Abstract. The present study investigated whether erythromycin (ERY) reduces cigarette smoke (CS)-induced emphysema in rats and aimed to determine the anti-inflammatory effect of ERY, which may identify potential treatments for chronic obstructive pulmonary disease. Furthermore, the current study focused on the potential effects on the imbalance between matrix metalloprotease (MMP) and anti-MMP activity, the phosphorylation of mitogen-activated protein kinases (MAPKs) and the nuclear factor-κB (NF-κB) signaling pathway. Wistar rats were divided into the following three groups (n=12 each): control (ERY vehicle only, without any CS exposure), CS (animals were exposed to CS for 12 weeks) and CS + ERY (animals were exposed to CS for 12 weeks and received 100 mg/kg/day ERY). The recruitment of inflammatory cells into the bronchoalveolar lavage fluid (BALF) and the histopathology of lung tissue from all groups was evaluated to grade the severity of the emphysema. The expression of MMP-2, MMP-9 and tissue inhibitor of metalloproteinase-1 was evaluated by immunohistochemistry and western blotting. The activation of MAPKs, NF-κB and inhibitor of NF-κB (IκBα), in lung tissues was examined by western blotting. Treatment with ERY resulted in fewer inflammatory cells and cytokines in the BALF, and fewer emphysema-associated changes in the lungs compared with control. The stimulus of CS promoted the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and p38, but not c-Jun NH2-terminal kinase, thereby inducing the activation of the ERK/MAPK signaling pathway in rats. Furthermore, CS exposure increased the expression of NF-κB and decreased the expression of IκBα. The levels of phosphorylated ERK1/2 and p38 were significantly reduced in rats with CS-induced emphysema when treated with ERY compared with the CS group. The results of the present study therefore indicate that oral administration of ERY may suppress CS-induced emphysema by regulating inflammatory cytokines and the MMP/anti-MMP imbalance via the MAPK/NF-κB pathway.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most significant chronic conditions and is now the fourth most common cause of death worldwide, which has an increasing economic and social burden (1,2). COPD affects >300 million people worldwide and accounts for 4 million deaths every year (3). However, unlike heart disease and malignancy, mortality due to COPD has increased progressively; without effective intervention, the number of deaths caused by COPD is predicted to increase by >30% between 2010 and 2020 (4). Smoking, the major risk factor for COPD (5), is a powerful inducer of inflammatory mediators, including oxidants and proteases (6). Emphysema is a characteristic of COPD, which is caused by chronic inflammation in the lung that contributes to lung tissue destruction and is mediated by a multistep pathway, which involves an imbalance of metalloproteases, including matrix metalloproteases (MMPs) and ADAM metallopeptidases, and anti-metalloproteases, which include tissue inhibitor of metalloproteinases (TIMPs) and α-2 macroglobulin. This imbalance leads to MMP overproduction that is not sufficiently counteracted by TIMPs (7,8). The imbalance of the metalloproteases and anti-metalloproteases may be a potential target for the treatment of the chronic...
inflammation and emphysema caused by cigarette smoke (CS). The term 'macrolide' is used to describe drugs with a macrocyclic lactone ring of ≥12 elements (9,10). In addition to antimicrobial properties, other properties of macrolides were suggested in the 1960s, particularly in chronic inflammatory diseases, and these actions are observed only for treatment with 14- and 15-membered macrolides, including erythromycin (ERY), clarithromycin, roxithromycin and azithromycin (11). The effects of macrolides in patients with chronic inflammatory airway disease appear to be independent of antimicrobial properties. A previous report demonstrated the anti-inflammatory effect of macrolides in CS-induced lung inflammation and emphysema, and also reported the effect of ERY on the imbalance of metalloproteases/anti-metalloproteases (12). However, the potential mechanism of ERY in the inhibition of the inflammation in CS-induced emphysema has not been clarified. Central to the inflammatory response induced by CS is an increased level of chemotactic cytokines, particularly interleukin (IL)-8. It is associated with an increase in the activation of intracellular signaling molecules, including mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) (13,14). MAPKs, together with NF-κB activation, are the most activated kinases in airway epithelial cells and macrophages exposed to CS extracts, and these kinases are important in inducing the expression of various pro-inflammatory chemokines and cytokines, and inducing the activation of cytosolic phospholipase A2 (15). The present study evaluated the imbalance of metalloproteases/anti-metalloproteases in CS-induced emphysema and investigated the upstream signaling that altered metalloprotease/anti-metalloprotease levels, via phosphorylation of the MAPK family and NF-κB overactivation. The current study also investigated whether ERY affects the metalloprotease/anti-metalloprotease imbalance in a dose-dependent manner, and if so, whether it does so by affecting signaling pathways dependent on MAPKs or NF-κB.

