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Chemically Modified Tetracyclines Inhibit Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production in Cultured Rat Mesangial Cells

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Tetracyclines inhibit matrix metalloproteinases (MMP) and attenuate connective tissue degradation in a wide variety of human and animal disorders. Chemically modified tetracyclines (CMT) have been synthesized in which the antibacterial potency has been eliminated but in which the anti-MMP efficacy is retained. Nitric oxide (NO) modulates MMP synthesis and activity in mesangial cells *in vitro.* Therefore, we examined whether CMT inhibit iNOS gene and protein expression and NO production in cultured rat mesangial cells. Mesangial cells were maintained in media containing IFN-γ and LPS for 24–72 h. Test media contained either no further additives or CMT-1, 3, 5, or 8 at concentrations of 1, 2.5, 5, and 10 μg/ml. iNOS gene and protein expression were assessed and NO production was determined by the Griess reaction. Incubation of mesangial cells with CMT-3 and CMT-8 resulted in time- and dose-dependent inhibition of NO production that was maximal at 48 h ($<$20% of control) and at a drug concentration of 5 μg/ml ($P<0.05$). Addition of CMT-1 had a modest (40%) inhibitory effect and CMT-5 did not alter NO production. The impact of CMT on NO production was directly related to their potency as collagenase inhibitors. Moreover, CMT-induced changes in NO synthesis were associated with parallel alterations in steady-state iNOS mRNA abundance and protein expression. These agents may be useful to ameliorate NO-dependent glomerular inflammation.

NO is a messenger molecule that serves many functions including smooth muscle relaxation, neurotransmission, platelet aggregation, immune cell activation, and tumor cell killing (1). NO is synthesized from the guanidino nitrogen of L-arginine by NOS (2,3). This enzyme has three isoforms: two constitutive species are present in neuronal (NOS1) and endothelial cells (NOS3) and an inducible species, iNOS (NOS2), is present in a wide variety of cells including macrophages, hepatocytes, and dermal fibroblasts (3,4). The inducible isoform of the enzyme is also expressed in renal cells including glomerular mesangial cells and tubular epithelial cells (3,5,6,7).

In glomerulonephritis induced by the intravenous injection of anti-thymocyte serum, administration of the NOS inhibitor, L-NMMA, reduces urinary nitrite and protein excretion and attenuates mesangial cell injury and matrix expansion (8). This suggests that NO contributes to renal damage in immune-mediated glomerulonephritis.

We recently demonstrated that NO stimulates the synthesis and activity of a 72-kDa neutral MMP in cultured rat mesangial cells (9). MMP activity is an important modulator of glomerular injury (10). Amin et al (11,12) recently demonstrated that several tetracyclines, notably minocycline and doxycycline, inhibit the expression of an osteoarthritis NOS isoform in human chondrocytes and iNOS in LPS-stimulated macrophages. CMT have been synthesized that are

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devoid of antibacterial activity but that retain the capacity to inhibit MMP, including collagenase (13). In view of these considerations and the impact of NO on MMP synthesis, we investigated the effect of CMT on iNOS gene and protein expression and NO synthesis in cultured rat mesangial cells.

METHODS

Culture of rat mesangial cells. Rat mesangial cells were isolated and characterized in accord with previously described methods (14). The cells were grown in standard Dulbecco's modified Eagle medium (DME) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum. The plastic flasks (25 cm^2) were kept in a 90% air-10% CO_2 environment at 37°C. Mesangial cells were passed by trypsinization and used between passages 5–12.

Study drugs. Four CMT, prepared as previously described (13) and supplied by CollaGenex Inc. (Newton, PA), were utilized in these experiments. CMT-1 is 2,4-dedimethylaminotetracycline, CMT-3 is 6-demthyl, 6-deoxy, 4-dedimethylaminotetracycline, and CMT-8 is 6α deoxy-5-hydroxy-4-dedimethylaminotetracycline. Studies were also conducted using CMT-5, the pyrazole derivative of tetracycline, which is generated by replacing the carbonyl oxygen at carbon 11 and the -OH at carbon 12 with nitrogens, thereby modifying the probable cation binding site of the tetracycline molecule. CMT-1, -3, and -8 are potent inhibitors of collagenases and gelatinases and are essentially devoid of antibacterial potency (13). CMT-5 is inactive *vis-a-vis* MMP inhibition.

