



Erythromycin regulates peroxisome proliferator-activated receptor γ to ameliorate cigarette smoke-induced oxidative stress in macrophages

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Background: Chronic obstructive pulmonary disease (COPD) is significantly influenced by oxidative stress. Recent studies have elucidated the anti-oxidative stress properties of peroxisome proliferator-activated receptors γ (PPAR γ), augmenting its known anti-inflammatory effects. The exact influence of PPAR γ on oxidative stress in COPD remains elusive. This study aimed to investigate the potential mechanism by which PPAR γ counteracts the oxidative stress instigated by cigarette smoke in macrophages.

Methods: Macrophages were cultured and exposed to 1% cigarette smoke extract (CSE), 1 μ g/mL erythromycin (EM), and 10 μ mol/mL GW9662 (a PPAR γ antagonist). Reactive oxygen species (ROS) in macrophages was identified using fluorescent microscopy. PPAR γ expression was ascertained through reverse transcription-polymerase chain reaction (RT-PCR) and Western blot techniques. The superoxide dismutase (SOD) in macrophage supernatant was measured by enzyme linked immunosorbent assay (ELISA), as was malondialdehyde (MDA).

Results: Our results shown that cigarette smoke stimulated macrophages to increase ROS release, decrease the expression of PPAR γ , increase the expression of MDA and decrease the expression of SOD. After PPAR γ inhibitor acted on macrophages stimulated by cigarette smoke, the expression of MDA was inhibited and the content of SOD increased. When EM was used to treat macrophages stimulated by cigarette smoke, the expression of ROS decreased, the expression of PPAR γ increased, the expression of MDA decreased and the expression of SOD increased.

Conclusions: This study suggests that PPAR γ plays an anti-oxidative role by inhibiting the expression of MDA and promoting the expression of SOD. Cigarette smoke induces oxidative stress by inhibiting PPAR γ pathway. EM inhibits oxidative stress by activating PPAR γ pathway.

Keywords: Chronic obstructive pulmonary disease (COPD); peroxisome proliferator-activated receptor γ (PPAR γ); oxidative stress; erythromycin (EM); cigarette smoke

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Introduction

Chronic obstructive pulmonary disease (COPD) stands as a prominent cause of human morbidity and mortality, gaining recognition as a major global health concern. COPD ranks as the third leading cause of death in China and the fourth globally (1). Oxidative stress is the main driving mechanism of COPD pathogenesis, which promotes chronic lung inflammation and deterioration in COPD patients (2).

Oxidative stress caused by cigarette smoke stimulates the release of inflammatory cells (especially macrophages), which infiltrate the lung and release a lot of reactive oxygen species (ROS), thus aggravating chronic airway inflammation and airway remodeling (3). Oxidative stress is characterized by an amplified intracellular ROS concentration, which in turn produces a substantial quantity of oxygen free radicals, inflicting damage on deoxyribonucleic acid (DNA), proteins, and lipids (4). These excessive oxygen free radicals can oxidize polyunsaturated acids, such as arachidonic acid, converting them to malondialdehyde (MDA), a notable lipid peroxidation product (5). Elevated MDA levels can stimulate inflammatory cells, prompting the release of inflammatory

cytokines and ROS. This sequence deteriorates the body's oxidative equilibrium, initiating or intensifying oxidative stress (6,7). Consequently, MDA serves as a primary indicator of oxidative stress, used to gauge its severity in cells and tissues (8,9). Superoxide dismutase (SOD) acts as a vital antioxidant enzyme that safeguards cells against oxidative damage (10). Through rapid scavenging of oxygen free radicals in tissues, SOD provides cells protection from ROS, thereby attenuating oxidative stress and cellular damage (11). Notably, exposure of lung tissue in mice to cigarette smoke resulted in an uptick in ROS and MDA levels and a decline in SOD and other radical scavengers, culminating in oxidative tissue damage (12). Hence, identifying therapeutic targets to mitigate macrophage oxidative stress emerges as a priority.

