glycoproteins, such as those of the brush border of proximal tubular cells; brush border loss indicates proximal tubular cell damage. Sirius red was used to examine the extent of fibrosis, as those specifically stains collagen fibers. Morphological injury was scored using H&E-stained kidney sections as previously described (Erdem et al., 2000): 0 = normal; 1 = areas of focal granulovascular epithelial cell degeneration and granular debris in the tubular lumina with or without evidence of desquamation in small foci (<1% of total tubule population involved by desquamation); 2 = tubular epithelial necrosis and desquamation easily seen but involving less than half of the cortical tubules; 3 = more than half of the proximal tubules showing necrosis and desquamation, but intact tubules are easily identified; and 4 = complete or almost complete proximal tubular necrosis. Immunohistochemistry was performed using the Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA), following the supplied instructions. Kidney sections were incubated at 4°C overnight with antibodies against ED-1 (Chemicon, Temecula, CA) to detect monocytes and macrophages, α-smooth muscle actin (α-SMA) (Dako, Glostrup, Denmark) to detect myofibroblasts or proliferating cell nuclear antigen (PCNA, Sigma) to identify proliferating cells.

For the following histological investigations, only cortical sections were examined. Neutrophils were identified by H&E staining and quantified. Quantification of monocytes/macrophages was determined by counting ED-1–positive cells in 10 fields at ×400 magnification. PCNA-positive cells were quantified in 20 fields per cross-section in the cortex, excluding glomeruli, at ×400 magnification. Using an in situ cell death detection kit (Roche, Indianapolis, IN), terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL), with propidium iodide counterstaining, was performed to detect apoptotic cells. The number of apoptotic cells was expressed as percentage of TUNEL-positive cells.

Measurement of myeloperoxidase activity and superoxide formation. Renal cortical tissue was homogenized in lysis buffer (25mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 2mM ethylenediamine tetraacetic acid) containing 1:100 protease inhibitor cocktail (Sigma) and centrifuged at 4°C for 30 min. Myeloperoxidase activity was determined as previously described (Suzuki et al., 1983). Superoxide production in cortical extracts was quantified by a spectrophotometric assay based on rapid reduction of myeloperoxidase activity and superoxide formation.
ferricytochrome c to ferrocytochrome c according to a modified previous protocol (Griendling et al., 1994). Reduction of ferricytochrome c independent of superoxide was corrected for by deducting the activity not inhibited by superoxide dismutase.

Quantitative real-time PCR for tumor necrosis factor-α, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1. Total RNA was extracted from the renal cortex using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was transcribed using a cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR reactions were carried out using 10 µl of 2× TaqMan Universal PCR Master Mix, 1 µl of 20× TaqMan Gene Expression Assay Mix (Mm00443258_ml for tumor necrosis factor-α [TNF-α], Rn01456716_gl for monocyte chemoattractant protein-1 [MCP-1], and Rn00564227_ml for intercellular adhesion molecule-1 [ICAM-1]), and 9 µl of cDNA in duplicate. Reactions were run on a 7300 real-time PCR system (Applied Biosystems) with the following thermal cycler conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Transcription of the housekeeping gene GAPDH was determined by specific primer/probe mix.

Cell culture study. Immortalized rat proximal tubular cells (a gift from Dr Julie Ingelfinger, Harvard Medical School, Boston, MA) were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 4mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1× nonessential amino acid, and 5% fetal calf serum at 34°C in an atmosphere of 95% air and 5% CO₂. Cells were serum starved overnight once they reached 70% confluency. Cells were then incubated with gentamicin (3mM), with or without TK (50nM) or bradykinin (BK) (1µM), for 24 h. Cellular apoptosis was determined by incubation with Hoechst 33342 (10 µg/ml) for 5 min in the dark. Apoptotic cells were identified by their distinct condensed nuclei. Caspase-3 activity in cell lysates was determined using a fluorometric caspase-3 assay kit (Oncogene, San Diego, CA) according to the manufacturer’s instructions. Reaction was monitored by a blue to green shift in fluorescence upon cleavage of 7-amino-4-trifluoromethylcoumarin. Samples were read with a fluorescence reader (PerkinElmer Life Sciences, Waltham, MA). Western blot analysis was performed to detect the total and phosphorylated forms of Akt (Cell Signaling Tech., Beverly, MA).

