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Peroxisome Proliferator-Activated Receptor (PPAR γ) Plays a Protective Role in Cigarette Smoking-Induced Inflammation via AMP-Activated Protein Kinase (AMPK) Signaling

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Cigarette smoking is a well-known risk factor in multiple chronic pulmonary diseases. This study aims to investigate the role of peroxisome proliferator-activated receptor (PPAR) γ in cigarette smoking-induced inflammation.

Material/Methods: Cigarette smoking extract (CSE) was employed to induce inflammation in bronchial epithelial cells (BECs). After CSE administration, several autophagy-related proteins (Beclin1, autophagy-related gene (ATG)5, ATG7, p62, and LC3) and PPAR γ levels were examined by western blot. Subsequently, PPAR γ agonists and antagonist were used to treat CSE-induced BECs, several inflammatory factors (interleukin (IL)-6, IL-8, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2) and autophagy-related proteins were detected to measure the inflammatory and autophagy levels. Then LC3 knockdown was performed to verify the role of autophagy in CSE-induced inflammation. Finally, the AMP-activated protein kinase (AMPK) and its downstream S6 kinase (S6K) were detected in CSE-stimulated BECs.

Results: CSE administration caused insufficient autophagy and the decrease of PPAR γ in BECs. The PPAR γ agonists ameliorate the CSE-induced inflammation and promote the autophagy development, evidenced by the changes of inflammatory factors and autophagy-related proteins. Loss-of-function experiments demonstrated that the PPAR γ played an anti-inflammatory role in an autophagy-dependent manner. In addition, CSE administration inactivated the AMPK signaling, which was restored by PPAR γ agonists. The effects of PPAR γ agonists on inflammation and autophagy could be abolished by AMPK inhibitor.

Conclusions: We demonstrated that PPAR γ played a protective role in CSE-induced inflammation and autophagy by activating AMPK signaling in BECs, which may provide investigation basis for clinical therapy of chronic pulmonary diseases.

MeSH Keywords: **AMP-Activated Protein Kinases • Autophagy • Inflammation • PPAR gamma • Smoking**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/909285>

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Background

Chronic obstructive pulmonary disease (COPD) is a common chronic pulmonary disease, causing 2.9 million deaths every year in the world [1]. COPD is characterized by persistent respiratory symptoms and airflow limitation, involves obstructive bronchiolitis and emphysema, and may result in respiratory failure and cardiovascular diseases [2]. The pathogenesis has not been completely elucidated, however, several risk factors have been identified, including smoking. There is a higher prevalence of respiratory symptoms and pulmonary dysfunction, a more obvious decline of forced expiratory volume in 1 sec (FEV₁) and a greater mortality among cigarette smokers than nonsmokers [3]. Passive cigarette smoking exposure causes experimental inflammation in animal pulmonary tissues and bronchial epithelial cells (BECs) [4,5].

Autophagy is a metabolic process that mediates degradation and recycle of cytoplasmic components. Autophagy is very important for maintenance of homeostasis as well as development [6]. It also performs an important role in pulmonary epithelial injury [7]. In the process of autophagy, LC3 plays a critical role in autophagosome formation. LC3 is cleaved to LC3 I by proteolytic at C-terminus. Then LC3 I binds with phosphatidylethanolamine anchored to the phagophore membranes, therefore producing LC3 II [8]. P62, as an autophagy receptor, interacts with ubiquitinated substrates and LC3 to deliver ubiquitinated proteins to degradation. P62 is thereby trapped within autophagosome and ultimately degraded [9]. Thus, this molecule is often used to monitor autophagy, and accumulating p62 is regarded as an indicator of insufficient autophagy.

It has been reported that peroxisome proliferator-activated receptor- γ (PPAR γ) is decreased in pulmonary tissues of COPD patients and cigarette smoking extract (CSE)-induced rats, and the PPAR γ agonist ameliorates CSE-induced inflammation and alveolar cell apoptosis in rats [10,11]. PPAR γ , a member of nuclear hormone receptor superfamily, plays anti-inflammatory and antioxidant roles via multiple mechanisms [11,12]. CSE also causes insufficient autophagy in BECs, and inhibition of the insufficient autophagy enhances the CSE-induced inflammation and senescence in BECs [13]. In another paper of this team, they use the sirtuin 6 to induce complete autophagy in BECs, and the autophagy activation attenuated the CSE-induced senescence in BECs [14]. In addition, PPAR γ agonist induces autophagy activation and promotes the degradation of p62 in pancreatic β cells [15], and the PPAR γ deficient suppresses autophagy and induces chondrocyte apoptosis in mice [16].

Nevertheless, the relationship between PPAR γ agonist-attenuated emphysema symptoms and autophagy remains unclear. In this study, we used CSE to treat BECs to mimic cigarette smoking-induced inflammation *in vivo*, to investigate

the roles of PPAR γ and autophagy, and explored the underlying mechanisms.

Material and Methods

CSE preparation

CSE was prepared according to the previously described [11]. Two filtered cigarettes were smoked using a peristaltic pump, and the cigarette smoke was bubbled through 20-mL serum free Keratinocyte medium. The solution was filtered through a 0.45- μ m pore membrane (Millipore, Boston, MA, USA), and regarded as 10% CSE solution.