It was found that macrophages were one of the main cellular targets for peroxisome proliferator-activated receptor γ (PPAR γ) activation. Activation of PPAR γ could promote macrophage polarization into macrophage 2 (M2), and could reverse macrophage 1 (M1) induced by cigarette smoke into M2, which was involved in inhibiting inflammatory reaction and antioxidant stress (13,14). Upon ligand activation, PPAR γ exhibits a spectrum of biological functions encompassing anti-inflammatory responses, anti-oxidative stress, anti-apoptosis, and lipid metabolism regulation (15). PPAR γ , an isomer of peroxisome proliferator-activated receptors (PPARs), has PPAR γ 2 primarily expressed in adipose tissue, implicating it in lipid metabolism (16). Experimental data reveals that cigarette smoke extract (CSE) exposure results in PPAR γ down-regulation in human bronchial epithelial cells. Moreover, rosiglitazone treatment can bolster PPAR γ and glutathione peroxidase 3 (an important antioxidant enzyme) level, diminish ROS production, and curb oxidative stress (17). Thus, PPAR γ could emerge as a pivotal therapeutic target in COPD prevention strategies, owing to its antioxidative properties.

In recent years, the antioxidant capabilities of macrolide drugs have garnered increasing interest. In patients with COPD, prolonged low-dose erythromycin (EM) administration has been shown to decrease neutrophil concentrations and elastase levels in sputum (18). Furthermore, macrolides exhibit anti-oxidative stress properties: for instance, azithromycin reduces ROS production in bronchial epithelial cells exposed to tobacco smoke, underscoring its anti-oxidative stress effects (19). Barnes postulated that macrolides may play a pivotal role as emerging therapeutic agents for COPD (20). The 2017 joint guidelines from the European Respiratory Society and American Thoracic Society endorsed the use of macrolides

Highlight box

Key findings

- This study suggests that peroxisome proliferator-activated receptors γ (PPAR γ) plays an anti-oxidative role by inhibiting the expression of malondialdehyde (MDA) and promoting the expression of superoxide dismutase (SOD). Cigarette smoke induces oxidative stress by inhibiting PPAR γ pathway. Erythromycin (EM) inhibits oxidative stress by activating PPAR γ pathway.

What is known and what is new?

- Chronic obstructive pulmonary disease (COPD) is significantly influenced by oxidative stress. Recent studies have elucidated the anti-oxidative stress properties of PPAR γ , augmenting its known anti-inflammatory effects.
- In this study, we found that PPAR γ played an antioxidant role by inhibiting the expression of MDA and promoting the expression of SOD. Cigarette smoke induced oxidative stress by inhibiting PPAR γ pathway, which might be one of the mechanisms of oxidative stress induced by cigarette smoke. EM inhibited oxidative stress by activating PPAR γ pathway, which might be the potential mechanism of EM in treating COPD.

What is the implication, and what should change now?

- These insights pave the way for a renewed technical perspective for deciphering the etiology of COPD, while also fortifying the theoretical foundation for employing EM in COPD therapeutic regimes.

to manage acute COPD exacerbations (21). Similarly, the Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report highlighted the utility of macrolides for addressing acute COPD episodes (22). Protracted administration of macrolides has been associated with a delay in subsequent COPD exacerbations, a reduction in airway inflammation frequency, and an improvement in patients' quality of life (23). The therapeutic advantages of macrolides in stable COPD patients are attributed to their anti-inflammatory and anti-oxidative stress properties, rather than their antibacterial activity, positioning them as promising candidates for COPD treatment (24). Our prior research indicated that EM modulates the PPAR/nuclear factor kappa-B (NF- κ B) signaling pathway in macrophages, attenuating inflammation and ROS expression induced by CSE (25). Nonetheless, the exact mechanism through which EM suppresses oxidative stress by modulating PPAR γ remains an area of exploration. This study aims to provide a comprehensive understanding of EM's role in countering oxidative stress, thereby laying the groundwork for its therapeutic application in COPD management. We present this article in accordance with the MDAR reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1647/rc>).

Methods

Experimental materials

Experimental materials included cigarettes (Guangxi Tobacco, China), PPAR γ antibody, GW9662 (a PPAR γ inhibitor), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, USA), reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara Bio, Japan), fluorescent secondary antibody goat anti-rabbit (EarthOx, USA), Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Corning, USA), phorbol myristate acetate (PMA) and EM powders (Sigma, USA), ROS detection kit (Beyotime, China), Fetal bovine serum (GIBCO, USA), PPAR γ and GAPDH primers (Bioengineering, China), radio immunoprecipitation assay (RIPA) protein lysate (Beyotime), SOD and MDA enzyme linked immunosorbent assay (ELISA) kits (Cloud Cloning Technology, China), and phosphatase inhibitor (Roche, Switzerland).

