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Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines

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Abstract **Chemically modified tetracyclines [CMT-3 (IC_{50} ~6–13 μM = ~2.5–5 μg/ml) and CMT-8 (IC_{50} ~26 μM = 10 μg/ml), but not CMT-1, -2 or -5], which lack anti-microbial activity, inhibited nitrite production in LPS-stimulated macrophages. Unlike competitive inhibitors of L-arginine which inhibited the specific activity of inducible nitric oxide synthase (iNOS) in cell-free extracts, CMTs exerted no such direct effect on the enzyme. CMTs could, however, be shown to inhibit both iNOS mRNA accumulation and protein expression in LPS-stimulated cells. Tetracyclines (doxycycline and CMT-3) unlike hydrocortisone had no significant effect on murine macrophages transfected with iNOS promoter (tagged to a luciferase reporter gene) in the presence of LPS. However, doxycycline and CMT-3 augmented iNOS mRNA degradation, in LPS-stimulated murine macrophages. These studies show a novel mechanism of action of tetracyclines which harbours properties to increase iNOS mRNA degradation and decrease iNOS protein expression and nitric oxide production in macrophages. This property of tetracyclines may have beneficial effects in the treatment of various diseases where excess nitric oxide has been implicated in the pathophysiology of these diseases.**

Key words: Nitric oxide; Macrophages; Tetracycline; Chemically modified tetracyclines (CMT); Collagenase; RNA

1. Introduction

Nitric oxide (NO), first identified as an endothelium-derived relaxation factor, is now recognized to regulate the functions of many mammalian cells and tissues [1]. NO is produced by the ubiquitous enzyme, nitric oxide synthase (NOS). The over-expression of inducible NOS (iNOS) in a variety of inflammatory tissues had led many to conclude that the modulation of NO synthesis and action could represent a new approach to treatment of inflammatory and autoimmune diseases [2,3], including osteoarthritis (OA) [4] and rheumatoid arthritis (RA) [5].

Various studies have shown that among the tetracycline group of broad-spectrum antibiotics, doxycycline and minocycline exert biological effects independent of their anti-microbial activity [6–9]. Such effects include inhibition of activity of matrix metalloproteases (MMPs), including collagenase (MMP-1), gelatinase (MMP-2) and stromelysin (MMP-3), and prevention of pathogenic tissue destruction [6]. Recent studies have also indicated that tetracyclines and inhibitors of MMPs block tumor progression [10], bone resorption [11] and angiogenesis [12]. In view of these diverse effects of tetracyclines, we have also observed that doxycycline and minocycline inhibit iNOS expression in murine macrophages [13]. The present study shows that: (a) some chemically modified tetracyclines (CMT-3 and CMT-8, but not CMT-1 or CMT-5) share the ability to inhibit iNOS expression in a similar fashion as observed with doxycycline and minocycline; (b) CMT-3 and doxycycline augment iNOS mRNA degradation and have no significant influence on iNOS gene transcription.

2. Materials and methods

Murine macrophage cells (RAW 264.7) were obtained from ATCC (Rockville, MD, USA). An anti-murine iNOS antibody was obtained from Transduction Laboratories (Lexington, KY, USA). Doxycycline, minocycline, hydrocortisone, *N*-acetyl imidazole (NAI), and lipopolysaccharide (LPS) were obtained from Sigma (St. Louis, MO, USA). The CMTs (designated as CMT-1, -2, -3, -5 and -8) were a generous gift from CollaGenex (Newtown, PA, USA).

Equal amounts of protein (25–50 μg) estimated by BCA reagent (Pierce, Rockford, IL, USA) were loaded onto SDS-PAGE gels and stained to verify the concentrations of various protein fractions by examining the intensities of the protein bands on the gels. Western blot analysis was carried out from the same cell extracts. The Western blot was probed with a specific anti-iNOS murine mAb as specified by Transduction Laboratories. Membranes with bound antibodies (e.g., iNOS) were stripped by submersion in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubating at 50°C for 30 min with occasional agitation. Membranes were then washed twice for 10 min at RT using large volumes of wash buffer. The same blot was also probed with an anti-actin antibody generously provided by Dr. James L. Lessard (Children's Hospital Medical Center, Cincinnati, OH, USA) using the standard protocol. Blots were developed using the ECL Western blot system (Amersham, Arlington Heights, IL, USA). Quantitation of the bands was performed using a densitometer from Molecular Dynamics (Sunnyville, CA, USA).

