Erythromycin Regulates Cigarette Smoke-Induced Proinflammatory Mediator Release Through Sirtuin 1-Nuclear Factor κ B Axis in Macrophages and Mice Lungs

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Keywords

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Abstract

Background: Macrolides have anti-inflammatory and anti-oxidative stress function, but their pharmacological regulation remains unclear. Sirtuin 1 (SIRT1) is redox-sensitive protein belongs to class III histone/protein deacetylases, SIRT1 regulates the acetylation/expression of nuclear factor κ B (NF- κ B) and is involved in the airway inflammation of chronic obstructive pulmonary disease. **Objectives:** The present study was designed to examine the effects of erythromycin (EM) on the SIRT1-NF- κ B axis and NF- κ B-dependent proinflammatory cytokines. **Methods:** Human macrophages were preincubated with EM and then treated with cigarette smoke extract (CSE). The mice were treated by injecting drugs to gastric with EM before cigarette smoke exposure. Reactive oxygen species (ROS) released by treated human macro-

phages were detected using flow cytometry. The expression of SIRT1 and NF- κ B was analyzed by western blotting. SIRT1 and the RelA/p65 subunits of NF- κ B interaction were detected by coimmunoprecipitation. We found that EM suppressed CSE-induced ROS released in human macrophages, which coincided with increases in SIRT1 protein expression in the macrophages and lungs of mice, resulting in suppressed NF- κ B acetylation and expression correlated with a reduction of inflammatory mediators. **Conclusion:** These findings suggest that EM increased SIRT1, leading to acetylation/expression of NF- κ B, and thereby decreasing cigarette smoke-driven NF- κ B-dependent proinflammatory cytokine.

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Introduction

There is increasing research proving the effects of macrolide therapy for chronic obstructive pulmonary disease (COPD) [1, 2]. Long-term treatment with macrolide antibiotics such as erythromycin (EM) reduces

COPD exacerbation [2–4]. Macrolides have anti-inflammatory effects [2, 5, 6]. Treatment with macrolide antibiotics is also used for cystic fibrosis and diffuse panbronchiolitis [7–9]. Macrolides demonstrate several immunomodulatory activities in vivo, including downregulation of inflammation, decreased production of reactive oxygen species (ROS), and inhibition of nuclear transcription factor activation [8, 10]. However, the molecular mechanism underlying the anti-inflammatory and anti-oxidative stress action of macrolides remains to be determined.

COPD is characterized by a progressive decline in lung function and chronic inflammatory responses [11]. Cigarette smoking is the major factor for the ongoing inflammation in the airways and lung parenchyma, and the severity of airflow limitation is correlated with the degree of pulmonary inflammation [12, 13]. Previous studies have demonstrated that one of the major etiologic factors driving inflammatory responses is oxidative stress in long-term exposure to cigarette smoke [14, 15]. Oxidative stress can activate the nuclear factor κ B (NF- κ B) pathway at many levels and NF- κ B expression and activation is increased in COPD and correlates with increased release of NF- κ B-dependent proinflammatory cytokines [16–18].

Sirtuins belong to class III histone/protein deacetylases (HDACs) and are members of the silent information regulator 2 family [19]. In humans, there are 7 members of this family of deacetylases (Sirtuin 1 [SIRT1]–7) [20]. There is increasing interest in investigating the role of SIRT1 in inflammation and stress responses [19–22]. SIRT1 is a NAD⁺-dependent HDAC essential for maintaining silent chromatin via the deacetylation of histones and regulating gene transcription and protein functions [21]. Increasing evidence indicates that SIRT1 is a link between oxidative stress, inflammation, and cell aging/senescence, all processes that are characteristic of COPD phenotypes, especially COPD/emphysema [13, 22]. Previous studies indicate that SIRT1 can directly bind to NF- κ B and deactivates NF- κ B RelA/p65 through deacetylation and proteasome degradation [23, 24]. In the lung tissues of chronic smokers and COPD patients, SIRT1 expression is reduced by cigarette smoke [25]. Decreased SIRT1 expression is associated with increased NF- κ B activation and the release of NF- κ B-dependent proinflammatory cytokines, consequently promoting the inflammatory reaction [25–27].

