Macrolide antibiotics inhibit nitric oxide generation by rat pulmonary alveolar macrophages

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Abstract: There is evidence that macrolide antibiotics are effective in the treatment of chronic airway inflammatory diseases, probably through actions other than their antibacterial properties.

To determine whether macrolides affect the nitric oxide-generating system in the respiratory tract, rat pulmonary alveolar macrophages (PAMs) were studied in vitro. The release of NO was assessed by direct measurement with a specific amperometric sensor for this molecule, and the expression of type II NO synthase (NOS) messenger ribonucleic acid (mRNA) was determined by Northern blotting.

Incubation of PAMs with lipopolysaccharide from Escherichia coli and recombinant human interferon-gamma caused release of NO, which was accompanied by induction of type II NOS mRNA. The release of NO was reduced by coincubation of cells with the macrolides erythromycin, clarithromycin and josamycin in a concentration-dependent manner, the maximal inhibition being 73±10, 81±6 and 84±9%, respectively, but was not altered by amoxicillin or cefaclor. These macrolides likewise inhibited the induction of type II NOS mRNA, whereas no inhibitory effects were observed with amoxicillin or cefaclor.

These results suggest that macrolide antibiotics specifically inhibit type II NO synthase gene expression and consequently reduce NO production by rat pulmonary alveolar macrophages, which might result in attenuation of airway inflammation.


Nitric oxide is a highly reactive gaseous free radical generated from the amino acid L-arginine by NO synthase (NOS), which exists in both constitutive and inducible isoforms [1, 2]. Endogenous NO may be involved in the regulation of airway and vascular smooth muscle tone, microvascular permeability, pulmonary neurotransmission and host defence [3]. Moreover, NO may have anti-inflammatory effects, such as protection against vascular leakage, leukocyte adherence and cellular damage [4, 5]. However, NO can exert deleterious effects when it is inappropriately generated or overproduced, and excessive amounts of NO and its metabolites such as peroxynitrite may contribute to the pathophysiology of inflammation and the resultant tissue damage [3, 6, 7]. In the respiratory tract, the existence of constitutive form of NOS (type I and type III NOS) has been shown in various cell types, including vascular endothelial cells, nerve cells and airway epithelial cells [8], indicating that these cells constitutively generate NO. Conversely, production of NO is upregulated in a variety of pulmonary inflammatory diseases [9–11], in which inducible NOS (type II NOS) in pulmonary alveolar macrophages (PAMs) may be involved.

There is some evidence to suggest that long-term administration of the macrolide antibiotic agent erythromycin may be effective in the treatment of chronic airway inflammation, including chronic bronchitis, bronchiectasis and diffuse panbronchiolitis [12, 13]. Although the mechanisms are uncertain, macrolides may exert these effects through actions other than their antimicrobial properties, such as immunomodulatory actions on inflammatory cells [13], reduction of airway secretion [14] and inhibition of cholinergic neurotransmission [15]. Because overproduction of NO by type II NOS may be associated with the development of airway inflammation, it was hypothesized that macrolides could affect the NO-generating system in inflammatory cells. Therefore, in the present study, the effects of macrolides and other antibiotics on the release of NO from rat PAMs by means of real-time measurement of NO levels using a highly specific amperometric sensor for this molecule and on the expression of the type II NOS gene by Northern blot analysis were examined.

