Minocycline reduces renal microvascular leakage in a rat model of ischemic renal injury

Timothy A. Sutton, K. J. Kelly, Henry E. Mang, Zoya Plotkin, Ruben M. Sandoval, and Pierre C. Dagher

Minocycline reduces renal microvascular leakage in a rat model of ischemic renal injury. Am J Physiol Renal Physiol 288: F91–F97, 2005. First published September 7, 2004; doi:10.1152/ajprenal.00051.2004.—Tetracyclines exhibit significant anti-inflammatory properties, inhibit matrix metalloproteinases (MMPs), and are protective in models of ischemia-reperfusion injury (IRI). Both inflammatory cascades and MMP activation have been demonstrated to modulate microvascular permeability. Because increased microvascular permeability occurs during IRI in a variety of organs, we hypothesized that minocycline, a semisynthetic tetracycline, would diminish microvascular leakage during renal IRI. To test this hypothesis, we used intravital 2-photon microscopy to examine leakage of fluorescent dextrans from the vasculature in a rodent model of IRI. Minocycline significantly reduced the extent of dextran (500 kDa) leakage from the renal microvasculature 24 h after ischemia. Although minocycline diminished leukocyte accumulation in the kidney following ischemia, areas of leukocyte accumulation did not correlate with areas of microvascular permeability in either the saline- or minocycline-pre-treated animals. Minocycline diminished the perivascular increase in MMP-2 and MMP-9, as well as the increase in MMP-2 activity 24 h after ischemia. ABT-518, a specific inhibitor of MMP-2 and MMP-9, also significantly reduced the extent of dextran (500 kDa) leakage from the renal microvasculature 24 h after ischemia. Our results indicate that minocycline mitigates the renal microvascular permeability defect following IRI. This effect is spatially distinct from the effect of minocycline on leukocyte accumulation and may be related to diminished activity of MMPs on the integrity of the perivascular matrix.

THE DEVELOPMENT OF ACUTE RENAL failure (ARF) in the hospitalized patient portends a significant increase in morbidity and mortality (25). Recent therapeutic interventions demonstrated to be successful in experimental models of ARF have failed to translate into successful clinical interventions in human ARF (19). Consequently, current therapeutic strategies remain primarily preventative and/or supportive. However, continued progress into understanding the pathophysiology of ARF provides a source of optimism toward future clinical advancements.

Ischemia-reperfusion injury (IRI) alone, or as a contributor in the setting of sepsis and/or multiorgan failure, is the most common cause of human ARF (24). Tubular epithelial cell injury has been of central importance in explaining the decrement in glomerular filtration rate that is the hallmark of ARF; however, the pathophysiology of ischemic ARF has evolved to include a complex interplay between tubular injury, inflammation, and altered renal microvascular function (5). Functional and morphological alterations of the renal vasculature have played a conceptual role in the pathophysiology of ARF for a number of years (9, 31). Recent studies have provided additional evidence for the role that vascular injury, in particular endothelial cell injury, plays in the pathophysiology of ischemic ARF (3, 6, 32). Endothelial cell swelling, disorganization of the actin-cytoskeleton in vascular smooth muscle and endothelial cells, and disruption of endothelial cell attachments are some of the morphological alterations that have been observed in the renal microvasculature following ischemic injury (6, 13, 23, 32). Functional consequences of these morphological alterations include disordered vascular reactivity, imbalanced intravascular hemostatic mechanisms, increased leukocyte adherence, and increased vascular permeability (31). Preventing or ameliorating the microvascular injury that occurs during ARF appears to be an increasingly important component in a multiprong therapeutic approach to ARF.