Total RNA was isolated using TRI Reagent (MRC, Cincinnati, OH, USA). Northern blot analysis was carried out as described earlier [14,15]. Briefly, 20 μg of RNA was subjected to electrophoresis in 1% agarose formaldehyde gel, and then transferred via capillary action

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Abbreviations: CMT, chemically modified tetracycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; MMP, matrix metalloprotease; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; OA, osteoarthritis; RA, rheumatoid arthritis

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onto a nylon membrane (Zeta Probe, Bio-Rad Laboratories, Melville, NY, USA). The membrane was hybridized with [^{32}P]dCTP-labelled iNOS cDNA (4 kb *Sma*I fragment), a kind gift from Dr. James Cunningham (Harvard Medical School, Boston, MA, USA). After hybridization, the blot was exposed to Kodak X-ray film (Kodak, Rochester, NY, USA) for 24–48 h with intensifying screens at −70°C. The GAPDH probe was purchased from ClonTech (Palo Alto, CA, USA) and probed as described above. Quantitation of the intensity of the iNOS and GAPDH bands was performed using a Personal Densitometer SI (Molecular Dynamics).

Specific activity of iNOS was determined in cell-free extracts by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described [15,16]. RAW 264.7 cells were induced with LPS (100 ng/ml) in the presence and absence of minocycline, CMTs or hydrocortisone for 14–20 h. Following induction, the cells were pelleted at 4°C and resuspended in Tris buffer (10 mM, pH 7.4) containing 10 μg/ml each chymostatin, antipain, leupeptin and pepstatin, 1 mM DTT and 1 mM PMSF [15]. Cells were lysed in a Polytron PT1200 homogenizer (Kinematica, Switzerland) after 3 cycles of rapid freeze-thawing. The lysate was centrifuged at 16 000 rpm for 60 min at 4°C, and the supernatants were used as cell-free extracts. The protein was measured by BCA assay reagent using BSA as standard [17]. The reaction mixture for iNOS assay consists of Tris 50 mM (pH 7.8); BSA 1 mg/ml; DTT 1 mM; $CaCl_2$ 2 mM; FAD 10 μM; BH_4 10 μM; L-arginine 30 μM; NADPH 1 mM. The reaction mixture was spiked with 1 μl (250 nM) of L-[^{3}H]arginine (Du Pont NEN, Boston, USA, MA) (1 mCi/ml = 37.0 MBq/ml). After 20 min the assays were terminated by heating the reaction mixture at 90°C for 5 min; 10 μl (≃100 000 cpm) of the supernatant was spotted on activated Avicel TLC plates (Analtech, Newark, DE, USA). The TLC plates were developed in a solvent system consisting of ethanol/water/ammonia (80:16:4). Quantitation of the spot for L-[^{3}H]citrulline was performed by a Bioscan System 200 Imaging Scanner.

RNA stability analysis was carried out as previously described [18]. Briefly, cells were stimulated with 100 ng/ml of LPS (± tetracycline) followed by addition of actinomycin D (5 μg/ml) 4 h post-stimulation. Total RNA was prepared at different time periods and analyzed by Northern blot analysis as described above. The data are represented as percentage of iNOS mRNA degraded after normalizing the values with GAPDH.

RAW 264.7 cells were transfected with the 1.7 kb murine iNOS promoter linked to a luciferase reporter gene [19] using the DEAE-dextran method as previously described [20]; 10 μg of the plasmid DNA was added to 10^7 cells in 1.0 ml of DMEM (without serum) containing DEAE-dextran (250 μg/ml) and 50 mM Tris (pH 7.4). The suspension was further incubated at 37°C for 60 min followed by a 1.0 min shock with 10% DMSO at room temperature. These cells were incubated for 45 min with the respective drugs and stimulated with 100 ng/ml LPS 24 h post-transfection. The cells were finally harvested 24 h after LPS stimulation and assayed for luciferase activity as described by the manufacturer's instructions (Promega, Madison, WI, USA).