Cell culture

Human bronchial epithelial cell line 16HBE was purchased from Zhongqiaoxinzhou (Shanghai, China), culture with Keratinocyte medium at 37°C in 5% CO₂. After adhered to the plates, the cells were transfected with LC3 siRNA or negative control (NC) sequence. The 16HBE cells were treated with PPAR γ agonist rosiglitazone or troglitazone, antagonist bisphenol A diglycidyl ether (BADGE) (Aladdin, Shanghai, China), or AMPK inhibitor compound C (MCE, NJ, USA).

RNA extraction, reverse transcription and real-time PCR

The total RNA was extracted with a TRIpure kit (BioTeke, Beijing, China) and reversely transcribed into cDNA with M-MLV reverse transcriptase (BioTeke) in the presence of Oligo(dT) and random primers (Sangon, Shanghai, China).

After concentration measurement, the cDNA was used for real-time PCR with 2 \times Power Taq PCR Master Mix (BioTeke) and SYBR Green (Solarbio, Beijing, China) to detect the mRNA level of PPAR γ , with β -actin as the internal control. The procedure was set as follow: 94°C for 5 min, 94°C for 10 sec, 60°C for 20 sec, 72°C for 30 sec, followed with 40 cycles of 72°C for 2 min 30 sec, 40°C for 1 min 30 sec, melting from 60°C to 94°C each 1°C for 1 sec, and finally incubated at 25°C for several minutes. The real-time PCR primers were purchased from Sangon, and the sequence information are shown in Table 1. The data was analyzed with 2^{- $\Delta\Delta$ Ct} method.

Western blot

The cellular protein was extracted with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China), separated with SDS-PAGE, and transferred onto PVDF membrane (Millipore). After blocking with 5% skim milk for 1 hour, the membrane was incubated with 1 of the following antibodies at 4°C overnight: rabbit anti-PPAR γ (Bioss, Beijing, China; 1: 500), rabbit anti-Beclin1

Table 1. Sequence information of real-time PCR primers used in this study.

Name	Sequence (5'-3')
PPAR γ F	5'-GGAGCAGAGCAAAGAGGT-3'
PPAR γ R	5'-TTGGTCGTTCAAGTCAAGAT-3'
β -actin F	5'-CCATCGTCCACCGCAAAT-3'
β -actin R	5'-GCTGTCACCTTCACCGTTC-3'

PPAR γ – peroxisome proliferator-activated receptor γ ;
F – forward; R – reverse.

(CST, Boston, MA, USA; 1: 1000), rabbit anti-autophagy-regulated gene (ATG)5 (CST; 1: 1000), rabbit anti-ATG7 (CST; 1: 1000), rabbit anti-microtubule associated protein 1 light chain 3 (LC3) (Proteintech, Wuhan, Hubei, China; 1: 1000), rabbit anti-p62 (CST; 1: 1000), rabbit anti-inducible nitric oxide synthase (iNOS) (Bioss; 1: 500), rabbit anti-cyclooxygenase (COX)-2 (Proteintech; 1: 1000), rabbit anti-AMP-activated protein kinase (AMPK) (Abcam, Cambridge, UK; 1: 2000), rabbit anti-p-AMPK (Abcam; 1: 2000), rabbit anti-S6 kinase (S6K) (CST; 1: 1000), rabbit anti-p-S6K (CST; 1: 1000), mouse anti- β -actin (KeyGEN, Nanjing, Jiangsu, China; 1: 500). After rinsing with TBST, the membrane was incubated with the corresponding secondary antibody at 37°C for 45 min, followed with a signal exposure with ECL reagent. β -actin served as the cellular internal control.

Immunofluorescence

The cells were cultured on glass slides to a confluence of 70–80%, and fixed with 4% paraformaldehyde (Sinopharm, Beijing, China) for 15 min. After permeation with 0.1% tritonX-100 (Sinopharm), the cells were blocked with goat serum for 15 min, and incubated with antibody against iNOS (Bioss; 1: 200) or COX-2 (Proteintech; 1: 200) at 4°C overnight. Subsequently, the cells were incubated with Cy3-labeled IgG (Beyotime), counterstained with DAPI, mounted with antifading reagent (Solarbio), and finally observed under a microscope at 400 \times magnification.

ELISA

The content of interleukin (IL)-6 and IL-8 in the culture supernatant was detected with an IL-6 ELISA kit or IL-8 ELISA kit (USCN, Wuhan, Hubei, China) according to the manufacturer's protocols.

Statistical analysis

Data in this study were presented as mean \pm SD and analyzed by one-way or two-way ANOVA test with post hoc Bonferroni's multiple comparisons. A *P* value was considered as statistically

significant when it was less than 0.05. (* *P*<0.05, ** *P*<0.01, *** *P*<0.001, ns, no significance)

Results

CSE induced the decrease of PPAR γ and insufficient autophagy in BECs

Cigarette smoking is well-known to induce bronchial inflammation, so the CSE was prepared from cigarettes and used to treat 16HBE cells. After application of 1% or 5% CSE, the mRNA and protein levels of PPAR γ showed significant decrease in 16HBE cells (Figure 1A). The expression levels of several autophagy-related protein, Beclin1, ATG5, ATG7, and p62, were increased after CSE treatment (Figure 1B, 1C), and the transition of LC3 I to LC3 II was also enhanced, suggesting the autophagy activation. Subsequently, the 16HBE cells were treated with 1% CSE for different hours. As shown in Figure 1D and 1E, the mRNA and protein levels of PPAR γ were decreased upon CSE stimulation, bottomed at 12 hours, and increased from 24 hours post CSE treatment (Figure 1D, 1E), suggesting a protective role after CSE stimulation. The autophagy-related protein levels were increased in a time-dependent manner (Figure 1F, 1G). Nevertheless, the p62 protein is also a substrate of autophagy, and degraded in the later stage [17]. The increase of p62 in CSE-treated BECs suggested that the CSE-induced autophagy was insufficient.