Macrophages are derived from monocytes and are considered to be important cells in perpetuating the inflammatory response of CS and play a key role in orches-

trating chronic inflammation in patients with COPD [25]. Our previous studies showed that EM upregulated the expression of HDAC, resulting in decreased cigarette smoke-driven NF- κ B activation and NF- κ B-dependent proinflammatory cytokines in human macrophages [9]. However, the precise molecular mechanisms of EM anti-inflammatory and anti-oxidative stress remain unclear. This study was designed to identify the effects of EM on the SIRT1-NF- κ B axis in human macrophages and the lungs of mice. The role of EM in the regulation of NF- κ B-dependent proinflammatory cytokines (TNF- α and IL-6 release) in human macrophage cells was also investigated. Our results provide a basis for the future development of EM as an effective treatment for COPD.

Materials and Methods

Cigarette Smoke Exposure in Mice

Specific pathogen-free inbred male BALB/c mice (8 weeks of age, 20–25 g body weight) were purchased from the Guangxi Medical University Laboratory Animal Center (Nanning, China). This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, National Research Council, 2011). All methods and experimental protocols in the present program were also approved by the laboratory animals ethics committee of Guangxi Medical University (the approval number: No. 20140403). All mice were housed in sterilized cages and maintained on a 12:12 h light:dark cycle and received sterilized food and water ad libitum.

Mice were randomly divided into 3 groups, 6 in each group. Briefly, in CS-exposed group, the mice were exposed whole body to 5 cigarettes (Nanning Zhenlong unfiltered cigarettes: 10 mg of tar and 0.9 mg of nicotine) 4 times a day with 30-min smoke-free intervals in a closed 0.75 m³ room 5 days a week for 24 weeks. The control groups (air-exposed mice) were exposed to 24 weeks of air. In the EM treatment group, mice were treated by injecting drugs to gastric with EM (100 mg kg⁻¹/day) 1 h before the cigarette smoke exposure [28].

Process of Bronchoalveolar Lavage Fluid

The mice were sacrificed with sodium pentobarbital anesthesia 24 h after the last air or smoke exposure. Their lungs were lavaged via a cannula inserted into the trachea, and the left lobe was tied off and then instilled with 1 mL aliquots of saline. The first and second aliquots were combined and centrifuged, and the cell-free supernatants were frozen for later analyses. One part of the right lungs (30–50 mg) was homogenized for western blotting after lavaging.

Tissue Processing for Morphological

The left lungs were inflated by instilling 10% formalin at a constant pressure of 25 cm H₂O (for 10 min) and then ligated and removed. Inflated lungs were fixed for 24 h before embedding in paraffin. After paraffin embedding, 5- μ m sections were cut and stained with hematoxylin and eosin for histological analysis. For each animal, 10 fields at a magnification of 200 $\times$ were captured

randomly from the 4 different zones of the left lung. We determined the enlargement of alveolar spaces by mean linear intercept (Lm) in the lungs of mice as described previously. Two investigators independently measured Lm in a blinded manner.

Cell Lines and Cell Culture

The human monocytic cell line U937 [29, 30] obtained from ATCC (CRL-1593.2; Shanghai, China) was grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Excell Bio, Shanghai, China), 2 mM L-glutamine, 100 µg/mL penicillin, 100 U/mL streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, 1 µg/mL human holotransferrin, and 1 mM oxaloacetic acid. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. The U937 cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (10 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), according to standard procedures [31]. Cell differentiation and adherence were assessed using light microscopy. In most cases, over 85% of the total cell population adhered to the surface with a distinctive altered morphology (macrophage like).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay

The effects of cigarette smoke extract (CSE) and EM on cell viability were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as previously described [9]. The human macrophage cells were cultured in 96-well plastic plates at a density of 5,000 cells/well at 37 °C, 5% CO₂, and 96% air saturation for 24–72 h in the presence or absence of CSE (0.1, 1, and 5%) or EM (0.1, 1, and 20 µg/mL). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (20 µL/well and 5 mg/mL) was added, and the cells were cultured for 4 h. After media removal, 100% DMSO (200 µL) was added to dissolve the formed crystals. The optical densities at 570 nm were measured with a spectrophotometric microtiter plate reader (BioTek, ELX800, Highland Park, Winooski, VT, USA). The morphology of the cultured human macrophage cells was evaluated under a microscope (Nikon, TS100-F, Tokyo, Japan) throughout the course of the experiments.

