

Ropivacaine has the potential to relieve PM2.5-induced acute lung injury

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Abstract. Ropivacaine is a commonly used local anesthetic in the clinic due to its low toxicity to the cardiovascular system or central nervous system, good tolerance and high clearance rate. The present study intended to investigate the effect of ropivacaine on PM2.5-induced acute lung injury (ALI) and reveal the underlying mechanism. After ropivacaine exposure, cell viability, oxidative stress and inflammation in PM2.5-induced BEAS-2B cells were assessed by Cell Counting Kit-8 and DCFH-DA staining, corresponding commercial kits and ELISA, respectively. The effects of ropivacaine on the expression of MMP9 and MMP12 and the proteins related to NLRP3/Caspase-1 signaling were then determined by western blot and reverse transcription-quantitative PCR analyses. In addition, NLR family pyrin domain containing 3 (NLRP3) agonist monosodium urate (MSU) was used to treat BEAS-2B cells followed by ropivacaine treatment and the effects on the above-mentioned cellular behaviors were determined again. The results indicated that the viability of BEAS-2B cells was decreased after PM2.5 induction, accompanied by aggravated oxidative stress and inflammation. However, ropivacaine alleviated oxidative stress and inflammation in PM2.5-induced BEAS-2B cells in a dose-dependent manner. Ropivacaine was also indicated to decrease the expression levels of NLRP3/Caspase-1 signaling-related proteins in PM2.5-induced BEAS-2B cells. Furthermore, cell viability was decreased, while oxidative stress and inflammatory response were aggravated, in PM2.5-induced BEAS-2B cells treated with MSU. In summary, the present results implied that ropivacaine exerted protective effects on PM2.5-induced ALI, and this effect may be related to NLRP3/Caspase-1 signaling.

Introduction

The lung is a complex and primary organ for gas exchange in mammals that has the largest epithelial surface in close contact with the external environment (1). Acute microbial (bacterial or viral) infections may trigger severe damage to the lungs, incurring the occurrence of lung-related diseases such as acute lung injury (ALI) (2). ALI is defined as increased alveolar-capillary permeability triggered by severe noncardiogenic factors, leading to severe tissue damage and even irreversible pulmonary damage in severe conditions (1,3). Recently, the number of patients with ALI due to long-time exposure to particulate matter has increased, which has gained worldwide attention (4).

Ropivacaine is a commonly used local anesthetic in the clinic, whose structural formula is presented in Fig. 1. It is currently regarded as one of the most potent anesthetics due to its low toxicity to the cardiovascular system and central nervous system, good tolerance and high clearance rate (5). It has been reported that ropivacaine is able to significantly improve the inflammatory response induced by tumor necrosis factor (TNF)- α , and it may reduce the activities of matrix metalloproteinases (MMPs), nuclear factor erythroid 2-related factor 2 and oxidative stress in brain microvascular endothelial injury induced by high glucose (6,7). In the model of acute hypertension, inhibition of endothelial nitric oxide synthase activity reduces the pressure-induced permeability of pulmonary vascular endothelial cells (8). Ropivacaine exerts anti-inflammatory effects by inhibiting the MAPK and NF- κ B signaling pathway in macrophages (9). Accumulating studies have focused on the effect of ropivacaine on lung-related diseases. It may reduce endotoxin-induced ALI by reducing neutrophil infiltration, block inflammatory SRC proto-oncogene, non-receptor tyrosine kinase (SRC) signal transduction and ICAM-1 expression, alleviate endotoxin combined with hyperinflation-induced ALI and improve pulmonary edema in N-formylmethionyl-leucyl-phenylalanine (fMLP)-induced ALI mice (10-12).

The above results have demonstrated that ropivacaine has obvious lung-protective effects, but the effect of ropivacaine on lung injury caused by particulate matter with a diameter of $\leq 2.5 \mu\text{m}$ (PM2.5) has remained elusive. Therefore, the present study aimed to explore whether ropivacaine is able to alleviate PM2.5-induced inflammation and oxidative stress in lung epithelial cells.

