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In vitro and *in vivo* assessment of the protective effect of sufentanil in acute lung injury

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Abstract

Objectives: To investigate the mechanisms underlying the protective effect of sufentanil against acute lung injury (ALI).

Material and Methods: Rats were administered lipopolysaccharide (LPS) by endotracheal instillation to establish a model of ALI. LPS was used to stimulate BEAS-2B cells. The targets and promoter activities of IκB were assessed using a luciferase reporter assay. Apoptosis of BEAS-2B cells was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling.

Results: Sufentanil treatment markedly reduced pathological changes in lung tissue, pulmonary edema and secretion of inflammatory factors associated with ALI *in vivo* and *in vitro*. In addition, sufentanil suppressed apoptosis induced by LPS and activated NF-κB both *in vivo* and *in vitro*. Furthermore, upregulation of high mobility group box protein 1 (HMGB1) protein levels and downregulation of miR-129-5p levels were observed *in vivo* and *in vitro* following sufentanil treatment. miR-129-5p targeted the 3′ untranslated region and its inhibition decreased promoter activities of IκB-α. miR-129-5p inhibition significantly weakened the protective effect of sufentanil on LPS-treated BEAS-2B cells.

Conclusion: Sufentanil regulated the miR-129-5p/HMGB1 axis to enhance IκB-α expression, suggesting that sufentanil represents a candidate drug for ALI protection and providing avenues for clinical treatment.

Keywords

Sufentanil, miR-129-5p, high mobility group box protein 1, NF-κB, IκB-α, acute lung injury, lipopolysaccharide

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Introduction

Acute lung injury (ALI) damages the alveolar epithelial cells or capillary endothelial cells, resulting in diffuse interstitial lung disease and alveolar edema and leading to acute hypoxic respiratory insufficiency. Sufentanil is a

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pure μ-opioid agonist. Pretreatment with sufentanil protects the liver from ischemia-reperfusion injury by inhibiting the inflammatory response.[1] Sufentanil treatment altered the expression of high mobility group box protein 1 (HMGB1) and NF-κB in rats with pancreatitis, and attenuated inflammation and oxidative stress induced by sepsis via kininogen-1. These findings suggested that sufentanil plays a potential protective role in inflammatory disease.[1,2] MicroRNAs (miRNAs) are a class of small non-coding single-stranded RNA molecules that participate in the regulation of gene expression by reducing mRNA stability or inhibiting mRNA translation.[3] In recent years, a number of studies have demonstrated that miRNAs are effective regulators of systemic inflammation and immune response.[4–6] Recent studies have shown that sufentanil can regulate the expression of miRNAs and reduce apoptosis.[7] miR-129-5p negatively regulates inflammatory responses,[8,9] and normal expression of this miRNA plays an important role in preventing excessive inflammation.[10] Some studies also indicated that miR-129-5p can target and negatively regulate HMGB1.[8]

Midazolam combined with sufentanil can influence the expression of HMGB1 and NF-κB,[11] but the role of sufentanil in ALI has not been well studied. Previous studies have shown that HMGB1 regulates the expression of NF-κB by binding to the receptor for advanced glycation endproducts.[12] A recent study suggested that HMGB1 can promote expression of NF-κB by inhibiting the activity of IκB.[13] Therefore, we aimed to investigate the protective effect of sufentanil on ALI and the mechanism of sufentanil inhibition of HMGB1 and NF-κB signaling.

Materials and methods

Animals

Thirty-two adult male specific pathogen free Sprague-Dawley rats (220–250 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). Before the experiment, rats were randomly divided (n = 8 rats per group) into a control group, a lipopolysaccharide (LPS) acute lung injury model group (LPS group), a sufentanil control group (sufentanil group) and a LPS + sufentanil treatment group (LPS + sufentanil group). ALI was established by intratracheal instillation of 2 mg/kg LPS. The sufentanil group and the LPS + sufentanil group received sufentanil (5 μg/mL, 0.2 mL) by intraperitoneal injection 1 hour before injection of saline solution or LPS. The study was approved by the ethical committee of Fuwai Hospital, Chinese Academy of Medical Sciences.

Hematoxylin-eosin (HE) staining. After LPS or sufentanil treatment, lung tissues of rats were collected to perform HE staining. After the right lung was removed, the tissue was washed with phosphate-buffered saline (PBS), then fixed at 4°C with 4% paraformaldehyde for 24 hours. The lung tissue was washed with PBS, then dehydrated in 30%, 50% and 70% alcohol for 10 minutes each. Paraffin-embedded sections were prepared from lung tissues and HE staining was performed according to the manufacturer's instructions (Beyotime, Shanghai, China) to observe pathological changes in lung tissues.

