Combination of erythromycin and dexamethasone improves corticosteroid sensitivity induced by CSE through inhibiting PI3K-δ/Akt pathway and increasing GR expression

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Erythromycin and dexamethasone combination restores corticosteroid sensitivity
ABSTRACT

Corticosteroid insensitivity, which is induced by cigarette smoke extract (CSE), is a significant barrier when treating chronic obstructive pulmonary disease (COPD). Erythromycin (EM) has been shown to have an anti-inflammatory role in some chronic airway inflammatory diseases, particularly diffuse panbronchiolitis (DBP) and cystic fibrosis. Here, we explored whether the combination therapy of EM and dexamethasone (Dex) reverse corticosteroid insensitivity and investigated the molecular mechanism by which this occurs. We demonstrated that the combination of EM and Dex restored corticosteroid sensitivity in peripheral blood mononuclear cells (PBMCs) from COPD patients and U937 cells after CSE exposure. Moreover, pre-treatment with 10 μg/ml, 50 μg/ml or 100 μg/ml EM reversed the HDAC2 protein reduction induced by CSE exposure in a dose-dependent manner. U937 cells exposed to CSE show a reduction in HDAC activity, which was potently reversed by EM or combination treatment. While 10% and 17.5% CSE increased PAkt expression in a concentration-dependent manner, pre-application of EM and the combination treatment in particular blocked this PAkt increase. Total Akt levels were unaffected by CSE or EM treatments. Furthermore, the combination treatment enhanced glucocorticoid receptor (GR)α expression. Our results demonstrate that the combination therapy of EM and Dex can restore corticosteroid sensitivity through inhibition of the PI3K-δ/Akt pathway and enhancing GRα expression.

Key words: Chronic obstructive pulmonary disease; Erythromycin; Corticosteroid insensitivity; Histone deacetylase 2; Glucocorticoid receptor
1. Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by limited airflow due to chronic lung inflammation caused by inhalation of particles and gases (3, 6), especially those produced by cigarette smoke (21). The chronic inflammation in COPD is attributed to decreased histone deacetylase2 (HDAC2) activity, thought to be induced by oxidative stress. It has previously been shown that a reduction in HDAC2 can lead to transcription of nuclear factor kappa B (NF-κB), a protein complex known to enhance the activity of the pro-inflammatory cytokines interleukin (IL)-8 and tumor necrosis factor α (TNF-α) (5, 17). Oxidants, released from cigarette smoke extract (CSE) and inflammatory cells, can also lead to the activation of PI3K-δ. Protein kinase B (AKT), a major effector molecule of PI3K and regulated by PI3Ks, plays an important role on cell signal transduction. Phosphorylated Akt (PAkt) at Ser437 represents an important marker of PI3K-δ activation (25, 31). Activation of the PI3K-δ/Akt pathway may reduce HDAC2 activity, weaken corticosteroid receptor function and ultimately result in the enhancement of inflammatory gene acetylation (26). The enhancement of inflammatory gene acetylation promotes the persistence of airway and lung inflammation through the increasing expression and production of inflammatory factors. Thus, increasing oxidative stress leads to decreased HDAC2 activity, ultimately promoting corticosteroid resistance and prolonged inflammation (26, 27).

Corticosteroids are a class of anti-inflammatory drugs that reduce expression of inflammatory genes through inhibition of NF-κB-induced histone acetylation (7, 8).
Although corticosteroids have an established therapeutic role in chronic inflammatory lung disorders such as asthma and diffuse panbronchiolitis (DPB), their effect on COPD-related lung inflammation remains controversial. Corticosteroid receptor function is decreased in COPD due to decreased HDAC2 levels, which may limit the efficacy of corticosteroid treatments. Cigarette smoke has been shown to be a strong risk factor to increase inflammatory response, which can also reduce HDAC2 activity and lead to corticosteroid insensitivity (6, 8).