PPAR γ agonists suppressed CSE-induced inflammation and enhanced the autophagy progress in BECs

Since the initial decline and compensatory increase of PPAR γ after CSE treatment, the PPAR γ agonists rosiglitazone (10 μ M) and troglitazone (5 μ M), or antagonist BADGE (100 μ M), were used to activate or inactivate the PPAR γ after 1% CSE stimulation. As shown in Figure 2A and 2B, the CSE-induced IL-6 and IL-8 secretion increase was attenuated by rosiglitazone and troglitazone but aggravated by BADGE (Figure 2A, 2B). The expression levels of two inflammation-related proteins, iNOS and COX-2, were increased by CSE stimulation, which was attenuated by rosiglitazone or troglitazone, and augmented by BADGE in 16HBE cells (Figure 2C, 2D), suggesting that CSE-induced inflammation was inhibited by PPAR γ agonists, and promoted by PPAR γ antagonist. The levels of autophagy-related protein, Beclin1, ATG5, ATG7, and LC3 II/I, were showed similar changes to inflammatory factors (Figure 2E, 2F). However, the p62 level was decreased after PPAR γ agonist treatment, and increased after PPAR γ antagonist treatment, suggested that the PPAR γ activation promoted the autophagy development.

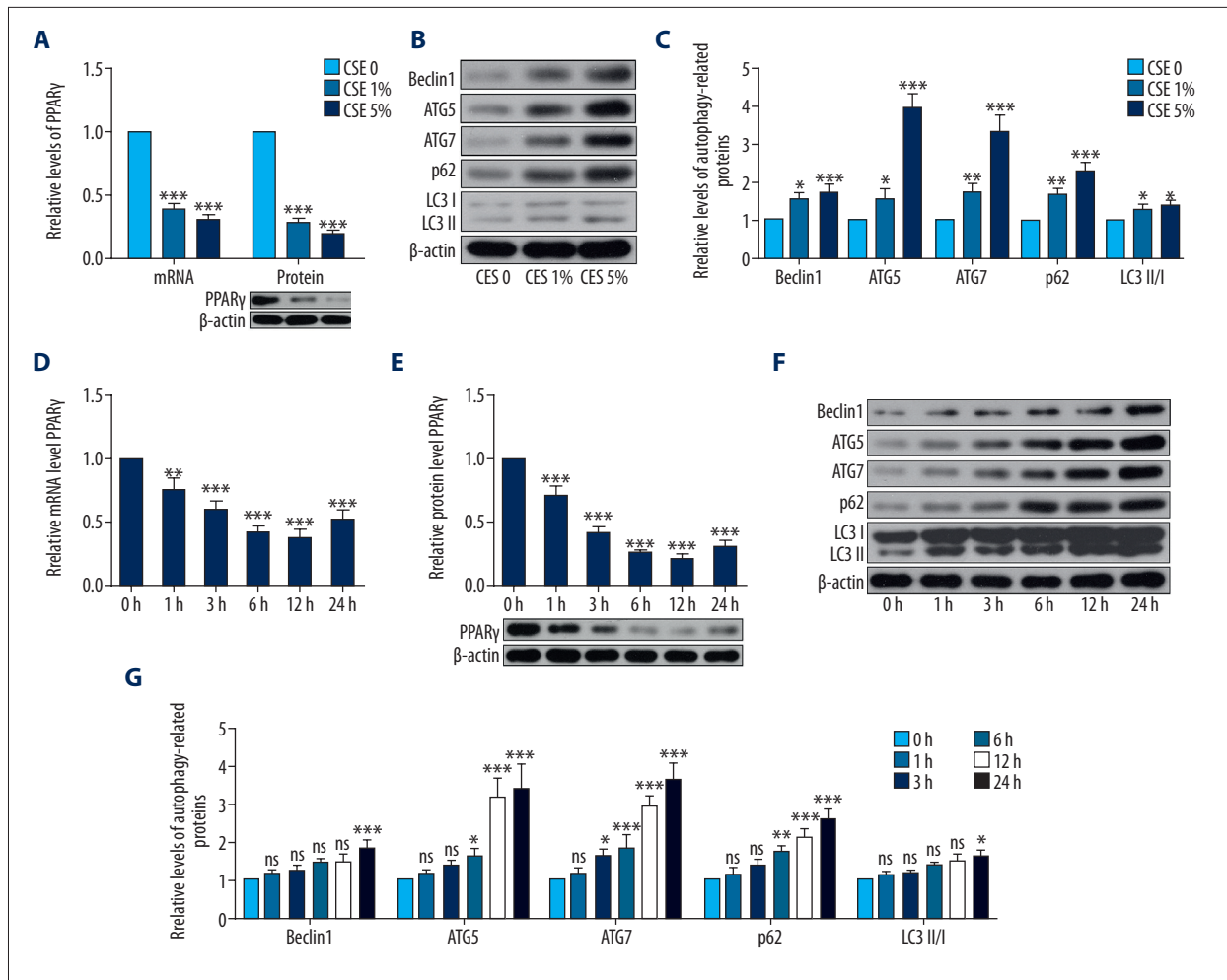


Figure 1. CSE induced the decrease of PPAR γ and insufficient autophagy in BECs. (A) The mRNA and protein levels of PPAR γ in 16HBE cells after treatment with CSE of different concentrations for 6 hours were detected by real-time PCR and western blot. (B, C) The expression levels of several autophagy-related proteins in 16HBE cells after treatment of CSE for 6 hours were detected by western blot. (D) The mRNA level of PPAR γ in 16HBE cells after treatment of 1% CSE for different hours was detected by real-time PCR. (E) The protein levels of PPAR γ in 16HBE cells after treatment of 1% CSE for different hours were detected by western blot. (F, G) The expression levels of several autophagy-related proteins in 16HBE cells after treatment of 1% CSE for different hours were detected by western blot. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significance, compared with CSE 0 or 0 hours group) (CSE – cigarette smoking extract; PPAR γ – peroxisome proliferator-activated receptor γ ; BECs – bronchial epithelial cells)

PPAR γ inhibited CSE-induced inflammation in an autophagy-dependent manner

The previous results demonstrated that CSE administration caused inflammation and insufficient autophagy in 16HBE cells, and the PPAR γ activation attenuated the inflammation and enhanced the autophagy. However, the relationship between inflammation and autophagy remained unclear. Therefore, the 16HBE cells were transfected with an LC3 siRNA sequence to inhibit autophagy. As shown in Figure 3A, after LC3 silencing, the LC3 levels were decreased (both I and II types, and LC3 II more obviously) and p62 level was increased (Figure 3A, 3B),

suggesting the blocked autophagy. The ELISA results showed that the secretion of IL-6 and IL-8 in the LC3-silenced cells was significantly inhibited, compared to NC cells (Figure 3C, 3D). The immunofluorescence and immunoblotting results revealed that the expression levels of iNOS and COX-2 were reduced by rosiglitazone or troglitazone administration, but the reduction was abolished after LC3 silencing (Figure 3E–3H). The results in this section revealed that the protective effect of PPAR γ on CSE-induced inflammation were depended on autophagy activation.