Wet and dry ratio of pulmonary tissue

The middle lobe of the right lung of rats was taken. Surface water and residual blood were quickly removed with filter paper. An electronic balance was used to measure the wet weight of lung tissue (W). The lung tissue was then baked at 80°C for 24 hours. The lung tissue was dried until a constant weight was reached. The dry weight (D) was determined, and the W/D ratio of the middle lobe of the right lung was calculated.

Assessment of tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels

After the trachea was separated, the right bronchus was clipped, and the hilum was ligated. Left bronchoalveolar lavage was performed with precooled PBS, and the bronchoalveolar lavage fluid (BALF) was recovered. The recovered BALF was centrifuged at 12,000 ×g at 4°C for 15 minutes. The supernatant was removed and stored at −80°C. Cytokine levels in BALF were assessed according to the ELISA kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Levels of TNF-α and IL-6 in BEAS-2B cell supernatants were assessed in the same manner.

Western blotting

Lung tissue was quickly placed in liquid nitrogen and then transferred to a −80°C freezer. Protein was extracted with 500 μL of precooled radioimmunoprecipitation buffer in a microcentrifuge tube. Tissue lysate was prepared by centrifugation at 12,000 ×g, 4°C for 15 minutes. Protein concentrations were determined using a bicinchoninic acid assay. The proteins were electrophoresed in 12% SDS-PAGE gels and then electrotransferred to polyvinylidene difluoride membranes. After the transfer, the membranes were blocked with 5% non-fat milk powder at room temperature for 1 hour, and then incubated at 4°C overnight with primary antibodies (Abcam, Cambridge, UK). The membranes were washed three times for 5 minutes per wash with Tris-buffered saline containing 0.1% Tween-20 (TBST), and then the secondary antibody (Abcam) was added and incubated at room temperature for 1 hour. The membranes were washed with TBST again three times for 5 minutes per wash.

Cell culture

BEAS-2B cells (ATCC, Manassas, VA, USA) were grown in high-glucose Dulbecco's Modified Eagle Media (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco). When the cells reached confluency, they were treated with 0.25% trypsin (Thermo Fisher Scientific) containing ethylenediaminetetraacetic acid, resuspended in medium and inoculated in different dishes for subsequent experiments

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol (Thermo Fisher Scientific). A miRNA reverse transcription kit (Takara Bio, Kyoto, Japan) was used for reverse transcription of miRNAs. The mRNA was reverse transcribed into cDNA using a reverse transcriptase kit (Thermo Fisher Scientific). The PCR reaction mixture was prepared in a 0.2-mL tube. U6 and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were used as internal references for miRNA and mRNA, respectively. qPCR was performed using SYBR Green mix (Novoprotein, Shanghai, China). Relative expression was calculated using the $2^{-\triangle\triangle Ct}$ method.

Dual luciferase reporter assay

HMGB1 3′ untranslated region (UTR) wild-type and mutant reporter plasmids were designed and synthesized by Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China). The cells were grown in 24-well plates, and each group of 3 wells was further cultured for 24 hours. Transfection was carried out when BEAS-2B confluency was between 50% and 70%. Cells without plasmids were transfected with miR-129-5p mimic or miR-NC (negative control) using Lipo6000™ (Beyotime, Shanghai, China). After culture for an additional 48 hours, luciferase activity was detected.

MiR-129-5P silencing assay

BEAS-2B cells were trypsinized, counted and seeded in six-well plates. The miR-129-5P-inhibitor was constructed by Guangzhou Ruibo Biotechnology Co., Ltd. BEAS-2B cells were transfected with miR-129-5P-inhibitor or miR-NC using Lipo6000^{TM}. After 24 hours, cells were treated with LPS and sufentanil.

Cell counting kit-8 (CCK8) assay

Cells were inoculated in 96-well plates at a density of 4000 cells per well. After each treatment, 10 μL of CCK-8 solution (Beyotime) was added to each well. The cells were incubated for 2 hours, and the absorbance of each well was determined at 450 nm.

ELISA

The cell supernatant in each group was collected. The concentrations of TNF-α and IL-18 were measured according to the manufacturer's instructions for the related kit (Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China). Absorbance was measured at 450 nm using a microplate reader.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic cells were stained using a TUNEL kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. BEAS-2B cells were fixed with 4% paraformaldehyde for 30 minutes and then incubated with PBS containing 0.3% Triton X-100 for 5 minutes. The samples were incubated in PBS containing 0.3% hydrogen peroxide at room temperature for 20 minutes to inactivate endogenous peroxidase. Sections were then three times with PBS. The samples were incubated for 60 minutes in the dark with 50 μL of biotin-labeled solution, and for 30 minutes at room temperature with 50 μL streptavidin-horseradish peroxidase solution. 3,3′-Diaminobenzidine was used as a substrate. Nuclei were stained with hematoxylin. Staining was observed under a fluorescence microscope.

