Carbon monoxide inhalation protects lung from lipopolysaccharide-induced injury in rat

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Abstract: Carbon monoxide (CO), a metabolite of heme catalysis by heme oxygenase (HO), has been proposed to have anti-oxidative, anti-inflammatory and anti-apoptotic functions. Lipopolysaccharide (LPS)-induced lung injury (LI) is characterized by oxidative stress, inflammatory reaction and excessive pulmonary cell apoptosis. So we supposed that CO might have protection against LI. LI in rats was induced by intravenous injection of LPS (5 mg/kg). To observe the effect of CO inhalation, LI rats were exposed to $2.5 \times 10^{-4} (V/V)$ CO for 3 h. CO-induced changes of lung oxidative stress parameters, inflammatory cytokines, cell apoptosis, HO-1 expression and histology were examined. Results revealed that expressions of the tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interlukin-6 (IL-6), activities of maleic dialdehyde (MDA) and myeloperoxidase (MPO), and cell apoptosis in LPS injection + CO inhalation group were $(0.91\pm0.25) \text{ pg/mg protein}, (0.64\pm0.05) \text{ pg/mg protein}, (1.02\pm0.23) \text{ nmol/mg protein}, (7.18\pm1.62) \text{ U/mg protein}$ and $(1.60\pm0.34)\%$, respectively, significantly lower than the corresponding values in LI group $(1.48\pm0.23) \text{ pg/mg protein}, (1.16\pm0.26) \text{ pg/mg protein}, (1.27\pm0.33) \text{ nmol/mg protein}, (8.16\pm1.49) \text{ U/mg protein}$ and $(3.18\pm0.51)\%$, respectively. Moreover, CO inhalation obviously increased the expressions of HO-1 and interlukin-10 (IL-10) and activity of superoxide dismutase (SOD) $(5.43\pm0.92), (0.26\pm0.07) \text{ pg/mg protein and (60.09\pm10.21) U/mg protein in LPS injection + CO inhalation group vs (3.08\pm0.82), (0.15\pm0.03) \text{ pg/mg protein and (50.98\pm6.88) U/mg protein in LI group, P<0.05}}$. LI was attenuated by CO inhalation. Our study demonstrates that inhalation of low concentration of CO protects lung against LPS-induced injury via anti-oxidant, anti-inflammation, anti-apoptosis and up-regulation of HO-1 expression.

Key words: carbon monoxide; heme oxygenase; lung injury; oxidative stress; inflammation; apoptosis

一氧化碳吸入对脂多糖诱导大鼠急性肺损伤的保护作用

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摘要: 血红素氧合酶(heme oxygenase, HO)降解血红素的主要代谢产物一氧化碳(carbon monoxide, CO)具有抗氧化、抗炎症和抑制细胞凋亡作用, 而脂多糖(lipopolysaccharide, LPS)诱导的肺组织过氧化、炎症性损伤及大量肺泡上皮和血管内皮细胞凋亡正是导致肺损伤(lung injury, LI)的关键。由此我们猜想, CO有可能通过上述机制对LI起保护作用。通过静脉注入LPS(5 mg/kg体重)诱导大鼠LI, 观察吸入室内空气或 $2.5 \times 10^{-4} (V/V)$ CO 3 h 后, 肺氧化酶学、炎症细胞因子、细胞凋亡、HO-1表达及组织形态学变化。结果显示, 静脉注入LPS诱导LI后, CO吸入组大鼠肺肿胀因子$\alpha$ (tumor necrosis factor-$\alpha$, TNF-$\alpha$)、白细胞介素6 (interlukin-6, IL-6)、丙二醛(maleic dialdehyde, MDA)、髓过氧化物酶(myeloperoxidase, MPO)和细胞凋亡分别如下：(0.91±0.25) pg/mg蛋白、(0.64±0.05) pg/mg蛋白、(1.02±0.23) nmol/mg蛋白、(7.18±1.62) U/mg蛋白、(1.60±0.34) %, 均显著低于LI组的(1.48±0.23) pg/mg蛋白、(1.16±0.26) pg/mg蛋白、(1.27±0.33) nmol/mg蛋白、(8.16±1.49) U/mg蛋白、(3.18±0.51) %, P<0.05。CO吸入组HO-1、白细胞介素10 (interlukin-10, IL-10)表达及超氧化物歧化酶(superoxide dismutase, SOD)活性分别为(5.43±0.92), (0.26±0.07) pg/mg蛋白、(60.09±10.21) U/mg蛋白, 它们均显著高于LI组的(3.08±0.82), (0.15±0.03) pg/mg蛋白、(50.98±6.88) U/mg蛋白。与LI组相比, CO吸入组肺损伤减轻。研究结果表明, 低浓度CO吸入通过抗氧化、抗炎症、抑制细胞凋亡、上调HO-1表达而减轻LPS诱导的肺损伤。

