

GSK-3 β Inhibitors Attenuate the PM_{2.5}-Induced Inflammatory Response in Bronchial Epithelial Cells

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Background and Purpose: PM_{2.5}-associated airway inflammation has recently been recognized as pivotal to the development of COPD. Aberrant glycogen synthase kinase (GSK)-3 β signaling is linked to the inflammatory response. Therefore, we investigated the effects of GSK-3 β inhibitors on the PM_{2.5}-induced inflammatory response in bronchial epithelial cells.

Methods: The production of phosphorylated GSK-3 β (p-GSK-3 β) was analyzed by immunohistochemistry with PM_{2.5}-induced mice. HBECs were treated with various inhibitors targeting GSK-3 β or JNK before PM_{2.5} stimulation. The production of GSK-3 β signaling was analyzed by Western blotting. Inflammatory cytokine production was detected by qRT-PCR and ELISA.

Results: PM_{2.5} exposure caused lung inflammation, upregulated serum concentrations of HMGB1 and IL-6, decreased IL-10 expression, and significantly attenuated p-GSK-3 β production in mice. HBECs exposed to PM_{2.5} showed significantly reduced p-GSK-3 β production, an increased ratio of p-JNK/JNK, increased NF- κ B activation and I κ B degradation, and upregulated the inflammatory cytokines HMGB1 and IL-6. Intervention with GSK-3 β inhibitors TDZD-8 and SB216763 significantly suppressed PM_{2.5}-induced outcomes. Moreover, the JNK inhibitor SP600125 also reduced the level of NF- κ B phosphorylation induced by PM_{2.5}. The differences in the levels of inflammation-related cytokines in the TDZD-8 groups were greater than those in the SB216763 groups.

Conclusion: Inhibition of GSK-3 β weakens the PM_{2.5}-induced inflammatory response by regulating the JNK/NF- κ B signaling pathway in bronchial epithelial cells.

Keywords: PM_{2.5}, COPD, GSK-3 β , inflammatory response, HBECs

Introduction

With the increasing speed of urbanization and industrialization, environmental pollution caused by PM_{2.5} has challenged public health and resulted in an enormous economic burden. Many epidemiological studies have shown that PM_{2.5} exposure is correlated with an enhanced risk of chronic obstructive pulmonary disease (COPD),^{1,2} which is characterized by progressive fixed airflow because of airway inflammation.³ The rates of morbidity and mortality of COPD have gradually increased over the past decade. Therefore, there is an urgent need to study the cellular mechanisms of PM_{2.5}-induced COPD.

Published studies have indicated that abnormal glycogen synthase kinase-3 (GSK-3) activity contributes to the pathological processes of COPD.⁴ GSK3 is a serine-threonine protein kinase that is constitutively active in cells and has two isoforms in mammals: GSK-3 α and GSK-3 β . Phosphorylation of an N-terminal

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serine residue (Ser9) in GSK-3 β suppresses GSK-3 activity and attenuates its activity to change cell function.⁵ GSK-3 β is a contributing factor in regulating the activity of numerous factors, including c-Jun,⁶ β -catenin,⁷ NF- κ B and STAT.⁸ Recently, GSK-3 β has emerged as a key regulator of the inflammatory response,⁹ and it may represent an important downstream effector of oxidant-mediated signaling during COPD inflammation.¹⁰ Moreover, an increasing number of studies have shown that PM2.5 exposure increases airway inflammatory cytokine production in COPD. Such discoveries are the basis for the hypothesis that GSK-3 β may play an important role in regulating the airway inflammation caused by PM2.5 in COPD.

Several previous studies showed that GSK-3 β inhibition alleviated the inflammatory response induced by a wide range of extracellular stimuli.¹¹ However, it is currently uncertain whether GSK-3 β inhibitors could suppress the airway inflammation of COPD caused by PM2.5. As the first line of defense against inhaled harmful particles, HBECs play a key role in the development of COPD.¹² Here, different GSK-3 β inhibitors, the small-molecule ATP competitive inhibitor SB216763 and the non-ATP competitive inhibitor TDZD-8, were employed to intervene in the GSK-3 β pathway in HBECs.

Materials and Methods

Cell Culture and Treatment

The 16HBE human bronchial epithelial cell line was obtained from Guangzhou Medical University (Guangzhou, China), an agent of ATCC. The 16HBECs were treated with DMEM (Sigma–Aldrich, Louis, USA) supplemented with 10% FBS and 1% penicillin (Sigma–Aldrich, Louis, USA), and then, the cells were pretreated with TDZD-8, SB216763 or SP600125 (Sigma–Aldrich, Louis, USA) for 30 mins and treated with different concentrations of PM2.5 (5–20 μ g/mL).

Cell Counting Kit-8 (CCK8) Cell Viability Assay

HBECs were treated with PM2.5 (5, 10, 20, or 40 μ g/mL) for 24 hours. HBEC viability was measured by CCK-8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions, and the optical density (OD) was measured at 490 nm.

Collection and Extraction of PM2.5

The details of the PM2.5 collection and analysis methods were obtained as previously described.¹³ Briefly, the collection of traffic ambient PM2.5 was prepared with aerodynamic impactors, and the extracts were obtained with DMSO. The physicochemical characterizations were analyzed through gravimetric analysis. The mean concentrations of polynuclear aromatic hydrocarbons (PAHs) and n-alkanes in PM2.5 were 108.453 μ g/g and 18,670.883 μ g/g, respectively, with a final PAH recovery of 44.62%; the mean concentrations of these DMSO extracts were 48.392 μ g/g and 164.675 μ g/g, respectively.