Preparation of Aqueous CSE

Research-grade cigarettes were smoked to 0.5 cm above the filter in a fume hood using the methods developed by Carp [32] and Se-Ran [33] with a slight modification as previously described [34]. CSE (10%) was prepared by bubbling smoke from 2 cigarettes into 20 mL of serum-free RPMI at a rate of 1 cigarette/min. The pH of the RPMI was adjusted to 7.4, optical densities at 350 nm were determined (0.81 ± 0.03), and the media were sterile filtered with a 0.45-µm membrane filter (25-mm Acrodisc; Pall, Ann Arbor, MI, USA). The CSE was freshly prepared on the day of the experiment. The patterns of absorbance at 320 nm (spectrogram) showed few differences between each CSE preparation. We prepared control media by bubbling air through 20 mL of the serum-free RPMI, adjusting the pH to 7.4, and sterile filtering as described above.

CSE and EM Treatments

Differentiated U937 cells were seeded at a density of $1-2 \times 10^6$ cells/well in 6-well culture plates in a total final medium volume of 2 mL and grown in RPMI containing 10% FBS to ~80 to 90% confluence. The cells were washed in phosphate-buffered saline (PBS)

and then exposed to various treatments in media containing 1% FBS. All treatments were performed in duplicate. The cells were divided into several groups: CSE (treated with 1% CSE), EM (pre-treated with 1 µg/mL of EM for 24 h before treated with 1% CSE), pyrrolidine dithiocarbamate (PDTTC; treated with NF-κB inhibitor PDTTC, 10 nM of PDTTC for 24 h) [35], and NAM (nicotinamide, a SIRT1 inhibitor, 20 mM, for 24 h) [36]. The cells were cultured at 37 °C with 5% CO₂. After culture with the drugs, the cells were washed with cold PBS and then lysed to measure the SIRT1 levels. Culture media from these macrophages were collected for TNF-α, IL-6, and TNF-α assays and stored at -80 °C until use.

Measurement of ROS by Flow Cytometry

ROS were measured by flow cytometry using 5-(-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) dye. The cells were seeded at a density of 2.5×10^5 cells/well in 6-well culture plates in a final volume of 2 mL of RPMI with 1% FBS and were grown to ~80 to 90% confluency. Freshly prepared CSE was added, and the cells were incubated for 24 h, washed 3 times with prewarmed PBS, resuspended in 1 mL of PBS, and incubated with or without H2DCFDA (10 µM) for 0.5 h. The cells were washed and resuspended in PBS, and flow cytometric analysis was performed with an Elite flow cytometer (FACSCanto II, BD Bioscience, San Jose, CA, USA). Dead cells detected with propidium iodide (10 µg/mL) by forward and side scattering were gated out. A minimum of 10,000 events was acquired for each sample. The debris was gated out, and analyses were performed only on the live monocytic populations [37, 38].

Western Blotting Analysis for SIRT1 and NF-κB

Cellular proteins were extracted from the treated U937 cells with 10% Nonidet P-40 lysis buffer (RIPA buffer, #89900; Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with 2 protease inhibitor cocktails (PMSF, ST506; Biyuntian, Shanghai, China; phosphatase inhibitor cocktail, Cat. No. 040906845001; Roche, Mannheim, Germany) [39, 40]. The mice lungs were homogenized and treated with 10% Nonidet P-40 lysis buffer (RIPA buffer, #89900; Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with protease inhibitor cocktail (PMSF, ST506; Biyuntian, Shanghai, China). After 30 min of incubation under 4 °C, the cell lysates were centrifuged, and supernatants containing the proteins were collected. Protein quantitation was performed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Middlesex, MA, USA) following the manufacturer's instructions. Isolated soluble proteins (20 µg) were electrophoresed on 10% PAGE gels and electroblotted onto 0.45-µm PVDF membranes. The membranes were blocked with 5% non-fat dry milk for 1 h, incubated with Tris-buffered saline (TBS) with 0.1% Tween 20 for 1 h, and then incubated with rabbit polyclonal anti-human-SIRT1 (1:300 diluted in TBS) or anti-NF-κB (1:1,000 diluted in TBS) antibody for 24 h (SIRT1 rabbit mAb, D1D7, NF-κB rabbit mAb, D14E12; Cell Signaling Technology, Danvers, MA, USA) [41]. After washing with TBS, the SIRT1 and NF-κB protein expressions were detected with goat anti-rabbit antibody (goat anti-rabbit IgG-HRP: sc-200; Santa Cruz Biotechnology, Dallas, TX, USA), conjugated to horseradish peroxidase (1:10,000 diluted in TBS) for 1 h, and visualized with enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Product #34077; Thermo Fisher, Middlesex, MA, USA).