关键词: 一氧化碳; 血红素氧合酶; 肺损伤; 氧化应激; 炎症; 凋亡

中图分类号: R683.42

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Sepsis-related lung injury (LI) is a major cause of morbidity and mortality in intensive care units. However, the precise mechanism of LI induced by lipopolysaccharide (LPS) remains vague and requires further elucidation. Previous studies suggest that disorders of oxidant/anti-oxidant and inflammation/anti-inflammation play an important role in the development of LPS-induced LI in animals[1]. Recent observations also demonstrate that excessive pulmonary epithelium cell apoptosis is involved in the pathogenesis of LPS-related LI[2]. LI is characterized by increase of oxygen free radicals, inflammatory cytokines, endothelial and alveolar epithelium cell apoptosis, which caused lung edema, hemorrhage and inflammatory cell sequestration. In response to these challenges, lung cells synthesize specific anti-oxidative enzymes and proteins such as heme oxygenase (HO)-1 and superoxide dismutase (SOD), anti-inflammatory cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10), anti-apoptotic molecules such as Bcl-2 and isozyme of alkaline phosphatase (IAP). It has been shown that reduction of oxygen free radicals and pro-inflammatory cytokines, suppression of cell apoptosis, increase of anti-oxidative enzymes and anti-inflammatory cytokines could attenuate LI[3-5]. Carbon monoxide (CO), a metabolite of heme catalysis by HO, was reported to confer lung protection against O₂ toxicity in rats continuously exposed to 2.5×10⁻⁴ (V/V) CO with balanced air [O₂ concentration 0.21 (V/V)] was compressed (Nanjing Special Gas Co., Nanjing, China). A 36-liter plexiglas chamber was pre-infused with 9 ml CO, and the CO concentration in this chamber was kept 2.5×10⁻⁴ (V/V) while the balanced 2.5×10⁻⁴ (V/V) CO (flow rate kept at 2 L/min) was continuously infused into the chamber. CO analyzer was used to measure CO levels continuously in the chamber. Gas samples were introduced to the analyzer through a port in the top of the chamber at a rate of 1 L/min and were analyzed by electrochemical detection. Concentration was measured every hour and there was no fluctuation in the CO concentration after the chamber had equilibrated (approximately 10 min).

1.2 CO exposure
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1.3 Arterial blood gas analysis and COHb measurement
Arterial blood samples (0.5 ml) were collected and heparinized at the observation time point after LPS injection or CO inhalation. COHb, partial pressure of arterial oxygen (Pao₂) and saturation of arterial oxygen (SaO₂) were measured with blood gas analyzer (Roche OMNI S6, USA).