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Materials and methods

Cell culture and treatment. The human bronchial epithelial cell line BEAS-2B was procured from the Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (MilliporeSigma) with 10% fetal bovine serum (MilliporeSigma), 100 mg/ml penicillin and 100 mg/ml streptomycin in an incubator with 5% CO₂ at 37°C.

The collection of PM2.5 was performed as previously described (13). A total of 50 mg PM2.5 particles were collected by high-volume impactors assembled with a glass fiber filter (Cytiva) from the mouth of the Yangtze River at China's central eastern coast in Shanghai at a flow rate of 1.13 m³/min for every 24 h between October 2019 and April 2020. PM2.5 samples were dissolved in DMSO at the concentrations of 100 µg/ml and stored at -80°C for subsequent use. Ropivacaine was diluted in VasuLife Basal Medium (Kurabo) at the concentrations of 1 nM and 1, 10 and 100 µM for further use. For the induction of ALI, the cultured BEAS-2B cells were collected and exposed to PM2.5. Furthermore, the cells were treated with monosodium urate (MSU; MilliporeSigma), an NLR family pyrin domain containing 3 (NLRP3) agonist, at 150 µg/ml 48 h to study the mechanism.

CCK-8 assay. Cells were exposed to PM2.5 in medium for 24 h. A CCK8 kit (Dojindo Laboratories, Inc.) was used to measure the cell viability of BEAS-2B cells in accordance with the manufacturer's protocol, and the absorbance at 490 nm was read by an EnSpire microplate reader (PerkinElmer, Inc.).

Measurement of reactive oxygen species (ROS), malondialdehyde (MDA) and major endogenous antioxidant glutathione (GSH). BEAS-2B cells were seeded in 96-well plates at a density of 2x10⁴ cells/well and cultured overnight with 5% CO₂ at 37°C. The cells were then treated with ropivacaine for 24 h prior to the addition of the dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 45 min. After washing with PBS, cells were fixed with 4% of polyformaldehyde at room temperature for 30 min. Finally, a confocal fluorescence microscope (EX/EM: 488/525; Leica Microsystems GmbH) was used to observe the cells. The fluorescence intensity was analyzed by using ImageJ v1.8 software (National Institutes of Health). The intracellular contents of MDA and GSH were measured by a commercial assay kit (Nanjing Jiancheng Bio Co., Ltd.) according to the manufacturer's protocol.

ELISA. The levels of interleukin (IL)-6 (cat. no. D6050), IL-8 (cat. no. D8000C) and TNF-α (cat. no. DTA00D) in the BEAS-2B cell supernatants were determined by a commercially available ELISA kits from R&D Systems, following the recommendations provided by the manufacturer.

Western blot analysis. Cells were washed in PBS and total protein was extracted in RIPA lysis buffer (Beyotime Institute of Biotechnology). Lysed cells were subjected to centrifugation at 16,100 x g for 10 min at room temperature. Quantification of the protein samples was performed using a BCA assay kit (Tiangen Biotech, Co., Ltd.). Protein extracts were electrophoresed on 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). The membranes were then blocked with 5% skimmed milk (Absin Bioscience, Inc.) in Tris-buffered saline

Table I. Primers used for quantitative PCR.

Name/direction	Primer sequence
MMP9	
Forward	5'-GAACCAATCTCACCGACAGG-3'
Reverse	5'-GCCACCCGAGTGTAAACCATA-3'
MMP12	
Forward	5'-AGTTTTGATGCTGCTCACTACCG-3'
Reverse	5'-CACTGGTCTTTGGTCTCTCAGAA-3'
NLRP3	
Forward	5'-GGTCCTCTTTACCATGTGCTTC-3'
Reverse	5'-AAGTCATGTGGCTGAAGCTGTA-3'
Caspase-1 p20	
Forward	5'-AGAAAAGCCATGGCCGACAA-3'
Reverse	5'-ACGTGCTGTCAGAGGTCTTG-3'
ASC	
Forward	5'-GATCCAGGCCCTCCTCAG-3'
Reverse	5'-GCATCTTGCTTGGGTTGGTG-3'
GAPDH	
Forward	5'-AGCCACATCGCTCAGACAC-3'
Reverse	5'-GCCCAATACGACCAAATCC-3'

