CCAAT/Enhancer-Binding Protein Mediates Carbon Monoxide-Induced Suppression of Cyclooxygenase-2

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Cyclooxygenase-2 (COX-2) is a key enzyme involved in the inflammatory process that is rapidly induced in macrophages in response to LPS. Carbon monoxide (CO), a byproduct of heme oxygenase-1, can suppress proinflammatory response in various *in vitro* and *in vivo* models of inflammation. This study was undertaken to examine whether CO can regulate (and if so, to delineate the mechanism by which CO regulates) LPS-induced COX-2 expression in macrophages. RAW 264.7 murine macrophages were stimulated with LPS (0–10 ng/ml) with or without CO (500 ppm). Northern and Western blot analysis was done. Prostaglandin E₂ and nitrite concentration was measured from cell culture supernatant. Electrophoretic mobility shift assay was performed to assess nuclear factor binding. CO downregulated LPS-induced COX-2 mRNA and protein expression. CO also inhibited LPS-induced prostaglandin E₂ secretion ($P < 0.05$). CO also decreased LPS-induced CCAAT/enhancer-binding protein (C/EBP) β and δ protein expression in LPS-treated RAW 264.7 cells. Gel shift analysis revealed that CO treatment decreased LPS-induced activation of protein binding to C/EBP consensus oligonucleotides of murine *cyclooxygenase-2* promoter. CO also decreased LPS-induced nitric oxide synthase-2 protein expression and nitrite production, and decreased LPS-induced activation of protein binding to C/EBP consensus oligonucleotides of murine *nitric oxide synthase-2* promoter. CO may act as an important regulator of inflammation by virtue of its ability to regulate C/EBPs.

Keywords: heme oxygenase; lipopolysaccharides; nitric oxide synthase

Heme oxygenase-1 (HO-1) is a microsomal enzyme responsible for degradation of heme, generating biliverdin, iron, and carbon monoxide (CO) (1). HO-1 can be induced by a wide variety of stimuli, and the enzyme is involved in cellular and tissue defense against oxidative stress possessing potent anti-inflammatory properties (2, 3). There is growing interest in the role of CO in the anti-inflammatory and cytoprotective function of HO-1 (4–7), but the pathways involved in the anti-inflammatory effect of CO are poorly understood. CO can modulate mitogen-activated protein kinase (5) and guanylate cyclase/3',5'-guanylate cyclic monophosphate (cGMP) pathway (8) to inhibit secretion of proinflammatory cytokines. CO also modulates several transcription factors, including NF- κ B (4, 6) and activating protein-1 (4), which are involved in inflammation. But whether other pathways or molecules are involved in the anti-inflammatory effect of CO is not known.

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Cyclooxygenase-2 (COX-2) is a key enzyme catalyzing the rate-limiting step in the inducible production of prostaglandins, and is induced by variety of stimuli in different cells (9). Prostaglandins secreted by inflammatory cells such as macrophages are thought to be important in early initiation of inflammatory response by increasing vascular permeability (10), vasodilation, recruitment of other inflammatory cells to the site of infection (11), and release of proinflammatory cytokines (12). Also COX-2-deficient mice are known to be resistant to LPS-induced inflammation and death (13), which indicates that COX-2 is an important molecule in this model of acute inflammation. Interestingly, COX-2-deficient mice showed a cytokine profile similar to that of CO-treated mice showing downregulation of proinflammatory cytokine TNF- α and upregulation of anti-inflammatory cytokine IL-10 (13). But whether CO can downregulate COX-2, and if so what signaling pathways are involved in CO-mediated downregulation of COX-2, is not known.

In the present study, we show that CO inhibits LPS-induced COX-2 expression and production of prostaglandin E₂ (PGE₂) in murine RAW 264.7 macrophages via modulation of transcription factor CCAAT/enhancer-binding proteins (C/EBPs). CO also downregulated LPS-induced nitric oxide synthase-2 (NOS-2), another molecule in which regulation by C/EBPs plays a prominent role. Thus, CO may act as an important regulator of inflammation by virtue of its ability to regulate C/EBPs.