Analysis of IκB-α promoter activities

The IκB-α promoter was cloned upstream of the luciferase gene. Then, miR-129-5p inhibitor, mimic or sufentanil was added to LPS-treated BEAS2B cells transfected with IκB-α-luciferase constructs. After 48 hours, luciferase activity was detected using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Data were reported as means ± standard deviations. Differences among groups were assessed using one-way analysis of variance. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of sufentanil on TNF-α and IL-6 levels in the BALF of rats treated with LPS

HE staining showed that alveolar wall structures were relatively complete in the lung tissues of rats in the control group and sufentanil group, with irregular shapes, different sizes of the lumen, and different vacuolar structure. In the LPS group, alveolar cavities disappeared, alveolar walls thickened, fibrosis was obvious, and many inflammatory cells infiltrated into the lung tissues. Following sufentanil treatment, the alveolar wall structures of rat lung tissue were relatively intact, with obvious lumens and cavitation-like structures (Figure 1a). The ALI model of sepsis in the LPS group

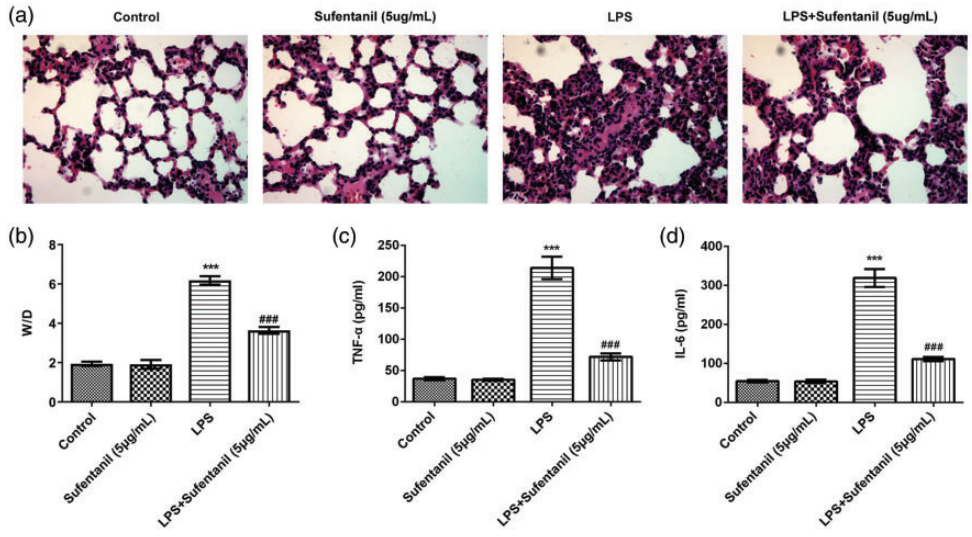

Figure 1. (a) Effects of sufentanil on pathological changes in lung tissue, wet/dry (W/D) ratio (b), tumor necrosis factor (TNF)-α levels (c) and interleukin (IL-6) levels (d) in bronchoalveolar lavage fluid following treatment of rats with lipopolysaccharide (LPS). Values represent means ± standard deviations. *** $P < 0.001$ vs control group, ### $P < 0.001$ vs LPS group.

was established by intratracheal instillation of LPS (2 mg/kg). Compared with the control group, the W/D ratio was increased significantly after exposure to LPS (Figure 1b). The inflammatory cytokines TNF-α and IL-6 play important roles in the development and progression of inflammatory responses. The concentrations of TNF-α and IL-6 were significantly higher in the LPS group compared with the control group (Figure 1c–d). Compared with the LPS group, the sufentanil group had significantly lower levels of TNF-α and IL-6. Furthermore, sufentanil had no effect on the TNF-α and IL-6 levels of control rats not treated with LPS.

Effect of sufentanil on expression of apoptosis-related proteins in LPS-treated rat lung tissue

To investigate the mechanism of sufentanil in protection against ALI in rats, we assessed the expression of Bcl2, Bax, cleaved caspase3 and caspase3 in rat lung tissue. Compared with the control group, expression of Bax and cleaved caspase3 in the lung tissues of the LPS group was significantly upregulated while the expression of Bcl-2 was markedly reduced. These expression changes were reversed in the LPS + sufentanil group (Figure 2).

