Minocycline inhibits apoptosis and inflammation in a rat model of ischemic renal injury

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Kelly, K. J., T. A. Sutton, N. Weathered, N. Ray, E. J. Caldwell, Z. Plotkin, and P. C. Dagher. Minocycline inhibits apoptosis and inflammation in a rat model of ischemic renal injury. Am J Physiol Renal Physiol 287: F760–F766, 2004. First published June 1, 2004; 10.1152/ajprenal.00050.2004.—Tetracyclines exhibit significant anti-inflammatory properties in a variety of rheumatologic and dermatologic conditions. They have also been shown to inhibit apoptosis in certain neurodegenerative disorders. Because ischemic renal injury is characterized by both apoptosis and inflammation, we investigated the therapeutic potential of tetracyclines in a rat model of renal ischemia-reperfusion. Male Sprague-Dawley rats underwent bilateral renal artery clamp for 30 min followed by reperfusion and received either minocycline or saline for 36 h before ischemia. Minocycline reduced tubular cell apoptosis 24 h after ischemia as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining and nuclear morphology. It also decreased cytochrome c release into the cytoplasm and reduced upregulation of p53 and Bax after ischemia. The minocycline-treated group showed a significant reduction in tubular injury and cast formation. In addition, minocycline reduced the number of infiltrating leukocytes, decreased leukocyte chemotaxis both in vitro and ex vivo, and downregulated the expression of ICAM-1. Serum creatinine 24-h posts ischemia was significantly reduced in the minocycline-treated group. We conclude that minocycline has potent antiapoptotic and anti-inflammatory properties and protects renal function in this model of ischemia-reperfusion. Tetracyclines are among the safest and best-studied antibiotics. They are thus attractive candidates for the therapy of human ischemic acute renal failure.

Tetracyclines; acute renal failure; cytochrome c; p53

ACUTE RENAL FAILURE (ARF) following ischemia-reperfusion (I/R) remains a major cause of mortality and morbidity in hospitalized patients (24). It frequently occurs in intensive care unit patients suffering from sepsis or following major surgical interventions. To date, treatment has been mostly supportive and relies predominantly on the control of fluids and electrolytes as well as dialytic intervention when needed. Indeed, many successful approaches for the prevention and treatment of renal I/R in animal models have failed to alter the outcome of patients when attempted at the bedside (3). Despite these drawbacks, renal I/R remains at the forefront of current research aiming at reducing the mortality from this very serious condition.

The pathophysiology of ischemic ARF is very complex but ultimately results from tubular destruction, back leak, obstructive cast formation, and widespread vascular damage. These are thought to occur secondary to an intense inflammatory response initiated by the infiltration of leukocytes and the production of a myriad of proinflammatory cytokines and chemokines (3). Tubular and vascular cell death ensues in the form of necrosis and apoptosis (19). Recently, tubular cell apoptosis has emerged as a primary and major contributor to the pathophysiology of renal I/R and can determine the outcome of renal damage independently of the concomitant inflammatory response (12, 13). Nevertheless, as both inflammation and apoptosis coexist in renal I/R, the ideal preventative or therapeutic approach would indeed target both processes.

Tetracyclines possess a wide repertoire of anti-inflammatory and immunomodulatory actions in addition to their well-characterized antimicrobial effects (18). Indeed, they have not only been successfully used in the management of a variety of rheumatologic and dermatologic conditions but are also being investigated in the treatment of ischemic cardiac and central nervous system disease (1, 6, 29). Their mechanism of action is complex and incompletely understood and includes inhibition of free radical and cytokine production, interference with protein synthesis, and modulation of matrix metalloproteinase activity (6, 18, 22). More recently, tetracyclines were also shown to possess potent antiapoptotic properties manifested by inhibition of caspase 1 and 3 expression and direct blockade of cytochrome c release from mitochondria (5, 34). Thus tetracyclines offer a unique and potentially powerful approach to the prevention and treatment of renal I/R as investigated in this paper.

METHODS

Renal ischemia. All animal experimentation was conducted in conformity with the “Guiding Principles for Research Involving Animals and Human Beings.” Minocycline (Sigma, St. Louis, MO), 45 mg/kg in 0.9% NaCl or an equal volume of 0.9% NaCl (placebo), was administered via intraperitoneal (IP) injection. This was given 36 h before surgery and was followed by 22.5 mg/kg IP every 12 h for a total of four doses. The dose of minocycline was based on the in vivo antiapoptotic efficacy reported in the literature (28). Male Sprague-Dawley rats weighing 180–220 g (Harlan, Indianapolis, IN) were anesthetized with IP pentobarbital sodium (40–70 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C. Both renal pedicles were occluded via a midline incision for 30 min followed by reperfusion. Sham surgery consisted of an identical procedure with the exception of the application of the microaneurysm clamps. Creatinine was determined by standard picric acid reaction in serum obtained from the tail vein or via cardiac puncture.

