



Original article

Inhibition of nuclear thioredoxin aggregation attenuates PM_{2.5}-induced NF-κB activation and pro-inflammatory responses

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ABSTRACT

Exposure to fine particulate matter (PM_{2.5}) can induce oxidative stress and proinflammatory cytokine production, which are central for the induction of PM_{2.5}-mediated adverse effects on public health. Nuclear factor kappa B (NF-κB) signaling is essential for inflammation. The subcellular distribution of thioredoxin (Trx) is related to the activation of NF-κB, but the mechanism involved is unclear. In the current study, we focused on the relationship between the antioxidant Trx and NF-κB in human bronchial epithelial cells (BEAS-2B) after PM_{2.5} exposure. We inhibited the nuclear translocation of Trx by cHCEU (4-cyclohexyl-1-[3-(2-chloroethyl)ureido] benzene) and subsequently increased the transcriptional activity of Nrf2 to upregulate the expression of Trx by t-BHQ. Our data suggest that PM_{2.5} exposure induces the activation of NF-κB and the expression of the downstream proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α in BEAS-2B cells. CHCEU alleviates inflammatory cytokines by blocking Trx nuclear translocation and inhibits the DNA binding activity of NF-κB. T-BHQ could promote the transcriptional activity of Nrf2 but failed to alleviate the production of inflammatory cytokines. Furthermore, the synergistic effect of t-BHQ and CHCEU on alleviating PM_{2.5}-induced inflammation is more effective than the use of CHCEU alone. Our findings characterize the underlying molecular mechanisms of proinflammatory responses induced by PM_{2.5} and show that the nuclear translocation and accumulation of Trx in nuclei play important roles in PM_{2.5}-induced NF-κB activation and proinflammatory responses.

1. Introduction

In recent years, air pollution has become the primary problem affecting the Chinese environment, and haze weather is also the focus of public attention worldwide. Ambient fine particulate matter (PM_{2.5}) is the main constituent of haze in China. PM_{2.5}, which has an aerodynamic diameter below 2.5 μm, is easily inhaled into the airways where it is deposited in the lung alveoli. Therefore, the lung is the initial site of PM_{2.5} deposition and is one of the targets of PM_{2.5} toxicity. In addition, PM_{2.5} contains heavy metal particles, acidic oxides, organic pollutants, bacteria, fungi, and viruses [1]. Numerous studies have shown that the detrimental components of PM_{2.5} cause airway inflammation, asthma, chronic obstructive pulmonary disease, arrhythmias and lung cancer [2]. Inflammatory responses have been linked to the initial steps involved in these pulmonary diseases. PM_{2.5} inhalation or instillation aggravates inflammatory responses in humans

and animals, which is characterized by increased vascular permeability and oxidative stress [3,4], and is accompanied by neutrophil recruitment together with nuclear factor kappa B (NF-κB) activation and the increased expression of genes encoding tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) [5–8].

NF-κB, a redox-sensitive transcriptional factor, is critical for the production of inflammatory cytokines and is usually quiescent in the cytoplasm, where it exists in an inactive multiunit complex bound to an inhibitory protein (I-κB). Many factors, such as excessive ROS production and heavy metals activate NF-κB signaling. Of note, PM_{2.5} activates this complex by inducing the phosphorylation and subsequent degradation of I-κB and translocation of the active dimer into the nucleus, where it binds to the promoter region of genes including many proinflammatory cytokines such as TNF-α and IL-6 to induce their expression [9]. During NF-κB activation, the redox state plays an important role in

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the cytoplasm and nucleus. It is well known that oxidative stress promotes the degradation of I- κ B, which dissociates from NF- κ B to promote its entry into the nucleus [10]. However, excessive oxidative stress in the nucleus results in the oxidation of a cysteine residue (cys-62) in the NF- κ B subunit (p50) that interferes with its binding to DNA and decreases inflammatory gene expression [11]. Under physiological conditions, thioredoxin (Trx) promotes the DNA binding of NF- κ B by maintaining the redox-active cysteine of p50 in cys-62 via the formation of a multi-molecular complex with NF- κ B in the nucleus. Trx is a small molecule protein containing a thiol/disulfide group that specifically reduces the oxidized cysteine of the target protein. Therefore, Trx functions by reducing cysteine residues that are critical for NF- κ B binding to DNA. NF- κ B has been used as a competitive target for the development of drugs for the treatment of pulmonary inflammation. However, the development of drugs that directly inhibit the components of NF- κ B itself for prolonged periods might induce adverse events because it plays a crucial role in immune and host defense responses. Whether it is possible to interfere with the activation of NF- κ B by inhibiting the entry of Trx into the nucleus is unclear.

It is well known that Trx is localized mostly in the cytoplasm and exerts a protective effect against cell injury by scavenging excess ROS [12]. In the cytoplasm, Trx has an inhibitory effect on I- κ B kinase, thus inhibiting NF- κ B translocation to the nucleus. Trx in different subcellular locations has dual and opposing roles in regulating oxidative stress signaling and inflammation: the overexpression of Trx regulated the redox state in the cytoplasm, yet also promoted the DNA binding of NF- κ B in the nucleus [13]. When oxidative stress occurs, nuclear factor erythroid 2-related factor 2 (Nrf2), an upstream transcription factor of Trx [14], is activated and transferred into the nucleus to regulate the antioxidant response element (ARE) mediated expression of antioxidant enzymes, including Trx, to protect against the adverse effects of oxidative stress [15]. Therefore, we investigated whether NF- κ B-mediated inflammatory responses caused by PM_{2.5} intervention could be inhibited by upregulating the transcriptional activity of Nrf2.

In the current study, we investigated the inflammatory mechanism of PM_{2.5} on human bronchial epithelial cells and the relationship between the antioxidant Trx and the inflammatory transcription factor NF- κ B. To examine the role of Trx subcellular distribution in inflammatory responses leading to NF- κ B activation, we used a specific nuclear inhibitor, cHCEU (4-cyclohexyl-[3-(2-chloroethyl)ureido]benzene), to interfere with the PM_{2.5}-induced activation of NF- κ B. We also compared the treatment of tert-butylhydroquinone (t-BHQ), an agent that could upregulate Nrf2 transcriptional activity, to observe its role in inflammation induced by PM_{2.5}. We attempted to explore the underlying molecular mechanisms of PM_{2.5}-induced inflammatory responses and determine whether blocking Trx nuclear translocation is a potential intervention target.

2. Methods

2.1. Collection and preparation of PM_{2.5}

PM_{2.5} samples were collected during the winter, 2016 in Beijing. A PM_{2.5} high volume air sampler (Tianhong, China, TH-1000CII) was placed on the rooftop of a building about 10 m tall. PM_{2.5} samples were collected in quartz fiber filters. These filters that loaded the samples were cut into pieces and sonicated in water. Then the PM_{2.5} suspensions were dried and stored at -80°C . The 30 mg PM_{2.5} particles supplemented with 30 mL PBS, then 1000 $\mu\text{g/mL}$ PM_{2.5} was stored at -80°C , treated with ultrasonic oscillation for 30 min every time.

