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ORIGINAL RESEARCH

Cigarette Smoke Extract Induces MUC5AC Expression Through the ROS/ IP3R/Ca^{2+} Pathway in Calu-3 Cells

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Background: Chronic obstructive pulmonary disease (COPD) is caused by exposure to noxious external particles, air pollution, and the inhalation of cigarette smoke. Airway mucus hypersecretion particularly mucin5AC (MUC5AC), is a crucial pathological feature of COPD and is associated with its initiation and progression. In this study, we aimed to investigate the effects of cigarette smoke extract (CSE) on MUC5AC expression, particularly the mechanisms by which reactive oxygen species (ROS) induce MUC5AC expression.

Methods: The effects of CSE on the expression of MUC5AC and mucin5B (MUC5B) were investigated in vitro in Calu-3 cells. MUC5AC and MUC5B expression levels were measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), immunofluorescence staining, and enzyme-linked immunosorbent assay (ELISA). Total cellular levels of ROS and Ca^{2+} were determined using DCFH-DA and Fluo-4 AM. Subsequently, the expression levels of IP3R, IRE1α, p-IRE1α and XBP1s were measured by Western blotting. Gene silencing was achieved by using small-interfering RNAs.

Results: Our findings revealed that exposure to CSE increased MUC5AC levels and upregulated ROS, IP3R/Ca^{2+} and unfolded protein response (UPR)-associated factors. In addition, knockdown of IP3R using siRNA decreased CSE-induced Ca^{2+} production, UPR-associated factors, and MUC5AC expression. Furthermore, 10 mM N-acetyl-l-cysteine (NAC) treatment suppressed the effects of CSE, including ROS generation, IP3R/ Ca^{2+}, UPR activation, and MUC5AC overexpression.

Conclusion: Our results suggest that ROS regulates CSE-induced UPR and MUC5AC overexpression through IP3R/ Ca^{2+} signaling. Additionally, we identified NAC as a promising therapeutic agent for mitigating CSE-induced MUC5AC overexpression.

Keywords: chronic obstructive pulmonary disease, mucin5AC, cigarette smoke extract, reactive oxygen species, unfolded protein response, IP3R, Ca^{2+}

Introduction

Chronic obstructive pulmonary disease (COPD) is a slow-developing heterogeneous lung condition characterized by persistent, frequently progressive airflow limitation caused by abnormalities in the airways or alveoli that cause persistent, progressive, airflow obstruction. COPD is now one of the top three leading causes of death worldwide, with an increasing economic and social burden.[1] Many people suffer from COPD for years and die of this disease or its complications. The COPD burden is thought to increase globally in the next decade due to aging populations and continued exposure to COPD risk factors.[2] COPD resulted from the interactions of gene and environment.[3] This is caused by a chronic inflammatory response following exposure to noxious external particles, air pollution and inhalation of cigarette smoke.[4] Both traditional and electronic cigarettes negatively impact the bronchial epithelium, but traditional cigarettes generally posing a greater risk due to their higher concentration of harmful chemicals. Cigarettes smoke exposure induced inflammation of the small airways by altering the differentiation of basal cells and caused respiratory

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function to decline.[5] As a consequence of exposure to over 5000 compounds or some cigarette smoke, manifestations of abnormalities in mucin production and secretion, oxidative stress, and inflammation are evident in the lung with COPD.[6]

Mucins are large glycoproteins, and two gel-forming mucin polymers, MUC5AC and MUC5B, dominate human airway mucus. In healthy condition, MUC5B is the major mucin in the airway and is necessary for mucociliary clearance.[7] On the contrary, MUC5AC is the minor mucin in the healthy airway and does not seem to be necessary for mechanical mucociliary clearance from the lung.[7] However, MUC5AC is mainly produced by the goblet epithelium in the airway and it seems to be mainly responsive to external environmental stress such as air pollution, tobacco smoking, as well as exposure to biomass fuel or infectious agents.[8] The study suggested that MUC5AC concentrations were low in healthy conditions, but increased significantly in COPD patients compared with MUC5B.[9] Thus, MUC5AC hypersecretion in the airways is an important pathogenesis of COPD.

The endoplasmic reticulum (ER) is comprised of some tubules and sheet-like structures extend the nuclear membrane spanning throughout the cytoplasm.[10] ER plays an important role in lipid and steroid synthesis, calcium storage and protein production, folding and transport.[11] To facilitate protein folding, the ER requires greater levels of calcium and must maintain an oxidizing environment to enable disulfide bond formation, which is one of the post-translational modifications of proteins occurring in the ER and is required for the generation of stable, correctly folded proteins.[12] A variety of stresses can influence protein folding in the ER, and this can result in the misfolded or unfolded proteins accumulated in the ER lumen, a state commonly called ER stress.[13] To maintain ER function and ensure protein folding, cells activated a series of cellular signaling pathways to reprogram gene transcription and protein modifications to maintained unfolded or misfolded proteins proper folding and restore the protein homeostasis, which is called the unfolded protein response (UPR).[14] The study demonstrated that the production and secretion of mucins by goblet cells depending on AGR2 regulating the goblet cell UPR by acting as a rheostat of IRE1β endonuclease activity.[15] However, the underlying specific molecular mechanism between UPR and mucins remains not fully deciphered.

Protein folding is influenced by the redox state of the ER; thus, ER and oxidative stress in cells are two closely interconnecting events. In fact, UPR activation and increased reactive oxygen species (ROS) levels in cells coincide during stress initiation. A previous study found that hypoxia can alter the homeostasis of mitochondrial ROS (mitoROS) and induce ER stress.[16] To respond to hypoxia, an adaptive response in cells, which is mediated by the activation of hypoxia-inducible factors, reprogramming of mitochondrial metabolism, increased ROS influx, UPR in the ER, and the subsequent integrated stress response (ISR). Tunicamycin and thapsigargin, which induce ER stress/UPR initiation in cells, also increased the levels of ROS.[17,18] Interestingly, studies demonstrated that ROS was in mutual action with UPR/ISR.[16,19] The AhR/ROS/ERK pathway activation plays a crucial role in benzo(a)pyrene-induced MUC5AC overexpression in human airway epithelial cells.[20] Kim et al found that intracellular ROS generation-induced MUC5AC overexpression by epidermal growth factor receptors in human nasal epithelial cells.[21] Therefore, we speculated that MUC5AC overexpression might be related to both ROS and UPR in cells.