Statistical analysis. Data were compared among experimental groups using ANOVA followed by Fisher’s protected least squared difference. Data are expressed as mean ± SE. Differences were considered statistically significant at a value of p < 0.05.

RESULTS

Study 1: Prevention of Gentamicin-Induced Nephrotoxicity

Effect of TK infusion on gentamicin-induced renal dysfunction and blood pressure. BUN and sCr levels were measured to evaluate the effect of gentamicin on renal function (Figs. 1A and 1B). After 10 days of gentamicin treatment, a significant increase in both BUN and sCr levels was observed compared to the saline-treated group. TK administration prevented the rise in BUN and sCr caused by gentamicin. The protective effect of TK on renal function was blocked by icatibant, indicating a kinin B2 receptor–mediated event. Systolic blood pressure, recorded on days 4 and 8 of gentamicin administration, was not altered among all four groups. Figure 1C shows blood pressure data on day 8 of gentamicin treatment.

FIG. 1. Renal dysfunction was determined indirectly by measuring (A) BUN and (B) sCr levels from blood collected 10 days after gentamicin treatment. (C) Systolic blood pressure measurements on day 8 of gentamicin administration. Values are expressed as mean ± SE (n = 6–8). *p < 0.05 versus saline and Gent/TK.
Effect of TK on gentamicin-induced kidney damage. Renal damage was assessed in PAS-stained kidney sections after 10 days of gentamicin treatment (Fig. 2A). Rats receiving saline injections had well preserved kidneys. Administration of gentamicin for 10 days caused considerable and widespread damage in the cortex. Extensive tubular necrosis, desquamation and dilatation, brush border loss, and occasional protein casts were observed in the cortical regions of the gentamicin group. Tubular morphology appeared normal in the medulla, except for a few protein casts. TK infusion attenuated gentamicin-induced kidney injury as indicated by fewer necrotic cells, dilated tubules and protein casts, and restoration of brush border. Tubular damage was quantified by a scoring method using H&E-stained slides (Fig. 2B). Rats receiving gentamicin had the most proximal tubular damage compared to the other groups. However, tubular injury score was significantly reduced in rats receiving TK infusion, and coinfusion of icatibant blocked TK’s effect.

TK prevents gentamicin-induced apoptosis in kidney and cultured proximal tubular cells. Tubular cells appeared to be the primary site of apoptosis in the cortex of rats receiving gentamicin, as determined by TUNEL staining (Fig. 3A). TK protein infusion in gentamicin-treated rats significantly decreased the amount of TUNEL-positive cells in the cortex compared to the gentamicin group, but icatibant blocked TK’s effect (Figs. 3A and 3B). Rats in the saline group had an insignificant number of TUNEL-positive cells. In addition, immortalized rat proximal tubular cells treated with gentamicin (3mM) underwent apoptosis, as determined by Hoechst 33342 staining (Fig. 3C). However, both TK (50nM) and BK (1lM) treatment significantly reduced the occurrence of cellular apoptosis induced by gentamicin. Few apoptotic cells were identified in the untreated control group. These findings were confirmed by quantification of apoptotic cells (Fig. 3D). Moreover, caspase-3 activity in proximal tubular cell lysates was increased upon gentamicin treatment compared to the other groups but reduced by addition of BK or TK (Fig. 3E). Furthermore, gentamicin caused a noticeable decrease in Akt phosphorylation compared to the untreated control group (Fig. 3F). However, phosphorylation of Akt was significantly elevated by both BK and TK above that of cells treated with gentamicin.

