Carbon monoxide ameliorates lipopolysaccharide-induced acute lung injury via inhibition of alveolar macrophage pyroptosis

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Abstract: Carbon monoxide (CO) has been reported to exhibit a therapeutic effect in lipopolysaccharide (LPS)-induced acute lung injury (ALI). However, the precise mechanism by which CO confers protection against ALI remains unclear. Pyroptosis has been recently proposed to play an essential role in the initiation and progression of ALI. Thus, we investigated whether pyroptosis is involved in the protection of CO against ALI and its underlying mechanism. First, an LPS-induced ALI mouse model was established. To determine the role of pyroptosis, we evaluated histological changes and the expression levels of cleaved caspase-11, N-gasdermin D (GSDMD), and IL-1β in lung tissues, which are the indicators of pyroptosis. Inhalation of CO exhibited protective effects on LPS-induced ALI by decreasing TNF-α and IL-10 expression and ameliorating pathological changes in lung tissue. In vitro, CO significantly reduced the expression of cleaved caspase-11, N-GSDMD, IL-1β, and IL-18. In addition, it increased nuclear factor E2-related factor 2 (NRF-2) expression in a time-dependent manner in RAW 264.7 cells and decreased N-GSDMD expression. The expression of cleaved GSDMD and release of LDH were increased after treatment with a specific NRF-2 inhibitor, ML385, indicating that NRF-2 mediates the inhibition of pyroptosis by CO. Taken together, these results demonstrated that CO upregulated NRF-2 to inhibit pyroptosis and subsequently ameliorated LPS-induced ALI.

Key words: acute lung injury (ALI), carbon monoxide (CO), gasdermin D (GSDMD), lipopolysaccharides (LPS), pyroptosis

Introduction

Acute lung injury (ALI) is a common critical illness and is characterized by an acute respiratory insufficiency, the symptoms of which include chest pain, chest tightness, and shortness of breath. In severe cases, hemoptysis, respiratory distress, respiratory failure, hypoxemia, hypercapnia, lethargy, coma, and even death may occur. Usually, the mortality from ALI remains between 30% and 40% [1]. ALI can be caused by various direct and indirect effects. Therefore, prevention and control of ALI are important.

Recent studies demonstrated that carbon monoxide (CO) may be a new therapeutic strategy for pneumonia and that inhalation of CO could effectively reduce the damage in many lung diseases, such as ALI [2]. However, its use is a double-edged sword, as it could lead to toxic effects even though its protective effect against lung injury has been validated, with its effect usually determined by the dose of inhalation [3]. Thus, unveiling...
the mechanism of the protective effect of CO will help in the identification of potential targets to focus on with respect to its protective effect. However, the underlying mechanism remains unclear. Recently, a breakthrough concerning inflammation has been made in relation to pyroptosis. Pyroptosis is a caspase-dependent programmed cell death process associated with the release of IL-1β and IL-18, which are usually considered as the critical and initial causes of inflammatory diseases, including ALI [4, 5]. In general, pyroptosis pathways mainly include canonical and non-canonical pathways. The canonical pathway is inherently dependent on caspase-1 activation and mediated by the NLRP3 inflammasome. In the non-canonical pathway, caspase-11 can specifically bind to the lipid A of lipopolysaccharide (LPS), leading to pyroptosis [6]. In 2015, Shi et al. [7] first reported that gasdermin D (GSDMD) is the common downstream executor of the canonical and non-canonical pathways, which offered new insights into the study of pyroptosis.

On the other hand, recent studies have shown that CO can increase nuclear factor E2-related factor 2 (NRF-2) expression, which plays a critical role in ameliorating intracellular and mitochondrial ROS production [8]. NRF-2 is a transcription factor that regulates the expression of antioxidant and detoxification enzymes [9]. Moreover, NRF-2 has been identified as an important regulator of activation of the NLRP3 inflammasome which is the mediator of pyroptosis in canonical pathway [10].

