Carbon monoxide inhibits IL-17-induced IL-6 production through the MAPK pathway in human pulmonary epithelial cells

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Carbon monoxide inhibits IL-17-induced IL-6 production through the MAPK pathway in human pulmonary epithelial cells. Am J Physiol Lung Cell Mol Physiol 289: L268–L273, 2005; doi:10.1152/ajplung.00168.2004.—Interleukin (IL)-17 is a proinflammatory cytokine that is produced by activated memory CD4 T cells, which regulates pulmonary neutrophil emigration by the induction of CXC chemokines and cytokines. IL-17 constitutes a potential target for pharmacotherapy against exaggerated neutrophil recruitment in airway diseases. As a cytoprotective and anti-inflammatory gaseous molecule, carbon monoxide (CO) may also regulate IL-17-induced inflammatory responses in pulmonary cells. Herein, we examine the production of cytokine IL-6 induced by IL-17 and the effect of CO on IL-17-induced IL-6 production in human pulmonary epithelial cell A549. We first show that IL-17 can induce A549 cells to release IL-6 and that CO can markedly inhibit IL-17-induced IL-6 production. IL-17 activated the ERK1/2 MAPK pathway but did not affect p38 and JNK MAPK pathways. CO exposure selectively attenuated IL-17-induced ERK1/ERK2 MAPK activation without significantly affecting either JNK or p38 MAPK activation. Furthermore, in the presence of U0126 and PD-98059, selective inhibitors of MEK1/2, IL-17-induced IL-6 production was significantly attenuated. We conclude that CO inhibits IL-17-stimulated inflammatory response via the ERK1/2-dependent pathway.

interleukin-17; interleukin-6; inflammation; mitogen-activated protein kinase

Carbon monoxide (CO), a gaseous molecule, at low concentration has been shown to exert cytoprotective and anti-inflammatory effects, while being toxic and lethal to living organisms at high concentrations (22). Our laboratory has demonstrated that CO [250 parts per million (ppm)] exerts potent anti-inflammatory effects with reduced inflammatory cell influx into the lungs and marked attenuation in the expression of proinflammatory cytokines (18). CO could differentially and selectively inhibit the expression of lipopolysaccharide (LPS)-induced proinflammatory cytokines TNF-α, interleukin (IL)-1β, and macrophage inflammatory protein (MIP)-1β and increased the LPS-induced expression of the anti-inflammatory cytokine IL-10 (19). We have also demonstrated that CO can mediate protection against endotoxemia in rodents via suppression of inducible nitric oxide synthase (iNOS) expression in the lung but accentuating iNOS expression in the liver (23). In a rat model of lung transplantation, we have also shown that CO exposure conferred cytoprotection against lung tissue rejection by exerting potent antiapoptotic and anti-inflammatory effects, in particular downregulating proinflammatory genes, including IL-6, MIP-1α, and macrophage migration inhibitory factor (27). Furthermore, CO also exhibits antiproliferative effects in T lymphocytes and thus inhibits the inflammatory process (29).

IL-17 is a proinflammatory cytokine produced by activated memory CD4 T cells, which regulates pulmonary neutrophil emigration in the context of both local gram-negative bacterial infection (33) and antigenic stimuli (4). Overexpression of IL-17 or the administration of recombinant IL-17 in the lung results in the induction of CXC chemokines, which recruit neutrophils to the airway (9). Recently, in mice with homozygous deletion of the ligand (16) or receptor (34), the role of IL-17 in regulating lung inflammation has been better defined. Mice with a homozygous deletion of the IL-17 receptor have markedly decreased recruitment of neutrophils into the lung when challenged with a gram-negative pathogen (34). This is likely due to downregulated CXC chemokine expression, as well as the decreased production of granulopoietic factors such as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (24, 34). Thus IL-17 is considered to be an attractive target for neutrophil-dominated inflammatory responses in the lung (5).

It appears that IL-17 family members signal through a family of unique cognate receptors, which belongs to type I transmembrane protein. However, the cytoplasmic domain of the IL-17 receptor lacks conserved signaling domains with other cytokine receptors (30), and thus the signaling pathway of IL-17 remains unclear. IL-17 has been reported to activate NF-κB and all three classes of MAPKs, including ERK1 and ERK2, JNK, and p38 MAPKs (1, 10, 25). The IL-17-induced activation of the MAPK pathways in bronchial epithelial cells results in the production of IL-6 and the CXC chemokine IL-8 in vitro, which is one likely mechanism by which recombinant IL-17 can induce the recruitment of neutrophils in the lung (10).

