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Carbon Monoxide Attenuates Lipopolysaccharides (LPS)-Induced Acute Lung Injury in Neonatal Rats via Downregulation of Cx43 to Reduce Necroptosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BDE 1 Wanwei Li
DF 2 Fang Wu
C 3 Long Chen
BC 1 Qian Li
BF 1 Juan Ma
B 1 Mengchun Li
AE 1,3 Yuan Shi

1 Department of Pediatrics, Daping Hospital, Army Medical University, Chongqing, P.R. China
2 Department of Neonatology, Chongqing Angel Women's and Children's Hospital, Chongqing, P.R. China
3 Department of Neonatology, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing, P.R. China

Corresponding Author: Yuan Shi, e-mail: Petshi530@163.com
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Background: Acute lung injury (ALI) is one of major causes of death in newborns, making it urgent to improve therapy. Administration of low dose carbon monoxide (CO) plays a protective role in ALI but the mechanisms are not fully understood. This study was designed to test the therapeutic effect of monoxide-releasing molecule 3 (MORM3) in lipopolysaccharide (LPS) induced neonatal ALI and the possibly associated molecular mechanisms.

Material/Methods: For this study, 3- to 8-day old Newborn Sprague-Dawley rats were subjected to intraperitoneal injection of 3 mg/kg LPS to induce ALI. Then animals received intraperitoneal injection of carbon monoxide-releasing molecules 3 (CORM3) (8 mg/kg) or inactive CORM3 (iCORM3) for 7 consecutive days. Lung tissues were collected for histological examination and total cell counts and protein content in bronchoalveolar lavage fluid (BALF) were measured. Expression of Cx43 and necroptosis-related markers were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot.

Results: LPS exposure induced significant lung injury indicated by histological damage, increased lung wet/dry weight ratio (W/D) and increased total cell counts and protein concentration in BALF. These changes were significantly ameliorated by administration of CORM3 but not iCORM3. LPS also increased necroptosis-related markers RIP1, RIP3, and MLKL and their elevation was blocked by CORM3. CORM3 administration ameliorated LPS induced elevation of Cx43 expression and adenoviral overexpression of Cx43 abolished lung protective effect of CORM3. CORM3 administration attenuated LPS induced activation of extracellular-signal-regulated kinase (ERK) and its protection against necroptosis was abolished by ERK inhibitor U0126.

Conclusions: CORM3 attenuates LPS-Induced ALI in neonatal rats and its lung protective effect might be through downregulation of Cx43 to attenuate ERK signaling and ameliorate necroptosis, suggesting CORM3 as a potential therapeutic drug for ALI in neonates.

MeSH Keywords: Acute Lung Injury • Carbon Monoxide • MAP Kinase Signaling System

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Background

Acute lung injury (ALI), which can lead to acute respiratory distress syndrome, exhibits high rates of morbidity and mortality [1], although many studies have been devoted to improvement of its therapy [2,3]. Especially, neonates are very susceptible to ALI, making ALI become one of major causes of death in newborns [4–6]. Despite advances in clinical practices in critical care medicine, few therapies showed efficacy except of the use of lung protective ventilatory strategies. Therefore, there exists an urgent need to develop and improve treatments.

Carbon monoxide (CO) has long been known as toxic due to its capacity to bind hemoglobin and disturb oxygen transport. However, growing evidences showed that low doses of CO have beneficial function in many diseases, especially lung injury induced by hyperoxic and ischemia-reperfusion condition [7,8]. It is reported that administration of low dose CO exerts anti-inflammatory and cytoprotective property in ALI [9,10], making it a promising therapy. However, the mechanism for CO inhalation therapy has not been fully understood and it has many limitations such as hampering oxygen transport by hemoglobin when the dose is not highly controlled [11]. Therefore, many carbon monoxide-releasing molecules (CORM) have been put forward as a valid alternative to CO gas in ALI [12]. Among these monoxide-releasing molecules, CORM3 has showed great effect in improving organ structural and functional recovery after acute injury [13,14]. These evidences indicate that CORM3 might have lung protective effect in lipopolysaccharide (LPS)-induced ALI of neonates.