In this study, we investigated the mechanism by which cigarette smoke extract (CSE) regulates MUC5AC expression in Calu-3 cells and explored potential drugs to inhibit the damage associated with CSE-induced abnormal expression of MUC5AC.

Materials and Methods

CSE Preparation

CSE was prepared as described previously.[22] In short, two non-filtered cigarettes (Hongtashan, China) were burned, and the smoke was bubbled with a vacuum pump through the 20 mL serum-free Dulbecco's modified Eagle's medium (DMEM). Then, the DMEM with CSE was filtered through a 0.2 μm pore-size filter and its pH was adjusted to 7.35–7.45 by using NaOH. The prepared solution was considered to have 100% CSE. Finally, 100% CSE was diluted with DMEM to the required concentration for further experiments.

Cell Culture and Treatment

Human mucoepidermoid carcinoma-3 (Calu-3) cells were purchased from the ATCC. Calu-3 cells were cultured in DMEM (Gibco, NY, USA) with 10% fetal bovine serum (PAN, Germany) and 1% penicillin-streptomycin at 37°C with 5% CO_2 in a humidified incubator. Calu-3 cells were stimulated with 10% CSE for 24 h and treated with the ROS inhibitor N-acetyl-l-cysteine (NAC; Sigma-Aldrich, 10mM).

Cell Viability Assay

1×10^4 Calu-3 cells were seeded in a 96-well plate and cultured for 24 h. Calu-3 cells were treated with different concentrations of CSE (0, 5, 10, 20, and 30%) or NAC (0, 5, 10,15 mM) when the cell density reached approximately 80%. After 24 h of treatment with CSE or NAC, the cells were washed three times with phosphate-buffered saline (PBS) and 100 μL fresh medium with 10 μL Cell Counting Kit-8 (CCK-8, Abclonal, China) was added. A blank control well containing only CCK-8 reagent and the DMEM medium was set. Then, the cells were cultured at 37°C in an incubator for 1 h and the value of optical density at 450 nm was measured using an enzyme-labeling instrument (Multiskan Sky 1530, Thermo Fisher Scientific, USA). The formula for calculating cell viability (%) is as follows: Cell viability (%)=(A1(Treatment)−A2(Blank)) /(A3(Control)−A2(Blank)×100%.

Western Blot Analysis

Calu-3 cells were lysed in a RIPA buffer containing protease and phosphatase inhibitors (Beyotime). Protein concentrations were measured using a BCA protein assay kit (Kingmorn, Shanghai, China). The proteins from each sample were separated by 10% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Merck, USA). And then membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBST) containing 0.1% Tween-20 for 1 h, subsequently the membranes incubated with the primary antibodies (1:1000 dilution for anti-IRE1α (Abcam, ab37073), anti-pIRE1α (Abcam, ab48187), anti-IP3R (Abcam, ab37073), and anti-XBP1s (CST, #40435); 1:5000 dilution for anti-β-actin antibody (proteintech, 20536–1); 1:10000 for anti-GAPDH antibody (proteintech, 60004–1)) by overnight at 4°C. The PVDF membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:2000 dilution) for 1 h at room temperature. Finally, enhanced luminol-based chemiluminescence (ECL) reagents were used to detect immunoreactive bands using a chemiluminescence detection system (Amersham Image 680; GE Healthcare, USA). Densitometry of all bands was performed using ImageJ 1.8.0.112 software.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from Calu-3 cells using TRIzol RNA Reagent (Ambion, Thermo Scientific, USA). All-in-one First-Stand Synthesis MasterMix (with dsDNase) (ABclonal Biotechnology Co., China) was used to synthesize cDNA. Quantitative real-time PCR analysis was performed using 2×SYBR Green PCR master mix (ABclonal Biotechnology Co., China) in an Applied BiosystemsTM I Cycler (Quantstudio 3, Thermo Fisher Scientific, USA). All the primer sequences used for PCR were synthesized by Sangon Biotech. The qPCR primers used for MUC5AC were 5′-GGCAACATCAAGAAGAGCGGAGAG-3′ (forward) and 5′-TGTGGAGGTGGTACTGTCTGTCTG-3′ (reverse). The qPCR primers used for MUC5B were 5′-CTGCTACGACAAGGACGGAAACTAC-3′ (forward) and 5′- CAGGCGGTGGTGAAGGTGAATG-3′ (reverse).

Measurement of Ca^{2+}

Calu-3 cells were plated at a density of 1×10^5 cells/well in 6-well plates containing DMEM with 10% fetal bovine, 24 h before the experiment. The cells were incubated with the fluorescent calcium assays Fluo-4AM at 2μM (Beyotime, Shanghai, China) for 30 min in a CO2 incubator when the cell density reached approximately 90%. To eliminate extracellular Fluo-4 AM, cells were washed three times with PBS. Fluorescence signals were detected and analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter, CA, USA).

Immunofluorescence Staining

Calu-3 cells in 6-well plate were fixed with 4% paraformaldehyde. The cells in the plates were washed 3 times with PBS. The cells were blocked with 10% goat serum for 30 min at room temperature to reduce non-specific antibody binding. Cells were then exposed to the primary antibodies against MUC5AC at a dilution of 1:500 overnight at 4°C for immunostaining. Following incubation with the primary antibodies, the cells were washed three times with PBS and incubated with FITC-conjugated Affinipure Goat Anti-Mouse IgG(H+L) at a dilution of 1:500 for 1 h at room temperature. Subsequently, the cells were washed 3 times with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min in the dark. Images were visualized using a fluorescence microscope (EVOS M5000, Invitrogen by Thermo Fisher Scientific, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

MUC5AC and MUC5B levels in the culture supernatant were quantified using the ELISA kit (Abcam, ab303761; ab77995). All procedures were performed according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate spectrophotometer (Multiskan Sky 1530, Thermo Fisher Scientific, USA).

siRNA Transfection in vitro

siRNA transfection was performed using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's instructions. IP3R and negative control siRNAs were obtained from Sangon Biotech (Shanghai, China). Calu-3 cells were seeded in 6-well plates at a density of 2×10^5 cells/well in DMEM containing 10% FBS without penicillin-streptomycin and incubated overnight. Calu-3 cells were transfected with IP3R-siRNA using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 6 h, whereas control cells were transfected with negative control siRNA. Following transfection, the cells were washed with PBS 3 times and then incubated in complete culture medium for further studies. Calu-3 cells were cultured for 48–72 hours and the efficacy of knockdown was evaluated by Western blotting.