Cell culture and treatment

The U937 human histiocytic lymphoma cell line (ATCC, China) was cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂. To differentiate U937 cells into macrophages, cells were treated with 100 ng/mL PMA for 24 hours. Subsequently, macrophages were incubated for 24 hours with 1% CSE, 1 μ g/mL EM, and 10 μ mol/mL GW9662. The experimental groups were denoted as: (I) control group (NC); (II) CSE; (III) CSE + EM; (IV) CSE + GW9662; (V) CSE + GW9662 + EM.

Preparation of CSE

CSE was prepared as described by Sheridan *et al.* (26). Ten cigarettes, with filters removed, were combusted, and the resultant smoke was drawn through an injector device, allowing it to permeate 10 mL of RPMI-1640 medium. The pH of this medium was adjusted to 7.4, yielding the CSE solution. This solution was subsequently filtered using a 0.22 μ m sterile filter membrane. The concentration of the CSE solution was determined by assessing its absorbance at 320 nm via a spectrophotometer.

Fluorescence microscopy and fluorescence microplate reader

ROS detection kit is the most commonly method to quantitatively detect the level of ROS in cells based on the change of fluorescence intensity of fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Macrophages were stained using the ROS-specific probe DCFH-DA. Post-staining, the intracellular ROS levels across all experimental groups were visualized using a fluorescence microscope. After the differentiation of U937 cells into macrophages, they were treated with a 1:1,000 dilution of DCFH-DA solution. Following a 20-minute incubation, cells were washed thrice with 1 $\times$ phosphate buffer solution (PBS). The resultant ROS fluorescence in macrophages was immediately captured using a fluorescence microscope. For quantitative assessment, the average fluorescence intensity of each group was determined using a fluorescence microplate reader set at excitation and emission wavelengths of 488 and 525 nm, respectively.

Western blot

Total protein was extracted from treated macrophages using radio immunoprecipitation assay (RIPA) protein lysate supplemented with a phosphatase inhibitor. The extracted protein was subsequently subjected to electrophoresis on a 10% polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk powder for an hour, then incubated with the anti-PPAR γ antibody (1:1,000) at 4 °C for 24 hours. This was followed by a 1-hour incubation with the fluorescent secondary goat anti-rabbit antibody (1:800) at room temperature. After thorough washing with tris buffered saline with tween (TBST), the expression bands of PPAR γ protein were detected using an infrared film scanner. The protein expression levels were quantified using ImageJ analysis software.

RT-PCR

Total RNA was extracted from macrophages using TRIzol reagent. Complementary DNA (cDNA) was synthesized utilizing reverse transcriptase (Promega Corporation, USA) and primers supplied by Sangon Biotech. The following primer sequences were used: PPAR γ , forward sequence 5'-TGGAATTAGATGACAGCGACTTGG-3' and reverse sequence 5'-TTGAATGTCTTCAATGGGCTTCAC-3'; GAPDH, forward sequence 5'-CAGGAGGCATTGCTGATGAT-3' and reverse sequence 5'-GAAGGCTGGGGCTCATTT-3'. RT-PCR was performed following the kit's recommended protocol using the provided primer sequences. Each RT-PCR reaction was replicated a minimum of three times. The expression level of PPAR γ was determined using the $2^{-\Delta\Delta C_q}$ method, with normalization to GAPDH.

The expression of MDA and SOD was detected by ELISA

The macrophage supernatant was added to the respective SOD and MDA ELISA kits. Assays were conducted following the manufacturer's protocol for the ELISA kits. The absorbance of SOD and MDA in the cell supernatant was measured at 450 nm using an enzyme-linked immunosorbent assay instrument. Concentrations of SOD and MDA were extrapolated based on the standard curve.

Statistical analysis

Data were analyzed using SPSS 22.0 software and presented as mean $\pm$ standard deviation. Differences between groups were evaluated using analysis of variance (ANOVA). A P value of less than 0.05 was considered statistically significant.

Results

Effect of EM on ROS release from human macrophages stimulated by CSE

Fluorescence microscopy was employed to directly visualize ROS release (Figure 1), and its expression levels were quantified using a fluorescent microplate reader (Figure 2). Human macrophages exhibited augmented ROS production post CSE stimulation (CSE group) compared to the control group ($P < 0.05$). Pre-incubation with EM for 24 hours, followed by CSE stimulation (CSE + EM group), resulted in diminished ROS levels ($P < 0.05$). Macrophages in the CSE + GW9662 (a PPAR γ inhibitor) group demonstrated elevated ROS production. However, the ROS levels in the CSE + GW9662 + EM group were attenuated compared to the CSE + GW9662 group ($P < 0.05$).