In the present research, we used CORM3 as source of CO, tested whether it protects against LPS-induced ALI in neonatal rats and explored underlying mechanisms. We found that administration of CORM3 exerted therapeutic effect against LPS induced ALI. For mechanisms, CO might ameliorate necroptosis through downregulation of Cx43, which activates extracellular-signal-regulated kinase (ERK) signaling to induce necroptosis.

Material and Methods

Neonatal rat and ALI model establishment

We used 3- to 8-day old Newborn Sprague-Dawley rats were obtained from the Experimental Animal Center of Daping Hospital affiliated to Third Military Medical University (Chongqing, China). All experiments were approved by the Animal Care and Use Committee of Third Military Medical University. The animals were housed in an artificial specific pathogen-free environment with 12-hour light/dark cycle and 25±5°C temperature. Intraperitoneal injection of 3 mg/kg LPS (Sigma-Aldrich) to neonatal rats was performed to induce ALI as previously reported [15]. Then animals received intraperitoneal injection of CORM3 or iCORM3 (8 mg/kg) for 7 consecutive days after LPS exposure.

Histological examination

Lung tissues of animals collected and fixed in 4% paraformaldehyde before being embedded in paraffin and sectioned at 4-μm thickness. Next the sections were stained with hematoxylin and eosin (HE, Sigma) and observed under a light microscope. Then severity of alveolar congestion, alveolar hemorrhage and infiltration or aggregation of neutrophils was examined to score the lung injury as previously described [16].

Lung edema evaluation

Lung edema was evaluated by the ratio of wet/dry weight. The lung tissues were harvested and weighed immediately to gain wet weight. Next blood on the lung surface was washed away and the lung was dried for 48 hours at 70°C. Then dry weight of lungs was gained the ratio of wet/dry weight was calculated.

Determination of total cell counts and protein content in bronchoalveolar lavage fluid (BALF)

The bronchoalveolar lavage fluid (BALF) was collected by lavaging the lung with sterile phosphate-buffered saline (PBS) by intratracheal injection 3 times. The BALF was centrifugation at 800 g at 4°C for 10 minutes and the supernatant was used to measure the protein content with bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Cell pellets were resuspended in 0.9% saline and stained with Wright-Giemsa for 8 minutes. Then total cell counts were determined using a hemocytometer as previously described [17].

Western blot analysis

Lung tissues were homogenized in commercialized lysis buffer (Beyotime, China). The supernatants of the lysates were collected after centrifugation (12 000g/minute, 30 minutes). Bradford protein assay kit was used to measure protein concentration. Then protein samples (50 μg) were subjected to SDS-PAGE with 10% polyacrylamide gel followed by electrotransfer into polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies over night at 4°C after being blocked with 5% non-fat milk at room temperature for 2 hours. Then membranes were washed with Tris buffer saline (TBS) and then incubated with secondary antibodies for 1 hour at room temperature. After being washed with TBS, Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) was used to visualize protein bounds. GAPDH was used to normalize the densitometric intensity of proteins.