MATERIALS AND METHODS

Cell Culture and Treatments

A murine macrophage cell line RAW 264.7 (ATCC, Rockville, MD) was used for all experiments for this study except when indicated otherwise. Cells were maintained in Dulbecco's modified Eagle media (Invitrogen Corp., Carlsbad, CA) containing 10% FBS and 100 μ g/ml gentamicin in a humidified atmosphere of 5% CO₂ in air with or without additional CO as described below. After 2 h pretreatment with either CO or air, cells were stimulated with LPS (1–10 ng/ml). For determination of PGE₂ and nitrite, 6-well plates were plated with 1×10^6 cells per well and allowed to grow overnight. Then cells were stimulated with LPS (2.5 ng/ml) with or without pretreatment with 500 ppm of CO.

COX-2 Promoter Luciferase Reporter Experiments

The cloning of the wild-type (WT) *cox-2* promoter and mutants of C/EBP (C/EBP-1 and C/EBP-2) were generated as previously described by site-directed mutagenesis (14). RAW264.7 cells were transiently transfected with either WT or C/EBP mutants (C/EBP-1 or C/EBP-2) overnight before stimulation with LPS (10 ng/ml) for 24 h in the absence or presence of CO (500 ppm). Luciferase assays were performed using Promega Dual Luciferase kit (Promega, Madison, WI) according to manufacturer's protocol.

Isolation of Alveolar Macrophages

The experimental protocols were performed in accordance with the guidelines of institutional animal care and use committee (IACUC) of University of Pittsburgh. Briefly, mice were tracheotomized after transection of the abdomen. A blunt 21-gauge needle was secured into the trachea. Bronchoalveolar lavage (BAL) was performed five

times with 0.3 ml PBS. Cell pellets were collected after centrifuging at $1,000 \times g$ for 10 min at 4°C . The supernatant was discarded and the cells were resuspended in 10% FBS DMEM. Unattached cells were washed off twice with PBS after 3 h culture, and the remaining macrophages were incubated overnight in DMEM supplemented with 10% FBS. On the following day, cells were washed twice again before being treated as indicated.

CO Exposures

RAW 264.7 cells were exposed to compressed air with varying concentrations of CO (250–500 ppm). Five percent CO_2 was present for buffering requirements. Details of CO administration have been previously described (5).

Measurement of PGE_2 and Nitrite

Cell media samples were analyzed for PGE_2 and nitrite with kits purchased from R&D Systems (Minneapolis, MN) according to manufacturer's instructions.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated and Northern blot analysis was performed as previously described (15). ^{32}P -labeled mouse COX-2 cDNA (Oxford Biomedical Research, Oxford, MI) was used in the analysis.

Nuclear Protein Extraction

Nuclear proteins were extracted with nuclear extraction kit from Active Motif (Carlsbad, CA) according to manufacturer's instructions, and were used in Western blot analysis and electrophoretic mobility shift assay (EMSA).

Western Blot Analysis

Whole cell protein and nuclear protein extracts were used for Western blot analysis as previously described (4). The following antibodies were

used for immunoblotting: anti-COX-2, anti-C/EBP β , anti-C/EBP δ (Santa Cruz, Santa Cruz, CA), anti-NOS-2 (inducible) (Alexis Biochemicals, San Diego, CA), and anti- β -actin (Abcam, Cambridge, UK).

EMSA

EMSA was performed as previously described (4). Sequences of oligonucleotides used were as follows: murine *cox-2* C/EBP element 5'-CTGCCGCTGCGGTTCTTGCGCAACTCACT-3' and its complementary sequence; murine *nos-2* C/EBP element 5'-CACAGAGTGATGTAATCAAGCA-3' and its complementary sequence; murine ATF/CRE-E-Box site of COX-2 promoter 5' G TCA CCA CTA CGT CAC GTG GAG TCC GCT T 3'.

For supershift experiments, 2 μl of polyclonal purified antibody (Santa Cruz) was incubated with nuclear extracts for 30 min before the probe addition.

Statistics

Data are expressed as mean $\pm$ SD. Data were analyzed by using SPSS for Windows 11 (SPSS Inc, Chicago, IL). Differences in measured variables between experimental and control groups were assessed using Student's *t* test. *P* values < 0.05 were considered significant.