Animals and Sample Lung Tissues

Twenty female C57BL mice were randomly fed in the Laboratory Animal Center of Guangzhou Medical University. The experiment was approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University and performed according to the regulations designated by the Chinese Association for Laboratory Animal Science Policy. The mice were randomly allocated to the PBS and PM2.5 concentration groups (100 μ g/20 μ L) twice per week for 4 weeks by tracheal drip. Mice were sacrificed by intraperitoneal injection of sodium pentobarbital (100 mg/kg), and then paraffin-embedded lung tissues were stained for hematoxylin and eosin (H&E) and immunohistochemistry using standard techniques.

Histology and Immunohistochemistry

These sections of lung tissue were stained by H&E for morphometric detection. The sections were stained with p-GSK-3 β antibodies (1:100, Santa Cruz, USA) and then with peroxidase (HRP)-conjugated anti-mouse IgG according to the manufacturer's protocols. Expression was evaluated by confocal microscopy at 200 \times magnification. We quantified the OD of p-GSK-3 β -positive cells using a semiquantitative scoring system.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from HBECs was extracted using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. GAPDH served as an internal standard. The primer sequences were as follows: GAPDH, 5'-ATCACTGCCACCCAGAAG-3' and 5'-TCCACGACGGACACATTG-3'; IL-6, 5'-GCCTTCGGTCCAGTTGCC-3' and 5'-GCGCAGAATGAGATGA

GTTGTCATG-3'; HMGB1, 5'-AATACGAAAAGGATATT GCT-3' and 5'-GCGCTAGAACCAACTTAT-3.

Western Blot Analysis

Total proteins were extracted using a protein extraction kit (Invitrogen, Carlsbad, USA), and Western blotting was performed according to the manufacturer's instructions. Antibody concentrations were applied as follows: p-GSK-3 β antibody (1:1000), p-JNK antibody (1:1000), p-NF- κ B p65 antibody (1:1000), I κ B α antibody (1:1000) and β -actin (1:1000, Santa Cruz, USA). The results are expressed relative to the control (control, set as 1.0).

ELISAs

The concentrations of IL-10, IL-6 and HMGB1 (R&D Systems, Minneapolis, MN, USA) in mouse serum and cell culture medium were detected using ELISA kits following the manufacturer's protocol. A standard curve was prepared with standard solution, and the absolute concentrations were detected according to the standard curve.

Statistical Analysis

Statistical analysis was performed with SPSS 25.0 statistical software. Group data are expressed as the means \pm standard deviations (SDs). For data with a normal distribution, comparisons among multiple groups were performed with ANOVAs. Two-group comparisons were performed by Student's *t*-test. $p < 0.05$ was considered to be significant.

Results

PM2.5 Induced Inflammatory Changes and Reduced the Expression of p-GSK-3 β in the Lungs of Mice

As depicted in [Figure 1A](#), H&E staining analysis showed that PM2.5 exposure caused the upregulation of inflammatory cells in the lungs of mice. ELISA results revealed that the expression levels of the inflammatory mediators HMGB1 and IL-6 were significantly higher in the PM2.5 group; however, the expression of the anti-inflammatory cytokine IL-10 displayed an opposite trend compared with that of HMGB1 and IL-6 in the serum ([Figure 1B](#)). Moreover, immunostaining and Western blot data revealed that phosphorylation of serine-threonine kinase (Ser9) of GSK-3 β (p-GSK-3 β) expression was markedly lower in the PM2.5 group than in the control group ([Figures 1C–E](#)).

Effect of PM2.5 on the HBEC Viability

HBEC viability was measured using a CCK-8 assay to evaluate the cytotoxicity of PM2.5. After incubation with various concentrations of PM2.5 (0, 5, 10, 20, and 40 μ g/mL) for 24 h, the CCK-8 assay results showed that the cell viability did not markedly change after 24 h of PM2.5 (5–20 μ g/mL) treatment, but the cell viability decreased after treatment with 40 μ g/mL PM2.5 ([Figure 2](#)). Concentrations of PM2.5 (5–20 μ g/mL) were selected for further experiments.

PM2.5 Reduced the Expression of p-GSK-3 β in HBECs

To assess the role of p-GSK-3 β in HBECs treated with PM2.5, we examined the relative protein expression levels of GSK-3 β and p-GSK-3 β in HBECs. Western blot data revealed that the expression of p-GSK-3 β did not change markedly after 24 h of PM2.5 (5–10 μ g/mL) treatment, whereas the expression was significantly decreased after PM2.5 stimulation at 20 μ g/mL compared with the control group ([Figure 3A](#)), which suggested that 24 h of exposure of HBECs to PM2.5 (20 μ g/mL) led to a decrease in p-GSK-3 β expression. Furthermore, Western blot data also revealed that the expression p-GSK-3 β did not change markedly after 3 h of PM2.5 (20 μ g/mL) treatment, but the expression was markedly decreased after 12 and 24 h of PM2.5 treatment, in a time-dependent manner ([Figure 3B](#)).

GSK-3 β Inhibitors Increased the Expression of p-GSK-3 β in HBECs

To gain mechanistic insight into the effects of the GSK-3 β inhibitors used, we examined the expression p-GSK-3 β in HBECs. As expected, Western blot data showed that the GSK-3 β inhibitors TDZD-8 and SB216763 significantly increased the expression of p-GSK-3 β exposed to PM2.5. ([Figure 4](#)). In addition, the altered level of p-GSK-3 β in the TDZD-8 group was stronger than that in the SB216763 group.