Macrolide antibiotics, including erythromycin (EM), roxithromycin and clarithromycin, have previously been shown to be effective in chronic airway diseases such as asthma, DBP and cystic fibrosis through reduction of HDAC2 expression. Macrolide administration not only inhibits inflammatory cytokine production by neutrophils and epithelial cells (2), but also reduces IL-8 levels and the numbers of neutrophils found in the bronchoalveolar lavage fluid (BALF) (5). Recent studies have shown that long-term macrolide administration reduces the frequency of COPD exacerbations (10, 24) and improves the clinical symptoms of asthma and bronchiectasis (15, 23). Previously, studies from our lab have shown that six months of EM treatment in COPD patients was sufficient to reduce airway inflammation and COPD-related exacerbations (16). Moreover, EM administration reversed the CSE-induced decreased expression of HDAC2 (22). Because corticosteroid receptor function is reduced in COPD patients, treatment with corticosteroids does not have a significant impact on inflammation or HDAC2 activity. A combination treatment of EM and corticosteroids may be more effective on restoring HDAC2 levels and
reversing corticosteroid insensitivity than either treatment alone. In the present study, a combination therapy including EM and dexamethasone (Dex) were used in an in vitro experimental approach to determine whether corticosteroid sensitivity was improved through inhibiting PI3K-δ/Akt pathway and increasing GR expression.

2. Materials and methods

2.1. Cell culture and treatment

Peripheral blood from each patient or healthy subjects was collected and peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation. All patients and healthy subjects provided written informed consent. PBMCs and U937 cells (human histiocytic lymphoma cell line; CRL-1593.2, ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and cultured at 37°C with 5% CO₂. U937 cells were treated with 10 μg/ml EM (Amresco, ID, USA), indicated concentration of Dex (Qian Jiang Pharmaceutical, Hubei, China), EM and Dex or 1 μM IC87114 PI3K-δ inhibitor (Selleckchem, Houston, TX, USA) prior to stimulation with 10% CSE (conditions listed in the section below). PBMCs were treated with EM, indicated concentration of Dex, EM and Dex or 1 μM IC87114 PI3K-δ inhibitor.

2.2. CSE preparation

CSE was prepared according to methods previously established by Yang (31) and
Carp (12). The smoke from two unfiltered cigarettes was slowly dissolved into 10 ml of RPMI-1640 medium. CSE at 10%, 17.5% and 20% concentrations, calculated by spectrophotometer, was used in the experiments. After adjusting the pH to 7.4, the CSE solution was filtered through a 0.22 μm filter and used within 1 hr for experiments.

2.3. BrdU cell proliferation assay

Proliferation of U937 cells was assayed with a BrdU cell proliferation kit (Millipore, Billerica, MA, USA). Cells were plated into sterile 96-well tissue culture plates at a concentration of $2 \times 10^5$ cell/ml in a volume of 200 μl per well and then incubated at 37°C for two days. U937 cells were then treated with CSE (10%, 17.5% and 20%) or EM (10 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml; molecular weight: 733.94 g/mol, purity quotient >99.99%, Sigma, St. Louis, MO, USA). PBMCs were treated with the same concentrations of EM but not with CSE. After 24 h of treatment with CSE or EM, a 10 μl BrdU solution was added to each sample. Samples were then fixed by addition of 200 μl BrdU fixing solution for 30 min at room temperature. The fixing solution was removed by aspiration and blotting, and plates were allowed to dry. Incorporated BrdU was detected by adding 100 μl per well of a pre-diluted anti-BrdU mouse monoclonal antibody for 1 h at room temperature (Roche, Basel, SWI). Samples were then washed three times in the provided wash buffer. A peroxidase-conjugated goat anti-mouse IgG secondary antibody was then added (1:2,000 dilution) in a final volume of 100 μl per well (Roche). After incubation for
30 min at room temperature, secondary antibody was removed and cells were washed. Colorimetric detection of BrdU incorporation was performed by adding 100 μl per well of 3,3’,5,5’-tetramethylbenzidine peroxidase substrate for 30 min at room temperature in the dark. Absorbance measurements were then recorded using a microplate spectrophotometer at a wavelength of 450 nm.

2.4. IL-8 assay

U937 cells were treated with EM (10 μg/ml), EM and Dex or 1 μM IC87114 PI3K-δ inhibitor prior to stimulation with 10% CSE. PBMCs were treated with EM, EM and Dex or 1 μM IC87114 PI3K-δ inhibitor. The cells were pretreated with Dex (10^{-12} - 10^{-6} M) for 1 hr and stimulated with 10 ng/mL TNF-α (Peprotech, Rocky Hill, NJ, USA) overnight. Culture media were collected after treatments and centrifuged at 1,000 rpm for 5 min to precipitate the cells. The supernatants were obtained and stored in -80°C prior to experimental use. IL-8 levels were quantified with an ELISA (R&D Systems, Minneapolis, MN, USA), as previously reported (22). The IL-8 inhibition ratio was calculated using Microsoft Excel, version 2003.

