Regulation of IL-1β-induced GM-CSF production in human airway smooth muscle cells by carbon monoxide

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Asthma is a chronic inflammatory disease associated with increased production and elaboration of the eosinophil-active cytokine granulocyte-monocyte colony-stimulating factor (GM-CSF). GM-CSF, a 23-kDa glycoprotein initially identified by its ability to promote in vitro proliferation and differentiation of hematopoietic progenitors into neutrophils and macrophages, stimulates the generation of eosinophils and erythrocytes. GM-CSF can also enhance the functions of mature hematopoietic cells, including antigen presentation, complement- and antigen-mediated phagocytosis, and anti-tumor immunity (8). In addition to its principal function as a stimulator of macrophage proliferation, GM-CSF may enhance the secretion of other inflammatory mediators, such as superoxide anion (O2−), E-series prostaglandins, leukotrienes, arachidonic acid, plasminogen activator, IFN-γ, IL-1, TNF-α, and other CSFs. Many diverse cell types may produce GM-CSF, including fibroblasts, endothelial cells, airway smooth muscle cells, macrophages, and T cells (32).

A number of proinflammatory cytokines and growth factors use MAPK as signaling intermediates to elicit pleiotropic receptor-mediated cellular responses. The highly conserved MAPK family of structurally related serine/threonine kinases participates in the regulation of diverse cellular processes such as growth, differentiation, apoptosis, and adaptive responses to environmental stress (19). The three known major subgroups of the MAPK family include ERK1 and ERK2, JNK/SAPK, and p38 MAPKs (19).

The inducible form of heme oxygenase (HO-1) responds to multiple stress stimuli, including prooxidative states and proinflammatory cytokines (i.e., TNF-α, IL-1β) (6). In addition to HO-1, the HO system includes at least one constitutively expressed isozyme (HO-2) (22). Collectively, HO activity provides the rate-limiting step in heme degradation, catalyzing the oxidative cleavage of heme-b to form ferrous iron, carbon monoxide (CO), and biliverdin-IXα. NAD(P)H:biliverdin reductase completes heme degradation by converting biliverdin-IXα to bilirubin-IXα (37). CO derived from the HO reaction may participate in the regulation of neural and vascular processes (15, 23, 27, 30, 34, 38), and furthermore, may promote cytoprotection by apoptotic and anti-inflammatory mechanisms (4, 5, 28, 34).

Recent studies showed elevated HO-1 expression in association with preclinical models of asthma and human asthma (7, 18, 21). Several laboratories also detected elevated levels of CO in the breath of patients with asthma (14). The physiological function of HO-1 and CO in the pathogenesis of asthma remains unknown. Our laboratory recently demonstrated that CO...
attenuates aeroallergen-induced airway inflammation in mice, implicating a potential important functional role of CO in human asthma (5). In the present study, we show that CO inhibits GM-CSF production in human airway smooth muscle cells (HASMC) via modulation of both ERK1/2 MAPK and guanylate cyclase/cGMP-signaling pathways. Thus, CO may act as an important mediator of human airway remodeling in asthma by virtue of its ability to regulate GM-CSF production.

MATERIALS AND METHODS

Chemicals. The cytokines TNF-α, IL-1β, and INF-γ were purchased from R&D systems (Minneapolis, MN). LPS and all other reagent chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The guanylate cyclase inhibitor IH-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1 one (ODQ), the selective MEK1 inhibitor PD-98059, and the MEK1/2 inhibitor UO-126 were purchased from Calbiochem-Novabiochem (San Diego, CA). All inhibitors were prepared as concentrated stock solutions in DMSO.