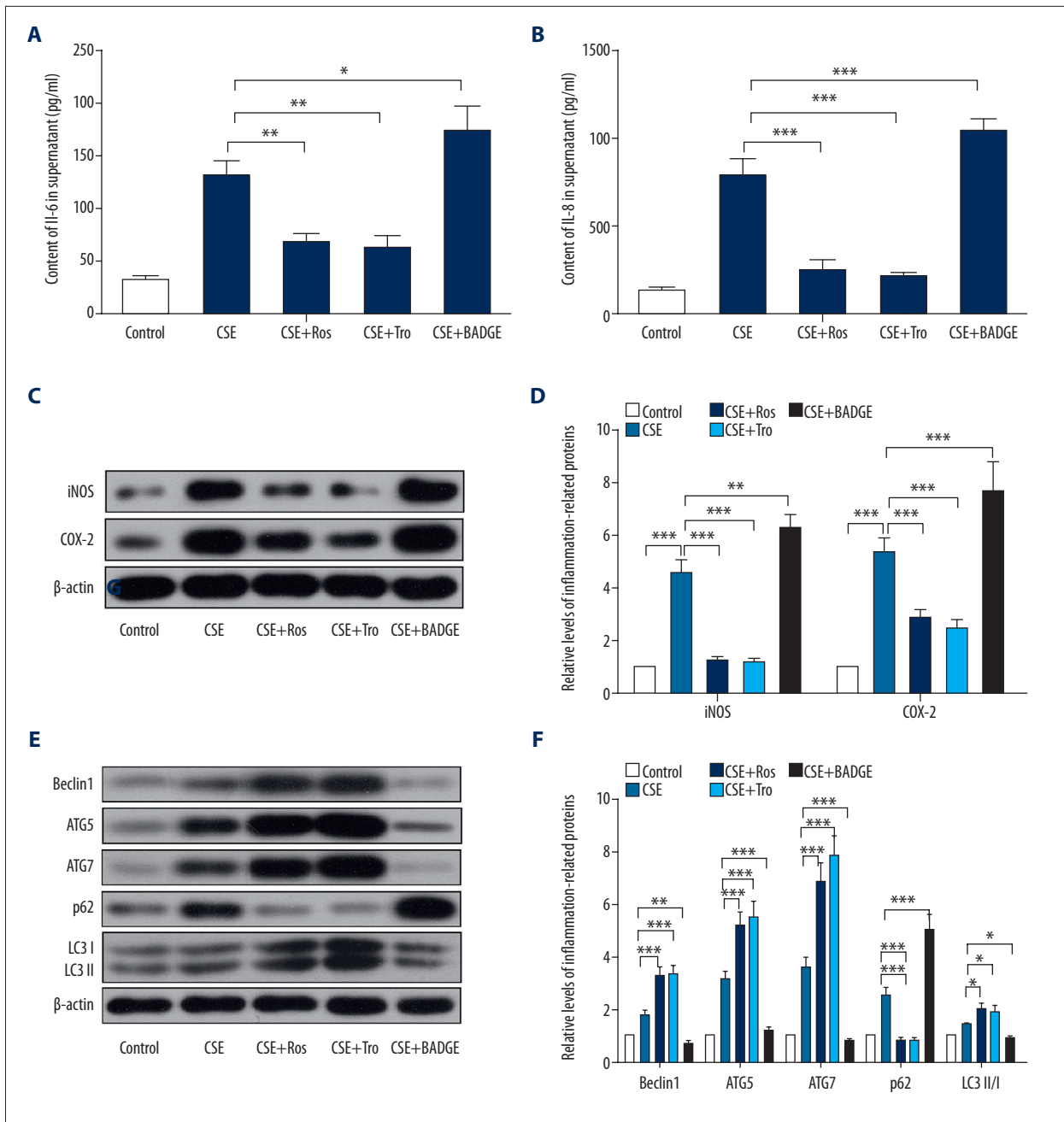


Figure 2. PPAR γ agonists suppressed CSE-induced inflammation and enhanced the autophagy progress in BECs. (A, B) The content of IL-6 and IL-8 in 16HBE cell culture supernatant was detected by ELISA kits after treatment of 10 μ M rosiglitazone/5 μ M troglitazone/100 μ M BADGE for 24 hours. (C, D) The expression levels of iNOS and COX-2 in 16HBE cells after treatment of 1% CSE and 10 μ M rosiglitazone/5 μ M troglitazone/100 μ M BADGE for 24 hours were detected by western blot. (E, F) The expression levels of several autophagy-related proteins in 16HBE cells after treatment of 1% CSE and 10 μ M rosiglitazone/5 μ M troglitazone/100 μ M BADGE for 24 hours were detected by western blot. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significance) (CSE, cigarette smoking extract; PPAR γ – peroxisome proliferator-activated receptor γ ; BECs – bronchial epithelial cells; IL – interleukin; iNOS – inducible nitric oxide synthase; COX-2 – cyclooxygenase-2; BADGE – bisphenol A diglycidyl ether).