GSK-3 β Inhibitors Alleviated PM2.5-Activated JNK Pathways in HBECs

Since GSK-3 β is an upstream regulator of the C-jun N-terminal kinase (JNK) signaling pathway, we explored the protein expression levels of JNK and p-JNK in HBECs treated with PM2.5. As shown in [Figure 5](#), PM2.5 treatment significantly enhanced the ratio of p-JNK/JNK, while treatment with TDZD-8 and SB216763 markedly

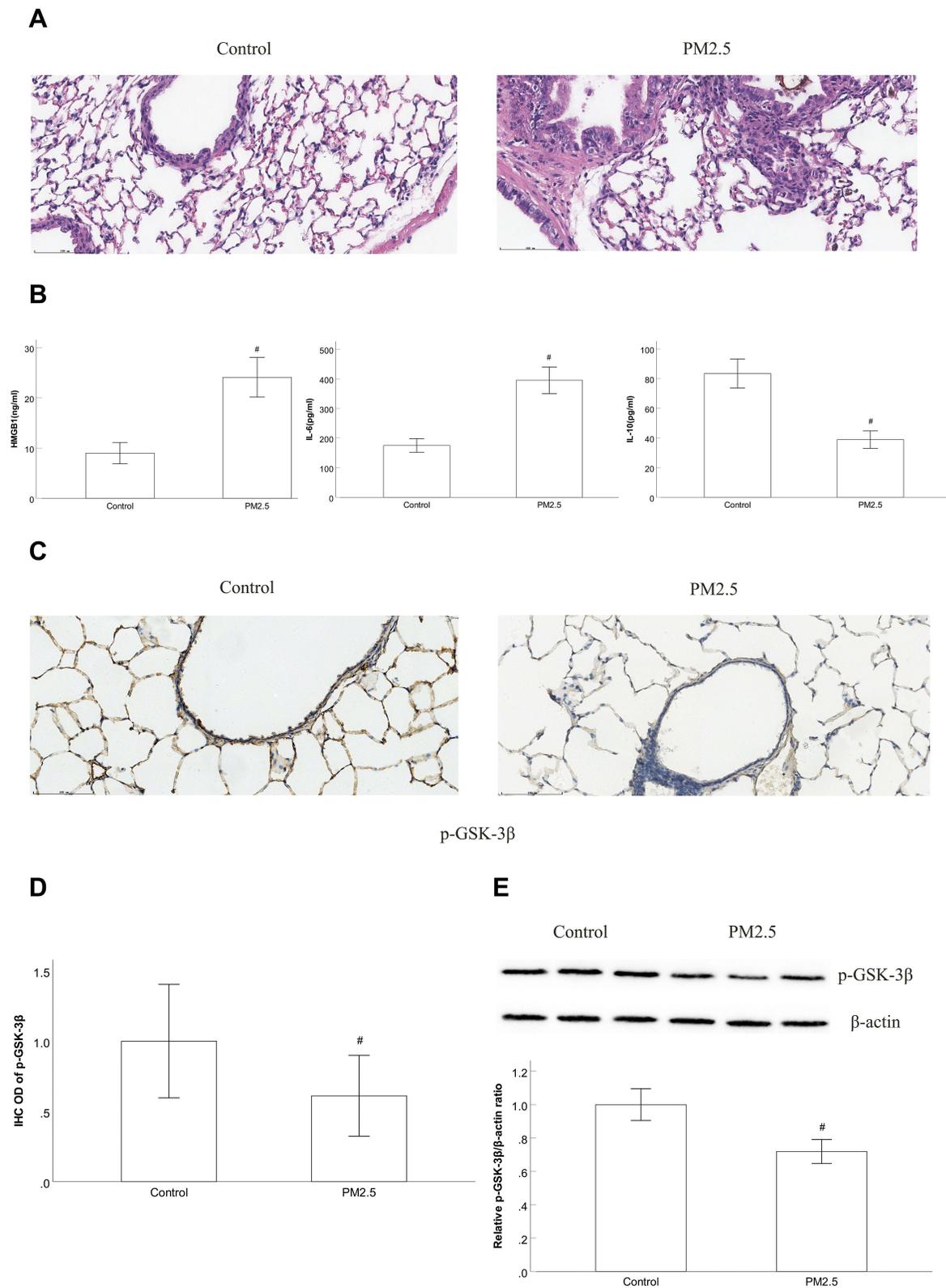


Figure 1 PM2.5 induced inflammatory changes and reduced the expression of p-GSK-3β in the lungs of mice.

Notes: (A) H&E staining showed that PM2.5 exposure caused the upregulation of inflammatory cells. (B) ELISA results showed that PM2.5 exposure increased the expression of HMGB1 and IL-6 and decreased the expression of IL-10. (C) Immunohistochemical staining showed that PM2.5 exposure reduced the expression of p-GSK-3β in lung tissues (original magnification 200×, bar 100 μm). (D) The OD of p-GSK-3β-positive cells were lower in PM2.5-treated mice than in untreated mice. (E) Western blot analysis showed that PM2.5 exposure reduced the expression of p-GSK-3β in lung tissues. #P<0.05, compared to control group, n=5.

Abbreviations: PM2.5, particulate matter<2.5 μm.