TK reduces gentamicin-induced inflammatory cell infiltration. Inflammatory cell accumulation in the kidney was determined by immunohistochemical staining against ED-1, a specific marker for monocytes and macrophages (Fig. 4A). These cells were detected primarily in the interstitium. TK infusion significantly reduced monocyte/macrophage infiltrates in the kidney compared to rats receiving gentamicin alone. ED-1 immunostaining was barely detectable in the saline group. Quantification of ED-1–positive cells verified these observations (Fig. 4B). Neutrophils were identified by H&E staining and quantified (Fig. 4C). Gentamicin administration caused an increase in neutrophil infiltration compared to saline-treated rats, whereas TK infusion significantly reduced gentamicin’s effect. Icatibant blocked TK’s inhibitory effects on both monocyte/macrophage and neutrophil accumulation in the kidney. In addition, myeloperoxidase activity was significantly higher in the gentamicin group than that of the saline group (Fig. 4D) but was significantly reduced by TK infusion. TK’s effect on myeloperoxidase activity was partially blocked by icatibant (p < 0.07).

TK infusion reduces superoxide formation and proinflammatory mediator expression. Gentamicin treatment caused an elevation of superoxide formation significantly above that of
FIG. 3. TK reduces renal cell apoptosis. (A) TUNEL with propidium iodide counterstaining in the renal cortex. Propidium iodide stains nuclei red. Magnification is ×200. (B) Quantification of apoptotic cells. (C) Hoechst 33342 staining of cultured immortalized rat proximal tubular cells. Magnification is ×200. (D) Quantification of apoptotic cells in vitro. (E) Caspase-3 activity in cultured proximal tubular cell lysates (n = 4). (F) Representative Western blots of phosphorylated and total Akt, and ratio of phospho- to total Akt determined by densitometry (n = 3). Values are expressed as mean ± SE. *p < 0.001 versus saline/control and Gent/TK; **p < 0.05 versus other groups; #p < 0.05 versus Gent/BK; †p < 0.001 versus Gent/TK.
FIG. 4. TK protein infusion reduces inflammatory cell infiltration and proinflammatory mediator expression. (A) Immunohistochemical staining for ED-1, a specific marker of monocytes and macrophages, in the cortex after 10 days of gentamicin treatment. Magnification is ×200. (B) Quantification of ED-1-positive cells. (C) Quantification of neutrophils in the cortex of kidney sections stained with H&E. (D) Myeloperoxidase (MPO) activity and (E) superoxide production in cortical extracts. Quantitative real-time PCR was performed to determine relative expression levels of (F) TNF-α, (G) MCP-1, and (H) ICAM-1 in cortical extracts. Values are expressed as mean ± SE (saline, n = 4; experimental groups, n = 5–7). *p < 0.005 and **p < 0.05 versus saline and Gent/TK.
the saline group (Fig. 4E). TK infusion in gentamicin-treated rats dramatically reduced superoxide production compared to rats receiving gentamicin alone. However, TK’s effect on the generation of superoxide was abolished by icatibant. We also investigated the potential involvement of proinflammatory mediator expression in gentamicin-induced renal injury. A significant upregulation of TNF-α, MCP-1, and ICAM-1 gene expression was induced upon gentamicin treatment, as determined by real-time PCR (Figs. 4F–H). Expression of these proinflammatory mediators was markedly reduced in gentamicin-treated rats receiving TK protein infusion. However, expression levels of these proinflammatory genes were elevated upon icatibant coinfusion. These observations implicate a role for a kinin B2 receptor signaling event in reducing oxidative stress and proinflammatory mediator gene expression.

Study 2: Kidney Repair/Regeneration after Gentamicin-Induced Acute Renal Injury

Effect of TK infusion on gentamicin-induced renal dysfunction. Rats treated with gentamicin had dramatically higher BUN and sCr levels at days 11 and 25 compared to the saline group (Table 1). At 2 weeks after termination of gentamicin treatment, TK infusion reduced BUN and sCr levels similar to those in the saline group. These results indicate that TK replacement promotes recovery from gentamicin-induced renal dysfunction.

