Tristetraprolin Mediates Anti-Inflammatory Effects of Carbon Monoxide on Lipopolysaccharide-Induced Acute Lung Injury

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Low-dose inhaled carbon monoxide is reported to suppress inflammatory responses and exhibit a therapeutic effect in models of lipopolysaccharide (LPS)-induced acute lung injury (ALI). However, the precise mechanism by which carbon monoxide confers protection against ALI is not clear. Tristetraprolin (TTP; official name ZFP36) exerts anti-inflammatory effects by enhancing decay of proinflammatory cytokine mRNAs. With the use of TTP knockout mice, we demonstrate here that the protection by carbon monoxide against LPS-induced ALI is mediated by TTP. Inhalation of carbon monoxide substantially increased the pulmonary expression of TTP. carbon monoxide markedly enhanced the decay of mRNA-encoding inflammatory cytokines, blocked the expression of inflammatory cytokines, and decreased tissue damage in LPS-treated lung tissue. Moreover, knockout of TTP abrogated the anti-inflammatory and tissue-protective effects of carbon monoxide in LPS-induced ALI. These results suggest that carbon monoxide-induced TTP mediates the protective effect of carbon monoxide against LPS-induced ALI by enhancing the decay of mRNA encoding proinflammatory cytokines. (Am J Pathol 2015, 185: 2867–2874; http://dx.doi.org/10.1016/j.ajpath.2015.07.002)

Acute lung injury (ALI) is a clinical problem induced by acute and excessive pulmonary inflammation and continues to cause high rates of morbidity and mortality despite modern clinical practices in critical care medicine. Therefore, there is an urgent need to develop effective treatment for ALI. Multiple studies have found that inhalation of low concentrations of carbon monoxide confers tissue protection in animal models of lung diseases, including those of oxidative and inflammatory lung injury and ischemia/reperfusion injury. Carbon monoxide exposure induces alterations in local production of cytokines, including reduced production of tumor necrosis factor (TNF)-α, IL-6, and IL-1β, increased production of IL-10, and reduced fibrosis. The anti-inflammatory effects of carbon monoxide involve the modulation of several signaling pathways, including the p38 mitogen-activated protein kinase (MAPK) and peroxisome proliferating-activated receptor-γ pathways.

However, the precise mechanism by which carbon monoxide confers protection against ALI is not clear. The inflammatory response is reported to be modulated by post-transcriptional control. The post-transcriptional control of inflammatory transcripts depends on AU-rich element-mediated mechanisms. The destabilizing function of AU-rich elements is believed to be regulated by AU-rich element-binding proteins. Tristetraprolin (TTP; official name ZFP36) is an AU-rich element-binding protein that promotes degradation of a number of inflammatory transcripts.
mediators, including TNF-α, granulocyte-macrophage colony-stimulating factor, IL-2, IL-3, IL-6, CCL-2, CCL-3, inducible nitric oxide synthase, and cyclooxygenase 2. TTP knockout mice develop severe inflammatory arthritis, autoimmune dysfunction, and myeloid hyperplasia, indicating the importance of TTP in limiting inflammatory responses.

We previously found that carbon monoxide induces the expression of TTP (official name ZFP36) in macrophages and suppresses the expression of inflammatory cytokines induced by lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. Given that LPS is considered to be a key molecule that induces acute inflammatory responses, resulting in development of ALI, we hypothesized that TTP mediates carbon monoxide’s protective role in ALI. Here, we evaluated the role of TTP in mediating the anti-inflammatory properties of carbon monoxide in a mouse LPS-induced ALI model. We show that carbon monoxide enhanced the decay of inflammatory cytokine mRNAs induced by LPS in the lung and protected the mice from acute pulmonary inflammation. Carbon monoxide failed to exert its anti-inflammatory effects in the absence of TTP expression, demonstrating a critical role for TTP in the anti-inflammatory activity of carbon monoxide.

Materials and Methods

Mice and Animal Research Ethics

TTP knockout mice were kindly provided by Dr. Perry J. Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Mice were bred in the animal facility at the University of Ulsan and were born and housed in the same room under specific pathogen-free conditions. In all experiments, sex- and age-matched littermates were used as controls. All mice were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Ulsan. All animal procedures were approved by the Institutional Animal Care and Use Committee of Immuno-modulation Research Center (permit number HTC-14-030). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