2.2. Cell culture and treatment

BEAS-2B (Human lung bronchial epithelial) cells were purchased

from the Cell Resource Center, Shanghai Institutes for Biological Science (SIBS), China. The cells were cultured in a DMEM medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) in an incubator (Sanyo, Japan), with a humidified atmosphere of 5% CO₂ in air at 37 $^{\circ}\text{C}$.

BEAS-2B cells were plated in plates, and when they reached 70–80% confluence, complete medium was replaced with starvation medium (DMEM with 1% FBS) and incubated for 12 h. For assessment of the effects of PM_{2.5}, cells were treated with PM_{2.5} either at 25, 50, 100, 200 $\mu\text{g/mL}$ for the dose gradient or at 6, 12, 24, 48 h for the time periods. For the studies testing the effects of chemicals and reagents, BEAS-2B cells were treated with PM_{2.5} in the presence of the t-BHQ (50 μM) and/or cHCEU (50 μM) simultaneously for 24 h incubation. The reagents were dissolved in dimethyl sulfoxide (DMSO), and the final concentrations of DMSO in the culture medium did not exceed 0.2%.

2.3. Cell viability assay

We used MTT ((4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay to measure cell viability and proliferation. Cells were exposed to PM_{2.5} (0, 25, 50, 100, 200 $\mu\text{g/mL}$) for 24 h to investigate cell proliferation. After incubating for 4 h at 37 $^{\circ}\text{C}$ with MTT, the cells were labeled, then DMSO solution was added, and the cells were shaken in the dark at 37 $^{\circ}\text{C}$ in a shaker. The plate was read at a wavelength of 492 nm on a microplate reader. The relative cell viability was calculated and compared with the absorbance of the control group. All MTT assays were accomplished thrice.

2.4. ROS detection

BEAS-2B cells were cultured in 12-well plates at a density of 1×10^5 cells/mL (1 mL/well). After overnight incubation, cells were treated with PM_{2.5} at concentrations of 25, 50, 100 and 200 $\mu\text{g/mL}$. After trypsinization, cell suspensions from each of the wells were collected into a tube and probed with 150 μL of 10 μM DCFH-DA (Sigma). The cells were incubated at room temperature away from light for 30 min. Subsequently the multimode reader (Berthold Technologies, Germany) was used for the detection of fluorescence signal.

RoGFP was a gift from Paul Schumacker (Addgene plasmid # 49435). The plasmid was transformed and amplified according to the previous method [16]. Plate BEAS-2B cells at the desired density in a 20-mm dish. Mix 3 μg roGFP plasmid and 4 μL Lipofectamine[®] 2000 Transfection Reagent (Invitrogen[™]) with DMEM serum-free medium. Incubate cells for 6 h and replace the transfected medium with complete medium. The cells were subjected to PM_{2.5} intervention 48 h after transfection. Analyze live cells using a fluorescent expression microscope equipped with imaging capabilities at two different excitations (405 and 488 nm), and emission at 515 nm.

2.5. RT-qPCR analysis

Cells total RNA were extracted by using TransZol Up (ET111, TransGen Biotech, Beijing, China) and were transcribed from mRNA to cDNA by the application of the Transcript First-Strand cDNA Synthesis SuperMix (AT301, TransGen Biotech, Beijing, China). RT-qPCR was performed with the CFX96[™] real-time quantitative PCR detection system (BioRad Inc., Hercules, CA), using the SYBR Green qRT-PCR kit (AQ141, TransGen Biotech, Beijing, China). The experiment was repeated at least three times with independent mRNA samples. The gene expression levels were determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase using the $2^{-\Delta\Delta\text{CT}}$ method. The primer sequences are listed below.

2.6. Primers for BEAS-2B

GAPDH F	TCAACGACCACTTTGTCAAGCT
GAPDH R	CCATGAGGTCCACCACCT
Nrf2 F	AACCACCCTGAAAGCACAGC
Nrf2 R	TGAAATGCCGGAGTCAGAATC
Trx F	CATAACGAGCCATTGGCTATT
Trx R	GCATAATGTTTATTGTCAGC
NF-κB F	AACAGCAGATGGCCATACCT
NF-κB R	ACGCTGAGGTCCATCTCCTTG
IL-1 F	GGATATGGAGCAACAAGTGG
IL-1 R	ATGTACCAAGTTGGGGAAGT
IL-6 F	TAGCCGCCACACAGACAG
IL-6 R	GGCTGGCATTTGTGG TTGGG
IL-8 F	GAGGGTTGTGGAGAAGTTTTTG
IL-8 R	CTGGCATCTCACTGATTCCTG
TNF-α F	TCTTCAGCTCTGGGAAAATGC
TNF-α R	TTTTGCTTGCCCTCCCAGG

2.7. Western blot analysis

Protein was extracted with Kit KGP2100 (KeyGen Biotech, China). The cells were washed twice with PBS and disrupted in lysis buffer. The concentration was assayed by using bicinchoninic acid (BCA, Thermo Scientific, Rockford, IL, USA). Total protein was extracted and sized into equal amounts of protein (20 µg) in 12% polyacrylamide SDS-gel, and subsequently transferred to the PVDF membranes (Millipore, Billerica, MA, USA), which were then blocked in 5% BSA solution and incubated overnight at 4 °C with primary antibodies against Nrf2 (ab31163, Abcam; 1:1000), Trx (ab26320, Abcam; 1:1000), NF-κB p65 (#8242, CST; 1:1000), p-NF-κB p65 (#3033, CST; 1:1000), NF-κB p50 (ab32360, Abcam; 1:1000), p-NF-κB p50 (ab194729, Abcam; 1:1000), IκBα (ab32518, Abcam; 1:1000), GAPDH (#2118, CST; 1:2000). Anti-rabbit IgG, HRP-linked antibody (#7074, CST; 1:1000) was used as the secondary antibody. The proteins mentioned above were detected using an enhanced chemiluminescence method (ECL, Thermo). Tanon-5200 system (Beijing Yuan Ping Hao Biotech Co. Ltd) was used to scanned the

