Doxycycline Modulates Nitric Oxide Production in Murine Lung Epithelial Cells

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Doxycycline Modulates Nitric Oxide Production in Murine Lung Epithelial Cells

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Many effective therapeutic agents exhibit effects that are different from their intended primary mode of action. Antibiotics such as doxycycline and erythromycin A are no exception. They also display anti-inflammatory activity. Using LA4 murine lung alveolar epithelial cells, effects of doxycycline and erythromycin A on inducible NO synthase (iNOS) NO production as well as iNOS protein and mRNA production were investigated. Induction of iNOS was accomplished by treatment with cytomix (TNF-α, IL-1β, and IFN-γ each at 5 ng/ml). Production of NO or iNOS was not detected in controls with or without erythromycin A. In the presence of cytomix, erythromycin A did not decrease NO, nitrite, iNOS protein, or mRNA production. In contrast, doxycycline caused a dose-dependent decrease in NO, nitrite, iNOS protein, and mRNA production in cytomix-treated cells. Doxycycline at 30 μg/ml produced a 90% decrease in nitrite and NO production and a 52% decrease in iNOS mRNA transcription compared with cytomix treatment alone. Actinomycin D treatment suggests that doxycycline decreases stability of iNOS mRNA in cytomix-treated cells. To determine a mechanism for the decrease in iNOS expression, NF-κB and AP-1 transcription regulatory systems and p38 MAPK were examined. Doxycycline treatment gave no statistically significant change in NF-κB activation but did decrease p38 MAPK protein in cytomix-treated cells by 50%, suggesting that p38 MAPK may be responsible for stabilization of iNOS mRNA. These results demonstrate that doxycycline decreases NO production from iNOS by destabilization of iNOS mRNA via decreased expression of p38 MAPK. The Journal of Immunology, 2006, 176: 567–572.

The common obstructive airway disorders such as asthma, chronic bronchitis, diffuse panbronchiolitis, and bronchiectasis are all invariably associated with chronic airway inflammation (1–3). Despite treatment with anti-inflammatory therapies, these disorders are often progressive, suggesting that the current anti-inflammatory therapies are inadequate.

Macrolides and tetracyclines are bacterial and fungal secondary metabolites that possess antibacterial action but also exhibit distinct and separate anti-inflammatory actions. These antibacterial compounds have been known to reduce inflammation associated with a variety of inflammatory diseases since the 1970s (4, 5). Among these compounds are erythromycin A and many of its derivatives such as azithromycin, clarithromycin, and roxithromycin, as well as tetracycline and several related compounds such as doxycycline. These compounds have exhibited anti-inflammatory activity against a variety of inflammatory airway disorders (5). However, concern has been raised about development of microbial antibiotic resistance limiting macrolide usefulness and suggesting a need for alternative anti-inflammatory therapies.

One marker of inflammation has been measurement of NO, a gas produced by lung epithelial cells, which has been shown to be elevated in several inflammatory airway conditions (6). We hypothesized that doxycycline possesses potent anti-inflammatory properties in murine lung epithelial cells, leading to a reduction of NO produced by inducible NO synthase (iNOS) and that this inhibition is due, at least in part, to decreased stabilization and expression of iNOS mRNA and its translation to form active iNOS enzyme. The effect of doxycycline on the NF-κB transcriptional regulatory system was examined as well as the effects of p38 MAPK on iNOS mRNA stability. The results confirm this hypothesis in vitro and suggest that doxycycline treatment may be an effective airway anti-inflammatory therapy in diseases such as asthma, chronic bronchitis, diffuse panbronchiolitis, and bronchiectasis.