TK accelerates recovery of renal damage. Administration of gentamicin for 10 days resulted in notable damage in the renal cortex, including tubular necrosis, tubular dilatation, accumulation of protein casts, and loss of brush border (Fig. 5A). Two weeks after termination of gentamicin treatment, renal morphology had improved, as evidenced by a partial restoration of the tubules and brush border, though not to the extent of the saline group. TK infusion, however, accelerated the recovery process as to be comparable to the kidneys in the saline group. Cells undergoing active proliferation were identified by immunohistochemical staining against PCNA (Fig. 5B). Renal cell proliferation was primarily localized to tubular cells. Gentamicin treatment for 10 days caused a dramatic increase in cell proliferation in the renal cortex. Two weeks after cessation of gentamicin administration, the number of PCNA-positive cells declined roughly 50%. Infusion of TK for 2 weeks after termination of gentamicin treatment caused a further reduction in cell proliferation.

TK infusion reduces myofibroblast and collagen accumulation. Myofibroblasts were identified by α-SMA immunohistochemistry (Fig. 6A). As expected, blood vessels stained positively for α-SMA. Ten days of gentamicin administration resulted in a dramatic accumulation of myofibroblasts in the cortical interstitium, as evidenced by numerous α-SMA-positive cells. At 2 weeks after cessation of gentamicin treatment, the number of myofibroblasts declined. However, infusion of TK for 2 weeks caused a marked decrease in

![FIG. 5. TK restores kidney morphology and histology. (A) Kidney histology was examined by PAS staining. Magnification is ×200. (B) Quantification of PCNA-positive cells in the renal cortex. Values are expressed as mean ± SE (saline, n = 3; experimental groups, n = 5–7). *p < 0.01 versus saline and Gent/TK.](https://academic.oup.com/toxsci/article-abstract/102/2/433/1674306)
myofibroblasts compared to the 2-week control group. Rats in the saline group had practically no α-SMA–positive staining in the kidney except for blood vessels. In order to determine the extent of fibrosis, kidney sections were stained with Sirius red, which specifically stains collagen fibers (Fig. 6B). Sirius red staining of kidneys from rats 1 day after cessation of gentamicin administration was comparable to that of the saline group. However, several foci of fibrosis were observed 2 weeks after termination of gentamicin treatment. Administration of TK dramatically reduced collagen accumulation compared to the 2-week control group.

**DISCUSSION**

In the current study, we examined the preventive and reparative effects of TK administration by protein infusion both during and after a 10-day regimen of gentamicin. In the preventive study, we found that TK replacement improved renal function and decreased renal apoptosis, inflammation and proinflammatory mediator expression, with no effect on blood pressure. Moreover, we demonstrated that TK and BK inhibited gentamicin-induced apoptosis in cultured proximal tubular cells. In the reparative/regenerative study, TK infusion for 2 weeks significantly improved renal function and completely restored kidney morphology by reducing gentamicin-induced accumulation of myofibroblasts and collagen molecules. As renal kallikrein levels are markedly reduced in rats after gentamicin administration (Higa et al., 1985), our findings indicate that TK replacement can accelerate the spontaneous reparative process of the kidney and reduce the incidence of fibrosis after an acute nephrotoxic insult induced by gentamicin. Taken together, our study suggests an important role for TK in the prevention and repair of kidney injury in a setting of acute and chronic renal failure.