Fig. 1. Photomicrographs of hematoxylin and eosin-stained lung tissue of air-exposed (a), smoke-exposed (b), and EM-treated (c) mice at 24 weeks (200×). Smoke exposure significantly induced alveolar wall destruction and airspace enlargement in the mice. Treated with EM relieved this effect. **d** Destruction of the alveolar

walls was determined by measuring the MLI. Smoke exposure significantly increased the MLI of the mice compared to control group, while treated with EM decreased the MLI compared to the smoke group ($n = 6$, * $p < 0.05$ compared to the control values; # $p < 0.05$ compared to the smoke). EM, erythromycin.

Coimmunoprecipitation of Protein Complexes

For coimmunoprecipitation of endogenous protein complexes from whole-cell extracts, the cells were lysed with 10% Nonidet P-40 lysis buffer supplemented with a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin, and PMSF, phosphatase inhibitor cocktail; Roche, Mannheim, Germany) for 30 min on ice and centrifuged at 12,000 g for 5 min at 4 °C [23]. Cellular lysates were incubated with an anti-NF- κ B antibody (1:100 dilution; Cell Signaling Technology, Danvers, MA, USA) and incubated at 4 °C overnight, followed by incubation with protein A-Sepharose overnight at 4 °C. The immunoprecipitation complex was isolated by centrifugation at 10,000 g for 5 min at 4 °C, and the immunoprecipitated proteins were detected by western blotting [23, 41]. The levels of SIRT1 adducts with RelA/p65 NF- κ B were analyzed by immunoblotting with anti-SIRT1 antibody (SIRT1 rabbit mAb, D1D7; Cell Signaling Technology, Danvers, MA, USA), and a specific acetylated lysine 310 residue (K310) antibody (Acetyl-NF- κ B p65 [Lys310] Antibody, Cell Signaling Technology, Danvers, MA, USA) was analyzed for RelA/p65, respectively.

ELISA for IL-6 and TNF- α

Culture media were collected after treatments and centrifuged at 1,000 g for 15 min. The supernatants were collected and stored at -20 °C until analysis. IL-6 and TNF- α levels in the supernatants of macrophages and bronchoalveolar lavage fluid of mice were determined by sandwich ELISA [42, 43] using the respective dual antibody kits (IL-6 ELISA Kit, TNF- α ELISA Kit; Wuhan Huamei Systems, Wuhan, China) according to the manufacturer's instructions [44].

Statistical Analysis

The data are presented as means $\pm$ SEM, and statistical analysis was performed with SPSS 16.0 for PC (SPSS Inc., Chicago, IL, USA) using a one-way ANOVA followed by the Friedman test and the Wilcoxon signed-rank test for multiple comparisons. Statistical significance was assigned at $p < 0.05$.

Results

Photomicrographs of Hematoxylin and Eosin-Stained Mice's Lung Tissue

Photomicrographs of hematoxylin and eosin-stained lung tissue of air-exposed (Fig. 1a), smoke-exposed (Fig. 1b), and treated with EM (Fig. 1c) mice at 24 weeks (the cells were observed under an inverted microscope at a magnification of $\times 200$). Smoke exposure clearly induced alveolar wall destruction and airspace enlargement in mice. Alveolar enlargement was still seen in Figure 1c (EM group), but the extent of alveolar destruction was lower than in Figure 1b. Destruction of alveolar walls was determined by measuring the MLI (mean linear intercept). The MLI of the smoke group was significantly increased compared to the control group, and treated with

EM decreased the MLI (Fig. 1d, $n = 6$, $p < 0.05$). Data are shown as mean $\pm$ SEM. * $P < 0.05$ compared to the control values; # $p < 0.05$ compared to the smoke (Student t test).