3. Results and discussion

Our recent studies show that doxycycline and minocycline inhibit iNOS expression at the level of iNOS mRNA accumulation in murine macrophages stimulated with LPS [13]. In view of these observations, we examined if chemically modified tetracyclines as shown in Fig. 1 and which lack anti-microbial activity as previously reported [21], could also modulate iNOS expression. We therefore compared the effects of CMTs and minocycline on nitrite accumulation in LPS-stimulated murine macrophages as shown in Fig. 2. CMT-3 > CMT-8 (but not CMT-1, -2, or -5) inhibited nitrite accumulation in a dose-dependent manner. The IC_{50} level of CMT-3 was < 2.5 μg/ml (< 6 μM) while that of CMT-8 was ∼10 μg/ml (∼26 μM). CMT-2 and CMT-5 at concentrations of 10 μg/ml did not show a significant effect on nitrite accumulation (Fig. 2), whereas a marginal effect (inhibition) was seen with minocycline and CMT-1 at similar concentrations. These experiments demonstrate that CMT-3 is more effective in its ability to inhibit nitrite accumulation than doxycycline, minocycline [13], and other CMTs tested in these studies.

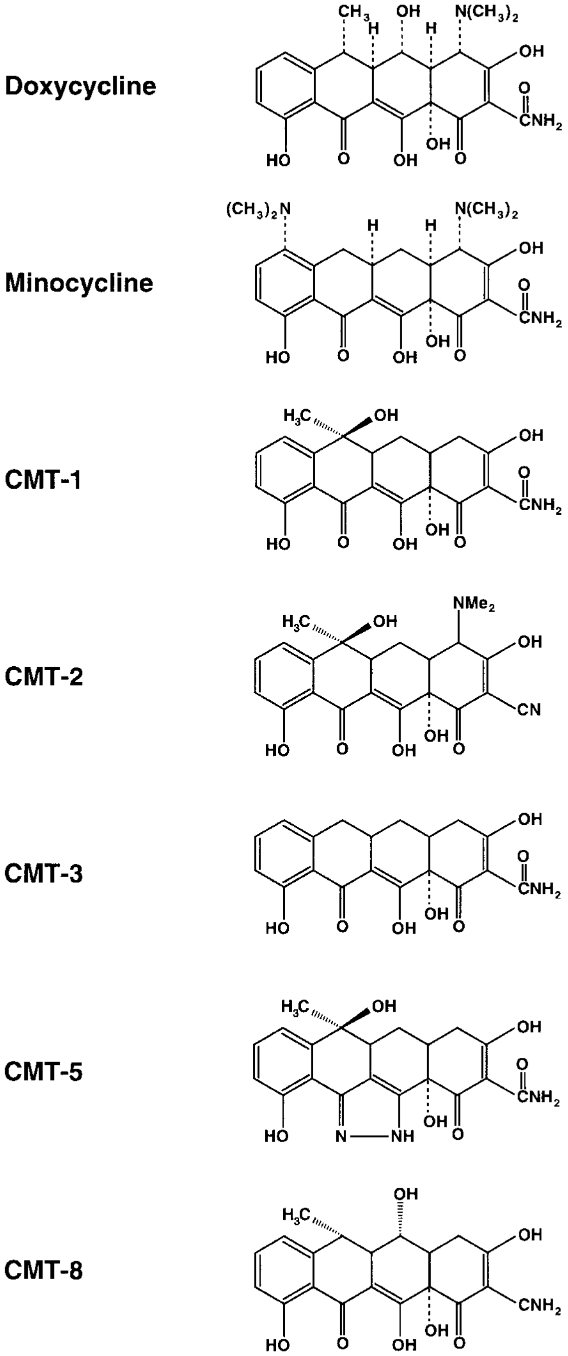

Fig. 1. Structures of doxycycline, minocycline and various chemically modified tetracyclines used in this study (provided by Collagenex, Inc.).