Apoptosis is a crucial early event that can initiate injury-induced inflammation and subsequent tissue damage. Indeed, treatment with antiapoptotic agents such as insulin-like growth factor-1 or caspase inactivators in animals with acute renal failure can block the early onset of not only apoptosis but also inflammation and tissue injury (Daemen et al., 1999). The aminoglycoside gentamicin specifically targets the proximal tubular cells in the kidney, typically resulting in their death, with the ensuing loss of other tubular cells in the cortex and medulla. We observed necrotic and apoptotic cells in the cortex 10 days after administration of gentamicin. TK infusion significantly suppressed the development of cellular apoptosis caused by gentamicin, but the effect was blocked by coinfusion of icatibant, a kinin B2 receptor antagonist. This suggests that the kinin B2 receptor mediates the antiapoptotic activity of TK. This observation is consistent with our previous study using human TK gene transfer in this rat model (Bledsoe et al., 2006a). In the current study, we further verified the antiapoptotic effect of kallikrein/kinin *in vitro*. Apoptosis of immortalized rat proximal tubular cells induced by gentamicin was prevented by TK and BK treatment. Inhibition of apoptosis occurred in conjunction with reduced caspase-3 activity and increased Akt phosphorylation. Akt is a key effector molecule in the survival pathway against apoptosis as phosphorylation of Akt blocks cytochrome c release and caspase-3 activation (Zhang et al., 2003). Our results show that both TK and BK inhibit gentamicin-induced tubular epithelial cell apoptosis and stimulate the Akt signaling pathway. It has been well documented that acute renal failure, whether induced by gentamicin or some other nephrotoxic agent, promotes oxidative stress in the form of ROS (Basnakian et al., 2002; Sha and Schacht, 1999). ROS can stimulate apoptosis by causing DNA strand breaks, either directly or indirectly (Basnakian et al., 2002), and activating MAPKs, particularly p38MAPK and JNK (Finkel, 1998; Rana et al., 2001). Thus, the decrease in apoptosis by TK and BK may be partially due to the suppression of superoxide production.

Kidney injury, via necrosis and/or apoptosis, predictably triggers an inflammatory response. All cell types in the kidney (e.g., endothelial, tubular epithelial, interstitial) are capable of synthesizing chemokines and their receptors (Segerer et al., 2000).
The cytokine TNF-α stimulates the expression of chemokines, particularly MCP-1, through ROS production (Chen et al., 2004; Volk et al., 2000). The chemokines are then secreted to attract circulating leukocytes to the site of injury. TNF-α also upregulates the expression of leukocyte adhesion molecules in renal tubular and endothelial cells (Spiecker et al., 1998; Wuthrich et al., 1993). ICAM-1 and other adhesion molecules are then displayed on the cell surface to recruit neutrophils and monocytes. After a firm interaction occurs between the leukocyte and the tubular or endothelial cell, the leukocytes undergo spreading, diapedesis, extravasation, and migration into the interstitial space (Segerer et al., 2000). Once in the damaged area, the inflammatory cells release a broad range of cytokines and chemokines that amplify the inflammatory response. In our study, we showed that rats receiving gentamicin had a marked accumulation of neutrophils and monocytes/macrophages in the kidney, in conjunction with increased myeloperoxidase activity. Elevated expression of TNF-α, MCP-1, and ICAM-1 correlated with the infiltration of inflammatory cells. Furthermore, the increase in renal inflammation induced by gentamicin treatment was associated with augmented superoxide levels. TK infusion in gentamicin-treated rats, however, suppressed superoxide production as well as proinflammatory mediator expression and inflammatory cell accumulation. Inhibition of the effects of TK by icatibant coinfusion indicates that TK’s actions against inflammation are mediated by the kinin B2 receptor.