Cell culture and treatments. Primary HASMC were purchased from Clonetics, BioWhittaker (Walkersville, MD) and cultured in smooth muscle cell basal medium containing 5% fetal bovine serum, insulin, hFGF, GA-1000, and hEGF (Clonetics). Cells were maintained in humidified incubators containing an atmosphere of 5% CO2 and 95% air at 37°C. HASMC were starved for 24 h and then treated with cytomix, a mixture of TNF-α (500 U/ml), IL-1β (100 U/ml), INF-γ (100 U/ml), LPS (0.1 μg/ml), final concentrations, or each cytokine individually at the indicated doses and exposure times. Media samples were collected at various time points and analyzed for GM-CSF levels by ELISA. For experiments using MAPK inhibitors, cells were preincubated for 1 h with PD-98059 (20 μM) or UO-126 (20 μM) before cytokine treatments. For experiments using ODQ, a guanylate cyclase inhibitor, HASMC were exposed to CO (250 ppm) in the absence or presence of ODQ (50 μM) and then treated with IL-1β (100 U/ml).

CO exposure. For cell culture experiments, CO at a concentration of 1% corresponding to 10,000 ppm in compressed air was mixed with compressed air containing 5% CO2 in a stainless steel mixing cylinder before being delivered into the exposure chamber. The flow rate into the chamber was 2 l/min. The chamber was humidified and maintained at 37°C. The CO levels in the chamber were monitored using a CO analyzer (Interscan, Chatsworth, CA). There were no fluctuations in the CO concentrations after the chamber had equilibrated.

Measurement of GM-CSF. The concentration of GM-CSF released by HASMC into the culture supernatant was measured by an ELISA as described by the manufacturer (R&D systems).

cGMP immunoassays. Cellular levels of cGMP were quantified using an Enzyme Immunoassay Kit (Biomol, Plymouth Meeting, PA), which is a competitive immunoassay for the quantitative determination of cGMP. HASMC were incubated in the presence or absence of CO (250 ppm). Cell lysates were analyzed for cGMP content, according to the manufacturer's instructions.

Western blot analysis. Total cellular extracts were obtained for the Western analyses by lysis of cells in SDS sample buffer containing 62.5 mM Tris·HCl (pH 6.8 at 25°C), 2% wt/vol SDS, 10% glycerol, 50 mM DTT, and 0.01% wt/vol bromophenol blue. Samples were boiled for 5 min. Protein samples (50 μl) were resolved by a 10–12.5% SDS-PAGE and then electroblotted onto nitrocellulose membranes (BioRad, Hercules, CA). The membranes were incubated with phospho-specific or non-phospho-specific rabbit polyclonal antibodies to p38 MAPK, ERK1/ERK2 MAPK, or SAPK/JNK (Cell Signaling, Beverly, MA) (1:1,000) for 1.5 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:5,000) for 1.5 h. The blots were treated with LumiGLO (New England Biolabs, Beverly, MA) for signal development and then exposed to X-ray film. All gels were repeated in triplicate. The level of phosphorylated p38 MAPK, JNK, and ERK1/ERK2 was normalized to the total level of p38 MAPK, JNK, or ERK1/ERK2 detected in the same membrane.

Statistical analysis. Data are presented as means ± SE. The significance of the differences was determined using ANOVA by the Student-Newman-Keuls method.

RESULTS

Effect of cytomix on GM-CSF production in HASMC. The basal level of GM-CSF in HASMC was below the detection limit of the ELISA assay. Treatment with cytomix, a mixture of TNF-α (500 U/ml), IL-1β (100 U/ml), INF-γ (100 U/ml), LPS (0.1 μg/ml), (final concentrations) strongly induced GM-CSF protein synthesis after 8-h exposure (Fig. 1). TNF-α or INF-γ, when administered individually, did not stimulate the level of GM-CSF within the detection limit of the assay. On the other hand, LPS and IL-1β each induced GM-CSF protein synthesis after 8-h exposure.