Given that IL-17 plays a key role of neutrophil-dominated inflammation in lung, and CO confers potent anti-inflammatory effects, we hypothesized that CO can protect lung cells from IL-17-stimulated inflammatory responses. In this study, we will examine whether CO can modulate IL-17-stimulated inflammatory responses and the signaling pathways by which CO inhibits IL-17-stimulated inflammatory responses.

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MATERIALS AND METHODS

Reagents. Recombinant human IL-17 and enzyme-linked immunosorbent assay (ELISA) kit were obtained from R&D Systems (Minneapolis, MN). The (anti-) phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p44/p42 MAPK (Thr202/Tyr204), p44/p42 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), and SAPK/JNK rabbit polyclonal antibodies were purchased from Cell Signaling Technology, New England Biolabs (Beverly, MA). The specific inhibitor of p38 MAPK SB-203580, as well as the MEK inhibitors U0126 and PD-98059, was from Calbiochem (San Diego, CA). A double-stranded oligodeoxynucleotide probe containing the consensus transcription factor-binding site for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was purchased from Promega (Madison, WI). All other reagent chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Cell culture. Human pulmonary epithelial cells, A549, derived from a lung carcinoma, were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and 100 μg/ml gentamicin in a humidified incubator at 37°C with atmosphere of 5% CO2 and 95% air. Cells were grown to 60–80% confluence and rendered quiescent in medium containing 0.5% FBS before the cytokine treatment. For experiments using MAPK and NF-κB inhibitors, cells were preincubated for 2 h with inhibitors before treatment with exogenous IL-17 (100 ng/ml). The concentrations used for the kinase inhibitors fall within the optimal range for inhibiting respective MAPK and NF-κB pathways without cytotoxicity. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) as recommended by the manufacturer. The vehicle contained the same concentration of DMSO as was present in the treatment group containing 0.1% of DMSO. The maximal DMSO content did not exceed 0.1%

CO exposures. CO exposure was manipulated according to our previous report (19). After 2 h of pretreatment with 250 ppm CO, cells were treated with IL-17 (100 ng/ml) by adding directly from stock to culture media, and the culture plates were returned to the chamber. For MAPK antagonist blocking experiments, cells were also pretreated for 2 h before CO exposure.

Cytokine analysis. Samples of media were collected at 16 h after IL-17 treatment or indicated time point. The production of IL-6 was analyzed with ELISA kits purchased from R&D Systems, using the manufacturer’s instructions.

RESULTS

IL-17 stimulates production of IL-6 in A549 cells. To confirm the effect of IL-17 on the production of IL-6, A549 cells were treated with IL-17 for up to 24 h. Medium samples were collected at the indicated time points, and the production of IL-6 was detected with ELISA method. As shown in Fig. 1, IL-17 challenge elicited a time-dependent increase of IL-6 production in a time-dependent manner. *P < 0.05 was considered significant vs. control.

IL-17 treatment or indicated time point. The production of IL-6 was elicited an increase of IL-6 production in a time-dependent manner. *P < 0.05 vs. control (Fig. 2). CO exposure blocked the induction of IL-6 by IL-17 in A549 cells. Cells were pretreated with 250 ppm CO before exposing to IL-17 challenge. Samples of media were collected at 16 h after IL-17 treatment and analyzed by ELISA. The production of IL-6 induced by IL-17 was inhibited by CO exposure. *P < 0.05 vs. control (control); **P < 0.05 vs. IL-17 treatment in air.
production. IL-17 significantly induced IL-6 production from 12 to 24 h.

**CO inhibits IL-17-induced IL-6 production.** After demonstrating that IL-17 could stimulate production of IL-6 in A549 cells, we determined whether CO could inhibit IL-17-induced IL-6 production. We exposed A549 cells to IL-17 in the presence or absence of 250 ppm of CO and assessed IL-6 production by ELISA. CO significantly decreased the production of IL-6 induced by IL-17 (Fig. 2). CO also produced similar inhibitory effects on the inflammatory cytokines IL-8 (data not shown).