1.4 HO-1 mRNA expression
HO-1 mRNA from pulmonary lobe was detected by semiquantitative reverse transcription-polymerase chain reaction (SqtRT-PCR) in the Molecular Medicine Institution of Nanjing Medical University. All detection was performed in accordance with the manufacturer’s instruction described by Lang et al[9]. RNase digestion was performed prior to transcription to cDNA, which was synthesized following the addition of 5 µmol/L random primers, 1 mmol/L dNTPs, and incubation at 37 °C with Moloney rat leukemia virus reverse transcriptase. Contamination with DNA was excluded by performing PCR from templates incubated without reverse transcriptase. The primers used for PCR amplification were 5’-ATGGATGATGATATCGCC-GCG-3’ (β-actin, 240 bp), as well as 5’-AAGATGTGCCC-AGAAAGCCCCTGGAC-3’ (HO-1, 395 bp). The PCR

1 MATERIALS AND METHODS

1.1 Animals and experimental design
Twenty-four adult healthy male Sprague-Dawley rats were purchased from Animal Experimental Centre of Nanjing Medical University, weighing 200–250 g. Rats acclimatized animal room for a week, and were given routine drink and diet. Rats were randomly divided into LI group, CO inhalation alone group, LPS injection + CO inhalation group and the control group. All rats were anaesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection, and then LPS or normal saline was administered through tail vein. The rats in LI group were placed in room air after an equal volume of saline injection. The rats in LPS injection + CO inhalation group were intravenously injected LPS (5 mg/kg) and exposed to 2.5×10⁻⁴ (V/V) CO continuously, and the rats in the control group were intravenously injected an equal volume of saline and exposed to room air. After 3-hour observation, all rats were abdominal aorta exsanguinated immediately under pentobarbital (20 mg/kg, intraperitoneal injection) anesthesia. Blood from abdominal aorta was collected for blood gas analysis and carboxyhemoglobin (COHb) measurement. Lung lobes were cleaned with 4°C normal saline to clear blood. The harvested tissues were put into liquid nitrogen immediately for following examination.

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reaction mixture (40 µl) contained 2 mmol/L MgCl₂, 0.2 mmol/L dNTP, 1 µmol/L primer and 1 U Taq DNA polymerase. Samples were amplified by means of 32 cycles of 3 min denaturation at 94 ºC, 1 min annealing at 54 ºC (HO-1) or 55 ºC (β-actin) and 1 min elongation in a Perli thermal cycles. The correlation between the expression level of β-actin and HO-1 was analyzed by SqRT-PCR. Signal intensity from SqRT-PCR was quantified by density scanning (Tiger 920G, USA) and analyzed. The quantitation of HO-1 mRNA expression was compared between groups respectively.

1.5 Cytokine measurements
Concentrations of the cytokines, tumor necrosis factor-α (TNF-α), IL-6 and IL-10, were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Jingmei Biochemistry Co., Shanghai, China). One pulmonary lobe was collected and washed in normal saline, and homogenized immediately on ice in 9 ml normal saline. The homogenates were centrifuged at 3 000 g at 4 ºC for 10 min. The homogenates were centrifuged at 3 000 g at 4 ºC for 10 min. The levels of TNF-α, IL-6 and IL-10 in supernatant were measured in the Molecular Medicine Institution of Nanjing Medicinal University. Protein content in the sample was determined by Comassie blue assay. Results were corrected per microgram of protein.

1.6 Maleic dialdehyde (MDA) content, myeloperoxidase (MPO) and SOD activity determination
Pulmonary MDA content, MPO and SOD activity were determined with chemical method described as the manufacturer’s instructions (Nanjing Jiancheng Biochemistry Co., Nanjing, China). Lung tissue was washed in normal saline, and homogenized immediately on ice in 9 ml icy normal saline. The tissue homogenate was centrifuged at 3 000 g at 4 ºC for 10 min. The MDA content, MPO and SOD activity in supernatant were measured with the corresponding kits. Light absorbance at 490 and 620 nm was read and compared with that of the standards. Protein content in the sample was determined by Comassie blue assay. Results were corrected per microgram of protein.