Effect of IL-1β on GM-CSF production in HASMC. IL-1β administration induced a time- and dose-dependant increase in the synthesis of GM-CSF in HASMC as assessed by ELISA (Fig. 2, A and B). An initial increase of GM-CSF synthesis occurred at 4 h and peaked at 20 h of IL-1β treatment. Later time points showed no further increase in the levels of GM-CSF (data not shown). The increase of GM-CSF protein synthesis also correlated with the increased dose of IL-1β administered.
Carbon monoxide (CO) inhibited cytokine-induced GM-CSF protein production. After starvation for 24 h, we administered cytokine, LPS, or IL-1β to HASMC in the absence and presence of 250 ppm CO and measured the GM-CSF protein levels in the media. Exposure to 250 ppm CO significantly attenuated the induction of GM-CSF production by cytokine, LPS, or IL-1β (Fig. 3A), as determined by ELISA.

**CO exerts its effect via inhibition of ERK MAPK.**

When HASMC were serum starved and stimulated by IL-1β, activation of phosphorylated p38 MAPK, JNK, and ERK1/ERK2 was observed, with peak expression at 15 min after stimulation (Fig. 4A). The activation of phosphorylated p38 MAPK, JNK, and ERK by IL-1β was compared in the absence and presence of CO (250 ppm). There was no difference in IL-1β-mediated p38 MAPK and JNK activation in room air or CO. However, ERK1/2 activation was significantly inhibited in the presence of CO (Fig. 4B). HASMC were starved for 24 h and then pretreated with PD-98059 (20 μM) and UO-126 (20 μM) (selective inhibitors of MEK1 and MEK1/2, respectively) for 1 h before treatment with IL-1β. GM-CSF levels were detected by ELISA in the media collected at 8 h. Figure 5 shows that PD-98059 and UO-126 significantly inhibited IL-1β-induced GM-CSF synthesis. These data indicate that the suppression of IL-1β-induced GM-CSF synthesis by CO involves inhibition of ERK1/2.

**Suppression of IL-1β-induced GM-CSF production by CO is dependent on the activation of guanylate cyclase and the generation of cGMP.** Exposure of HASMC to CO (250 ppm) for 8 h increased the levels of intracellular cGMP (Fig. 6A). HASMC were exposed to CO (250 ppm) in the presence or absence of ODQ and then treated with IL-1β (100 U/ml). The inhibitory effect of CO on IL-1β-induced GM-CSF synthesis was reversed by ODQ (Fig. 6B). The inhibitory effect of CO...
on ERK1/2 activation was also blocked in the presence of ODQ (Fig. 6C). These data indicate that the suppression of IL-1β-induced GM-CSF synthesis by CO depends on the activation of guanylate cyclase and the generation of cGMP.

DISCUSSION

Asthma is characterized by an increased expression of components of the inflammatory cascade, including cytokines, chemokines, growth factors, enzymes, receptors, and adhesion molecules (3). An enhanced production of inflammatory mediators promotes the infiltration and activation of eosinophils in the airways (1, 13). Experiments using in situ hybridization and immunohistochemistry revealed the increased expression of Th2 cytokine mRNA, including IL-3, IL-4, IL-5, and GM-CSF, in asthmatic airways (3). In the studies presented here, we show that IL-1β treatment induced a time- and dose-dependent increase in GM-CSF production from HASMC (Figs. 1 and 2).

Fig. 4. CO inhibits IL-1β-induced GM-CSF protein production via a mechanism that requires the suppression of ERK MAPK. A: HASMC were starved for 24 h and stimulated with IL-1β. The phosphorylation of ERK, JNK, and P38 MAPK was measured at 0–30 min by Western blotting using corresponding phospho-specific anti-MAPK antibodies. Membranes were subsequently probed with the corresponding non-phospho-specific antibodies to ensure equal loading. B: HASMC were starved for 24 h and stimulated with IL-1β in the presence and absence of CO. Phosphorylation of ERK, JNK, and P38 MAPK was measured at 30 min by Western blot. The figures are representative of 3 independent experiments.