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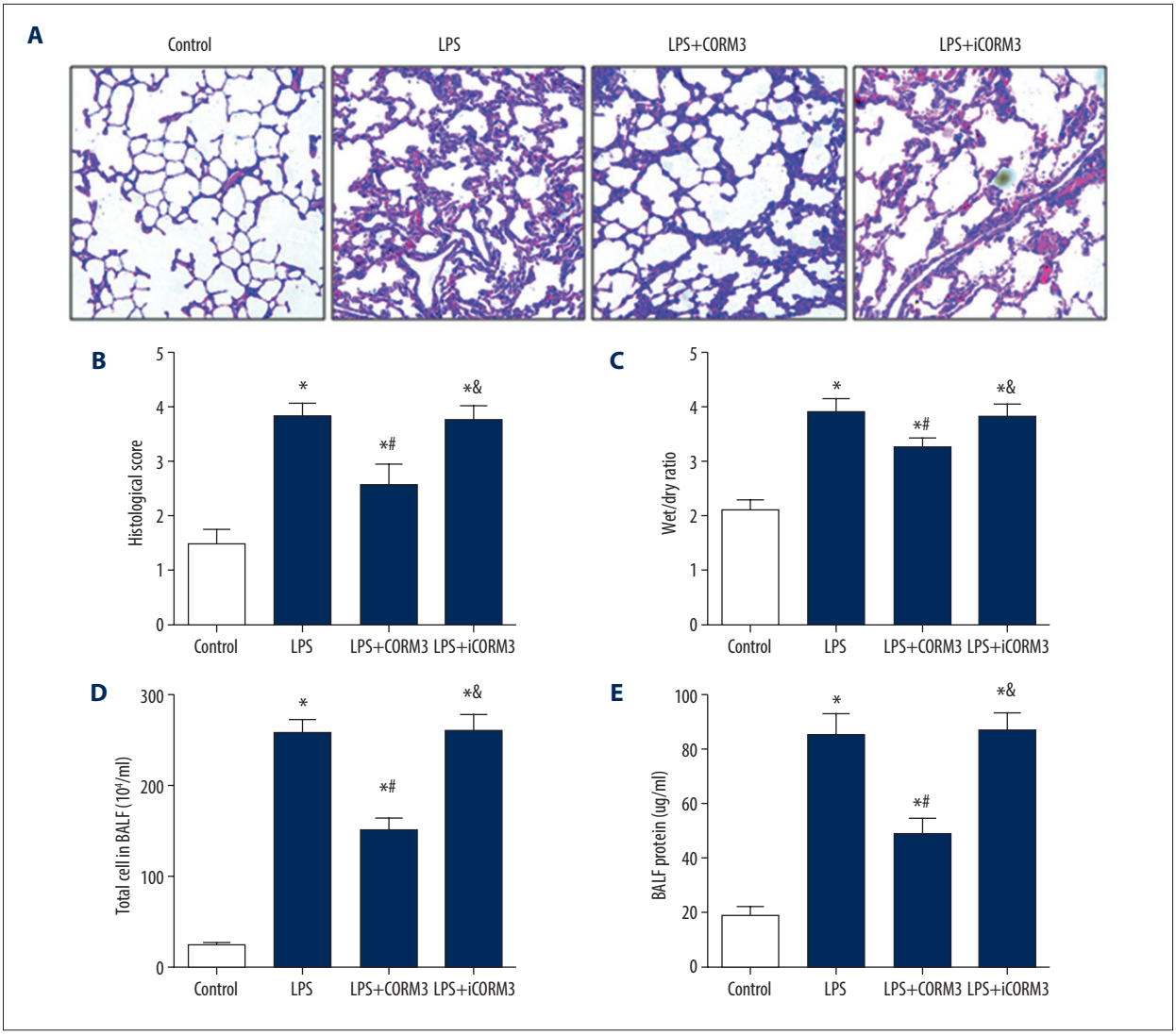

Figure 1. CO ameliorated LPS-induced ALI. Neonatal rats were treated with control vehicle, LPS, LPS+CORM3, or LPS+iCORM3. (**A**) Histologic alterations of lung tissues examined by hematoxylin and eosin staining (magnification, 200×). (**B**) Pathological score of lung tissues. (**C**) The lung wet/dry ratio. (**D**) Total cell number in BALF. (**E**) Protein concentrations in BALF. & $P<0.05$ versus LPS+CORM3; # $P<0.05$ versus LPS; * $P<0.05$ versus control; n=8–10 for all. CO – carbon monoxide; LPS – lipopolysaccharide; ALI – acute lung injury; CORM3 – carbon monoxide-releasing molecules 3; iCORM3 – inactive CORM3; BALF – bronchoalveolar lavage fluid.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (ThermoFisher, Waltham, MA, USA) were used to isolate total RNA from lung tissues following the manufacturers' instructions. Reverse transcription was performed before Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Select Master Mix (Thermo Fisher). GAPDH was selected to normalized gene expression and double delta Cq method was used to calculate the relative expression of mRNA.

Statistical analysis

SPSS 18.0 statistical package was used to analyze sample data, which was expressed as mean ± standard deviation (SD). Significant differences among more than 2 groups were analyzed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) post hoc test. Significant differences between 2 groups were analyzed using Student's *t*-test. $P<0.05$ was considered as statistically significant.

