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## Carbon Monoxide Decreases the Level of iNOS protein and Active Dimer in IL-1 $\beta$ -Stimulated Hepatocytes

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### Abstract

There is evidence that NO can regulate CO production, however less is known about CO regulation of NO synthesis. Our studies were undertaken to define how CO regulates iNOS in cultured hepatocytes. CO (250 ppm) exposure resulted in a significant decrease in iNOS protein, nitrite production, level of active iNOS dimer and cytosolic iNOS activity in cells stimulated with cytokines (IL-1 $\beta$ ) or transfected with the human iNOS gene. However, IL-1 $\beta$ -stimulated iNOS mRNA expression was unaffected by CO. These effects of CO on iNOS protein levels were inhibited when CO was scavenged using hemoglobin. HO-1 induction with an adenoviral vector carrying HO-1 showed a decrease in total iNOS protein, nitrite production, and iNOS dimer level from cells stimulated by IL-1 $\beta$ . iNOS protein level was significantly higher in lung endothelial cells isolated from HO-1 knockout mice compared to wild type cultures stimulated with cytokines mixture. CO was found to increase p38 phosphorylation and p38 inhibition using SB203580 increased iNOS protein levels in response to IL-1 $\beta$ . Interestingly, proteasome inhibitors (MG132 and Lactacystin) and an autophagy inhibitor (3-Methyladenine) reversed CO influence iNOS levels. Our results imply that CO exposure decreases NO production by suppressing dimer formation and increasing iNOS degradation through a process involving p38 activation.

### Keywords

Heme oxygenase; Carbon monoxide; Inducible nitric oxide synthase; Nitric oxide; proteasome

### Introduction

The inducible nitric oxide synthase (iNOS) gene is expressed by hepatocytes in a number of physiologic and pathophysiological conditions affecting the liver including sepsis and hemorrhagic shock (1–3). iNOS protein and the CO product of its enzymatic activity, nitric oxide (NO) modulates several aspects of liver injury. iNOS expression potentiates the hepatic oxidative injury in warm ischemia/reperfusion (4), while NO protects against hepatic apoptotic cell death seen in models of sepsis and hepatitis. Anti-apoptotic actions are either cyclic nucleotide dependent or independent, and include the induction of heat shock protein 70 expression, prevention of mitochondrial dysfunction, and inhibition of caspase activity (5–8).

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Heme oxygenase (HO) catalyzes the breakdown of heme into equimolar concentrations of carbon monoxide (CO), biliverdin, and iron. The inducible isoform, HO-1, is potentially involved in the control of intracellular heme levels and is believed to play an important role in attenuating tissue injury caused by inflammatory stimuli (9). Notably, up-regulation of HO-1 has been shown to protect against lipopolysaccharide (LPS)-induced cardiovascular collapse, and minimizes ischemic liver damage (10).

CO, like NO, is a second messenger gas involved in a number of physiological processes (11). Both CO and NO activate soluble guanylate cyclase to increase cyclic GMP (cGMP) levels. It is becoming increasingly clear that iNOS/NO and HO-1/CO can modulate each other's activity. These two systems are linked in that NO can up-regulate HO-1 expression leading to the formation of endogenous CO (10;12;13), and CO can bind to the heme group in the iNOS protein and influence the production of NO (14;15). Although much is known about the HO-1/CO and iNOS/NO pathways, how these two important systems interact is less understood.

It has been postulated that CO serves as an intracellular signal to modulate the tissue stress response and interacts with iNOS to control its expression and activity (13;16–19). Specifically, CO could act as a feedback inhibitor of iNOS when the concentrations of NO exceed a critical threshold.

Interleukin-1beta (IL-1 $\beta$ ) is a key mediator in a variety of inflammatory responses and the most effective cytokine for the induction of the iNOS gene expression (20;21). In this report we investigated the effect of CO on iNOS expression and NO production in IL-1 $\beta$ -treated hepatocytes. Exogenous CO decreased iNOS expression associated with suppressed iNOS dimer formation. Our findings suggest CO is a feedback inhibitor of iNOS expression and activity.

## Material and Methods

### Materials

Williams Medium E, penicillin, streptomycin, L-glutamine, HEPES were purchased from Life Technologies, Inc. Mouse recombinant IL-1 $\beta$  was obtained from R & D Systems. Polyclonal antibodies towards the following: phospho-ERK 1/2 (Thr202/Tyr204), phospho-Akt (Ser473), phospho-p38, p38, phospho-JNK and I $\kappa$ B were purchased from Cell Signaling. Polyclonal anti-HO-1 anti-NF- $\kappa$ B antibodies were purchased from StressGen Biotechnologies and SantaCruz. KT5823, LY294002, PD 98059, SP600125 and wortmannin are from Calbiochem. [ $\gamma$ -32P]ATP was obtained from NEN Life Science Products. 1H-(1,2,4)-oxadiazole [4,3-a] quinoxalin-1-one (ODQ) was purchased from Promega. Cobalt protoporphyrin (CoPPIX), and tin protoporphyrin (SnPPIX) were acquired from Porphyrin Products. All other reagents are from Sigma, unless otherwise indicated.

### Cell Culture

Primary hepatocytes were isolated and purified from rat and cultured as described previously (22). Highly purified hepatocytes (>98% purity and >98% viability by trypan blue exclusion) were suspended in Williams medium E supplemented with 10% calf serum, 1  $\mu$ M insulin, 2 mM L-glutamine, 15 mM HEPES (pH 7.4), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of  $2 \times 10^5$  cells/well in 12-well plates for cell viability analysis or  $3 \times 10^6$  cells/60-mm dish for Western blot and EMSA. After 18 h fresh medium containing 5% calf serum was added including designated treatments.

### Preparation of Whole Cell Lysate

Cells were cultured in medium with or without serum for 24 h and then stimulated with CO or other specified reagent. Monolayers were rinsed twice with ice-cold PBS, and crude cell lysates were isolated with 300  $\mu$ l of whole cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu$ g/ml leupeptin, and 1 mM PMSF) was then added to each plate. The cells were lysed by three freeze and thaw cycles. Cytosolic fractions were obtained by centrifugation of the ruptured crude lysates at 12,000 $\times$ g for 20 min at 4°C and aliquots were stored at -80 °C until use. Protein concentration was determined by BCA (Pierce).

### Western blot Analysis

Proteins were separated on 10% SDS-PAGE in denaturing or non-denaturing conditions and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies for iNOS (1:1000), HO-1 (1:2000), p-Akt (1:1000), p-ERK (1:1000), p-p38 (1:500), p-JNK (1:500), beta-actin (1:5000) in PBS-Tween (0.01%) (PBS-T) containing 1% nonfat milk for 1 h. The immunoblots were developed following a 2 min incubation with enhanced chemiluminescent (ECL) solution (Pierce), and exposure to x-ray film.

