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Tetracycline inhibits the nitric oxide synthase activity induced by endotoxin in cultured murine macrophages

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Abstract

Here we investigate the effects of tetracycline base and of a semi-synthetic tetracycline derivative, doxycycline, on the induction of inducible nitric oxide synthase and, hence, on the production of nitric oxide (NO) by lipopolysaccharide in J774 macrophage cultured in vitro. The treatment of J774 line with tetracycline base (6.25–250 μM) or doxycycline (5–50 μM) dose-dependently decreased the lipopolysaccharide-stimulated (1 μg/ml) inducible NO synthase activity and, consequently, nitrite formation. For instance, the inhibition was 70% for tetracycline base at 250 μM and 68% for doxycycline at 50 μM. The inhibitory effect of tetracyclines was due neither to a reduction in the viability of the cells, studied as colorimetric 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, nor to an indiscriminate inhibition of total protein synthesis, but to a specific decrease in inducible NO synthase protein content in the cells, as attested by the significant reduction of the expression of inducible NO synthase, assayed by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. However, no effect of tetracyclines on inducible NO synthase mRNA accumulation could be demonstrated in lipopolysaccharide-stimulated macrophage line, suggesting that the inhibitory effect of tetracyclines on NO synthesis involves post-transcriptional events. The reduction in lipopolysaccharide-stimulated nitrite accumulation produced by tetracyclines was significantly less when they were applied 6 h after lipopolysaccharide and absent 12 h after lipopolysaccharide, indicating that tetracyclines modify an early event in inducible NO synthase activation operating after mRNA transcription. The findings presented in this study indicate that the modulation of NO synthesis is another possible pathway by which tetracyclines may function as anti-inflammatory compounds. © 1998 Elsevier Science B.V.

Keywords: Nitrate; Tetracycline; Doxycycline; J774 cell line

1. Introduction

Tetracyclines are used as antibiotics to treat a variety of infections. Recently, it has been demonstrated that tetracyclines have anti-inflammatory properties not related to the antimicrobial efficacy of the drug (Golub et al., 1992). In fact, tetracyclines are effective in the treatment of skin diseases not associated with microbial etiology (e.g., rosacea, pyoderma gangrenosum, dermatitis herpetiformis, epidermolysis bullosa, bullous pemphigoid and α_1-antitrypsin deficiency panniculitis) (Golub et al., 1992; Humbert et al., 1989, 1991). Furthermore, this inhibitory effect was seen in germ-free animals (Golub et al., 1983) and in sterile in vitro systems (Golub et al., 1983, 1984). The mechanisms of action of tetracyclines on these diseases have been related to the ability of tetracyclines to inhibit the activity of mammalian collagenases and other related matrix collagenases and matrix metallo-proteinases (Golub et al., 1983, 1984, 1992; Humbert et al., 1989, 1991). The second-generation semi-synthetic tetracycline derivatives, such as doxycycline and minocycline, are more effective inhibitors of collagenases than the parent compound tetracycline (Golub et al., 1983, 1987, 1992; Humbert et al., 1989, 1991).

The modulation of inflammatory cells and mediators by tetracyclines is attested by the fact that they inhibit neutrophil-mediated tissue damage by inhibiting their migra-

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tion and degranulation (Gabler and Creamer, 1991; Martin et al., 1974) and, what is potentially more important, by suppressing the synthesis of oxygen radicals (Gabler and Creamer, 1991), or scavenging for reactive oxygen metabolites, such as hypochlorous acid, thus preventing the hypochlorous acid conversion of the osteoblast proenzyme to active collagenase (Ramamurthy et al., 1993). Moreover, tetracyclines strongly block the synthesis of arachidonic acid (Vadas et al., 1991) and of prostaglandin E_2 (El Attar et al., 1988).

Increasing evidence indicates that nitric oxide (NO) may play an important role in inflammation having a relevant part in tissue damage (Ialenti et al., 1992; Vane et al., 1994). NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine (Ialenti et al., 1992; Moncada and Higgs, 1993) by NADPH-dependent enzyme, NO synthase. NO is cytostatic or cytotoxic both for invading microorganisms, for the cells that produce it and for neighbouring cells (Hibbs et al., 1990). Thus, pathogens close to the source of NO are destroyed selectively, even though sustained high levels of NO could be damaging to the host cells and tissues.