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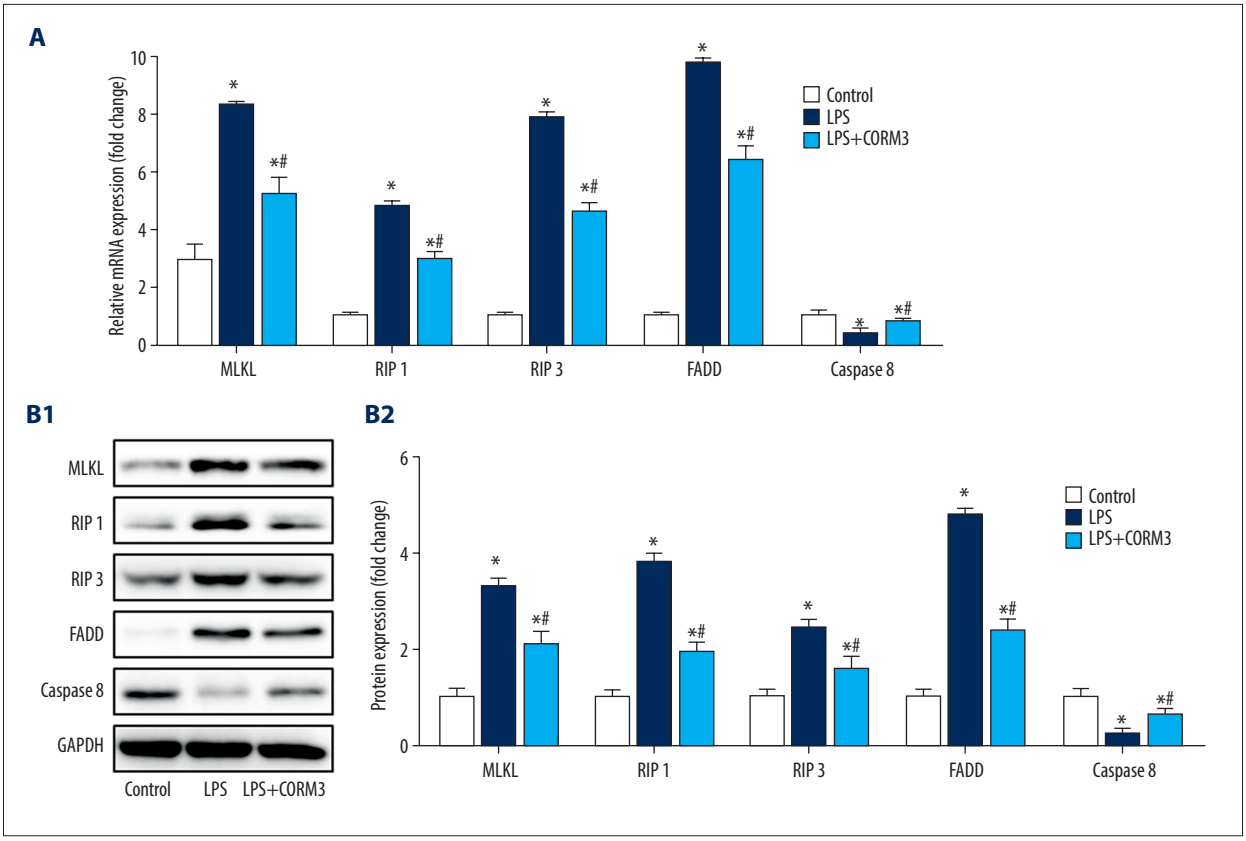

Figure 2. CO ameliorated RIP3-mediated necroptosis in LPS-induced ALI. (**A**) The mRNA expression of MLKL, RIP1, RIP3, FADD, and caspase-8 in the lung tissues (n=5). (**B**) Representative blots (**B1**) and quantification (**B2**) of MLKL, RIP1, RIP3, FADD, and caspase-8 protein expression in lung tissues (n=5). # $P<0.05$ versus LPS; * $P<0.05$ versus control. CO – carbon monoxide; LPS – lipopolysaccharide; ALI – acute lung injury.