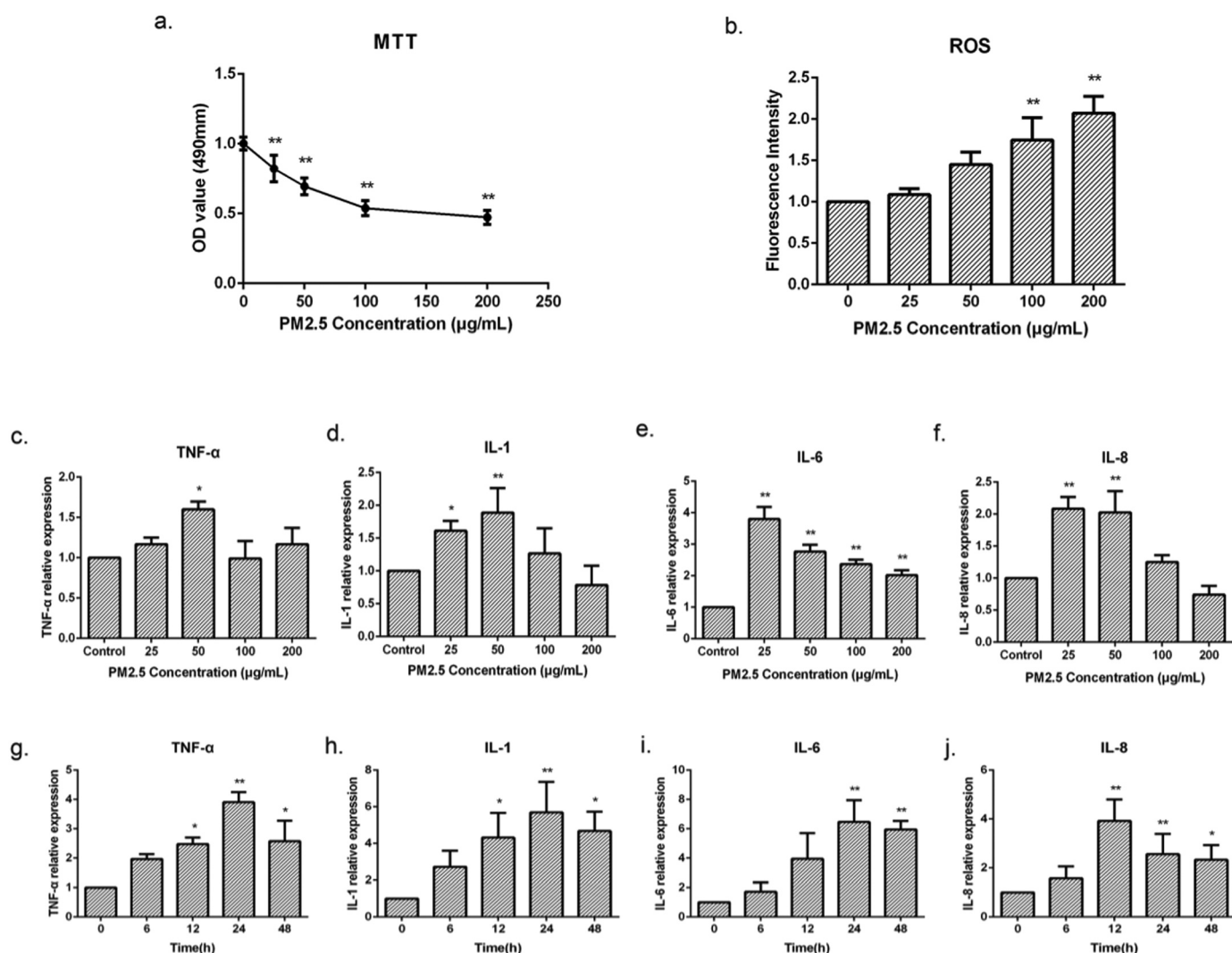


Fig. 1. PM_{2.5} decreases cell viability, induces oxidative stress and proinflammatory responses in BEAS-2B cells. (a) PM_{2.5} exposure for 24 h concentration-dependently decreased BEAS-2B cell viability, as determined by MTT assay. The data shown are the means ± SEM from three independent experiments. *p < 0.05, **p < 0.01 compared with the control group. (b) Increased ROS production measured by DCFH-DA was observed in response to PM_{2.5}. **p < 0.01 compared with the control group. (c–f) Dose-dependent effects of PM_{2.5} on TNF-α, IL-1, IL-6 and IL-8 mRNA expressions. The cells were incubated for 24 h with increasing concentrations of PM_{2.5}. TNF-α, IL-1, IL-6 and IL-8 mRNA expressions were expressed relative to β-actin mRNA expression (mean ± SD from 3 different experiments). (g–j) Time-dependent effects of PM_{2.5} on TNF-α, IL-1, IL-6 and IL-8 mRNA expressions. The cells were stimulated with PM_{2.5} (50 µg/mL) for the pre-determined times. TNF-α, IL-1, IL-6 and IL-8 mRNA expressions were expressed relative to β-actin mRNA expression (mean ± SD from 3 different experiments).

blots and then Image J software was used to analyze the intensity of grayscale images.

2.8. Immunofluorescent assay

BEAS-2B cells grew on round chamber slides and were exposed to the supernatant. Slides were then washed with PBS, cells were fixed in 4% paraformaldehyde for 20 min, and then fixed with 0.3% Triton X-100 for 10 min at room temperature (RT). Subsequently they were washed with PBS more than three times and blocked with 5% bovine serum albumin (BSA) for 10 min at RT. Then, the cells were incubated with the primary antibody against Trx (1:500, Abcam, CA, USA) at 4 °C overnight. The slides were washed with PBS and finally incubated with goat anti-rabbit antibody (CST, 4412s) conjugated with fluorescein isothiocyanate (FITC) for 1 h at RT on the second day. After finishing the above steps, 4',6-Diamidino-2-phenylindole (DAPI) was applied for nuclear staining. Immunofluorescence staining of BEAS-2B cells was examined by laser scanning confocal fluorescence microscopy.

2.9. Co-immunoprecipitation (COIP)

The COIP protocol is described by SINOBLE biotechnology center. Protein extraction was performed with Kit KGP2100 (KeyGen Biotech, China) and protein concentration was determined using bicinchoninic acid (BCA, Thermo Scientific, Rockford, IL, USA). Immunoprecipitations were performed using IgG (TDY Biotech, China) and protein A/A-agarose (GE) at 4 °C. Western blot was applied to detect the lysates and immunoprecipitates by using the NF- κ B or Trx primary antibodies and appropriate secondary antibody.

2.10. Electrophoretic mobility shift assay (EMSA)

Preparation of a polyacrylamide gel for EMSA: assemble the gel (70 × 80 mm) by using 1.5 mm spacers and a comb. To prepare a 6.5% polyacrylamide gel, use the stock solutions which includes 3.3 mL of 40% acrylamide, 1 mL of 50% Glycerol, 1 mL of 10xTBE and 14.8 mL

double distilled H₂O. Add 12 μ L of 10% freshly prepared ammonium persulfate (APS) and 20 μ L tetramethylethylenediamine (TEMED). Immediately pour the acrylamide solution to the gel and let it polymerize. Wait for approximately 30 min. Assemble the electrophoresis apparatus with the gel, fill the tanks with 0.5X TBE and connect with the power supply. Set up an EMSA reaction by adding 0.5 μ L of the radiolabeled probe to the protein extract. Incubate for 20 min at room temperature. Add 1 μ L of the sample to the reaction. Run the gel for 60 min at 180 V. Electrophoretic Transfer of Binding Reactions to Nylon Membrane. Soak the nylon membrane in 0.5X TBE for 10 min. Sandwich the gel and nylon membrane in a clean electrophoretic transfer unit. Use 0.5X TBE, cooled to ~10 °C with a circulating water bath. Transfer at 380 mA (~100 V) for 30 min. Typical transfer times are 30–60 min at 380 mA. When the transfer is complete, put the membrane with the bromophenol blue side up on a dry paper towel. Cross-link Transferred DNA to Membrane, cross-link at a distance of approximately 0.5 cm from the membrane for 5–10 min with a hand-held UV lamp equipped with 254 nm bulbs. Detect Biotin-labeled DNA by Chemiluminescence. Subsequently expose to a film and develop autoradiography. Exposure time may range from 5 to 10 min.