Fig. 5. MAPK inhibitors decrease GM-CSF production by IL-1β. HASMC were starved for 24 h and then pretreated with PD-98059 (PD; 20 μM) and UO-126 (UO; 20 μM), selective inhibitors of MEK1 and MEK1/2, for 1 h before treatment with IL-1β (100 U/ml). DMSO was vehicle control. Media were collected at 8 h and analyzed for GM-CSF levels by ELISA. Data represent means ± SE of samples from 3 independent experiments. *P < 0.05 vs. IL-1β treatment.

Fig. 6. Role of cGMP in the inhibitory effect of CO on GM-CSF activation. A: HASMC were exposed to CO (open bar, 250 ppm) for 8 h, and cell extracts were analyzed for cGMP content, as described in MATERIALS AND METHODS. Results shown are means ± SE of samples from 3 independent experiments. *P < 0.05 vs. room air (RA, filled bars) control group. cGMP was increased in HASMC exposed to CO. B: HASMC were then exposed to CO (250 ppm) in the presence or absence of IH-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 50 μM), a guanylate cyclase inhibitor. HASMC were then treated with IL-1β (100 U/ml). Media were collected at 8 h and analyzed for GM-CSF levels by ELISA. Data represent means ± SE of samples from 3 independent experiments. *P < 0.05, significant decrease vs. RA (open bars) control; #P < 0.05, significant increase vs. CO (filled bars) without ODQ control. ODQ reversed the inhibitory effect of CO on IL-1β-induced GM-CSF production. C: phosphorylation of ERK1/ERK2 was measured at 15 min by Western blotting using corresponding non-phospho-specific anti-MAPK antibodies. Membranes were subsequently probed with the corresponding non-phospho-specific antibodies to ensure equal loading. The figure is representative of 3 independent experiments. ODQ blocked the inhibitory effect of CO on IL-1β-induced ERK1/ERK2 activation.

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Increased levels of CO have been detected in the breath of asthmatics (14). We previously reported that CO can markedly attenuate aeroallergen-induced airway inflammation (5). In this report, we demonstrate that CO could play a novel and important role in human asthma by inhibiting the synthesis of GM-CSF in airway smooth muscle. We show that HASMC exposed to CO (250 ppm) exhibited significant attenuation of IL-1β-induced GM-CSF synthesis, as detected by ELISA (Fig. 3).

Recent studies demonstrate increased expression of the 32-kDa stress protein HO-1, the principal biological source of CO, in preclinical models of asthma and in human asthma (7, 18, 21). HO-1 responds to induction by a variety of agents, including ultraviolet A radiation, thiol-reactive substances, nitric oxide, and pro-inflammatory cytokines (6, 17). Such agents promote oxidative stress either by increasing intracellular reactive oxygen species production or diminishing reducing potential through the complexation and/or depletion of intracellular reduced glutathione. These observations led to the hypothesis that HO-1 participates in cellular defense mechanisms against oxidative stress (17, 20). Over the last decade, a number of laboratories including ours demonstrated that induction of endogenous HO-1 or exogenous administration of HO-1 via gene transfer provides protection against oxidative stress in many in vivo and in vitro models, including hyperoxia- or endotoxin-mediated lung injury, ischemia/reperfusion injury, and atherosclerosis (9, 11, 16, 24, 28, 29).

The mechanisms by which HO-1 provides cytoprotection remain elusive, but they likely relate to the biological activities of its enzymatic reaction products. HO-dependent effects on intracellular iron homeostasis, anti/prooxidant balance, and CO-dependent signal transduction all possibly contribute to cellular protection (32). Prooxidant iron released from HO activity may increase intracellular iron storage capacity by triggering the de novo synthesis of ferritin, a possible cytoprotectant (39). Biliverdin-IXα and its metabolite bilirubin-IXα have known in vitro antioxidant and radical scavenging properties (36). Increasing evidence suggests that CO by virtue of anti-apoptotic and anti-inflammatory effects also plays a role in cytoprotection (4, 5, 28, 34).