LPS-Induced ALI and Carbon Monoxide Exposure

Mice were exposed to carbon monoxide at a concentration of 250 ppm in an exposure chamber (LB Science, Daejeon, Republic of Korea) at room temperature for 4 hours per day for 5 days. ALI was induced by intranasal administration of LPS (2.5 mg/kg body weight; Sigma-Aldrich, St. Louis, MO). The mice were divided into three groups: a sham group, an LPS-induced lung injury group, and an LPS-induced lung injury group with carbon monoxide inhalation. Twenty-four hours after LPS, bronchoalveolar lavage (BAL) fluid was collected by flushing the lung with 1 mL of phosphate-buffered saline (Figure 1A). Protein concentrations in BAL fluid were determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL), and the number of total cells in BAL fluid was determined with a hemocytometer. BAL cells were also stained with fluorochrome-conjugated antibodies against Siglec-F, Gr-1, CD4, CD8, B220, F4/80, CD11b, or Ly6G (BD PharMin, San Diego, CA). Flow cytometric analysis was performed with a FACSCanto II (BD Biosciences, San Jose, CA).

Determination of Inflammatory Cytokine Levels

The concentrations of IL-1β, TNF-α, and IL-6 in BAL fluid and lung were determined with DuoSet ELISA (enzyme-linked immunosorbent assay) Development Systems kits (R&D Systems, Minneapolis, MN). ELISA results were normalized with the use of a standard curve. Dissected left lungs were homogenized in phosphate-buffered saline and centrifuged to obtain supernatant fluids for ELISA. Total protein concentrations in the lung tissue homogenates were determined with bicinchoninic acid protein assay kit (Pierce Biotechnology).

Evaluation of Lung Injury Severity

Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-mm thick sections. Lung injury severity was scored by inspection of hematoxylin and eosin-stained whole lung sections, as previously described. Briefly, two
separate investigators (J.W.Y., J.H.L.) evaluated and scored the lung injury severity in a blinded manner on the basis of four aspects, namely alveolar capillary congestion, hemorrhage, infiltration or aggregation of neutrophils in the air space or the vessel wall, and thickness of the alveolar wall/hyaline membrane formation. Each of the four components was categorized from 0 to 4, whereby a higher number is more severe. These scores were added to generate a clinical score that ranged from 0 to 16. The average sum of each field score was compared among groups.

**Actinomycin D—Based RNA Kinetic Analysis**

BAL cells were collected from mice exposed to carbon monoxide and LPS as described in *LPS-Induced ALI and Carbon Monoxide Exposure*. Cells were incubated with 5 μg/mL actinomycin D (Sigma-Aldrich) to stop transcription, and cells were collected at 0, 15, 30, 45, 60, and 120 minutes after addition of actinomycin D and analyzed for TNF-α and IL-1β mRNA content by quantitative RT-PCR.

**Quantitative Real-Time RT-PCR**

Total RNA was extracted from cells and the accessory lobe of lung of WT and TTP knockout mice with the use of the RNeasy Mini Kit (Qiagen, Hilden, Germany). For RNA kinetic analysis, 3 μg of DNase I-treated total RNA was reverse transcribed with oligo-dT and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RT-qPCR was performed by monitoring the increase in fluorescence in real time of SYBR Green dye (Qiagen) with the use of StepOnePlus Real-time PCR kits (Applied Biosystems, Foster City, CA) with the use of the following PCR primer pairs: glyceraldehyde-3-phosphate dehydrogenase, forward, 5'-ATGACAACTTTGGCATTGTG-3' and reverse, 5'-CATACTGGCAGGTTCCTCC-3'; IL-1β, forward, 5'-TTGTGCGAAAAGAAGTGCA-3' and reverse, 5'-TACAAACACCAGCCTCCACA-3'; IL-6, forward, 5'-ACAATTCACTTACCAG-3' and reverse, 5'-TGGCCATTGCAACATCTTTTC-3'; and TNF-α, forward, 5'-ACTGGCCG-3' and reverse, 5'-GACTTTCTCTGGAATAAGGTT-3'.

**SDS-PAGE Analysis and Immunoblotting**

Proteins were resolved by SDS-PAGE, transferred onto Hybond-P membranes (Amersham Biosciences Inc., Piscataway, NJ), and probed with appropriate dilutions of the following antibodies: rabbit anti-mouse TTP (T5327; Sigma-Aldrich) and anti-β-actin (A2228; Sigma-Aldrich).
Immunoreactivity was detected with the electro-chemiluminescence detection system (Amersham Biosciences Inc.). Films were exposed at multiple time points to ensure that the images were not saturated.

Statistical Analysis

For statistical comparisons, *P* values were determined with the Student’s *t*-test.