NO could react with the Fe–S groups forming an iron–nitrosyl complex, causing the inactivation and degradation of the Fe–S prosthetic groups of aconitase and complex I and complex II of the mitochondria electron transport chain (Beckman, 1990; Hibbs et al., 1990; Moncada and Higgs, 1993; Stadler et al., 1993). Alternatively, NO may react with the oxygen anion radical ($O_2^{\cdot}$) to form peroxynitrite anion ($ONOO^-$) which decays once protonated to form the highly reactive hydroxyl radical ($HO^{\cdot}$) and the stable free radical $NO_2^{\cdot}$ (Beckman, 1990; Hibbs et al., 1990; Moncada and Higgs, 1993). Furthermore, the involvement of NO in inflammatory reactions is also confirmed by its role in the induction of the septic shock syndrome by bacterial endotoxin (lipopolysaccharide) (Cunha et al., 1994; Milano et al., 1997), and in the inhibition (Stadler et al., 1993) or in the activation of cyclooxygenase enzymes (Salvemini et al., 1993). On the other hand, prostaglandin E_2 is able to regulate inducible NO synthase (Milano et al., 1995).

Recently, it has been demonstrated that tetracyclines inhibit NO synthesis by lipopolysaccharide-stimulated murine macrophages (Amin et al., 1996; Milano et al., 1997) and, furthermore, we have shown that they are able to protect mice from lipopolysaccharide-induced shock down-regulating inducible NO synthase in various organs and cytokines and nitrate secretion in the blood (Milano et al., 1997).

In this work, we have further studied the mechanism by which tetracyclines inhibit NO synthesis, analysing the effect of these drugs in vitro on inducible NO synthase activity and protein in cellular extracts from J774 macrophage line stimulated with lipopolysaccharide. Furthermore, studies on the effects of tetracyclines on inducible NO synthase mRNA levels were carried out.

2. Materials and methods

2.1. Macrophage line

The murine macrophage cell line J774 was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were maintained in culture medium.

2.2. Reagents

Lipopolysaccharide (from *E. coli*, serotype 026:B6), tetracycline base and doxycycline were obtained from Sigma (Poole, UK). Tetracycline base was dissolved in ethanol and diluted in phosphate buffer saline (PBS), doxycycline was dissolved in PBS. Tissue culture medium consisted of RPMI-1640 (TechGen International, Les Ulis, France) supplemented with glutamine (2 mM), antibiotics (penicillin and streptomycin) and 2% foetal calf serum (Seromed, Biochrom KG, Berlin). Tissue culture plasticware was purchased from NUNC (Roskilde, Denmark). N^G-monomethyl-L-arginine hydrochloride (L-NMMA) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). L-2,3,4,5-[^{3}H]arginine monohydrochloride (62 Ci/mmol) and L-4,5-[^{3}H]leucine (167 Ci/mmol) were from Amersham International (Amersham, Amersham, UK). Other reagents were purchased from Sigma.

2.3. Induction of NO synthase activity and NO synthesis

J774 were cultured for 2 h at 37°C in an atmosphere of 5% CO_2 in 24-well Costar plates at 2×10^6 cells in 2 ml of culture medium. Nonadherent cells were then removed by washings and the adherent ones cultured with various stimuli as detailed in the legends to the figures. At various time intervals, culture supernatants were collected and NO measurement carried out; the cells were washed and treated with extraction buffer (250 μl of 0.1 M HEPES, pH 7.4, with 1 μM diithiothreitol: Sigma).

The cells were frozen and thawed three times and then harvested with a rubber policeman. They were transferred into Eppendorf tubes and centrifuged at $10\,000 \times g$ for 30 min at 4°C and in the supernatants the activity of NO synthase, extracted from the cells, was assayed.

2.4. Measurement of NO_2^-

NO_2^- in the culture supernatant was determined by Griess reaction (Ding et al., 1988). Briefly, 100 μl/well of the sample was incubated with an equal volume of Griess solution (1% sulfanilamide in 5% phosphoric acid + 1% alpha-naphthyl-amine in distilled water) at room temperature for 10 min. The absorbance was evaluated with a Titertek ELISA reader (Flow, Rockville, MD) at 550 nm. The level of NO_2^- reflects NO synthesis.