Tetracyclines are a class of compounds that have been well characterized for their antibiotic properties. Additionally, tetracyclines have recently been demonstrated to have protective effects during IRI in skeletal muscle, liver, heart, brain, and kidney (2, 7, 8, 20, 27, 30, 33, 37, 40, 41). The protective mechanisms of tetracyclines in IRI are not fully understood, although a variety of potential mechanisms have been studied including inhibition of matrix metalloproteinases (MMPs), modulation of inflammatory cascades, and inhibition of apoptosis (2, 20, 27, 33, 37, 41). The potential protective effect of tetracyclines on the microvasculature following IRI has not been investigated in any organ. Consequently, we hypothesized that minocycline, a semisynthetic, second-generation tetracycline, would have a protective effect on the renal microvasculature in ischemic ARF. In this paper, we used intravital 2-photon microscopy to demonstrate that minocycline ameliorates renal microvascular leakage in a rat model of ischemic ARF and that this effect is mediated, in part, by modulation of MMPs.

MATERIALS AND METHODS

Animal model of renal ischemia. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 180–220 g (Harlan, Indianapolis, IN) were administered minocycline (Sigma, St. Louis, MO) 45 mg/kg in 0.9% NaCl or 0.9% saline (vehicle) intraperitoneally 1 h prior to the onset of the ischemic insult and repeated daily for duration of the study. Animals were randomized into two groups: saline (n=16) and minocycline (n=16). 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.
an equal volume of 0.9% NaCl (placebo) via intraperitoneal 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 dosing regimen for minocycline was derived, in part, from prior in vivo studies demonstrating a beneficial effect of minocycline in IRI (41). In separate experiments, male Sprague-Dawley rats were administered ABT-518 (kind gift of Dr. D. H. Albert, Abbott Laboratories, Abbott Park, IL), an orally active inhibitor of MMP-2 and MMP-9 (15, 36), at 100 mg/kg in olive oil or an equal volume of olive oil (placebo) by gavage. This dose was given daily for 2 days before surgery and on the day of surgery. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C. A midline incision was made, the renal pedicles were isolated, and bilateral renal ischemia was induced by clamping the renal pedicles for 45 min followed by reperfusion. Sham surgery consisted of an identical procedure with the exception of immediate release of the microaneurysm clamps. For experiments involving live 2-photon microscopic imaging of rat kidneys, a flank incision was made over the left kidney, the renal pedicle was isolated, and unilateral renal ischemia was induced by clamping the left renal pedicle for 45 min as previously described (12).

**Intravital 2-photon microscopy.** For studies examining renal microvascular permeability, 100 µl of rhodamine-conjugated dextran (3,000 Da, 20 mg/ml in 0.9% saline; Molecular Probes, Eugene, OR), 500 µl of FITC-conjugated dextran (500,000 Da, 7.5 mg/ml in 0.9% saline; Molecular Probes), and 400 µl of Hoechst 33342 (1.5 mg/ml in 0.9% saline; Molecular Probes) were injected via the tail vein into anesthetized rats just before imaging. The left kidney of the anesthetized rat was imaged through a retroperitoneal window via a left-flank incision using a Bio-Rad MRC-1024MP laser-scanning confocal/multiphoton scanner (Hercules, CA) with an excitation wavelength of 800 nm attached to a Nikon Diaphot inverted microscope (Fryer, Huntley, IL) as described by Dunn et al. (12). Image processing was performed using Metamorph software (Universal Imaging, West Chester, PA). Images obtained were analyzed in a 4 × 4 grid, and each grid section (16 per image) was scored for the presence or absence of dextran extravasation for each dextran. The extravasation score for each dextran was calculated by summing the number of grid segments with extravasation of the dextran of interest and dividing by the total number of grid segments scored. Approximately 30 images were collected for each animal examined.

For studies examining the spatial relationship of leukocyte infiltration to renal microvascular permeability, leukocytes were isolated from two normal rats and red blood cells were lysed in an ammonium chloride solution. Leukocytes were labeled with acridine orange (30 µg/ml; Molecular Probes) for 10 min at 37°C. Rhodamine was conjugated to dextran (500,000 Da; Molecular Probes) using 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes). Leukocytes (10^6) labeled as above were injected intravenously 24 h after renal ischemia. Approximately 30 min after leukocyte injection, 500 µl of the rhodamine-conjugated dextran (500,000 Da, 7.5 mg/ml in 0.9% saline) were injected. This time differential was chosen because accumulation of labeled leukocytes reached a steady state by ~20 min. Imaging was performed following injection of dextran as described in the preceding paragraph.