The above two aspects concerning the therapeutic use of CO suggest that the protective effect of CO on ALI may be mediated by regulating pyroptosis and the involved pathway. Thus, in this study, we investigated the role of pyroptosis in the development of ALI and proposed that CO could block pyroptosis and thereby attenuate the lung injury induced by LPS. Moreover, NRF-2 was also identified as the critical mediator in this process.

Materials and Methods

Materials and reagents

LPS was purchased from MedChemExpress LLC (Cat No. HY-D1056, Monmouth Junction, NJ, USA). CORM-3 was purchased from Sigma-Aldrich Inc. (Product No. SML0496, St. Louis, MO, USA). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay kits were purchased from Roche., Ltd. (Basel, Switzerland). DAPI was purchased from Beyotime Biotechnology (Cat No. C1002, Shanghai, China). ML385 was purchased from Selleck Chemicals (Cat No. S8790, Shanghai, China). Rabbit monoclonal antibody to caspase-11 (ab180673, 1:1,000), rabbit monoclonal antibody to GSDMD (ab209845, 1:1,000), and rabbit polyclonal antibody to NRF-2 (ab31163, 1:1,000) were all purchased from Abcam (Shanghai) Trading Co., Ltd. (Shanghai, China). Rabbit monoclonal antibody to GAPDH (#2118) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Experimental animals and design

Female 8-week-old C57BL/6 mice (weighing 19 ± 2 g) were purchased from Lingchang Biological Technology (Shanghai, China). Mice were housed in cages with a 12 h dark and light cycle and provided certified rodent diet with filtered water ad libitum. They were randomly divided into six groups (n=10 per group): normal Saline (NS), LPS (1 mg/kg, 2.5 mg/kg, 5 mg/kg), CO (250 ppm), and LPS+CO groups. For induction of ALI, LPS (1 mg/kg, 2.5 mg/kg, or 5 mg/kg) was applied to mice once by intravenous tail injection as reported previously [11]. In the LPS+CO group, CO (250 ppm) was inhaled for 2 h prior to LPS injection. At the 4 h, 12 h, and 24 h time points, the serum was collected to detect TNF-α and IL-10 levels by ELISA. After 24 h, the right lung tissues of mice were taken for hematoxylin and eosin (H&E) staining and immunohistochemical staining of IL-1β. The proteins of left lung tissues were extracted to detect the expression of caspase-11 and GSDMD. For the immunohistochemical staining of NRF-2 expression, CO (250 ppm) was inhaled for 2–8 h, and at 2 h, 4h, and 8h, the lung tissues were collected to perform immunohistochemical staining and analysis. All animal studies were carried out according to the requirements of the Animal Care Committee at the Institute of Tongji University (No. 7663217).

Cell culture and experimental design

The murine macrophage RAW 264.7 cell line was obtained from Shanghai Life Academy of Sciences Cell Library (Shanghai, China). RAW 264.7 cells were cultured in DMEM-high glucose with 10% (v/v) fetal bovine serum (FBS) and incubated at 37°C in a humidified 5% CO₂ atmosphere.

After pretreatment with the water-soluble CO-releasing molecule CORM-3 (200 µM), a water-soluble CO-releasing molecule for 12 h, the RAW 264.7 cells were subjected to LPS (200 ng/ml) treatment. At 0 h, 1 h, 4 h, 8 h, and 12 h, the levels of IL-1β and IL-18 in cell supernatants were detected by ELISA. At 12 h after LPS treatment, RAW 264.7 cells were harvested to determine the expression of caspase-11 and GSDMD.
Samples and tissue preparation

At the 4 h, 12 h, and 24 h after LPS challenge, the mice were anesthetized, and blood (500 µl per mice) was collected from the heart and then placed at room temperature for 1.5 h. The serum was harvested by centrifugation at 3,500 rpm for 15 min at 4°C to perform ELISA. Simultaneously, lung tissues were collected. At 24 h after LPS challenge, the mice were sacrificed, and right lung tissues were collected, immersed in 4% paraformaldehyde for 24 h, and then embedded in paraffin for H&E staining and immunohistochemical assay. The proteins of left lung tissues were extracted, and the levels of GS-DMD, caspase-11, and other proteins were detected by western blotting.