Materials and Methods

Culture of LA4 cells

The clonal murine lung alveolar epithelial cell line LA4 was purchased from the American Type Culture Collection (7). LA4 cells were grown in 25-cm² tissue culture flasks (Corning Costar) in Ham’s F-12 containing 10% FCS, 2 mM l-glutamine, and penicillin-streptomycin (100 U/ml, 100 μg/ml) until confluent. After washing with serum-free medium doxycycline, dissolved in distilled water, or erythromycin A, dissolved in DMSO, was added to confluent LA4 cultures for 3 h, and the cells were cultured for 18 h in serum-free Ham’s F-12 with or without cytomix (CM). CM is a combination of recombinant human TNF-α, human IL-1β, and murine IFN-γ each at 5 ng/ml concentration (all cytokines from R&D Systems), that is used to stimulate the expression of iNOS (8, 9). Concentrations of tested chemicals ranged from 0.3 to 30 μg/ml for doxycycline and 0.3 to 60 μg/ml for erythromycin A based on reports of clinically relevant serum and tissue levels (10, 11). Serum levels of doxycycline are reported to be up to 79 μg/ml (depending on the individual) (10) and for erythromycin in the range of 1.9 to 6.5 μg/ml (11). Ampicillin (50 μg/ml) and sparsomycin (0.5 μg/ml) were used as control compounds. All control cultures contained the appropriate amount of carrier solvent alone.

Cell viability assays

Cell viability was determined by assay for release of lactate dehydrogenase (LDH) into cell culture supernatants using a commercially available LDH assay kit (Sigma-Aldrich).

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Determination of NO concentration in culture flask head space and nitrite in culture flask supernatants

After 18 h of stimulation with CM, culture flasks were tightly sealed and incubated at 37°C. After 3 h head space gas was withdrawn using a needle and syringe. NO concentrations were measured using a NO chemiluminescence analyzer (Sievers) with a detection sensitivity of <1 parts per billion (ppb). The analyzer was calibrated daily according to the manufacturer’s directions using a gas of known NO concentration. Nitrite concentrations of the cell culture supernatant were determined spectrophotometrically using the Griess reaction (12).

Protein determination

Protein concentrations were determined spectrophotometrically using the Bradford assay (13).

Cell lysis, SDS-PAGE, and Western blotting

Cell extracts were prepared for Western blotting using a modification of previously described methods (14). Cells were lysed by using 10 mM Tris-HCl (pH 7.4) containing 1% SDS and 1 mM EDTA and a premade mixture of protease inhibitors (Sigma-Aldrich). Cell lysates were clarified by centrifugation before electrophoresis. SDS-PAGE was performed using a 4% T acrylamide stacking gel and a 7.5% T acrylamide separating gel for iNOS or a 10% T separating gel of p38 MAPK (15). After electrophoresis was complete, the separated proteins were transferred to a polyvinylidene difluoride membrane for further analysis (16). Transfer was performed using a Tris-HCl (25 mM), glycine (192 mM), and methanol (20% v/v) buffer system. The transfer was performed at 4°C for 1 h at 30 V and then for 30 min at 60 V. Abs for iNOS (Transduction Laboratories) or p38 MAPK (Santa Cruz Biotechnology) were used to immunocn dentify iNOS or p38 MAPK protein on the transfer membranes using goat anti-rabbit IgG alkaline phosphatase as the secondary Ab-enzyme conjugate and NBT/5-bromo-4-chloro-3-indolyl phosphate phosphate substrate tablets (Sigma-Aldrich) in 0.1 M Tris- HCl (pH 9.1) as the substrate system. Five micrograms of Ab were used at each step during the development procedures. The transfer membranes were blocked with BLOTTO (20 mM Tris base, 180 mM NaCl, 4% nonfat dry milk, 0.02% NaN3) to minimize nonspecific binding.

RNA isolation and RT-PCR

iNOS mRNA was analyzed by RT-PCR. Total cellular RNA was extracted from adherent cells using a modification of the methods of Chomczynski and Sacchi (17). The RNA was reverse transcribed using a commercially available RT-PCR kit (Promega) using the sense and antisense primers (Table I) added at 0.2 μM final concentration. Using a PerkinElmer model 480 thermal cycler, reverse transcription was performed on total cellular RNA at 48°C for 45 min. PCR for iNOS was performed in the same sample tubes at 94°C for 2 min followed by 28 cycles consisting of 94°C for 45 s, 60°C for 45 s, 72°C for 2 min, followed by 72°C for an additional 7 min. β-Actin (primers shown in Table I) was used as a “housekeeping gene” and RT-PCR was performed in a similar manner as for iNOS except for an annealing temperature of 50°C. The DNA fragments were separated by electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, 2 mM Na2 EDTA, pH 8.5) and then analyzed and quantitated by densitometry. Increasing PCR cycles gave increasing amounts of transcription. RNA was isolated from cultures at various times. RT-PCR was used to determine the amount of iNOS mRNA present at each time.