**Western blotting.** Kidneys were removed without fixation, washed with ice-cold saline, minced, and rapidly transferred into 400 µl of ice-cold PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgSO4, pH 6.9) containing 0.5% Triton X-100, 10 µg/ml chymostatin, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 mM 1,4-dithiothreitol (DTT). Samples were sonicated and then allowed to extract on ice for 10 min. Subsequently, the samples were centrifuged at 4°C and the supernatants were carefully removed for protein determinations and Western blotting. Proteins were measured by a Coomassie blue assay (Coomassie Plus; Pierce Chemical, Rockford, IL) and resolved on a 15% Tris·HCl gel by electrophoresis. An equal amount of protein was loaded in each lane for a given experiment. After electrophoresis, proteins were transferred to a PVDF filter membrane and probed with either mouse monoclonal anti-rat MMP-9 (Oncogene, San Diego, CA), mouse monoclonal anti-rat MMP-2 (Oncogene), or rabbit polyclonal anti-human MMP-1 (Calbiochem, San Diego, CA) followed by horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse IgG or HRP-conjugated polyclonal goat anti-rabbit IgG (Bio-Rad). Immunoreactive bands were detected by chemiluminescence (SuperSignal West Dura Extended, Pierce Chemical). Blots were scanned on a Bio-Rad Fluor-S Multimeter to determine band densities.

**Gelatin zymography.** Kidneys were removed without fixation and proteins were extracted using standard techniques. Equal amounts of protein were loaded and resolved on 10% Tris-HCl gel containing 1 mg/ml gelatin (Type A from porcine skin; Sigma). The gels were then processed and stained as previously described (17, 35) to detect gelatinolytic activity. Blots were scanned on a Bio-Rad Fluor-S Multimeter to determine band densities.

**Fluorescence confocal microscopy.** Kidney sections from anesthetized mice were fixed ex vivo in 4% paraformaldehyde, and 50-µm vibratome sections were obtained. Sections were stained with DAPI (Molecular Probes), mouse monoclonal anti-rat MMP-9, or mouse monoclonal anti-rat MMP-2 followed by polyclonal Texas red-labeled sheep anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Images were collected with an LSM-510 Zeiss confocal microscope (Heidelberg, Germany) equipped with argon and helium/neon lasers. Image processing was performed using Metamorph software.

**Statistical analysis.** All results represent an n = 3 unless noted otherwise. Results are expressed as means ± SE. Homogeneity of dextran extravasation from images within the same animal and from animals under the same experimental condition was analyzed by a χ²-test. Subsequently, pooled dextran extravasation data from experimental and control animals were analyzed for significance by a χ²-test. Correlation of the spatial relationship between leukocyte extravasation and dextran leakage was analyzed by Spearman’s rank order correlation. Band density data were analyzed for significance by unpaired Student’s t-test and ANOVA. A P value of 0.05 was considered to be statistically significant.

**RESULTS**

Minocycline diminishes the increase in microvascular permeability following renal ischemia. To investigate the effect of minocycline on the integrity of the renal microvasculature during IRI, we used intravital 2-photon microscopy to examine changes in renal microvascular permeability. We chose to examine renal microvascular permeability at 24 h following ischemia because this is when the defect has been demonstrated to be most extensive (32). We observed no increase in renal microvascular permeability in the sham-operated rats irrespective of treatment with minocycline. Twenty-four hours after ischemia, we observed leakage of both the low-molecular-weight (3,000 Da) dextran (LMWD) and the high-molecular-weight (500,000 Da) dextran (HMWD) from the renal microvasculature in the saline-treated animals (Fig. 1, A–C). The extent of LMWD leakage was greater than that of the HMWD. Extravasation scores following ischemia in the saline-treated animals were 2.71 ± 0.36 and 1.21 ± 0.25, respectively, for LMWD and HMWD. Leakage of both dextrans was also observed following ischemia in the minocycline-treated animals (Fig. 1, D–F). However, the extravasation score (0.03 ± 0.01) and the extent of leakage of the HMWD in the minocycline-treated animals ischemia were significantly less.
than that observed in the saline-treated animals following ischemia (n = 2 animals and 30 images per animal, P < 0.001). There was no significant difference in the extravasation score (2.47 ± 0.27) and the extent of LMWD leakage in the minocycline-treated animals compared with the saline-treated animals following ischemia.