**CO exerts effects by an NF-κB-independent manner.** It has been reported that IL-17 exerted its effect through NF-κB (1, 10, 25). Thus we first examined the involvement of NF-κB pathway using the selective inhibitors of NF-κB. We pretreated A549 cells with SN50, a cell-permeable inhibitor peptide and an inactive control peptide SN50M of NF-κB, or another NF-κB inhibitor BAY 11-7082 before exposing cells to IL-17. The production of IL-6 was measured with ELISA before and after IL-17 treatment. Both SN50 and SN50M showed similar negligible effects of IL-17’s induction of IL-6 production (Fig. 3A). Using another NF-κB inhibitor BAY 11-7082 (Fig. 3B), we also show that inhibition of NF-κB did not affect IL-17-induced IL-6 production.

We further examined whether IL-17 activates NF-κB DNA binding activity in A549 cells by EMSA and the effect of CO on NF-κB DNA binding activity. As demonstrated in Fig. 4, neither IL-17 nor CO affected NF-κB activity in A549 cells. These data suggest that CO inhibits IL-17-induced IL-6 via an NF-κB-independent pathway.

**CO inhibits IL-17-induced IL-6 via the MAPK pathway.** Given our observation that CO exerts anti-inflammatory effects through MAPK pathway (15, 18, 19) and that CO inhibits IL-17-induced IL-6 production via an NF-κB-independent pathway, we investigated whether the MAPK signaling pathway may be involved in the inhibition of CO in IL-17-induced IL-6 production. We pretreated A549 cells with SB-203580, a specific inhibitor of p38, or U0126 and PD-98059, two MEK inhibitors before exposing cells to IL-17. The production of IL-6 was examined by ELISA before and after IL-17 treatment. As shown in Fig. 5, pretreatment of A549 cells with SB-203580, a specific inhibitor of p38, or U0126 and PD-98059, two MEK inhibitors before exposing cells to IL-17. The production of IL-6 was measured with ELISA before and after IL-17 treatment. As shown in Fig. 5, pretreatment of A549 cells with SB-203580 did not affect the induction of IL-6 by IL-17 (Fig. 5A), but U0126 and PD-98059 inhibited IL-17-induced IL-6 production (Fig. 5B and C). These data suggest that the ERK1/ERK2 MAPK pathway may be involved in IL-17-induced IL-6 production.

Furthermore, we observed that IL-17 induced ERK1/ERK2 MAPK activation in A549 cells but did not affect p38 and JNK MAPK activation (Fig. 6). In the presence of 250 ppm CO, both JNK and p38 MAPK activation was not affected; however, IL-17-induced activation of the ERK1/ERK2 MAPKs was significantly attenuated (Fig. 6). Exposure to CO induced...
a delay of ERK1/ERK2 activation that peaked at 30–60 min, compared with peak activation at 15 min after IL-17 treatment alone. The results suggest that CO can inhibit IL-17-induced IL-6 production through altering the activation pattern of MEK-ERK1/2 MAPK pathway in human pulmonary epithelial A459 cells.

**DISCUSSION**

It has recently been reported that IL-17 can induce the secretion of cytokines and chemokines that stimulate neutrophil generation and recruitment in the airway when produced by activated memory CD4+ T cells (9). Due to the ubiquitous distribution of the receptor of IL-17, IL-17 exerts pleiotropic biological activities in a variety of pulmonary cells. When secreted from CD4+ T cells, IL-17 can act on airway smooth muscle cells (32), fibroblast cells (14), epithelial cells (6, 8, 21), and endothelial cells (12, 31) to produce a variety of proinflammatory cytokines and chemokines such as IL-2, IL-6, IL-8, transforming growth factor-β, monocyte chemoattractant protein-1, growth-related oncogene-α, granulocyte chemoattractant protein-2, G-CSF, ICAM-1, etc. (6, 8, 12, 14, 21, 31, 32). In animal in vivo model, both endotoxin exposure (13) and allergen inhalation (4) can cause the release of IL-17 from activated T lymphocytes. Once released, endogenous IL-17 can at least act in part by inducing local release of neutrophil-mobilizing cytokines, and this contributes to recruitment of neutrophils in the airways. Because neutrophils may be important in airway remodeling during inflammation, IL-17-related mechanisms constitute potential targets for pharmacotherapy against exaggerated neutrophil recruitment in airway disease.