Sufentanil regulated HMGB1, NF-κB and miR-129-5p expression in LPS-treated rats

Levels of HMGB1, P-IκB-α and P-NF-κB p65 were significantly increased in the LPS group compared with the control group (Figure 3). Compared with the LPS group, sufentanil effectively reduced levels of HMGB1, p-IκB-α and P-NF-κB p65. Expression of IκB-α and miR-129-5p was significantly decreased in the LPS group compared with the control group. Both expression changes were reversed in the LPS + sufentanil group (Figure 3). These results showed that the protective effect of sufentanil against lung injury induced by

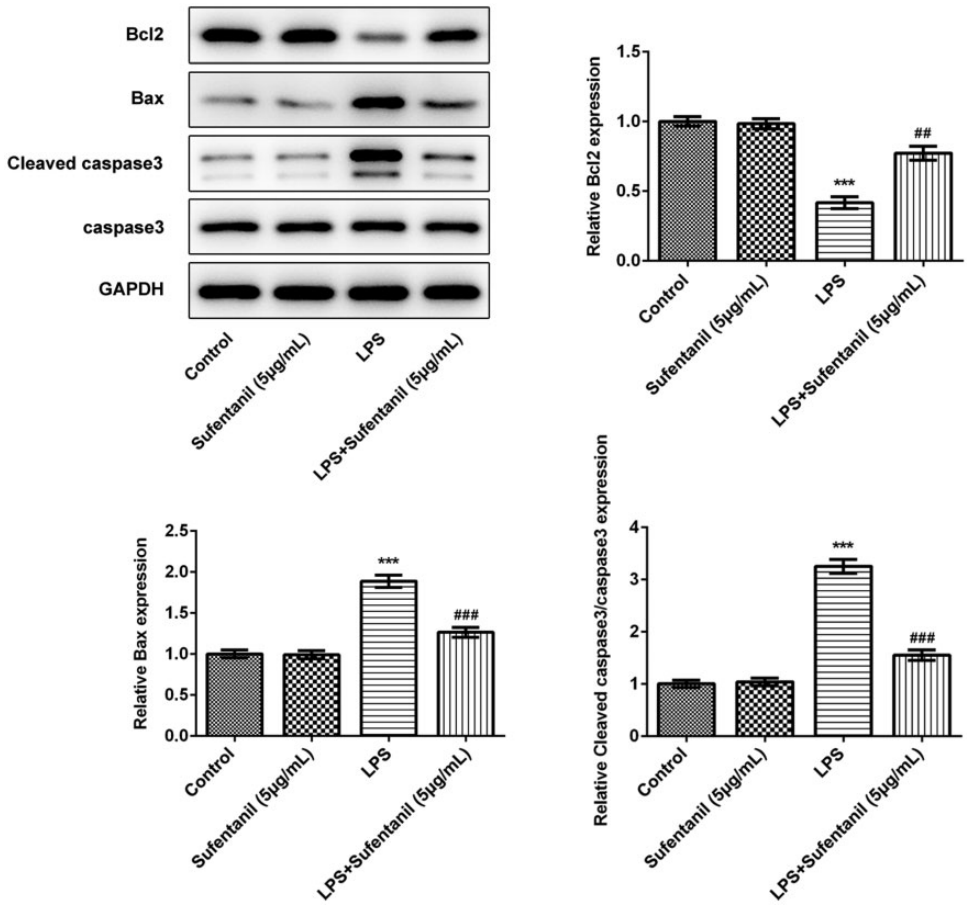

Figure 2. Effects of sufentanil on Bcl2, Bax, cleaved caspase3 and caspase3 expression in lipopolysaccharide (LPS)-treated rat lung tissue. Expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values represent means ± standard deviations. *** $P < 0.001$ vs control group, ##$P < 0.01$, ###$P < 0.001$ vs LPS group.

LPS could be related to HMGB1 expression, activation of NF- κB and miR-129-5p expression.