2.5. IC50-Dex calculation and corticosteroid sensitivity

The half maximal inhibitory concentration (IC50) of Dex (IC50-Dex) was used as a gauge for corticosteroid sensitivity. The IC50-Dex was determined using IL-8 inhibition ratio levels and different Dex concentrations. Microsoft Excel, version 2003, was used to calculate the IC50-Dex.
2.6. Western blot assay

Nuclear proteins were extracted from U937 cells from each treatment group using lysates (containing 0.5% NP40 and 0.25 M sucrose). Quantification of protein levels was performed using a bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). To examine HDAC2, PAkt, Ser473, Akt and GR, 20 μg of each sample was loaded and run on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and 0.1% Tween-20 (TBST). Membranes were then incubated for 2 hrs with rabbit anti-human polyclonal antibodies against HDAC2 (dilution 1:2,000; Abcam, Cambridge, MA, USA), PAkt Ser473 (dilution 1:1,000; Cell Signaling Technology, Danvers, MA, USA), total Akt (dilution 1:1,000; Cell Signaling Technology) and GR (dilution 1:1,000; Cell Signaling Technology) (20). After several washes with TBST, HDAC2, PAkt, Akt and GR were detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution: 1:10,000 in 5% nonfat dry milk in PBS; Abcam). Blots were visualized using enhanced chemiluminescence (Thermo Scientific Pierce, Rockford, IL, USA). Densitometry to quantify the blots was performed using a Bio-Rad gel image analyzer (California, USA). A mouse anti-human GAPDH antibody (dilution 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control.
2.7. HDAC activity

U937 cells were seeded at 1 - 5 × 10^6 cells/ml, pretreated with 10 μg/ml EM, EM and
Dex or IC87114 for 2 hrs before being exposed to CSE-infused media overnight.
Nuclear proteins were extracted from treated cells using RIPA buffer. HDAC activity
in these extracts was measured using a Fluo-Lys HDAC activity assay kit (Enzo Life
Sciences, Farmingdale, NY, USA). Nuclear extract from HeLa cells was used as a
positive control.

2.8. Statistical analysis

All data were analyzed using the SPSS statistical software, version 16.0 (SPSS Inc.,
Chicago, IL, USA). All quantitative data were expressed as the mean ± standard
development (SD). Multiple comparisons were analyzed using a one-way analysis of
variance (ANOVA) followed by the Friedman and Wilcoxon Signed-Rank tests. A \( P \)
value less than 0.05 was set as the threshold for statistical significance.

3. Results

3.1 Effects of CSE and EM on the growth of U937 cells

To determine whether exposure of U937 cells to CSE was cytotoxic, cellular growth
assays were performed in the presence of a range of CSE concentrations. Treatment of
U937 cells with a 10% or 17.5% CSE solution did not reduce cell proliferation over a
72 h time period. A 20% CSE solution, however, slowed U937 cell growth when
compared to untreated control samples. A 10% concentration of CSE was therefore
used in subsequent experiments to avoid any cytotoxic effects. No decrease in proliferation and no significant morphological changes were observed by microscopy in U937 cells or PBMCs treated with 10 μg/ml, 50 μg/ml, 100 μg/ml or 200 μg/ml EM. While U937 cells exposed to CSE (10%) released IL-8, addition of 10 μg/ml EM significantly decreased IL-8 release (Fig. 1).