2.5. Measurement of NO synthase activity

NO synthase activity was assayed measuring the conversion of L-[^{3}H]arginine to [^{3}H]citrulline as described by Salter et al. (1991). Briefly, 20 μl of lysate were incubated with KH_2PO_4 (50 mM), valine (59.8 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.4 mM), EDTA (1.2 mM), diithiothreitol (0.8 mM), NADPH (0.2 mM), L-arginine (38 μM), L-citrulline (2 mM), TH_4Biopterin (50 mM, 50 μl), FAD (1 mM, 500 μl) and [^{3}H]arginine (25 μCi). In some tubes L-NMMA, NO synthase inhibitor, (1 mM) was added to the other reagents. After 15-min incubation at 37°C, the reaction was stopped by adding 0.5 ml HEPES-Na (20 mM), pH 6, containing 2 mM EDTA. The whole reaction mixture was applied to 1 ml columns of Dowex. The radioactivity corresponding to [^{3}H]citrulline contents in 450 μl eluate was measured by liquid scintillation counting (Beckman, Milan, Italy). The protein content of the supernatants was determined by the Coomassie blue binding method according to the manufacturer's recommendations (Pierce, Rockford, IL, USA). NO synthase activity was expressed as pmol NO (mg protein)$^{-1}$ min^{-1}.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Western blot

J774 cells were rapidly rinsed with PBS, lysed in boiling lysis solution (1% SDS–10 mM Tris pH 7.4) and then boiled for an additional 5 min. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay method (Pierce).

SDS-PAGE was performed as described by Laemmli (1970), using 7.5% (w/v) polyacrylamide gel. An appropriate amount of total protein from the cell lysate was loaded on to the gel with 6 × SDS sample buffer (Tris–Cl pH 6.8 0.35 M, glycerol 30% v/v, SDS 10% w/v, β-mercaptoethanol 6% v/v, bromophenol blue 0.012% w/v). After electrophoresis the proteins were transferred onto an immobilon membrane (Millipore, Bedford, MA, USA) using a wet electrotransfer system (Biorad, Hercules, CA, USA). The membrane was then blocked in tris buffer saline–Tween (TBS–T: 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% dried nonfat milk and subsequently incubated with a mouse monoclonal anti-inducible NO synthase antibody (1.5 μg) (Transduction Laboratories, Lexington, KY, USA), diluted in TBS–T. An anti-mouse horseradish peroxidase labelled antibody was used as second antibody (Amersham). Bands were visualized by autoradiography using the enhanced chemiluminescence detection system (Pierce) following the manufacturer's instructions. A mixture of different color proteins was used as protein molecular weight markers (Sigma, St. Louis, MO, USA).

2.7. Northern-blot analysis

Cellular cytoplasmic RNA was isolated from both treated and untreated cells as described above, according to Berger and Birkenmeier as described previously (Giallongo et al., 1986). RNA (20 μg) was electrophoresed on 2.2 M formaldehyde–1% agarose gel and transferred to nylon membrane (Hybond, Amersham, UK) according to the manufacturer's instructions. Hybridizations were carried out with oligolabeled probes in: 6 × saline sodium citrate (SSC) (1 × SSC is: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0); 50% formamide; 5% Denhardt's solution; 0.5% SDS; 140 μg/ml denatured salmon sperm DNA at 42°C for 24 h. A final wash was performed in 0.2 × SSC, 0.1% SDS at 55°C and the filter was autoradiographed for 24–48 h at −70°C with intensifying screen. The inducible NO synthase probe was a 0.5 kb DNA fragment obtained by polimerase chain reaction amplification from a mouse inducible NO synthase cDNA and was kindly provided by Dr. Xu Damo and Prof. F.Y. Liew. As a control for the amount of RNA loaded per lane the filter was further hybridized with a 1.4 kb *Bam*HI DNA fragment, derived from the human ribosomal gene cluster, which specifically hybridizes to the 28s rRNA (De Leeuw et al., 1989).