Leukocyte accumulation is spatially distinct from areas of increased microvascular permeability in the rat kidney following renal ischemia. Tetracyclines have been demonstrated to have generalized anti-inflammatory properties (22, 26). Because leukocytes interacting with the microvascular endothelium have been demonstrated to play a role in enhancing endothelial permeability in IRI (11, 14), modulating leukocyte accumulation in the kidney may be a potential mechanism by which minocycline imparts a protective effect on the integrity of the renal microvasculature following ischemia. To investigate the effect of minocycline on leukocyte accumulation and its relationship with microvascular permeability during renal IRI, we used intravital 2-photon microscopy to examine the spatial relationship of leukocyte accumulation to areas of increased renal microvascular permeability following ischemia. As we previously reported (20), fewer leukocytes per field were observed in the minocycline-treated animals compared with the saline-treated animals following ischemia; however, we did not observe a spatial correlation (r = 0.05) of labeled leukocytes with areas of HMWD leakage in either the minocycline-treated or the saline-treated animals following ischemia (Fig. 2).

Minocycline diminishes the increase in MMP-2 and MMP-9 following renal ischemia. Inhibition of MMPs is a well-recognized function of tetracyclines (18). Activation of MMP-2 and MMP-9 following IRI has been implicated in the disruption of microvascular integrity leading to increased permeability in the brain (1, 28). To further elucidate the mechanism by which minocycline may preserve renal microvascular integrity, we used Western blotting, zymography, and immunofluorescence confocal microscopy to examine the effect of minocycline on MMPs in the kidney during IRI. After ischemia, protein levels of both MMP-2 and MMP-9 increased above the levels observed in the sham-operated animals (Fig. 3A).
was observed. There was no change in the level of MMP-1 following renal ischemia. Treatment with minocycline diminished the increase in MMP-2 and MMP-9 protein levels following ischemia to 48 ± 6% (n = 3) and 51 ± 8% (n = 3), respectively, of that observed in the saline-treated animals. Minocycline had no effect on MMP-1 levels. Evaluation of MMP activity by zymography revealed a 340 ± 8% (n = 3) increase in a ~62-kDa band consistent with the active form of MMP-2 following ischemia (Fig. 3B). Treatment with minocycline diminished the increase in MMP-2 activity following ischemia to 46 ± 4% (n = 3) of that observed in the salinetreated animals. Interestingly, an increase in MMP-9 activity was not observed 24 h after ischemia.

Consistent with the changes in MMP-2 and MMP-9 observed with Western blotting, examination of immunostained kidney sections by confocal microscopy revealed an increase in MMP-2 and MMP-9 following ischemia compared with the sham-operated animals (Fig. 4). This increase was much less pronounced in the minocycline-treated animals following ischemia compared with the saline-treated animals. Both MMP-2 and MMP-9 immunostaining were most pronounced in the outer medullary region of the ischemic kidney and both demonstrated an interstitial pattern of staining consistent with perivascular localization. Outside of an overall decrease in the

Fig. 2. Leukocyte accumulation is spatially distinct from areas of increased microvascular permeability in the rat kidney following renal ischemia. Rhodamine-labeled dextran (500,000 Da; red), the nuclear stain Hoechst 33342 (blue), and acridine orange-labeled leukocytes (green) freshly isolated from the buffy coat of littermate rats were injected via the tail vein into MI rats or SI rats 24 h after 45 min of ischemia. A, B, and C: representative images from a SI rat that demonstrate the distribution of labeled leukocytes following ischemia. D: representative image taken from a SI rat that demonstrates an area of extravasation of the rhodamine-labeled HMWD following ischemia. Spatial correlation of leukocytes (arrows) to areas of HMWD extravasation (*) was not observed in either the MI or SI rats (r = 0.05). Bar = 20 μm.