Results

CO ameliorated LPS-induced ALI

Growing evidence has demonstrated that low dose of CO has protective effects against oxidative stress [18], inflammation [19], and apoptosis [20] thus exerting beneficial effects in ALI. However, CO inhalation therapy has many limitations such as hampering oxygen transport by hemoglobin when the dose is not highly controlled. Therefore, many CORMs have been put forward as a valid alternative to CO gas in ALI. In the present study, CORM3 was intraperitoneally injected to neonatal rats to test its effects on LPS induced acute long injury. As is shown in Figure 1A, the alveolar structure was clear and there was no infiltration of inflammatory cells in lung tissue of control group. In LPS group, there was edema in the lung interstitium, and the alveolar structures were significantly damaged with significant infiltration of inflammatory cells, indicating LPS induced ALI was successfully established. Notably, these histological alterations were significantly ameliorated by administration of CORM3 instead of iCORM3, indicating CO released from CORM3 exerted therapeutic effect against LPS induced ALI (Figure 1A). Consistent with these changes, the histological score in LPS group was significantly higher than control group, but the increase was effectively blocked by administration of CORM3 instead of iCORM3 (Figure 1B). Furthermore, LPS administration resulted in a significant elevation in lung W/D ratio indicating elevated lung water content, and this elevation also significantly mitigated by CORM3 (Figure 1C). In addition, LPS also caused a significant elevation in the total cell counts and protein content in BALF, further confirming the lung injury and inflammation, and this change was also ameliorated by CORM3 instead of iCORM3 (Figure 1D, 1E). Collectively, these results suggested that CORM3, through releasing CO, had a protective effect against LPS induced ALI.

CO ameliorated necroptosis in LPS-induced ALI

Previously studies have demonstrated that necroptosis plays essential role in LPS induced ALI and it has been regarded as a promising target for the treatment of lung injury [21,22]. Therefore, we examined whether CO mitigated necroptosis in LPS-induced ALI by qPCR and western blot. Our results showed that both mRNA expression of RIP1, RIP3, MLKL, and

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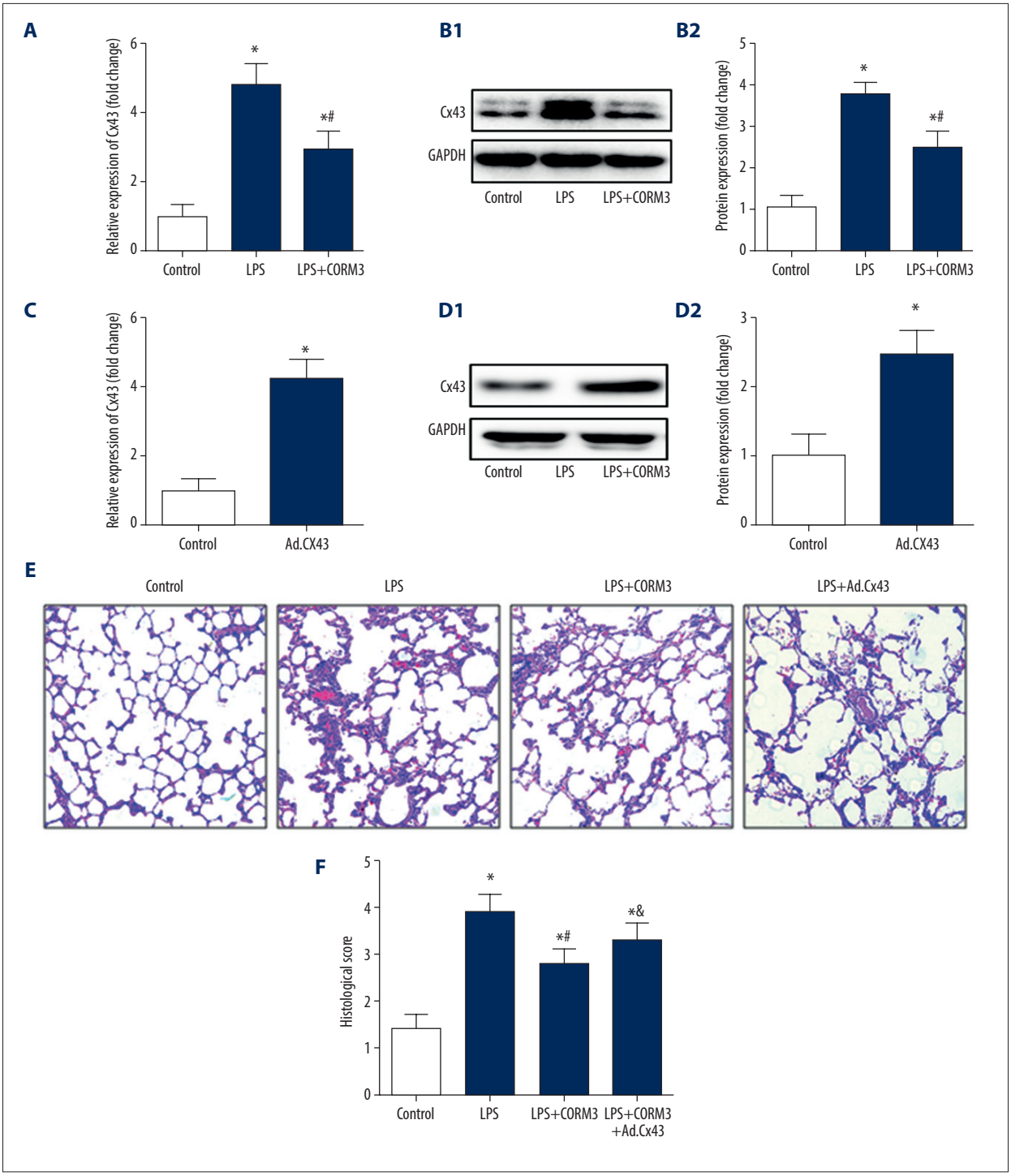