Experimental conditions. Mesangial cells were plated in 96 well plates, 2.5×10^4 cells/well, for determination of nitrite production. They were maintained in 25 cm^2 flasks for Western analysis or 75 cm^2 flasks for Northern analysis. The plates were assigned to one of the following experimental conditions: (1) Control: DME containing IFN-γ (50 U/ml) and LPS (10 μg/ml) and no further additives; (2) Control + CMT-1; (3) Control + CMT-3; (4) Control + CMT-5; and (5) Control + CMT-8. The following concentrations of the CMT—1, 2.5, 5, and 10 μg/ml—were tested. Cells were maintained in the test media for 24–72 h.

Nitrite assay. Nitrite production was measured using the Griess assay (15). Briefly, 125 μl of a solution containing 1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% phosphoric acid was added to 125 μl of conditioned media. Samples were incubated at 25°C for 10 min and absorbance was measured at 550 nm. Nitrite production was normalized to the number of viable cells and expressed as a percentage of the value in control DME media containing no further additives.

Cell viability assay. A colorimetric method was used to determine the number of viable RMC following exposure to the various test reagents and experimental conditions. 100 μl of phenazine methosulfate (PMS) was added to 2 ml of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) in the dark. 25 μl of the mixture was added to each well in 96 well plates. Plates were wrapped in foil and incubated for 1 h at 37°C in the 10% CO_2-90% air atmosphere. Absorbance was read at 490 nm in the samples and the solution blanks.

Western analysis. Rat mesangial cells were harvested in PBS and centrifuged at 1,000 rpm for 5 min to sediment cells. The pellet was dissolved in 100 μl lysis buffer (50 mM Tris/HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM PMSF and 1% Triton X-100). The suspension was freeze-thawed three times and equal aliquots of the lysates (25 μg protein) were loaded onto a 7.5% acrylamide-SDS gel. Gels were run at 200 V and 70 mA for 45 min and protein was then transferred from the gel onto a nitrocellulose membrane electrophoretically. After blocking the membrane with buffer containing 0.25% gelatin and 0.05% Tween 20, the membrane was exposed to a primary murine monoclonal antibody to iNOS (Transduction Labs., Lexington, KY) followed by a secondary antibody (horseradish peroxidase-linked anti-mouse IgG). Immunoblots of the iNOS protein were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Northern analysis. Total mRNA was isolated from rat mesangial cells by homogenization in a phenol-guanidinium thiocyanate mixture (RNA STAT-60, Friendswood, TX), dissolved in 10 mM Tris-EDTA buffer and quantitated by the ratio of absorbance at 260 and 280 nm. Total RNA (20 μg) was subjected to electrophoresis through a 1.2% agarose/0.7 M formaldehyde gel in 1× morpholinopropanesulfonic acid (MOPS) buffer. RNA was blotted onto GeneScreen Plus (Dupont/NEN, Boston, MA) and fixed to the membrane by baking at 80°C for 2 h. The blot was prehybridized for 2–4 h at 42°C in 50% deionized formamide, 5× SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4-H_2O, 0.005 M EDTA), 5× Denhardt's solution (2% polyvinylpyrrolidone, bovine serum albumin, and Ficoll 400) 1% SDS, 10% dextran sulfate, and 10μg/ml denatured salmon sperm DNA. For detection of iNOS mRNA, an 817-base pair cDNA probe was used that was kindly provided by Drs. Carl Nathan and Qiao-Wen Xie. The cDNA insert used for hybridization was removed from the plasmid by digestion with *EcoR* I and Hind *III* and radiolabelling was carried out using the Prime-a-Gene System (Promega, Madison, WI). The membrane was incubated in 5×10^5 cpm/ml of labelled iNOS cDNA at 42°C for 16 h. The blot was then washed in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 23°C, followed by 0.1× SSC, 0.1% SDS at 50°C. After exposing the blot to X-OMAT film (Kodak) at −70°C for 24–72 h with an intensifying screen, the membrane was stripped by boiling in sterile distilled water for 10 min and then rehybridized with labelled GAPDH cDNA as described.