Effect of doxycycline on NF-κB

The effect of doxycycline on the NF-κB transcription regulatory system was determined using a commercially available ELISA kit (Active Motif). The primary Ab used in this kit detects an epitope of p50/p65 that is accessible only when NF-κB is activated and bound to its consensus DNA-binding sequence. Cell extracts were prepared according to manufacturer’s instructions. LA4 cells were treated with 30 μg/ml doxycycline for 3 h followed by addition of CM for 1 h before preparation of cell extracts for NF-κB analysis. For each analysis each well contained 5 μg protein.

Results

LA4 cell culture supernatants from all treatment types were assayed for LDH release as a measure of cell viability. There were no significant differences in the LDH activity in the culture supernatants from all treatment types (data not shown).

Doxycycline, but not erythromycin A, decreased NO production in CM (5 ng/ml each of IL-1β, TNF-α, and IFN-γ)-stimulated LA4 cells (Table II). Doxycycline markedly inhibited NO production at the highest concentration used. NO production was also inhibited at the lower doxycycline concentrations, in a concentration-dependent manner, but the decrease was not as dramatic. The differences in NO production between CM-treated and CM plus 3 μg/ml, 10 μg/ml, or 30 μg/ml doxycycline was statistically significant (p < 0.05). The difference with CM plus 0.3 μg/ml doxycycline was not statistically significant (p > 0.05). In contrast, erythromycin A inhibited NO production only at the highest concentration tested (10 μg/ml) and this decrease was not statistically significant (p > 0.05).

Stability of iNOS mRNA

LA4 cells were treated with CM or CM plus doxycycline for 18 h as described above and then treated with actinomycin D (10 μg/ml) to stop transcription. Data were analyzed by one-way ANOVA with Fisher’s protected least significant difference (Fisher’s PLSD). In all cases, a p < 0.05 was considered to be significant. The data are expressed as mean ± SD.

Table II. Effect on NO production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doxycycline (SD)</th>
<th>Erythromycin A (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 ppb NO (1)</td>
<td>21 ppb NO (5)</td>
</tr>
<tr>
<td>CM alone</td>
<td>2680 (237)</td>
<td>1980 (76)</td>
</tr>
<tr>
<td>30 μg/ml alone</td>
<td>10 (6)</td>
<td>8 (2)</td>
</tr>
<tr>
<td>30 μg/ml + CM</td>
<td>8 (2)</td>
<td>1800 (172)</td>
</tr>
<tr>
<td>10 μg/ml alone</td>
<td>8 (0.8)</td>
<td>—</td>
</tr>
<tr>
<td>10 μg/ml + CM</td>
<td>1094 (277)</td>
<td>—</td>
</tr>
<tr>
<td>3 μg/ml alone</td>
<td>7 (1)</td>
<td>8 (2)</td>
</tr>
<tr>
<td>3 μg/ml + CM</td>
<td>1200 (78)</td>
<td>1900 (643)</td>
</tr>
<tr>
<td>0.3 μg/ml alone</td>
<td>9 (2)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>0.3 μg/ml + CM</td>
<td>2400 (144)</td>
<td>1870 (333)</td>
</tr>
</tbody>
</table>