Figure 3. Downregulation of Cx43 was involved in lung protective effect of CO. Animals with or without Cx43 overexpression were treated with control vehicle, LPS, or LPS+CORM3. (**A**) Lung Cx43 mRNA expression (n=5). (**B**) Representative blots (**B1**) and quantification (**B2**) of Cx43 protein expression in lung tissues (n=5). (**C**) Representative blots (**B1**) and quantification (**B2**) of Cx43 protein expression in lung tissues from animals treated with control adenovirus or adenoviral Cx43 (n=5). (**D, E**) Hematoxylin and eosin staining images (**D**), and pathological score (**E**) of lung tissues from animals in control, LPS, LPS+CORM3, or LPS+CORM3+Ad. Cx43 group (n=5). & $P<0.05$ versus LPS+CORM3; # $P<0.05$ versus LPS; * $P<0.05$ versus control. CO – carbon monoxide; LPS – lipopolysaccharide; CORM3 – carbon monoxide-releasing molecules 3.

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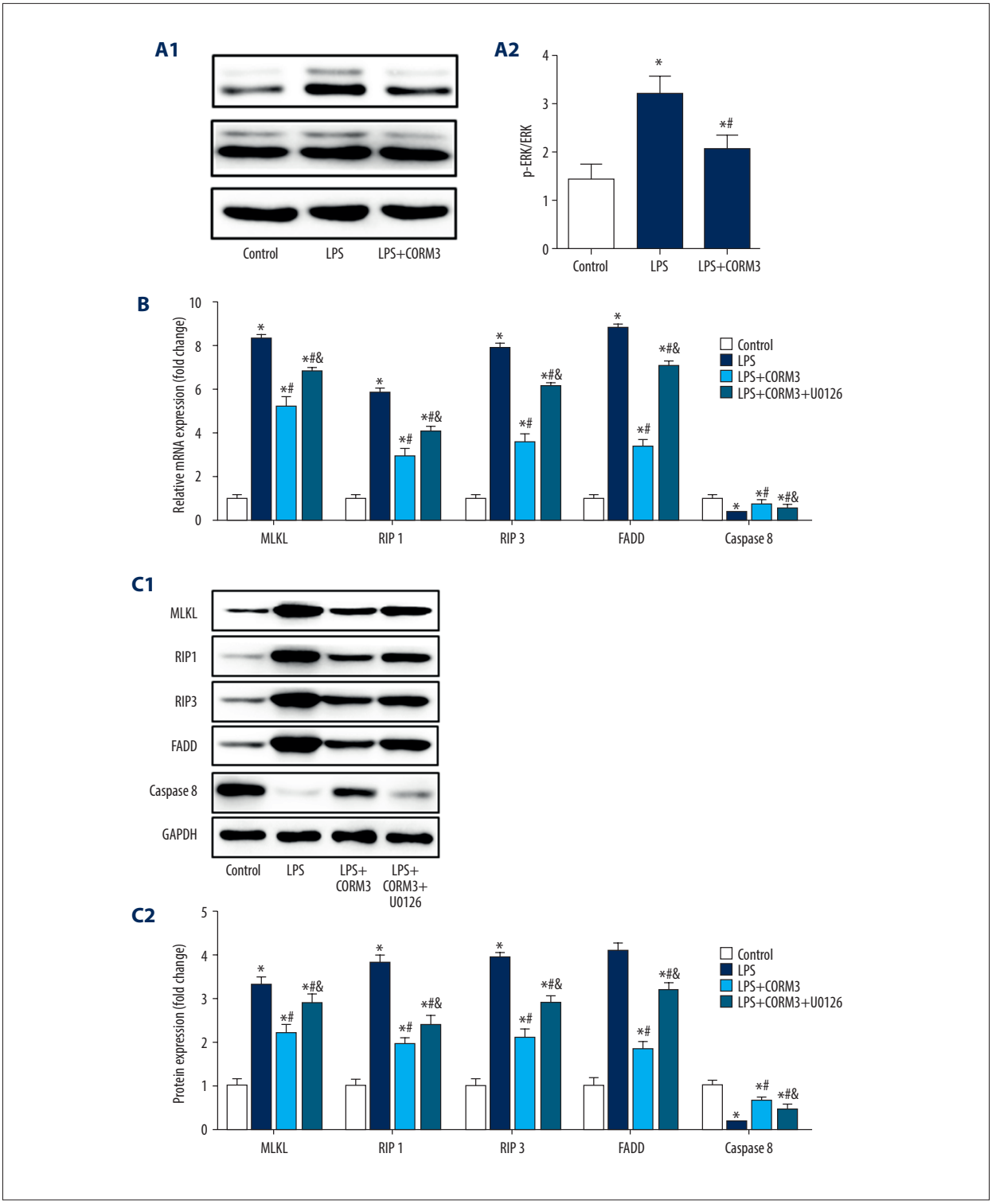