3.2 A combination of EM and Dex improved corticosteroid sensitivity in PBMCs from COPD patients

PBMCs isolated from 10 COPD patients and 10 healthy control subjects were pretreated with EM (10 μg/ml) or EM and Dex for 2 hrs. The cells were then treated with Dex ($10^{-12}$ - $10^{-6}$ mol/L) for 1 hr. Cells were stimulated overnight with 10 ng/ml TNF-α. The anti-inflammatory effect of the different treatments was analyzed using the IC50-Dex (Methods section), determined through IL-8 levels. The IC50-Dex of COPD patients was approximately 270 fold higher than the IC50-Dex of healthy controls ($5.5 \pm 2.2 \times 10^{-5}$ M versus $2.0 \pm 0.3 \times 10^{-7}$ M, respectively; $P < 0.001$). Pre-treatment of PBMCs with EM decreased the IC50-Dex of PBMCs from COPD patients ($5.7 \pm 1.1 \times 10^{-6}$ M; $P < 0.001$). Pretreatment of cells with a combination of EM and Dex significantly increased the IC50-Dex ($1.0 \pm 0.1 \times 10^{-6}$ M; $P < 0.05$). These results suggest that PBMCs from COPD patients were less sensitive to Dex than PBMCs from healthy controls and that the combination therapy, in comparison to EM alone, improved corticosteroid sensitivity in PBMCs from COPD patients (Table 1, Fig. 2A).
3.3 The combination of EM and Dex improved corticosteroid sensitivity in CSE-exposed U937 cells

U937 cells were pretreated with EM (10 μg/ml) or IC87114 (PI3K-δ inhibitor, 1 μM) for 2 hrs prior to an overnight incubation with 10% CSE. The cells were then treated with Dex (10^{-6} - 10^{-12} M) for 1 hr and stimulated with TNF-α (10 ng/ml) overnight. Treatment of U937 cells with CSE significantly increased the IC50-Dex in comparison to untreated controls (7.6 ± 1.6 × 10^{-4} M versus 5.7 ± 1.1 × 10^{-6} M, respectively; \( P < 0.001 \)). Pretreatment with EM reduced the IC50-Dex in comparison to CSE alone (1.1 ± 0.2 × 10^{-4} M; \( P < 0.001 \)). Similarly, pretreatment of U937 cells with IC87114 also decreased the IC50-Dex in comparison to CSE treatment alone (1.4 ± 0.3 × 10^{-5} M; \( P < 0.001 \)). Pretreatment with the EM and Dex combination therapy significantly decreased the IC50 (IC50: 2.4 ± 0.2 × 10^{-5} M; \( P < 0.05 \)) when compared with the CSE group and the EM alone group. Similarly, IC87114 significantly restored corticosteroid sensitivity (IC50: 1.4 ± 0.1× 10^{-5} M; \( P < 0.05 \)) when compared with the CSE group. Pretreatment with Dex alone did not decrease the IC50-Dex (\( P > 0.05 \)). These results suggest that the EM and Dex combination treatment improved corticosteroid sensitivity in U937 cells (Fig. 2B,C).

3.4 Combination of EM and Dex increased HDAC2 protein expression

U937 cells, exposed to CSE overnight, were first pretreated with EM, Dex (10^{-6} M), EM and Dex or IC87114 for 2 hrs. CSE-exposed U937 cells showed a significant
reduction in HDAC2 protein expression. Addition of EM, EM and Dex or IC87114 blocked the HDAC2 decrease, especially in the EM and Dex combination treatment group (all $P < 0.05$). Dex alone, however, was unable to prevent the CSE-induced decrease in HDAC2 protein levels ($P > 0.05$; Fig. 3A,B). We observed a dose-dependent effect of EM on HDAC2 levels after CSE exposure, with 50 μg/ml EM increasing HDAC2 levels by 55% in comparison to 10 μg/ml EM and 100 μg/ml EM increasing HDAC2 levels by 100% in comparison to 10 μg/ml EM ($P < 0.05$; Fig. 3C).

3.5 Total HDAC activity was enhanced by the combination of EM and Dex

We analyzed total HDAC activity using the Fluo-Lys HDAC activity assay. In U937 cells treated with CSE alone, total HDAC activity was significantly reduced. Pretreatment with Dex alone was insufficient in increasing total HDAC activity after CSE exposure ($P > 0.05$; Fig. 4A). Pretreatment with EM and Dex, EM alone or IC87114 restored HDAC activity to control levels (all $P < 0.05$). No statistically significant differences in increased HDAC activity were observed between the different treatment groups. Moreover, EM alone did not dose-dependently increase HDAC activity. Thus, the changes in total HDAC activity cannot solely be explained by changes in HDAC2 levels (Fig. 4B).