EM's impact on PPAR γ expression in CSE-stimulated human macrophages

Each group's cells' expression of the PPAR γ protein was determined using a Western blot

CSE-stimulated human macrophages (CSE group) manifested reduced PPAR γ protein expression relative to the control group ($P < 0.05$). Macrophages pre-incubated with EM for 24 hours and subsequently stimulated with CSE (CSE + EM group) displayed enhanced PPAR γ protein levels ($P < 0.05$). The CSE + GW9662 group revealed diminished PPAR γ protein expression, whereas the CSE + GW9662 + EM group exhibited increased PPAR γ protein levels compared to the CSE + GW9662 group ($P < 0.05$) (Figure 3).

RT-PCR was used to detect the content of PPAR γ messenger ribonucleic acid (mRNA) in macrophages of each group

CSE-stimulated human macrophages (CSE group) demonstrated a decline in PPAR γ mRNA levels compared

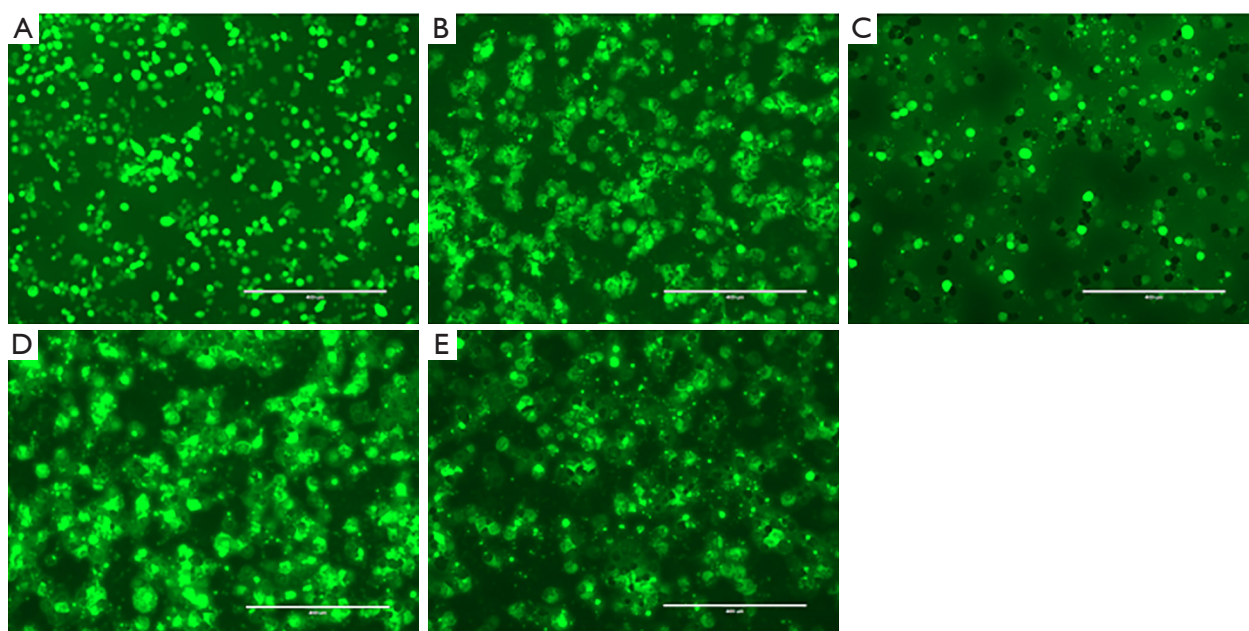


Figure 1 Fluorescence intensity of ROS released by human macrophages in each group (fluorescence microscope $\times 200$, scale 400 μm): (A) control group; (B) CSE group; (C) CSE + EM group; (D) CSE + GW9662 group; (E) CSE + GW9662 + EM group. ROS, reactive oxygen species; CSE, cigarette smoke extract; EM, erythromycin.