We further examined the effects of CMT-3 and CMT-8 on the enzyme activity of iNOS. Cells were stimulated with LPS in the presence of equal amounts of CMT-3 or -8 for 16 h. The medium was assayed for nitrite and iNOS enzyme in cell extracts in an L-arginine-to-L-citrulline conversion assay. As expected, CMT-3 and hydrocortisone inhibited nitrite accumulation significantly more than CMT-8. These data were substantiated by a significant decrease in specific enzyme ac-

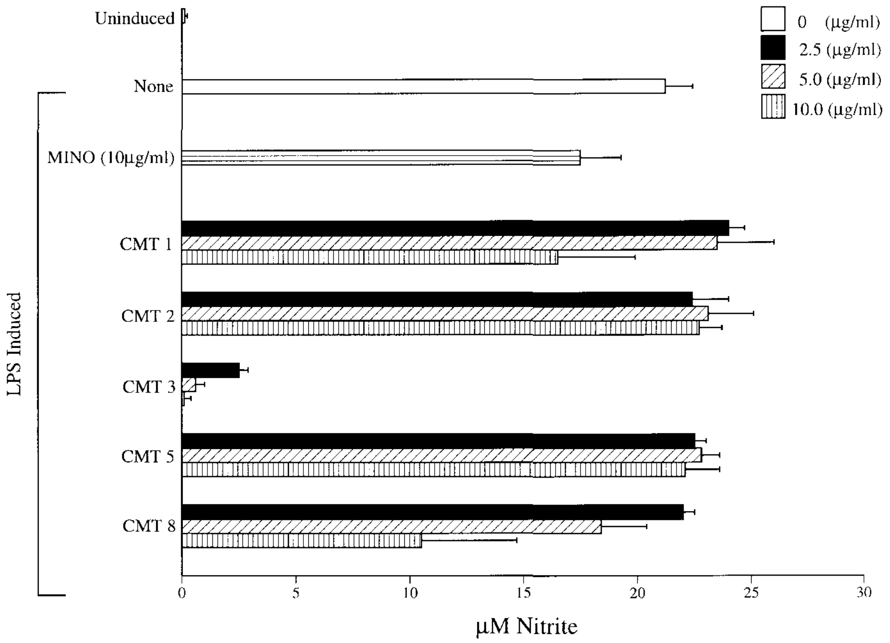

Fig. 2. Effect of CMTs on nitrite accumulation in murine macrophages stimulated with LPS. Murine macrophages (RAW 264.7 cells) were stimulated with LPS (100 ng/ml) in the presence of 10 μg/ml minocycline (MINO) and various concentrations of CMTs in triplicate for 48 h. The levels of nitrite were estimated by the modified Griess method [31]. Data are expressed as μM nitrite accumulated of triplicate determinants ($n = 3$). Statistics were derived using unpaired Student's t-test. Data represent 1 of 3 similar experiments.

tivity of iNOS in the intact cells, as shown in Table 1. These experiments demonstrated that the decrease in nitrite accumulation by the CMTs could be partially to the decrease in iNOS enzyme activity within the cells.

Various investigators have shown that tetracyclines inhibit collagenase activity via direct effects on the enzyme [22–24]. Another mechanism proposed for this phenomenon is that procollagenase is reduced to inactive fragments upon activation in the presence of doxycycline [23]. We have recently shown that acetylating agents such as aspirin and *N*-acetyl imidazole [15], as well as competitive inhibitors of L-arginine (NMA), inhibit iNOS catalytic activity in cell-free extracts. In view of these observations, we examined the direct effect of CMTs on the ability of iNOS to convert radiolabelled L-[^{3}H]arginine to L-citrulline in cell-free extracts in vitro. RAW 264.7 cells were stimulated with LPS for 16 h and cell-free extracts were made. Separate aliquots of equal amounts of enzyme were preincubated with various concentrations of CMTs (6–24 μM), NMA (200 μM) or NAI (1 mM) for 20 min before the enzyme reaction was initiated after the addition of co-factors. As expected, NMA and NAI showed 75 and 45% inhibition of iNOS enzyme activity, respectively, but there was no significant effect (< 5% inhibition) by the CMTs (data not shown). These experiments demonstrate that the action of these CMTs, like the doxycycline and minocycline [13], seems to be distinct from those reported for MMPs such as procollagenase at similar concentrations [22,23,25]. In view of the above observation and our previous experiments which indicate that iNOS protein is decreased by doxycycline and minocycline [13], we tested the effects of CMT-3 and −8 at various concentrations on iNOS protein expression. RAW 264.7 cells were stimulated with LPS in the presence and absence of CMTs for 16 h; cell-free extracts were prepared and examined for iNOS expression by Western blot analysis. Fig. 3 shows that, like minocycline [13], CMT-3 > CMT-8 inhibited 133 kDa iNOS expression. The effect of CMT-3 at 2.5 μg/ml was similar to that observed with 20 μg/ml of minocycline. The inhibition in the accumulation of nitrite in the same experiment was substantiated with the data shown in the Western blot analysis. Hydrocortisone, as expected, inhibited iNOS expression as previously reported [15]. Furthermore, there was no significant difference in the constitutively expressed pro-