CO exerts physiological effects in neuronal and cardiovascular regulation. The latter may include the inhibition of vascular smooth muscle proliferation and platelet aggregation and the stimulation of vasodilation (10, 25–27). These previously described functions of CO have largely been attributed to increases in cGMP synthesis, resulting from stimulation of guanylate cyclase by direct physical binding of CO to the heme moiety of the enzyme (12). Consistent with this paradigm, our current studies show that exposure of HASMC to CO for 8 h increased the levels of intracellular cGMP (Fig. 6A). Administration of ODQ, a guanylate cyclase inhibitor, reversed the inhibitory effect of CO on IL-1β-induced GM-CSF synthesis (Fig. 6B).

Cytokines and growth factors regulate a number of cellular processes through the intermediary of MAPK-dependent signal transduction pathways (19). Recently, we showed that the anti-inflammatory effects of HO-1 and CO involve the downregulation of proinflammatory cytokine synthesis, which in turn depends on selective modulation of the p38 MAPK pathway (28). In efforts to examine the mechanism by which CO inhibited GM-CSF synthesis, we compared the phosphorylation of p38 MAPK, JNK, and ERK1/2 by IL-1β in the absence and presence of CO. No apparent difference occurred between IL-1β-mediated p38 MAPK and JNK activation in the absence or presence of CO. However, CO significantly inhibited ERK1/2 activation in response to IL-1β (Fig. 5). Furthermore, inhibition of ERK by CO was blocked in the presence of ODQ (Fig. 6C). These data indicate that the inhibition of IL-1β-induced ERK1/2 activation, and ultimately of GM-CSF synthesis by CO treatment, depended on the activation of guanylate cyclase and the generation of cGMP.

In this study, we used a low concentration (250 ppm) of CO, designed to evoke physiological responses. Although the majority of endogenous CO arises principally from heme degradation, a small fraction may arise from other sources, including lipid peroxidation and xenobiotic metabolism (40). CO, a gaseous molecule, also occurs in nature as a ubiquitous air pollutant. Exposure to CO can be lethal at high concentrations in the context of industrial or accidental exposure (31). Against this paradigm of CO toxicity, recent interest has been accumulating regarding the possibility that CO, at low concentration, behaves as a regulatory molecule in cellular and biological processes (11, 28, 35). It is important to note that the CO concentration used for this study is the same or lower than the CO concentration used in previous studies (11, 28, 35) and that although CO inhibits GM-CSF synthesis as shown in this study, CO does not inhibit other cytokines such as MIP-2, JNK, IFN-γ, or KC (28) or MIP-1α (unpublished data). Interestingly, CO can augment the levels of the anti-inflammatory cytokine such as IL-10 (28). These data strongly suggest that CO does not merely exert a global inhibitory effect on all secreted cytokines or proteins. Further studies are needed to delineate the precise mechanisms by which CO exerts differential effects on various cytokines and how CO induces effective, nontoxic, intracellular signaling in airway cells such as HASMC.

In this study, we demonstrated that CO inhibits GM-CSF production in HASMC via the ERK MAPK- and cGMP-dependent pathways. Interestingly, and in contrast to this observation, our laboratory also recently demonstrated that CO inhibits HASMC proliferation via an ERK1/2 MAPK-dependent, but cGMP-independent pathway (35). Thus, it appears that in HASMC, CO signals anti-proliferative effects via the ERK MAPK pathway in a cGMP-independent fashion, whereas CO-induced anti-inflammatory effects occur via the ERK MAPK pathway in a cGMP-dependent fashion. In vascular smooth muscle cells, however, the anti-proliferative effects of CO apparently require cGMP-dependent signaling (25). These observations suggest that variations in signaling pathways occur in...
cell type-specific fashion, and furthermore, that cooperation between ERK1/2 and cGMP pathways may vary with cellular process, even in the same cell type. Nevertheless, our data clearly demonstrate that CO can inhibit the production of GM-CSF by HASMC. CO at low concentrations may therefore play an important protective role in inflammatory diseases such as asthma and thus has potential therapeutic implications.

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