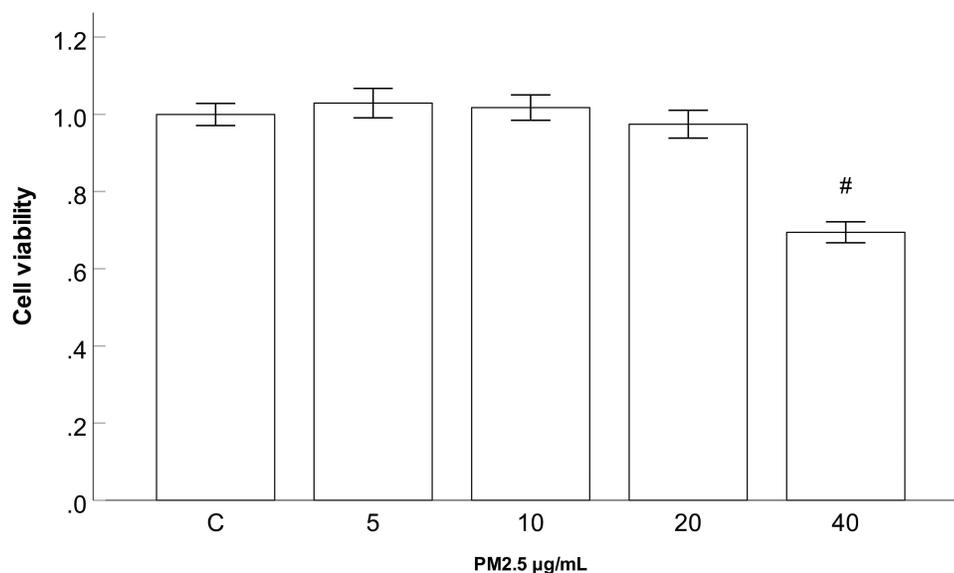


Figure 2 Effect of PM2.5 on HBEC viability.

Notes: CCK-8 assay results showed that cell viability did not markedly change after 24 h of PM2.5 (5–20 µg/mL) treatment, but cell viability decreased after treatment with 40 µg/mL PM2.5. #P<0.05, versus PM2.5 group, n=3.

Abbreviations: PM2.5, particulate matter<2.5 µm.

decreased the ratio of p-JNK/JNK induced by PM2.5. The altered ratios of p-JNK/JNK in the TDZD-8 groups were higher than those in the SB216763 groups.

GSK-3β Inhibitors and JNK Inhibitors Suppressed PM2.5-Induced NF-κB Activation and IκBα Degradation in HBECs

NF-κB plays a critical role in the pathogenesis of airway inflammation, and GSK-3β can modulate the activity of NF-κB. To clarify the mechanism by which PM2.5 induces the expression of inflammatory cytokines, the effects of GSK-3β inhibitors on NF-κB activation and IκBα degradation were measured. As depicted in Figures 6A and B, stimulation with PM2.5 significantly enhanced the levels of phosphorylated NF-κB and IκBα and decreased the levels of IκBα in HBECs. However, treatment with TDZD-8 or SB216763 markedly diminished PM2.5-induced NF-κB activation and IκBα degradation, respectively. Moreover, the altered levels of phosphorylated NF-κB and IκBα in the TDZD-8 groups were greater than those in the SB216763 groups. Because the JNK signaling pathway is an upstream signaling pathway of NF-κB, we explored the level of phosphorylated NF-κB in HBECs treated with PM2.5 and the JNK inhibitor SP600125. As shown in Figure 6C, the JNK inhibitor

SP600125 markedly abated the level of phosphorylated NF-κB induced by PM2.5.

GSK-3β Inhibitors Blocked PM2.5-Induced Inflammatory Responses in HBECs

Consistent with the animal experiment results, the results of qRT-PCR and ELISA showed that PM2.5 increased the mRNA expression levels of the inflammatory cytokines HMGB1 and IL-6 and induced the secretion of HMGB1 and IL-6 in cell supernatants. To further assess the role of GSK-3β inhibitors in PM2.5-induced inflammatory responses in HBECs, HBECs were treated with the GSK-3β inhibitors TDZD-8 or SB216763. As shown in Figure 7A–C, TDZD-8 and SB216763 treatment significantly reversed PM2.5-induced outcomes at both the mRNA and protein levels. Furthermore, the altered levels of inflammation-related cytokines in the TDZD-8 groups were greater than those in the SB216763 groups.

Discussion

PM2.5 exposure has recently been reported to be associated with the development and progression of COPD. Here, we investigated the effects of GSK-3β inhibition on the PM2.5-associated airway inflammatory response in HBECs. We demonstrated that short-term exposure to PM2.5 induced an imbalance between inflammatory

