BIOCHEMICAL PHARMACOLOGY 71 (2006) 307–318

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

CO from enhanced HO activity or from CORM-2 inhibits both O_2^- and NO production and downregulates HO-1 expression in LPS-stimulated macrophages

Klaokwan Srisook [a,1], *Shan-Shu Han* [a], *Hyung-Sim Choi* [a], *Mei-Hua Li* [b], *Hideo Ueda* [c], *Chaekyun Kim* [a,**], *Young-Nam Cha* [a,*]

[a] *Department of Pharmacology and Toxicology, College of Medicine, Inha University, Incheon 400-712, Republic of Korea*
[b] *National Research Laboratory, College of Pharmacy, Seoul National University, Seoul, Republic of Korea*
[c] *Mitleben R&D Associates, Division of Taiyo Instruments, Inc., Osaka, Japan*

ARTICLE INFO

Article history:
Received 19 July 2005
Accepted 21 October 2005

Keywords:
Carbon monoxide
CORM-2
Heme oxygenase
Superoxide
Nitric oxide

ABSTRACT

Carbon monoxide (CO) arising from heme degradation, catalyzed particularly by the stress-inducible heme oxygenase-1 (HO-1), has recently been demonstrated to provide cytoprotection against cell death in macrophages stimulated with bacterial lipopolysaccharide (LPS). In the present study, we determined the effects of CO on the production of reactive oxygen species (ROS) and nitric oxide (NO) by the LPS-stimulated RAW 264.7 macrophages. In addition, effect of CO-exposure on the production of superoxide (O_2^-) in the phorbol myristate acetate (PMA)-stimulated PLB-985 neutrophils was determined. Production of ROS by the LPS-stimulated macrophages pretreated with 50 μM $[Ru(CO)_3Cl_2]_2$, a CO-releasing molecule (CORM-2), was abolished and the production of O_2^- by the PMA-stimulated neutrophils pretreated with the CORM-2 was decreased markedly. The CORM-2 (50 μM) was not cytotoxic to both the unstimulated and LPS-stimulated macrophages when determined by employing mitochondrial reductase function test (MTT assay). In macrophages pretreated with increasing doses of CORM-2, both the LPS-derived upregulations of iNOS (NO production) and HO-1 expression (CO production) were suppressed in a dose-dependent manner. Alternatively, when the macrophages were treated with LPS and CO-donor together, the LPS-derived increase in NO production was decreased. Conversely, when the control and LPS-stimulated macrophages were treated with zinc protoporphyrin IX (ZnPP) to inhibit the HO activity blocking endogenous production of CO (basal and enhanced), macrophages died extensively. Interestingly, production of NO in the LPS-stimulated macrophages increased significantly following the ZnPP treatment. Addition of CORM-2 to the LPS-treated cells that were being treated additionally with ZnPP did not prevent the cell death. However, endogenous overproduction of CO by super-induction of HO-1 (obtained by pretreatment of macrophages with either buthionine sulfoximine or hemin) decreased the LPS-derived iNOS expression without affecting cell survival.

* *Corresponding author*. Tel.: +82 32 890 0961; fax: +82 32 885 8303.
** *Corresponding author*. Tel.: +82 32 890 0976; fax: +82 32 885 8303.
E-mail addresses: chaekyun@hotmail.com (C. Kim), youngnam@inha.ac.kr (Y.-N. Cha).
[1] Present address: Department of Biochemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand.
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.10.042

Combined, these results indicated that enhanced HO activity is essential for the survival of LPS-stimulated macrophages. Thus, upregulation of HO-1 and overproduction of CO may allow the survival of LPS-stimulated macrophages; first, by eliminating the free heme to prevent Fenton reaction, second, by limiting the availability of free heme required for induction of NO-producing heme enzyme (i.e., iNOS), third, by limiting additional production of O_2^- and NO via CO-derived inhibition on the activities of heme enzymes like NADPH oxidase and iNOS, respectively. CO may allow the LPS-activated macrophages to return back to the normal quiet state insensitive to additional stimuli causing oxidative stress.