3.6 PAkt, but not total Akt, levels decreased after combination treatment with EM and Dex
U937 cells exposed to CSE overnight were first pretreated with EM, Dex (10⁻⁶ M), EM and Dex or IC87114. It is important to note that total Akt expression was unaffected by 10% CSE, Dex alone, 10 μg/ml EM, the combination treatment of EM and Dex or IC87114. When we examined PAkt levels, CSE exposure significantly increased PAkt expression. Pretreatment with Dex alone did not affect PAkt expression after CSE exposure (P > 0.05; Fig. 5). The increase in PAkt after CSE exposure could be reversed by pretreatment with EM and Dex, EM alone or IC87114 (all P < 0.05). If CSE was increased from 10% to 17.5%, PAkt expression was further increased (P < 0.05) while total Akt levels remained unchanged (P > 0.05). Pretreatment with 50 μg/ml or 100 μg/ml EM decreased PAkt expression in a dose-dependent manner (P < 0.05), but did not alter total Akt expression levels (P > 0.05). These results demonstrate that while total Akt protein levels do not change after CSE exposure or pretreatment with EM, EM and Dex or IC87114 inhibitor, PAkt levels are significantly affected (Fig. 6).

3.7 The combination treatment increased GRα protein expression

After pretreatment with EM, Dex (10⁻⁶ M) or EM and Dex for 2 hrs, U937 cells were exposed to CSE overnight. CSE-exposed U937 cells showed a significant reduction in GRα protein expression. EM or EM and Dex blocked the GRα decrease, particularly in the combination treatment group (all P < 0.05). Dex alone, however, was unable to prevent the CSE-induced decrease in GRα protein level (P > 0.05; Fig 7).
4. Discussion

In this study, we demonstrated that PBMCs from COPD patients or CSE-exposed U937 cells treated with EM and Dex resulted in improvement of corticosteroid sensitivity. EM alone could increase the HDAC2 activity and decreased PAkt expression for CSE-exposed U937 cells with a dose-dependent manner. The combination of EM and Dex for U937 cells exposed CSE further increased the efficacy when compared to EM alone. Moreover, the combination treatment of EM and Dex could increase the GR expression.

Previous research has shown that inflammatory mediators such as IL-8 and TNF-α are released in COPD patients who smoke (13). Because there is reduced activity of corticosteroid receptors in COPD patients who smoke, corticosteroid therapy is ineffective and represents a significant barrier to patient treatment. It is currently thought that the decreased corticosteroid response results from reduced HDAC2 activity through PI3K-δ activation (1, 27).

Examination of the IC50-Dex in PBMCs from COPD patients revealed the presence of corticosteroid insensitivity. Exposure to CSE increased the IC50-Dex in U937 cells, indicating that CSE could induce corticosteroid insensitivity in U937 cells in vitro, similar to what we observed in PBMCs. Previous studies have shown that treatment with either theophylline or IC87114 was able to reverse corticosteroid insensitivity in cigarette-smoke-exposed mice in vivo (25, 30). Research from our own lab has shown that treatment with EM reduced smoking-induced inflammation
in rat lungs (4). In the present study, we sought to determine if the combination treatment of EM and Dex more effectively improved corticosteroid sensitivity, acting directly on HDAC2 or indirectly through the PI3K-δ/Akt pathway.

HDACs are critical proteins that repress the production of pro-inflammatory cytokines (9, 11) and HDAC2 has been reported to be required for corticosteroid-mediated anti-inflammatory activity (18). HDAC2 expression and activity is regulated through PI3K-δ/Akt and, specifically, a decrease in PAkt levels may increase the expression of HDAC2 (25). We have previously shown that pretreatment of U937 cells with EM reversed CSE-induced reductions in HDAC1, -2 and -3 protein expression, with the most prominent effects observed on HDAC2 (22). Decreased HDAC2 activity via CSE exposure may be related to corticosteroid insensitivity. Our and other articles have shown that macrolide treatment is effective in COPD patients and in patients with other chronic lung diseases (14, 17). The effects on HDAC2 of a combined therapy containing EM and the corticosteroid Dex, however, have not yet been evaluated.