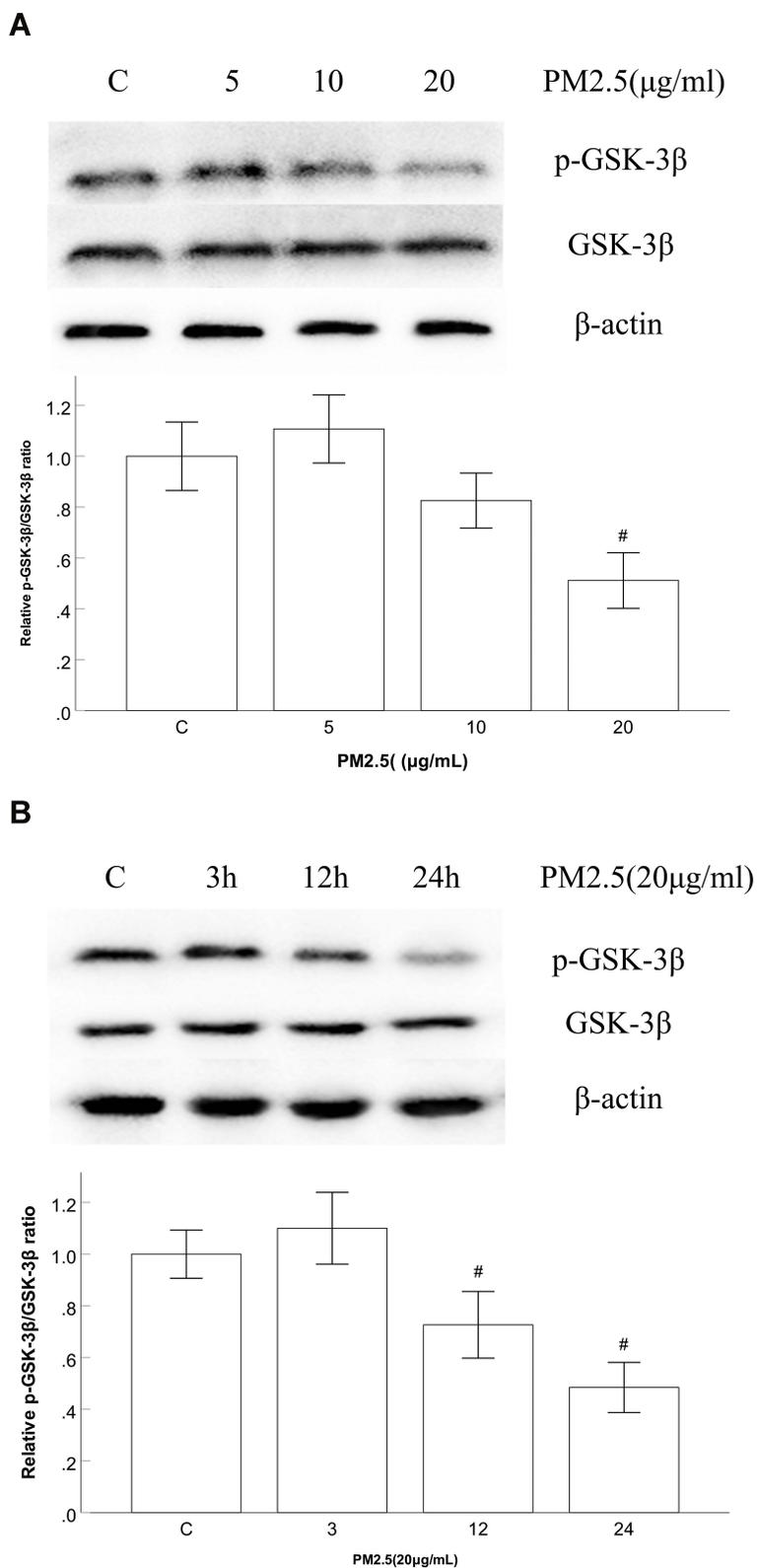


Figure 3 PM2.5 reduced the expression of p-GSK-3β in HBECs.

Notes: (A) Western blot analysis revealed that the expression of p-GSK-3β did not change markedly after 24 h of PM2.5 (5–10 μg/mL) treatment, whereas the expression was significantly decreased after PM2.5 stimulation at 20 μg/mL. (B) Western blot analysis showed that the expression of p-GSK-3β did not change markedly after 3 h of PM2.5 (20 μg/mL) treatment, but the expression was substantially decreased after 12 and 24 h of PM2.5 treatment. #P<0.05, compared to control group. n=3.

Abbreviations: PM2.5, particulate matter<2.5 μm.

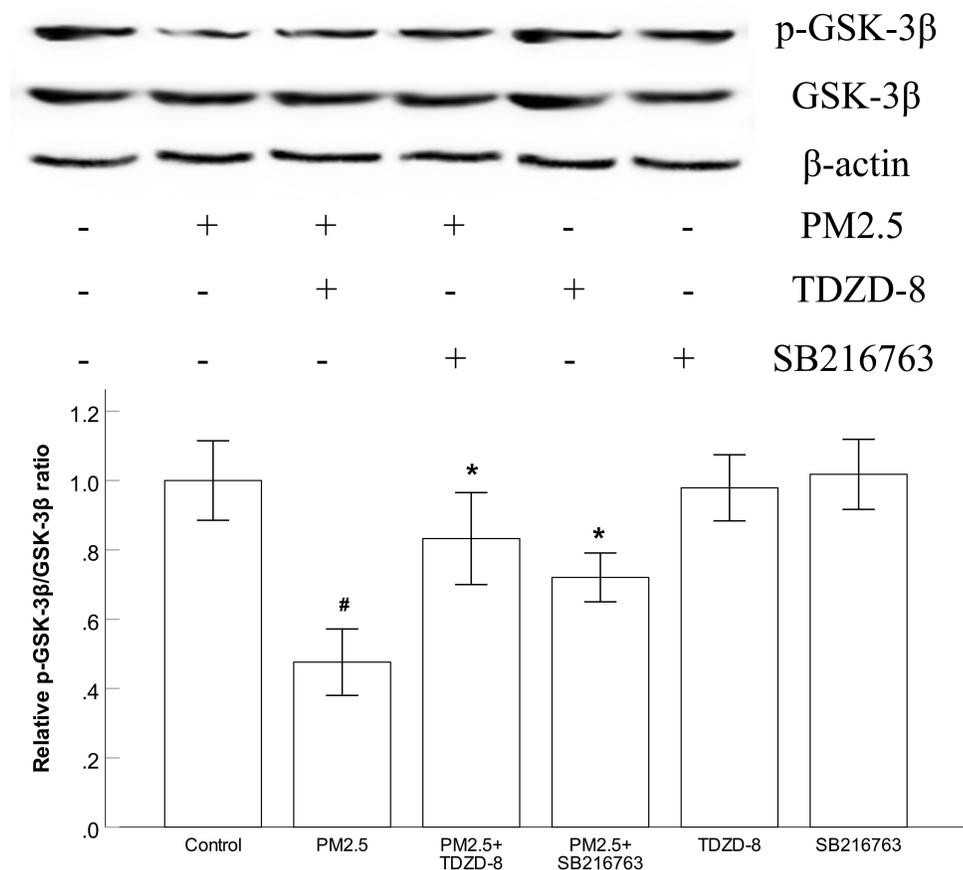


Figure 4 GSK-3 β inhibitors increased the expression of p-GSK-3 β in HBECs.