### Luciferase Reporter Assays

Evaluation of NF- $\kappa$ B activation was performed using a luciferase reporter assay. In brief, hepatocytes were co-transfected with NF- $\kappa$  B reporter constructs (100 ng/well; pGL3-NF- $\kappa$  B luciferase; provided by D. Golenbock, University of Massachusetts, Worcester, MA) and pIEP-Lac-z (0.5  $\mu$ g/well) using Lipofectin (Invitrogen) according to the manufacturer's instructions (23). Evaluation of iNOS expression was performed using a luciferase reporter assay with transient transfection of hepatocytes being accomplished with an iNOS promoter reporter constructs (1  $\mu$ g/well; pXP2; provided by C. Lowenstein, Johns Hopkins University, Baltimore, MD) or pIEP-LacZ (0.5  $\mu$ g/well) described in published work (24). Hepatocytes were treated with various stimuli after transfection for 24 h. Luciferase activity (reported as arbitrary units [A.U.]) was assayed 6 h post treatment using a luciferase assay kit (Promega) as per the manufacturer's instructions and measured on a Berthold Luminometer. Results were corrected for transfection efficiency and protein concentration.

### Northern Blot

Total RNA isolated from cultured hepatocytes using the method of RNazol adapted as described by Chomczynski and Sacchi (25). RNA (20  $\mu$ g) was loaded onto a 1% agarose gel containing 3% formaldehyde, transferred onto a nylon membrane and UV auto-crosslinked (UV Stratalinker 1800, Stratagene). The membranes were pre-hybridized at 43°C overnight as previously described and hybridized by random priming (Boehringer Mannheim) with [ $^{32}\text{P}$ ] dCTP-iNOS probe ( $\sim 2 \times 10^6$  cpm/ml), a 2.7 kb Not I fragment of the murine macrophage cDNA effective for identifying iNOS mRNA in rat hepatocytes (26). The hybridized filters were washed three times, and exposed to autoradiography film.

### Measurement of Nitrite

Nitrite was assayed by the Griess reaction. Briefly, 100  $\mu$ l of Griess reagent was added to 100  $\mu$ l of culture supernatant in a 96-well plate. Absorbance was measured at 550 nm using a 96-well plate reader, and nitrite concentration was calculated from a standard curve of sodium nitrite.

## Transfection of Adenovirus

Adenoviral vectors carrying the genes for bacterial  $\beta$ -galactosidase (Ad-LacZ), human iNOS (Ad-iNOS) and recombinant HO-1 (Ad-HO-1) were prepared as described previously (27; 28). Following overnight culture, hepatocytes were washed with Hanks' buffered saline twice prior to transduction at multiplicity of infection of 1 in a volume of 2 ml of Opti-MEM (Life Technology). Following a 6 h infection, the medium was changed to fresh Williams E containing 5% calf serum. After 24 h, cells infected with adenovirus were treated with CO or IL-1 $\beta$ . Samples were isolated at the indicated time points.

## Statistical Analysis

Data are presented as means  $\pm$  S.D. of the mean of at least three separate experiments.

## Results

### CO decreased the level of iNOS protein in hepatocytes stimulated by cytokines

To examine the effects of CO on hepatocyte iNOS expression and activity we first measured the consequences of exogenous CO exposure on iNOS protein levels and NO production in cytokine-stimulated hepatocytes (29). We have previously shown that IL-1 $\beta$  is the most effective cytokine stimulator of hepatocyte iNOS expression (30). Consistent with this previous report, Fig. 1A shows that iNOS protein levels at 24 hrs following cytokine exposure are highest with IL-1 $\beta$  alone with weak induction by TNF or IFN $\gamma$ . A combination of the three cytokines shows the expected synergy with greater iNOS expression. CO exposure for 24 hr markedly suppresses iNOS protein levels and decreases nitrite release by over 50% (Fig. 1B). The reduction in iNOS protein expression for IL-1 $\beta$  stimulated hepatocytes was confirmed by immunohistochemistry (Fig. 1C).

### CO decreases the level of active iNOS dimer and iNOS activity in response to IL-1 $\beta$

iNOS is active only as a homodimer (31) and CO may alter iNOS activity by interfering with dimer formation. Using non-denaturing SDS PAGE and Western blotting we found that CO exposure for 24hr markedly decreased the levels of dimeric iNOS (Fig. 2A). Doubling the CO levels to 500 ppm had no additional effect. To determine how CO affects the dimer to monomer ratio we quantified levels by UN-SCAN-IT gel for Windows 5.1 (Silk Scientific Corporation) at 12 hrs following IL-1 $\beta$  exposure. The ratio of iNOS dimer to monomer was 1 to 1 in air treated cells, but 0.5 to 1 in CO exposed cells (Fig. 2B). Fig. 2C shows the corresponding decrease in nitrite production at 12 hrs.

### Upregulation of HO-1 expression by CoPP or adenoviral vector decreased the level of iNOS protein and nitrite accumulation

Catabolism of cellular heme by HO-1 has been reported to limit new iNOS synthesis due to the requirement for heme to form functionally competent iNOS (16;31–37). HO-1 expression was measured in CO-treated cells (Fig. 3A) and was found to be induced to a greater degree by CO in cytokine-treated cells. To test whether enhanced HO-1 expression reduced iNOS expression, cells were treated with cobalt protoporphyrin (CoPP) to up-regulate HO-1 expression. CoPP treatment decreased IL-1 $\beta$  induced iNOS protein expression in a dose dependent manner (Fig. 3B). Cells treated with CoPP (100  $\mu$ M) and IL-1 $\beta$  for 12 hrs had both a decrease in the level of active iNOS dimer and nitrite accumulation (Fig. 3C and D). Overexpression of HO-1 using an adenoviral vector for 24 hrs followed by IL-1 $\beta$  stimulations also resulted in lower iNOS dimer protein levels and a significant decrease in nitrite accumulation (Fig. 4A and B) compared to control  $\beta$ -galactosidase-transduced cells.

### **Endothelial cells from HO-1 knockout mouse express higher levels of iNOS protein in response to cytokine stimulation**

Lung endothelial cells isolated from HO-1 knockout animals were used to determine if iNOS dimer levels and subsequent nitrite generation are modulated by HO-1. As shown in Fig. 5, cells from the HO-1 knockout mouse expressed higher levels of iNOS protein compared with that of cells from wild type mice in response to cytokine stimulation. Exogenous CO resulted in a modest reduction in the level of active iNOS dimer in the wild type cells. These findings support the conclusion that endogenous HO-1 modulates iNOS expression.

### **CO regulation of iNOS protein levels occurs at the translational or post-translational level**

We investigated iNOS mRNA levels from cells stimulated with IL-1 $\beta$  in the presence or absence of CO. Northern blot experiments showed no effect of CO on IL-1 $\beta$ -induced iNOS mRNA steady state levels (Fig 6A). As seen in Fig 6B, iNOS promoter activity was similarly unaffected by CO treatment.

To express iNOS independent of cytokine stimulation, hepatocytes were transduced using an adenoviral vector carrying iNOS. As shown in Fig 7A and B, CO exposure resulted in lower levels of iNOS protein and nitrite production in cells transfected with adenoviral vector carrying the human iNOS gene compared to cells not exposed to CO.