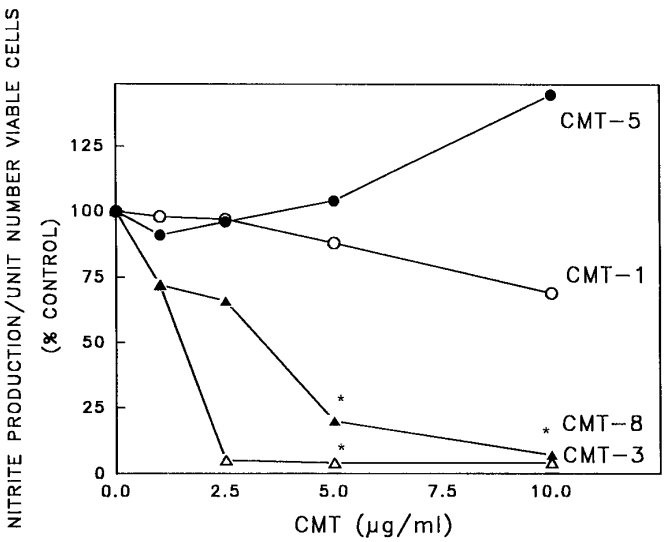

FIG. 1. NO production (nitrite/unit number viable cells) by rat mesangial cells measured by Griess assay (N=5). The experimental conditions are indicated by the following symbols: CMT-1, open circles; CMT-3, open triangles; CMT-5, closed circles; CMT-8, closed triangles. * $P<0.05$ *versus* control media.

Protein assay. Mesangial cells were scraped and dissolved in 0.2 N NaOH. The protein content of each sample was determined using a Coomassie blue reagent with bovine serum albumin standards (BioRad, Richmond, CA).

Materials and reagents. All plasticware was obtained from Corning Costar (Cambridge, MA) or Fisher Scientific (Pittsburgh, PA). The tissue culture materials were purchased from GIBCO BRL Life Technologies (Grand Island, NY) while the γ-interferon was obtained from Genzyme (Cambridge, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. Each experimental condition was studied 4–5 times and the results are presented as mean±SEM. Groups were compared using an analysis of variance and the student t-test with the Bonferroni correction and differences were considered significant if the P value was less than 0.05.

RESULTS

In rat mesangial cells that were grown in DME without the two cytokines, IFN-γ and LPS, there was no demonstrable NO production. Addition of CMT had no effect on nitrite accumulation under these basal conditions without IFN-γ and LPS (data not shown). Moreover, in the absence of iNOS activity and cytokine-induced stimulation of NO production, CMT did not alter mesangial cell viability (data not shown).

Exposure of rat mesangial cells to IFN-γ/LPS consistently enhanced NO production and this action was maximal after 48–72 h incubation (2–5 nmol/well) (Figure 1). Addition of CMT-1 caused a modest inhibition of NO production and the amount of nitrite per unit number of viable cells was approximately 60% of the level in the control condition after 48–72 h incubation. CMT-3 and CMT-8 both caused a significant reduction in NO synthesis ($P<0.05$). Although the effect of the two drugs was similar, CMT-3 appeared to more potent than CMT-8. The CMT-mediated reduction in NO synthesis was dose-dependent and was maximal at 5–10 μg/ml. Both CMT-3 and CMT-8 were substantially more potent inhibitors of NO production than doxycycline which reduced nitrite accumulation by only 46% at a concentration of 100 μg/ml. CMT-5, which has no inhibitory effect on collagenase activity, did not alter NO production by the mesangial cells during the entire course of the incubation. Cell viability was not altered by any of the CMT when the ambient concentration was 5 μg/ml or less (data not shown).

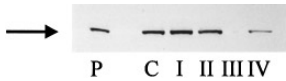

FIG. 2. Representative Western analysis (25 μg protein/lane) for iNOS protein expression in rat mesangial cells after 48 h incubation in the test media. The concentration of the CMT was 5 μg/ml in the experimental conditions. The arrow indicates the gel location of the 130 kDa molecular form of iNOS. Lane P, positive iNOS control; Lane C, control media; Lane I, CMT-1; Lane II, CMT-5; Lane III, CMT-3; Lane IV, CMT-8.

The effect of the CMT on NO production was paralleled by changes in the rat mesangial cell content of iNOS protein. Thus, after 48 h incubation with CMT (5 μg/ml), CMT-3 and CMT-8 caused a marked reduction in iNOS protein expression (n=3) (Figure 2). Exposure to CMT-1 resulted in a marginal decrease in mesangial cell iNOS content and CMT-5 did not alter iNOS expression compared to the control conditions.