Materials and methods

Isolation and culture of rat pulmonary alveolar macrophages

Pathogen-free Sprague-Dawley rats weighing 300–350 g were anaesthetized with intraperitoneal sodium pentobarbital (40 mg·kg body weight−1) and sacrificed by cutting the abdominal aorta. Bronchoalveolar lavage cells were collected by lavaging whole lungs with five instillations of 10 mL saline via a tracheal canula and withdrawing the fluid slowly while gently massaging the thorax. The lavage cells were pelleted by centrifugation (10 min, 200 × g), washed twice with phosphate-buffered saline and resuspended in RPMI-1640 medium. More than 95% of the
cells in this preparation were PAMs, as determined morphologically by May-Giemsa staining. Their viability was >95%, as determined by trypan blue exclusion. The PAMs were adjusted to 10^6 cells mL^{-1} with RPMI-1640 medium containing 10% heat-inactivated foetal bovine serum, penicillin (50 U mL^{-1}) and streptomycin (50 μg mL^{-1}), and seeded on 60-mm-diameter plastic culture dishes. After incubation in 5% carbon dioxide/95% air at 98% relative humidity for 120 min at 37°C, nonadherent cells were removed by aspiration. The adherent cells were cultured with 4 mL serum-free RPMI-1640 medium and stimulated for 12 h with lipopolysaccharide (LPS) from Escherichia coli (50 ng mL^{-1}) and recombinant human interferon gamma (IFN-γ, 50 U mL^{-1}) in the presence of various concentrations of erythromycin, clarithromycin, josamycin, amoxicillin, cefaclor or dexamethasone. In the control experiment, the cells were incubated for 12 h with the culture medium containing the vehicle of the drug (sterile saline) alone. A preliminary experiment showed that the PAMs incubated for 12 h with LPS and IFN-γ consistently showed positive staining for type II NOS-like immunoreactivity, the final concentration of PAMs in the culture medium was 6.6±1.2×10^5 cells mL^{-1}(n=16), and there were no significant differences in the number of PAMs between control, cytokine-treated, antibiotic-treated and dexamethasone-treated groups.

**Measurement of nitric oxide release**

The concentration of NO in the medium containing cultured PAMs was determined using an NO meter (Model NO-501; Inter Medical Co., Tokyo, Japan) by measuring the redox current between the following two electrodes [16]. The counter electrode was made of carbon fibre, and the NO-selective working electrode of platinum/iridium alloy (0.2 mm in diameter; Pt 90%, Ir 10%) coated with a three-layered membrane that consisted of potassium chloride, NO-selective resin and normal silicone membranes. The working electrode was supplied with 0.4–0.8 V for the electrochemical oxidization of NO. The NO diffusing through the membrane was detected as an electrical current. NO-selective resin and normal silicone membranes. Three-layered membrane that consisted of potassium chloride, NO-selective resin and normal silicone membranes. The working electrode was supplied with 0.4–0.8 V for the electrochemical oxidization of NO. The NO diffusing through the membrane was detected as an electrical current. NO-selective resin and normal silicone membranes.

The concentration of NO in the medium containing cultured PAMs 12 h after LPS and IFN-γ stimulation by means of the guanidinium/phenol/chloroform method. The total RNA (2.5 μg) was fractionated via 1% formaldehyde agarose gel electrophoresis, transferred to nylon membranes (Hybond N+, Amersham, UK) and immobilized with ultraviolet cross-linking.

A rat type II NOS partial complementary deoxyribonucleic acid (cDNA) probe (685 base pairs (bp)) was prepared by means of the reverse transcriptase polymerase chain reaction (PCR). RNA prepared from PAMs was reverse transcribed into cDNA, and PCR performed to amplify the 685-bp cDNA fragment. The sequences of the forward (5'-GAGATCAATGACGCTCGTGC-3') and reverse (5'-GCTTCTGTGCGATGTCAAGAAA-3') primers were based on the published type II NOS cDNA sequence [18, 19]. The authenticity of the PCR products was confirmed via dideoxy sequencing methods. The cDNA probe for human β-actin was purchased from Clonetech (Palo Alto, CA, USA).

The probes for rat type II NOS and human β-actin were labelled with [α-32P] deoxycytidine triphosphate (Amersham) using a random prime labelling method. Then hybridization and autoradiography were performed. In all experiments, the values obtained by densitometric scanning of the blots hybridized with the probe for type II NOS were corrected using the values obtained from hybridization with the β-actin probe.
Reagents

The following drugs were used: LPS from E.coli, L-arginine, erythromycin, dexamethasone, aminoguanidine (Sigma Chemical Co., St Louis, MO, USA); recombinant human IFN-γ (Otsuka Pharmaceutical Co., Tokyo, Japan); clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan); josamycin (Yamanouchi Pharmaceutical Co., Tokyo, Japan); amoxycillin (Takeda Pharmaceutical Industries, Osaka, Japan); cefaclor (Shionogi Pharmaceutical Co., Osaka, Japan); SNAP (Inter Medical Co., Tokyo, Japan); and PTIO (Tokyo Kasei Kogyo Co., Tokyo, Japan). Oxyhaemoglobin was prepared by reducing bovine haemoglobin (Sigma) using sodium dithionite and purified on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden).