Light microscopy. Twenty-four hours following surgery, kidneys were fixed with 4% paraformaldehyde, paraffin embedded, sectioned and stained using standard techniques with hematoxylin and eosin. Light microscopy was used to quantify casts and tubular injury and to assess proteinuria.

Immunohistochemistry. As a measure of apoptosis, kidney sections were stained with anti-TUNEL and with a polyclonal antibody to cleaved poly (ADP-ribose) polymerase (PARP) as described previously (23). This antibody is specific for the C-terminal degradation product of PARP. The number of TUNEL-positive cells was counted, and nuclei were analyzed for the presence of PARP using a blinded protocol.

Address for reprint requests and other correspondence: P. Dagher, Dept. of Medicine, Division of Nephrology, 950 W Walnut St, R2-202A, Indianapolis, IN 46202 (E-mail: pdaghe2@iupui.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Minocycline improves the morphological features of injury after renal I/R. Sham-operated animals with or without minocycline treatment showed normal renal morphology (Fig. 1A). In contrast, animals subjected to renal ischemia showed characteristic disruption of architecture, loss of tubular cells, and an abundance of cast formation (Fig. 1, B and C). These changes were predominant in the outer medulla and corticomedullary junction. Ischemic animals treated with minocycline had significantly improved architecture with good preservation of tubular cells and a remarkable reduction in the frequency of casts (Fig. 1, D and E; $P < 0.01$ when the minocycline-treated group was compared with the placebo saline group, $n = 20$ fields in each group).

Minocycline inhibits the infiltration of leukocytes into the ischemic kidney. To study leukocyte infiltration, we used two complementary approaches. First, fixed kidney sections were stained with a leukocyte-specific esterase that predominantly targets neutrophils and macrophages. As shown in Fig. 2A, at 4 μm, and stained using hematoxylin and eosin. The percentage of tubules in the cortex and outer medulla that showed disrupted architecture, tubular cell loss, or luminal casts was quantified [3 to 4 sections per kidney and 10 to 12 fields per section and compared using Fisher’s test as previously described (12, 13)]. Some sections were stained for leukocytes using a Naphtol AS-D Chloroacetate Esterase kit (Sigma) following the manufacturer’s instructions and counterstained with hematoxylin.

**Fluorescence microscopy.** Pieces from the fixed kidneys were preserved in 30% sucrose before 10-μm frozen sections were obtained. Some sections were stained for cytochrome c with a sheep polyclonal antibody (Ab-1, EMD Biosciences, San Diego, CA) followed by an unlabeled rabbit anti-sheep IgG (Zymed Lab, San Francisco, CA) and a Texas red-labeled tertiary donkey anti-rabbit (Jackson Lab, West Grove, PA). ICAM-1 was stained with an anti-ICAM-1 monoclonal antibody (27) (IA29, a kind gift from Dr. M. Miyasaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) followed by a FITC-labeled goat anti-mouse (Jackson Lab). Finally, all sections were counterstained with the nuclear dye DAPI (Molecular Probes, Eugene, OR). Images were collected with a Zeiss LSM 510 confocal microscope and analyzed with Zeiss LSM software and MetaMorph (Universal Imaging).

Separate sections were stained with terminal transferase-mediated dUTP nick end-labeling (TUNEL) reagent (Promega, Madison, WI) and DAPI for in situ apoptosis detection. In brief, 10-μm frozen sections were treated with 20 μg/ml proteinase K and then incubated in a nucleotide mixture containing fluorescein-12-dUTP and terminal deoxynucleotidyl transferase (TdT). Positive controls were pretreated with 1 U/ml DNAse, and negative controls were incubated without TdT. TUNEL-positive nuclei were expressed as a percentage of total nuclei (DAPI positive) per field. Six to eight fields per section and 2 to 3 sections per kidney were examined in each experiment.