### **CO suppressed iNOS expression in IL-1 $\beta$ stimulated hepatocytes through modulation of p38 kinase and the proteasome degradation pathway**

CO has been reported to modulate cell signaling through MAP kinase and guanylate cyclase pathways (38;39). We carried out experiments to determine if CO modulation of MAP or Akt accounted for the inhibition of IL-1 $\beta$ -induced iNOS expression. CO enhanced IL-1 $\beta$  induced p38 activation, had no effect on phosphorylation of ERK and JNK, and suppressed the phosphorylation of Akt (Fig. 8A)(40). To determine whether the enhanced activation of p38 MAPK contributed to the inhibitory effect of CO on iNOS expression, cultured hepatocytes were treated for 24 h with IL-1 $\beta$  and CO in the absence or in the presence of SB203580 (10  $\mu$ M), a selective inhibitor of p38 MAPK. SB203580 reversed the inhibitory effect of CO on IL-1 $\beta$ -induced iNOS protein expression. As expected the ERK inhibitor, PD98059, had no effect on IL-1 $\beta$  induced iNOS protein levels (Fig. 8B). Inhibitors of guanyl cyclase (ODQ) and Akt (wortmannin) likewise had no effect on the CO-inhibition of iNOS expression (data not shown).

iNOS degradation is known to be proteasome dependent upon stable expression of human iNOS in human epithelial kidney (HEK293) cells (40). To determine if the effects of CO required proteasome activity, IL-1 $\beta$  stimulated hepatocytes were exposed to CO in the presence or absence of the proteasome inhibitor MG132 or Lactacystin. The presence of proteasome inhibitors (MG132; 1 and 5  $\mu$ M or Lactacystin; 0.25 and 2.5  $\mu$ M) prevented the reduction of iNOS expression in IL-1 $\beta$  treated hepatocytes resulting from CO exposure (Fig. 9).

## **Discussion**

This study was undertaken to determine how CO modulates iNOS protein expression and NO production induced by IL-1 $\beta$  in hepatocytes. CO (250 ppm) down regulated iNOS protein and suppressed the level of active iNOS dimer formation in IL-1 $\beta$ -stimulated hepatocytes. Overexpression of HO-1 using CoPP or adenovirus also effectively decreased the level of iNOS protein, and enzymatically active iNOS. Our results implicate p38 in the CO mediated effects on iNOS expression. The failure to see the reduction in iNOS in response to CO in the presence of proteasome inhibitors suggests that targeting NOS for degradation may be one mechanism

by which CO lowers iNOS expression. The suppression of dimer formation raises the possibility that CO might also directly impact on iNOS activity.

CO has been shown to be cytoprotective by modulating NF- $\kappa$ B and iNOS in several types of cells including endothelial cells, hepatocytes and macrophages. We reported that CO increased level of iNOS protein following TNF $\alpha$ /D-galactosamine treatment *in vivo*, resulting in liver protection (29). Our study presented here shows that CO decreased iNOS protein levels in IL-1 $\beta$  stimulated cells. The interplay between CO/HO-1 and NO/iNOS has been the subject of much debate, not only because of the possibilities of exploiting these pathways as therapeutic targets, but also the possible ramifications of interfering with either CO or NO production. The CO/HO-1 and NO/iNOS pathways are linked in that NO can up regulate HO-1 expression leading to the formation of endogenous CO (41), and CO can bind heme within iNOS and influence the enzyme activity of iNOS and the production of NO (42). Both pathways are expressed in a number of stress conditions, including ischemia/reperfusion and endotoxemia (43). Thus, it is likely that the interplay between CO and NO production takes place. For example, NO can induce HO-1 expression at moderate levels by iNOS. CO produced by HO-1 could then suppress iNOS activity limiting NO production to reduce the risk of iNOS-mediated toxicity.

Our results suggest that the effects of CO on iNOS protein levels are mediated through p38 (13) modulation of protein turnover(16;17). Interplay between p38 and CO to modulate inflammatory cytokines and induction of stress proteins has been established in clinically relevant *in vivo* and *in vitro* models (38). For example, CO induced cell death is prevented by blocking p38 activity. Our results suggest that CO promotes iNOS protein turnover through the activation of p38 as seen in other models (44–46). Post-translational modification of iNOS protein by ubiquitination and phosphorylation are mechanisms reported to modulate iNOS protein level (47;48). These investigators performed co-transfection in HEK293T cells with plasmids for both human iNOS and ubiquitin or an ubiquitin mutant (K48R) to demonstrate that iNOS ubiquitination precedes protein degradation. iNOS monomers and dimers had similar half-lives but both increased in the presence of proteasome inhibitors (48). The relatively short half-life of iNOS was a rather surprising finding because iNOS is known for its prolonged production of large amounts of NO. Interestingly in our studies, the effect of protein turnover inhibitors on iNOS protein levels, proteasome inhibitors (MG132 and Lactacystin) and an autophagy inhibitor (3-Methyladenine), superceded the effect of CO on iNOS levels. Our results imply that CO exposure decreases NO production by suppressing dimer formation and increasing iNOS degradation through a process involving p38. Specifically, CO could act as a feedback inhibitor of iNOS when the concentrations of NO gaseous molecule exceed a critical threshold and the products of heme degradation might play a role in this process.

Dimerization is necessary for catalytic activity for each of the three NOS isoforms. Dimerization of iNOS is distinct from the constitutive isoforms (nNOS and eNOS) in that it is independent of increase in intracellular Ca<sup>+2</sup> levels and subsequent calmodulin binding for NO synthesis (49). iNOS is synthesized initially as a monomer, which is enzymatically inactive and dependent on the concomitant cytokine stimulated synthesis of BH<sub>4</sub> and heme in order to dimerize and generate NO (50). In aqueous solutions, NO reacts to form NO<sub>2</sub><sup>-</sup>, which, in the presence of heme-containing proteins will be further oxidized to form NO<sub>3</sub><sup>-</sup> (51). These properties of NO allow for an indirect measure of the redox environment in which iNOS is found. The generation of NO contributes to the regulation of iNOS enzymatic activity: a high level of NO down regulates heme and BH<sub>4</sub> synthesis, thereby creating a mixed population of iNOS monomer and dimer. Panda *et al* (2002) found in IL-1 $\beta$  treated hepatocytes exposed to exogenous CO had diminished iNOS total protein and active dimer due to the above mentioned feedback inhibition of iNOS/NO generation system. These authors concluded that the differences in the three NOS isoform dimer strength results from the varied cellular

environments in which each isozyme is expressed, specifically iNOS is BH<sub>4</sub> dependent to form stable dimers (52).

While it is not clear how CO differently regulates iNOS expression in different organs or different stimuli, CO exerts anti-inflammatory and anti-apoptotic effects by modulating iNOS. Considering that pathophysiological conditions such as hemorrhagic shock and ischemic reperfusion injury result in increased overproduction of NO (53;54), our findings reveal a potential interaction between CO/HO-1 and NO/iNOS in mediating protection against NO/iNOS-mediated liver damage. Depending on the circumstances, sustained NO production up-regulates HO-1. CO generated by HO-1 would then act to suppress initially iNOS activity and then promote iNOS degradation in an important feedback mechanism.