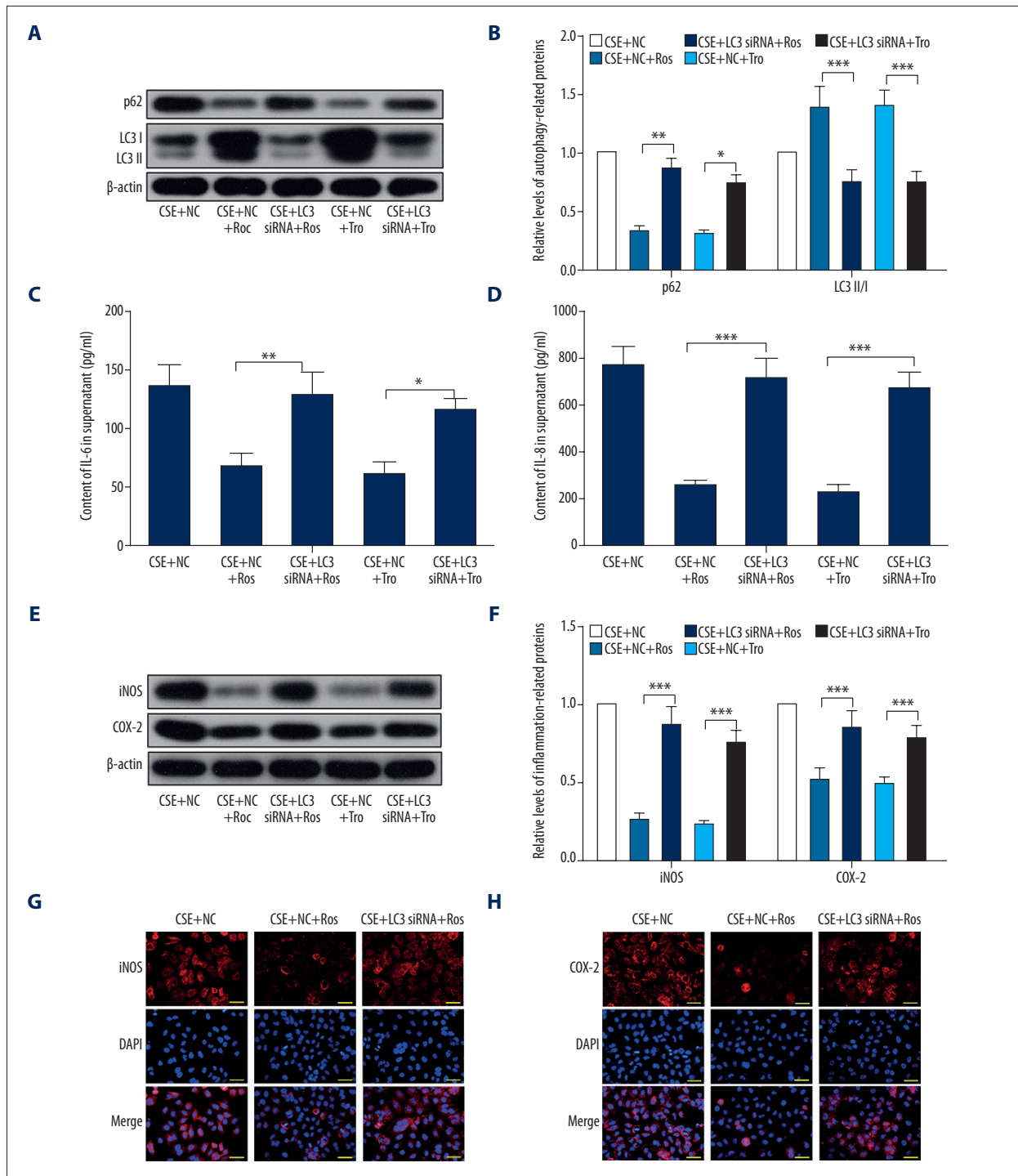


Figure 3. PPAR γ inhibited CSE-induced inflammation in an autophagy-dependent manner. **(A, B)** The expression levels of p62 and LC3 in 16HBE cells after treatment of 10 μ M rosiglitazone/5 μ M troglitazone or/and LC3 siRNA for 24 hours were detected by western blot. **(C, D)** The content of IL-6 and IL-8 in 16HBE cell culture supernatant was detected by ELISA kits after treatment of 10 μ M rosiglitazone/5 μ M troglitazone or/and LC3 siRNA for 24 hours. **(E, F)** The expression levels of iNOS and COX-2 in 16HBE cells after treatment of 10 μ M rosiglitazone/5 μ M troglitazone or/and LC3 siRNA for 24 hours. **(G, H)** The immunofluorescence was performed to detect the expression of iNOS and COX-2 in 16HBE cells after treatment of 10 μ M rosiglitazone/5 μ M troglitazone or/and LC3 siRNA for 24 h (the scale presented 50 μ m). (* P <0.05, ** P <0.01, *** P <0.001, ns, no