CO exposure

In vivo CO exposure was conducted with continuous CO inhalation at 250 ppm, as reported by Nakao et al. [12]. The concentration was confirmed by the CO concentration detector of Huazhong University of Science and Technology. For in vitro experiments, CO exposure was conducted with the CO-releasing agent CORM-3, at the concentration of 200 µM, as reported by Lee et al. [13].

Histopathology and immunohistochemistry staining

The fixed lungs were thoroughly washed with 0.01M PBS (pH 7.4) and embedded in paraffin. Paraffin sections were cut at a thickness of 4 µm for H&E staining. Changes in histopathology were visualized by optical microscope (×200). Severity of alveolitis was evaluated by Ashcroft score according to the method of Szapiei et al., who mainly observed and analyzed the thicknesses of alveolar or bronchial walls [14]. Briefly, Grade 0 = no alveolitis; Grade 1 = mild alveolitis characterized by a lesion area<20% of the lung; Grade 2 = moderate alveolitis characterized by a lesion area of 20–50%; Grade 3 = diffuse alveolar inflammation characterized by a lesion area>50%. Fifteen slices were obtained for each group, with three images each obtained in the upper, middle, and lower fields for each slice for a total of 45 images. We evaluated the degree of lung injury by quantifying interstitial fibrosis among the groups using the Ashcroft score [15].

Immunohistochemical staining was performed using paraffin sections. IL-1β expression was investigated. The tissues were incubated in primary antibodies overnight at 4°C and then in horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive cells were visualized using 3,3'-diaminobenzidine (DAB). Antibody dilutability was 1:50 for IL-1β and 1:100 for NRF-2.

ELISA

TNF-α and IL-10 expression levels were analyzed by ELISA (Anogen, Toronto, Canada) according to the manufacturer’s protocol. The contents of TNF-α and IL-10 in the serum samples were calculated according to the standard curve.

Western blot analysis

Proteins from cells were mixed with RIPA buffer (Beyotime, Shanghai, China). The total proteins were quantified by bicinchoninic acid assay. Equal amounts of soluble proteins were loaded onto SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 0.05% Tween and 5% BSA in Tris-buffered saline for 2 h at room temperature and incubated overnight at 4°C with primary antibodies against pro-caspase-11, cleaved caspase-11, full-GSDMD, N-GSDMD, NRF-2, and GAPDH. The preparative membranes were reacted with appropriate secondary antibodies conjugated to HRP. The immunological complexes were visualized with electrochemiluminescence. Band intensities were analyzed with the Image J software.

LDH release

An LDH release assay was used to evaluate pyroptosis. After LPS/CO treatment, supernatants of cultured cells were collected to detect the content of an LDH using the LDH assay kit (Cat. C0016, Beyotime Biotechnology). Percentage of LDH release was calculated as 100 × (experimental LDH − spontaneous LDH) / (maximum LDH release-spontaneous LDH).

Statistical analysis

Results are presented as the mean ± SD. Significant differences in means between two samples were analyzed using LSD t-tests. One-way analysis of variance (ANOVA), followed by Bonferroni’s or Tukey’s multiple comparisons test, was used when comparing more than two sets of data. A P-value <0.05 was considered significant.