Effects of EM on CSE-Induced SIRT1 and NF- κ B Protein Expression

The effects of EM on CSE-induced SIRT1 protein expression in the mice lungs and human macrophages were measured using Western blotting. Preincubated with EM (1 μ g/mL) for 24 h increased SIRT1 protein expression in human macrophages, as a negative control, NAM decreased SIRT1 protein expression (Fig. 2a, b; $n = 5$). Smoke decreased SIRT1 expression, while EM increased SIRT1 expression in the mice lungs (Fig. 2e, f; $n = 5$).

In human macrophages, treated with 1% CSE for 24 h increased NF- κ B protein expression. However, pretreatment with EM (1 μ g/mL) for 24 and 48 h attenuated CSE-induced NF- κ B protein expression. As a negative control, PDTC inhibited NF- κ B activation (Fig. 2c, d; $n = 5$). Our data also indicated that smoke increased NF- κ B expression and EM attenuated this effect in lung of mice (Fig. 2g, h; $n = 6$).

Further experiments were performed to determine whether EM regulates the acetylation of NF- κ B via SIRT1 by coimmunoprecipitation. CSE treatment decreased SIRT1, resulting in increased acetylation of RelA/p65 NF- κ B. EM significantly increased SIRT1 leading to decreased acetylation of RelA/p65 NF- κ B in human macrophages (Fig. 2i, j; $n = 3$). Data are shown as mean $\pm$ SEM. * $p < 0.05$ compared to the control values; # $p < 0.05$ compared to the smoke/CSE (Student t test).

Effect of EM, CSE on Cell Viability and ROS Generation

The human macrophage cells were exposed to 0.1, 1, and 20 μ g/mL EM for 24, 48, and 72 h. Human macrophage cell viability was decreased when the cells were treated with 20 μ g/mL EM for 24 h and 1 μ g/mL EM for 72 h. EM at a concentration of 0.1 μ g/mL for 24, 48, and 72 h or 1 μ g/mL for 24 and 48 h did not affect cell viability; at a concentration of 20 μ g/mL, human macrophage cell viability decreased significantly. Human macrophage cell viability was decreased when the cells were treated with 5% CSE for 24 h. CSE at a concentration of 0.1% for 24, 48, and 72 h or 1% for 24, 48, and 72 h did not affect cell viability; at a concentration of 5%, cell viability decreased significantly (Fig. 3a, $n = 5$).

We determined whether EM attenuated CSE-induced ROS generation by flow cytometry (Fig. 3b). Cells were seeded in 6-well plates (2.5×10^5 cells/well), preincubated

with EM (1 μ g/mL) for 24 or 48 h, and then treated with CSE (1%) for 24 h. The cells were washed with PBS and incubated with H2DCFDA for 30 min. The cell lysates were resuspended in PBS, and the ROS concentrations were measured using flow cytometry (B) [37, 45, 46]. Our data showed that CSE increased ROS production significantly, which was attenuated by EM (Fig. 3c, $n = 3$, * $p < 0.05$ compared to the control values; # $p < 0.05$ compared to 1% CSE), suggesting that EM inhibits CSE-induced ROS generation in vitro.

Effects of EM on the Release of CSE-Induced Inflammatory Mediators in Cultured Human Macrophages and Lung of Mice

We next determined the effects of EM on the release of CSE-induced inflammatory mediators in cultured human macrophages and lung of mice. We measured IL-6 and TNF- α the culture media using ELISA. Treated with 1% CSE for 24 h increased the release of these inflammatory mediators. However, preincubation with EM (1 μ g/mL) for 24 h inhibited CSE-mediated proinflammatory cytokine release. In the mice BAL, smoke increased TNF- α release and treated with EM significantly decreased smoke-driven TNF- α release (Fig. 4c).