RESULTS

CO Suppresses COX-2 mRNA and Protein Expression in RAW 264.7 Macrophages

We assessed the expression of COX-2 mRNA in RAW 264.7 macrophages exposed to 2.5 ng/ml LPS in the presence or absence of CO. Cells were harvested sequentially from 0 min to 3 h after LPS treatment for RNA extraction and analysis by Northern blotting. As shown in Figure 1A, the untreated cells and those treated with CO did not have detectable COX-2 mRNA expression by Northern blotting before stimulation. LPS

Figure 1. CO suppresses COX-2 expression in LPS-stimulated RAW 264.7 cells. (A) Total RNA was extracted from cells stimulated with LPS (2.5 ng/ml) for indicated times in the absence or presence of 500 ppm of CO, and Northern blot analysis was performed. Ethidium bromide staining was done to show equal loading. Bar graph represents mean $\pm$ SD of densitometry value of three independent experiments. (B) Total cellular proteins were extracted 4 h after stimulation with various doses of LPS in the presence and absence of 500 ppm of CO and Western blot analysis was performed for COX-2 expression. Data representative of three independent experiments. RA, room air; CO, 500 ppm CO. **P* < 0.05 . (C) Alveolar macrophages were harvested with or without LPS treatment for 4 h in the absence or presence of CO (500 ppm) and COX-2 production was determined by Western blot analysis. Findings were representative of three independent experiments.

Figure 2. CO dose-dependently suppresses LPS-induced COX-2 protein expression. Whole cell lysates were harvested at 4 h after LPS (2.5 ng/ml) treatment with or without two different doses of CO treatment (250 and 500 ppm) for Western blot analysis. Data are representative of two independent experiments.

markedly increased COX-2 mRNA expression (23.1 ± 2.8 arbitrary units [AU]) at 3 h, which was decreased significantly by 500 ppm of CO (11.3 ± 4.8 AU) at 3 h ($P < 0.05$).

We then assessed the effect of CO (500 ppm) treatment on LPS (0–10 ng/ml)-induced COX-2 protein expression in RAW 264.7 macrophages. As shown in Figure 1B, CO decreased the expression of COX-2 protein in a dose-dependent manner at 4 h. These data demonstrate that CO suppresses COX-2 expression at both mRNA and protein levels. We also performed similar experiments in alveolar macrophages of mice, and observed that CO can markedly attenuate LPS-induced COX-2 expression (Figure 1C).

CO Dose-Dependently Suppresses LPS-Induced COX-2 Protein Expression

We examined effect of two different doses of CO in LPS-induced COX-2 protein expression in RAW 264.7 cells. Whole cell lysates were harvested at 4 h after LPS (2.5 ng/ml) treatment with or without two different doses of CO treatment (250 and 500 ppm) for Western blot analysis. As shown in Figure 2, LPS-induced COX-2 protein expression was inhibited by CO in a dose-dependent manner. LPS also caused a slight induction in C/EBP β and strong induction of C/EBP δ protein expression, both important transcription factors in COX-2 expression, which was also inhibited by CO dose dependently.

CO Suppresses LPS-Induced PGE₂ Production

Cell culture media was assayed for PGE₂, one of the downstream products of COX-2. Four hours of LPS (2.5 ng/ml) treatment increased PGE₂ from 172.4 ± 103.8 pg/ml to $2,013.9 \pm 624.2$ pg/ml, which was significantly decreased by CO (500 ppm) to 696.4 ± 185.8 pg/ml (Figure 3, $P < 0.05$).

CO Suppresses LPS-Induced Nuclear C/EBP β and C/EBP δ Expression

Given the importance of C/EBP β and C/EBP δ as transcriptional factors responsible for COX-2 induction, we sought to determine

Figure 3. CO decreases LPS-induced prostaglandin(PG)E₂ production in RAW 264.7 macrophages. Cells were treated for 4 h with LPS (2.5 ng/ml) in the presence or absence of CO (500 ppm). PGE₂ level was measured by immunoassay. Data represent means $\pm$ SD of samples from three independent experiments. RA, room air; CO, 500 ppm CO. * $P < 0.05$.

the levels of these C/EBPs in the nucleus of RAW 264.7 cells exposed to LPS with or without CO treatment. Nuclear protein extracts were harvested at 0, 1, 2, and 4 h after stimulation with LPS (2.5 ng/ml) in the presence or absence of 500 ppm of CO. As shown in Figure 4, LPS increased nuclear C/EBP β isoforms (38, 35, and 19 kD); this increase was slightly downregulated by 500 ppm of CO. The most striking difference was seen with C/EBP δ , which showed marked suppression by CO.