NLRP3, NLR family pyrin domain containing 3; ASC, apoptosis-associated speck-like protein.

for 2 h at room temperature, and incubated overnight at 4°C with primary antibodies against MMP9 (cat. no. ab76003; 1:1,000 dilution; Abcam), MMP12 (cat. no. ab52897; 1:1,000 dilution; Abcam), NLRP3 (cat. no. IMG-6668A; 1:200 dilution; Novus Biologicals, LLC), apoptosis-associated speck-like protein (ASC; cat. no. sc-514414; 1:200, Santa Cruz Biotechnology, Inc.), caspase-1 p20 (cat. no. PA5-99390; 1:500 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) and GAPDH (cat. no. #5174; 1:1,000 dilution; Cell Signaling Technology, Inc.). HRP-conjugated anti-rabbit IgG (cat. no. 14708; 1:15,000 dilution; Cell Signaling Technology, Inc.) or anti-mouse IgG (cat. no. ab205719; 1:2,000 dilution; Abcam) was used as a secondary antibody for incubation at room temperature for 2 h. Immunoreactive bands were visualized using an Pierce enhanced chemiluminescence detection kit (cat. no. 32209; Thermo Fisher Scientific, Inc.). Gray values were analyzed by using ImageJ software (v1.8; National Institutes of Health).

Reverse transcription-quantitative (RT-q)PCR assay. Total RNA from BEAS-2B cells was extracted by TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (2 µg) was then reverse-transcribed into cDNA with the Transcriptor 1st strand cDNA Synthesis Kit (Roche Diagnostics). Real-time qPCR was performed using the OneStep TB Green RT-PCR Kit (Takara Bio, Inc.). The primers used are listed in Table I. The thermocycling program was 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The relative expression of each gene was calculated by the 2^{-ΔΔCq} method (13) and GAPDH was used as the internal control.