Measurement of Total ROS

1×10^5 Calu-3 cells were seeded in 6-well plates containing DMEM with 10% fetal bovine. After the administration of CSE or NAC, Calu-3 cells were washed three times with PBS. Subsequently, for the assessment of total cellular ROS, an oxidative stress detection assay(Beyotime, Shanghai, China), was applied to the cells. Cells were incubated in DMEM containing 10 μM of DCFH-DA for a duration of 30 min at 37°C in a 5% CO_2 air incubator. Next, the cells were washed thrice with PBS before the images were captured using a fluorescence microscope (EVOS M5000, Thermo Fisher Scientific, USA) for total ROS. The fluorescence intensity was analyzed using ImageJ software.

Statistical Analysis

All values are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 21.0; SPSS Inc., USA). Differences between the two groups were analyzed using the Student's *t*-test, while comparisons among more than two groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was set at $P< 0.05$.

Results

Effects of CSE on Cell Viability

To assess the effects of CSE exposure concentration, Calu-3 cells viability was evaluated by CCK-8 assays. Cells were treated with various concentrations of CSE (0%, 5%, 10%, 20%, and 30%) for 24 h. As shown in Figure 1, 20% and 30% CSE significantly decreased Calu-3 cell viability after exposure to CSE for 24 h compared to that in the control group ($P<0.001$). Therefore, exposure to 10% CSE for 24 h was selected as the optimal concentration for subsequent experiments.

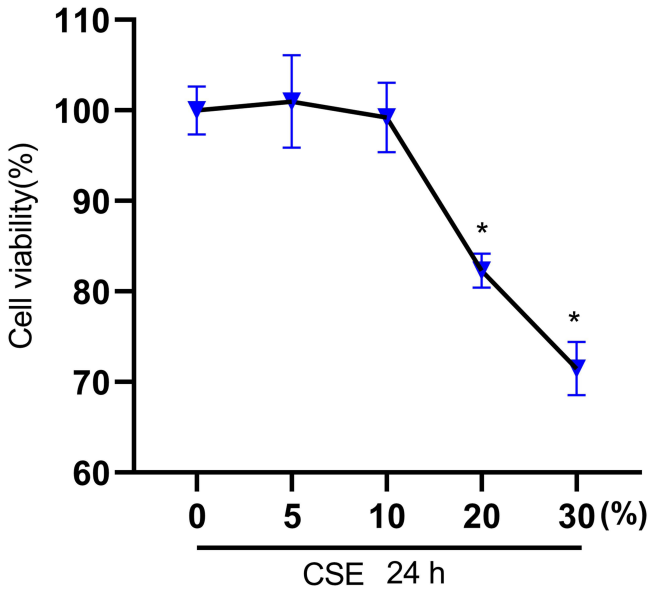

Figure 1 Measurement of cell viability under CSE stimulation. Cell proliferation measurement of Calu-3 cells after exposure to 5%, 10%, 20%, or 30% NE for 24 h by CCK-8 assay(n=3). *$P < 0.01$ compared with the control group. The Calu-3 cells in control group were without CSE exposure.

CSE Exposure Induced MUC5AC Expression in Calu-3 Cells

As we all know, cigarette smoking is an important environmental risk factor for COPD. To investigate the role of CSE in MUC5AC expression in Calu-3 cells, we examined the expression of MUC5AC by immunofluorescence after CSE stimulation. CSE exposure significantly increased the protein expression of MUC5AC (Figure 2A and B). Moreover, qRT-PCR revealed that the levels of MUC5AC mRNA in the CSE group were markedly higher than those in the control group (Figure 2C), which was consistent with the immunofluorescence results. We further measured the MUC5B expression under CSE exposure by ELISA and qRT-PCR. We found that the MUC5B levels were also increased under CSE stimulation (Figure 2D and E). However, the changes of MUC5AC were higher than MUC5B after CSE stimulation, which suggests that MUC5AC plays a major role in COPD.

CSE Induced ROS and UPR in Calu-3 Cells

It is well known that oxidative stress is a significant risk factor for inflammatory reactions in COPD, which can lead to the pathological characteristics of emphysema and small airway fibrosis.[23] Smoking exposure often damages the proteasome itself, leading to the accumulation of insoluble proteins in airway epithelial cells and alveolar epithelial cells, ultimately causing UPR in COPD. Next, we investigated the potential molecular mechanisms underlying CSE-induced MUC5AC expression, with a special focus on the ROS and UPR signaling pathways. We examined the expression of intracellular ROS and UPR-associated factors following CSE exposure. As expected, the total intracellular ROS levels were higher in the CSE group than in the control group (Figure 3A, D). Western blot analysis showed that the expression of phosphorylated IRE1α, XBP1s, and IP3R was upregulated after CSE exposure (Figure 3B, E). Furthermore, we assessed the relative changes in intracellular Ca^{2+} levels using Fluo-4AM. We found that intracellular Ca^{2+} levels also increased after CSE exposure (Figure 3C, F).

IP3R/Ca^{2+} Signaling and UPR Involved in the Production of MUC5AC

Inositol 1,4,5-trisphosphate receptors (IP3Rs) mediated Ca^{2+} flux across ER membranes increases cytosolic Ca^{2+} concentration and regulates biological effects.[24] We performed siRNA on Calu-3 cells to investigate whether IP3R/Ca^{2+} signaling is involved in the expression of MUC5AC. siRNA knocked down the protein expression levels of IP3R (Figure 4A, D). We found that the intracellular Ca^{2+} levels decreased after IP3R knockdown (Figure 4B, F). We further evaluated the changes in UPR-associated factors and MUC5AC expression using Western blotting and immunofluorescence. The results showed that IP3R knockdown