CO Attenuates Nuclear Protein Binding to C/EBP Element of Murine COX-2 Promoter

To confirm that CO can inhibit activation of C/EBP transcription factors, EMSAs were performed using nuclear extracts from RAW264.7 macrophages treated with LPS, with or without CO (500 ppm). Cells were harvested 0, 2, and 4 h after treatment. As shown in Figure 5, LPS (2.5 ng/ml) induced nuclear protein binding to C/EBP element of murine COX-2 promoter. Decreased binding to C/EBP element by CO was seen starting at 2 h (Figure 5, lane 3 versus lane 6) and was evident by 4 h (lane 9 versus lane 12).

Specificity of binding was examined by carrying out supershift assay by adding specific antibodies to C/EBP β and C/EBP δ . At 2 h, most of the supershifted complexes represented C/EBP β , while at 4 h both C/EBP β and δ contributed to DNA–protein complex. CO did not seem to have preferential effect on either C/EBP β or C/EBP δ . Interestingly, we observed that CO does not decrease binding of nuclear protein to ATF/CRE-E-Box site of COX-2 promoter, demonstrating the specificity of CO (data not shown).

CO Downregulates LPS-Induced COX-2 via C/EBP

In order to directly examine whether C/EBP regulates CO's ability to attenuate LPS-induced COX-2 expression, we performed reporter analysis in RAW264.7 using the *cox-2* promoter. We demonstrated that LPS markedly activates WT *cox-2* promoter (> 8 -fold) and CO significantly attenuates LPS-induced WT *cox-2* promoter (Figure 6). Interestingly, mutational analysis of the *cox-2* promoter in RAW264.7 cells demonstrated that the mutated C/EBP DNA binding sites were critical to CO's ability to attenuate LPS-induced *cox-2* promoter activation (Figure 6).

Figure 4. CO decreases C/EBP expression in the nucleus of LPS-stimulated RAW 264.7 cells. Nuclear protein extracts were harvested at 0, 1, 2, and 4 h after stimulation with LPS (2.5 ng/ml) in the presence or absence of 500 ppm of CO. Data are representative of three independent experiments. RA, room air; CO, 500 ppm CO.

CO Downregulates Inducible NOS Expression through C/EBPs

To confirm the importance of C/EBP modulation in the anti-inflammatory effect of CO, we examined the effect of CO in NOS-2 expression, a protein in which C/EBPs play an important role in regulation of transcription (16). RAW 264.7 macrophages were stimulated with LPS (2.5 ng/ml) in the presence or absence of CO. Whole cellular extract was examined for NOS-2 protein expression by Western blot, nitrite production was measured in culture media, and nuclear protein binding activity was evaluated by EMSA. Figure 7A shows that NOS-2 protein expression induction by LPS was downregulated by CO (500 ppm). Also, CO downregulated NOS-2 protein expression dose dependently (Figure 7B). CO (500 ppm) inhibited nitrite production at 6 h from 50.6 $\mu\text{mol/l}$ to 20.0 $\mu\text{mol/l}$ ($P < 0.05$) (Figure 7C). This reduction of NOS-2 activity by CO was accompanied by decreased nuclear protein binding activity to C/EBP element of murine *nos-2* promoter at 4 h (Figure 8). Supershift assay showed

that most of the binding was attributable to C/EBP β and C/EBP δ , both of which were attenuated by CO (Figure 8).