Western blots. In some experiments, the kidney was removed without fixation and proteins were extracted immediately from the cortex or medulla using standard techniques. Proteins were then measured by Coomassie blue assay (Pierce Chemical, Rockford, IL) and resolved on a 15% Tris-HCl gel by electrophoresis. An equal amount was loaded in each lane for a given experiment. After electrophoresis, proteins were transferred to a polyvinylidene difluoride filter membrane and probed for p53 (Ab-1, EMD Biosciences) and Bax (B-9, Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate horseradish peroxidase-conjugated secondary antibodies were used along with ECL labels.

**Intravital imaging of leukocytes.** Leukocytes were isolated from two normal rats and red blood cells (RBC) were lysed in an ammonium chloride solution. They were then labeled with acridine orange (30 μg/ml) for 10 min at 37°C. Labeled leukocytes (10⁵) were injected intravenously 24 h after renal ischemia. Imaging of the kidney in the live animal was performed using a Bio-Rad MRC-1024MP Laser-Scanning Confocal/Multiphoton Scanner (Hercules, CA), as previously described in detail (8, 10). After initial imaging, 500 μg of the nuclear dye Hoechst 33342 were administered intravenously over 2 min to allow identification of all cells and examination of nuclear morphology.

**Leukocyte chemotaxis.** Leukocytes were isolated from rats by centrifugation, and RBC were lysed by serial washes with ammonium chloride. White blood cells (WBC) were resuspended in HBSS, and chemotaxis in response to the chemotactic fMLP (10⁻⁶ M) was determined in a standard 48-well microchamber with polycarbonate filters of 3-μm pore size (Nuclepore, Pleasanton, CA) separating upper and lower chambers. In some experiments, WBC were resuspended in RPMI medium and incubated with 10, 1, 0.1, or 0.01 μM minocycline for 60 min before the assay. These concentrations were chosen based on serum levels of 0.5 to 2 μM reported in the literature using protocols similar to ours. Of note is the fact that tetracyclines achieve tissue levels several folds higher than serum and these tissue levels correlate best with activity (11, 17, 21, 25, 26, 33).

**RESULTS**

Minocycline improves the morphological features of injury after renal I/R. Sham-operated animals with or without minocycline treatment showed normal renal morphology (Fig. 1A). In contrast, animals subjected to renal ischemia showed characteristic disruption of architecture, loss of tubular cells, and an abundance of cast formation (Fig. 1, B and C). These changes were predominant in the outer medulla and corticomedullary junction. Ischemic animals treated with minocycline had significantly improved architecture with good preservation of tubular cells and a remarkable reduction in the frequency of casts (Fig. 1, D and E; $P < 0.01$ when the minocycline-treated group was compared with the placebo saline group, $n = 20$ fields in each group).

Minocycline inhibits the infiltration of leukocytes into the ischemic kidney. To study leukocyte infiltration, we used two complementary approaches. First, fixed kidney sections were stained with a leukocyte-specific esterase that predominantly targets neutrophils and macrophages. As shown in Fig. 2A,
sham-operated animals showed no staining in the kidneys. In contrast, animals subjected to renal ischemia had an average of four to six leukocytes per field (Fig. 2, B and C). Some of the leukocytes had morphology suggestive of neutrophils (Fig. 2B, inset). In the minocycline-treated group, the number of infiltrating leukocytes was reduced to zero to two per field (Fig. 2D; \( P < 0.01 \), \( n = 15 \) fields in each group).

We also used a different approach in which prelabeled leukocytes were injected and images were acquired in the live animal using two-photon microscopy. As shown in Fig. 3A, no leukocytes were observed in the sham-operated kidneys. In contrast, labeled leukocytes localized to the ischemic kidney within 10 to 20 min of the injection time (Fig. 3B) with a mean of 2.35 \( \pm 0.5 \) leukocytes per field. These leukocytes were retained in the ischemic kidney for several hours as determined by sequential imaging of the same fields. In minocycline-treated animals, the retention of leukocytes in the ischemic kidneys decreased to 0.39 \( \pm 0.09 \) leukocyte per field (Fig. 3C; \( P < 0.001 \) vs. saline/I/R; \( P = \) not significant vs. sham).

Minocycline reduces leukocyte chemotaxis ex vivo and in vitro and downregulates the expression of renal ICAM-1. The inhibitory effects of minocycline on leukocyte infiltration into the kidney could result from a direct effect on leukocyte activation and/or from an effect on leukocyte adhesion molecules in the kidney. We therefore investigated the effects of minocycline on leukocyte chemotaxis using a Boyden chamber assay. As shown in Fig. 4, leukocytes exposed in vitro to minocycline concentrations as low as 0.01 \( \mu \)M had severely impaired chemotaxis in the Boyden chamber assay. Furthermore, leukocytes harvested ex vivo from minocycline-treated animals also showed decreased chemotaxis compared with leukocytes from saline-treated controls (data not shown).