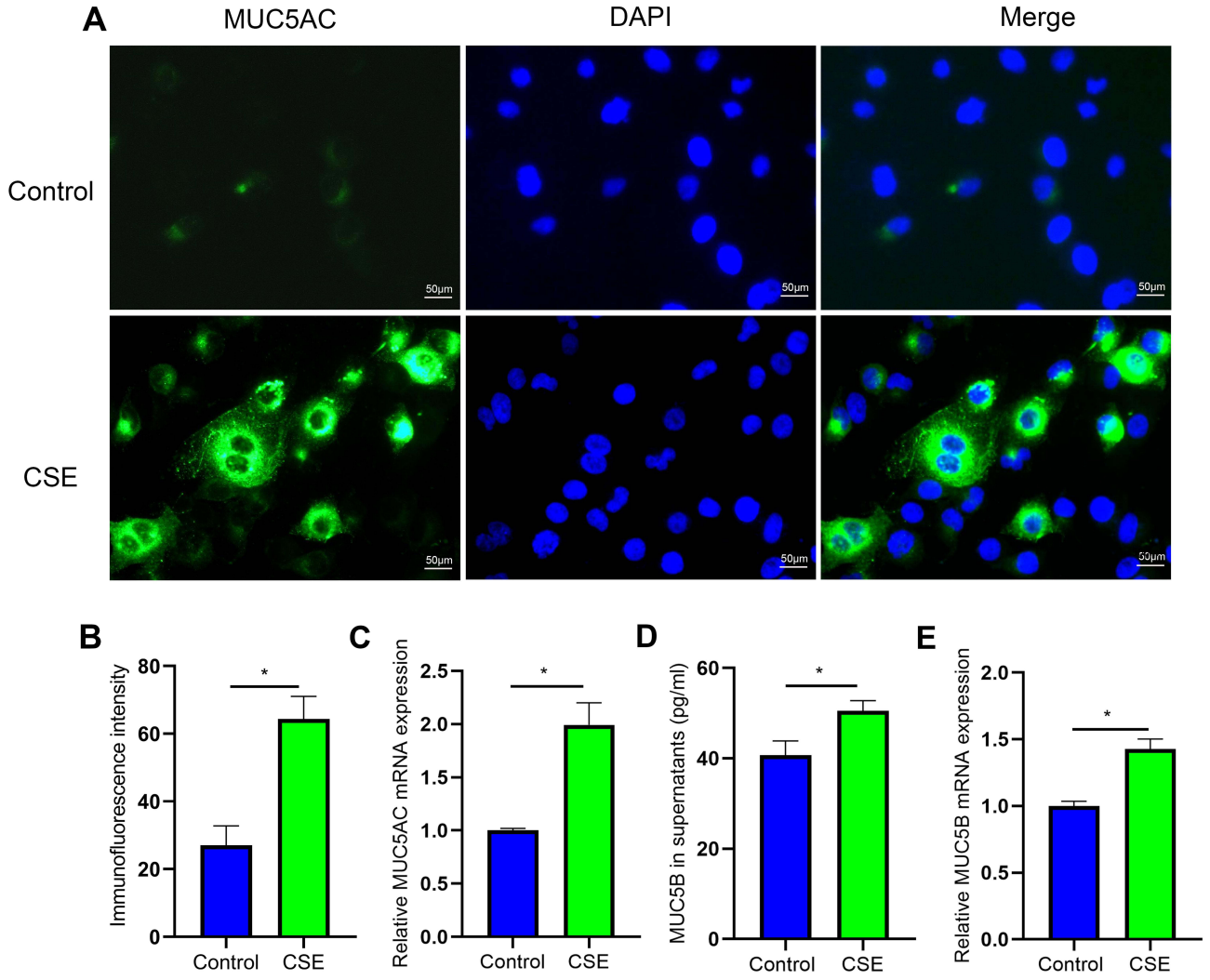

Figure 2 Effects of CSE on the expression of MUC5AC in Calu-3 cells. (**A**) The expression of MUC5AC in Calu-3 cells by immunofluorescence staining. (**B**) Quantitative analysis of MUC5AC protein levels. (**C**)The level of MUC5AC mRNA expression determined by RT-qPCR in Calu-3 cells with or without CSE exposure. Scale bars: 50 μm. (**D**) The expression of MUC5B in culture supernatants was determined by ELISA. (**E**) The level of MUC5B mRNA expression measured by RT-qPCR. All values are presented as the mean ± SD(n=3).
Notes: *$P < 0.05$ versus control.

inhibited the increased phosphorylation of IRE1α, XBP1s, and MUC5AC induced by CSE(Figures 4A, C, E, and 5), but not affect the expression of MUC5B. These data demonstrate that the IP3R/Ca^{2+} signaling pathway and UPR may regulate the production of MUC5AC induced by CSE.

NAC Alleviated MUC5AC Expression Induced by CSE

To investigate the relationship between ROS and IP3R/ Ca^{2+} and its function in the expression of MUC5AC, we treated Calu-3 cells with the ROS inhibitor NAC. A 10 mM NAC was selected as the treatment concentration (Supplementary Figure 1). As shown in Figure 6, treatment with NAC significantly decreased the levels of cellular ROS induced by CSE. We further found that the intracellular Ca^{2+} levels decreased after NAC treatment (Figure 6C, H). Next, we explored whether ROS regulates the activation of IP3R and UPR-related factors. Our results showed that CSE activated the phosphorylation of IRE1α, XBP1s, and IP3R, whereas pre-treatment with NAC attenuated the upregulation of these proteins induced by CSE (Figure 6A, D, E, F). We evaluated the expression levels of MUC5AC after treatment with NAC. We found that increased levels of MUC5AC stimulated by CSE were attenuated by NAC (Figure 6I and J).