## Abbreviations

<b>HO</b>	heme oxygenase
<b>iNOS</b>	inducible nitric oxide synthase
<b>IL-1<math>\beta</math></b>	interleukin-1beta MAPK, mitogen-activated protein kinase
<b>NOS</b>	nitric oxide synthase
<b>NF-<math>\kappa</math>B</b>	nuclear factor $\kappa$ B

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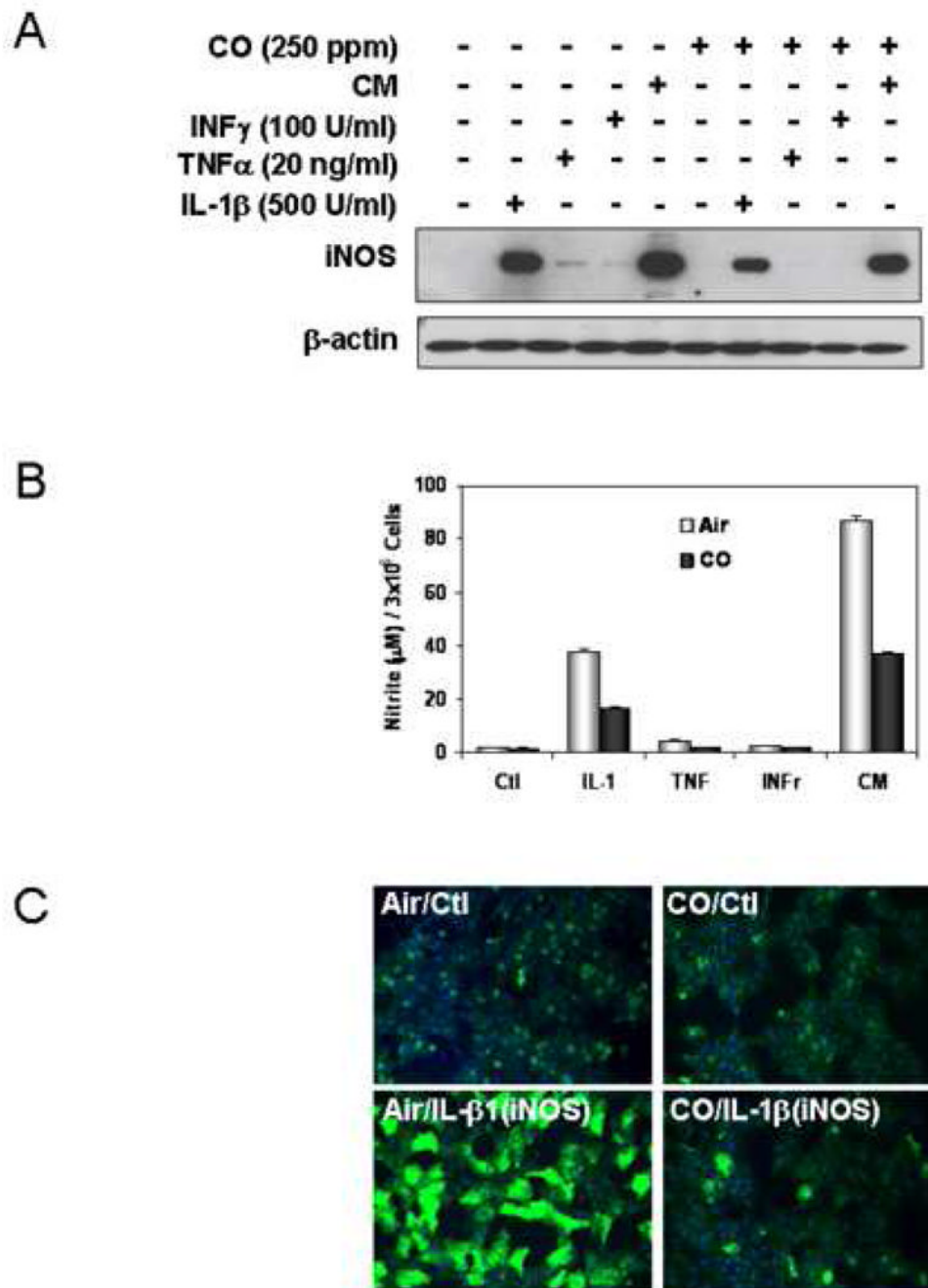
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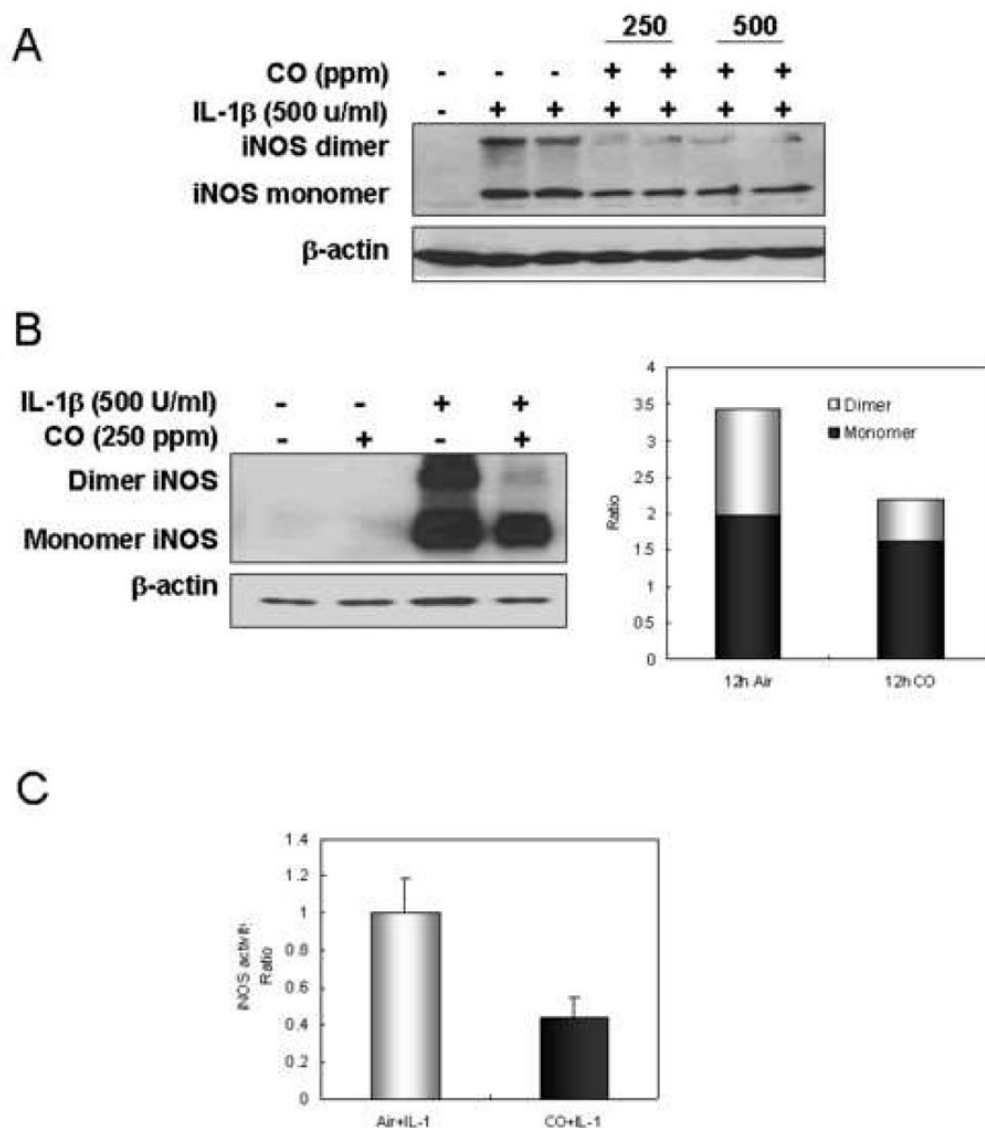
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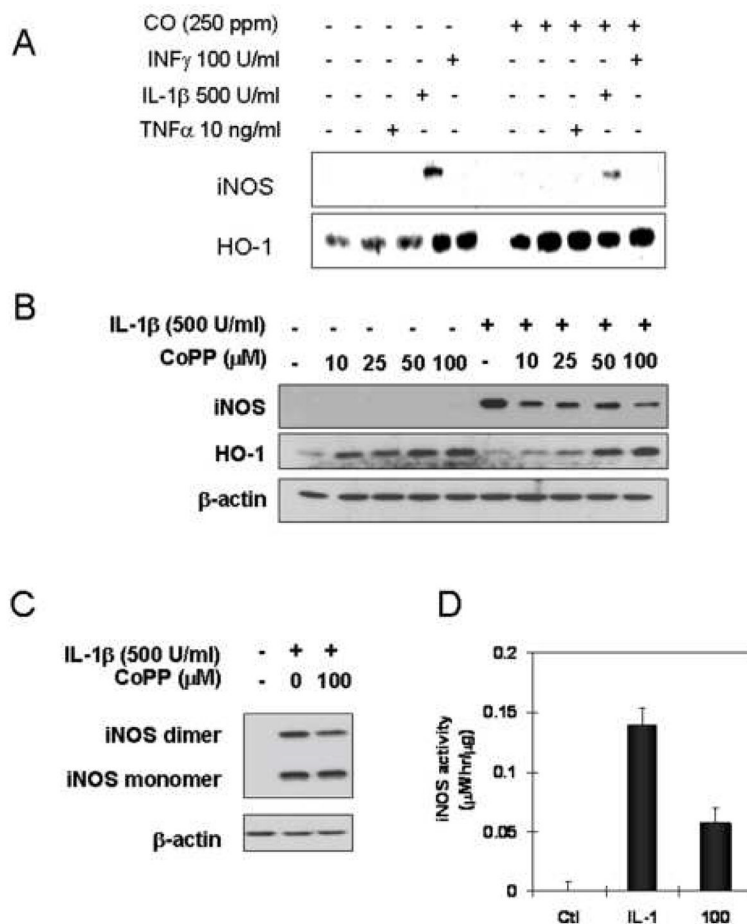
**Figure 1.**