We also examined the effect of minocycline on the expression of ICAM-1. As shown in Fig. 5, A and B, there was an increased staining for ICAM-1 24 h after I/R. The staining was predominantly in a vascular pattern. Animals treated with minocycline had a markedly reduced staining for ICAM-1 in their ischemic kidneys (Fig. 5C). Thus minocycline has both a direct effect on leukocytes and an indirect effect through downregulation of vascular ICAM-1 expression.

Minocycline prevents tubular cell apoptosis after renal I/R. The effects of minocycline on tubular cell apoptosis were examined next. We used the combined criteria of TUNEL positivity and nuclear changes to quantitate apoptosis in multiple sections. We previously determined the sensitivity and specificity of the TUNEL assay through correlations among live imaging, propidium iodide uptake, nuclear morphology, and the TUNEL stain. In ischemic injury, the specificity and sensitivity of the TUNEL were 99 and 61\%, respectively (14).

As shown in Fig. 6, C and D, sham-operated animals with or without minocycline treatment showed on average 0 to 1\% TUNEL-positive nuclei in all fields examined. In contrast, TUNEL positivity increased to 22\% in ischemic kidneys (Fig. 6E). These were predominantly localized to the outer medullary region. Minocycline treatment reduced TUNEL positivity to less than 4\% (Fig. 6F). In Fig. 7, we show the strong correlation between TUNEL positivity (Fig. 7A) and nuclear fragmentation and condensation (Fig. 7B) that are quite characteristic of apoptosis. Figure 7, A and B, shows the same field but Fig. 7B has only the blue channel on. Of note also is the...
presence of multiple tubular casts composed predominantly of condensed nuclear fragments strongly suggestive of an apoptotic origin (Fig. 7C).

Minocycline reduces the upregulation of p53 and Bax and inhibits the release of cytochrome c into the cytoplasm after I/R. We previously showed that apoptotic cell death after renal I/R is, in part, mediated by p53. We therefore proceeded to determine whether the antiapoptotic effects of minocycline also involved p53. As shown in Fig. 8, ischemia significantly increased medullary p53 protein levels at 24 h and minocycline treatment significantly reduced this increase. Additionally, minocycline prevented the increase in Bax protein, a proapoptotic transcriptional target of p53. Of note is the slight increase in p53 seen in minocycline-treated sham-operated animals. The significance of this increase is unknown, but it was not accompanied by an increase in Bax, as shown in Fig. 8.

Finally, we examined the effects of minocycline on cytochrome c release, a process central to the intrinsic apoptotic pathway. As shown in Fig. 9A, cytochrome c had a punctate and granular perinuclear distribution characteristic of a mitochondrial localization in sham kidneys. After renal ischemia, many fields examined showed cytochrome c staining in a diffuse cytoplasmic pattern indicating release from mitochondria (Fig. 9B) (9, 20). This diffuse cytoplasmic staining of cytochrome c corresponded to areas of intense and widespread nuclear fragmentation and condensation characteristic of apoptosis (Fig. 9C). In ischemic rats treated with minocycline, cytochrome c was found again in a predominantly granular and perinuclear distribution suggestive of a mitochondrial distribution (Fig. 9D). The mitochondrial localization of cytochrome c was also confirmed by colocalization with cytochrome c oxidase staining as we previously demonstrated (13).

Minocycline treatment improves renal function after I/R. Finally, we show that the antiapoptotic and anti-inflammatory effects of minocycline were accompanied by a remarkable improvement in renal function as determined by serum creatinine 24 h after I/R (Fig. 10). Minocycline treatment given once at the time of renal artery clamp resulted in partial protection. In this group, there was less tubular necrosis (P < 0.04) evident in histological sections, but improvements in mean serum creatinine, frequency of casts, TUNEL-positive nuclei, and esterase-positive cells in kidney sections did not reach statistical significance (compared with the saline/I/R group).

Fig. 3. Effects of minocycline on leukocyte infiltration into the kidneys of live animals after I/R. Leukocytes were harvested from donor rats and loaded with acridine orange. They were then injected intravenously into rats subjected to sham surgery or I/R. The kidneys were imaged 24 h after surgery in the live animal with 2-photon microscopy as detailed in METHODS. All nuclei were stained blue with an intravenous injection of Hoechst 33342. Acridine orange-loaded leukocytes show green fluorescence.