Notes: (A) Western blot analysis showed that the GSK-3 β inhibitors TDZD-8 and SB216763 significantly increased the expression of p-GSK-3 β . # $P < 0.05$ compared to the control group. * $P < 0.05$, compared to PM2.5 group, $n = 3$.

Abbreviations: PM2.5, particulate matter < 2.5 μm .

cytokines and anti-inflammatory cytokines and reduced p-GSK-3 β expression in both mice and HBEC models. Reduced p-GSK-3 β expression activated JNK pathways, caused NF- κB activation and I κB degradation, and then increased the levels of inflammatory cytokines in HBECs. Targeting GSK-3 β inhibition may be a new strategy for the prevention or therapy of PM2.5-associated airway inflammation in COPD.

GSK-3 β is a serine-threonine protein kinase that plays a key role in regulating the inflammatory response by converging several signaling pathways, but its role in the regulation of PM2.5-related airway inflammation is not clear. An epidemiological study showed that PM2.5 exposure led to airway inflammation in COPD.^{13,14} Long-term inflammatory mediators, including TNF- α , IL-1 β , and IL-6, are strongly associated with the processes in chronic inflammatory diseases.¹⁵ Moreover, HMGB1 is implicated as a late inflammatory mediator that can irritate the release of many inflammatory mediators, such as IL-1 β , IL-6, and

TNF- α .¹⁶ IL-10 plays an anti-inflammatory role by reducing the production of inflammatory mediators. A study reported that GSK-3 β inhibitors prevented mouse lung inflammation and injury caused by bleomycin.¹⁷ Consistently, our present research also observed that PM2.5 increased the levels of the inflammatory cytokines HMGB1 and IL-6 and decreased the expression of the anti-inflammatory cytokines IL-10 and p-GSK-3 β in mice. Inflammatory cytokine and p-GSK-3 β expression also displayed the same trend in HBECs exposed to PM2.5. Intervention with the GSK-3 β inhibitors TDZD-8 and SB216763 significantly suppressed the PM2.5-induced inflammatory response in HBECs, and TDZD-8 showed a stronger anti-inflammatory effect than B216763, which suggested that inhibition of GSK-3 β activity led to potent anti-inflammatory effects in cell models caused by PM2.5.

What are the molecular mechanisms by which GSK-3 β inhibitors decrease the inflammatory response caused by PM2.5? GSK3 β phosphorylation at Ser9 will cause its

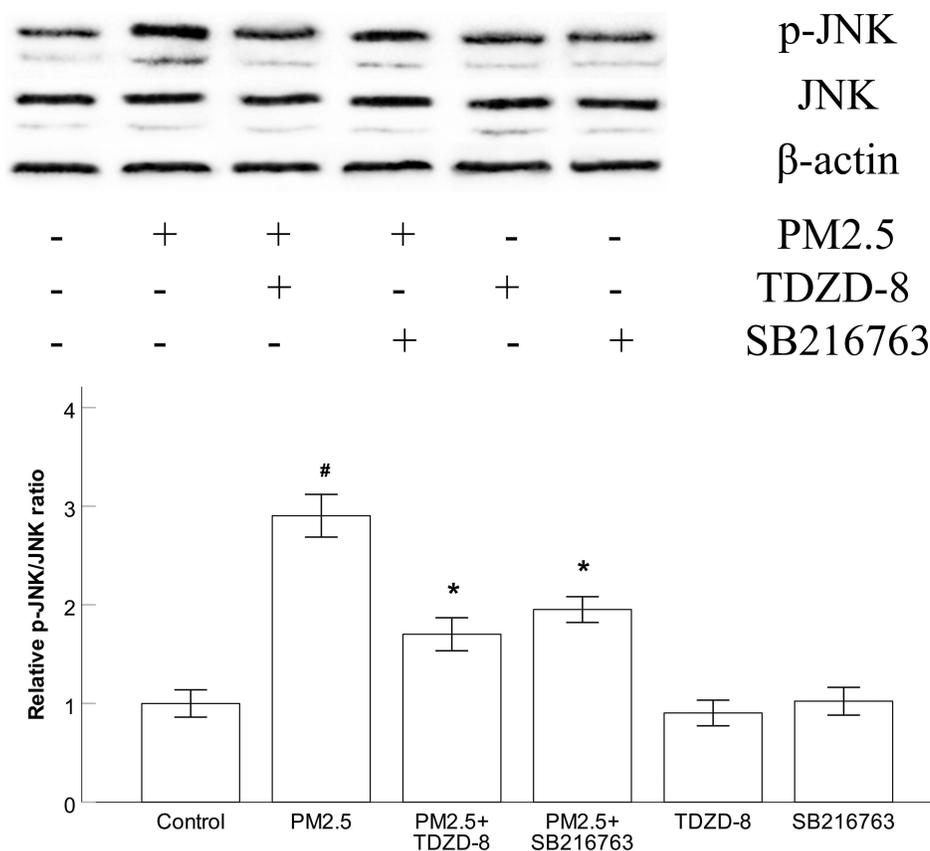


Figure 5 GSK-3 β inhibitors alleviated PM2.5-activated JNK pathways in HBECS.

Notes: (A) Western blot analysis showed that the GSK-3 β inhibitors TDZD-8 and SB216763 markedly decreased the PM2.5-induced ratio of p-JNK/JNK. [#] $P < 0.05$, compared to control group. ^{*} $P < 0.05$, compared to PM2.5 group, $n = 3$.