DISCUSSION

CO, one of the byproducts of the HO-1 pathway, has been shown to be anti-inflammatory and cytoprotective in various models of inflammation and tissue injury, including sepsis (4–6), asthma (17, 18), chronic graft rejection (19), and ventilator-induced lung injury (20). The signaling pathways and transcription factors involved in CO-mediated anti-inflammatory and cytoprotective effects remain elusive. One of the major pathways involved seems to be p38 mitogen-activated protein kinase (MAPK) pathway (5). CO acts through p38 MAPK to inhibit LPS-induced production of TNF- α in mice (5). CO modulates IL-1 β -induced granulocyte-monocyte colony-stimulating factor production in human airway smooth muscle cells by modulating guanylate cyclase/cGMP pathway (8). CO also modulates several

Figure 5. CO attenuates nuclear protein binding to C/EBP element of murine *cox-2* promoter. EMSA was performed using nuclear extracts from RAW264.7 macrophages treated with LPS (2.5 ng/ml) with or without CO (500 ppm). Cells were harvested 0, 2, and 4 h after treatment. Specificity of binding was examined by carrying out supershift assay by adding specific antibodies to C/EBP β and C/EBP δ . Data are representative of three independent experiments. RA, room air.

Figure 6. CO attenuates LPS-induced *cox-2* promoter via C/EBP. RAW264.7 cells were transiently transfected with either WT or C/EBP mutants (C/EBP-1 or C/EBP-2) overnight before stimulation with LPS (10 ng/ml) for 24 h in the absence or presence of CO (500 ppm). Luciferase assays were performed using Promega Dual Luciferase kit according to manufacturer's protocol. Experiments performed in triplicate in 96-well plates. Open bars, RA + LPS; shaded bars, CO + LPS.

transcription factors, as CO decreases LPS-induced NF- κ B activation (4, 6) and LPS-induced activating protein-1 activation (4) in macrophages. In this study, we have uncovered another signaling pathway by which CO modulates its anti-inflammatory effect. CO can modulate induction of COX-2 through its effect on activation of C/EBP transcription factors. CO also inhibited induction of NOS-2, which was also accompanied by decreased transcription factor binding to C/EBP consensus sequence of murine *nos-2* promoter. It is important to note, however, that the specificity of CO responses depends heavily on cell type and stimuli (1, 4–8). For example, while CO attenuates many of the proinflammatory cytokines, it has negligible effect on LPS-induced MIP-2 in macrophages (5).

C/EBPs are a family of basic leucine zipper (bZIP) transcription factors involved in regulation of various aspects of cellular functions (21). Thus far, six C/EBP members have been cloned from several species (22–29). All C/EBP isoforms share substantial sequence identity in the C-terminal 55–65 amino acid residues, which contains the bZIP domain. Different C/EBP proteins are able to form homodimers or heterodimers with other members of the C/EBP family and with the exception of C/EBP ξ (29), interact with an identical recognition sequence (30). The cellular functions of C/EBPs are diverse. C/EBPs are implicated in differentiation of many cell types, including adipocytes (31, 32), myelomonocytic cells (33–35), hepatocytes (36), and mammary epithelial cells (37). C/EBP β and δ are induced by a number of inflammatory agents, including LPS and various cytokines, and are implicated in the regulation of various proinflammatory genes (22, 38). C/EBPs are also involved in control of metabolism (39, 40) and cellular proliferation (41, 42).

The regulation of COX-2 synthesis occurs mainly at the transcriptional level, although mRNA stabilization is also involved in response to specific agents (43, 44). The stimuli, signal transduction pathways, and transcription factors involved in the induction of *cox-2* gene expression are extremely diversified and cell specific. In macrophages, C/EBPs are very important in inducing COX-2. In C/EBP β –/– macrophages, COX-2 was not induced by LPS, which was reversed by expression of C/EBP β in those same cells (43). Caivano and colleagues showed that COX-2 induction by LPS could be divided into an initial phase that was independent of *de novo* protein synthesis, which requires C/EBP β , and a late phase that requires *de novo* protein synthesis and both C/EBP β and δ (45). In this study, we show that CO decreased COX-2 mRNA and protein induction, as well as one of its end products, PGE₂. CO also downregulated induction of C/EBP β and δ proteins and nuclear protein binding to C/EBP consensus sequence of *cox-2* promoter, which suggests that downregulation of COX-2 by CO occurs at least in part through modulation of C/EBPs.