Fig. 4. Effect of minocycline on leukocyte chemotaxis in vitro. Leukocytes harvested from donor rats were assayed for chemotaxis in a Boyden chamber in the presence of 0, 0.01, 0.1, 1, or 10 μM minocycline. Bars represent means ± SE, n = 6, *P < 0.001.

Fig. 5. Effect of minocycline and I/R on renal ICAM-1 expression. A-C: representative kidney sections obtained 24 h after sham surgery or I/R and pretreatment with saline or minocycline. Sections are stained with a monoclonal anti-ICAM-1 antibody and a FITC-labeled secondary antibody (green). Nuclei are stained with DAPI (blue).
In this paper, we show that minocycline, a member of the tetracycline family of antibiotics, protects the kidney from the serious consequences of I/R. It does so through potent anti-apoptotic and anti-inflammatory properties that target various components of these two pathophysiological processes commonly at work during I/R. The anti-inflammatory properties of tetracyclines have been demonstrated in ischemic conditions like stroke and myocardial infarction (1, 31). Their antiapoptotic effects were also shown in certain neurodegenerative conditions (5, 30, 32, 34). To our knowledge, this is the first demonstration of their efficacy in the setting of renal I/R through both antiapoptotic and anti-inflammatory effects.

Inflammation is recognized as an important component of renal I/R (3). Neutrophils, lymphocytes, and macrophages have all been proposed to participate in the inflammatory process (3, 23). Our study does not attempt to differentiate between various types of infiltrating cells. Rather, we show a global effect of minocycline on reducing the number of infiltrating leukocytes. Only occasionally was the identification of cells with neutrophil or macrophage morphology certain with the esterase stain.

Our studies with acridine orange-labeled leukocytes in live animals not only complement the esterase assay but they also offer a dynamic temporal and spatial view of the infiltration process. Indeed, leukocytes seem to localize to ischemic kidneys within minutes and are retained there for at least several hours. Despite the possibility of activating leukocytes during the harvesting process, the presence of ischemic injury is necessary for their localization to the kidney. Furthermore, the protective effects of minocycline seem to target both leukocytes and their vascular receptors as shown with the Boyden chamber assay and ICAM-1 staining, respectively. The importance of ICAM-1 molecules in facilitating inflammation is firmly established, and tetracyclines now offer a powerful way of downregulating them (4, 15, 16, 23).

In addition to its anti-inflammatory effects, minocycline also inhibits tubular cell apoptosis after I/R with great efficacy. Apoptosis is increasingly recognized as a major form of cell death during I/R and can even impact the functional outcome independently of inflammation (2, 7, 19). Indeed, we recently showed that inhibition of apoptosis with guanosine or a p53 inhibitor protected glomerular filtration rate (GFR) despite a lack of effect on the inflammatory phenotype (12, 13). Thus apoptosis of tubular cells can be a direct consequence of factors like hypoxia and nucleotide depletion and, when quantitatively significant, can affect GFR by contributing to decreased tubular cell mass, cast formation, and tubular back leak.
Although the antiapoptotic properties of minocycline could be simply secondary to the reduced inflammation, our studies with cytochrome c release also raise the possibility of a direct effect. Indeed, the inhibition of apoptosis was accompanied by a significant reduction of cytochrome c release into the cytoplasm. Recently, such an effect of tetracyclines was demonstrated in neuronal tissues and was shown to result from a direct interaction of tetracyclines with mitochondria (34). In addition, minocycline prevented the upregulation of p53 and Bax following ischemia. Thus cytochrome c release could be inhibited directly or indirectly secondary to p53 inhibition, the two mechanisms being not mutually exclusive. In turn, inhibition of p53 and cytochrome c release will prevent activation of downstream caspases that normally execute the apoptotic program. Whether minocycline also inhibits the expression of caspases was not investigated but has been reported by others (5).

In this paper, the examined outcome was restricted to renal function 24 h after I/R. As we previously demonstrated, inhibition of apoptosis alone can account for this effect. However, the added benefit of reduced inflammation should not be minimized. Indeed, inflammation is likely to be an important determinant of long-term effects of I/R such as fibrosis. These, however, will require more chronic models to be investigated adequately.

In conclusion, tetracyclines possess both antiapoptotic and anti-inflammatory properties in addition to an excellent safety record. They provide significant functional protection in this model of ischemic renal injury. Thus they offer a new and unique tool with which to interfere with the complex pathophysiology of renal I/R.

REFERENCES