Materials and methods

Animal model for cigarette smoke exposure. All of the experiments were conducted in accordance with the guidelines of the Research Ethics Committee of China Medical University (Shenyang, China). Male Wistar rat weanlings (6-7-weeks-old; 70-210 g) were obtained from the Laboratory Animal Center of China Medical University and were maintained in a pathogen-free environment. They were fed laboratory rodent chow and water ad libitum and were kept on a 12 h light/dark cycle. Rats were randomly assigned into the following three groups: control (n=12), CS exposure (n=12) and CS+ERY (n=12). Rats in the CS group were exposed to smoke from 16 commercial cigarettes (Marlboro; Philip Morris USA, New York, NY, USA; each cigarette contains 0.9 mg nicotine, 14 mg carbon monoxide and 12 mg tar oil; the smokescope v/V% was 8%) with the filters removed for 30 min twice a day, 6 days per week for 12 weeks. The rats in the ERY group were also exposed to CS for 12 weeks and treated with an orally administered injection of ERY (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 100 mg/kg, once daily. The drug treatments were performed 0.5 h prior to CS exposure from day 1 of the 12-week exposure period. The control group received a daily orally administered injection of saline, which was the vehicle for ERY, without any smoke exposure. At the end of week 12, rats were sacrificed by exsanguination under anesthesia (40 mg/kg pentobarbital intraperitoneally; Sigma-Aldrich; Merck KGaA) and lungs were excised. The right upper lobes were taken and stored at -80°C. The middle lobes of the right rat lungs, which were not lavaged, were briefly washed in ice-cold saline containing 5 mM CaCl2+1 mM MgCl2, fixed in ExCell Plus™ fixative (American MasterTech Scientific, Inc., Lodri, CA, USA) at room temperature for 24 h. Subsequently, the middle lobes of the right rat lungs were embedded in paraffin blocks and sectioned (4 μm) for conventional hematoxylin and eosin staining. The left lungs were infused with 2 ml PBS four times. The bronchoalveolar lavage fluid (BALF) was centrifuged for 10 min at 900 X g and 4°C. The cell-free supernatants were stored at -80°C.

Morphologic and morphometric analyses of lung tissue. Hematoxylin and eosin staining was performed on paraffin-embedded lung tissue sections (4 μm). The measure of lung tissue morphology was determined by light microscopy at a magnification of x100. The mean linear intercept (MLI) (16), a measure of inter-alveolar wall distance, was defined by the total length of the cross-line/the numbers of the alveolar walls intersecting the test lines. The mean alveolar number (MAN) (16), an indicator of alveolar density, was calculated by counting the numbers of alveoli in each field. Five sections were analyzed per animal, and four images were acquired from a randomly selected location in each slide.

Measurement of IL-8 and leukotriene B4 (LTB4). The protein expression of IL-8 and LTB4-α in the BALF supernatant of rats in each group was determined by ELISA kits purchased from EnzoLife Sciences, Inc. (Farmingdale, NY, USA; cat nos. ADI-900-074 and ADI-900-068, respectively), according to the manufacturer's instructions.

Characterization of inflammatory cells in BALF. The collected BALF samples from the left lung tissues were centrifuged at 1,400 X g for 10 min at 4°C, and their supernatants were stored at -80°C until ELISA analysis. The pelleted cells were resuspended in PBS, and 1x10⁵ cells were subjected to cytoospin centrifugation on glass slides and fixed with 100% methanol for 15 min, followed by staining with May-Grünwald-Giemsa solution for 15 min. A differential cell count of BALF cell pellets was performed under a light microscope at a magnification of x200 according to morphological characteristics.