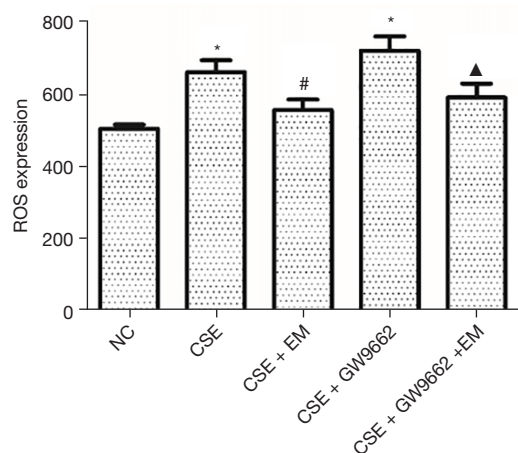


Figure 2 Fluorescent microplate reader was used to determine the ROS release level. NC: control group. *, $P < 0.05$ vs. NC group; #, $P < 0.05$ vs. CSE group; ▲, $P < 0.05$ vs. CSE + GW9662 group; $n = 7$. CSE, cigarette smoke extract; EM, erythromycin; ROS, reactive oxygen species.

to the control group ($P < 0.05$). In contrast, the CSE + EM group exhibited augmented $\text{PPAR}\gamma$ mRNA expression following a 24-hour pre-incubation with EM ($P < 0.05$). The CSE + GW9662 group showed reduced $\text{PPAR}\gamma$ mRNA

levels, whereas the CSE + GW9662 + EM group presented elevated $\text{PPAR}\gamma$ mRNA expression in comparison to the CSE + GW9662 group ($P < 0.05$) (Figure 4).

MDA expression in human macrophages was detected using ELISA

MDA expression was augmented in CSE-stimulated human macrophages (CSE group) compared to the control group ($P < 0.05$). Following a 24-hour pre-incubation with EM and subsequent CSE stimulation, MDA levels diminished ($P < 0.05$). However, the CSE + GW9662 group revealed heightened MDA levels. Notably, the CSE + GW9662 + EM group manifested reduced MDA expression compared to the CSE + GW9662 group ($P < 0.05$) (Figure 5).

SOD expression in human macrophages was detected using ELISA

Human macrophages stimulated by CSE (CSE group) demonstrated reduced SOD expression relative to the control group ($P < 0.05$). A 24-hour pre-incubation with EM followed by CSE stimulation led to augmented SOD expression in the CSE + EM group ($P < 0.05$). Conversely, the CSE + GW9662 group exhibited a decline in SOD

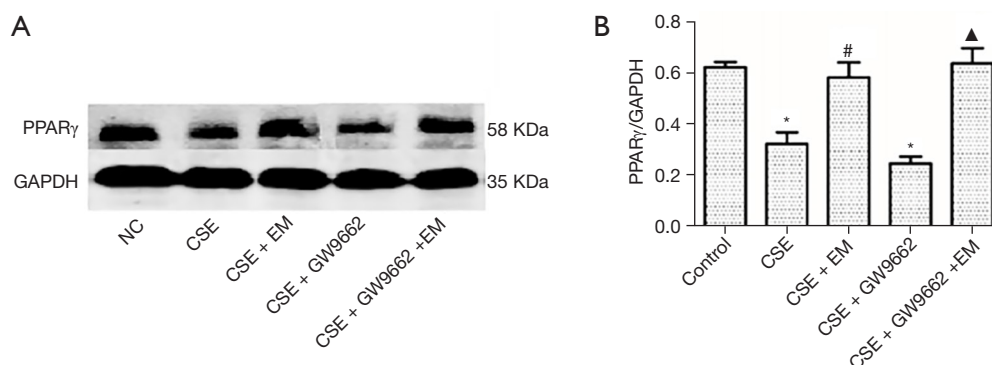


Figure 3 Each group's human macrophages' PPAR γ protein expression. (A) PPAR γ protein expression in each group's cells. (B) Quantification of PPAR γ protein in cells of each group. NC: control group. *, $P < 0.05$ vs. NC group; #, $P < 0.05$ vs. CSE group; $\blacktriangle$, $P < 0.05$ vs. CSE + GW9662 group; $n = 4$. PPAR γ , peroxisome proliferator-activated receptors γ ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CSE, cigarette smoke extract; EM, erythromycin.