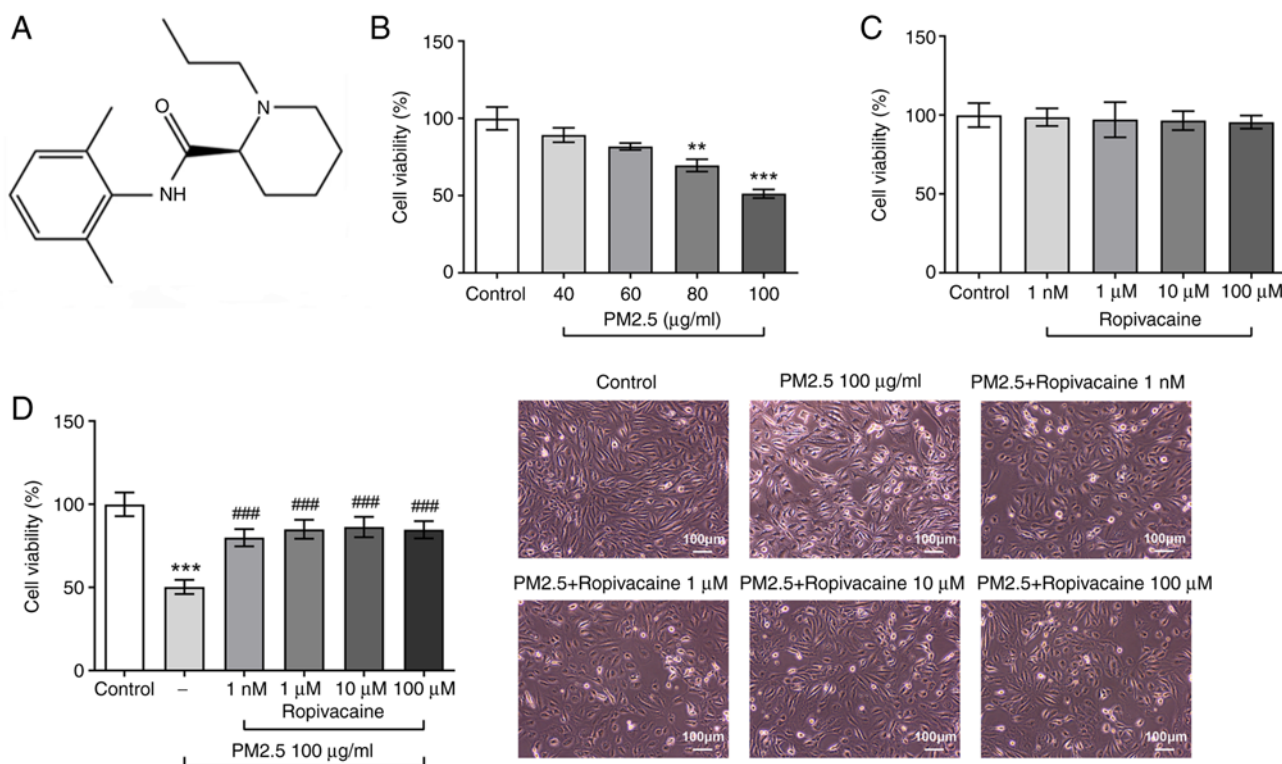


Figure 1. Impact of ropivacaine on the viability of PM2.5-induced BEAS-2B cells. (A) Chemical structure of ropivacaine. (B) Viability of BEAS-2B cells induced by PM2.5. (C) Viability of BEAS-2B cells treated with ropivacaine. (D) Viability and morphology of PM2.5-induced BEAS-2B cells treated with ropivacaine (scale bars, 100 µm). **P<0.01, ***P<0.001 vs. control; ###P<0.001 vs. PM2.5. PM2.5, particulate matter with a diameter of ≤ 2.5 µm.

Statistical analysis. Quantitative data were expressed as the mean \pm standard deviation. GraphPad Prism 6 (GraphPad Software Inc.) was used for data analysis. Data among groups were analyzed by ANOVA, followed by Tukey's post-hoc test. P<0.05 was considered to indicate statistical significance.

Results

Impact of ropivacaine on the viability of PM2.5-induced BEAS-2B cells. To determine the impact of ropivacaine on ALI, the changes in the viability of BEAS-2B cells induced by PM2.5 were first assessed. As clearly presented in Fig. 1B, the viability of BEAS-2B cells was decreased by PM2.5 in a dose-dependent manner. However, no significant change in viability was observed in BEAS-2B cells exposed to different doses of ropivacaine, demonstrating the relative nontoxicity of ropivacaine used on BEAS-2B cells (Fig. 1C). Subsequently, BEAS-2B cells were induced with PM2.5 and treated with ropivacaine. In comparison with the control, PM2.5 markedly reduced the viability and altered the morphology of BEAS-2B cell, which was rescued by ropivacaine (Fig. 1D).

Impact of ropivacaine on oxidative stress and inflammation of PM2.5-induced BEAS-2B cells. Since oxidative stress and inflammation are well-recognized hallmarks of ALI, the changes of oxidative stress and inflammation in PM2.5-induced BEAS-2B cells exposed to ropivacaine were then determined. The DCFH-DA staining results indicated that the ROS content in BEAS-2B cells was stimulated by

PM2.5, while it was reduced by different concentrations of ropivacaine (Fig. 2A). A similar effect was observed in terms of MDA levels, whereas GSH exhibited the opposite trend. With regard to inflammatory response, the inflammatory factors IL-6, IL-8, IL-1 β and TNF- α shared similar expression changes in PM2.5-induced BEAS-2B cells exposed to ropivacaine, as demonstrated by the result that PM2.5 induced high levels of inflammatory factors, while ropivacaine led to lower levels of these factors (Fig. 2B). Collectively, ropivacaine relieves oxidative stress and inflammation of PM2.5-induced BEAS-2B cells.

Impact of ropivacaine on the expression levels of MMPs in PM2.5-induced BEAS-2B cells. MMPs have an important role in regulating cellular homeostasis by degrading extracellular matrix (ECM), particularly MMP12 and MMP9, which are related to airway remodeling and tissue damage. PM2.5 may significantly increase the expression of MMP9 and MMP12 (14-16). Thus, the expression of MMP-12 and MMP-9 was detected in the present study. It was observed that PM2.5 induced abnormally high levels of MMP12 and MMP9, whereas these effects were partially abolished by ropivacaine (Fig. 3A and B). These results implied the potential effects of ropivacaine on the restoration of airway remodeling and tissue damage.