Measurement of MMP-2, MMP-9 and TIMP-1. The right upper lobes of lungs were lysed by RIPA buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) at a ratio of 100 mg of tissue to 1 ml of lysis buffer. This was followed by centrifugation at 1,500 X g for 20 min at 4°C. Total protein concentration was determined using the Bradford assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and equal amounts of protein (30 μg) were separated by SDS-PAGE (4% stacking gel and 10% separating gel). Protein was subsequently transferred onto a nitrocellulose membrane by electroblotting. The membrane was blocked for 1 h at room temperature with...
blocking buffer [3% non-fat milk in TBS (pH 7.4) containing 0.1% Tween-20] and was subsequently incubated overnight at 4°C with mouse antibodies against MMP-2 (1:500; cat. no. ab7033; Santa Cruz Biotechnology, Inc.), MMP-9 (1:500; cat. no. ab38898), TIMP-1 (1:500; cat. no. ab61224) and β-actin (1:2,000; cat. no. ab6276; all from Abcam, Cambridge, UK). The membrane was washed three times and incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (IgG; 1:5,000; cat. nos. sc-516102 and sc-2357, respectively; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The bound secondary antibody was detected using the Enhanced Chemiluminescence Plus kit (GE Healthcare Life Sciences, Chalfont, UK) and analyzed using the Las-3000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). The band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The fold changes in protein expression were calculated relative to the levels in the control group. The average values for each group were determined by 3 independent experiments. Lung tissues were sectioned (4 µm), cleared of paraffin and endogenous peroxidases were blocked by incubation with 3% H2O2 for 10 min at room temperature, followed by three washes with PBS for 3 min. Tissue sections were subsequently incubated with rabbit serum (Sigma-Aldrich; Merck KGaA) for 10 min at ambient temperature. Sections were incubated overnight with mouse MMP-2 (1:50; cat. no. ab7033; Abcam), mouse MMP-9 (1:50; cat. no. ab38898; Abcam) or rabbit TIMP-1 (1:50; cat. no. ab61224; Abcam) antibodies at 4°C, which was followed by the addition of biotinylated rabbit anti-mouse (1:2,000; cat. PA1-28567; Thermo Fisher Scientific, Inc.) or mouse anti-rabbit and anti-mouse IgG secondary antibody (1:2,000; cat. nos. sc-2357 and sc-516102; Santa Cruz Biotechnology, Inc.). To verify the binding specificity for MMP-2, MMP-9 and TIMP-1, certain sections were incubated with only a primary or secondary antibody. In these situations, no positive staining was identified in the sections, indicating that the immunoreactions were positive in all of the experiments. Immunohistochemistry staining was performed in accordance with the manufacturer’s instructions and visualized by the use of diaminobenzidine staining. Digital photos were analyzed with Image-Pro Plus 4.1 (MediaCybernetics, Inc., Rockville, MD, USA) by an observer who was blind to the group classification.

Measurement of phosphorylated (p)-MAPK and NF-κB. Total protein was extracted from the upper right lobes of the lungs by lysis with RIPA buffer (100 mg of tissue to 1 ml of lysis buffer). This was followed by centrifugation at 1,500 x g for 20 min at 4°C. The protein concentration was determined using the Bradford assay and equal amounts of protein (50 µg) were separated by SDS-PAGE (4% stacking gel and 10% separating gel) for detection of extracellular signal-regulated kinase (ERK)1/2, c-Jun NH2-terminal kinase (JNK) and p38. Following electrophoresis and electrophoretic transfer of proteins to nitrocellulose membranes, membranes were blocked with 3% non-fat milk in TBS (pH 7.4) containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then rinsed three times in TBST and incubated at room temperature with mouse monoclonal antibodies against activated diphosphorylated ERK1/2 (1:500; cat. no. sc-81492; Santa Cruz Biotechnology, Inc.), p-JNK (1:500; cat. no. sc-293137; Santa Cruz Biotechnology, Inc.) and p-p38 (1:500; cat. no. sc-17852-R; Santa Cruz Biotechnology, Inc.) for 1 h. β-actin levels were also assessed as a loading control using a mouse β-actin antibody (1:2,000; cat. no. ab6276; Abcam). The blots were processed as described for the measurement of MMP-2, MMP-9 and TIMP-1. To assess the activation of NF-κB, nuclear extracts were analyzed by western blot analysis for the p65 fraction of NF-κB and the NF-κB inhibitor α (IκBα). Nuclear extracts were isolated as previously described (17). The protein concentration was determined using the Bradford assay and equal amounts of protein (30 µg) were separated by SDS-PAGE (4% stacking gel and 10% separating gel). NF-κB was detected with NF-κB p65 rabbit polyclonal antibody (1:500; cat. no. ab16502; Abcam) and IκBα rabbit polyclonal antibody (1:500; cat. no. ab7217; Abcam), β-actin (1:2,000) served as the control. The blots were processed as described for the measurement of MMP-2, MMP-9 and TIMP-1.