CO, at low concentration, is believed to act as a potent anti-inflammatory molecule, which can selectively modulate the proinflammatory/anti-inflammatory cascade of cytokines. CO could inhibit the expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, MIP-1β, and granulocyte/macrophage colony-stimulating factor and increase the LPS-induced expression of the anti-inflammatory cytokine IL-10 (18, 19, 25, 27). Chapman et al. (2) examined the role of CO administered to allergen-challenged mice. They found when the mice were challenged with aerosolized ovalbumin, the eosinophil influx was blocked and the production of IL-5 was reduced by CO exposure. Furthermore, the effects of CO in this model were selective, having no effect on other allergen-induced cytokine production such as eotaxin and IL-12. These data provide evidence to the notion that CO has a functional role in models of inflammation. Our study here that CO can inhibit the...
production of proinflammatory cytokine IL-6 induced by IL-17 adds further insights to the anti-inflammatory armamentarium of CO.

It has been reported that all three MAPKs participate in IL-17-induced release of the IL-6 cytokine and C-X-C chemokines (9). For example, IL-17-induced release of C-X-C chemokines was sensitive to inhibition of the p38 MAPK pathway in human bronchial epithelial (HBE) cells (21). However, Kawaguchi and colleagues (6) showed that only ERK1/2, but not p38 or JNK, was activated by IL-17 in primary bronchial epithelial cells. In transformed human bronchial epithelial (16HBE) cells, however, both p38 and ERK MAPK pathways play a role in IL-17-induced release of neutrophil-mobilizing cytokines (10). Here we demonstrate that in A549 pulmonary epithelial cells, the ERK1/2 MAPK pathway, but not p38 and JNK MAPK, regulated IL-17-induced IL-6 production, demonstrating the cell-specific effect of IL-17 on the MAPK pathway. Cell specificity could also account for NF-κB activation in response to IL-17. Although NF-κB has been shown to modulate IL-17-stimulated inflammatory response in human colonic myofibroblasts (3), it is not the case in A549 cells. The exact mechanisms by which CO acts at the molecular level remain incompletely understood. The involved signaling pathways of CO included MAPK, NF-κB, guanylyl cyclase-cGMP, and the nitric oxide (NO) pathway (15, 19, 20, 22, 35). The anti-inflammatory effects of CO are mediated through a pathway involving the MAPK pathway, but not through a guanylyl cyclase-cGMP or NO pathway when the mice macrophages were challenged with LPS (19). The effect of CO is also shown to be mediated via the JNK signaling pathway and then provides protection against sepsis through modulation of inflammatory cytokine production in a murine model (15). However, the protective effect of CO from arteriosclerotic lesions associated with chronic graft rejection and with balloon injury requires the activation of guanylate cyclase, the generation of cGMP, and as well as the activation of p38 MAPKs and the expression of the cell cycle inhibitor p21Cip1 (20). A recent report by Zuckerbraun et al. (35) showed that activation of both NF-κB and the NO pathway is required for the protective effects of CO, but how CO modulates this signaling pathway still remains unknown. CO could directly bind to the heme iron of soluble guanylyl cyclase, which causes a conformational change leading to an enhancement of enzymatic activity and guanosine 3’5’-monophosphate cGMP production (22). Since MAPKs do not possess hemoprotein components, the activating mechanism of CO affecting MAPK still remains elusive. One possibility is that CO could increase the production of reactive oxygen species, which shifts the redox state of the cell and then affects signaling cascades like the MAPKs.

Here we found that CO’s effect against IL-17-stimulated inflammatory response was mediated by ERK1/2 MAPK, but not by the NF-κB pathway. But unlike in other reports in which ERK1/2 activation is totally inhibited (26), we observe that only the activation pattern is changed by CO. Exposure to CO induced a delay of ERK1/ERK2 activation. The similar delayed response of MAPK was also observed in CO-treated airway smooth muscle cells (28). Perhaps the delay is long enough for the activation of an alternative pathway that regulates a different set of genes that inhibit IL-6. The delay in activation of ERK1/2 might also change the response of cells and inhibit the production of IL-17-induced cytokine production.

Recently, Miljkovic et al. (12) reported that IL-17 could also induce NO production, which involved p38 MAPK or iNOS transcription factor NF-κB activation. This study indicated that IL-17-triggered iNOS activation in endothelial cells might participate in regulation of the T cell-dependent inflammatory response. Because CO can bind to the heme group in the iNOS protein and influence the production of NO (7, 11) and NO can upregulate heme oxygenase (HO)-1 expression, leading to the formation of endogenous CO (17), Zuckerbraun et al. (35) hypothesized that CO requires upregulation of iNOS and production of NO in mediating protective effects and that subsequent NO generation requires HO-1 induction to mediate its therapeutic effects. Thus further studies are needed to study the interaction of two gaseous molecules, NO and CO, in IL-17-induced inflammatory processes.

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