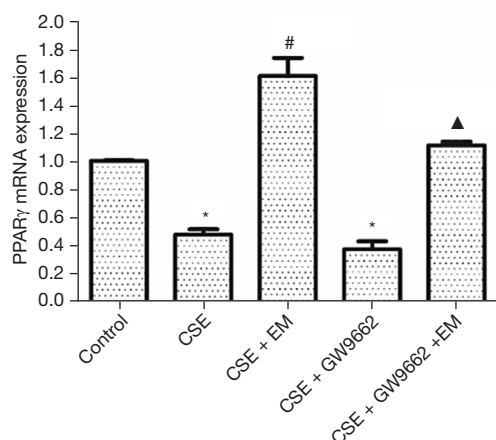


Figure 4 EM's impact on PPAR γ mRNA expression in CSE-stimulated human macrophages. *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. CSE group; $\blacktriangle$, $P < 0.05$ vs. CSE + GW9662 group; $n = 4$. CSE, cigarette smoke extract; EM, erythromycin; PPAR γ , peroxisome proliferator-activated receptors γ ; mRNA, messenger ribonucleic acid.

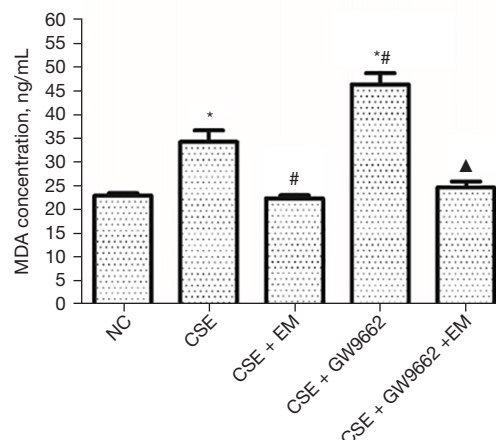


Figure 5 The expression of MDA in the supernatant of human macrophages in each group. NC: control group. *, $P < 0.05$ vs. NC group; #, $P < 0.05$ vs. CSE group; $\blacktriangle$, $P < 0.05$ vs. CSE + GW9662 group; $n = 8$. CSE, cigarette smoke extract; EM, erythromycin; MDA, malondialdehyde.

levels. The SOD expression in the CSE + GW9662 + EM group was elevated in comparison to the CSE + GW9662 group ($P < 0.05$) (Figure 6).

Discussion

PPAR γ is a nuclear receptor with anti-inflammatory and antioxidant properties (16). In order to clarify the molecular mechanism of PPAR γ antioxidant function, the relationship between PPAR γ and MDA and SOD was explored in

this experiment. We found that the expression of PPAR γ was negatively correlated with the expression of MDA and positively correlated with the expression of SOD in macrophages. This revealed that SOD and MDA might be the targets of PPAR γ mediating antioxidant stress. We also found that EM may activate PPAR γ pathway to play an antioxidant stress role.

Oxidative stress serves as a central pathogenic mechanism underlying COPD. Our findings reveal that EM mitigates the ROS release from human macrophages. Conversely, ROS emission escalates in macrophages under

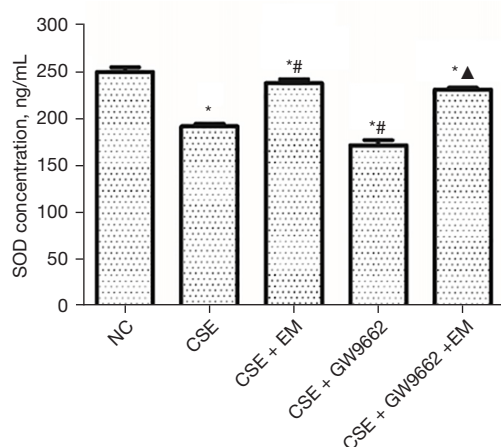


Figure 6 SOD expression in supernatant of human macrophages in each group. NC: control group. *, $P < 0.05$ vs. NC group; #, $P < 0.05$ vs. CSE group; ▲, $P < 0.05$ vs. CSE + GW9662 group; $n = 8$. CSE, cigarette smoke extract; EM, erythromycin; SOD, superoxide dismutase.

CSE stimulation. These observations imply that tobacco smoking can potentiate macrophage-mediated ROS production. EM appears to counteract the oxidative stress instigated by smoking by attenuating ROS levels. Notably, smoking-induced oxidative stress is instrumental in COPD pathogenesis (27). Existing literature underscores the phenomenon that the lung parenchyma of COPD patients releases a multitude of inflammatory cells, predominantly macrophages. When activated, these macrophages and other inflammatory cells typically discharge copious amounts of ROS (28). In alignment with the aforementioned studies, our results intimate that EM can ameliorate oxidative stress by curbing ROS release.