Statistical analysis. Statistical analyses were performed with SPSS 11.5 (SPSS, Inc., Chicago, IL, USA). All data were expressed as the mean ± standard deviation. Differences between multiple groups were compared with a one-way analysis of variance. Differences between two groups were tested for significance by the least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of ERY on CS-induced lung damage. Histopathological studies were performed to assess the morphological damage caused by CS and the effect of ERY treatment. To investigate the effect of ERY on the emphysema induced by CS, MLI and MAN were measured. The control group demonstrated no evident histological changes; the structure of the alveoli was intact with regular ciliary arrangement in the control group (Fig. 1A). Compared with the morphology of the control group, CS induced significant morphological changes characterized by conspicuous inflammation accompanied with focal emphysema; the alveolar walls were broken and merged, and the cavities of the alveoli were enlarged (Fig. 1A). MLI was significantly increased (P<0.01; Fig. 1B), and MAN was significantly lower (P<0.01; Fig. 1B) in the CS group compared with the control group. ERY demonstrated a protective effect on emphysematous changes induced by CS, with a significantly reduced MLI (P<0.05 vs. the CS group; Fig. 1B) and a significantly higher MAN (P<0.05 vs. the CS group; Fig. 1B) in the CS + ERY group compared with the CS group. Compared with the morphological changes in the CS group, the structural damage induced by CS was partially recovered in the CS + ERY group (Fig. 1A); however, the MLI remained higher than in the control group (P<0.01; Fig. 1B) and MAN remained lower that the control group (P<0.01; Fig. 1B).

ERY reduces the number of inflammatory cells, and the levels of IL-8 and LTB4 present in the BALF following CS exposure. As presented in Table I, CS significantly increased the number of neutrophils (P<0.01) and lymphocytes (P<0.01), and significantly decreased the number of macrophages (P<0.01) compared with the control group. Additionally, as presented in Fig. 2, rats in the CS group had significantly increased levels of...
IL-8 (P<0.01) and LTB4 (P<0.01) compared with the control group. Compared with the CS group, ERY treatment led to a significant reduction in the total cell count (P<0.05; Table I), the relative count of neutrophils (P<0.01; Table I) and lymphocytes (P<0.01; Table I), and a significant reduction in the levels of IL-8 (P<0.01) and LTB4 (P<0.01; Fig. 2), and increased the macrophage count (P<0.05; Table I).

**Effects of ERY on lung MMP-2, MMP-9 and TIMP-1 protein expression.** To evaluate the effect of ERY on the regulation of the imbalance of metalloproteases/anti-metalloproteases, the present study performed immunohistochemical staining (Fig. 3A) and western blotting for MMP-2, MMP-9 and TIMP-1 (Fig. 3B). The protein expression levels of MMP-2 and MMP-9 in the CS group were significantly increased compared with

Table I. Cytological count in bronchoalveolar lavage fluid of rats from different groups.

| Group   | Total cell count (x10⁶/l) | Neutrophil (%) | Lymphocyte (%) | Macrophage (%) |
|---------|--------------------------|----------------|----------------|----------------|
| Control | 4.20±2.41                | 3.02±1.96      | 3.96±2.02      | 86.52±12.49    |
| CS      | 14.6±4.37                | 21.64±10.73    | 8.38±4.82      | 63.73±12.55    |
| ERY     | 10.3±3.67                | 12.74±5.16     | 10.54±5.52     | 73.65±15.69    |

*P<0.05 and *P<0.01 vs. control group; †P<0.05 and ‡P<0.01 vs. the CS group. Data are presented as the mean ± standard deviation (n=12).

Control rats were exposed to normal air and received daily saline injections. CS rats were exposed to CS for 12 weeks. CS + ERY rats were exposed to CS for 12 weeks and received daily injections of 100 mg/kg ERY. CS, cigarette smoke; ERY, erythromycin.
the control group (P<0.05 and P<0.05, respectively; Fig. 3B), while the level of TIMP-1 was significantly reduced compared with the control group (P<0.05; Fig. 3B). While the expression of MMP-2 and MMP-9 were reduced significantly by ERY treatment compared with the CS group (P<0.05 and P<0.05, respectively; Fig. 3B), the expression of TIMP-1 was significantly increased by ERY treatment compared with the CS group (P<0.05; Fig. 3B). The ratio of MMP-9/TIMP-1 was increased significantly following CS exposure compared with the control group (P<0.05; Fig. 3B) but significantly reduced in the CS + ERY group compared with the CS group (P<0.05; Fig. 3B).

**Effect of ERY on NF-κB activation.** To investigate the mechanism of ERY-mediated regulation of NF-κB in vivo, the present study investigated the effect of ERY on IκBα (Fig. 4). Compared with the control group, the level of IκBα was significantly decreased in the CS group (P<0.05; Fig. 4), while treatment with ERY increased the expression of IκBα compared with the CS group (P<0.05; Fig. 4). Thus, this indicated that CS-induced IκBα degradation was significantly blocked by treatment with ERY. Additionally, the activation of NF-κB p65 was decreased significantly in the CS + ERY group compared with the CS group (P<0.05; Fig. 4).