1. Introduction

Macrophage serves as the first-line defense against invading pathogens by undergoing immediate oxidative burst to overproduce superoxide anion (O_2^-) and delayed overproduction of nitric oxide (NO). The overproduced O_2^- and NO radicals combine rapidly to generate peroxynitrite ($ONOO^-$), a strongly oxidizing and cytotoxic product that can kill the invading pathogens directly. In the stimulated macrophages, the heme-containing enzymes like NADPH oxidase (PHOX) and inducible nitric oxide synthase (iNOS) catalyze the production of O_2^- and NO radicals, respectively. Activities of these radical-producing heme enzymes increase in macrophages upon stimulation with bacterial lipopolysaccharide (LPS) and inflammatory cytokines. While these O_2^- and NO radicals are not sufficiently reactive to oxidize the cellular GSH directly, they combine rapidly (6.7×10^9 M/s) to generate $ONOO^-$ which is highly reactive (toxic) and oxidizes GSH directly [1]. While the $ONOO^-$ can kill invading pathogens, it can also kill macrophages themselves and surrounding host tissues. Production of $ONOO^-$ is known to be involved in the pathogenesis of inflammation and rejection of grafted tissues. However, macrophages and host cells can protect themselves from the toxicity of $ONOO^-$ by enhancing the expression of heme oxygenase-1 (HO-1, EC 1.14.99.3). Heme oxygenases (HO) catalyze the oxidative degradation of free heme and release iron, biliverdin and carbon monoxide (CO). Among the three isoforms of HOs expressed in most mammalian tissues, HO-1 is the only inducible form (reviewed in [2]). In this connection, induction of HO-1 expression occurs not only by its substrate free heme but also by various other pro-inflammatory non-heme stimulants like H_2O_2, NO, bacterial LPS, cytokines, heavy metals and other oxidants [2–4]. Most commonly, these inducers of HO-1 expression cause oxidation and depletion of GSH and cause oxidative cell damage. In the current study, we investigated the protective role of upregulating the HO-1 expression against the cytotoxicity caused by excessive production of O_2^- and NO radicals. Results obtained in this study suggest that heme degradation occurring simultaneously with CO production provided by the overexpressed HO-1 is involved in the cytoprotective effect in macrophages stimulated with LPS.

Heme oxygenases (HO) are localized in the microsomal membrane and are the rate-limiting enzymes involved in oxidative degradation of free heme to generate equimolar Fe^{++}, biliverdin and CO [5]. As a regulator of intracellular pool of free heme, the enhanced HO activity resulting from HO-1 induction has been shown to deplete heme [6]. This limits the availability of free heme to be incorporated into newly synthesized apo-protein, thus preventing the assembly and activity of several heme-containing enzymes like cyclooxygenase-2 [7,8], iNOS [9] and cytochrome p450 [10], among others. Recent study by Taille et al. [6] showed that HO-1 induction caused suppression of $gp91^{phox}$ expression, the heme-containing catalytic subunit of NADPH oxidase. Thus, upon induction of HO-1, the NADPH oxidase activity was suppressed and O_2^- production was decreased. Next, as the producer of bile pigment antioxidants, the enhanced HO activity has been shown to decrease cellular content of O_2^-. This was caused by the bile pigment-dependent scavenging of the overproduced O_2^- [11–13]. Furthermore, as the producer of CO, enhanced HO activity has been shown to provide protective effects in several experimental animal models of diseases caused by oxidative stress. As CO is known to bind avidly to the heme-iron contained in heme enzymes like NADPH oxidase and iNOS, CO can inhibit the electron transfer reactions required for additional production of O_2^- and NO, the reactive species causing oxidative stress. Thus, as with the NO produced by NOS, CO produced by HO has been shown to decrease vascular tone [14], alter smooth muscle cell proliferation [15], and inhibit the expression of endothelial adhesion molecules [16]. In addition, the CO overproduced from enhanced HO activity has been demonstrated to provide cytoprotective effects against the toxicity caused by overproduction of NO in multiple experimental models such as endotoxemia [17], shock [18], ischemia/reperfusion [19] and xenograft rejection [20].