Results

Carbon Monoxide Increases TTP Level in the Lung Tissues

To test whether the anti-inflammatory function of TTP is required for carbon monoxide’s protective effect against LPS-induced lung injury, we first determined whether carbon monoxide increases the level of TTP in lung tissues. The lung tissues of wild-type mice exposed to either carbon monoxide or LPS showed slight increases in TTP protein and mRNA levels (Figure 1, B and C). However, mice exposed to both carbon monoxide and LPS showed dramatic increases in TTP levels (Figure 1, B and C). These results suggest that carbon monoxide and LPS synergistically increase TTP level in lung tissues of mice.

*TTP* Deficiency Abrogates the Protective Effects of Carbon Monoxide against LPS-Induced Lung Injury

Next, we analyzed the role of TTP in LPS-induced lung injury with the use of *TTP*−/− mice. Under normal conditions, BAL cell number and BAL protein concentrations were increased 1.7-fold and 1.3-fold, respectively, in *TTP*−/− mice, although these levels were not statistically different from those in wild-type mice (Figure 2, A and B). These results indicate that *TTP*−/− mice do not exhibit severe preexisting lung tissue damage. However, after LPS treatment, both BAL cell number and BAL protein concentrations were increased 3.5-fold and 3.7-fold, respectively, in *TTP*−/− mice compared with those in wild-type mice (Figure 2, A and B). The most abundant cells in BAL after LPS treatment were neutrophils in both wild-type (85%) and *TTP*+/+ (91%) mice (Figure 2, C and D). These results indicate that TTP plays a role in protecting mice from LPS-induced lung injury. We next determined whether TTP deficiency affects the protective effect of carbon monoxide against LPS-induced lung injury. Although carbon monoxide exerted protective effects in wild-type mice and decreased the BAL protein concentration and the numbers of BAL cells, this protection was absent in *TTP*−/− mice (Figure 2, A and B). In addition, we found that, although carbon monoxide decreased the percentage of BAL neutrophils to 40% in wild-type mice, it had no effect in *TTP*−/− mice (Figure 2, C and D). Lung histology as determined by hematoxylin and eosin staining of lung sections also yielded similar results. Under normal conditions, the lung histology of *TTP*−/− mice was comparable with that of wild-type mice (Figure 2E). However, LPS administration to *TTP*−/− mice led to much more severe lung damage, reflected by thickening of alveolar septae and cellular infiltration, than was seen in wild-type mice (Figure 2E). In addition, although the administration of carbon monoxide substantially reduced LPS-induced lung damage in wild-type mice, it did not protect *TTP*−/− mice from LPS-induced lung damage at all (Figure 2E). Severity of lung injury was also scored with the use of a semiquantitative histopathology score system, which evaluates lung injury for four aspects: alveolar capillary congestion, hemorrhage, infiltration, or aggregation of neutrophils in the alveoli, and thickness of the alveolar wall/hyaline membrane formation. Although the treatment with carbon monoxide significantly reduced LPS-induced lung injury scores in wild-type mice, it had no effect in *TTP*−/− mice (Figure 2F). These results indicated that TTP is required for the protective effect of carbon monoxide against LPS-induced lung injury.

Inhibitory Effect of Carbon Monoxide against LPS-Induced Lung Inflammation Depends on TTP

Lung inflammation was reported to be a key factor in LPS-induced lung injury.30–33 We also observed that LPS treatment strongly increased the expression of the inflammatory cytokines, TNF-α, IL-1β, and IL-6, in BAL fluids and lung tissues of wild-type mice, which were further increased in *TTP*−/− mice, relative to wild-type mice (Figure 3). These results indicate that TTP plays an inhibitory role in LPS-induced lung inflammation. Carbon monoxide administration was reported to suppress LPS-induced lung inflammation.11,35 Consistently, we found that carbon monoxide application significantly reduced LPS-induced inflammatory cytokine levels in BAL fluids and lung tissues of wild-type mice (Figure 3). To further evaluate the role of TTP in the anti-inflammatory effects of carbon monoxide, we measured the levels of LPS-induced inflammatory cytokines in BAL fluid and lung tissues of *TTP*−/− mice exposed to carbon monoxide. In *TTP*−/− mice, administration of carbon monoxide did not reduce LPS-induced inflammatory cytokine levels at all (Figure 3). Of particular note, although carbon monoxide decreased both the protein (Figure 3, A–F) and mRNA (Figure 3, G–I) levels of inflammatory cytokines in wild-type mice, it had no effect in *TTP*−/− mice. These findings suggest that the ability of carbon monoxide to block LPS-induced lung inflammation is TTP dependent.