Table III. Effect of doxycycline and erythromycin on nitrite production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doxycycline (SD)</th>
<th>Erythromycin A (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.2 μM nitrite</td>
<td>0.1 μM nitrite</td>
</tr>
<tr>
<td>CM alone</td>
<td>5.9 (0.7)</td>
<td>8.9 (1.7)</td>
</tr>
<tr>
<td>30 μg/ml alone</td>
<td>0.3 (0.2)</td>
<td>0.1 (0.2)</td>
</tr>
<tr>
<td>30 μg/ml + CM</td>
<td>0.4 (0.1)</td>
<td>0 (0.9)</td>
</tr>
<tr>
<td>10 μg/ml alone</td>
<td>0.4 (0.1)</td>
<td>—</td>
</tr>
<tr>
<td>10 μg/ml + CM</td>
<td>5.5 (0.7)</td>
<td>—</td>
</tr>
<tr>
<td>3 μg/ml alone</td>
<td>0.2 (0.4)</td>
<td>0.1 (0.07)</td>
</tr>
<tr>
<td>3 μg/ml + CM</td>
<td>6.2 (1.6)</td>
<td>8.5 (2.4)</td>
</tr>
<tr>
<td>0.3 μg/ml alone</td>
<td>1.4 (0.2)</td>
<td>0.1 (0.4)</td>
</tr>
<tr>
<td>0.3 μg/ml + CM</td>
<td>5.8 (0.2)</td>
<td>8.8 (0.9)</td>
</tr>
</tbody>
</table>

Materials and Methods

Table I. Oligonucleotides used for PCR amplification

<table>
<thead>
<tr>
<th></th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>sense 5’-CCCTTCCAGATTTTCTGCAGCAG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense 5’-GGTCTGACGAGCCCTCTGCTTG-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>sense 5’-TGACCCAGATCACTGGTGAG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense 5’-TCAGGGATGTACGCATTG-3’</td>
</tr>
</tbody>
</table>

*a* Primer pairs used for RT-PCR experiments. Experimental procedures were performed as described in Materials and Methods.
erythromycin A exhibited little inhibition of NO production at all concentrations tested. Assays for NO and nitrite were performed on at least two sets of samples in triplicate.

In similar experiments, cell culture supernatants were examined for nitrite content (Table III). In the absence of superoxide, LA4 cells produce mostly nitrite (9, 18, 19). Furthermore, nitrite and nitrate concentrations correlate in LA4 cells even in the presence of superoxide (9, 18, 19). Doxycycline, at a concentration of 30 \( \mu \text{g/ml} \), decreased production of nitrite in CM-stimulated LA4 cells (Fig. 3, \( p < 0.05 \) when compared by scanning densitometry).

Erythromycin A did not reduce production of iNOS mRNA in CM-stimulated cells (Fig. 4). These findings are consistent with the insignificant change in NO and nitrite production seen after erythromycin treatment. Expression of the \( \beta \)-actin gene and production of its mRNA was not altered during any of the treatments discussed (data not shown). Erythromycin A at a concentration of 60 \( \mu \text{g/ml} \) did not alter iNOS mRNA expression or production of nitrite (data not shown).

Doxycycline treatment does not result in a statistically significant decrease in NF-\( \kappa \)B in CM-stimulated cells with respect to CM control (no treatment) (Fig. 5). In a companion experiment, treatment of LA4 cells with erythromycin A results in a statistically significant decrease in NF-\( \kappa \)B after CM treatment (data not shown), but there is no effect on iNOS mRNA transcription.

Expression of p38 MAPK protein was affected by doxycycline in CM-treated LA4 cells (Fig. 6). The presence of doxycycline in CM-treated cells decreased the amount of p38 MAPK protein by 50% of that produced relative to either treatment alone. The presence of 10 \( \mu \text{g/ml} \) actinomycin D in CM-treated LA4 cell cultures results in a decrease in iNOS mRNA decay in the presence of doxycycline over a 6-h time course (Fig. 7). The absence of doxycycline results in no significant decrease in iNOS mRNA production.