In order to ascertain whether the differences in iNOS protein content were associated with alterations in iNOS gene expression, Northern analysis was performed in rat mesangial cells exposed to the four CMT for 24 h (n=2). These studies demonstrated that the iNOS mRNA abundance was significantly reduced by CMT-3 and CMT-8, minimally decreased by CMT-1 and unaltered by exposure to CMT-5 (Figure 3). These changes in iNOS mRNA levels were correlated with CMT-induced alterations in mesangial cell NO production and iNOS protein content. The intensity of the GAPDH gene signal was comparable in the experimental samples indicating that equal aliquots were loaded onto the gel (data not shown).

DISCUSSION

These data indicate that select CMT have the capacity to inhibit iNOS gene and protein expression and decrease NO production in cultured rat mesangial cells. The findings, which are in accord with the observations of Amin et al (11,12), extend the effect of tetracyclines on iNOS activity to a second cell type and to chemically modified derivatives that are devoid of antibacterial potency. Although many biological agents including TGF-β, PDGF, interleukin 13, and dexamethasone inhibit iNOS activity (2,3,16), there is a paucity of medications that exert as profound an effect as CMT on iNOS expression and NO synthesis. Because these drugs are safe and well tolerated, they may have potential clinical uses in the treatment of disorders associated with excessive NO production.

The CMT used in these experiments vary in their effect on collagenase activity with CMT-3 and CMT-8 being the most potent inhibitors, CMT-1 exerting only modest activity, and CMT-5 having no impact on collagenolysis (17). The impact of the CMT on iNOS directly paralleled their *in vitro* and *in vivo* potency as collagenase inhibitors. In view of the inhibitory effect of NO on MMP activation in bovine and human articular cartilage (18) and rat mesangial cells (9), this raises the possibility that the actions on CMT on extracellular matrix (ECM) degradative enzymes may be mediated by changes in iNOS expression and NO production.

The mechanism by which CMT alter iNOS activity appears to be at the level of gene expression because of the parallel changes in the steady state level of iNOS mRNA, iNOS

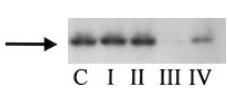

FIG. 3. Representative Northern analysis (20 μg RNA/lane) for steady state iNOS mRNA abundance in rat mesangial cells after 24 h incubation in the test media. The concentration of the CMT was 5 μg/ml in the experimental conditions. The arrow indicates the gel location of the iNOS mRNA. Lane C, control media; Lane I, CMT-1; Lane II, CMT-5; Lane III, CMT-3; Lane IV, CMT-8.

protein content, and NO production. Additional studies are needed to ascertain whether CMT are acting directly to modify gene transcription or post-translationally by altering mRNA stability. There are precedents for altered iNOS activity originating at each of these three levels (2,3). Finally, CMT may exert their effects secondarily via other mediators including reactive oxygen molecules or cytokines such as TGF-β (19,20).

NO serves many functions within the kidney including regulation of afferent arteriolar tone and glomerular hemodynamics (21,22) and control of mesangial cell proliferation and contraction (23,24). Animals studies and *in vitro* experiments suggest that NO also modulates release of renin (25), tubular cell Na^+-K^+ ATPase activity (26), and maintenance of external salt balance (15). In addition, NO regulates renal accumulation of ECM by modulating the synthesis and degradation of matrix proteins (9,14). In view of this broad range of physiological functions, the role of NO in the pathogenesis of renal disease is increasingly complex. During circumstances of acute immunologically-mediated glomerular injury, NO acts as a pro-inflammatory agent to foster glomerular damage. Thus, in rats with glomerulonephritis induced by injection of anti-thymocyte serum, proteinuria, glomerular expression of TGF-β, and accumulation of ECM proteins are suppressed by treatment with the iNOS inhibitor, L-NMMA (8). It is unclear whether NO is exerting its effect by altering ECM synthesis, MMP activity or the generation of reactive free radical derivatives of NO such as peroxynitrite (3,27,28). Our data raise the possibility that administration of select CMT may inhibit iNOS activity and NO generation within the kidney and attenuate renal injury in experimental models of immune-mediated glomerulonephritis associated with enhanced NO production.

In summary, we have demonstrated that select CMT inhibit iNOS gene and protein expression and NO production by rat mesangial cells *in vitro*. The action of the CMT on NO synthesis mirrored their potency as collagenase inhibitors. These findings suggest that inhibition of iNOS with CMT may be a novel therapy to ameliorate certain clinical forms of glomerulonephritis.

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