2.8. Cell viability assay

A modification of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) reduction assay of Mosmann (1983) was used. Cultures were incubated for 24 h and pulsed with 100 μl of 0.2 mg/ml MTT reagent for 2 h at 37°C, followed by 15 min incubation at 37°C with 100 μl dimethyl sulfoxide (DMSO, Sigma). After this period, microtiter plates were read at 595 nm in a ELISA plate reader. The results are expressed as absolute optical density (O.D.) readings. The standard error of the mean (S.E.M.) never exceeded 15% of the mean O.D. value and is omitted.

2.9. Determination of total protein synthesis

Cells were seeded at a density of 10^7/ml in 96 well plates for varying time periods in RPMI 1640 medium supplemented with 2% foetal calf serum containing or lacking lipopolysaccharide and/or tetracyclines. Total protein synthesis was measured as leucine incorporation using a 4-h labelling interval with [^{3}H]leucine. After six washings in RPMI supplemented with nonradioactive leucine, the cells were further cultured in complete RPMI medium in 5% CO_2 atmosphere. At the end of culture periods acid-insoluble macromolecules were precipitated with ice-cold trichloroacetic acid (10%). The precipitate was washed to remove soluble precursors and it was dissolved in SDS/NaOH followed by the measurement of radioactivity by scintillation counting (Garg and Hassid, 1993). The aforementioned parameters were corrected for cell protein content per well.

2.10. Statistical analysis

All experiments were performed three or four times and the results are expressed as the mean ± S.E.M. Some data are reported as the mean ± S.E.M. of three or four individual experiments, others are reported as the mean ± S.E.M. of a single representative experiment. This was done in those experiments in which the levels of NO and NO synthase were variable. This is probably due to the non-synchronous growth phases of the J774 cell line. However, within each single experiment, the S.E. was within 10%. Significance was tested by Student's *t*-test by variance analysis (Student–Newmann–Keuls test).

3. Results

3.1. Effect of tetracycline base and doxycycxline on the viability of lipopolysaccharide-activated J774 line

We studied the viability of J774 line cultured with lipopolysaccharide, with or without tetracycline base and doxycycline, to evaluate whether these drugs really affected the activity of viable cells. To determine the loss of viability, we used a colorimetric MTT reduction assay. J774 cells were cultured for 24 h with increasing doses of tetracycline base and doxycycline with or without 1 μg/ml of lipopolysaccharide and were compared with drug-untreated cultures by measuring cell viability. J774 macrophage line remained viable after 24 h culture in the presence of tetracycline base and doxycycline in the unstimulated cultures at all doses used, apart from doxycycline 400 μM and tetracycline base 500 μM. Lipopolysaccharide activation determined a significant reduction ($P < 0.01$) in the MTT signal respect to the untreated cells, but the addition of tetracycline base and doxycycline did not further decrease the MTT signal with the exception of higher concentrations (e.g., 500 μM for tetracycline base or 400 μM for doxycycline) (Table 1). This means that lipopolysaccharide-activation reduces the viability of the cells and tetracyclines modify NO synthesis without inducing a further increase in the death of the cells. As 500 μM (tetracyclines) and 400 μM (doxycycline) appear to increase cell mortality, we did not use them in further experiments. The trend was confirmed with direct viable cell counts (trypan bleu), even though we found 15% variations compared with MTT evaluation.