Regulatory role of ropivacaine in immune response and oxidative stress by NLRP3/Caspase-1 signaling. A previous study reported that PM2.5 triggered the immune response in the lung by activating NLRP3/Caspase-1 signaling (17),

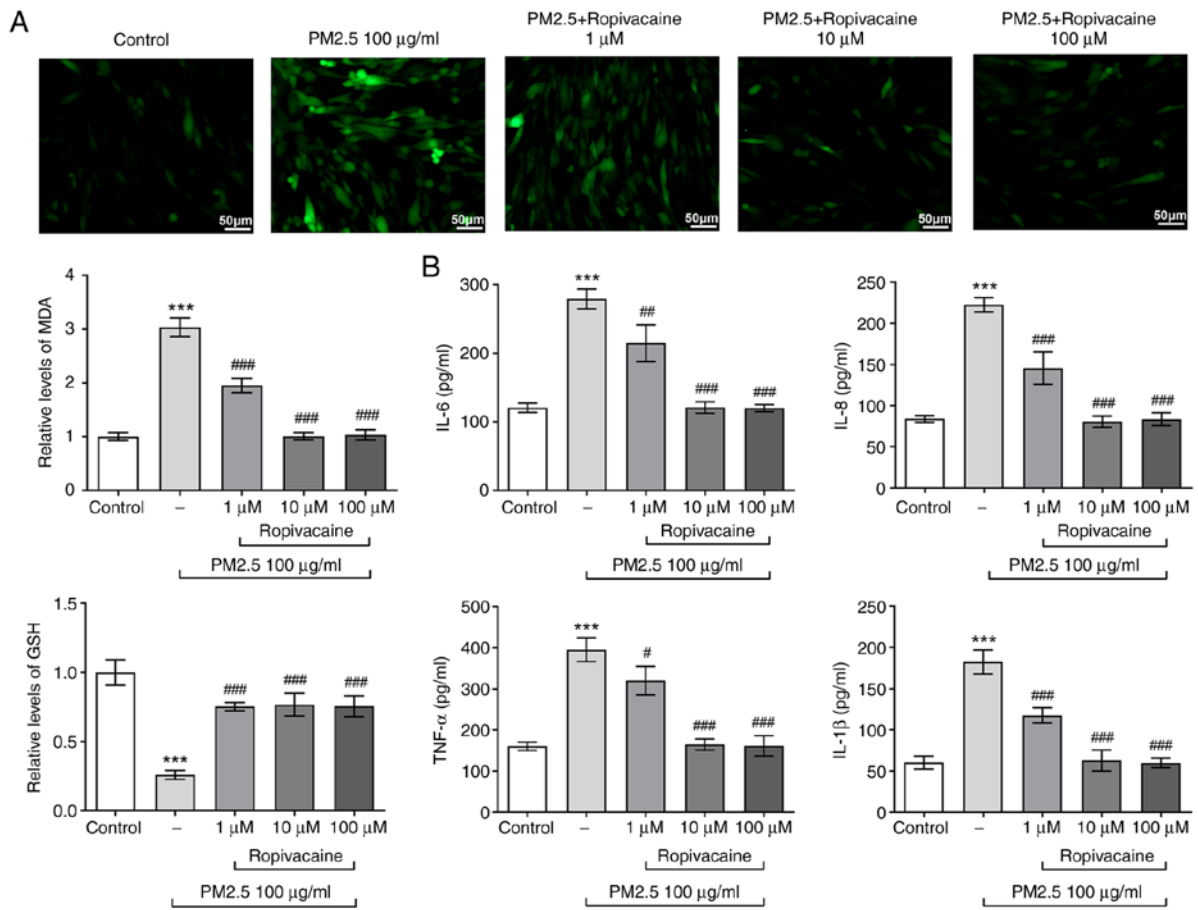


Figure 2. Impact of ropivacaine on the oxidative stress and inflammation of PM2.5-induced BEAS-2B cells. Levels of (A) reactive oxygen species (scale bar, 50 μ m), MDA and GSH and (B) inflammatory cytokines in PM2.5-induced BEAS-2B cells treated with ropivacaine. *** P <0.001 vs. control; # P <0.05, ## P <0.01, ### P <0.001 vs. PM2.5. MDA, malondialdehyde; GSH, glutathione; PM2.5, particulate matter with a diameter of ≤ 2.5 μ m.