CO decreases hepatic iNOS protein and nitrite production in response to IL-1 $\beta$  and cytokine mixture (CM). Rat hepatocytes were cultured for 24h in the presence of CM (IL-1 $\beta$ ;500 U/ml, INF $\gamma$ ;100U/ml, TNF $\alpha$ ;10ng/ml) with or without a 2h pre-exposure of CO (250 ppm), (A) Western blot was performed using denaturing SDS-PAGE. (B) Accumulated nitrite for 24h was measured in the culture medium using Griess reagent. Data are means $\pm$ S.D of three separate experiments. (C). Immunostaining for iNOS, cells were treated with or without CO (250 ppm) for 1 h, stimulated with IL-1 $\beta$  (500 U/ml) for 24h in the presence or absence of CO, monolayers were washed 2 $\times$  with cold PBS and then cells were fixed in 2% paraformaldehyde. Fixed cells were incubated with the primary monoclonal anti-iNOS antibody, and the primary

antibody staining was detected with the FITC-conjugated anti mouse IgG. Nuclei of cells were stained with Hoechst 33342. Images were taken at a magnification of 20× using a Olympus Provis microscope.

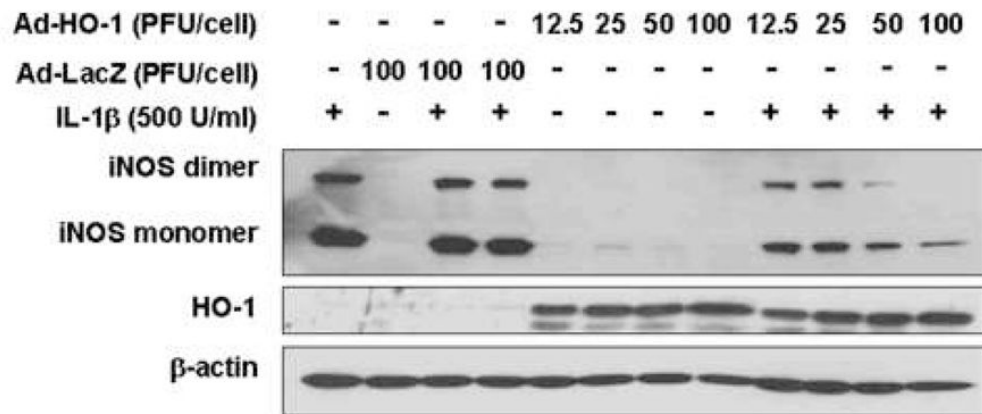
**Figure 2.**

CO decreases the level of active iNOS dimer and iNOS activity in response to IL-1 $\beta$ . (A) Hepatocytes were stimulated with IL-1 $\beta$  (500 U/ml) for 24h at different concentration of CO (250 or 500 ppm). After 24h, cells were harvested, washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at 12,000 $\times$ g for 20 min and then subjected to western blot. (B) Hepatocytes were treated with or without CO (250 ppm) for 2h, and stimulated with IL-1 $\beta$  (500 U/ml) for 12h in a presence or absence of CO. After 12h, monolayers were washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at 12,000 $\times$ g for 20 min and then western blot was performed using non-denaturing SDS-PAGE. Graph displays the ratio of active dimer to monomer from western blot. (C) Cytosolic iNOS activity was measured in the presence of 1 mM NADPH, 20  $\mu$ M FAD, 20  $\mu$ M FMN, and 4 mM L-arginine, with exogenous 100  $\mu$ M BH<sub>4</sub> at 37°C for 2h. Accumulated nitrite of cytosolic protein was measured using the Griess reagent. Data represent the mean  $\pm$  S.D. of more than three independent experiments.