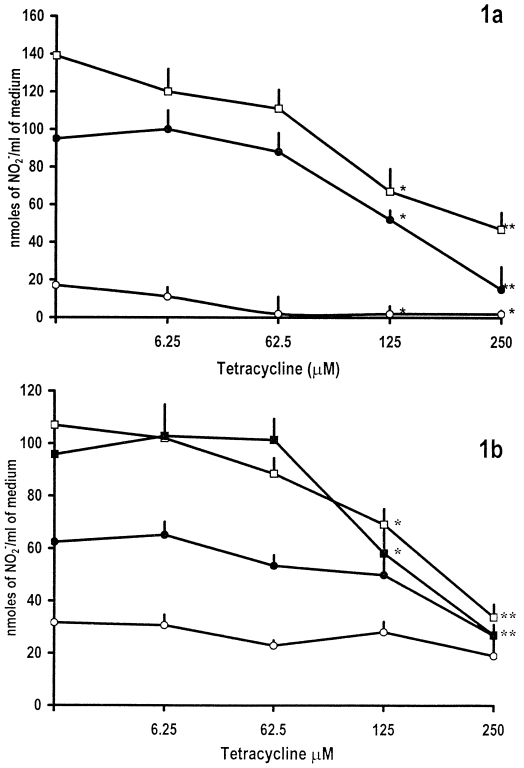

Fig. 1. (a) Levels of NO_2^- produced by J774 macrophage line after culturing for 48 h with LPS (○: 10 ng/ml; ●: 100 ng/ml; □: 1 μg/ml) in the presence of different doses of tetracycline. Data are expressed after background subtraction (18 ± 8 nmol/ml). (b) Levels of NO_2^- produced by J774 macrophage line after culturing for various times (○ 12 h; ● 24 h; □ 48 h; ■ 72 h) with 1 μg/ml of lipopolysaccharide in the presence of different doses of tetracycline base (6.25–250 μM). Data are expressed after background subtraction (10 ± 3 nmol/ml). Vertical bars = S.E.M., $n = 5$. Results shown in (a) and (b) are representative of five separated experiments. $*\ P < 0.05$ and $**\ P < 0.01$ represent significant inhibition.

3.2. Effect of tetracyline base and doxycycline on NO synthesis

J774 line was incubated with different doses of tetracyclines (6.25–250 μM) in the presence of various

Table 1
Effect of tetracycline base and doxycycline on the viability of J774 cell line studied by MTT reduction assay

		Tetracycline base (μM)			Doxycycline (μM)		
		62.5	250	500	5	50	400
RPMI	0.780 ± 0.05	0.919 ± 0.01	0.745 ± 0.02	0.520 ± 0.08*	0.692 ± 0.09	0.680 ± 0.05	0.480 ± 0.1*
Mitogen	0.537 ± 0.05	0.581 ± 0.05	0.566 ± 0.01	0.380 ± 0.05*	0.708 ± 0.04	0.515 ± 0.02	0.320 ± 0.08*

J774 cell line (10^6/ml) was incubated with RPMI–2% foetal calf serum or lipopolysaccharide (1 μg/ml) for 24 h in the presence of different doses of tetracycline base and doxycycline.
The viability is expressed as the mean O.D. ± S.E.M. of six wells from three independent experiments.
$*\ P < 0.05$ significantly different from untreated cells.

amounts of lipopolysaccharide for 48 h and supernatants tested for NO synthesis. According to previous data (Milano et al., 1995), the most significant cell stimulations were obtained with 0.1 and 1 μg/ml of lipopolysaccharide (113 ± 8 and 157 ± 18 nmol/ml, respectively), whereas the dose of 10 ng/ml of lipopolysaccharide was not very effective in inducing significant production of NO compared with medium alone (45 ± 10 vs. 18 ± 8 nmol/ml). Tetracycline base significantly ($P < 0.01$) reduced NO production, measured as NO_2^-, induced by all doses of lipopolysaccharide in a dose-dependent manner, starting from the dose of 125 μM (Fig. 1 a). Tetracycline base-induced inhibition of NO production was low after 12 h incubation, but it became clearer as the incubation times increased, reaching the peak between 48 and 72 h of culture (Fig. 1b). Doxycycline effects were similar at a lower dosage (50 μM) (Table 2). In further experiments, we have used 1 μg/ml of lipopolysaccharide, since this amount of stimulus gave the highest production of NO.

3.3. Effect of tetracycline base and doxycycline on the intracellular activity of NO synthase

To determine the effect of tetracyclines on inducible NO synthase activity we studied the profile of NO synthase expression in J774 line activated with lipopolysaccharide in the presence of different doses of tetracyclines. The enzyme synthesis was time-dependent. It was first detectable at around 9–10 h after stimulus, peaked between 12 and 24 h, and decreased thereafter being undetectable by 72 h. Fig. 2 shows data after 24 h of activation and indicates that tetracycline base was a potent inhibitor of cellular NO synthase activity in a dose-dependent manner, starting from 125 μM.