Table 1
Effect of CMTs on the specific activity of iNOS

Modulating agent	Nitrite released		Specific activity	
	μM	% inhibition	pmol/min per mg protein	% inhibition
Control (uninduced)	< 0.1	N/A	< 5	N/A
LPS induced	22.9	N/A	90.8	N/A
CMT-3 (10 μg/ml)	12.2	47	37.6	58.6
CMT-8 (10 μg/ml)	17.0	26	79.2	12.7
Hydrocortisone (10 μM)	11.8	49	51.5	43.3

Murine macrophage cells were stimulated with 100 ng/ml of LPS in the presence of CMTs or hydrocortisone for 16 h. The nitrite accumulated in the medium was examined and the enzyme activity assayed from cell-free extracts. Percent inhibition of nitrite/specific activity was calculated after comparing the values with LPS-stimulated cells. 10 μg/ml of CMT-3/8 was equivalent to 27 μM. The data represent one of two similar experiments. N/A, not applicable.

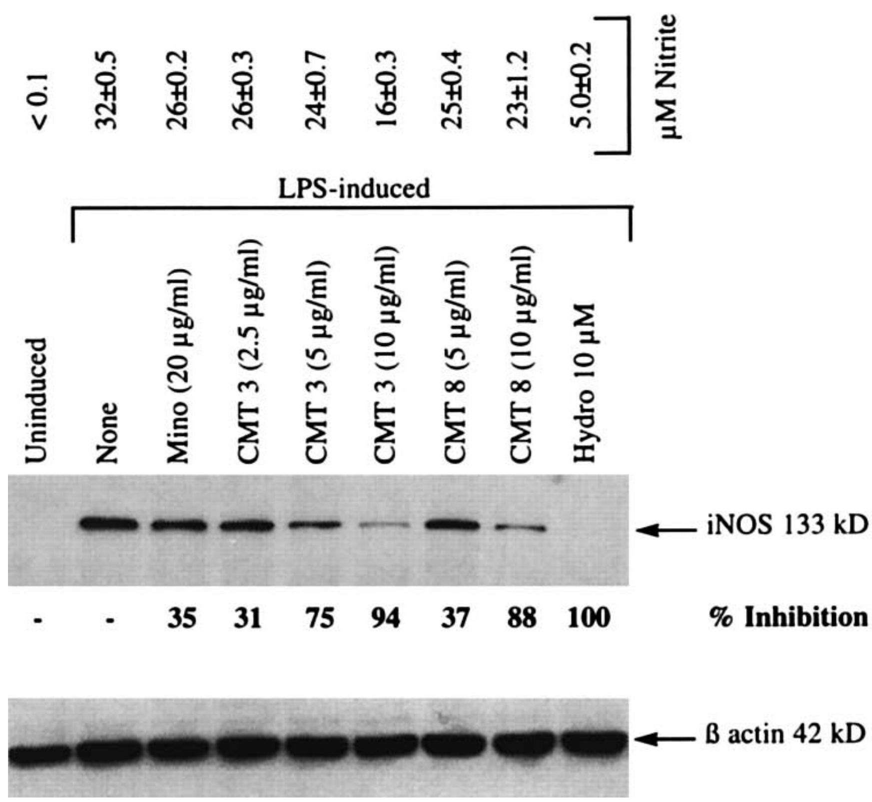

Fig. 3. Western blot analysis of iNOS in RAW 264.7 cells exposed to minocycline, CMTs and hydrocortisone in the presence of LPS for 16 h. Mino: represents minocycline (20 µg/ml equivalent to 40 µM). Hydro: represents hydrocortisone (10 µM equivalent to 3.4 µg/ml). CMT-3 (2.5, 5 and 10 µg/ml equivalent to 27, 13 and 6 µM, respectively) and CMT-8 (5 and 10 µg/ml equivalent to 27 and 13 µM, respectively). Cell-free extracts were prepared and aliquots were blotted and probed with a specific α-iNOS mAb. The percent inhibition of iNOS expression was compared to LPS-stimulated cells, as determined by a densitometer scan, after normalizing the values with β-actin in the same blot. The upper panel shows the representative nitrite values in this particular experiment. The data represent 1 of 2 similar experiments.