Fig. 3. Minocycline diminishes the increase in matrix metalloproteinase (MMP)-2 and MMP-9 following ischemia. Metalloproteinase levels and activity were measured following sham and renal artery clamp surgeries to determine the effect of minocycline on MMPs. A: representative Western blots of renal medullary tissue for MMP-1, MMP-2, and MMP-9 from saline-treated rats 24 h after ischemia (SI) or sham (SS) surgery and from MI rats 24 h after ischemia (n = 3). B: representative gelatin zymography of renal medullary extracts from saline-treated rats 24 h after SI or SS surgery and from MI rats 24 h after ischemia. Left: molecular weights (kDa). The ~62-kDa band is consistent with the active form of MMP-2 (n = 3).
extent of immunostaining, minocycline treatment did not appear to alter the general localization of MMP-2 or MMP-9 in the ischemic kidney.

To further examine the role that attenuation of MMP-2 activity has on the renal microvascular integrity following ischemia, we examined the effect of ABT-518, an orally active inhibitor of MMP-2 and MMP-9, on HMWD extravasation following renal ischemia. The extravasation score (0.05 ± 0.03) and the extent of leakage of the HMWD in the ABT-518-treated animals were significantly less than that observed in the saline-treated animals following ischemia (n = 3 animals and 20 images per animal, P < 0.001).

**DISCUSSION**

Alteration of microvascular permeability can be a critical component of tissue injury during IRI. Our finding that minocycline exerts a protective effect on the renal microvasculature during renal IRI by ameliorating the leakage of high-molecular-weight substances from the microvasculature provides a novel effect of minocycline and an additional therapeutic mechanism by which minocycline may exert a protective effect in ischemic ARF and IRI in general. The implication of selective protection against leakage of higher molecular weight substances is not fully elucidated. However, in a previous study, we observed that diminished renal microvascular flow following ischemia was most often adjacent to areas where leakage of the higher molecular weight dextran occurred (32). Leakage of the higher molecular weight dextran may indicate areas where microvascular barrier function is most compromised and thus where the renal microvasculature has sustained the greatest damage during IRI. Although our observations were limited to the cortical area of rats due to technical considerations, presumably the permeability defect in the corticomedullary area would be even more pronounced than what we observed in the cortical microvasculature (16). Altered microvascular permeability may have important functional and/or mechanistic significance in the overall pathophysiology of ischemic ARF. Increased microvascular permeability can contribute to extending ischemic injury through compressing peritubular capillaries thereby further compromising medullary blood flow (21). Additionally, leakage of plasma from the vascular space can contribute to hemoconcentration that can lead to stasis and serve to further diminish perfusion as has been observed in other organs (34). Furthermore, leakage of plasma proteins into the interstitium may have important consequences for the modulation of renal injury, renal recovery, and, ultimately, renal function (4).