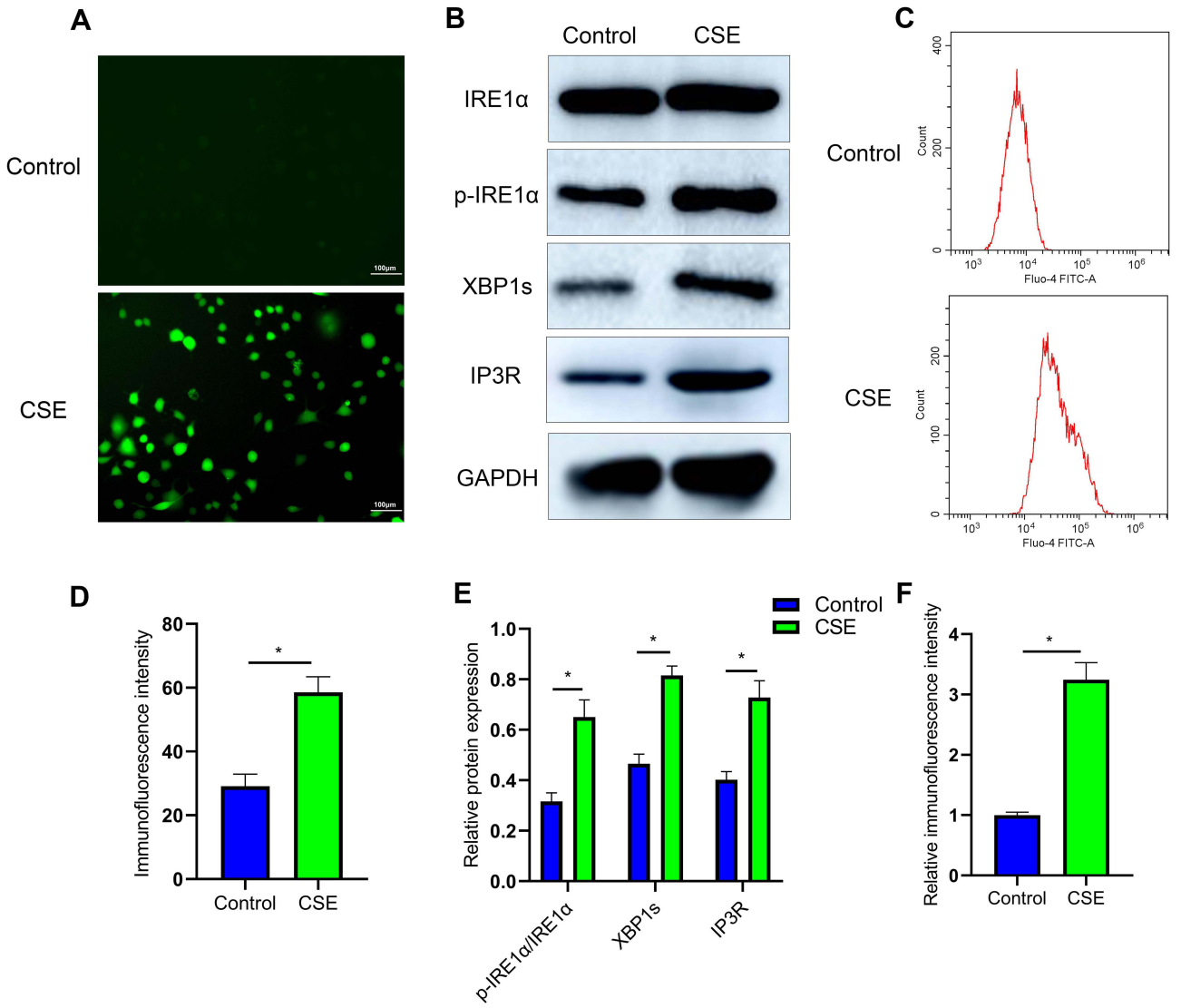

Figure 3 CSE-induced Oxidative stress and UPR in Calu-3 cells. (**A**) Total ROS were measured in Calu-3 cells after CSE exposed. (**B**)Western blot analysis showed the expression levels of IRE1α, p-IRE1α, XBP1s and IP3R in Calu-3 cells. (**C**) The measurement of intracellular Ca^{2+} by Fluo-4AM under CSE exposure. (**D**) Quantitative analysis of total ROS levels. (**E**) Quantitative analysis of IRE1α, p-IRE1α, XBP1s and IP3R levels by Western blot. (**F**) Relative immunofluorescence intensity in control group and CSE group by flow cytometry. Scale bars: 100 μm. All values are presented as the mean ± SD of three independent experiments (n = 3).
Notes: *$P < 0.01$ compared with control group.

Collectively, these data suggested that reducing ROS activity attenuated CSE-induced MUC5AC production via the IP3R/ Ca^{2+} and UPR pathways in Calu-3 cells.

Discussion

Cigarette smoke, identified as the predominant risk factor for COPD and lung cancer, induces airway mucus hypersecretion, which contributes significantly to the initiation and progression of COPD. In this study, we demonstrated CSE induced MUC5AC overexpression in Calu-3 cells. Calu-3 cells are human airway epithelial cell line commonly used in research to study respiratory physiology and pathophysiology, including the production and regulation of mucins. In our study, utilizing Calu-3 cell cultures, which consist of up to 40% goblet-like cells expressing mucin granules, we achieved a substantial accumulation of mucin granules within the cytosol.[25] Calu-3 cells are particularly valuable for studying mucin production and secretion due to their ability to produce mucins, especially MUC5AC and MUC5B, which are the primary mucins found in the respiratory tract.[26,27] We further investigated the possible involvement of IP3R/Ca^{2+} in