Statistical analysis

All data are expressed as mean±SEM. Statistical analysis was performed by analysis of variance or the Newman-Keul multiple comparison test, and a p-value of <0.05 was considered statistically significant.

Results

Release of nitric oxide synthesy synthe gene expression

The rat PAMs were incubated for 12 h before L-arginine was added. The output current of the NO-selective electrode in the medium bathing the cultured PAMs is demonstrated in figure 2. Immersion of the electrode in the medium did not produce electrical current in PAMs that had been incubated with the solvent of LPS and IFN-γ (saline) alone, even in the presence of L-arginine (10⁻³ M) (fig. 2a). When the cells were incubated with LPS (50 ng·mL⁻¹) plus IFN-γ (50 U·mL⁻¹), baseline current was not detected, but application of L-arginine caused an increase in the current with a lag time of 10 s–3 min. Between 15 and 30 min after the addition of L-arginine, the response showed the maximal value of 147±25 pA (p<0.001, n=11), which corresponds to an NO concentration of 116±20 nM in the medium [16], and the subsequent electrical current remained elevated during a 2-h observation period (fig. 2b). To assess whether the observed response was associated with type II NOS-mediated release of NO, after the response of electrical current reached a plateau, aminoguanidine rapidly decreased the current by 76±9% (p<0.001, n=9) in PAMs treated with LPS plus IFN-γ.

The rat PAMs were incubated for 12 h with LPS plus IFN-γ in the presence of erythromycin (10⁻²–10⁻⁵ M), clarithromycin (10⁻³–10⁻⁴ M), josamycin (10⁻⁷–10⁻⁴ M), amoxycillin (10⁻⁴ M), cefaclor (10⁻⁵ M) or dexamethasone (10⁻⁷ M). As shown in figure 2d and figure 3, exposure of cells to erythromycin, clarithromycin or josamycin at 10⁻⁵ M reduced the electrical current induced by LPS plus IFN-γ by 73±10, 81±6%, or 84±9%, respectively (p<0.001, n=11 for each drug). Dexamethasone (10⁻⁷ M) likewise inhibited the production of NO by 96±4% (p<0.001, n=11), but amoxycillin and cefaclor at 10⁻⁴ M had no effect. The inhibitory effects of the macrolide antibiotics on NO release were dose-dependent, the concentra-
CCL

PAMs are induced by LPS and IFN-γ, expression of the type II NOS gene and release of NO by rat e.g., in the respiratory tract. Inhibition of NO generation by macrolides might be effects can be inhibited by macrolide antibiotics. The present in vitro studies demonstrate that the expression of the type II NOS gene and release of NO by rat PAMs are induced by LPS and IFN-γ, and that these effects can be inhibited by macrolide antibiotics. The inhibition of NO generation by macrolides might be associated with their anti-inflammatory actions in the respiratory tract.

Based on molecular cloning and sequencing analyses, there are at least three main isoforms of NOS [1]: type I (primarily soluble, e.g., in brain); type II (primarily soluble, e.g. in macrophages) and type III (primarily particulate, e.g. in endothelium). Compared with type I and type III NOS, which require Ca²⁺ and calmodulin for enzyme activation, type II NOS may spontaneously synthesize and release NO, once it is induced [23]. However, it has not been known how much NO is released from type II NOS-containing cells. In this study, NO release from rat PAMs was measured using an NO-selective electrode [16, 24]. Even when the electrode was immersed in medium containing PAMs which had been incubated with LPS and IFN-γ, no current was detected. Although the concentration of L-arginine in the medium after the 12-h incubation period was not measured, the failure to detect NO might be due to depletion of L-arginine, a substrate of NOS, during the incubation, since addition of L-arginine caused an increase in the NO-selective current. Indeed, Jorens et al. [25] showed that the production of nitrite by rat PAMs required the presence of L-arginine in the culture medium. It was also found that the subsequent addition of aminoguanidine, a specific inhibitor of type
Figure 6. Effects of antibiotics and a steroid on type II nitric oxide synthase (NOS) messenger ribonucleic acid (mRNA) expression by rat pulmonary alveolar macrophages (PAMs) stimulated with lipopolysaccharide (LPS, 50 ng/mL) and interferon gamma (IFN-γ, 50 U/mL) (stim). The PAMs were incubated in the absence and presence of clarithromycin (CAM, 10⁻⁷–10⁻⁴ M), erythromycin (EM, 10⁻⁴ M), josamycin (JM, 10⁻⁷ M), amoxicillin (AM, 10⁻⁷ M), cefaclor (CCL, 10⁻⁴ M) or dexamethasone (DEX, 10⁻⁷ M). The mRNA levels of independent experiments were quantified by densitometric scanning, normalized for the corresponding β-actin signals and expressed as a percentage of those obtained for LPS plus IFN-γ. Data are presented as mean±SEM; n=4-6. ∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001 versus LPS plus IFN-γ alone.