Our intravital imaging of fluorescent-labeled leukocytes following ischemia provided a dynamic method for the spatial comparison of leukocyte accumulation and microvascular per-
meability defects in the kidney during IRI. The interaction of activated leukocytes and the endothelium has been demonstrated to play a role in enhancing endothelial permeability (11, 14). As anticipated, we did not observe accumulation of leukocytes or permeability defects in the kidney of the sham-operated animals despite the possibility of activating leukocytes during the harvesting procedure. Interestingly, we also did not observe colocalization of leukocytes with areas of dextran leakage in kidneys rendered ischemic regardless of treatment with minocycline. Our finding suggests that direct, ongoing interaction of leukocytes with the renal microvascular endothelium is not a prerequisite for the increase in microvascular permeability at 24 h. In addition, our finding also suggests the converse: that vascular permeability is not a prerequisite for leukocyte accumulation 24 h after renal ischemia. Although increased vascular permeability during IRI has been documented to occur in the absence of leukocytes (29), our observation does not exclude the possibility that inflammatory cascades contribute to renal microvascular permeability during IRI. Initiation of paracrine inflammatory cascades by accumulating leukocytes in the kidney may result in spatially distinct areas of increased microvascular permeability during IRI. Furthermore, the temporal interval of our study was limited due to the nature of intravital imaging. Consequently, we chose to examine leukocyte accumulation at the peak of microvascular permeability. Certainly, a temporal lag between the accumulation of leukocytes, inflammatory injury to the microvasculature, and initiation of microvascular leakage could exist which our study would not have captured. In addition, our observation does not exclude modulation of inflammatory pathways as a mechanism by which minocycline ameliorates renal microvascular permeability during IRI. Minocycline could indirectly alter paracrine inflammatory cascades initiated by accumulating leukocytes by decreasing the overall number of accumulating leukocytes or by directly inhibiting paracrine inflammatory cascades. Finally, our results do not exclude the possibility that activation of unlabeled, endogenous leukocytes could be spatially related to areas of HMWD extravasation, although it would seem unlikely that there would be a complete segregation of the effects of endogenous vs. exogenous leukocytes. Overall, further studies are needed to clarify the full extent that leukocytes play in increasing renal microvascular permeability during IRI.

Another potential mechanism by which minocycline may protect microvascular integrity is through modulation of MMPs. Indeed, recent data suggest that MMPs play an important role in the disruption of cerebral microvascular integrity following ischemia (1, 28). Critical constituents of the vascular matrix, including collagen IV, are known to be substrates of MMP-2 and MMP-9. In the brain, degradation of the perivascular matrix has been demonstrated to weaken vessels and lead to vascular leakage. Our observation in this study that both minocycline and ABT-518 mitigate the renal microvascular permeability defect following ischemia provides evidence that MMPs are important regulators of renal microvascular integrity following renal ischemia. A very recent study demonstrated that MMP-2 and MMP-9 protein levels increased 24 h following renal ischemia and that this increase was localized to the interstitium and thereby in close proximity to the renal microvasculature (4). This study also demonstrated an increase in MMP-2 activity 24 h after ischemia but a delay in the increase in MMP-9 activity until 48–72 h after ischemia. Although we only examined MMP protein levels, localization, and activity 24 h after ischemia to correspond with the maximal microvascular permeability defect, our findings are consistent with this study and suggest that MMP-2 may play a more important role in renal microvascular permeability following ischemia. In addition to breaking down the perivascular matrix, MMPs may be involved in cleaving endothelial cell-cell contacts, providing an additional mechanism by which MMPs could promote an increase in microvascular leakage (38). Interestingly, we observed that minocycline treatment diminished MMP-2 activity and both MMP-2 and MMP-9 protein levels following renal ischemia. We did not observe an effect of ischemia or minocycline on MMP-1 protein levels, suggesting that our observation is not a generalized effect for all MMPs. Although tetracyclines are well recognized for their effect as direct MMP antagonists, the ability of tetracyclines to transcriptionally and posttranslationally regulate MMPs, including MMP-2 and MMP-9, has just recently been appreciated (10). This combination of regulatory mechanisms may be a particularly effective strategy for inhibiting MMPs following ischemia.

In summary, we demonstrated that minocycline mitigates the renal microvascular permeability defect following IRI. This protective effect appears to be spatially distinct from the effect of minocycline on leukocyte accumulation and may be related to diminished activity of MMPs on the integrity of the perivascular matrix. In our previous study (20), minocycline has been demonstrated to inhibit tubular cell apoptosis, diminish inflammation, and provide an overall protective effect on renal function following ischemia. Our finding that minocycline exerts an additional protective effect on the renal microvasculature following ischemia is likely to be an important component of the overall protection provided by minocycline during renal IRI.

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