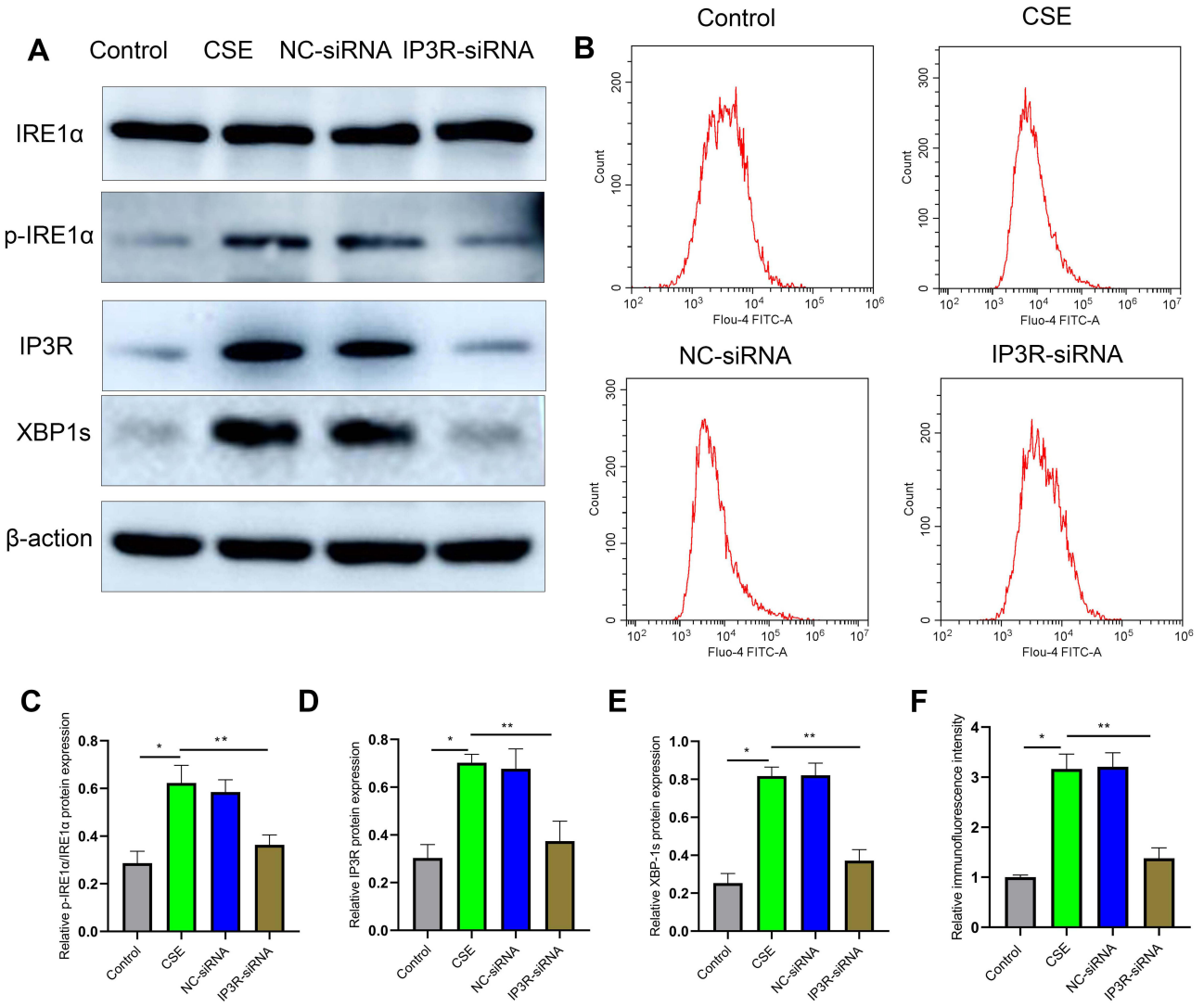

Figure 4 IP3R is necessary for CSE-induced intracellular Ca^{2+} and UPR. (**A**) Transfection with IP3R-siRNA decreased the protein levels of IRE1α, p-IRE1α, IP3R and XBP1s. (**B**)Transfection with IP3R-siRNA decreased the levels of intracellular Ca^{2+}. (**C**) Quantitative analysis of IRE1α and p-IRE1α levels by Western blot. (**D**) Quantitative analysis of IP3R levels by Western blot. (**E**) Quantitative analysis of XBP1s levels by Western blot. (**F**) Relative immunofluorescence intensity by flow cytometry after transfection with IP3R-siRNA. All values are presented as the mean ± SD of three independent experiments (n = 3). *P < 0.01 compared with control group.
Notes: *P < 0.01 compared with control group.**P < 0.01 compared with CSE group.

MUC5AC expression and its underlying molecular mechanisms. We found that oxidative stress, IP3R/Ca^{2+} ratio, and UPR were upregulated in CSE-induced MUC5AC expression. Furthermore, our study demonstrated that knockdown of IP3R/ Ca^{2+} suppressed CSE-induced UPR and MUC5AC expression in Calu-3 cells. Moreover, CSE-induced MUC5AC production, oxidative stress, and UPR progression were reversed by NAC treatment. These results demonstrated that CSE exposure resulted in oxidative stress and IP3R/ Ca^{2+}signaling activation, which activated the UPR and induced MUC5AC expression.

Abnormalities in mucus secretion, including the elevated expression of the mucins MUC5AC and MUC5B, are key features of COPD. COPD is regarded as a muco-obstructive disease characterized by an increased production of mucins, particularly MUC5AC, which may uniquely alter the properties of mucus.[28] Our study demonstrated that the changes of MUC5AC were greater compared with MUC5B under CSE exposure, which was consistent with the study of Radicioni G et al.[29] Thus, increased MUC5AC concentrations play an important role in the pathobiological component of COPD progression. Several signaling pathways have been identified in the regulation of MUC5AC expression, which include