3.4. Effect of tetracycline base and doxycycline on total protein cell content

Since the reduction in NO synthesis by tetracycline base and doxycycline could be related to an inhibition of total protein synthesis, we studied the effect of these drugs on protein synthesis evaluated by [^{3}H]leucine incorporation method. Data indicate that tetracycline base did not influence this parameter significantly (Fig. 3). Data were confirmed using doxycycline (data not shown).

Table 2
Effect of doxycycline on NO production induced by lipopolysaccharide

Doxycycline μM	Time of incubation (h)		
	12	24	48
−	32 ± 4	65 ± 4	85 ± 5
5	31 ± 3	55 ± 4	91 ± 5
50	16 ± 4*	21 ± 6*	26 ± 7**
100	16 ± 5*	18 ± 4**	21 ± 7**

J774 cell line (10^6/ml) was incubated with complete RPMI plus 2% foetal calf serum in the presence of an optimal dose of lipopolysaccharide (1 μg/ml) for various times in the presence of different doses of doxycycline.
The amounts of NO (measured as NO_2^-) released in the supernatants were determined as indicated in Section 2.
Values are given as nmol of NO_2^-/ml of medium (mean ± S.E.M. of four experiments).
* $P < 0.05$; ** $P < 0.01$ (Student's t-test) significantly different from untreated cells.

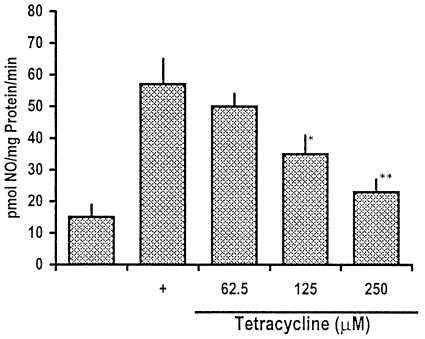

Fig. 2. Levels of inducible NO synthase produced by J774 cell line after culturing for 24 h with lipopolysaccharide (1 μg/ml) in the presence of different doses of tetracycline base. Inducible NO synthase activity is expressed as pmol NO (mg protein)$^{-1}$ min^{-1} (mean ± S.E.M. of three experiments) and measurements were performed as indicated in Section 2. (−) Negative control, medium alone; (+) lipopolysaccharide alone. * $P < 0.05$ and ** $P < 0.01$ represent significant inhibition.

3.5. Effect of tetracyclines on inducible NO synthase protein content, and on the expression of inducible NO synthase mRNA

We further examined whether tetracyclines decrease the inducible NO synthase protein in the total cell extracts. Therefore, J774 cells were stimulated with lipopolysaccharide for 12 h and cell-free extracts were examined for 130 kDa inducible NO synthase SDS-PAGE and Western blot. Fig. 4A shows that the expression of inducible NO synthase protein was decreased in J774 cells stimulated with

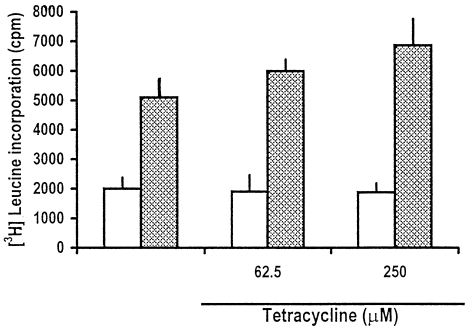

Fig. 3. Influence of tetracycline base on total protein synthesis. Total protein synthesis was determined as the incorporation of radio-labelled leucine in untreated (white column) and lipopolysaccharide-treated (1 μg/ml; shaded column) J774 cells after culturing for 24 h with different doses of tetracycline base. The results are representative of three experiments.