2.11. Statistical analyses

All data were assessed by SPSS 20.0 software using at least three independent experiments. Data were expressed as mean $\pm$ standard deviations (SD), and the data were evaluated by one-way analysis of variance (ANOVA) or Student's *t*-test. *P* < 0.05 values were considered as statistically significant for group differences.

3. Results

3.1. Characterization of PM_{2.5}

The morphology of PM_{2.5} was observed by TEM and the TEM pictures revealed that PM_{2.5} manifested irregular shape without uniform diameter and relatively favorable dispersibility (Supplementary

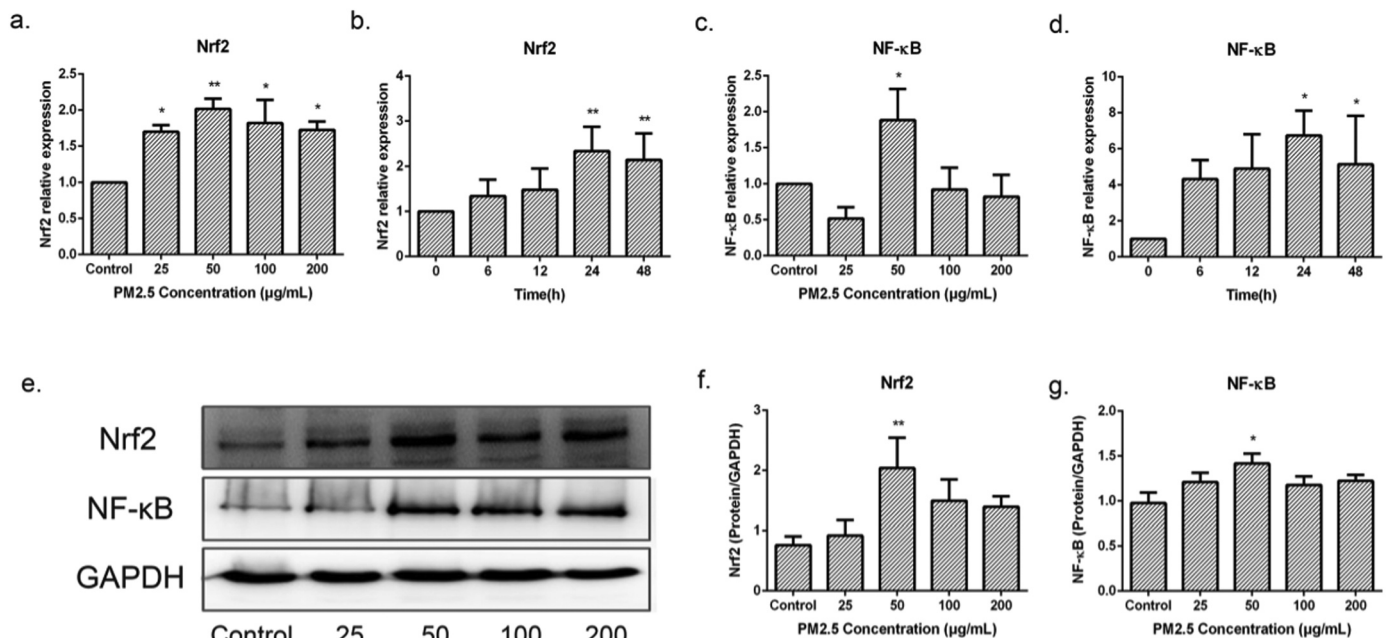


Fig. 2. PM_{2.5} induces activation of the transcription factors Nrf2 and NF- κ B. (a-d) PCR analysis of dose or time dependent Nrf2 and NF- κ B mRNA expression. **p* < 0.05, ***p* < 0.01 compared with the control group. (e-g) Western blot analysis of Nrf2 and NF- κ B (p65) protein levels with varying doses of PM_{2.5}. **p* < 0.05, ***p* < 0.01 compared with the control group.

material: Fig. S1). The distilled water was selected as reserve media and DMEM as media for cell culture, the hydrodynamic size of PM_{2.5} were detected at various time points (Supplementary material: Table S1). Our results suggested that hydration particle size of PM_{2.5} was less than 2.5 μ m in the liquid phase with good dispersibility.

PM_{2.5} decreases cell viability, and induces oxidative stress and proinflammatory responses in BEAS-2B cells.

To determine the effect of PM_{2.5} on cell survival, cell viability assays were performed in BEAS-2B cells treated with or without PM_{2.5} over a dose curve (25–200 μ g/mL). PM_{2.5} significantly decreased cell viability in a dose-dependent manner (Fig. 1a). We also examined the effect of PM_{2.5} on ROS production in BEAS-2B cells by roGFP plasmid transfection and DCFH-DA staining assay. Real-time observation of roGFP showed that BEAS-2B began to accumulate ROS gradually after transfection of PM_{2.5} (Supplementary material: Fig. S5 & Video S1.). We also obtained static images of intracellular ROS after 24 h of stimulation with different concentrations of PM_{2.5}. Confocal imaging demonstrated that roGFP expressions both in Cytoplasm and nucleus were increased with concentration dependent (Supplementary material: Fig. S6). PM_{2.5}-induced oxidative stress proven by roGFP sensor is consistent

with by DCFH-DA sensor (Fig. 1b). In conclusion, we believe that PM_{2.5} can cause progressive oxidative stress in cells. In addition, representative factors of inflammation including TNF- α , IL-1, IL-6, and IL-8 were determined by RT-qPCR. With an increased dosage of PM_{2.5}, the mRNA expressions of these factors increased and then decreased. When treated with 50 μ g/mL of PM_{2.5}, the mRNA expressions of TNF- α , IL-1, IL-6, and IL-8 were significantly ($p < 0.01$ or $p < 0.05$) increased compared with the control group (Fig. 1c–f). PM_{2.5} also stimulated the production of these proinflammatory cytokines in a time-dependent manner (Fig. 1g–j). After 24-h treatment with PM_{2.5}, the expressions of TNF- α , IL-1 and IL-6 reached a maximum ($p < 0.01$). Collectively, these data demonstrated that PM_{2.5} decreased cell viability and induced oxidative stress and inflammation in BEAS-2B cells.

Supplementary material related to this article can be found online at [doi:10.1016/j.freeradbiomed.2018.10.438](https://doi.org/10.1016/j.freeradbiomed.2018.10.438).