Figure 4. Cx43 activated ERK signaling pathway to induce necroptosis in LPS-induced ALI. Animals were treated with control vehicle, LPS, LPS + CORM3, or LPS+CORM3+U0126. (**A**) Representative blots (**A1**) and quantification (**A2**) of phosphorylated ERK versus total ERK protein expression. (**B**) The mRNA expression of MLKL, RIP1, RIP3, FADD, and caspase-8 in the lung tissues. (**C**) Representative blots (**C1**) and quantification (**C2**) of MLKL, RIP1, RIP3, FADD, and caspase-8 protein expression in lung tissues. # $P<0.05$ versus LPS; * $P<0.05$ versus control. ERK – extracellular-signal-regulated kinase; LPS – lipopolysaccharide; ALI – acute lung injury; CORM3 – carbon monoxide-releasing molecules 3.

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FADD was upregulated and caspase-8 downregulated in LPS group, reflecting necroptosis in the lung (Figure 2A). A similar alteration was found on the protein expression of RIP1, RIP3, MLKL, FADD, and caspase-8 (Figure 2B). These changes were effectively blocked by administration of CORM3 (Figure 2B), indicating necroptosis in LPS-induced ALI was ameliorated by CO. Taken together, these results suggested that amelioration of necroptosis might account for lung protective effect of CO in neonates.

Downregulation of Cx43 was involved in lung protective effect of CO

Gap junction composed of Cx43, has reported to involve in ALI through intercellular communication, thus Cx43 was regarded as an important target for ALI [23]. Therefore, we investigated whether Cx43 plays essential role in protective effect of CO in LPS-induced ALI. As is shown in Figure 3A and 3B, mRNA and protein expression of Cx43 were elevated in LPS group, and their elevation was blocked in CORM3 group, suggesting CO might protect lung injury through downregulation of Cx43. We next adenovirally overexpressed Cx43 in lung tissues, and results showed that Cx43 was effectively overexpressed (Figure 3C, 3D). Notably, Cx43 overexpression effectively attenuated protective effect of CORM3 in LPS induced histological alterations in lung tissue (Figure 3E). In addition, the impact of Cx43 overexpression on protective effect of CORM3 was further confirmed by quantification of histological scores (Figure 3F). These results indicated that down-regulation of Cx43 might be involved in protective effect of CO in LPS-induced ALI.