II NOS [20], reduced the current, indicating that the observed production of NO was type II NOS-mediated. This notion was further confirmed by the corresponding induction of type II NOS mRNA by stimulation with LPS plus IFN-γ.

In contrast to rat PAMs, the existence of type II NOS in human PAMs has been controversial. Although there is indirect evidence for the expression of type II NOS in human PAMs [26], many laboratories have had difficulties in inducing human PAMs to express this protein in vitro [27, 28]. Recent studies have shown that strong immunoreactivity can be detected with the anti-type II NOS antibody in PAMs from patients with bronchiectasis, acute bronchopneumonia and pulmonary tuberculosis but not from normal subjects [29, 30]. These data indicate that human PAMs are also capable of expressing type II NOS, and probably of producing NO under certain conditions. There is evidence that the concentration of NO in exhaled air is increased in patients with asthma [9] and bronchiectasis [11], and that long-term administration of erythromycin or clarithromycin may be clinically effective in the treatment of chronic bronchitis, bronchiectasis and diffuse panbronchiolitis [12, 13]. The efficacy of these macrolide antibiotics may not be accounted for by their antimicrobial properties but rather by antisecretory and/or anti-inflammatory actions. For example, macrolides inhibit the accumulation of neutrophils in the airways [31], generation of reactive oxygen species by neutrophils [32], release of tumour necrosis factor-α from PAMs [33] and interleukins-6 and 8 from bronchial epithelium [34] and expression of endothelin-1 in bronchial epithelial cells [35]. In addition, the present experiments show that macrolides decrease NO production from PAMs. These effects may lead to the suppression of airway inflammatory responses. In fact, SATO et al. [36] have recently shown that erythromycin attenuates virus-induced injury in mice by inhibiting inflammatory cell responses and suppressing overproduction of NO in the lung.

In the present study, coincubation of PAMs with the 14-membered ring macrolides, erythromycin and clarithromycin, and the 16-membered ring macrolide, josamycin, inhibited the expression of type II NOS mRNA induced by LPS plus IFN-γ, and the resultant release of NO into the incubation medium. Conversely, amoxicillin and cefaclor, the penicillin and cephalosporin antibiotics, respectively, had no such effects even at high concentration. Thus, the inhibitory effects of antibiotics on the NO-generating system appear to be specific to the macrolides. Moreover, the potency of the inhibition of type II NOS gene expression and NO release was not different between macrolides, suggesting that these drugs possess suppressive actions against NO production regardless of the structure of the lactone ring.

The subcellular mechanism of macrolide action on type II NOS gene expression remains unknown. Recent studies indicate that nuclear factor-kB (NF-kB) is one of the most important nuclear proteins involved in the cytokine-induced transcriptional induction of the type II NOS gene in various cell types including macrophages [37, 38], and that promoter regions of the rat type II NOS gene contain NF-kB-binding sites [39]. Thus, further studies are required to determine whether NF-kB is the target molecule for macrolides in the signal transduction pathway.

In conclusion, the expression of type II NO synthase messenger ribonucleic acid was assessed and the NO release from rat pulmonary alveolar macrophages directly measured, and it was found that macrolide antibiotics inhibited type II NO synthase gene expression and the consequent production of NO induced by lipopolysaccharide plus interferon gamma. Therefore, the inhibitory actions of macrolides on the NO-generating system might reflect the efficacy of the drugs in the treatment of airway inflammatory diseases. However, it is also possible that the reduced NO generation could be detrimental to an important host defence in the lung [40].

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References