3.2. PM_{2.5} induces the activation of the transcription factors Nrf2 and NF- κ B

Nrf2 and NF- κ B are key transcription factors that regulate cellular

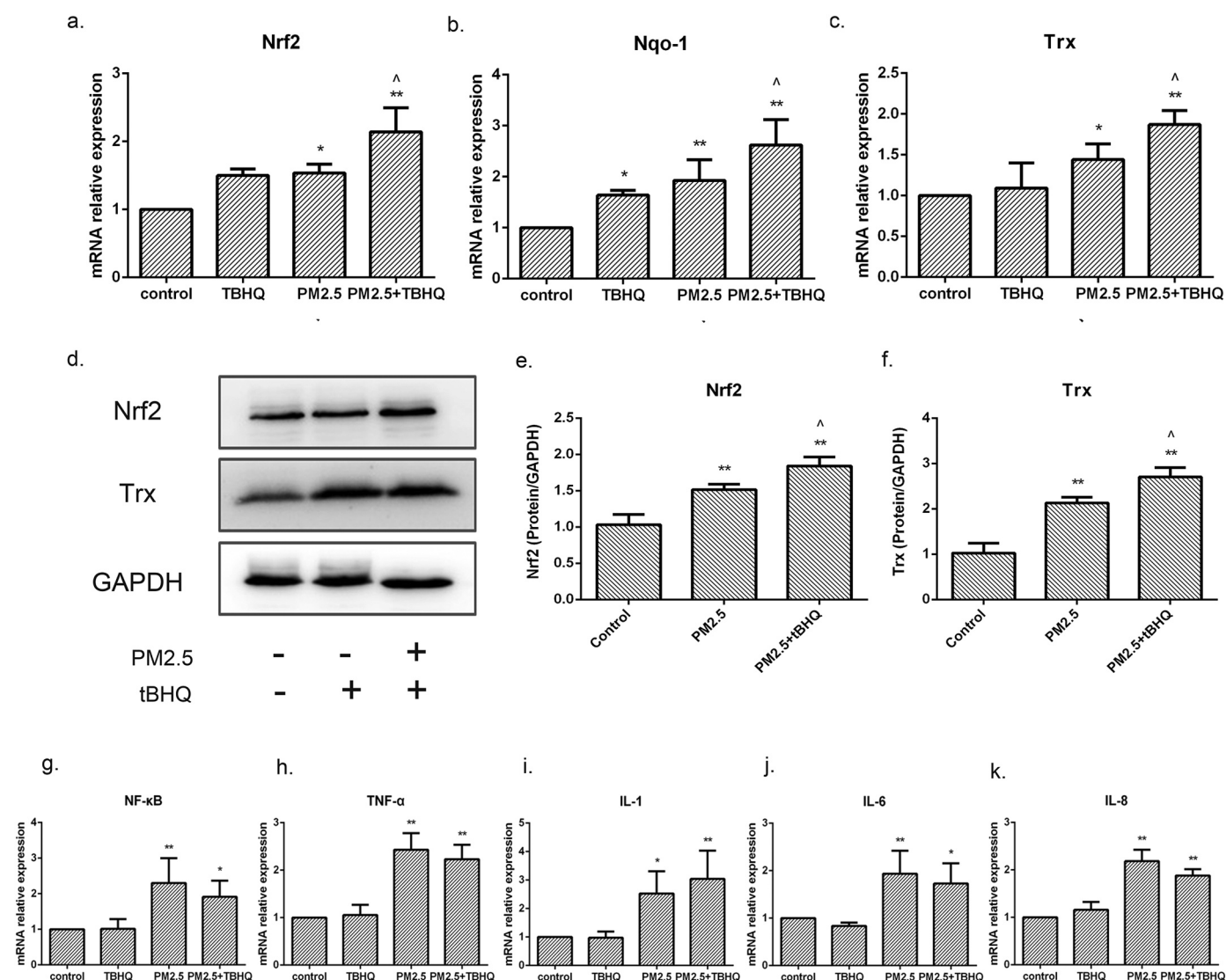


Fig. 3. T-BHQ promotes the transcriptional activity of Nrf2 but fails to alleviate inflammatory cytokine production. (a–f) T-BHQ induces the mRNA expressions or protein levels of Nrf2 and Nrf2-regulated genes in BEAS-2B cells. The results are shown as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ compared with the control group. ^ $p < 0.05$ compared with the PM_{2.5} group. (g–k) T-BHQ induces the mRNA expressions of NF- κ B and NF- κ B-regulated genes (TNF- α , IL-1, IL-6 and IL-8) in BEAS-2B cells. The results are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ compared with the control group. ^ $p < 0.05$ compared with the PM_{2.5} group.

responses to oxidative stress and inflammation, respectively. Nrf2 plays a vital role in activating antioxidant responses that decreases ROS [17]. We speculated that Nrf2 activation is involved in PM_{2.5}-induced ROS generation in BEAS-2B cells. By RT-qPCR analysis, we found that the Nrf2 mRNA level was increased after PM_{2.5} treatment in a dose- or time-dependent manner (Fig. 2a–b). Consistent with this finding, the protein expression of Nrf2 was significantly upregulated at a dose of 50 µg/mL (Fig. 2e–f). We also examined the expression levels of NF-κB gene and protein (NF-κB p65) stimulated by different concentrations of PM_{2.5}. It is suggested that the mRNA level of NF-κB peaks under the stimulation of 50 µg/mL of PM_{2.5} (Fig. 2c–d, e–g). Using it as an intervention dose, we found that PM_{2.5} significantly down-regulated the expression of IκBα and significantly up-regulated the total and phosphorylated proteins expression both of p65 and p50 (Supplementary material: Fig. S2). It was suggested that PM_{2.5} could cause degradation of IκB and phosphorylation of NF-κB. Knowing the amount of expression, we also wondered if phosphorylated p65 and p50 are transferred into the nucleus. Immunofluorescence results showed that the expressions of phosphorylated p65 and p50 were increased under PM_{2.5} stimulation, and a large amount of expression was expressed in the nucleus (Supplementary material: Figs. S3–4). Therefore, PM_{2.5} induces the activation of the transcription factors Nrf2 and NF-κB.

3.3. t-BHQ promotes the transcriptional activity of Nrf2 but does not alleviate inflammatory cytokine production

We applied t-BHQ, an inducer of Nrf2 and phase II detoxification enzyme, to explore the capability of Nrf2 and downstream genes to protect against PM_{2.5}. Nrf2 mRNA was upregulated indicating that t-BHQ affects Nrf2 transcription (Fig. 3a). Two important genes (NQO1 and Trx) regulated by Nrf2 were examined and RT-qPCR analysis showed that NQO1 and Trx mRNA levels were upregulated after PM_{2.5} treatment. Additionally, the administration of t-BHQ further enhanced NQO1 and Trx expressions compared with the PM_{2.5}-treated group (Fig. 3b–c). NQO1 is a conserved Nrf2 downstream target gene and was used as a positive control for Nrf2 transcription. Trx is a major cellular antioxidant that regulates cellular redox homeostasis and we previously confirmed the relationship between Nrf2 and Trx [14]. Consistent with the changes in mRNA expression, the protein levels of Nrf2 and Trx were upregulated after t-BHQ treatment (Fig. 3d–f). These results suggested that t-BHQ upregulated Nrf2 and downstream Trx to protect against cytotoxicity induced by oxidative stress in BEAS-2B cells. We further examined the effect of t-BHQ on the expressions of inflammatory cytokines. However, our RT-qPCR analysis showed that t-BHQ did not alleviate the expression of inflammatory cytokines (TNF-α,