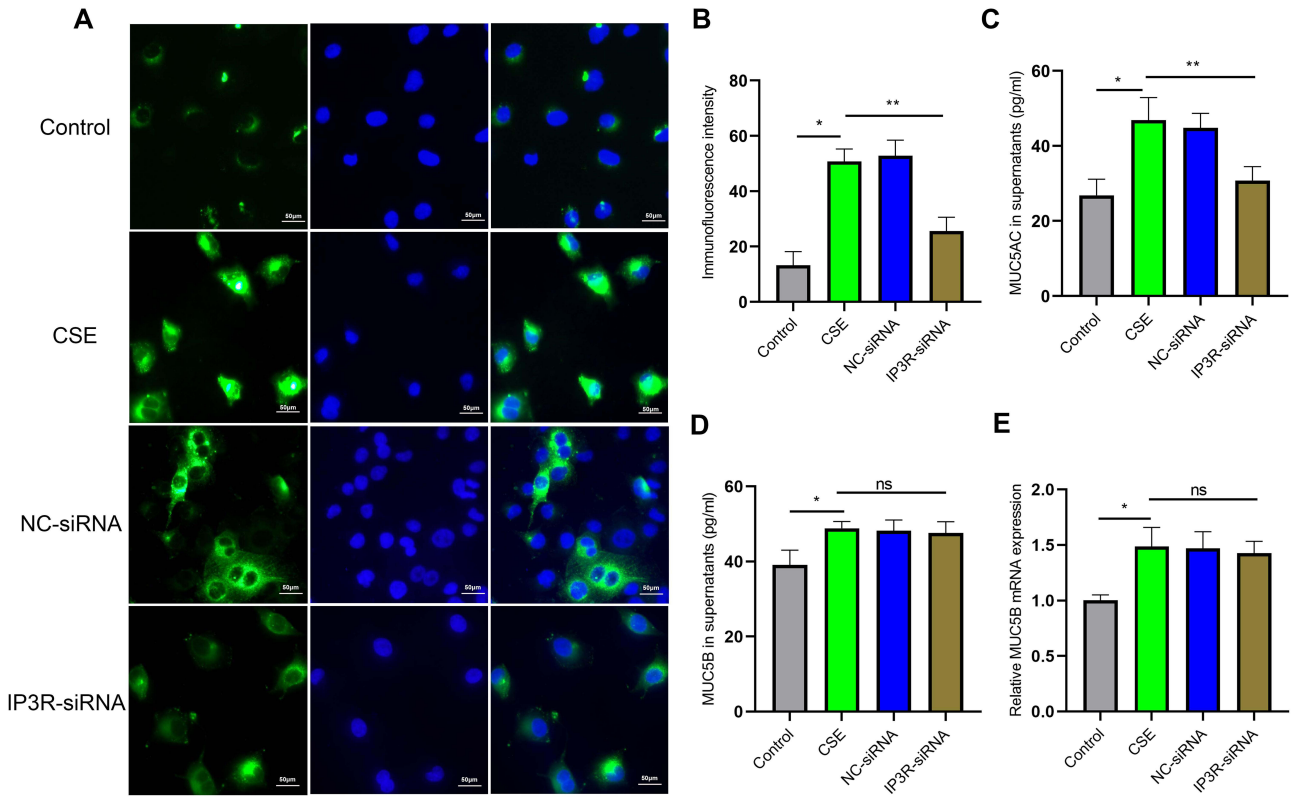

Figure 5 Transfection with IP3R-siRNA decreased the levels of MUC5AC. (**A**) The expression of MUC5AC in Calu-3 cells by immunofluorescence staining after transfection with IP3R-siRNA. Scale bars: 50 μm. (**B**) Quantitative analysis of MUC5AC immunofluorescence intensity. (**C**) The expression of MUC5AC in culture supernatants was determined by ELISA after transfection with IP3R-siRNA. (**D**) The expression of MUC5B in culture supernatants was measured by ELISA after transfection with IP3R-siRNA. (**E**) The levels of MUC5B mRNA expression measured by RT-qPCR after transfection with siRNA. All values are presented as the mean ± SD of three independent experiments (n = 3).

Notes:.*$P < 0.05$ compared with control group. **$P < 0.01$ compared with CSE group. ns, not significant.

NF-κB, ERK, oxidative stress pathways.[30,31] However, the specific molecular mechanisms involving ROS and the UPR in the production of MUC5AC remain unclear.

Oxidative stress occurs within the pulmonary system during COPD and is a significant predisposing factor that contributes to the inflammatory response associated with COPD. Oxidative stress is likely to be linked to the pathology and severity of COPD. Mitochondrial respiration in inflammatory cells was recognized as a crucial origin of ROS and cigarette smoke exacerbated ROS production through mitochondrial dysfunction.[23,32] In this study, we assessed the impact of cigarette smoke on ROS in vitro. Our experiments demonstrated that CSE exposure induces ROS production in Calu-3 cells. Excessive ROS generation induced by cigarette smoke exacerbated chronic inflammation, airway remodeling, fibrosis, and emphysema in COPD.[33] Recent study demonstrated that an increase in mitochondrial ROS production and activation of the ERK pathway played a pivotal role in the upregulation of MUC5AC induced by benzo(a)pyrene (BaP).[20] Tian et al also found that neutrophil elastase (NE)-induced ROS was actively involved in the upregulation of MUC5AC production.[34] Therefore, ROS represents a crucial pathway in MUC5AC production, and further investigation is needed to determine whether ROS in other intracellular compartments is associated with MUC5AC overexpression.

IP3R is a calcium ion channel situated within both the endoplasmic reticulum and sarcoplasmic reticulum. It consists of a tetrameric structure composed of four glycoproteins, possesses a relative molecular weight of approximately 260 kDa.[35] IP3R plays a crucial role as a key receptor channel in regulating intracellular calcium stores. Its widespread expression across most cell types plays an important role in eliciting various cellular processes including embryonic development, gluconeogenesis, and neuronal plasticity by finely modulating Ca^{2+} signals.[36] The ROS generated from NADPH oxidase can directly influence the conformational state of the IP3R located on the ER. This interaction induces