Sufentanil regulated miR-129-5p/HMGB1 expression in LPS-treated BEAS-2B cells

Bioinformatic analysis predicted a targeting relationship between miR-129-5p and HMGB1 (Figure 4a). Treatment of BEAS-2B cells with the miR-129-5p mimic upregulated the level of miR-129-5p by nearly two-fold (Figure 4b). To confirm the targeting relationship between miR-129-5p and HMGB1, HMGB1 3′UTR wild type and mutant plasmids were constructed and co-transfected with mimics in a double luciferase reporter assay. The results showed that miR-129-5p significantly downregulated the luciferase activity of the wild type HMGB1 3′UTR, but had no significant effect on the luciferase activity of the mutant HMGB1 3′UTR (Figure 4c). To assess whether sufentanil could ameliorate the decline in BEAS-2B cell viability following LPS

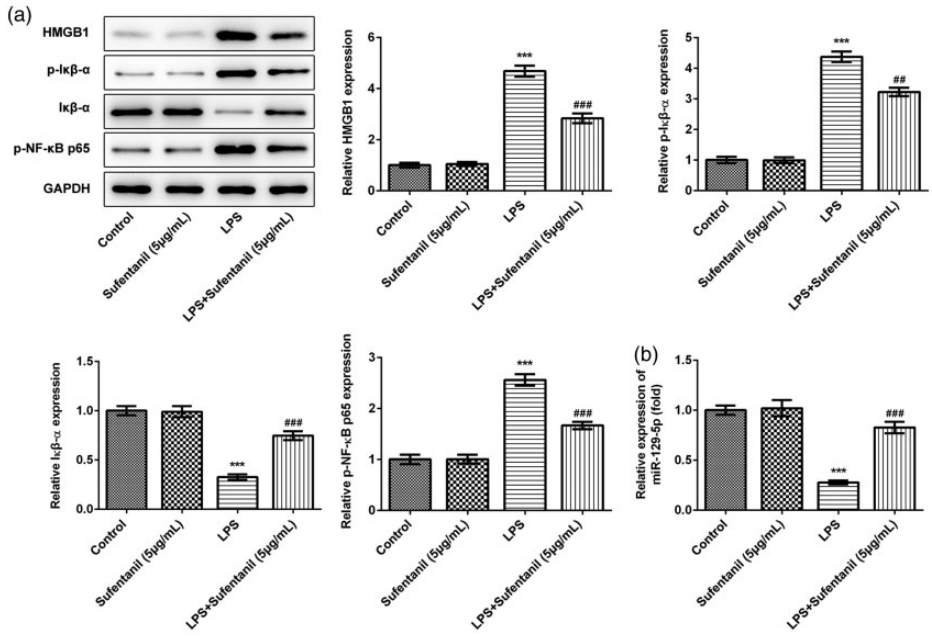

Figure 3. Effects of sufentanil on the expression of high mobility group box protein 1 (HMGB1), NF-κB signaling and miR-129-5p in lipopolysaccharide (LPS)-treated rat lung tissue. Expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values represent means $\pm$ standard deviations. *** $P < 0.001$ vs control group, ##$P < 0.01$, ###$P < 0.001$ vs LPS group.

stimulation, BEAS-2B cells were treated with different concentrations of sufentanil (5, 10 and 20 μM), and cell viability was assessed 24 hours later. At concentrations of 10 μM and 20 μM, sufentanil significantly increased the viability of cells compared with the LPS group (Figure 4d). The concentration of 10 μM sufentanil was selected for further study. RT-qPCR results showed that compared with the control group, LPS group cells had downregulated miR-129-5p levels and increased HMGB1 mRNA levels. These changes were reversed in the sufentanil group (Figure 4e–f).

A miR-129-5p inhibitor reversed the effects of sufentanil on LPS-treated BEAS-2B cells

To investigate the sufentanil mechanism of action in LPS-treated BEAS-2B cells, a miR-129-5p inhibitor was used to silence the expression of miR-129-5p. After knockdown of miR-129-5p using the miR-129-5p inhibitor, the expression of miR-129-5p was significantly decreased (Figure 5a). After BEAS-2B cells were treated with LPS, TNF-α and IL-6 levels in supernatants were quantitated by ELISA. Levels of TNF-α and IL-6 in the LPS group were enhanced compared with the control group (Figure 5b). Compared with the LPS group, sufentanil treatment significantly inhibited cytokine production by BEAS-2B cells. However, this effect was markedly counteracted by addition of the miR-129-5p inhibitor. We also found that the apoptosis induced by LPS was decreased in the LPS + sufentanil group, while this trend was reversed by addition of the miR-129-5p-inhibitor (Figure 5c).