Results

LPS induces ALI and pyroptosis in lung tissues

First, ALI was induced in mice with LPS and verified by H&E staining. Different doses of LPS treatment resulted in extensive damage to lung tissues, which was indicated by destroyed alveoli, thickening of alveolar walls, infiltration of inflammatory cells into alveolar spaces, and formation of hyaline membranes. The 5 mg/kg dose of LPS led to the most obvious injury (Figs. 1A and B). To detect pyroptosis, the levels of C-caspase-11 and N-GSDMD expression were determined by Western
blotting. LPS treatment resulted in upregulation of the expression of C-caspase-11 and N-GSDMD in a time-dependent manner compared with the NS group (Fig. 1C). Immunohistochemical analysis showed positive IL-1β staining in the LPS group (Fig. 1D). These data demonstrated that LPS led to ALI and induced pyroptosis in lung tissues.

**Inhalation of CO attenuates LPS-induced ALI in mice**

Next, we investigated whether CO attenuates LPS-induced ALI. Our ELISA results demonstrated that the levels of TNF-α and IL-10 expression were drastically upregulated after LPS treatment; however, mice that were pre-exposed to CO showed obvious attenuation (Figs. 2A and B). H&E staining showed that administration of CO substantially reduced LPS-induced lung in-
Fig. 2. Inhalation of carbon monoxide (CO) attenuates lipopolysaccharide (LPS)-induced acute lung injury (ALI). (A, B) Mice were exposed to CO at a concentration of 250 ppm for 2 h prior to administration of LPS (5 mg/kg) and at the 4 h, 12 h and 24 h time points, and the TNF-α and IL-10 levels were analyzed by ELISA assay. (C, G) Mice were injected with LPS (5 mg/kg), and at 24 h, lung sections were stained with H&E and subjected to immunohistochemical staining of IL-1β. (D) Quantitative analysis of histologic lung sections by lung injury score for four experimental groups. (E, F) Simultaneously, the proteins of left lung tissues were extracted to detect the expression of caspase11 and gasdermin D (GSDMD) by Western blotting and quantified. (G, H) The right lungs of mice were subjected to immunohistochemical staining for IL-1β at 24 h after LPS treatment, and the positive area of staining was quantified. Statistical results are presented as the mean ± SD. *P<0.05, **P<0.01. n=10.
jury (Figs. 2C and D) and simultaneously inhibited the activation of caspase-11 and GSDMD (Figs. 2E and F). Besides, CO exposure also blocked the release of IL-1β induced by LPS (Figs. 2G and H). These results indicated that pre-inhalation of CO could significantly attenuate LPS-induced lung injury and inhibit pyroptosis.

CO inhibits LPS-induced pyroptosis in macrophages

We further investigated the role of CO in pyroptosis in vitro. Pyroptosis occurs in both lung parenchymal cells and immune cells, but the latter are established as critical executors [16, 17]. Therefore, the RAW 264.7 cell line was used in this investigation. As shown in Figs. 3A and B, LPS induced cleavage of caspase-11 and GSDMD, while LDH release (Fig. 3C) and the contents of

Fig. 3. Carbon monoxide (CO) reduces lipopolysaccharide (LPS)-induced pyroptosis. RAW 264.7 cells were pretreated with CORM-3 (200 µM), and then added LPS (200 ng/ml) for designed time. (A, B) Cell lysates were subjected to Western blotting for the detection of pro-caspase11, C-caspase11, full-gasdermin D (GSDMD) and N-GSDMD, and C-caspase-11 and N-GSDMD were quantified (B). (C) Released LDH level was detected at 0 h, 1 h, 4 h, 8 h, and 12 h. (D, E) The levels of IL-1β and IL-18 in cell supernatants were detected. Statistical results are presented as the mean ± SD. *P<0.05, **P<0.01. Each experiment was repeated three times. For detection of the levels of IL-1β and IL-18, n=6.
IL-1β (Fig. 3D) and IL-18 (Fig. 3E) in the supernatants also rapidly increased, indicating that LPS induced caspase 11-mediated pyroptosis. However, pretreatment with CORM-3, which is a common carbon monoxide slow-release agent [18], significantly reduced the expression of cleaved caspase-11 and N-GSDMD and inhibited release of LDH, IL-1β and IL-18. These data highlighted a specific role of CO in caspase-11-mediated pyroptosis.