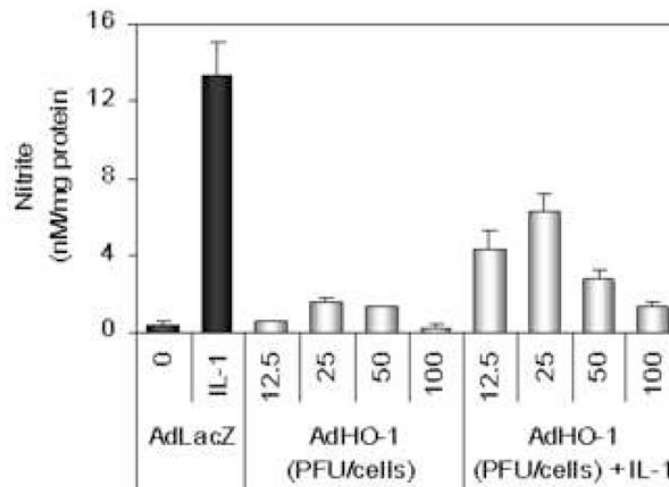
**Figure 3.**

HO-1 induction by CoPP suppresses the level of iNOS protein and active iNOS dimer in response to IL-1 $\beta$ . (A) Rat hepatocytes were cultured for 24h in the presence of cytokines (IL-1 $\beta$ ;500 U/ml, INF $\gamma$ ;100U/ml, TNF $\alpha$ ;10ng/ml) with or without CO (250 ppm) following the 2h pre-exposure of CO. Western blot for iNOS or HO-1 was performed using denaturing SDS-PAGE. (B). Cells were treated with CoPP at indicated concentration (10, 25, 50, 100  $\mu$ M) for 20h and further incubated with treatment of IL-1 $\beta$  (500 U/ml) for 24h. Cytosolic fractions were prepared by centrifugation at 12,000 $\times$ g for 20 min and western blot was performed using denaturing SDS-PAGE. (C). Cells were incubated with CoPP (100  $\mu$ M) for 20h prior to addition of IL-1 $\beta$  (500 U/ml). After 12h cells were washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at 12,000 $\times$ g for 20 min and western blot was performed using non-denaturing SDS-PAGE. (D). Cytosolic iNOS activity was measured in the presence of 1 mM NADPH, 20  $\mu$ M FAD, 20  $\mu$ M FMN, and 4 mM L-arginine, with exogenous 100  $\mu$ M BH<sub>4</sub> at 37°C for 2h. Accumulated nitrite of cytosolic protein was measured using Griess reagent. Data represent the mean  $\pm$  S.D. of more than three independent experiments