Abbreviations: PM2.5, particulate matter <2.5 μm .

inactivation.¹⁸ We clearly showed that GSK-3 β inhibitors increased the expression of p-GSK-3 β in HBECS exposed to PM2.5. Growing evidence has demonstrated an association between GSK-3 β and NF- κ B activation. NF- κ B is an important transcription factor because it promotes the secretion of inflammatory cytokines in HBECS after external stimulation. Active GSK-3 β increases NF- κ B phosphorylation and promotes the phosphorylation and degradation of the inhibitory protein κ B (I κ B) and then enhances the expression of inflammatory genes.¹⁹ In contrast, GSK-3 β inhibitors decreased the activation of NF- κ B, which in turn caused a decreased formation of inflammatory mediators.^{20,21} Consistent with previous reports, our results indicated that inhibitors of GSK-3 β can suppress NF- κ B activation and I κ B degradation in HBECS after PM2.5 treatment.

Previous studies have demonstrated that inactivation of GSK-3 β regulates the phosphorylation of JNK in normal bronchial epithelial cells activated by quercetin.⁶ JNK is a serine/threonine protein kinase that can regulate the development of inflammation. Very recently, a study

indicated that the activation of JNK promoted the activation of NF- κ B, further increasing the release of inflammatory factors.²² Moreover, the JNK inhibitor SP600125 inhibited PM2.5-induced expression of the inflammatory mediator IL-6 in human osteoarthritis synovial fibroblasts.²³ As predicted, in our study, we also found that GSK-3 β inhibitors inhibited the ratio of p-JNK/JNK in HBECS caused by PM2.5. Therefore, we showed that PM2.5 induced JNK phosphorylation and NF- κ B activation via GSK-3 β in HBECS, promoting the development of airway inflammation.

Additionally, to confirm whether the two signaling pathways of JNK and NF- κ B were functionally connected or separately induced by PM2.5, we suppressed JNK expression through the JNK inhibitor SP600125. In accordance with previous studies, our results showed that pretreatment with SP600125 markedly abated the level of phosphorylated NF- κ B induced by PM2.5, indicating that JNK plays an essential role in PM2.5-induced NF- κ B activation and the inflammatory response. However, the

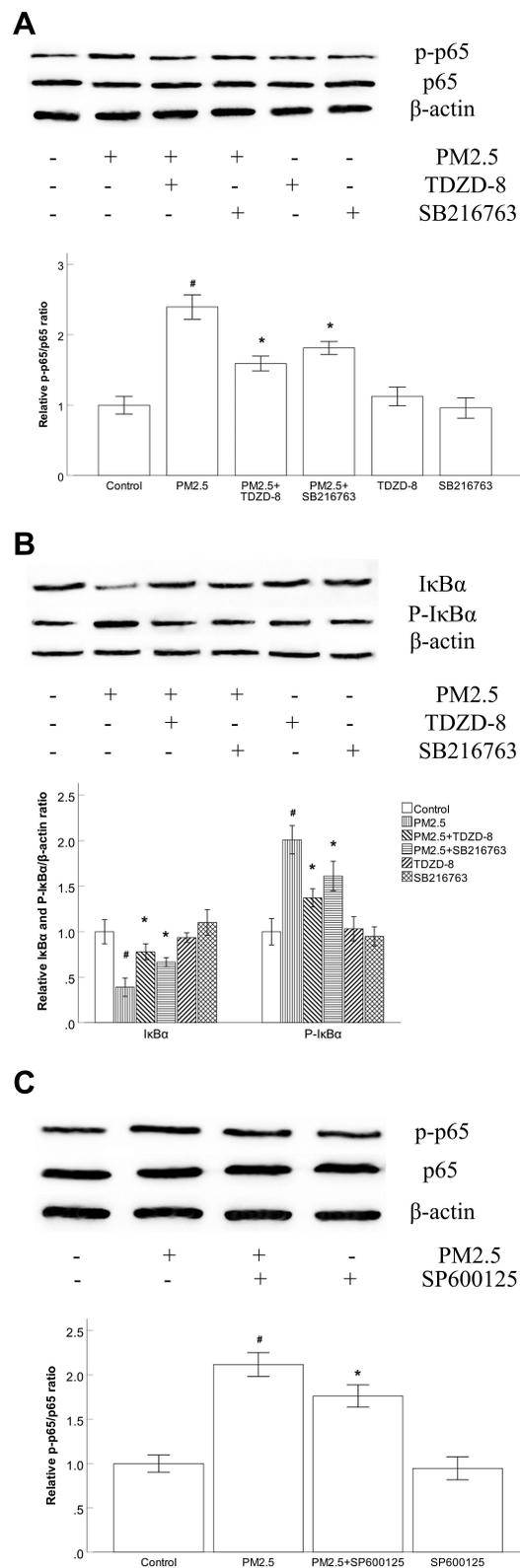


Figure 6 GSK-3 β inhibitors and JNK inhibitors suppressed PM2.5-induced NF- κ B activation and I κ B α degradation in HBECs.

Notes: (A) Western blot analysis revealed that the GSK-3 β inhibitors TDZD-8 and SB216763 markedly diminished PM2.5-induced NF- κ B phosphorylation. (B) Western blot analysis showed that TDZD-8 and SB216763 markedly suppressed PM2.5-induced I κ B α phosphorylation and I κ B α degradation, respectively. (C) The JNK inhibitor SP600125 markedly abated the level of phosphorylated NF- κ B induced by PM2.5. #P<0.05, compared to control group. *P<0.05, compared to PM2.5 group, n=3.

Abbreviations: PM2.5, particulate matter<2.5 μ m.