Discussion

Macrolides exert immunodulatory and anti-inflammatory effects that may benefit patients with COPD [47]. Long-term use of macrolides or antibiotics can suppress bronchial colonization and consequent exacerbations [48]. In the clinic, EM treatment (250 mg twice daily for 1 year) leads to a significant reduction (35%) in the frequency of exacerbations compared to placebo [48]. Macrolides have various anti-inflammatory effects, including reducing the production of cytokines such as TNF- α and IL-6 [8, 17, 49], which are time- and dose dependent [8]. Our previous study shows that the mechanism of the anti-inflammatory effect of EM was related to the regulation of HDAC protein in macrophages, but the particular mechanism needs further study [9]. The present study focused on the role of SIRT1, which is a NAD⁺-dependent HDAC, that regulates proinflammatory cytokines, apoptosis, stress resistance, metabolism, senescence, differentiation, and aging, all of which are linked to COPD [50, 51]. NF- κ B protein is the master regulator of inflammation, and SIRT1 also controls inflammation through regulating NF- κ B signaling by deacetylating the p65 subunit of the complex, thereby inhibiting NF- κ B signaling [19].

Fig. 2. **a** Representative western blotting and analyses of SIRT1 protein levels in human macrophages. **b** Relative densities (% of control) of SIRT1 in human macrophages after treated with CSE or NAM for 24 h alone or in combination with EM pre-incubated. Treatment with 1% CSE for 24 h reduced SIRT1 protein expression. Pre-incubated with EM (1 μ g/mL) for 24 h increased SIRT1 protein expression. As a negative control, NAM decreased SIRT1 protein expression ($n = 5$). **c** Representative western blotting and analyses of NF- κ B in the mice lungs levels in human macrophages. **d** Relative densities (% of control) of NF- κ B in human macrophages after treated with CSE for 24 h alone or in combination with EM preincubated. Treated with 1% CSE for 24 h increased NF- κ B protein expression. Preincubated with EM (1 μ g/mL) for 24 h attenuated CSE-induced NF- κ B protein expression. As a negative control, PDTC decreased NF- κ B protein expression. Treated with 1% CSE and NAM 24 h in combination increased NF- κ B protein expression ($n = 3$). **e** Representative western blotting and analyses of SIRT1 protein levels in the mice lungs. **f** Relative densities (% of control) of SIRT1 in the mice lungs; smoke decreased SIRT1 expression and EM attenuated this effect ($n = 5$). **g** Representative western blotting of NF- κ B protein levels in the mice lungs. **h** NF- κ B protein levels and relative densities (% of control) of NF- κ B in the mice lungs; smoke increased NF- κ B expression and EM attenuated this effect ($n = 6$). **i** Representative of NF- κ B/SIRT1 protein bands. CSE (1%) significantly increased Ac-RelA/p65 NF- κ B levels in the SIRT1-Ac-RelA/p65 complex. EM pre-incubated (1 μ g/mL) for 24 h attenuated CSE-induced Ac-RelA/p65 expression. Equal amounts (30 μ g) of immunoprecipitated SIRT1 protein was used for western blotting. **j** Relative intensity (% of Ac-RelA/p65 NF- κ B) of SIRT1 protein bands. The data are presented as means $\pm$ SEM. * $p < 0.05$ versus control; # $p < 0.05$ versus Smoke/CSE (Student t test). SIRT1, Sirtuin 1; CSE, cigarette smoke extract; EM, erythromycin; PDTC, pyrrolidine dithiocarbamate; NF- κ B, nuclear factor κ B; Co-IP, co-immunoprecipitation.

Fig. 3. a Effects of CSE and EM on the growth of human macrophage cells. Proliferation levels of human macrophage cells were determined in relation to the growth curve based on the measurement of the OD at each time point ($n = 5$). **c** Treated with CSE significantly increased ROS generation; EM inhibited the CSE-induced ROS generation, especially at 48 h. NAM significantly in-

creased ROS generation. **b** Flow cytometry of ROS generation, by measuring FITC-A (fluorescein isothiocyanate-area) and SSC-A (side scatter-area). * $p < 0.05$ vs. CSE; # $p < 0.01$ vs. control. CSE, cigarette smoke extract; EM, erythromycin; ROS, reactive oxygen species.