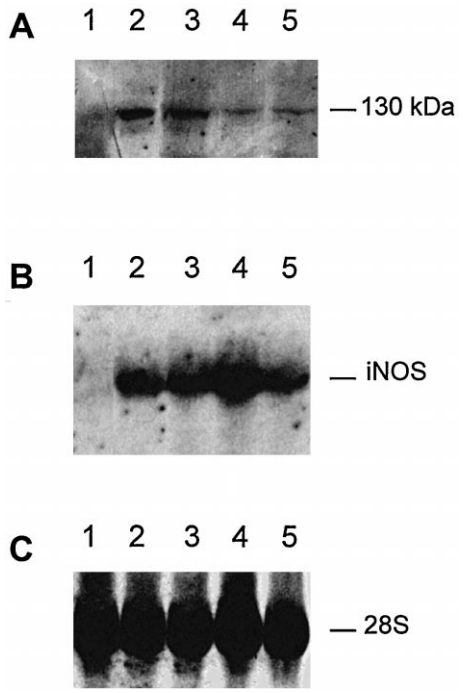

Fig. 4. Effect of tetracycline base on inducible NO synthase protein and inducible NO synthase mRNA accumulation. J774 cell line was cultured in medium alone or stimulated with lipopolysaccharide (1 μg/ml) with or without different doses of tetracycline base (62.5–125–250 μM). Total cell lysates from macrophages were processed for the determination of inducible NO synthase enzyme by SDS-PAGE and Western blot analysis (A). For mRNA accumulation cytoplasmic RNA (20 μg) was electrophoresed, transferred to nylon membrane and hybridized with an inducible NO synthase specific cDNA probe (B) or a 28s rRNA specific probe (C). Lane 1, RPMI alone; lane 2, lipopolysaccharide; lane 3, 62.5 μM tetracycline base plus lipopolysaccharide; lane 4, 125 μM tetracycline base plus lipopolysaccharide; lane 5, 250 μM tetracycline base plus lipopolysaccharide. The results are representative of three experiments.

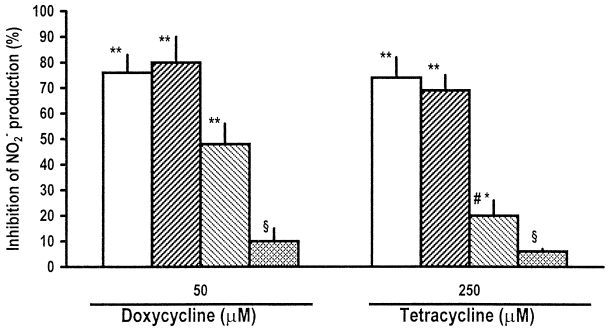

Fig. 5. Effect of tetracycline base and doxycycline when applied as 6 h pretreatment (□), or contemporaneously (square with right-diagonal lines), or as 6 h (square with left-diagonal lines) or 12 h (square with dots) post-treatment on NO production at 48 h after lipopolysaccharide (1 μg/ml) in J774 macrophages. Data (% inhibition of NO synthesis) are expressed as the mean ± S.E.M. of six wells from four independent experiments. $*P < 0.05$ and $**P < 0.01$ represent significant inhibition. ‡$P < 0.05$ and $P < 0.01$ represent significant differences between the post-treatment groups and the pre-treatment ones.

lipopolysaccharide by the treatment with tetracyclines in a dose-dependent manner. To determine whether tetracyclines inhibited inducible NO synthase protein expression at the level of mRNA accumulation, J774 cells were treated with lipopolysaccharide for 6 h (the peak time of mRNA accumulation) in the presence or absence of tetracyclines and analyzed for inducible NO synthase mRNA by Northern blot analysis. Tetracyclines had no significant effects on inducible NO synthase mRNA accumulation in lipopolysaccharide-stimulated J774 (Fig. 4B and C). The same results were obtained using doxycycline (data not shown).

3.6. Effect of tetracyclines on inducible NO synthase specific activity

Next, we investigated if the reduction in NO synthesis by tetracyclines was due to a direct inhibitory effect on inducible NO synthase enzyme activity. For this reason we have studied the effect of tetracycline base and doxycycline on NO synthesis administered 6 h before or 6 and 12 h after lipopolysaccharide stimulation, when the enzyme is already active. As shown in Fig. 5, when tetracycline base was administered 6 h before lipopolysaccharide, its inhibitory effect was extremely significant, but its inhibitory effect was significantly smaller when applied 6 h after lipopolysaccharide and was absent 12 h after lipopolysaccharide. Doxycycline continued to exert a clear inhibitory effect on NO synthesis 6 h after mitogen stimulation. Altogether, these experiments indicate that tetracyclines modify an early event in inducible NO synthase activation, operating after mRNA transcription.