**Effect of ERY on MAPK pathway activation.** The levels of phosphorylated MAPKs were measured by western blot analysis (Fig. 5). Total protein levels of MAPKs were used as controls for potential fluctuations in the overall protein levels. ERK1/2 and p38 phosphorylation was significantly increased in the CS group compared with the control group (P<0.05 and P<0.05, respectively; Fig. 5), butp38 and ERK1/2phosphorylationwas significantly reduced in the CS + ERY group compared with the CS group (P<0.05; Fig. 5). No significant changes in JNK phosphorylation levels in the CS group and CS + ERY group compared with those in the control group (Fig. 5).

**Discussion**

COPD is a lung disease characterized by progressive airflow limitation due to the inflammation-driven destruction of
The increased expression of MMPs is considered to be a key factor in the development of COPD and emphysema (8,19). Because CS exposure is the biggest risk factor for the development of COPD, the present study aimed to investigate the effect of CS on the imbalance of metalloproteases/anti-metalloproteases, and the effect of ERY on the destruction of lung tissue and the inhibition of the imbalance of metalloproteases/anti-metalloproteases. In the present study, the expression of MMP-2 and MMP-9 was demonstrated to be increased by stimulation with CS in a rat model of CS-induced emphysema, while TIMP-1 expression was decreased. Previous studies have demonstrated that the inhibition of MMPs by macrolides may reduce the extracellular spread of inflammation (9,20). In the current study, the imbalance of metalloproteases/anti-metalloproteases was accompanied by altered levels of inflammatory cells and cytokines in BALF, while ERY reduced the imbalance of metalloproteases/anti-metalloproteases and the changes in the inflammatory cells and cytokines in BALF to some extent, which was also associated with the amelioration of the emphysema. The potential signaling pathway of ERY in the inhibition of the inflammation in CS-induced emphysema has not been previously clarified. One previous study demonstrated the protective role of ERY on CS-induced emphysema and its involvement in the reduction of inflammation, the imbalance of MMP-9/TIMP-1 and the apoptosis of lung structural cells (7). The results of the present study provided novel evidence for the protective effect of ERY on the development of emphysema induced by CS via regulation of the expression of MMP-2, MMP-9 and TIMP-1, and improvement of the inflammation of the lungs via MAPK/NF-κB activation, thus indicating its potential use as a therapeutic agent for the treatment of COPD. Previous studies have demonstrated that NF-κB is regulated by macrolides (27-29). For example, azithromycin inhibits pro-inflammatory mediators, including CXCL-8, via inhibition of NF-κB in in vitro models (28,29). Solithromycin, another macrolide, inhibits activation of NF-κB under oxidative stress (30). Macrolides suppress the degradation of IkBα, an inhibitor of NF-κB, and/or affect the downstream dissociation from IkBα in the NF-κB signaling pathway (31). In the
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2. The results of the present study do not conclusively demonstrate whether MAPK and NF-κB are upstream or downstream of the signaling pathway. However, the changes identified in the present study indicated that there may be an association between the amelioration of emphysema when the emphysema model was treated with ERY and the changes in MAPK and NF-κB. In a previous study of acute and chronic CS exposure, the activation and expression of p38 MAPK in the lungs were compared between the following two mouse strains: C57BL/6 emphysema-susceptible mice and NZW emphysema-resistant mice: the selective p38 MAPK inhibitor, SB203580, ameliorated CS-induced lung inflammation and injury (32). ERY and clarithromycin inhibited CXCL-8 with inactivation of NF-κB and/or activator protein-1 in human bronchial epithelial cells in vitro (33,34). In one in vitro experiment, CS extract significantly induced ERK1/2 phosphorylation. PD98059, a specific inhibitor of ERK-MAPK, significantly blocked the effect of CS extract on ERK1/2 phosphorylation. Furthermore, PD98059 significantly inhibited the effect of CS extract on MMP-1 production and mRNA expression (35). These results indicate that CS may stimulate the production of and potentially activates MMP-1 through activation of the ERK1/2 signal transduction pathway. By inducing MMP-1, CS may result in excessive tissue destruction and may contribute to the development of emphysema (35).

In conclusion, CS may induce emphysema by simultaneously disturbing the balance of metalloproteases/anti-metalloproteases, increasing airway inflammation and upregulating MAPKs together with increased NF-κB activation signaling. Treatment with ERY reduced the development of emphysema via inhibition of the phosphorylation of MAPKs and the activation of NF-κB. These results revealed the anti-inflammatory role of ERY in CS-induced emphysema and airway inflammation, indicating that macrolides may have therapeutic potential for chronic airway inflammation and the associated emphysema caused by exposure to CS.

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