Cx43 activated ERK signaling pathway to induce necroptosis in LPS-induced ALI

It is reported that activation of extracellular regulated MAP kinase (ERK) plays important role in necroptosis [24]. In addition, Cx43 is upstream of ERK and downregulation of Cx43 can attenuate activation of ERK signaling in many biological progresses [25]. Therefore, we explored whether downregulation of Cx43 by CO attenuates ERK signaling and thus ameliorating necroptosis in LPS-induced ALI. Our results showed that ERK phosphorylation was enhanced in LPS group and its elevation was blocked by CORM3 (Figure 4A), indicating ERK signaling was activated in LPS induced ALI and its activation was attenuated by CO. We used EKR inhibitor U0126 to reverse ERK inactivation and found U0126 significantly diminished protective effect of CORM3 against necroptosis indicated by mRNA and protein expression of RIP1, RIP3, MLKL, FADD and caspase-8 (Figure 4B, 4C). Taken together, these results suggested that Cx43 might activate ERK signaling pathway to induce necroptosis, thus CO downregulates Cx43 to attenuate ERK signaling and thus ameliorating necroptosis in LPS-induced ALI.

Discussion

Recently, many studies demonstrated that CO, a by-product of heme catabolism, possesses various physiological effects including anti-inflammatory, anti-apoptotic, antioxidant, and anti-proliferative property *in vitro* and *in vivo* [26,27]. Therefore, CO was applied to treat organ stress including acute myocardial injury, acute spinal cord injury [28], acute kidney injury [29], and ALI [30]. Encouragingly, CO showed therapeutic effect against these organ injuries. But CO inhalation therapy has many limitations such as hampering oxygen transport by hemoglobin when the dose is not highly controlled. Therefore, many CORMs have been put forward as a valid alternative to CO gas and reported effective in organ protection against stress and injury [31]. However, few studies reported the effect of CORMs in neonatal lung injury. Among the CORMs, CORM-3 is water-soluble and it releases equimolar amount of CO. In addition, CORM3 has shown protective effects in various models such as acute myocardial infarction and liver failure [13,14]. Therefore, CORM3 was selected as the source of CO in this study. In the present study, we show that CORM3 exerts lung protective effect in LPS-induced acute injury in neonatal rats. Thus, together with previous studies, we further highlighted CO as a therapeutic strategy for neonatal lung injury.

Though growing evidences have showed lung protective of CO in LPS-induced ALI, the mechanisms remain not fully understood. Necroptosis, a different form of programmed cell death than apoptosis, consists of mechanism of many diseases including cancer and organ injury [32]. For example, liver injury in cholestasis can be protected against through miRNA-21 ablation, which attenuates necroptosis [33]. In addition, it is reported that receptor-interacting protein kinase 1 mediated necroptosis contributes to renal ischemia/reperfusion injury and acts as a target for treatment of renal injury [34]. Therefore, intensive research has studied necroptosis as a potential therapeutic target in multiple organ dysfunction. Especially, necroptosis was also reported to play essential role in various forms of lung injury including hyperoxia-induced lung injury and LPS-induced lung injury, making necroptosis as a therapeutic target for lung injury [35–37]. However, whether necroptosis also involves in lung protective effect of CO is unknown. Our study found CORM3 administration reduced necroptosis-related markers in LPS-induced neonatal lung injury, thus we speculate that CO might protects lung injury through inhibition of necroptosis.

Cx43 is one of the most important connexins involves in forming gap junction and plays important role in direct signal transfer between neighboring cells. Notably, Cx43 also is reported to involve in the development of lung injury [38,39]. For example, previous studies showed that Cx43 is upregulated in lung injury induced by endotoxin and exacerbates lung vascular

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permeability [40]. Consistently, we found that expression of Cx43 was elevated in LPS-induced ALI in neonatal rats. We also showed that CORM3 administration blocked Cx43 upregulation and adenoviral overexpression of Cx43 diminished lung protective effect of CO, suggesting that CO might protect against lung injury through downregulation of Cx43. It is reported that activation of ERK plays important role in necroptosis [41,42]. In addition, Cx43 is upstream of ERK and downregulation of Cx43 can attenuate activation of ERK signaling in many biological progresses [25]. Our results showed that administration of ERK inhibitor U1026 abolished necroptosis inhibitive and lung protective effect of CO. Thus, we speculated that CO downregulates Cx43 to inactivate ERK signaling to attenuate necroptosis.

Conclusions

CORM3 exerted therapeutic effect against LPS induced ALI in neonatal rats. For mechanisms, CO might downregulate Cx43 to inactivate ERK signaling to attenuate necroptosis. Additional experimental studies are needed to further reveal underlying mechanism of lung protective effect of CO and improve the efficacy of CO therapy.

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