In support of these observations, a recent study from this laboratory has demonstrated that C6 neuroblastoma cells pretreated with NO-donor had enhanced expression of HO-1 and were protected from the toxicity of high dose $CdCl_2$ [21]. Also, in macrophages stimulated with LPS, a biphasic induction of HO-1 was observed, first, weakly by the O_2^- overproduced from activated NADPH oxidase (oxidative burst) and second, strongly by the NO overproduced from upregulated iNOS [22]. When the elevated HO activity of these LPS-stimulated macrophages was inhibited using zinc protoporphyrin IX (ZnPP), cells did not survive. Conversely, when HO-1 was super-induced, LPS-driven induction of iNOS and overproduction of NO was suppressed and cell survival was increased [23]. This suggested that induction of HO-1 expression causing enhanced depletion of toxic free heme with overproduction of bile pigments and CO is involved in the adaptive cytoprotective effect. In this connection, many recent findings suggest that one mechanism by which the enhanced HO activity can protect cells from oxidative stress is

via generation of CO that results from enhanced heme degradation (reviews [24–27]).

Under stressful conditions, however, endogenous production of CO can increase significantly by prompt induction of HO-1 and the elevated CO production can be detected in the breath of patients with acute inflammatory diseases [28]. Such an increase of CO production resulting from HO-1 induction is viewed as a normal adaptive cytoprotective response of aerobic cells encountered with stressful inflammatory conditions. In support, there have been extensive studies dealing with the cytoprotective effects of HO-1 induction and overproduction of CO (reviewed in [26,27]). For example, low dose of CO delivered as gas (250 ppm) has been demonstrated to have vasodilatory, anti-inflammatory, and anti-apoptotic actions, in a manner similar to NO (reviewed in [26,27]). Alternatively, the CO generated from CORM-2 has been applied successfully to produce vasodilatation in vitro, attenuation of coronary vasoconstriction ex vivo, and reduction of acute hypertension in vivo [29].

Thus, in the present study, we employed a CORM-2 to demonstrate that CO can inhibit ROS production, down-regulate iNOS expression, inhibit NO production and thus, reduce the LPS-inducible expression of HO-1. We also demonstrate that CO inhibits O_2^- production in the PMA-stimulated neutrophils and suggest that this may be responsible for the secondary decrease in LPS-derived induction of iNOS expression and NO production.

2. Materials and methods

2.1. Chemicals and reagents

Monoclonal antibody of HO-1 was purchased from Stressgen. Monoclonal antibody against iNOS and horseradish peroxidase-conjugated mouse- and rabbit-IgG antibodies were obtained from BD Biosciences Transduction Laboratories. Oligonucleotide primers were bought from Takara. D,L-buthionine-[S,R]-sulfoximine (BSO), hemin, phorbol myristate acetate (PMA), tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$, CORM-2), lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4), *N*,*N*-dimethylformamide (DMF), diphenylene iodonium (DPI), superoxide dismutase (SOD), ferricytochrome c, β-actin monoclonal antibody, β-NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma Chemical. Fetal bovine serum (FBS) was purchased from HyClone. Penicillin–Streptomycin was obtained from Invitrogen/Gibco. ZnPP was obtained from Tocris.

2.2. Cell culture

Murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC). Cells suspended in DMEM containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated FBS were cultured at 37 °C in a humidified air containing 5% CO_2. The RAW 264.7 cells were then treated with CORM-2, ZnPP or hemin (oxidized heme), all dissolved in DMSO at the indicated times and incubated in the complete medium for the indicated durations before harvesting. Alternatively, a human promyelocytic cell line, PLB-985 (a kind gift from M.C. Dinauer, Indiana University School of Medicine), maintained in RPMI 1640 medium containing 10% FBS with antibiotics were employed to determine the effect of the CO-donor CORM-2 on the PMA-stimulated overproduction of O_2^-. For the granulocytic differentiation, the PLB-985 cells at a starting density of 2×10^5 cells/ml were exposed to 0.5% DMF for 5 days [30].