Fig. 4. a, b Treated with 1% CSE for 24 h increased the release of these inflammatory mediators. Preincubation with EM (1 µg/mL) for 24 h inhibited CSE-mediated proinflammatory cytokine release ($n = 5$). The data are presented as means $\pm$ SEM, * $p < 0.05$ vs. control; # $p < 0.05$ vs. CSE. **c** Smoke increased TNF-α release

and treated with EM significantly decreased smoke-driven TNF-α release. The data are presented as means $\pm$ SEM, * $p < 0.05$ vs. control; # $p < 0.05$ vs. smoke. IL-6, interleukin-6; CSE, cigarette smoke extract; EM, erythromycin.

It is well known that CS-induced oxidative stress is responsible for proinflammatory cytokine release in the lungs [16, 25]. The results of the present study showed that cigarette smoke induced ROS levels in cultured human macrophages. EM pretreatment reduced CSE-induced ROS generation, indicating that ROS reduction is

the major mechanism of EM's protective role in cell damage [9]. Cigarette smoke causes oxidative stress by generating ROS, thus causing cell injury and lung tissue damage [16, 52]. The results of the present study showed that cigarette smoke induced ROS levels in cultured human macrophages. EM preincubated reduced CSE-in-

duced ROS generation, indicating that ROS reduction is the major mechanism of EM's protective role in cell damage. CSE damage to mitochondrial proteins by carbonyl stress, which drives further endogenous ROS production by the damaged mitochondria [16, 52]. Previous research demonstrated that EM inhibited mitochondria induced by human lactoferrin in yeast, resulting in a reduction of ROS released by mitochondria [53]. Our study was consistent with previous research [9]. EM restrained CSE-mediated induction of ROS release in human macrophages. However, the mechanism of EM on macrophage mitochondria still needs further research. Our study showed that as a SIRT1 inhibitor, NAM increased the release of ROS in human macrophages. Eric reported that the decrease in SIRT1 protein levels is correlated with the downregulation of deacetylation and activation of PGC-1 α . Transactivation of estrogen-related receptor α by PGC-1 α decreases transcription of SIRT3, resulting in an increase in ROS [54]. Thus, it is tempting to speculate that NAM increased ROS release in human macrophages via the SIRT1-SIRT3 axis; the molecular mechanism needs further study. CSE-induced oxidative stress causes tissue damage resulting in the formation of carbonyl stress [15] and damage to mitochondrial proteins by carbonyl stress, which both drive further endogenous ROS production by the damaged mitochondria [16, 52]. We therefore speculated that protecting the mitochondria from carbonyl stress might be the molecular mechanism underlying the EM-induced reduction in ROS levels.

We further studied the effect of pharmacological regulation of EM on SIRT1 expressed and the role of SIRT1 in proinflammatory cytokine release in response to CS exposure in BAL of mice lungs and human macrophages. Many inflammatory cytokines are significantly increased in the BAL of smokers [55]. Alveolar macrophages are considered to be important cells in the inflammatory response of CS [17]. Our data demonstrated that CSE treatment significantly upregulated the release of proinflammatory mediators such as TNF- α concomitant with decreased levels of SIRT1 protein in mice lungs and human macrophages. Yang and Saravanan reported that CS-mediated decreased SIRT1 levels were associated with increased inflammatory cytokines in the lung homogenate of smokers and MonoMac6 cells [25, 26]. The mechanism by which CSE reduces SIRT1 is not fully understood; however, it is possible that CSE-derived oxidants lower the Michaelis-Menten constant (K_m) of SIRT1, alter the NAD $^+$ /NADH ratio, and induce oxidative modification of SIRT1 [26]. Our data are in agree-