4. Discussion

Here we demonstrate that tetracyclines are able to attenuate or inhibit the production of NO by lipopolysaccharide in cultured J774 macrophages. This inhibitory effect was observed both with tetracycline base and with its semi-synthetic derivate doxycycline, that, although structurally similar, is the most potent of commercially available tetracycline inhibitors of collagenase (Golub et al., 1987; Humbert et al., 1991).

The inhibitory mechanism of NO by tetracyclines appears to be specific enough, since tetracycline base was not able to inhibit in vitro those cytokines which mediate the induction of inducible NO synthase by lipopolysaccharide, like TNF-α and IL-1α (Milano et al., 1997), and participate in the cytotoxic and inflammatory properties of macrophages. The absence of significative modifications in MTT reduction and protein synthesis, evaluated by leucine incorporation signal in the first 24 h, indicates that the modulation and the suppression of NO production are not to the detriment of cell viability or due to an indiscriminate inhibition of total protein synthesis. It appears to be due to

a specific inhibition of inducible NO synthase protein content in the cells, as attested by the significant reduction in the expression of inducible NO synthase, assayed by SDS-PAGE and Western blot. The observation that tetracyclines do not inhibit inducible NO synthase mRNA accumulation in J774 cell line after 6 h of stimulation with lipopolysaccharide suggests that these drugs are involved in the blocking of post-transcriptional events. Our data are in agreement with those published by other investigators using RAW 264.7 cell line (Amin et al., 1996). In fact, for Amin et al. (1996) the stimulation of the cells with lipopolysaccharide in the presence of tetracyclines for 4 h did not induce any variation in the levels of inducible NO synthase mRNA, as obtained by us in 6 h stimulation. Although, we cannot exclude after 16 h of stimulation in the presence of tetracyclines, there may be a degradation of inducible NO synthase mRNA (Amin et al., 1996), further experiments are required to confirm that the susceptibility of inducible NO synthase mRNA to tetracycline degradation is specific. Furthermore, tetracyclines were also able to reduce inducible NO synthase enzyme activity and hence NO synthesis when they were used in culture 6 h before or together with lipopolysaccharide. When applied 6 or 12 h after lipopolysaccharide, the inhibitory effect of tetracyclines on NO synthesis was diminished or almost completely absent, showing that these drugs inhibit early event in inducible NO synthase expression, but not its activity.

The inhibitory activity of tetracyclines on NO release could provide a further explanation for the anti-inflammatory action of tetracyclines, clearly observed in several experimental models (El Attar et al., 1988; Gabler and Creamer, 1991; Golub et al., 1987, 1991; Martin et al., 1974; Ramamurthy et al., 1993; Suomalainen et al., 1992a,b), since the down-regulation of NO secretion could be one of the mechanisms by which tetracyclines control the inflammation. In fact, it has been demonstrated that NO up-regulates the release of inflammatory mediators (Ialenti et al., 1992; Marcinkiewicz et al., 1995; Milano et al., 1995; Salvemini et al., 1993) and, on the other hand, it has already been shown that tetracyclines strongly block the synthesis of some of them, like phospholipase A_2 (Suomalainen et al., 1992a,b) and prostaglandin E_2 (El Attar et al., 1988). Altogether, these data indicate that further studies are required to clarify the aspects of these regulatory circuits.

However, the anti-inflammatory potential of tetracyclines is held to be related essentially to the ability of these compounds to inhibit mammalian collagenases and several matrix metalloproteinases by a mechanism independent of the microbial activity (Golub et al., 1983, 1985, 1991; Greenwald et al., 1992). On the basis of these actions tetracyclines and their derivatives have been used with good results in vivo in the treatment of periodontal disease (Suomalainen et al., 1992a), rheumatoid arthritis (Breedveld et al., 1990; Farrel et al., 1992; Greenwald et al., 1990) and septic shock syndrome (Shapira et al., 1996; Milano et al., 1997). The use of tetracyclines in arthritis treatment is intriguing too, because in this disease there is an increased concentration of nitrite in the synovial fluid and serum samples (Farrel et al., 1992).

The inhibition of NO synthesis by tetracyclines is another possible pathway by which tetracyclines may function as anti-inflammatory compounds and could explain the interesting results obtained with tetracyclines in the treatment of septic shock (Shapira et al., 1996; Milano et al., 1997).

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