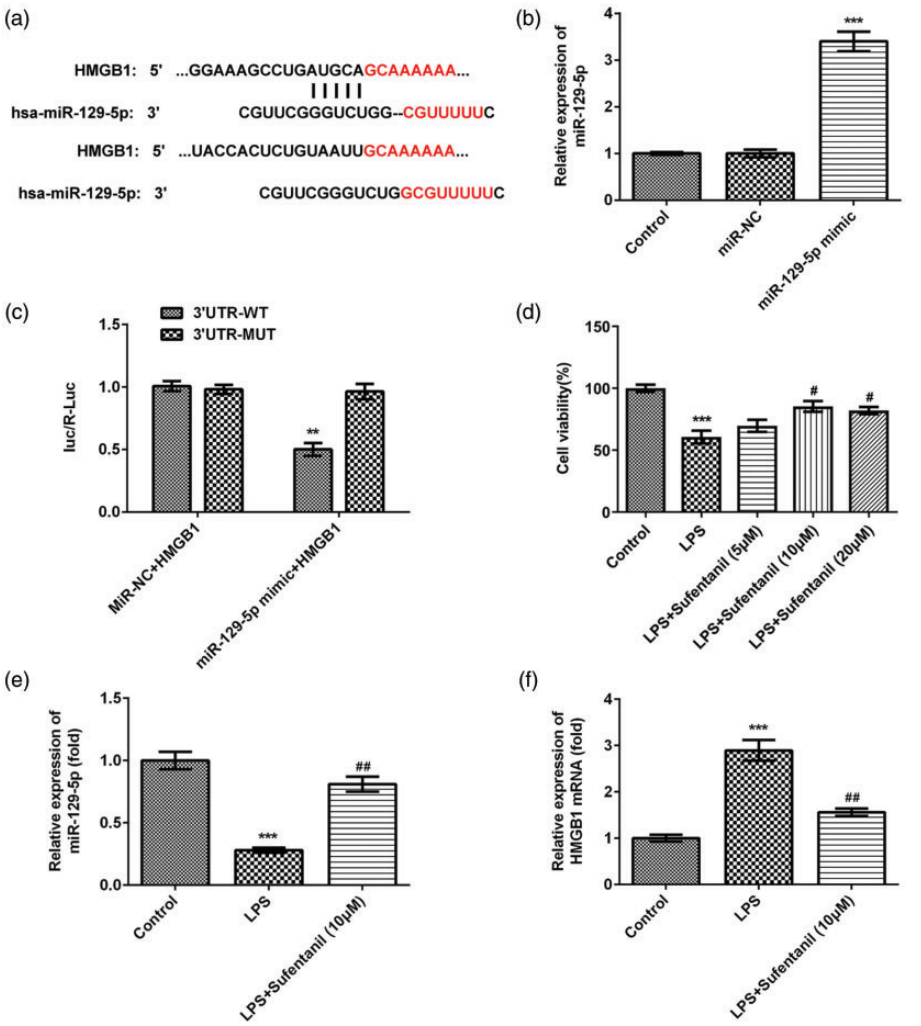

Figure 4. Regulation of the miR-129-5p/high mobility group box protein 1 (HMGB1) axis by Sufentanil *in vitro* following treatment with lipopolysaccharide (LPS). (a) Schematic diagram of miR-129-5p binding site in the HMGB1 3′ untranslated region (UTR). (b) Reverse transcription quantitative polymerase chain reaction was used to assess the transfection efficacy of a miR-129-5p mimic. *** $P < 0.001$ vs miR-NC (negative control). (c) A double luciferase reporter assay verified the targeting relationship between miR-129-5p and HMGB1. ** $P < 0.01$ vs 3′UTR mutant. (d) Effects of different concentrations of sufentanil on cell viability. (e) Sufentanil (10 μM) treatment increased miR-129-5p expression. (f) HMGB1 mRNA levels were upregulated following sufentanil treatment. Values represent means ± standard deviations. *** $P < 0.001$ vs control group, $^{\#}P < 0.05$ vs LPS group.

Sufentanil regulated the miR-129-5p/HMGB1/NF-κB pathway

Further experiments showed that LPS promoted HMGB1 expression in BEAS-2B cells as shown by western blotting (Figure 6a). Phosphorylation levels of IκB-α and NF-κB p65 were also assessed by western blotting. The results are shown in Figure 6a. Compared with the control