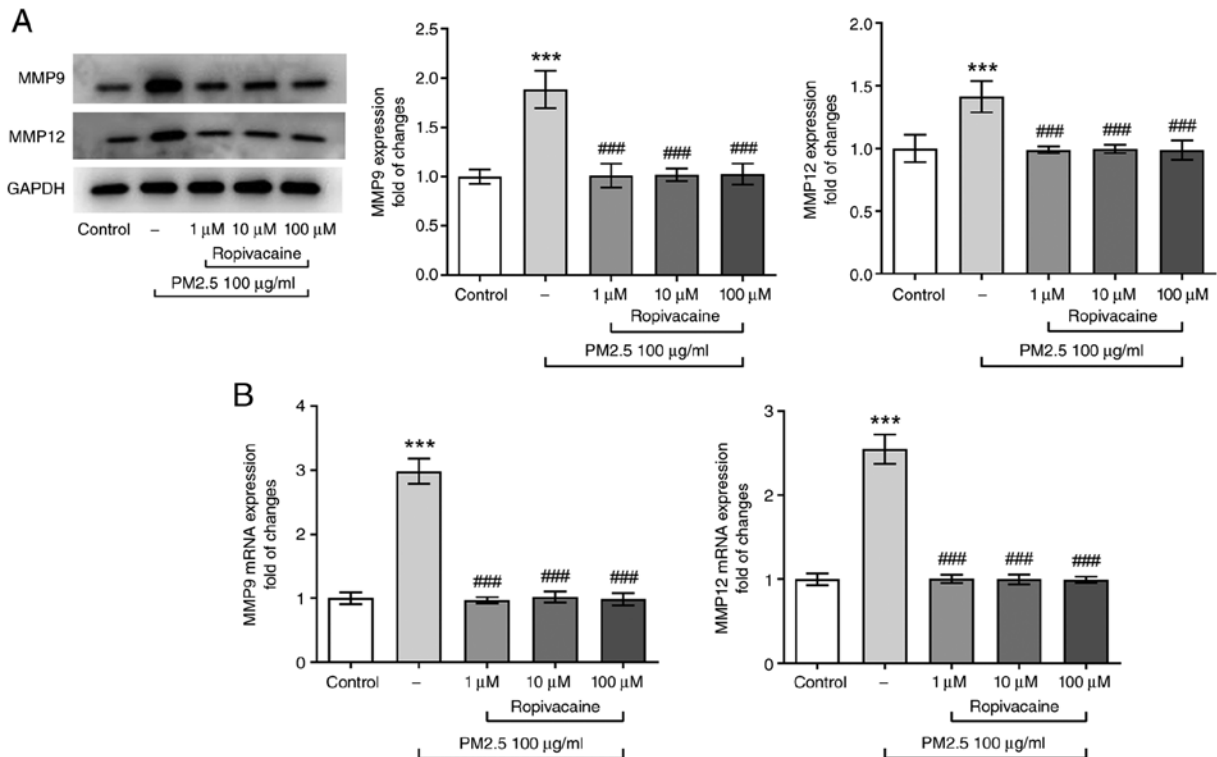


Figure 3. Impact of ropivacaine on the expression levels of MMPs in PM2.5-induced BEAS-2B cells. (A) Protein and (B) mRNA levels of MMP9 and MMP12 in PM2.5-induced BEAS-2B cells treated with ropivacaine. *** P <0.001 vs. control; ### P <0.001 vs. PM2.5. PM2.5, particulate matter with a diameter of ≤ 2.5 μ m.