**CO reduces LPS-induced pyroptosis via NRF-2 upregulation**

Since NLRP3 inflammasomes are involved in the process of pyroptosis, we hypothesized that CO may regulate NRF-2 and ameliorate pyroptosis. To confirm this hypothesis, we first investigated the expression of NRF-2 after CORM-3 treatment. The results showed that pretreatment with CORM-3 could significantly upregulate Nrf2 expression in RAW 264.7 cells in a time-dependent manner and that this was most obvious at 12 h (Fig. 4A). Meanwhile, the nuclear translocation of NRF-2 was also observed by confocal microscopy (Fig. 4B), and the results demonstrated that CORM-3 stimulated the nuclear translocation, indicating an activation of the NRF-2 pathway. Next, the effect of CORM-3 on pyroptosis was determined using ML385, a novel and spe-
cific NRF-2 inhibitor that mainly inhibits downstream transcription [19]. CORM-3 blocked the LPS-induced cleavage of GSDMD (Figs. 4C and D) and release of LDH (Fig. 4E), but ML385 partly counteracted these effects. These results clearly demonstrated that NRF-2 was involved in the CO’s suppression of LPS-induced pyroptosis.

Inhibition of NRF-2 eliminates the protective effect of CO on ALI

The above results suggested that CO may affect NRF-2 expression in vitro. Subsequently, the effect was investigated in vivo. Mice inhaled CO, and then the lungs were collected to perform NRF-2 immunohistochemical staining and detected the expression of downstream genes of Nrf-2, including Hmox1, Me1, and Nqo1. The results demonstrated that CO also stimulated the expression of Nrf-2 in vivo, and Hmox1, Me1, and Nqo1 were also upregulated (Figs. 5A and B). Next, mice were injected with ML385 (10 mg/kg) while simultaneously inhaling CO. After 2 h, the mice were intravenously injected with LPS (200 ng/ml) via the tail. After 24 h, lung tissues were collected to perform H&E staining and detect the expression of GSDMD. The results showed that ML385, the NRF-2 inhibitor, eliminated the protective effect of CO on ALI (Figs. 5D and E) and simultaneously counteracted the inhibitory effect of CO on pyroptosis (Figs. 5F and G).

Discussion

Carbon monoxide, a toxic gas that can lead to neurological disorders and even death, has been recently considered as a potential treatment for many diseases, including ALI [20–22]. However, the underlying mechanism and effective target remain unclear.

In this study, we investigated the role of pyroptosis in the process of ALI and performed in vivo and in vitro experiments to verify whether CO can regulate pyroptosis and thereby attenuate ALI.

First, we established an LPS-induced ALI mouse model and verified that LPS could induce the cleavage of caspase-11 and GSDMD, which are indicators of pyroptosis. These results were consistent with most of the studies concerning LPS and pyroptosis [23–25]. In fact, the pyroptosis includes canonical and non-canonical pathways, with the canonical pathway being mediated by caspase-1 and non-canonical pathway being mediated by caspase-11. However, in most studies, LPS has widely been considered to activate the non-canonical pathway, so we mainly investigated caspase-11. In agreement with other studies, we also concluded that LPS could activate GSDMD. Nevertheless, it is worth mentioning that the most obvious cleavage of GSDMD was found at 24 h after LPS treatment. To date, many studies suggest that LPS-induced pyroptosis is a rapid process with fast cleavage of GSDMD [6, 23]. Our data demonstrated a delayed upregulation of GSDMD and caspase-11 expression. However, we do not think this delayed activation for GSDMD as specific because the uptake of LPS is a slow process, which usually limits the LPS concentration to within a narrow range in vivo.