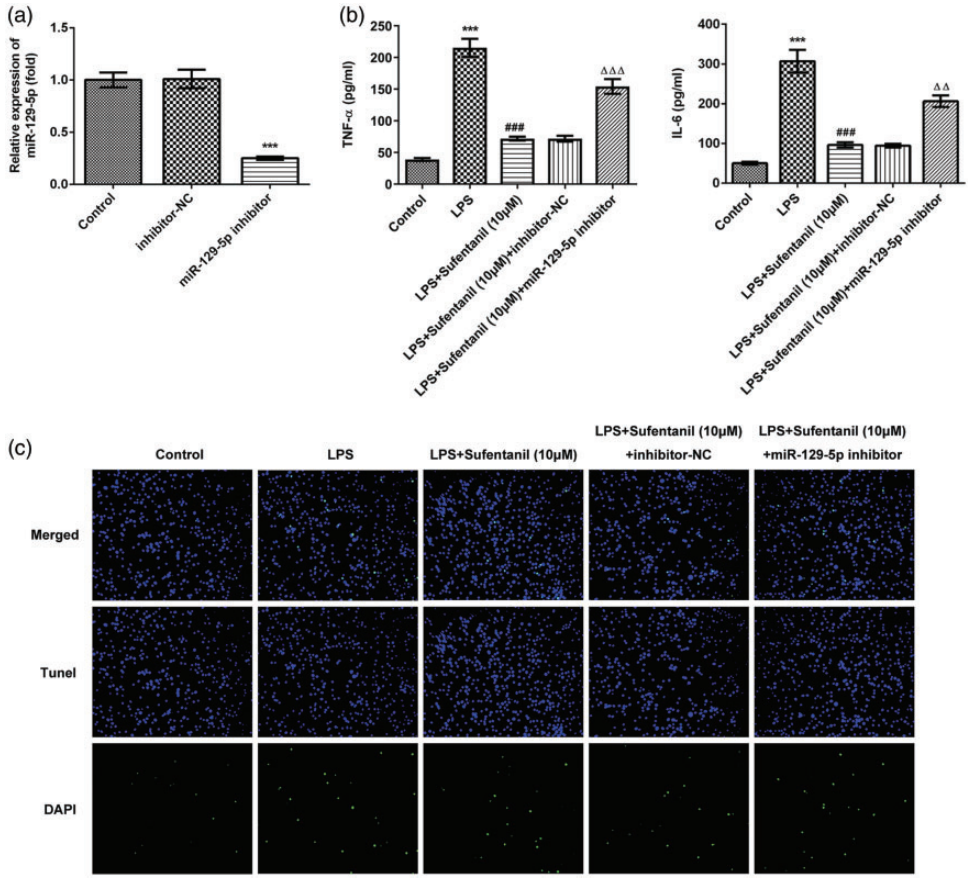

Figure 5. Sufentanil suppressed levels of pro-inflammatory cytokines and apoptosis through miR-129-5p following treatment with lipopolysaccharide (LPS). (a) Reduction in miR-129-5p expression following treatment with a miR-129-5p inhibitor. (b) ELISA was used to measure levels of tumor necrosis factor-α and interleukin-6 in BEAS-2B cell supernatants. (c) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to assess BEAS-2B apoptosis. DAPI, 4′, 6-diamidino-2-phenylindole. *** $P < 0.001$ vs control group, ### $P < 0.001$ vs LPS group, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ vs LPS+sufentanil (10 μM) + inhibitor NC (negative control).

group, the LPS group showed an increase in IκB-α and NF-κB p65 and a decrease in IκB-α. Sufentanil treatment reversed these changes. Moreover, the miR-129-5p inhibitor effectively counteracted the effects of sufentanil. A luciferase reporter construct under the control of the IκB-α promoter was produced. The inhibitor and the mimic of miR-129-5p were used to transfect BEAS2B cells. The miR-129-5a mimic significantly enhanced the promoter activities of IκB-α. By contrast, the miR-129-5a inhibitor reduced its promoter activities. Furthermore, we found that inhibition by miR-129-5a markedly blocked the effects of sufentanil in promoting IκB-α promoter activities in LPS-treated BEAS2B cells. These findings indicated that sufentanil enhanced IκB-α promoter activities by inducing the expression of miR-129-5p.