As was seen in other studies, C/EBP δ levels were very low in unstimulated cells, while C/EBP β was already present in appreciable amounts before cell stimulation (43, 45). The magnitude

Figure 7. CO suppresses nitric oxide synthase-2 (NOS-2) expression and nitrite production in LPS-stimulated RAW 264.7 cells. (A) Total cellular proteins were extracted 0, 2, 4, 6, and 10 h after stimulation with LPS (2.5 ng/ml) in the presence and absence of 500 ppm of CO. Data are representative of three independent experiments. (B) Whole cell lysates were harvested at 4 h after LPS (2.5 ng/ml) treatment with or without two different doses of CO treatment (250 and 500 ppm) for Western blot analysis. Data are representative of two independent experiments. (C) Cells were treated for 4 h with LPS (2.5 ng/ml) in the presence or absence of CO (500 ppm). Nitrite production in culture supernatant was measured by Griess reaction. Data represent means $\pm$ SD of samples from three independent experiments. RA, room air; CO, 500 ppm CO.

Figure 8. CO attenuates nuclear protein binding to C/EBP element of murine *nos-2* promoter. EMSA was performed using nuclear extracts from RAW264.7 macrophages treated with LPS (2.5 ng/ml) with or without CO (500 ppm). Cells were harvested 0 and 4 h after treatment. Specificity of binding was examined by carrying out supershift assay by adding specific antibodies to C/EBP α , C/EBP β , C/EBP δ , and C/EBP ϵ . Data are representative of three independent experiments.

of induction of C/EBP β and its inhibition by CO was not as prominent as C/EBP δ , thus raising the possibility that at least in the early phases of LPS stimulation where nuclear proteins bound to C/EBP consensus sequence was mostly C/EBP β , CO may be inhibiting other aspects of C/EBP β activation such as acetylation (46) and/or phosphorylation (47), although in one study, phosphorylation of C/EBP β in macrophages by LPS could not be discerned (45).

Another mechanism by which CO may modulate COX-2 induction is through its influence in MAPK cascades. It is known that LPS-induced MAPK cascades and inhibitors of p38 MAPK and extracellular signal-regulated kinase (ERK) pathways decrease COX-2 induction through inhibiting phosphorylation of cyclic AMP response element-binding protein. c-Jun and c-Jun N-terminal kinase activation also can induce transcription of *cox-2* gene through activation of cAMP response element of murine *cox-2* promoter (48). But modulation of these molecules by CO is unlikely because CO did not inhibit activation of p38 MAPK or ERK, nor inhibit nuclear protein binding to cAMP response element consensus sequence of murine *cox-2* promoter (data not shown). There is also a possibility that inhibition of COX-2 induction by CO may occur through its influence in NF- κ B activation, as was shown in LPS-induced cytokine induction in macrophages (6). The relative importance of NF- κ B and C/EBPs in modulation of COX-2 induction by CO is not known.

Another gene implicated in the inflammatory process that is regulated by C/EBPs is *nos-2* (15). COX-2 and NOS-2 are known to be regulated in the same manner in some systems through common mediators including C/EBPs (49, 50). We show that CO inhibited NOS-2 mRNA and protein induction, as well as nitrite production, which was accompanied by decreased binding activity of C/EBP consensus sequence of the murine *nos-2* promoter, strengthening the argument that CO modulation of C/EBPs is important in its anti-inflammatory effect.

In conclusion, CO modulates LPS-induced COX-2 and NOS-2 induction in RAW264.7 macrophages by virtue of its ability to regulate C/EBP isoforms. These studies provide further evidence that CO can modulate many key effector molecules, as has been reported previously (1, 4–8). Although many of these reports point toward an anti-inflammatory function of CO in many systems, we still poorly understand the increasingly complex regulation and function of CO in pathophysiologic states. Recent studies suggesting the lack of cytoprotective effect of CO in some systems highlight this complexity (51–53). This current study attempts to examine one mechanism by which CO may regulate the inflammatory responses. Further studies on

how activation of C/EBP isoforms are inhibited by CO are needed to better understand the mechanism of the anti-inflammatory effect of CO.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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