Carbon Monoxide Enhances Degradation of Inflammatory Cytokine mRNAs in a TTP-Dependent Manner

The requirement of TTP for the anti-inflammatory effects of carbon monoxide led us to test whether these results reflected a function of TTP to enhance degradation of inflammatory cytokine mRNAs. We first analyzed the ability of carbon monoxide to enhance degradation of LPS-induced inflammatory cytokine mRNAs in BAL cells of wild-type mice by calculating the half-lives of these mRNAs. Although the
Half-lives of TNF-α and IL-1β were 68 minutes and 64 minutes, respectively, after actinomycin D administration to BAL cells collected from wild-type mice treated with LPS alone (Figure 4, A and B), in BAL cells from wild-type mice treated with both carbon monoxide and LPS, these half-lives were reduced to 26 and 25 minutes, respectively (Figure 4, A and B). These results indicate that carbon monoxide enhances the decay of LPS-induced inflammatory cytokine mRNAs in lung tissues. However, in BAL cell from TTP−/− mice, the half-lives of TNF-α and IL-1β were >2 hours after actinomycin D and carbon monoxide did not enhance the decay of LPS-induced inflammatory cytokine mRNAs (Figure 4, A and B). Because alterations in BAL reflect changes in the lung parenchyma, these results indicate that Ttp-mediated destabilization of inflammatory cytokine mRNAs contributes to carbon monoxide’s anti-inflammatory function in LPS-induced lung inflammation.

**Discussion**

Despite several reports that indicated the tissue-protective function of carbon monoxide in animal models of ALI, the precise mechanism by which carbon monoxide confers...
In the present study, we found in the present study that carbon monoxide treatment resulted in a decrease in mRNA and protein expression of the LPS-induced inflammatory cytokines TNF-α, IL-1β, and IL-6. Our results clearly suggest that carbon monoxide down-regulates LPS-induced inflammatory cytokines at the transcript level. In addition, we found that, although carbon monoxide protected wild-type mice from LPS-induced lung inflammation, this was no longer the case in TTP−/− mice, indicating that TTP is a key mediator for carbon monoxide’s inhibitory effect on LPS-induced lung inflammation. Because TTP exerts its anti-inflammatory roles by enhancing the degradation of inflammatory cytokine mRNAs, we speculated that carbon monoxide treatment likewise enhances the degradation of mRNAs of inflammatory cytokines. Indeed, carbon monoxide enhanced the degradation of LPS-induced inflammatory cytokine mRNAs in wild-type mice, but TTP deficiency completely abrogated the carbon monoxide effect on these mRNAs. Our results suggest that carbon monoxide exerts its inhibitory effect on LPS-induced inflammation by enhancing the degradation of LPS-induced inflammatory cytokine mRNAs in a TTP-dependent manner.

It was reported that LPS treatment induces the expression of TTP. Even in the absence of carbon monoxide, LPS-induced TTP seems to exert an inhibitory function against LPS-induced lung inflammation, because TTP deficiency aggravates LPS-induced lung inflammation. However, TTP induced by LPS could not protect mice from LPS-induced lung injury. When TTP levels were substantially enhanced by carbon monoxide treatment, mice were protected from LPS-induced lung injury. Our results raise the question of how carbon monoxide elevates TTP levels in lung tissues. Although we did not determine the precise mechanism for the induction of TTP by carbon monoxide in lung tissues, we did find that carbon monoxide up-regulates TTP mRNA levels. Importantly, carbon monoxide alone induced a moderate increase in TTP level, but combined treatment with carbon monoxide, and LPS was required for strong increase of TTP in lung tissues, indicating that carbon monoxide and LPS synergistically increase TTP level in lung tissue. p38 MAPK is required for TTP induction by LPS, and the anti-inflammatory effects of carbon monoxide are modulated by p38 MAPK, suggesting the possibility that carbon monoxide increases TTP level synergistically with LPS via the p38 MAPK pathway. Carbon monoxide was reported to exert a protective function by enhancing STAT3 activation via a p38 MAPK-dependent pathway. Previously, we reported that STAT3 induces TTP transcription. Combining these findings, it may be hypothesized that the carbon monoxide/p38 MAPK/STAT3 pathway enhances TTP transcription. Further studies will be required to clarify the mechanisms involved in increase of TTP by carbon monoxide.

**Conclusion**

In summary, our study indicates that TTP has an inhibitory function against LPS-induced lung inflammation. Although
TTP levels are elevated by LPS alone, they are insufficient to protect mice from LPS-induced lung injury. Carbon monoxide treatment substantially enhances TTP levels in lung tissues of LPS-treated mice, and the carbon monoxide–carbon monoxide treatment substantially enhances TTP levels in lung tissues of LPS-treated mice, and the carbon monoxide–

**Acknowledgment**

*TTP* knockout mice were kindly provided by Dr. Perry J. Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, NC).

**References**