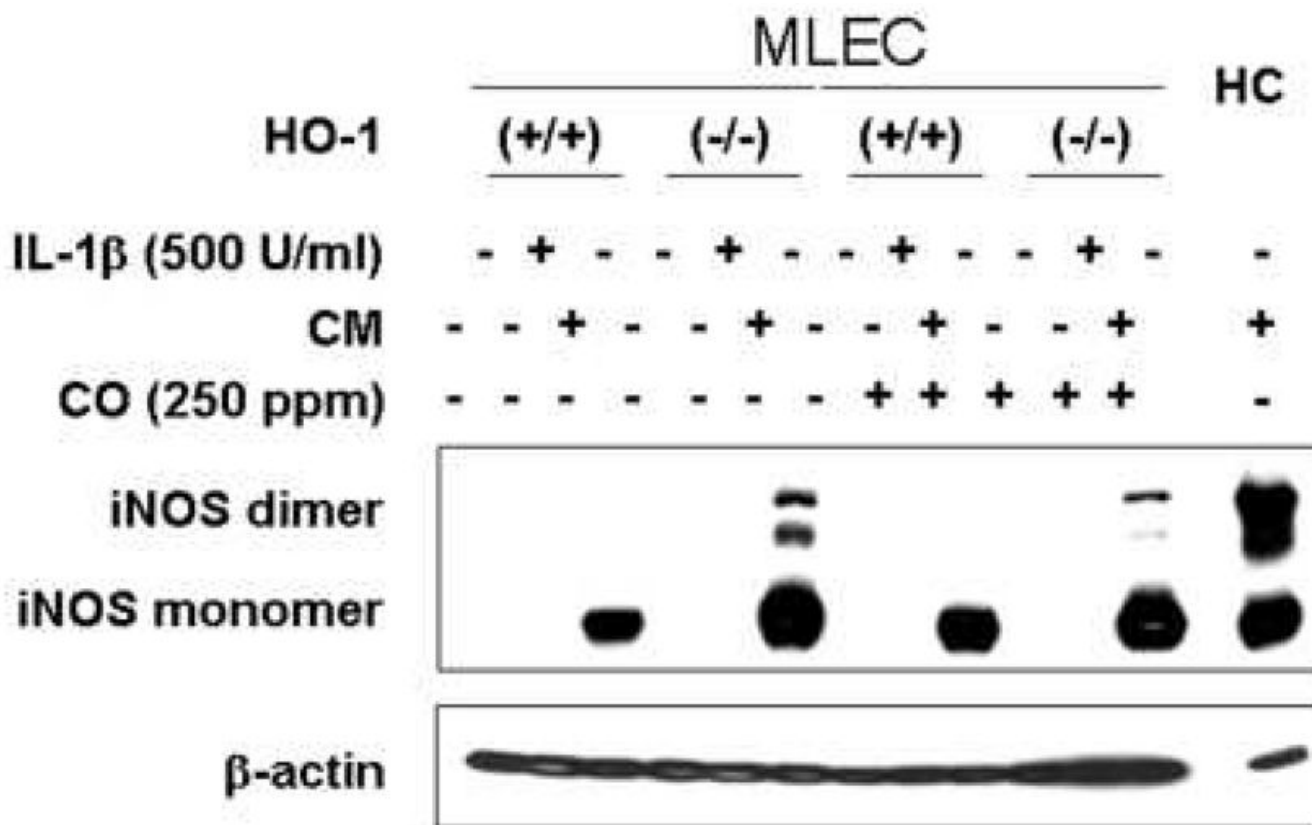
A



B

**Figure 4.**

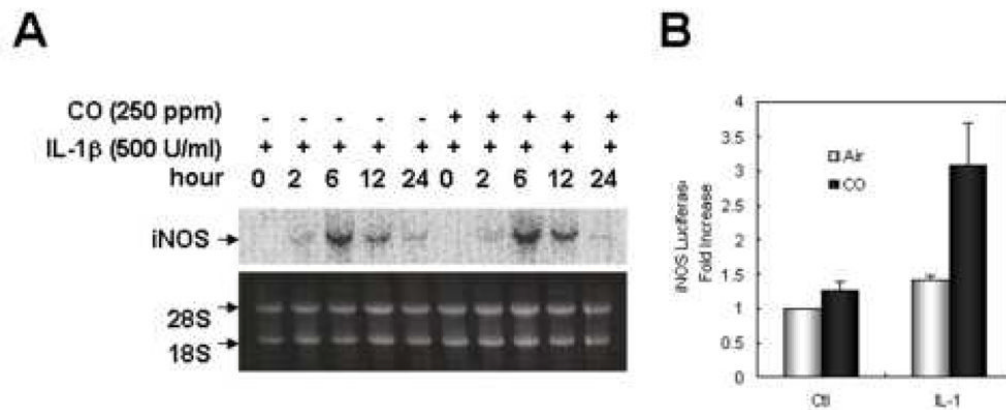
HO-1 overexpression by infection of a recombinant HO-1 adenovirus decreases iNOS protein in response to IL-1 $\beta$ . (A). Modified adenoviral vectors expressing HO-1 or  $\beta$ -galactosidase transduced in hepatocytes at the indicated multiplicity of infection (12.5, 25, 50, 100 PFU/cell). The infected hepatocytes were recovered overnight prior to changing to fresh medium and initiating iNOS expression by treatment with IL-1 $\beta$  (500 U/ml) for 24h. Monolayers were harvested after being washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at 12,000 $\times g$  for 20min and then western blot was performed using non-denaturing SDS-PAGE. (B) Accumulated nitrite was measured in the culture medium using Griess reagent. Data represent the mean  $\pm$  S.D. of more than three independent experiments



**Figure 5.**

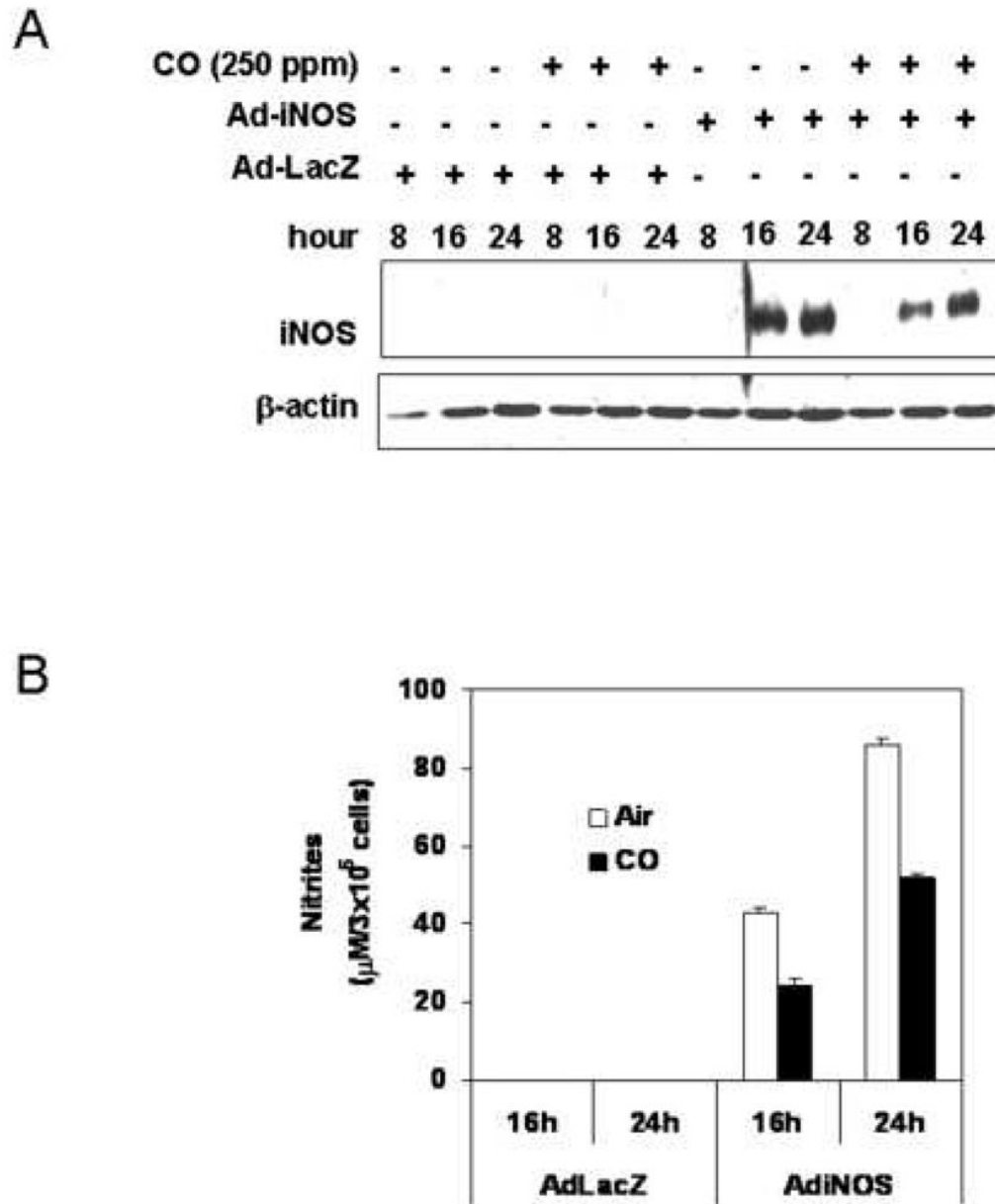
Endothelial cells from HO-1 knockout mouse increased the level of iNOS protein in response to cytokine mixture. Cells were incubated with IL-1 $\beta$  (500 U/ml) or cytokine mixture (CM; IL-1 $\beta$  (100 U/ml) INF $\gamma$  (100 U/ml) and TNF $\alpha$  (20 ng/ml)) in the presence or absence of CO (250 ppm). After 24h, monolayers were harvested after being washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Cytosolic fractions were prepared by centrifugation at 12,000 $\times$ g for 20min and then western blot was performed using non-denaturing SDS-PAGE. MLEC; mouse lung endothelial cell, HC; hepatocyte