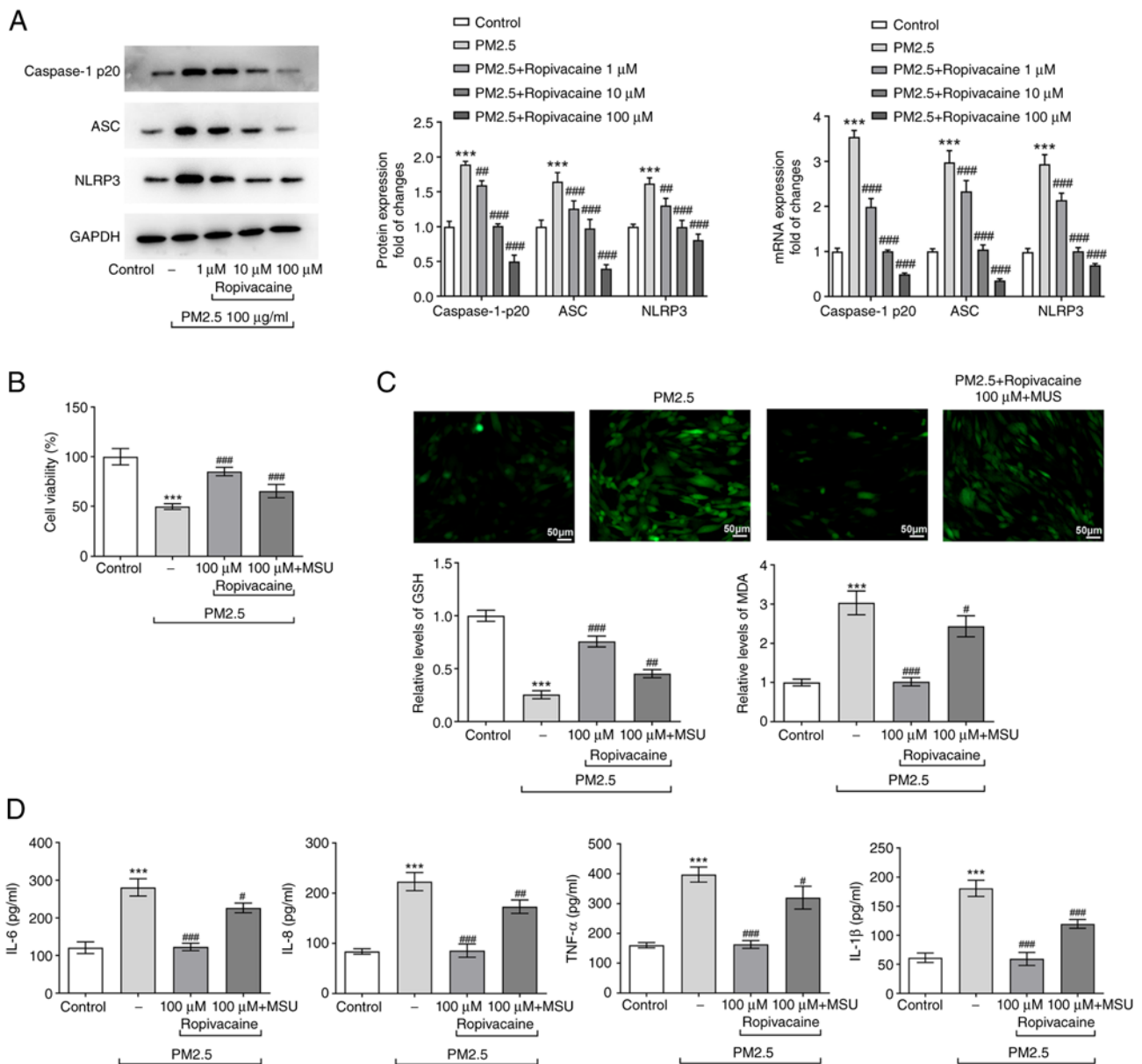


Figure 4. Regulatory role of ropivacaine in immune response and oxidative stress by NLRP3/Caspase-1 signaling. (A) The expression of proteins in the NLRP3/Caspase-1 signaling pathway in PM2.5-induced BEAS-2B cells treated with ropivacaine was assessed by western blot analysis. (B) Cell viability, (C) oxidative stress (scale bar, 50 µm) and (D) inflammation in PM2.5-induced BEAS-2B cells treated with MSU. *** $P < 0.001$ vs. control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. PM2.5. NLRP3, NLR family pyrin domain containing 3; MSU, monosodium urate; PM2.5, particulate matter with a diameter of ≤ 2.5 µm.