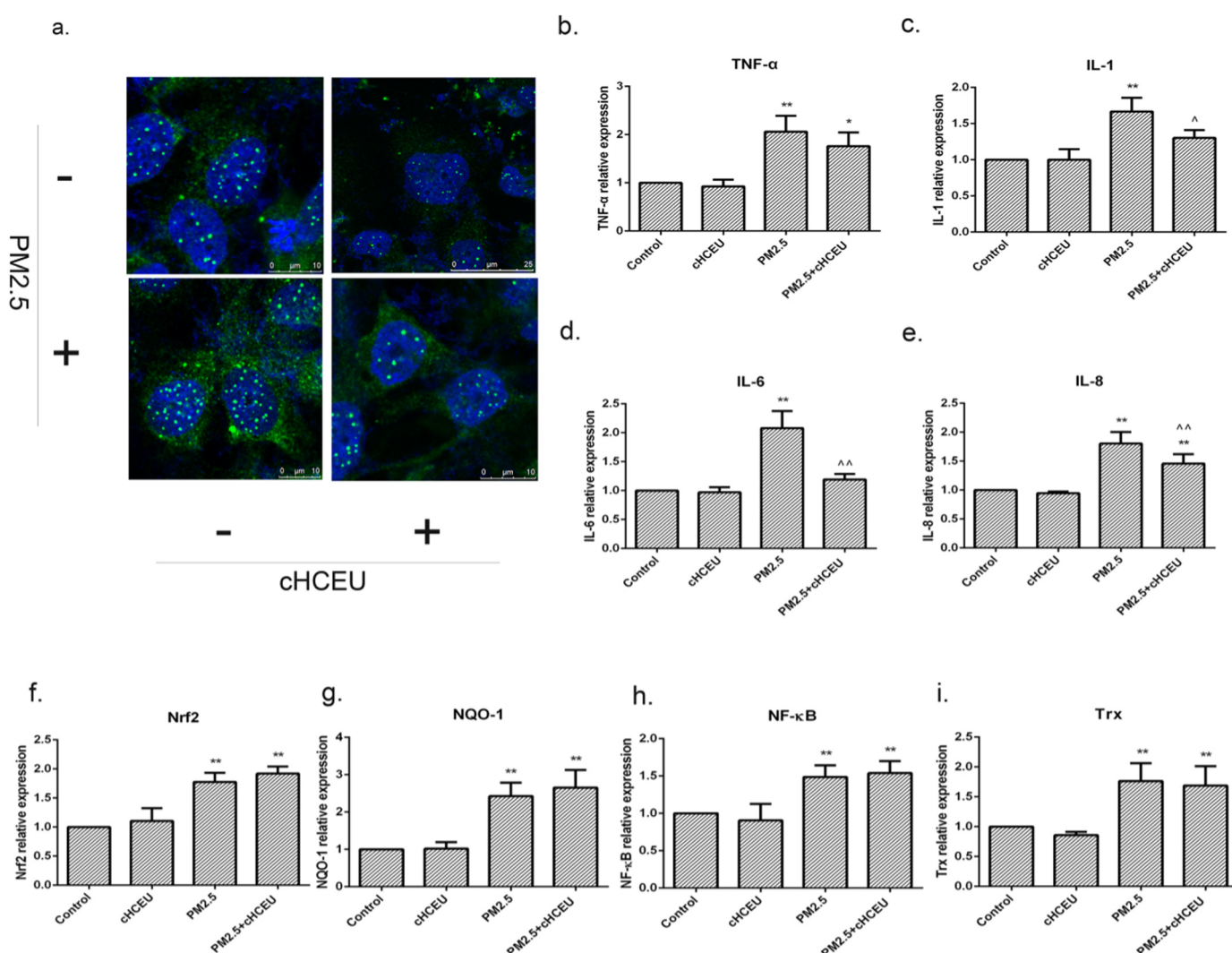


Fig. 4. chCEU alleviates inflammatory cytokines by inhibiting Trx nuclear translocation. (a) The subcellular distribution of Trx was monitored by confocal microscopy and indicated by green fluorescence in BEAS-2B cells treated with/without PM_{2.5} or chCEU. Magnification, ×63. (b–i) chCEU induces the mRNA expressions of inflammatory genes (TNF-α, IL-1, IL-6 and IL-8), transcription factor genes (Nrf2 and NF-κB) and related genes (NQO1 and Trx) in BEAS-2B cells. The results are presented as the mean ± SEM. *p < 0.05, **p < 0.01 compared with the control group. ^p < 0.05 compared with the PM_{2.5} group.

IL-1, IL-6 and IL-8) compared with the PM_{2.5}-treatment group (Fig. 3g-k).

3.4. cHCEU alleviates inflammatory cytokine production by inhibiting Trx nuclear translocation

Because increased nuclear Trx potentiates proinflammatory signaling in cells, we speculated that the subcellular distribution of Trx might have a significant role in inflammation. Immunofluorescence staining suggested that PM_{2.5} upregulated Trx expression both in the cytoplasm and nucleus. In particular, it promoted the nuclear presence of Trx (Fig. 4a). This prompted us to search for a new method to inhibit Trx nuclear translocation. cHCEU, a nucleating inhibitor of Trx, inhibits Trx present in the cell nucleus. Using immunofluorescence staining, we observed that cHCEU abrogated the presence of Trx in the nucleus with/without PM_{2.5}. To evaluate the effect of cHCEU responsiveness to inflammation induced by PM_{2.5}, we measured the mRNA expressions of TNF- α , IL-1, IL-6 and IL-8. We found that these inflammatory cytokines were decreased by cHCEU (Fig. 4b-e). Interestingly, cHCEU did not alter the gene expressions of NF- κ B or oxidative-related genes such as Nrf2, NQO-1 or Trx (Fig. 4f-i). Therefore, cHCEU suppresses the expression of inflammatory cytokines by modulating the subcellular localization of Trx rather than affecting the gene expression of the transcription factor NF- κ B.

3.5. cHCEU attenuates the NF- κ B DNA binding activity

Although the expression of NF- κ B was unchanged by cHCEU, the expressions of downstream inflammatory cytokines were downregulated. Therefore, we hypothesized that the suppression of Trx nuclear translocation by cHCEU attenuates the NF- κ B DNA binding activity. NF- κ B binding to specific sequences of nucleic acids was detected by electrophoretic mobility shift assay (EMSA) in BEAS-2B. We found a marked increase in NF- κ B DNA-binding activity during incubation with PM_{2.5} (Fig. 5a). The treatment of BEAS-2B cells with cHCEU followed by incubation with/without t-BHQ significantly reduced NF- κ B activation. This suggested that cHCEU reduced the activation of NF- κ B, which may be attributed to the inhibition of Trx nuclear translocation. Interestingly, t-BHQ regulated redox homeostasis by upregulating the expression of Trx both in the cytoplasm and nucleus, but was unable to reduce NF- κ B activation. This might explain the inability of t-BHQ to alleviate inflammatory cytokine production. Trx is a potential mediator of NF- κ B activation, which prompted us to investigate whether Trx undergoes nuclear translocation to interact with NF- κ B in the presence

of PM_{2.5}. Nucleoproteins from BEAS-2B cells were prepared and co-immunoprecipitated with either anti-NF- κ B monoclonal antibody or Trx monoclonal antibody. As shown in Fig. 5b, we observed specific complex formation between NF- κ B and Trx when they were co-expressed, demonstrating that a direct physical protein-protein interaction occurs between Trx and NF- κ B. These data indicate that cHCEU inhibits the binding of Trx to NF- κ B, thereby affecting the binding of NF- κ B to DNA.