Our data illustrate that PPAR γ expression in human macrophages diminishes post-CSE stimulation. Recent studies have elucidated reduced PPAR γ levels in both COPD-afflicted mice and human patients compared to healthy counterparts. Upon activation, PPAR exhibits anti-inflammatory properties in the context of COPD (29,30). Aligning with these findings, our prior research authenticated that cigarette smoke suppresses PPAR γ expression in macrophages (25). Such concordance buttresses the notion that COPD onset correlates with a decline in macrophage PPAR γ expression. Intriguingly, our observations also highlight the phenomenon that EM administration elevates PPAR γ expression in CSE-stimulated macrophages. Application of GW9662 (a PPAR γ

inhibitor) diminishes PPAR γ expression in CSE-challenged macrophages, whereas EM counters this effect. This pattern accentuates EM's potential to safeguard against PPAR γ degradation, offering protective implications.

Our analysis denotes that ROS levels in human macrophages augment upon CSE stimulation, especially when pre-treated with the PPAR γ inhibitor, GW9662. Evidence suggests that PPAR γ agonists can thwart ROS generation incited by cigarette smoke (31). Such findings emphasize the intimate association between PPAR γ and oxidative stress. The conspicuous ROS elevation upon cigarette smoke exposure, coupled with a pronounced PPAR γ downturn, suggests that excessive ROS might be instrumental in inhibiting PPAR γ expression. Introducing GW9662 in conjunction with EM resulted in a further decline of ROS levels in CSE-stimulated macrophages, accompanied by a resurgence in PPAR γ levels. This pattern elucidates EM's role in ROS inhibition and PPAR conservation, reinforcing its stance as an anti-oxidative stress agent. To delve deeper into the PPAR γ -mediated anti-oxidative stress mechanism, we assessed macrophage MDA and SOD expressions.

Our findings delineate an enhanced expression of MDA in human macrophages post CSE stimulation. Upon application of the PPAR γ inhibitor, GW9662, MDA expression augments in CSE-stimulated macrophages, but this increment is mitigated upon the introduction of EM. MDA serves as a pivotal marker for lipid peroxidation within the cell membrane and stands as the predominant indicator for oxidative stress assessment (5). Elevated oxidative stress markers, such as MDA, have been documented in COPD patients, underscoring the importance of curbing lipid peroxidation in COPD management (32). Literature suggests that PPAR γ is implicated in cellular lipid metabolism and manifests anti-inflammatory as well as anti-fatty acid oxidation properties (33). Collectively, these insights posit that PPAR γ 's anti-fatty acid oxidation effect is intertwined with MDA modulation, implying that PPAR γ potentially mitigates oxidative stress by inhibiting MDA expression.

Our analysis further reveals a diminished expression of SOD in human macrophages following CSE exposure. The introduction of the PPAR γ inhibitor, GW9662, further suppresses SOD expression in CSE-stimulated macrophages, yet this suppression is countered by subsequent EM administration. SOD is recognized as a vital antioxidant enzyme pivotal for shielding cells from oxidative detriment (34). It has been reported that SOD expression

undergoes downregulation, both transcriptionally and translationally, in PPAR γ gene knockout mice, thereby amplifying oxidative stress levels. Promoter analyses have indicated SOD as a direct target of PPAR γ (35). These revelations suggest that SOD may operate as the direct modulatory target through which PPAR γ exerts its anti-oxidative effects.

In this study, we found that PPAR γ played an antioxidant role by inhibiting the expression of MDA and promoting the expression of SOD. Cigarette smoke induced oxidative stress by inhibiting PPAR γ pathway, which might be one of the mechanisms of oxidative stress induced by cigarette smoke. EM inhibited oxidative stress by activating PPAR γ pathway, which might be the potential mechanism of EM in treating COPD. However, there are some limitations in this experiment, such as the lack of PPAR γ agonist and animal experiments. In the future, we will add PPAR γ agonists and animal experiments to further explore the mechanism of PPAR γ in anti-oxidative stress.

Conclusions

To sum up, our results support that PPAR γ can inhibit oxidative stress reaction by reducing the expression of MDA and increasing the expression of SOD. Cigarette smoke activates oxidative stress by regulating PPAR γ pathway, which may be an important target for cigarette smoke to activate oxidative stress. EM inhibits oxidative stress by activating PPAR γ pathway, which provides a new research target for macrolides in the treatment of COPD.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1647/rc>

Data Sharing Statement: Available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1647/dss>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1647/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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