1.7 Cell apoptosis observation
Pulmonary cell apoptosis rate was observed with flow cytometer in the Cell Detection Center of Nanjing Medical University. Lung cell apoptosis was determined either by propidium iodide (PI, BD PharmMingen Co., USA) nuclei staining or by flow cytometry which detects annexin V (BD PharmMingen Co., USA) binding described as Lang et al.⁹. Single cell suspension (0.5 ml) was fixed for 30 min with 70% alcohol at 4 ºC. Centrifuged at 1 500 g for 4 min, the supernatant containing cleaved chromation was digested with RNase 0.2 ml (50 pg/ml) and cultured for 30 min at 37 ºC. Cells were washed with phosphate buffered saline, and received 1 ml PI (50 mg/L) for staining. PI and annexin V binding was detected to identify cell apoptosis. 10 000 events of cell flowing through cytometer were applied to count pulmonary cell apoptosis rate. The apoptotic extent was quantified and analyzed by ModFit 2.3 program.

1.8 Histological photograph
Right lower pulmonary lobe was harvested and flushed with normal saline, and then was fixed with 10% formalin for 24 h. Embedded in paraffin, sections of 6 µm were stained with hematoxylin and eosin (HE staining) for light microscope observation. The pathological observation procedures were completed in the Department of Pathology, People’s Hospital of Jiangsu Province.

1.9 LI Evaluation
LI was scored as described previously[7]. Lung tissue was fixed in 10% formalin and sections were stained with HE. The injury was scored with a semi-quantitative grading system based on the structure changes including edema, alveolar and interstitial hemorrhage, and inflammatory cell sequestration. Alveolar edema was an index of water penetrating into alveolar space. Alveolar and interstitial hemorrhage was an index of erythrocyte infiltrating into alveolar space and interstitial. Inflammatory cell sequestration was the potent index of inflammatory cells aggregating into alveolar space and interstitial. Semi-quantitative grading system was a 0- to 4-point scale calculating system: 0=no injury; 1=25% injury in a light microscope field; 2=50% injury in a light microscope field; 3=75% injury in a light microscope field; 4=almost 100% injury in a light microscope field. Ten light microscope fields were analyzed for a pathological section and 12 sections were calculated for a specific specimen to determine rat LI score. Injury score reflects the extent and severity of LI. A percentage of tissue area [% tissue area=(cellular area/total area)×100] was used to express LI degree. The results were statistically compared.

1.10 Statistical analysis
Results were presented as mean±SD and analyzed with SPSS 11.0 statistic software. Differences were assessed with one way analysis of variance followed by the LSD test. Statistical significance was accepted at P<0.05.

2 RESULTS
2.1 CO inhalation increased COHb, but did not af-
fect arterial oxygenation

Although CO is easy to combine with hemoglobin and may affect arterial oxygenation, there was no report about measurable effect at the dose used in these studies on arterial blood gas analysis. Compared with the control group and LI group, PaO₂ and SaO₂ of rats in both CO inhalation alone group and LPS injection + CO inhalation group did not decrease. However, the arterial COHb levels in LI group and LPS injection + CO inhalation group significantly increased. COHb in CO inhalation alone group also increased significantly, and there was a significant difference compared with that in LI group, while there was no difference between LPS injection + CO inhalation group and CO inhalation alone group (Table 1).

2.2 CO inhalation up-regulated HO-1 mRNA expression

RT-PCR analysis showed that HO-1 mRNA expression in lung tissue was up-regulated markedly in LI group, and semi-quantitative analysis showed an increase of 3.08-fold and 2.93-fold compared with that in the control group and CO inhalation alone group. When LPS was administered in the presence of CO inhalation, HO-1 mRNA expression was further up-regulated compared with that in LI group. Semi-quantitative analysis of HO-1 mRNA showed a 1.76-fold increase compared with that in LI group. CO inhalation alone did not affect HO-1 mRNA expression.