3.6. The synergistic effect of t-BHQ and cHCEU alleviates PM_{2.5}-induced inflammation

Trx has dual roles in redox regulation and inflammation responses dependent upon its subcellular localization. The overexpression of Trx in the nucleus enhanced NF- κ B activity, whereas its cytoplasmic overexpression had the opposite effect [18]. Therefore, we simultaneously applied t-BHQ to upregulate the expression of Trx and cHCEU to inhibit Trx nuclear translocation. RT-qPCR analysis revealed that the synergic application of t-BHQ and cHCEU upregulated anti-oxidative genes such as Nrf2, NQO1 and Trx, but had no effect on the expression of NF- κ B mRNA compared with the cHCEU alone treatment group (Fig. 6a-d). This was consistent with the protein expression results (Fig. 6e-h). We determined the relative expression of inflammatory cytokines (Fig. 6i-l). TNF- α , IL-1, and IL-6 mRNAs were downregulated by synergic treatment with t-BHQ and cHCEU compared with cHCEU alone.

4. Discussion

Exposure to PM_{2.5} has a number of adverse effects on human health, such as respiratory, cardiovascular, cardiopulmonary, and reproductive systems, and may even lead to cancer. Epidemiological data demonstrate a consistent correlation between levels of PM in the ambient air and increases in respiratory and cardiovascular morbidities [19]. The main purpose of the study was to study the toxicity mechanism of PM_{2.5} on the respiratory system and to try to find possible therapeutic insights. The inhalation of PM_{2.5} into the lungs causes oxidative stress and inflammatory reactions, which are pathological underlying events leading to many lung diseases. However, the exact mechanisms are not yet elucidated. Trx is an important participant in the antioxidant system, and has a significant role in NF- κ B activation, DNA binding and the initiation of downstream inflammatory gene transcriptional expression. When oxidative stress occurs, I- κ B kinase (I-KK) is activated to degrade I- κ B, thereby allowing NF- κ B into the nucleus [12]. In the cytoplasm, reduced Trx has an inhibitory effect on I-KK, thus inhibiting NF- κ B translocation to the nucleus. However, nuclear specific oxidative

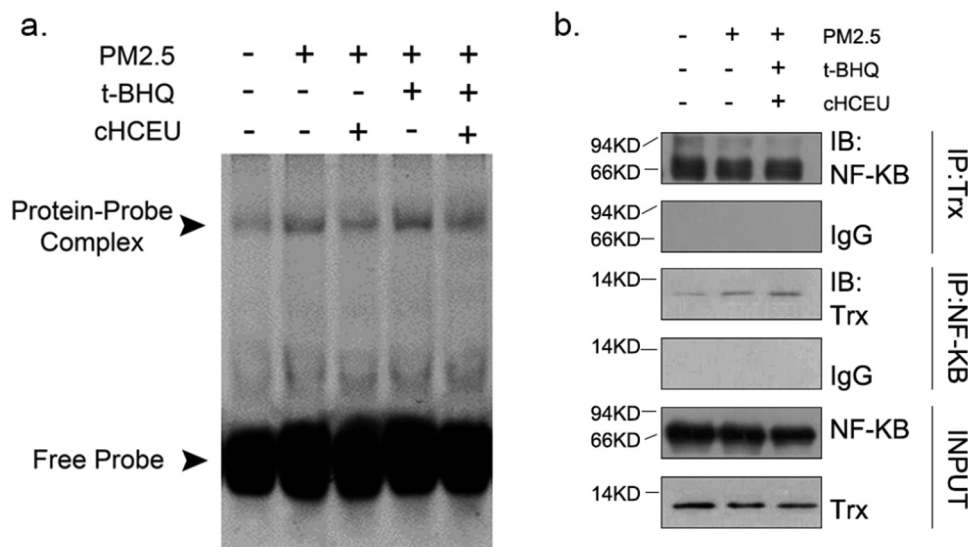


Fig. 5. cHCEU attenuates NF- κ B DNA binding activity. (a) The DNA binding activity of NF- κ B was assessed by Electrophoretic Mobility Shift Assay (EMSA) using nuclear extracts obtained from BEAS-2B cells that were untreated (Control) or treated with PM_{2.5}, PM_{2.5} plus t-BHQ, PM_{2.5} plus t-BHQ + cHCEU, or PM_{2.5} plus t-BHQ + cHCEU. (b) The results of the co-immunoprecipitation (CoIP) of endogenous Trx and NF- κ B using NF- κ B antibodies. Western blot analysis was carried out to detect Trx and NF- κ B. Reverse CoIP confirmed interaction between NF- κ B and Trx. CoIPs were also performed with Trx antibodies. Western blot analysis was carried out for Trx and NF- κ B.