Renal regeneration is a common mechanism following ischemia or toxicant exposure. Thus, interventions to promote or accelerate tubular cell repair would be beneficial for human patients. A previous study showed that TK expression was specifically diminished in the posts ischemic kidney after recovery from ischemia/reperfusion injury, implicating an important role of renal kallikrein in altering long-term renal function (Basile et al., 2005). Houghton et al. (1976) reported that kidneys of rats injected with gentamicin had undergone extensive regeneration and were morphologically comparable to control rats 4 weeks after termination of drug treatment, except for occasional interstitial fibrosis and tubular atrophy. In order to determine if TK could accelerate the regenerative process after gentamicin-induced nephrotoxicity, we examined rat kidneys 2 weeks after gentamicin exposure. Two weeks after cessation of drug treatment, kidney histology was improved but was not equivalent to kidneys of rats injected with saline. Infusion of TK during the 2-week period resulted in a complete restoration of renal histology as kidneys were structurally comparable to kidneys in the saline group. In addition, the regenerative effect of TK was accompanied by a return to normal BUN and sCr levels. This indicates that TK can accelerate the regenerative process of the kidney and restore renal function after acute renal failure, which may prove useful in the clinical setting.

Many quiescent cells enter the cell cycle in order to repopulate/replace kidney cells after acute renal injury. Previous reports have attributed proximal tubules and interstitial fibroblastic cells as sources of proliferating cells during the tissue repair process following aminoglycoside-induced kidney damage (Laurent et al., 1988; Nonclercq et al., 1988). The proliferation of these cells can be stimulated by a variety of growth factors, including epidermal growth factor and hepatocyte growth factor (Mene et al., 2003). In the present study, we observed a dramatic increase in PCNA-positive cells in the renal cortex 1 day after gentamicin treatment termination, which declined 2 weeks later. Administration of TK during the tissue recovery process resulted in a significantly lower number of proliferating cells compared to the gentamicin group at day 25. As TK accelerated renal regeneration after gentamicin treatment, the decrease in PCNA-positive cells may reflect that TK promotes cell proliferation until kidney histology and morphology is restored. Whether TK’s regenerative effects are due to stimulation of renal cell proliferation, either directly or through up-regulation/activation of growth factors, or some other mechanism, have yet to be elucidated.

In addition to expressing proinflammatory mediators, interstitial monocytes/macrophages also produce profibrotic factors, such as TGF-β, as well as ECM components, including fibronectin and collagens. The presence of myofibroblasts, as a result of epithelial-mesenchymal transition and/or the activation of resident fibroblasts by factors such as TGF-β, was determined by α-SMA immunohistochemical staining. These cells actively secrete large amounts of ECM proteins and TGF-β. We found that the number of α-SMA-positive cells decreased 2 weeks after termination of gentamicin treatment. Therefore, the occurrence of a large number of myofibroblasts 1 day after the 10-day gentamicin regimen suggests that these cells may contribute to the fibrotic condition observed 2 weeks later. However, TK administration abrogated the buildup of ECM in the kidney. TK’s protective effects against renal fibrosis have also been observed in other animal models, including Dahl salt-sensitive rats and deoxycorticosterone-ace tate salt rats (Bledsoe et al., 2006b; Xia et al., 2005). These data indicate that TK infusion offers a protective effect against the development of kidney fibrosis after an acute renal insult.

In summary, our findings show that TK protein infusion elicits renal protective effects against aminoglycoside-induced nephrotoxicity. TK administration during gentamicin treatment improved renal function and prevented kidney damage, oxidative stress, apoptosis, inflammation, and proinflammatory mediator expression via stimulation of the kinin B2 receptor. Our cell culture studies using proximal tubular epithelial cells suggest that activation/phosphorylation of the prosurvival effector Akt may contribute to the antiapoptotic action of TK. Moreover, TK accelerated the reparative/regenerative process and inhibited the development of fibrotic lesions as evidenced by a full restoration of kidney morphology in association with reduced myofibroblast and collagen accumulation 2 weeks after termination of gentamicin treatment.
In conclusion, our studies demonstrate that TK has a protective role in the prevention and recovery of kidney injury after an acute nephrotoxic insult.

FUNDING

National Institutes of Health grants (HL-29397 and DK-066350) to J.C.; Extramural Research Facilities Program of the National Center for Research Resources grant (C06 RR015455) to J.C.; SC IDeA Network of Biomedical Research Excellence grant (P20 RR016461) to J.C. and G.B.

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