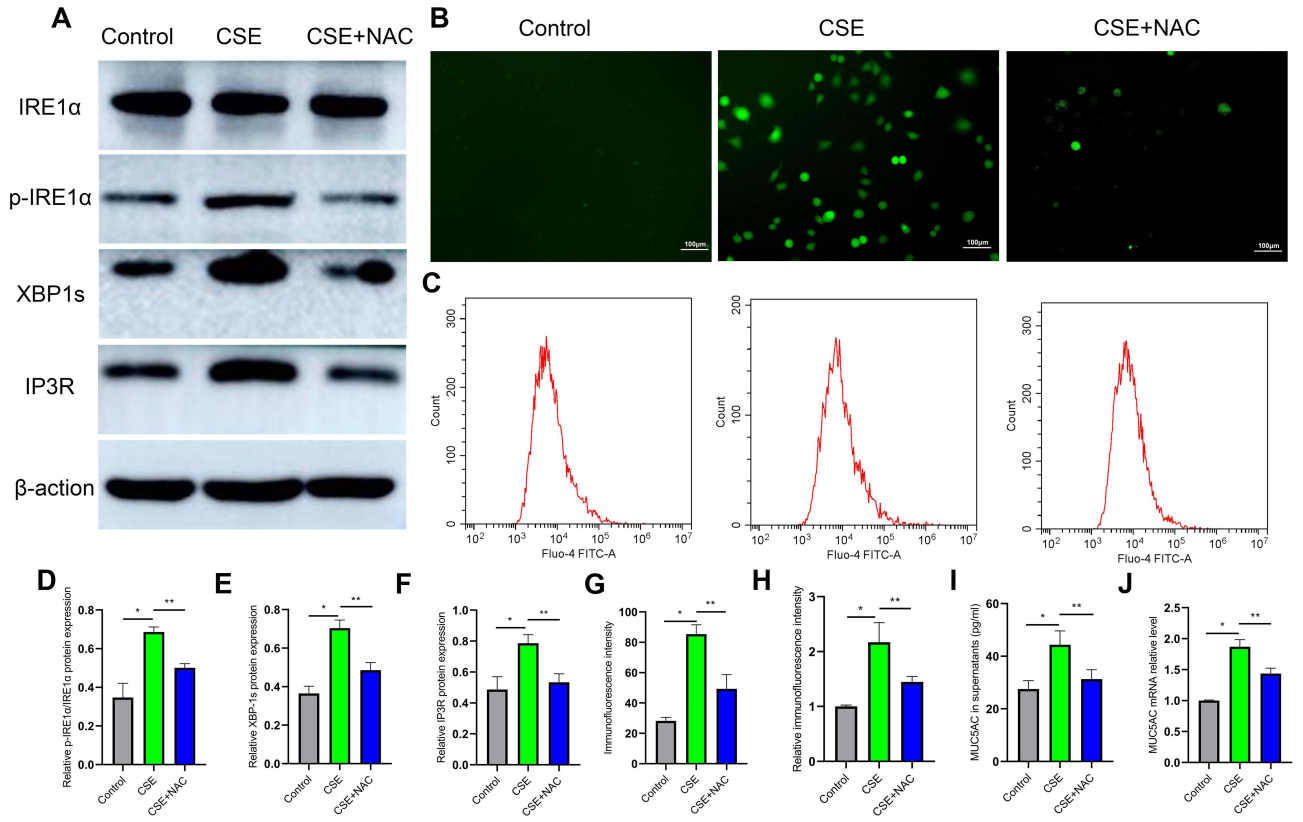

Figure 6 NAC alleviated oxidative stress and UPR in CSE-exposed Calu-3 cells. (**A**) The protein levels of IRE1α, p-IRE1α, XBP1s and IP3R measure by Western blot after NAC treatment. (**B**) Total ROS were measured in Calu-3 cells after NAC treatment. Scale bars: 100 μm. (**C**) Treatment with NAC decreased the levels of intracellular Ca^{2+}. (**D**) Quantitative analysis of IRE1α and p-IRE1α levels by Western blot. (**E**) Quantitative analysis of XBP1s levels by Western blot. (**F**) Quantitative analysis of IP3R levels by Western blot. (**G**) Quantitative analysis immunofluorescence intensity of ROS levels. (**H**) Relative immunofluorescence intensity by flow cytometry after treatment with NAC. (**I**) The expression of MUC5AC in culture supernatants was determined by ELISA after treatment with NAC. (**J**) The level of MUC5AC mRNA expression determined by qRT-PCR in Calu-3 cells after treatment with NAC. There are three independent experiments (n = 3).
Notes: *$P < 0.001$ compared with control group. **$P < 0.01$ compared with CSE group.

structural changes in IP3R, leading to an elevation in cytosolic free Ca^{2+} concentration, which initiates an intricate intracellular Ca^{2+} signaling cascade and regulates various biological effects mediated by the activity of Ca^{2+}-dependent protein kinase.[37] Another study also found that ROS-sensitive calcium channels like the IP3R and ryanodine receptors (RyR) situated on the ER membrane can be activated during oxidative stress, resulting in the release of stored Ca^{2+}.[38] In this study, we found the expression of ROS and IP3R/Ca^{2+} was upregulated after CSE exposure. A decrease in CSE-induced ROS and IP3R/Ca^{2+} levels was observed following NAC treatment, suggesting that ROS might be a potential activator of IP3R/Ca^{2+} in MUC5AC overexpression.

ER acts as a multifunctional organelle crucial for various cellular processes, including maintaining Ca^{2+} homeostasis within both the ER lumen and other cellular compartments, as well as facilitating lipid and protein biosynthesis, protein folding, and post-translational modification.[39] Trychta et al found that depleting ER calcium upregulated unfolded protein response genes as an adaptive response.[40] Some previous studies also found that disturbances in Ca^{2+} homeostasis within the ER triggered the activation of ER stress mechanisms and the UPR.[41] In the present study, CSE exposure promoted activation of ROS/IP3R/Ca^{2+} signaling, which further contributed to the occurrence of UPR and the activation effect could be reversed by IP3R knockdown and NAC treatment. These results demonstrated that CSE exposure leaded to an increase in ROS, which activated IP3R. This activation then triggered the release of Ca^{2+} from the ER and subsequently activated the UPR. Increasing evidence indicated that goblet cells express IRE, a distinctive sensor involved in the UPR, which constitutes an adaptive pathway regulating the mucin production and secretion.[15] Grey et al demonstrates that the formation of mature goblet cells, which are capable of producing adequate amounts of mucus, necessitates a basal level of UPR, accompanied by elevated levels of chaperones and expansion of the ER.[42] At the same time, our study found

that the MUC5AC expression was upregulated along with the increase of UPR-associated factors under CSE exposure. To further verify the role of UPR in MUC5AC, we observed that the expression of MUC5AC decreased when the UPR was inhibited by IP3R-siRNA and NAC treatment. In addition, how UPR regulates MUC5AC production and whether UPR affects other potential mechanisms require further investigation. In COPD, NAC is used as an antioxidant to provide GSH and mucus-dissolving agents. In a recent study, the cigarette smoke-induced CFTR dysfunction impaired the phagocytosis in the alveolar macrophages and the cigarette smoke also inhibited the mitochondrial respiration while inducing glycolysis and the generation of ROS, which were mitigated by NAC.[43] Other study found that NAC treatment enhanced pulmonary functions, decreased COPD-induced increases in TNFα and IL-6, reduced the inflammatory infiltration of the lung, reduced the tissue injury and destruction of the alveolar septum, decreased the thickness of the bronchiolar wall, and reduced the collagen and α-SMA levels in a rat model of COPD.[44] A trial by Zheng et al showed that the long-term use of NAC 600 mg twice daily may prevent exacerbations, particularly in patients with moderate-to-severe COPD.[45] These data suggest that NAC treatment plays an important role in antioxidant, anti-inflammatory effects and reducing airway mucus secretion in COPD.

Conclusions

In summary, we demonstrated that increased ROS production, which resulted in IP3R/Ca^{2+} signaling and UPR activation, is crucially involved in CSE-induced MUC5AC overexpression. Additionally, NAC treatment could inhibit the CSE effects on ROS and MUC5AC production via the IP3R/ Ca^{2+} and UPR pathways. This study provides a new perspective for understanding the regulation of MUC5AC, and suggests a potential target for further understanding the mechanisms of airway mucus hypersecretion in COPD.

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Disclosure

The authors declare that they have no competing interests in this work.

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