significance) (CSE – cigarette smoking extract; PPAR γ – peroxisome proliferator-activated receptor γ ; IL – interleukin; iNOS – inducible nitric oxide synthase; COX-2 – cyclooxygenase-2).

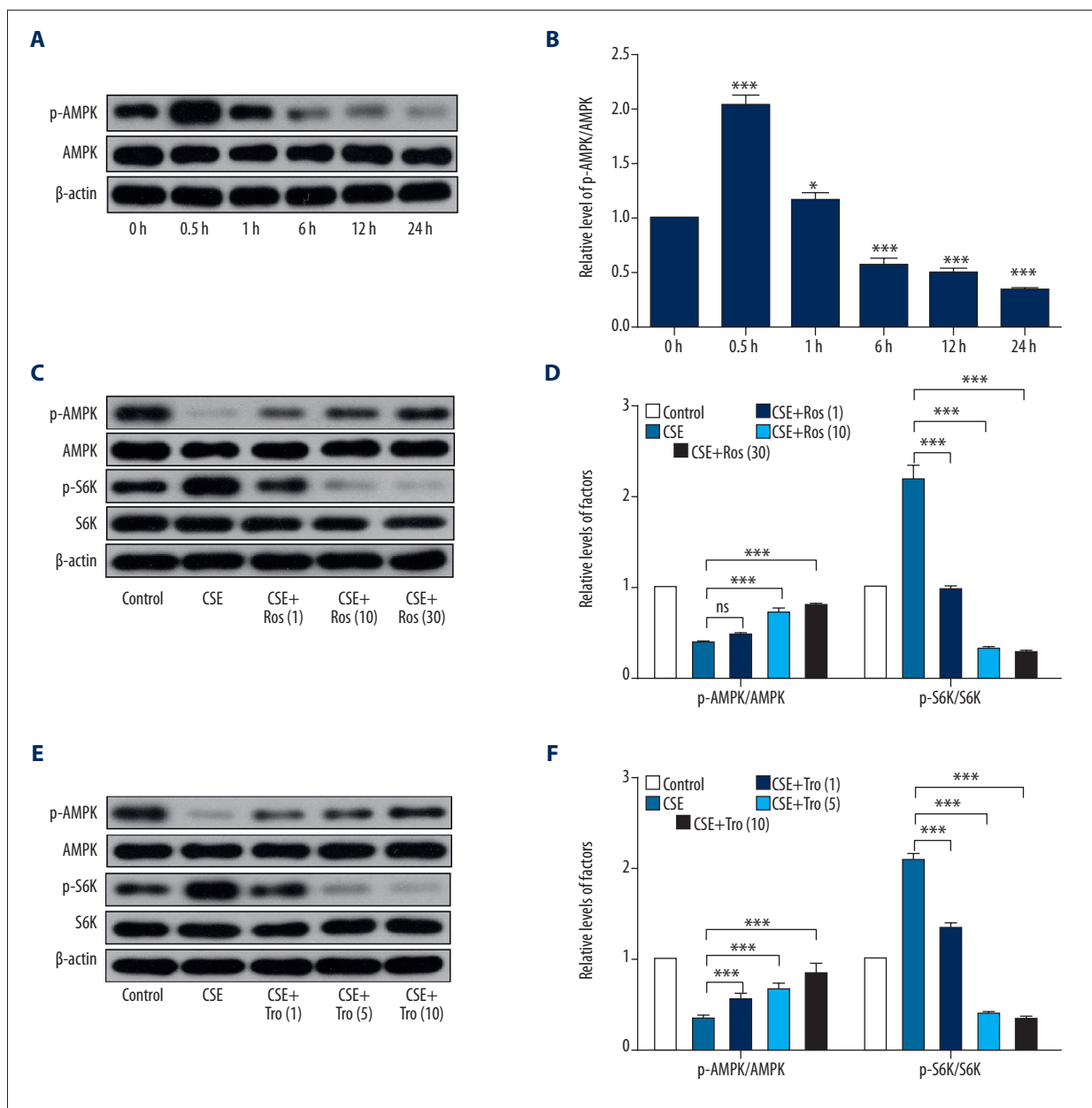


Figure 4. PPAR γ agonist activated the AMPK signaling. (A, B) The levels of p-AMPK and AMPK in 16HBE cells after treatment of 1% CSE for different hours were detected by western blot. (C, D) The levels of p-AMPK, AMPK, p-S6K, S6K in 16HBE cells after treatment of rosiglitazone (1, 10, 30 μ M) were detected by western blot. (E, F) The levels of p-AMPK, AMPK, p-S6K, and S6K in 16HBE cells after treatment of troglitazone (1, 5, and 10 μ M) were detected by western blot. (* $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$, ns, no significance) (CSE – cigarette smoking extract; PPAR γ – peroxisome proliferator-activated receptor γ ; AMPK – AMP-activated protein kinase; S6K – S6 kinase)