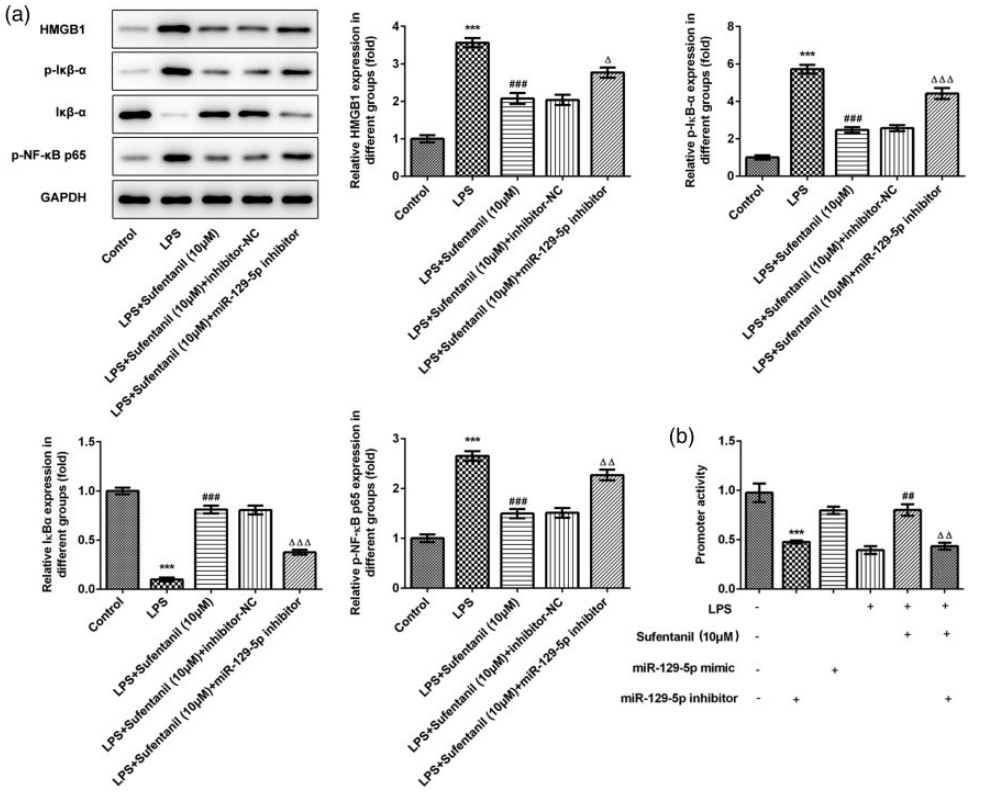

Figure 6. Regulation of the miR-129-5p/high mobility group box protein 1 (HMGB1)/NF-κB pathway by sufentanil in lipopolysaccharide (LPS)-treated BEAS-2B cells. (a) Sufentanil suppressed HMGB1 expression and NF-κB signaling through miR-129-5p. Expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (b) Activation of the IκB-a promoter region was detected using a microplate reader. *** $P<0.001$ vs control group, ### $P<0.001$ vs LPS group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, $^{\triangle\triangle\triangle}P<0.001$ vs LPS + sufentanil (10 μM) + inhibitor NC (negative control).

Discussion

In septic mice, elevated levels of pro-inflammatory cytokines, including TNF-a and IL-6, led to inflammatory response activation and pathological damage.[14] In sufentanil-treated rats, the lung W/D ratio was significantly reduced, indicating that sufentanil had a significant inhibitory effect on pulmonary edema in ALI. Recent studies suggested that the root cause of ALI was systemic inflammation after infection or trauma. The systemic inflammatory response to severe infection was mainly mediated by activation of NF-κB.[15,16] In the present study, increased P-IκB-α and P-NF-κB p65 protein levels in LPS-stimulated rats was rescued by sufentanil administration. This finding suggested that sufentanil inhibited the activation of NF-κB and reduced the production of TNF-α and IL-6, thus playing an anti-inflammatory and protective role in ALI.

To further reveal whether the protective effect of sufentanil on ALI was related to miR-129-5p and HMGB1, *in vitro* experiments were performed to investigate the mechanism of sufentanil action in LPS-stimulated BEAS-2B cells. The results of luciferase reporter experiments suggested

that miR-129-5p bound to the 3′UTR of HMGB1, indicating that there was a regulatory relationship between miR-129-5p and HMGB1. The miR-129-5p/HMGB1 axis is widely involved in biological processes such as inflammation, cell proliferation and autophagy, and affects the progression of several diseases including chronic constriction injuries, cerebral hemorrhage and lung cancer.[8,17–21]

We further found that a miR-129-5p inhibitor blocked the inhibitory effects of sufentanil on apoptosis and the NF-κB pathway. Recent studies have demonstrated that the NF-κB pathway regulates apoptosis in ALI.[22,23] The miR-129-5p/HMGB1 axis has been reported to regulate Toll-like receptor 4/NF-κB signaling.[19] Collectively, our data show that sufentanil suppressed the NF-κB pathway through the miR-129-5p/HMGB1 axis. A luciferase reporter gene assay further demonstrated that a miR-129-5p inhibitor blocked the effects of sufentanil in enhancing IκB-α promoter activity in BEAS-2B cells treated with LPS. HMGB1 has been reported to act as a transcription factor to negatively regulate the expression of IκB-α,[13] ultimately enhancing NF-κB signaling. Therefore, sufentanil regulates the NF-κB pathway through the miR-129-5p/HMGB1 axis to enhance the expression of IκB-α, and exerting a protective role in ALI.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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