\[ \text{FIGURE 1. Effect of doxycycline on iNOS protein. Lane 1, Molecular mass markers (kDa); lane 2, control (no treatment); lane 3, CM alone; lane 4, 30 \mu g/ml doxycycline; lane 5, 50 \mu g/ml doxycycline plus CM; lane 6, 10 \mu g/ml doxycycline; lane 7, 10 \mu g/ml doxycycline plus CM; lane 8, 3 \mu g/ml doxycycline; and lane 9, 5 \mu g/ml doxycycline plus CM.} \]

\[ \text{FIGURE 2. Effect of erythromycin A on iNOS protein. Lane 1, Molecular mass markers (kDa); lane 2 and 3, control (no treatment); lane 4 and 5, CM alone; lane 6 and 7, 30 \mu g/ml erythromycin A; and lane 8 and 9, 30 \mu g/ml erythromycin A plus CM.} \]

\[ \text{FIGURE 3. Effect of doxycycline on iNOS mRNA. Lane 1, bp markers; lanes 2–4, control (no treatment); lanes 5–7, CM alone; lanes 8–10, 30 \mu g/ml doxycycline; lanes 11 and 12, 30 \mu g/ml doxycycline plus CM; lane 13, bp markers; lanes 14–16, 3 \mu g/ml doxycycline; lanes 17–19, 3 \mu g/ml doxycycline plus CM; lanes 20–22, 0.3 \mu g/ml doxycycline; and lanes 23–25, 0.3 \mu g/ml doxycycline plus CM.} \]

\[ \text{FIGURE 4. Effect of erythromycin A on iNOS mRNA. Lane 1, bp markers; lanes 2 and 3, control (no treatment); lanes 4 and 5, CM alone; lanes 6 and 7, 30 \mu g/ml erythromycin A; lanes 8 and 9, 30 \mu g/ml erythromycin A plus CM; lane 10, bp markers; lanes 11 and 12, 3 \mu g/ml erythromycin A; lanes 13 and 14, 3 \mu g/ml erythromycin A plus CM; lanes 15 and 16, 0.3 \mu g/ml erythromycin A; and lanes 17 and 18, 0.3 \mu g/ml erythromycin A plus CM.} \]
DOXYCYCLINE MODULATES NO PRODUCTION

Discussion

Doxycycline effects NO production catalyzed by iNOS in CM-stimulated LA4 murine lung epithelial cells. Both production of NO itself and its oxidation end product, nitrite (NO$_2^-$) are significantly decreased by doxycycline (Tables II and III). In addition, doxycycline decreases the amount of iNOS mRNA (Fig. 3) and its translation to form iNOS protein (Fig. 1). Erythromycin A had no effect of either NO or nitrite production (Tables II and III) or on expression of iNOS mRNA (Fig. 4) or its translation to form iNOS protein (Fig. 2). The unrelated antibiotics, ampicillin and sparsomycin used as controls, did not affect NO production or expression of iNOS mRNA. This suggests that the observed effects are specifically related to doxycycline exposure and are not general, nonspecific effects of exposure to antibiotic compounds.

Analysis of the NF-κB transcription regulatory signaling pathway suggests that it may not play a significant role in attenuating expression of iNOS mRNA after exposure to doxycycline (Fig. 5). The primary Ab in the NF-κB ELISA kit (Active Motif) that was used detects an epitope of p50/p65 that is accessible only when NF-κB is activated and bound to its consensus DNA-binding sequence. This would provide a reliable measure of the degree of NF-κB activation in the presence or absence of doxycycline in CM-treated cells.

The p38 MAPK pathway plays an important role in the inflammatory response (20). A primary function of p38 MAPK is to regulate the stability of inflammatory protein mRNAs such as MCP-1 (21), GM-CSF and vascular endothelial growth factor (22, 23), TNF-α, IL-1, cyclooxygenase 2, IL-6, IL-8 (24–27), and iNOS (28). The p38 MAPK pathway influences stability of various mRNAs by promoting translation via AU-rich elements in the 3’-untranslated region of these molecules (24, 29). Doxycycline decreases the amount of p38 MAPK protein produced in CM-treated cells (Fig. 6). In addition, doxycycline appears to decrease the stability of iNOS mRNA in CM-treated cells (Fig. 7). These data suggest that the doxycycline-induced decrease in p38 MAPK protein may be responsible for the observed decrease in iNOS mRNA stability.