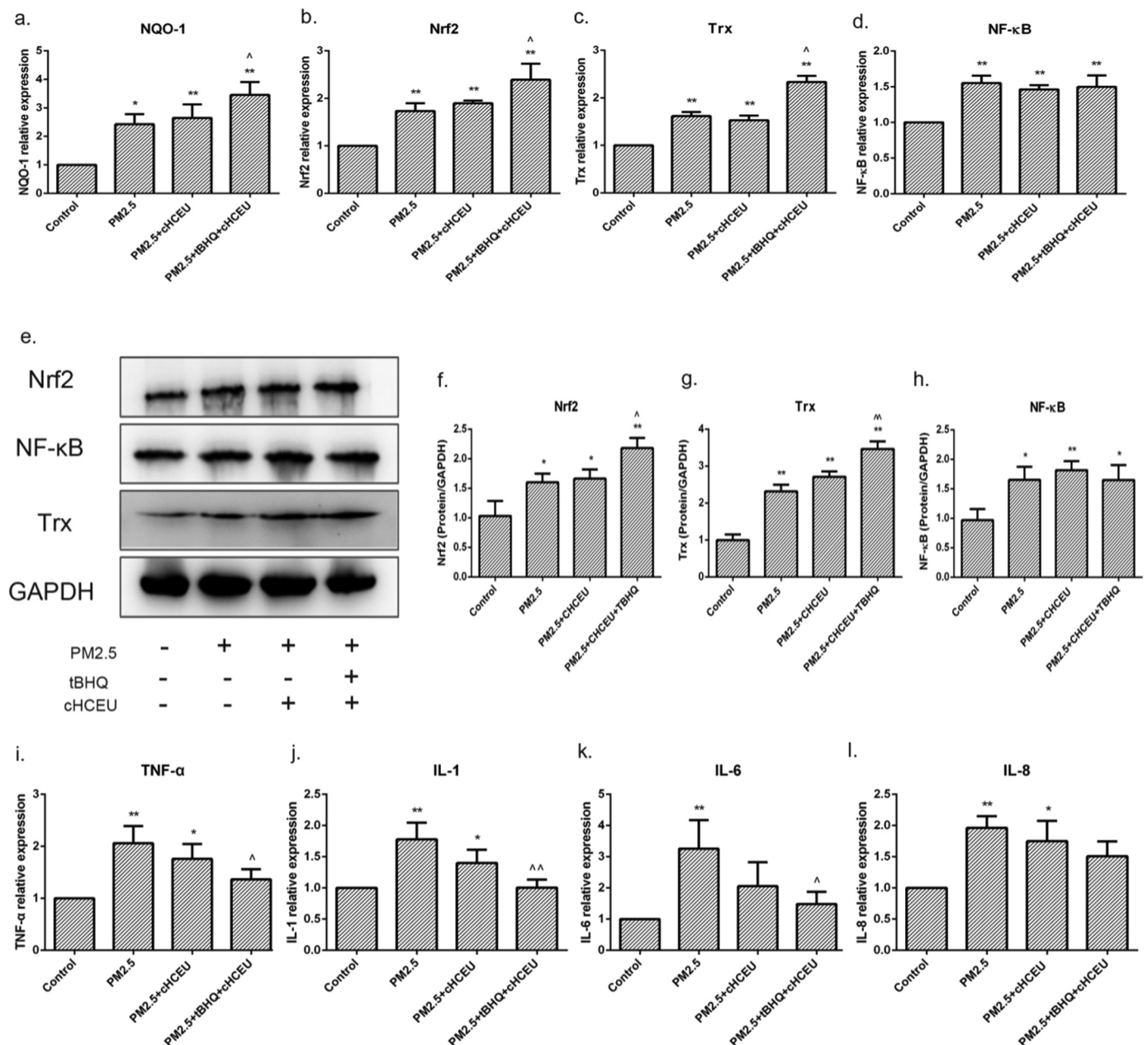


Fig. 6. Synergistic effect of t-BHQ and cHCEU on alleviating PM_{2.5}-induced inflammation. (a-d) The synergistic effect of t-BHQ and cHCEU on the mRNA expressions of Nrf2 and Nrf2-regulated genes and NF-κB in BEAS-2B cells. The results are presented as the mean ± SEM. *p < 0.05, **p < 0.01 compared with the control group. [^]p < 0.05 compared with the PM_{2.5} plus cHCEU group. (e-h) Synergistic effect of t-BHQ and cHCEU on the protein levels of Nrf2, Trx and NF-κB (p65) in BEAS-2B cells. The results are presented as the mean ± SEM. *p < 0.05, **p < 0.01 compared with the control group. [^]p < 0.05 compared with the PM_{2.5} plus cHCEU group. (i-l) Synergistic effect of t-BHQ and cHCEU on the mRNA expressions of inflammatory genes (TNF-α, IL-1, IL-6 and IL-8) in BEAS-2B cells. The results are presented as the mean ± SEM. *p < 0.05, **p < 0.01 compared with the control group. [^]p < 0.05 compared with the PM_{2.5} plus cHCEU group.

stress inhibits NF-κB transcriptional activity in the absence of nuclear Trx [13]. The oxidation of some cysteine residues in the NF-κB dimer decreases its DNA-binding activity. In the nucleus, Trx promotes the DNA binding of NF-κB by maintaining the redox-active cysteines of the NF-κB dimer in a reduced state [20]. It was reported that the over-expression of nuclear Trx promoted the DNA binding of NF-κB and redox modification of Cys62 in the dimer, which controlled the DNA binding [21]. Therefore, we speculate that the presence of Trx in the nucleus is critical for the transcriptional activity of NF-κB. The current study showed that altering PM_{2.5} in BEAS-2B cells promoted significant oxidative stress and upregulated Trx expression in the nucleus. Furthermore, EMSA analysis demonstrated that NF-κB DNA binding activity was significantly increased during PM_{2.5} incubation. Based on this

finding, we investigated a new method to inhibit the nuclear translocation of Trx. cHCEU inhibited Trx translocation into the nucleus by selective covalent alkylation [22]. Furthermore, cHCEU is used as an adjunct chemical or radiosensitizer for cancer chemotherapy because of its strong chemical resistance mechanisms that interfere with Trx translocation into the nucleus [23]. The current study showed that cHCEU inhibited NF-κB DNA binding activity by blocking Trx nuclear translocation to reduce the expression of inflammatory cytokines. A recent study also found that the excessive expression of Trx in the nuclei of cells and the kidneys of transgenic mice aggravated Cd-stimulated NF-κB activation suggesting that the nuclear translocation and accumulation of Trx in nuclei has an important role in Cd-induced inflammation and cell death [24]. Consistent with this, we suggest that

inhibiting Trx entry into the nucleus may cleave the NF- κ B-dependent inflammatory response pathway.

Based on the dual role of Trx in the cytoplasm and nucleus [25], we speculate that the increased expression of Trx might attenuate PM_{2.5} induced oxidative stress and inhibit inflammatory responses by preventing Trx from entering the nucleus. We used t-BHQ to enhance the activity of Nrf2 and upregulate the expression of Trx and other antioxidant genes, such as NQO1, to overexpress Trx and exert antioxidant effects. Our results showed that t-BHQ upregulated Nrf2 and its downstream antioxidant molecules (Trx and NQO1) against cell injury caused by oxidative stress in PM_{2.5} treated BEAS-2B cells, but did not alleviate inflammatory cytokine expressions. This suggests that anti-oxidation therapy targeting Nrf2 and upregulating Trx expression levels are not suitable for the treatment of inflammation caused by PM_{2.5} intervention. Furthermore, we simultaneously used t-BHQ to upregulate the expression of Trx and CHCEU to inhibit Trx nuclear translocation. This synergistic application significantly upregulated the expression of Trx, attenuated the DNA binding capability of NF- κ B, and downregulated the expression of inflammatory factors induced by PM_{2.5}. Anti-inflammatory treatment based on Trx nuclear transfer control may be a new treatment target.

5. Conclusions

In summary, the current study showed that increasing Trx in cell nuclei, induced by the disruption of cellular redox, potentiates PM_{2.5} toxicity in BEAS-2B cells. The translocation of Trx into nuclei was stimulated by PM_{2.5}, and NF- κ B activity was increased by the increase in nuclear Trx. Our results show that the translocation of Trx into nuclei is an important event that contributes to PM_{2.5}-induced inflammatory responses. CHCEU alleviated inflammatory cytokines by blocking Trx nuclear translocation and by inhibiting NF- κ B DNA binding activity, indicating that nuclear Trx translocation might be a potential therapeutic target to prevent or delay the adverse effects of PM_{2.5}.

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Conflicts of Interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.10.438.

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