whereas ropivacaine prevented the activation of NLRP3 (18). Thus, in the present study, it was hypothesized that ropivacaine exerted effects on ALI via NLRP3/Caspase-1 signaling. As presented in Fig. 4A, PM2.5 activated the expression of NLRP3, Caspase-1 p20 and ASC, which was reversed by increasing doses of ropivacaine. Subsequently, BEAS-2B cells were treated with the NLRP3 agonist MSU (150 µg/ml). The cell viability was decreased, while oxidative stress and inflammatory response were aggravated in BEAS-2B cells treated with MSU, which was abrogated by ropivacaine exposure (Fig. 4B-D). Of note, the cellular changes in MSU-treated BEAS-2B cells were consistent with those in PM2.5-treated BEAS-2B cells upon ropivacaine exposure. Thus, it was concluded that ropivacaine regulates the immune response and oxidative stress in PM2.5-induced BEAS-2B cells by NLRP3/Caspase-1 signaling.

Discussion

The lung is a sensitive organ that may easily suffer damage from inhaling substances such as PM2.5 (19). Compelling evidence has suggested the significance of PM2.5 in inducing various lung-related diseases. Lung parenchymal injury and lung ischemia-reperfusion injury were observed in rodents even after short-term exposure to PM2.5, indicating the threat of PM2.5 to lung function (20). It was also previously indicated that PM2.5 promotes the progression of allergic asthma in mice by enhancing inflammation and suppressing autophagy (21). Consistently, PM2.5 induced inflammation and oxidative stress in BEAS-2B cells, as demonstrated by elevated contents of ROS and MDA, and suppressed activity of GSH. These observations coincide with the fact that redundant ROS release is one of the essential factors for PM2.5-induced cell damage (19).

Accumulating evidence has demonstrated the protective effects of ropivacaine against lung-associated diseases. A study has indicated that ropivacaine attenuates inflammatory response in experimental endotoxin-induced lung damage *in vivo* (10). Concurrent with this, the present study indicated that the release of inflammatory factors in the supernatant of BEAS-2B cells was inhibited, accompanied by suppressed oxidative stress. Thus, ropivacaine may function as a potent anti-inflammatory substance for the treatment of lung injury.

The MMP family, which is able to degrade ECM and other substrates, participates in inflammation and tissue remodeling, which are essential for the pathogenesis of various lung-associated diseases (22). Well-orchestrated expression of MMPs, particularly MMP9 and MMP12, is required for the stabilized development of the lung. In the present study, PM2.5 stimulation elevated the expression of MMP9 and MMP12, thereby promoting the development of ALI. However, ropivacaine controlled their expression to relatively normal levels, which may inhibit this condition.

Inflammation induced by a large number of immune cells is critical for pulmonary inflammation upon challenge with PM2.5 (23,24). Once activated, the NLRP3 inflammasome enhances the expression of caspase-1 and convert pro-IL-1 β and pro-IL-18 into their mature bioactive forms (24). Furthermore, inhibition of the NLRP3/caspase-1 pathway was conducive to the reduction of inflammatory response in a PM2.5-induced mouse model (17). Thus, the present study suggested that ropivacaine exerted protective effects on the PM2.5-induced *in vitro* ALI model by modulation of the NLRP3/caspase-1 pathway. To validate this notion, MSU was substituted for PM2.5 as the inducer of the ALI model. An essential finding was that MSU led to decreased cell viability and promoted oxidative stress and inflammation, which was consistent with the effects of PM2.5. Furthermore, ropivacaine partially abolished the effect of MSU on oxidative stress and inflammation of BEAS-2B cells, demonstrating that ropivacaine exerted protective effects on PM2.5-induced ALI via NLRP3/Caspase-1 signaling.

In conclusion, the present study indicated that ropivacaine exerts protective effects on PM2.5-induced ALI and this effect may be relative to NLRP3/Caspase-1 signaling. The present study provided evidence that targeting this signaling may be a feasible strategy for ALI treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RZ wrote the manuscript and participated in the planning and execution of the experiments. XYL and YGH participated in

the major experiments and analyzed the results. RZ and XYL confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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