Regulation of inflammatory gene transcription represents a complex interplay between transcription factors and other factors mediating stability of mRNA. Previous studies suggest that NF-κB plays an important role in regulating iNOS transcription (30). However, our results suggest that NF-κB plays at best a minor role in the doxycycline-modulated decrease in iNOS-catalyzed NO production in murine LA4 cells. It seems likely that the decrease in iNOS mRNA and protein that is observed upon doxycycline treatment is due to decreased stability of iNOS mRNA that is a function of a concomitant decrease in p38 MAPK. This concept is supported by the recent work of Fechir et al. (31) that demonstrates that inhibition of p38 MAPK decreases stability of iNOS mRNA, ultimately leading to decreased iNOS expression. The effects of erythromycin on p38 MAPK have been previously reported elsewhere (32, 33).

Tetracyclines and similar antibiotics such as doxycycline have long been known to effectively decrease inflammation in rheumatoid arthritis (34, 35). The present results support the possibility that doxycycline may have potential as an anti-inflammatory treatment for inflammatory lung diseases such as asthma, diffuse panbronchiolitis, chronic bronchitis, and bronchiectasis.

Antibiotics such as the macrolides (erythromycin A and derivatives) (36, 37) and tetracycline derivatives such as doxycycline (38–40) have been successfully used to treat inflammation in a variety of clinical settings. However, successful treatment of inflammation requires long-term drug therapy which includes the possibility of the induction of resistance to antibiotics, such as erythromycin A, in potentially pathogenic bacteria. The possible increase in antibiotic-resistant Mycobacterium spp. (41, 42), Nocardia spp. (43, 44), and other bacterial pathogens is a serious problem that is often encountered when treating inflammatory lung diseases with erythromycin-type antibiotics. Therefore, other potential anti-inflammatory therapies which might not induce resistance, such as doxycycline, would be beneficial for treatment of chronic inflammatory lung diseases. The data presented in this article suggest that doxycycline may be useful as an anti-

FIGURE 5. Effect of doxycycline on NF-κB activation in CM-stimulated LA4 cells. Left column, CM treatment alone; right column, CM plus 30 μg/ml doxycycline. Total cell extracts, 5 μg of protein per assay well, were used in each case. Assay for NK-κB was performed with at least two samples each assayed in triplicate. SDs are shown.

FIGURE 6. Effect of doxycycline of p38 MAPK. Lane 1, molecular mass markers (kDa); lane 2, Control; lane 3, doxycycline (30 μg/ml) alone; lane 4, CM (5 ng/ml for each component) alone; lane 5, CM plus doxycycline. Twenty micrograms of protein was loaded per lane.

FIGURE 7. Stability of iNOS mRNA in the presence of 10 μg/ml actinomycin D.
inflammatory therapy in inflammatory lung diseases where emergence of resistance to doxycycline may not be a serious clinical problem.

In addition to its prominent role in tissue damage during chronic inflammation, NO is also believed to have roles in which its reduction would be detrimental, such as in the defense against microbial pathogens (45). iNOS has been demonstrated to be an important antimicrobial defense mechanism (46). The NO produced can react with superoxide produced by macrophages or neutrophils, forming peroxynitrite (45), which can damage a variety of microbial targets including lipids, heme clusters, aromatic amino acids, thiols, and DNA (47).

A reduction in iNOS expression and NO production is also seen in cystic fibrosis (48). This observed decrease in iNOS expression and NO production may be, in part, responsible for the continued infection and colonization of the cystic fibrosis lung by Pseudomonas aeruginosa (49).

In support of the clinical use of antibiotics as anti-inflammatory agents, recent studies have shown that chronic administration of azithromycin is beneficial in the obstructive lung disease, cystic fibrosis (50). Although the mechanism is unclear, azithromycin is an ineffective antibiotic against bacteria which infect the lungs of cystic fibrosis patients, indicating a presumed anti-inflammatory effect. In conclusion, we have shown that doxycycline reduces cytokine-induced NO production from murine epithelial cells. These results suggest that doxycycline may provide a potential benefit in the management of in vivo inflammation in certain chronic pulmonary diseases.

Disclosures
The authors have no financial conflict of interest.

References