PPAR γ inhibited the CSE-induced inflammation via the AMPK signaling

Next the AMPK and its downstream regulator, S6K, were detected. As shown in the Figure 4, after transient elevation, the AMPK phosphorylation was declined upon CSE stimulation in 24 hours (Figure 4A, 4B). Then PPAR γ agonist, rosiglitazone or troglitazone,

was used to treat the 16HBE cells. After treatment of rosiglitazone (1, 10, or 30 μ M) for 24 hours, the CSE-induced downregulation of p-AMPK was restored in various degrees (Figure 4C, 4D). The phosphorylation level of S6K was upregulated by CSE administration, which was ameliorated by rosiglitazone treatment (Figure 4C, 4D). The similar changes of AMPK and S6K were observed after troglitazone application (1, 5, or 10 μ M) (Figure 4E, 4F).

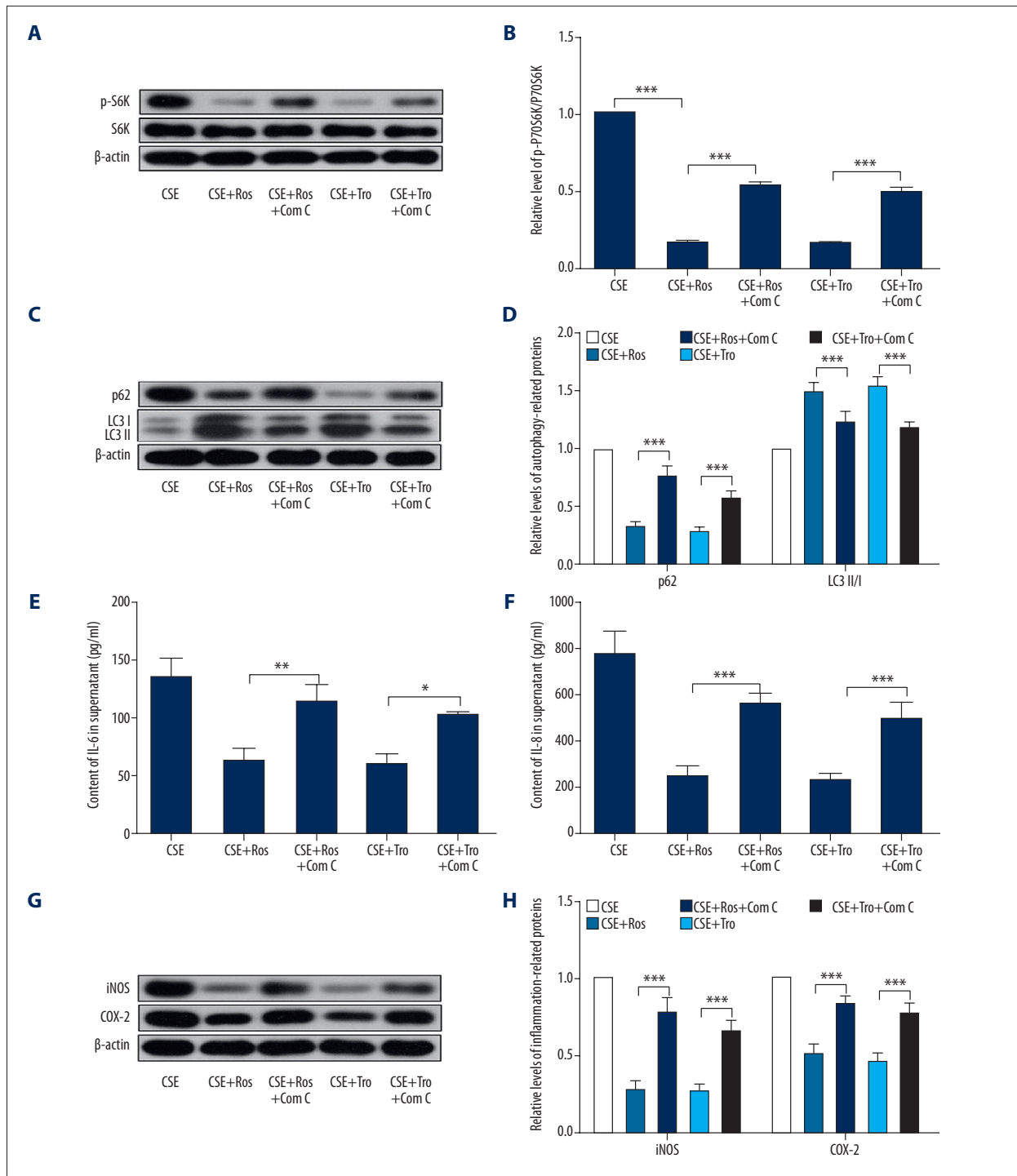


Figure 5. The effect of PPAR γ agonist on inflammation and autophagy was abolished by the AMPK inhibitor. **(A, B)** The levels of p-S6K and S6K in 16HBE cells after treatment of rosiglitazone/troglitazone or/and compound C were detected by western blot. **(C, D)** The levels of p62 and LC3 in 16HBE cells after treatment of rosiglitazone/troglitazone or/and compound C were detected by western blot. **(E, F)** The content of IL-6 and IL-8 in 16HBE cell culture supernatant after treatment of rosiglitazone/troglitazone or/and compound C was detected by ELISA kits. **(G, H)** The levels of iNOS and COX-2 in 16HBE cells after treatment of rosiglitazone/troglitazone or/and compound C were detected by western blot. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significance) (CSE – cigarette smoking extract; PPAR γ – peroxisome proliferator-activated receptor γ ; IL – interleukin; iNOS – inducible nitric oxide synthase; COX-2 – cyclooxygenase-2; AMPK – AMP-activated protein kinase; S6K – S6 kinase).

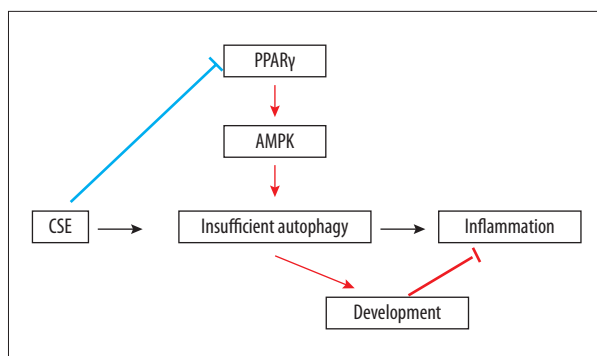


Figure 6. The role of PPAR γ in cigarette smoking-induced inflammation.

To verify the effect of AMPK on CSE-induced inflammation, an AMPK inhibitor, compound C (5 μ M), was used to treat 16HBE cells. As shown in Figure 5, the rosiglitazone or troglitazone-caused decreased in S6K phosphorylation was reversed by compound C in 16HBE cells (Figure 5A, 5B). Similarly, rosiglitazone- or troglitazone-induced autophagy was suppressed by compound C (Figure 5C, 5D). In addition, rosiglitazone- or troglitazone-induced decreased secretion of IL-6 and IL-8, and the downregulation of iNOS and COX-2 expression levels, were all recovered to some extent after compound C administration (Figure 5E–5H). The results in this section suggested that PPAR γ ameliorated the CSE-induced inflammation by inhibiting AMPK signaling (Figure 6).

Discussion