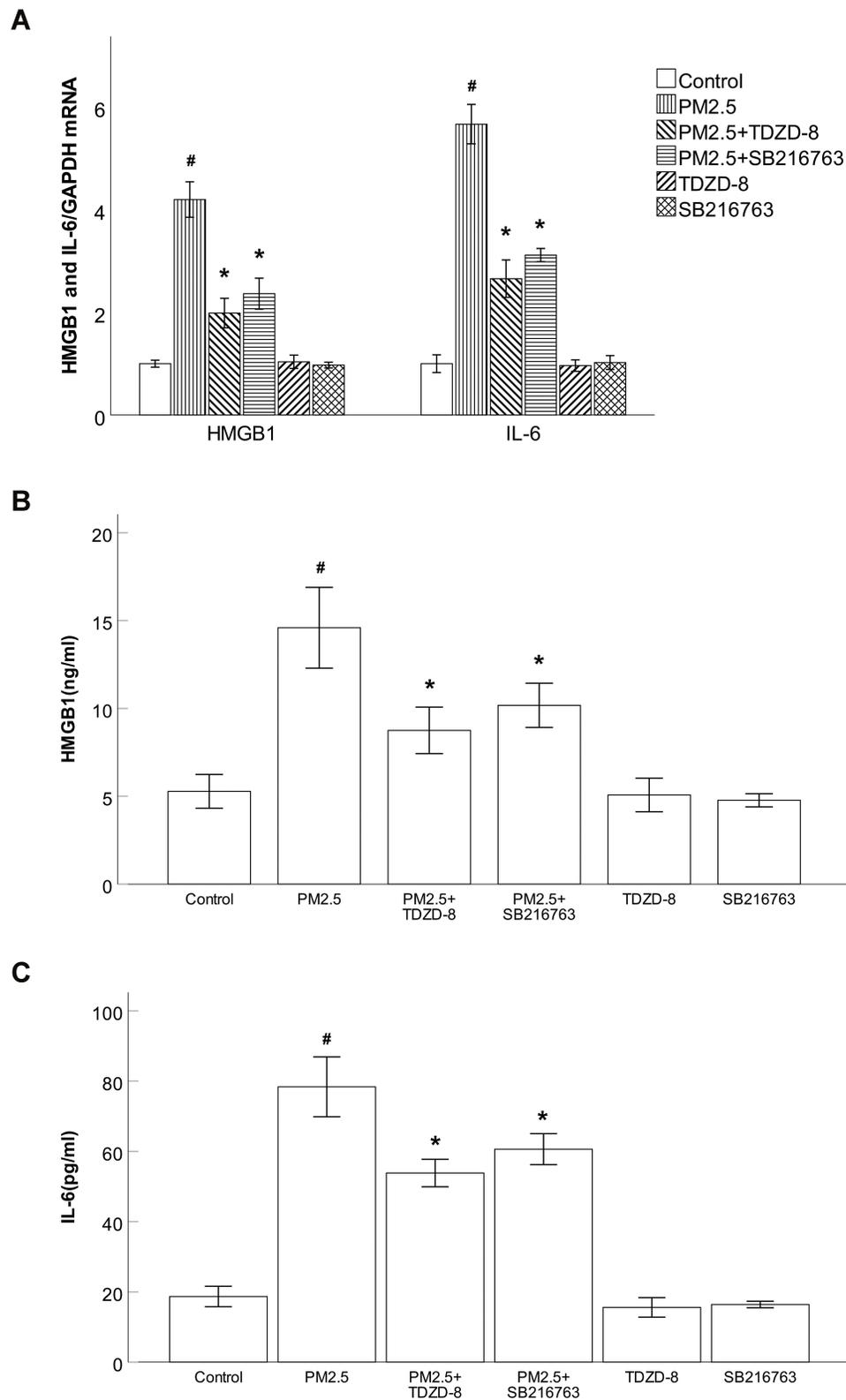


Figure 7 GSK-3 β inhibitors blocked PM2.5-induced inflammatory responses in HBECS.

Notes: (A) The results of qRT-PCR showed that the GSK-3 β inhibitors TDZD-8 and SB216763 markedly attenuated the production of HMGB1 and IL-6 induced by PM2.5. (B) ELISA results showed that the GSK-3 β inhibitors TDZD-8 and SB216763 markedly prevented the production of HMGB1 induced by PM2.5. (C) ELISA results showed that TDZD-8 and SB216763 markedly prevented the production of IL-6 induced by PM2.5. # $P < 0.05$, compared to control group. * $P < 0.05$, compared to PM2.5 group, $n = 3$. **Abbreviations:** PM2.5, particulate matter $< 2.5 \mu\text{m}$.

level of phosphorylated NF- κ B reduced by SP60025 was still higher than that in the GSK-3 β inhibitor group. These data suggested that in addition to JNK, NF- κ B might also be regulated by other signaling pathways.

Limitations

Our study has certain limitations. In our PM2.5-induced chronic model in vivo, a GSK-3 β inhibitor (TDZD-8 or SB216763) was not administered to confirm the direct effect of GSK-3 β in a PM2.5-related mouse model. However, this will be the focus of our next study.

Conclusion

In summary, the results of this study suggested beneficial effects of GSK-3 β inhibitors in attenuating inflammatory responses in PM2.5-induced HBEC models. The mechanism is involved in the suppression of JNK phosphorylation, the inhibition of NF- κ B activation, and the prevention of NF- κ B-dependent proinflammatory gene expression. Thus, inhibition of GSK-3 β may be a potential preventive and therapeutic strategy against PM2.5-associated airway inflammation in COPD. Further studies are required to verify these results.

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Disclosure

The authors report no conflicts of interest in this work.

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