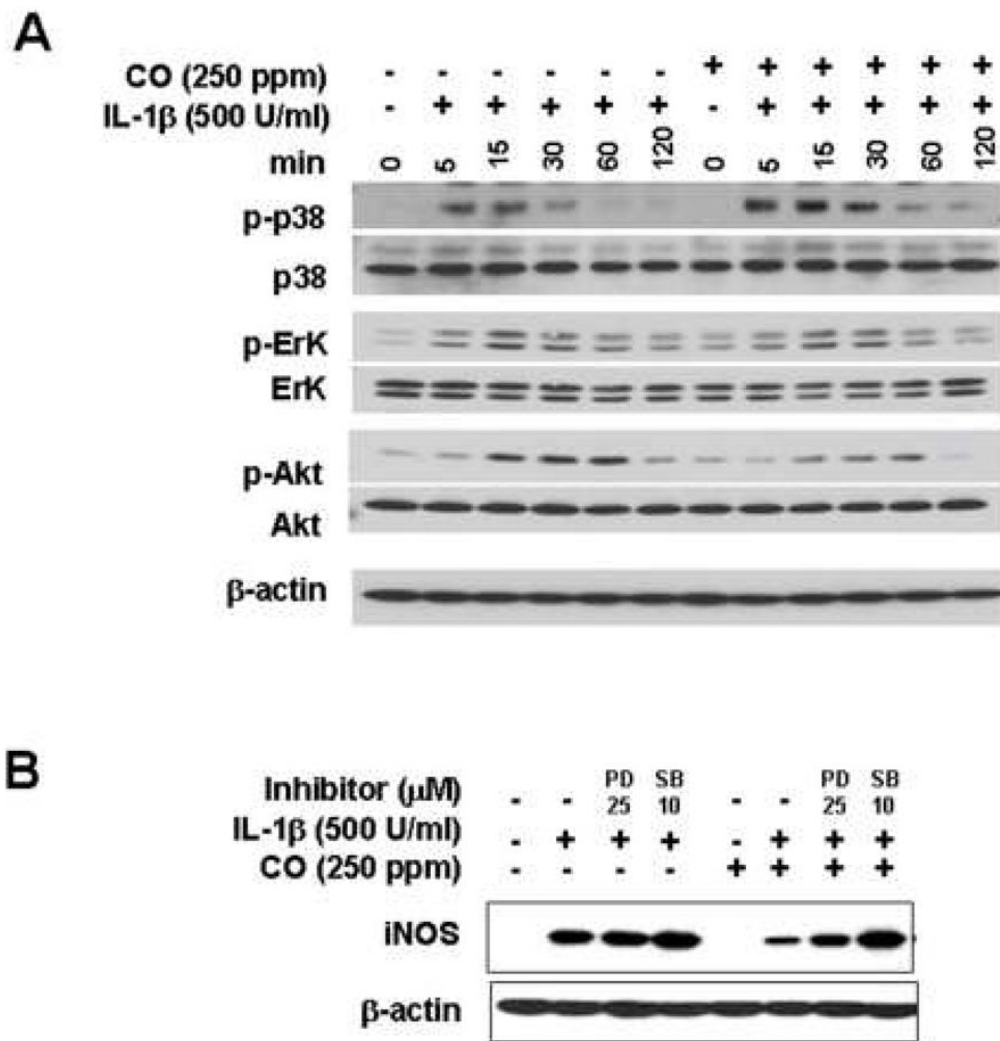
**Figure 6.**

CO does not increase the level of iNOS mRNA significantly. (A) Hepatocytes were incubated with IL-1 $\beta$  (500 U/ml) in the presence or absence of CO (250 ppm) for the indicated times (2, 6, 12, 24h). Total RNA was extracted from hepatocytes by the RNAzol method. The RNA (20  $\mu$ g) was electrophoresed on 1% agarose gel containing 1% formaldehyde, transferred onto a nylon membrane, and prehybridized with whale sperm DNA at 43°C overnight. The membrane was hybridized with  $^{32}$ P-labeled probes ( $\sim 2 \times 10^6$  cpm/ml) of murine macrophage iNOS, washed three times, and exposed to autoradiography film. (B) Evaluation of iNOS promoter activity was performed using a luciferase reporter assay. Hepatocytes were transfected with human iNOS promoter reporter construct for 24 h. Cells were incubated with IL-1 $\beta$  (500 U/ml) in a presence or absence of CO for 6h. Luciferase activity (reported as arbitrary units [A.U.]) was assayed 6 h after initiation of treatment using a luciferase assay kit (Promega) according to the manufacturer's instructions and measured on a Berthold Luminometer. Results were corrected for transfection efficiency and protein concentration. Data represent the mean  $\pm$  S.D. of more than three independent experiments.

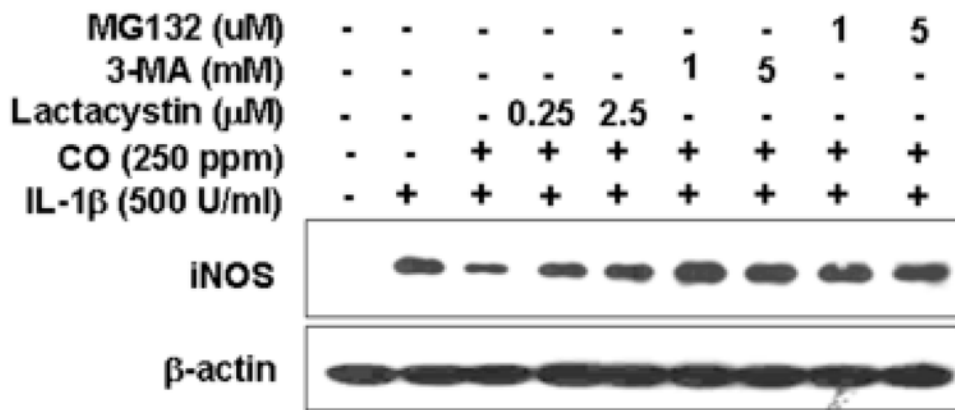


**Figure 7.**

CO decreases nitrite production and iNOS protein from hepatocytes transfected with adenoviral vector carrying a human iNOS gene. Modified adenoviral vectors (MOI of 3) carrying a human iNOS gene or  $\beta$ -galactosidase were prepared. (A). The hepatocytes were incubated with CO (250 ppm) for 4h prior to infection of adenoviral vector. The cells were transfected with adenoviral vector in a serum-free medium for 6h, and recovered for indicated time (8, 16, 24h) in fresh medium with 5 % serum in the absence or presence of CO. Monolayers were washed with ice-cold PBS, and lysed by three cycles of freeze and thaw. Western blot was performed from cytosolic fractions collected by centrifugation at  $12,000\times g$  for 20min. (B). Accumulated nitrite was measured in the culture medium using Griess reagent. Data represent the mean  $\pm$  S.D. of two independent experiments.

**Figure 8.**

CO enhances phosphorylation of p38 MAPK, while suppressing Akt phosphorylation in IL-1 $\beta$ -treated hepatocytes. (A) Cells were starved for 24h in medium without serum and stimulated with IL-1 $\beta$  (500 U/ml) in the presence or absence of CO (250 ppm). Cell lysed in lysis buffer containing with inhibitor of phosphatase and protease. Western blot was performed from cytosolic fraction using denaturing SDS-PAGE. The SB203580 reversed the inhibitory effect of CO on IL-1 $\beta$ -induced iNOS expression and nitrite production. (B) Cells were incubated with inhibitors of p38 or ERK (SB;10  $\mu$ M, PD; 25  $\mu$ M) for 1h and further incubated with IL-1 $\beta$  for 24h in a presence or absence of CO (250 ppm).



**Figure 9.**

Proteasome inhibitors increased the level of iNOS protein suppressed by CO. Cells were treated with IL-1 $\beta$  for 24h in a presence or absence of CO (250 ppm) following administration of proteasome inhibitor (3-Methyladenine; 1 and 3 mM, MG132; 1 and 5  $\mu$ M or Lactacystin; 0.25 and 2.5  $\mu$ M), western blot was performed from cytosolic fraction.