PPARs are members of nuclear hormone receptor family, including 3 isoforms: PPAR α , PPAR β , and PPAR γ [18], wherein the PPAR γ is the most extensively studied. PPARs are ligand-activated transcription factors, they react as heterodimers with retinoid X receptors (RXRs) and recognize PPAR response elements in the promoter of target genes that consist of repeat sequence “AGGTCA” separated by 1 nucleotide called DR-1 [19,20]. PPARs could be activated by multiple endogenous or pharmacological ligands, and rosiglitazone and troglitazone have been identified as PPAR γ agonists [18]. In our study, CSE administration caused inflammation and decreased PPAR γ in 16HBE cells. Further treatment with PPAR γ agonists ameliorated the CSE-induced inflammation. PPAR γ was also reported to regulate inflammatory reaction in asthma patients and

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experimental colitis mice [21,22]. It has been shown that autophagy plays opposite roles in different environments [23,24]. In CSE-treated 16HBE cells, loss-of-function experiments demonstrated that autophagy activation inhibited inflammatory and promoted cellular survival. In our study, CSE administration also induced insufficient autophagy, and PPAR γ activation promoted autophagy development. Notably, PPAR γ 's anti-inflammatory role is autophagy-dependent.

In addition, we also found that PPAR γ agonists enhanced the AMPK phosphorylation. AMPK is a serine/threonine kinase, comprised of 1 catalytic subunit α , and 2 regulatory subunits, β and γ [25]. The heterotrimer could be activated by AMPK kinase via phosphorylation [26]. AMPK cascade plays significant roles in glucose and lipid metabolism, cell growth and apoptosis, autophagy and inflammation [27]. CSE administration was reported to induce IL-8 production and AMPK phosphorylation in macrophages, and the AMPK inhibitor attenuated inflammation induced by CSE [28]. Whereas, in our study, we found the phosphorylation of AMPK α was transiently increased within 1-hour post CSE treatment, and then rapidly decreased, indicating a compensation response. Additionally, it has been reported that PPAR γ agonist activated AMPK, and then inhibited mTOR signaling and dephosphorylated a downstream factor, S6K [29]. Consistent with this report, in our study, we also found that after treatment of rosiglitazone or troglitazone, AMPK phosphorylation level was increased and S6K phosphorylation level was decreased, indicating that the decreased activity of AMPK was restored by PPAR γ agonists. Moreover, the inhibition of PPAR γ suppressed the CSE-induced autophagy, which is beneficial for the alleviation of inflammation.

Conclusions

In this study, we demonstrated that PPAR γ agonists ameliorated the CSE-induced inflammation by activating AMPK signaling in 16HBE cells, and the effects of PPAR γ agonists depended on autophagy. These findings may provide investigation basis for clinical therapy of COPD.

Conflict of interest

None.

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