2.3 CO inhalation decreased TNF-α, IL-6 expressions and increased IL-10 expression

LPS-induced expressions of numerous pro-inflammatory cytokines including TNF-α and IL-6 were observed in lung tissues after LPS challenge. TNF-α and IL-6 contents in lung tissues in LI group were 3.15-fold and 2.37-fold compared with those in the control group and CO inhalation alone group. However, CO inhalation alone did not affect HO-1 mRNA expression (Fig.1, Table 2).
fold increased respectively, and the contents were significantly higher than that in the control group. TNF-α and IL-6 contribute to LI. After CO inhalation, TNF-α and IL-6 expressions in LI rat lung tissues reduced significantly by 39.5% and 44.8%, respectively, compared with that in LI group. IL-10 is an inhibitor of pro-inflammatory cytokine synthesis and can limit the inflammatory process including TNF-α and IL-6 production. Therefore, the decrease in LPS-induced production of IL-10 might be responsible for LI, and rats with LI treated with CO inhalation might ameliorate lung damage. As expected, IL-10 expression after LPS administration decreased and was 0.64-fold lower than that in the control group. The decrease of IL-10 expression resumed in those rats with LI in the presence of CO inhalation. Compared with LI group, CO inhalation increased IL-10 level by 1.73-fold in lung tissues. CO inhalation alone did not decrease TNF-α and IL-6 expressions or increase IL-10 expression (Table 2).

2.4 CO inhalation reduced MDA production and MPO activity, but increased SOD activity

We assessed the effect of CO inhalation on oxidative stress. MDA level and MPO activity in the rat lung were measured. LPS challenge resulted in a significant increase of MDA level and MPO activity (1.61-fold and 1.36-fold higher than that in the control group, respectively). CO inhalation significantly reduced MDA and MPO accumulation in lung (decreased by 19.7% and 12.1%, respectively), which indicated that CO reduced oxygen free radicals and polymorphonuclear neutrophils (PMN) to get into lungs. LPS injection led to a marked decrease in SOD activity (decreased by 20.4% compared with that in the control group). CO inhalation increased SOD activity by 19.8% compared with that in LI group. However, CO inhalation alone group neither decreased MDA production and MPO activity nor increased SOD activity (Table 2).

2.5 CO inhalation suppressed cell apoptosis

LPS challenge led to a significant increase in lung cell apoptosis. Quantitative analysis showed a 26.5-fold increase compared with that in the control group (Fig.2C, Table 2). However, CO inhalation resulted in a marked decrease in rat cell apoptosis. Quantitative analysis showed there was a decrease of 49.7% in LPS injection + CO inhalation group (Fig.2D, Table 2) compared with that in LI group. Cell apoptosis wasn’t affected in CO inhalation alone group (Fig.2B, Table 2).

2.6 CO inhalation ameliorated LPS-induced lung damage

With LPS injection, morphological study clearly revealed that the epithelial and endothelial cell structure was severely destructed. Lung edema, alveolar hemorrhage and inflammatory cell sequestration appeared in the lung in 3 h (Fig. 3C). In LPS injection + CO inhalation group it was dem-
onstrated that lung edema, hemorrhage and inflammatory cell infiltration changed slightly (Fig.3D). In CO inhalation alone group there was no any changes in lung tissue structures (Fig.3B).

2.7 CO inhalation decreased injury score

LI score markedly increased after LPS injection. There was an increase of 14.4-fold compared with that in the control group. CO inhalation reduced injury score significantly and there was a decrease of 49.7% compared with that in LI group. However, CO inhalation alone did not affect score of normal rats (Table 2).

3 DISCUSSION

In our study, the measurements of MDA content, MPO activity and TNF-α, IL-6 levels showed that exogenous CO reduced oxidative stress and decreased pro-inflammatory cytokine expression. The measurements of apoptosis index showed that CO inhalation suppressed pulmonary cell apoptosis of LPS-induced LI rats. Evaluated by pathology and injury scores, CO inhalation attenuated damage induced by LPS. Furthermore, CO inhalation also increased IL-10 expression and SOD activity in LPS-induced LI rats. Additionally, the HO-1 expression was up-regulated in response to LPS, and CO inhalation up-regulated HO-1 expression more. However, these effects were not observed in CO inhalation alone group.