Based on above model, next the effects of CO on ALI and pyroptosis were subsequently investigated. Inhalation of an appropriate concentration of CO resulted in the LPS-induced ALI and pyroptosis being significantly attenuated. However, the target cells should be confirmed, as CO can diffuse into a variety of localities. Using the RAW 264.7, a murine macrophage cell line, we also verified the inhibitory effect of CO on pyroptosis. Carbon monoxide is an important reactive gas, and many studies have shown a variety of effects on macrophages, including the regulation of their activity [26], alteration of differentiation [27], and regulation of the production of reactive oxygen species [28], etc. However, the role of CO in regulating pyroptosis is still rarely reported. Thus, our findings will broaden the understanding of pyroptosis and carbon monoxide.

Regarding the mechanism of CO, several studies have indicated that CO alleviates the level of reactive oxygen species (ROS) through NRF-2 pathway. NRF-2 is closely associated with CO and also plays a key role in the regulation of NLRP3 inflammasome activation, which occurs upstream of GSDMD cleavage. Therefore, we investigated whether NRF-2 plays a role in the CO-mediated inhibition of pyroptosis. Ultimately, we confirmed that inhibition of NRF-2 with ML385, an NRF-2 inhibitor, could eliminate the protective effect of CO on ALI in vivo and in vitro.

In fact, many studies have reported the roles of NRF-2 in ALI and clarified that NRF-2 activation can provide protection against or prevent ALI [29–31]. In addition, the effect of CO on NRF-2 has also been reported [8]. However, to the best of our knowledge, this is the first study to demonstrate that CO suppresses LPS-induced pyroptosis via NRF-2 activation. This study may be instructive for ALI therapy. Though many studies have demonstrated the therapeutic effect of CO on various diseases, including ALI, the toxicity of CO limits its extensive application [32]. Therefore, clarification of its therapeutic mechanism and identification of critical targets are important. In this study, inhibition of pyroptosis was identified as the mechanism of CO-mediated protection against ALI, and NRF-2 was found to be a critical
Fig. 5. Inhibition of nuclear factor E2-related factor 2 (NRF-2) eliminates the protective effect of carbon monoxide (CO) on acute lung injury (ALI). (A, B) Mice were persistently exposed to CO at a concentration of 250 ppm, and lung tissues were collected to perform NRF-2 immunohistochemical staining at 2, 4 and 8 h. (C) The expression levels downstream genes of NRF-2 were detected by RT-PCR at 8 h after CO inhalation. (D-G) Inhaling CO, mice were intraperitoneal injected with ML385 (10 mg/kg) for 2h, and then ALI was induced by intravenous tail injection of lipopolysaccharide (LPS) (5 mg/kg). After 24h lung sections were collected to stained with H&E (D, E), and proteins were extracted to detect the expression of gasdermin D (GSDMD) and NRF-2 by Western blotting (F, G). Quantitative analysis of histologic lung sections by lung injury score for six experimental groups. Statistical results are presented as the mean ± SD. **P<0.01. For the animal experiments, n=6, and the Western blotting was repeated three times.
regulator. Therefore, NRF-2 could be a critical target that mediates the effect of CO on pyroptosis. Through the administration of NRF-2, the CO dosage could be reduced to minimize its cytotoxicity and maximize its protective effects.

Though the effect of CO on ALI and pyroptosis was verified, this study had some limitations. First, further studies are required to clarify whether CO could directly regulate the expression of NRF-2 and whether other factors mediate this relationship. Second, we only investigated the non-canonical pyroptosis pathway, and the influence of CO on the canonical pathway remains unclear. Finally, the ALI model was induced by tail intravenous injection of LPS into the tail, and various tissues and cells might be the targets of CO’s protective effect. Therefore, the effect of CO on other tissues requires further investigation.

In summary, this study demonstrated a protective effect of CO on LPS-induced ALI by inhibiting pyroptosis, which involved the upregulation of NRF-2.

### Conflict of Interests

The authors declare that they have no competing interests.

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### Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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