ment with previous studies. NAM is known to down-regulate the expression of SIRT1 [56]. Our experimental results show that the increased release of proinflammatory mediator is associated with NAM inhibition of SIRT1 expression. Decreased expression of SIRT1 promoted NF- κ B acetylation and transcription of IL-6 and TNF- α [27, 57, 58]. Our findings support these observations and verify the importance of SIRT1 in the regulation of proinflammatory mediators such as IL-6 and TNF- α in vivo and vitro. Furthermore, our current results demonstrate that EM increased SIRT1 levels are correlated with a reduction of proinflammatory cytokines in mice lungs and human macrophages. Previously, we had shown that EM inhibits oxidative stress which is induced by CSE. It has been reported that SIRT1 is a redox-sensitive deacetylase, which is post-translationally modified by oxidants and carbonyl stress, and that CSE can reduce SIRT1 protein levels through regulating oxidants and carbonyl stress [41]. In contrast, resveratrol is known to simulate SIRT1 activity and expression [18, 59] and significantly inhibits CSE-induced cytokine release and increases SIRT1 activity and expression in MonoMac6 cells [26]. In light of our data, it can thus be construed that EM increase SIRT1, resulting in decreased inflammatory CSE-driven cytokines. However, further studies are required to determine whether EM increased SIRT1 is directly associated with the decline of ROS in macrophages.

It has been well documented that SIRT1 is involved in the regulation of NF- κ B [24, 25, 60], participating in the regulation of inflammatory responses [25, 61]. Our data show that SIRT1 reduction induced by CSE leads to an increase in the acetylation of RelA/p65 NF- κ B and the induction of proinflammatory mediators. Previous studies demonstrated that SIRT1 physically interacts with the RelA/p65 subunit of NF- κ B and inhibits transcription by deacetylating RelA/p65 at lysine 310 [25, 62, 63]. Therefore, increased posttranslational modifications of SIRT1 lead to a disruption of the RelA/p65-SIRT1 complex, which culminates in acetylation and persistent activation of NF- κ B in response to CSE in macrophages [25]. EM increased SIRT1 activation, leading to deacetylation of NF- κ B and reduction of proinflammatory cytokines in human macrophages. Activated SIRT1 first directly binds and deactivates NF- κ B RelA/p65 through deacetylation and proteasome degradation [20]. Resveratrol is known to stimulate SIRT1 activity and expression [64] and could inhibit the NF- κ B inflammation pathway through activating SIRT1 in MC3T3-E1 cells [65]. Yang et al. [26] demonstrated that

resveratrol activated SIRT1 leading to reduced cigarette smoke-driven NF- κ B-dependent proinflammatory cytokine release in macrophages in vitro. Thus, our study shows for the first time that EM upgrade SIRT1 would have an inhibition on the acetylation of NF- κ B RelA/p65.

In conclusion, the present study demonstrated that CSE decreased SIRT1 expression, thereby disrupting the SIRT1-RelA/p65 complex and increasing activation of RelA/p65 NF- κ B, resulting in increased levels of proinflammatory cytokines. As an inhibitor of SIRT1, NAM increased the levels of proinflammatory mediators, suggesting that SIRT1 is related to the release of proinflammatory cytokines induced by CSEs. Our study shows for the first time that EM increased SIRT1 expression which was decreased by cigarette smoke, leading to the acetylation of RelA/p65 and thereby decreased cigarette smoke-driven NF- κ B-dependent proinflammatory cytokines released in macrophages in vitro and in mice lungs in vivo. Considering its convenience and low cost, EM has the potential to be used for the treatment of COPD.

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Disclosure Statement

No conflicts of interest, financial, or otherwise are declared by the author(s).

Author Contributions

M.-H.L. had full access to all of the data in the present study and takes full responsibility for the integrity of the data and data analysis; Nan Ma served as the principal investigator; X.-N.Z. and M.-H.L. contributed to the conception of the study; Z.-Y.H. and M.-H.L. contributed to the study design; M.-H.L., N.M., T.-T.D., Q.W., Z.-L.L., C.-F.Z. J.-F.Q., X.-J.T., and M.H. contributed to the laboratory investigations; N.M. and Z.-L.L. contributed to the data analysis and interpretation; M.-H.L. and N.M. contributed to the manuscript preparation; M.-H.L., J.B., X.-N.Z. and Z.Y. he contributed to the revision and editing of the manuscript.

Data Availability Statement

All data used to support the findings of this study are included within the article.

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