teins (such as β-actin) when the same blot was reprobed with anti-β-actin antibodies (Fig. 3). These experiments indicate that the decrease in NOS activity could be due to a decrease in the expression of iNOS protein.

Based on our previous studies with doxycycline and minocycline, we tested the ability of CMTs to inhibit iNOS mRNA accumulation in RAW 264.7 cells stimulated with LPS for 16 h. Fig. 4 shows a dose-dependent inhibition of iNOS mRNA accumulation by CMT-3. CMT-8 at 5 and 10 µg/ml showed a significant decrease in iNOS mRNA accumulation as compared to LPS-stimulated cells, whereas CMT-1 at 10 µg/ml showed no significant effect when the values were normalized with the respective GAPDH signals. The effect of hydrocortisone in this particular experiment was relatively less when the

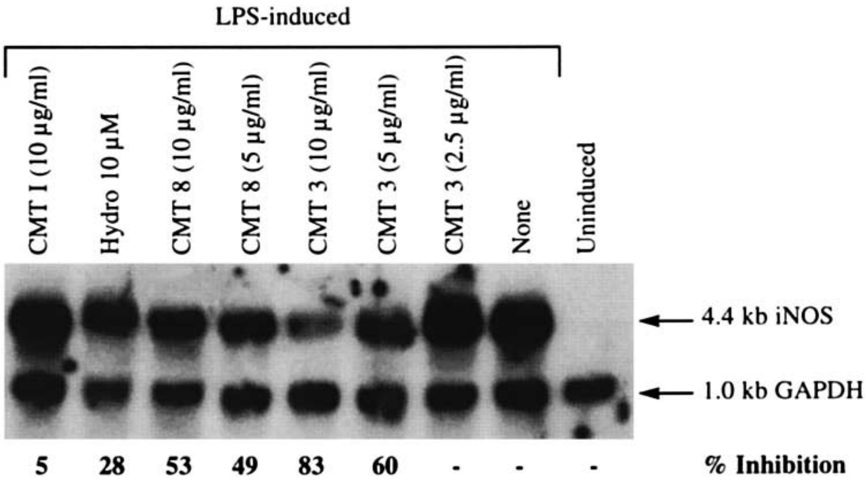

Fig. 4. Northern blot analysis of iNOS mRNA expression in RAW 264.7 cells stimulated with LPS in the presence of CMTs at 16 h. Total RNA was extracted and analyzed by Northern blot using α-iNOS and α-GAPDH probes. The iNOS/GAPDH signal was quantitated using a phosphoimager. The percent inhibition of iNOS mRNA expression was normalized with the GAPDH signal and compared with the values of the LPS-stimulated cells. Data represent 1 of 2 similar experiments.