Exposure of U937 cells to CSE caused a significant reduction in HDAC activity and HDAC2 protein expression. These reductions could be reversed by pretreatment with either EM or an IC87114. When cells were pretreated with the combination therapy of EM (10 μg/ml) and Dex, we observed a better restoration of corticosteroid sensitivity on CSE-exposed U937 cells compared to EM alone. In contrast, a recent study showed that 100 μM (approximately 73 μg/ml) could significantly increase the IL-8 inhibition ratio and did increase total HDAC activity by 300% in U937 cells
stimulated with \( \text{H}_2\text{O}_2 \) (19). There were, however, significant differences in the experimental conditions between the previous study and our current study. In our study, U937 cells were maintained in a monocyte state while the U937 cells from the previous study were exposed to phorbol 12-myristate 13-acetate (PMA), causing them to differentiate into an adherent macrophage-like morphology. The PMA-exposed U937 cells from the previous study were pre-incubated with \( \text{H}_2\text{O}_2 \), which may explain the difference in their results compared to our study.

In our study, we also compared the effects of different doses of EM on HDAC2 protein expression levels. Pretreatment of U937 cells with EM dose-dependently prevented the HDAC2 protein decrease induced by CSE exposure. It is important to note, however, that EM was unable to dose-dependently increase total HDAC activity, which indicates that the change in total HDAC activity cannot be solely explained by HDAC2 levels.

Phosphorylation of Akt at Ser437 is an important indicator of PI3K-\( \delta \) activation (30). It has been hypothesized that the reduction of HDAC2 expression observed in COPD patients is due to increased PI3K-\( \delta \)/Akt signaling, which results from an increase in oxidative stress in COPD patient lungs (28, 29). When a PI3K-\( \delta \) inhibitor was used as a therapy in CSE-exposed U937 cells, we observed an improvement of corticosteroid sensitivity as similar to treatment with EM and Dex. Treatment with different doses of EM reversed this PAkt increase but did not affect total Akt protein expression levels. We also assayed GR\( \alpha \) protein expression and found that the combination treatment enhanced GR\( \alpha \) expression, which was reduced in the
CSE-alone treatment group. These results indicated that the combination of EM and Dex reversed corticosteroid insensitivity induced by CSE via the PI3K-δ/Akt pathway and increased GRα expression.

In summary, this study demonstrates that administration of a combination treatment of EM and Dex restored corticosteroid sensitivity on U937 cells exposed to CSE and PBMCs obtained from COPD patients, through regulation of PI3K/Akt pathway and GRα expression. These results provide clinically relevant evidence for the use of marcolides and corticosteroids in the treatment of COPD patients.

5. Acknowledgments

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6. Grants

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6. References


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Table 1. PBMC subject characteristics

<table>
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Abbreviations: COPD: chronic obstructive pulmonary disease; FEV1/FVC: Forced expiratory volume in one second/Forced vital capacity; FEV1 % pred: Forced expiratory volume in one second, percent predicted.
Figure legends

Figure 1. Effects of EM (10 μg/ml) on IL-8 levels in U937 cells exposed to 10% CSE. U937 cells treated with CSE (10%) for 24, 48 or 72 hrs were pretreated with EM for 2 hrs. IL-8 levels were measured in culture supernatants via ELISA. Exposure to 10% CSE increased the release of IL-8, which could be inhibited by EM at 10 μg/ml. # P < 0.05. The histograms represent the means and the bars represent the SD from three independent experiments.

Figure 2. A combination treatment of EM and Dex increased anti-inflammatory effects in PBMCs from COPD patients. (A) Effects of EM and the combination treatment on corticosteroid sensitivity in PBMCs from COPD patients. PBMCs were pretreated with 10 μg/ml EM or EM and Dex for 2 hrs. The cells were treated with Dex (10^-6 - 10^{-12} M) for 1 hr and then stimulated with TNF-α (10 ng/ml) overnight. The IC50-Dex of COPD patients was more than 270 fold higher than that of healthy subjects (P < 0.001). Pretreatment with EM decreased the IC50-Dex of PBMCs from COPD patients (P < 0.001). Pretreatment with Dex alone did not decrease the IC50-Dex (P > 0.05). The combination treatment of EM and Dex significantly increased anti-inflammatory effects (P < 0.05). (B) U937 cells treated with 10% CSE overnight were pretreated with EM or EM and Dex for 2 hrs. The cells were treated with Dex (10^-6 - 10^{-12} mol/L) for 1 hr and then stimulated with TNF-α (10 ng/ml) overnight. CSE significantly increased the IC50-Dex (P < 0.001), which could be reversed by pretreatment with EM (P < 0.001). The combination of EM and Dex
significantly increased anti-inflammatory effects ($P < 0.05$). (C) U937 cells were pretreated with IC87114 prior to 10% CSE exposure overnight. The cells were treated with Dex ($10^{-6}$-$10^{-12}$ mol/L) for 1 hr and then stimulated with TNF-α (10 ng/ml) overnight. CSE significantly increased the IC50-Dex ($P < 0.001$), which was inhibited by pretreatment with IC87114 ($P < 0.001$). The individual symbols represent the means and the bars represent the SD from three independent experiments.