The precise mechanisms by which CO mediates protection is still not clear. Our observation has demonstrated several mechanisms that CO attenuated LPS-induced LI. One important mechanism is that CO protects lung from LPS-induced injury through down-regulating the end products of fatty acid peroxidation MDA and inhibiting MPO activity. LPS induced an increased oxidative burden, leading to the damage of lipids, proteins, DNA and inflammatory cytokine production including TNF-α, IL-6, PAF and ICAM-1 with subsequent aggravate damage[8]. A low concentration of CO provides protection from LPS-induced injury via directly inhibiting lipid peroxidation and decreasing reactive oxygen species. The other important mechanism is that the protective effects of CO against LPS-induced injury is mediated by reducing pro-inflammatory mediators (TNF-α and IL-6) and subsequent tissue injury, while up-regulating anti-inflammatory cytokine IL-10[10].

Otterbein et al.[8] has demonstrated that the administration of exogenous CO inhibited LPS-induced TNF-α expression while increased IL-10 production both in vitro and in vivo. Morse et al.[11] described serum IL-6 and IL-1β induced by LPS was markedly attenuated by exogenous CO administration. They postulated that CO had a direct action on cytokine production, and this effect was mediated via the transcriptional factor AP-1 at transcriptional level.

Furthermore, the suppression of cell apoptosis by CO inhalation may represent an additional mechanism by which CO provides protection against LPS-induced injury[7,14-16]. Although the precise physiological mechanism of anti-apoptosis by CO has not yet been established, it is possible that this effect of CO may be related to its powerful antioxidant and anti-inflammatory functions. CO might limit the generation of reactive oxygen species, lower the presence of free metal ions[2], and down-regulate pro-inflammatory cytokines[17]. Additionally, recent evidences suggested that CO-treated animals showed an early up-regulation of anti-apoptotic gene Bcl-2 and down-regulation of pro-apoptotic genes (e.g. Fox and Bax)[12,18]. Up-regulation of anti-apoptotic gene and down-regulation of pro-apoptotic genes can suppress apoptosis of pulmonary cells.

Our study showed that pulmonary cell apoptosis in LI rats decreased after CO inhalation. Cell apoptosis serves as a useful marker of LI in response to oxidative stress and inflammation such as acute LI and ischemia/reperfusion injury. The anti-apoptotic effects of CO may be ascribed to its anti-oxidation and anti-inflammation. Therefore, this pathway may be an important healing strategy for the prevention of LPS-induced injury.

To evaluate whether the protective roles of CO on LI are related with HO-1, we examined the expression of HO-1. Several previous studies reported that HO-1 could be up-regulated by oxidative stress, inflammatory cytokines and LPS[6,10]. We supposed that CO might increase HO-1 expression by which it protects lung against LPS-induced injury via influencing oxidative stress and inflammatory reaction. As we expected and Moore et al.[19] described, LPS induced up-regulation of HO-1 expression and CO further amplified HO-1 expression. HO-1 protects cell partly because free heme is toxic. HO-1 degrades heme to bilivertion, iron and CO, and these byproducts were believed to be the effective molecules with the potent cytoprotection, which was observed in the heme oxygenase system[4,6].

It is well known that exposure to high concentration of CO is lethal. Against this paradigm of CO toxicity, recent data clearly show that low concentration of CO can be a regulatory gas molecule in cellular signal transduction and biological processes. The CO concentration used for the current study is less than one-tenth of the dose of CO administered to human during measuring CO diffusion in pulmonary function. CO can combine with heme easily to
form COHb, and therefore affects arterial oxygenation. Excessive COHb impairs lung oxygenation because of hypoxia, therefore we measured PaO2 and SaO2. Surprisingly, no significant differences were found between the rats exposed to room air, LPS and $2.5 \times 10^{-4}$ ($v/v$) CO, which suggested that CO inhalation did not cause hypoxia-induced LI.

In conclusion, inhalation of CO at a low concentration powerfully protects lung from LPS-induced injury by antioxidant, anti-inflammatory, anti-apoptosis and up-regulation of HO-1 expression in LPS-induced LI rats.

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