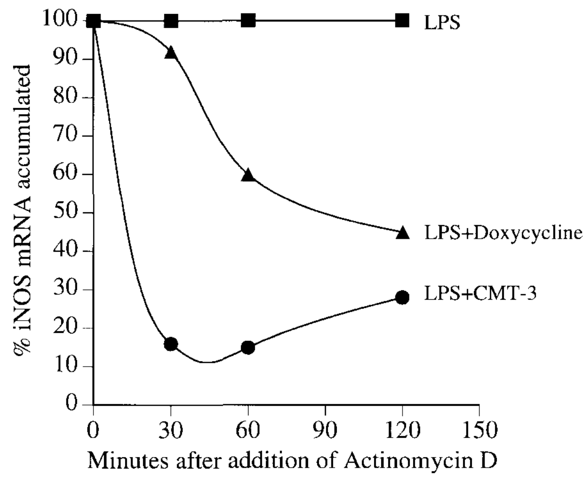

Fig. 5. Effect of doxycycline and CMT-3 on iNOS mRNA stability. RAW 264.7 cells were stimulated with 100 ng/ml of LPS in the presence or absence of doxycycline (40 μg/ml) or CMT-3 (10 μg/ml) for 4 h, followed by addition of actinomycin D (5.0 μg/ml). The cells were then harvested at different time intervals (0–120 min) and analyzed for iNOS mRNA accumulation as described in Section 2. The values for iNOS mRNA accumulation were normalized with GAPDH for equal loading. The percent inhibition of iNOS mRNA in tetracycline-treated cells was calculated against LPS-stimulated cells alone using a densitometer.

mRNA was in its steady state [15]. The effects of hydrocortisone have been seen to be more significant when the iNOS mRNA is examined at 4 h post-stimulation where it blocks NF-κB activation and iNOS transcription.

To examine the mechanism by which tetracyclines (doxycycline and CMT-3) decrease iNOS RNA accumulation, we evaluated the effects of these compounds on iNOS transcription and mRNA stability. Murine macrophages were transfected with a full-length iNOS promoter (tagged to a luciferase reporter gene) and stimulated with LPS in the presence and absence of doxycycline, CMT-3 and hydrocortisone. Stimulation of these cells with LPS alone increased the relative light units of luciferase activity from 0.22 to 35.0 which was designated as 100%. In a parallel experiment, 10 μM of hydrocortisone showed 88% inhibition of luciferase activity. Doxycycline at 20 and 40 μg/ml (41 and 82 μM, respectively) and CMT-3 at 10 μg/ml (25 μM) which markedly inhibited nitrite accumulation had no significant effect on the luciferase activity in the presence of LPS (data not shown). These experiments indicate that, unlike hydrocortisone, tetracyclines have no significant effect on iNOS transcription when stimulated with LPS.

We next examined the effects of tetracyclines on iNOS mRNA stability. Murine macrophage cells were stimulated with LPS (±doxycycline or CMT-3) for 4 h followed by incubation with actinomycin D. Total RNA was extracted at different time intervals after the addition of actinomycin D and examined for iNOS mRNA expression by Northern blot analysis. Fig. 5 shows that both CMT-3 > doxycycline augments iNOS mRNA instability when compared to cells incubated with LPS alone. Previous studies have shown that murine iNOS mRNA is modulated at post-transcriptional levels, by TGFβ [18], $Fe(3+)$ [26] and cycloheximide [27].

In summary, these experiments demonstrate that CMT-3 and -8 (both devoid of anti-microbial activity), like doxycycline and minocycline, can inhibit iNOS activity in murine macrophages. CMT-3 > CMT-8 at concentrations used in this study have been reported to inhibit collagenase activity, whereas CMT-5 showed no appreciable effect [28]. Furthermore, CMT-3 > CMT-8 were found to be more potent in their ability to inhibit iNOS expression when compared to effects of doxycycline and minocycline as previously reported [13]. This study also demonstrates that the mechanism of action of at least two tetracyclines (doxycycline and CMT-3) is similar: they render the iNOS mRNA susceptible to degradation and thereby decrease iNOS expression and nitric oxide production. These experiments support the previous hypothesis that tetracyclines, independent of their anti-microbial activity, exert pleiotropic functions including inhibition of MMPs, NOS expression, protection against peroxynitrite-dependent injury, angiogenesis, tumor progression, bone resorption and inflammation [10,12,29,30]. We hypothesize that the multifunctional properties of tetracyclines may be partly attributed to their ability to target another pleiotropic signalling molecule, NO, that has been shown to exert similar effects on many of the pathological conditions and manifestations.

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