**Figure 3. The effects of the combination treatment and IC87114 on HDAC2 protein expression after CSE exposure.** (A, B) U937 cells treated with CSE overnight were pretreated with EM, Dex ($10^{-6}$ M), EM and Dex or IC87114 for 2 hrs. CSE exposure reduced HDAC2 expression. This reduction was reversed by pretreatment with a combination of EM and Dex, EM alone or IC87114 (all $P < 0.05$). Pretreatment with Dex alone could not reverse the HDAC2 reduction induced by CSE ($P > 0.05$). (C) U937 cells treated with 10% CSE overnight were pretreated with different doses of EM (10 μg/ml, 50 μg/ml or 100 μg/ml) for 2 hrs. HDAC2 protein levels of cells pretreated with EM were the highest with 100 μg/ml EM; pretreatment with 50 μg/ml EM was approximately 55% higher than treatment with 10 μg/ml EM ($P < 0.05$). ($n = 3$ independent experiments).

**Figure 4. Effects of the combination treatment on total HDAC activity in U937 cells.** (A) CSE exposure in U937 cells significantly reduced total HDAC activity. Pretreatment with 10 μg/ml EM, EM and Dex or IC87114 ($P < 0.05$) reversed the
reduction in HDAC activity (all $P < 0.05$). There were no significant differences in the effectiveness on increasing HDAC activity between the combination treatment, EM or IC87114 ($P > 0.05$). Treatment with Dex alone did not reverse the reduction in total HDAC activity induced by CSE (Dex group vs. CSE group $P > 0.05$). (B) Cells treated with 10% CSE overnight were pretreated with 10 $\mu$g/ml, 50 $\mu$g/ml or 100 $\mu$g/ml EM for 2 hrs. The histograms represent the means and the bars represent the SD of three independent experiments ($n=3$ independent experiments; # $P < 0.05$; * $P > 0.05$).

**Figure 5. Effects of the combination treatment of EM and Dex on PAkt and Akt in CSE-exposed U937 cells.** (A) U937 cells were pretreated with EM (10 $\mu$g/ml), Dex ($10^{-6}$ M) or EM and Dex for 2 h prior to overnight treatment with CSE (10%). Western blotting was used to determine PAkt and total Akt levels. (B) U937 cells were pretreated with IC87114 for 2 h prior to overnight treatment with CSE (10%). Western blotting was used to determine PAkt and total Akt levels. ($n=3$ independent experiments; # $P < 0.05$).

**Figure 6. Effects of EM and CSE on PAkt and Akt levels in CSE-exposed U937 cells.** (A) Effects of different CSE concentrations on total Akt levels. Cells were treated with CSE (10%) overnight. Total Akt levels were compared to GAPDH levels. (B) Effects of different doses of EM on total Akt levels in cells exposed to CSE. Cells were pretreated with EM (10 $\mu$g/ml, 50 $\mu$g/ml or 100 $\mu$g/ml) for 2h prior to overnight
treatment with CSE (10%). Total Akt levels were compared to GAPDH levels. (C)

Effects of different CSE concentrations and different doses of EM on PAkt expression. Cells were pretreated with EM (10 μg/ml, 50 μg/ml or 100 μg/ml for 2 h prior to overnight treatment with CSE (10%, 17.5%). PAkt expression was compared to GAPDH levels and measured by western blot.

Figure 7. The effect of the combination treatment on GRα protein expression after CSE exposure. U937 cells treated with CSE overnight were pretreated with EM, Dex (10^{-6} M) or EM and Dex for 2 hrs. CSE exposure reduced GRα protein expression. This reduction was reversed by pretreatment with a combination of EM and Dex or EM alone (P < 0.05), particularly with the combination treatment. Pretreatment with Dex alone did not reverse the GRα reduction induced by CSE (P > 0.05). (n= 3 independent experiments; * P > 0.05).