2.3. Intracellular accumulation of reactive oxygen species (ROS)

To monitor the accumulation of ROS in macrophages resulting from LPS-stimulation (oxidative burst) and to determine the effect of CORM-2 on LPS-derived accumulation of ROS, a fluorescent probe dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA) was used. After treating the macrophages with LPS (1 μg/ml) for 4 h in the absence and presence of 50 μM CORM-2 dissolved in DMSO, the cells (1×10^4 cells/800 μl in four-well chamber slides) were rinsed with Kreb's Ringer solution and treated with 15 μM DCF-DA. Following 15 min incubation at 37 °C, cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 mW).

2.4. Measurement of NADPH oxidase activity (O_2^- production)

Rate of O_2^- production was determined as a measure of NADPH oxidase activity by employing a quantitative kinetic assay based on the SOD-inhibitable reduction of cytochrome c as reported previously [31,32]. Briefly, PLB-985 cells were suspended (2.5×10^5 cells/well) in 250 μl PBSG (PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose) containing 75 μM ferricytochrome c and then activated by adding 200 ng/ml PMA). The reduction of ferricytochrome c was followed by the change of its absorbance at 550 nm at every 30 s over a 30 min time course using a microplate reader with the associated SOFTMAX software (Molecular Devices). To determine the effect of CO on the production of O_2^-, neutrophils were incubated for 30 min at 37 °C with 50 μM CORM-2 dissolved in DMSO, a CO-releasing condition [29], prior to activation with PMA.

2.5. Cell viability test using MTT assay

Effects of various experimental modulations on cell viability were evaluated by determining the mitochondrial reductase function on the basis of their ability to reduce tetrazolium salt, MTT, into formazan crystals [33]. The formation of formazan is proportional to the number of functional mitochondria in living cells. This method was performed as described previously [22]. RAW 264.7 macrophages (1.5×10^5 cells) were plated into each well of 24-well plates and 10 μl of 5 mg/ml MTT was added into each well for 2 h and then the solution was aspirated. Subsequently, DMSO was put into each well to solubilize the blue formazan crystal and absorbance was read in a microplate reader. The absorbance of each well was

measured at 550 nm and the % viability of treated cells was compared with that of untreated control cells.

2.6. Western blot analysis

At various times after treating the RAW 264.7 macrophages with various chemicals, cells were washed with ice-cold PBS and then, scraped in the presence of ice-cold lysis buffer as was performed previously [22]. The cell lysate was sonicated and protein concentrations were determined by using the BCA protein assay kit (Pierce). Equal amount of proteins were mixed with loading buffer and subjected to electrophoresis using 10% SDS–polyacrylamide gels. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane and the membrane was then incubated further with primary antibodies of HO-1 (1:500 dilution), iNOS (1:1000) and β-actin (1:5000). The duration of incubations employed to determine the contents of HO-1 and iNOS were 2 h and 1 h for β-actin. Subsequently, the membrane was incubated with either goat anti-rabbit IgG or horseradish peroxidase secondary antibodies for 1 h at room temperature. The specific protein bands on the PVDF membrane were visualized on X-ray film activated by chemiluminescence using Western blotting luminol reagent (Santa Cruz Biotechnology). The intensities of each band signal were determined by densitometry using BIO-PROFIL software version 99.04 (Vilber Lourmat). The image densities of specific bands for iNOS and HO-1 were normalized with the density of β-actin band.

2.7. Nitrite determination (NO production)

Production of nitrite, a stable end product of NO oxidation, was used as a measure of iNOS activity. Nitrite present in the conditioned media was determined by spectrophotometric assay based on Griess reaction [34]. One hundred microliters of the conditioned media was incubated with the same volume of Griess reagent (0.1% (w/v) N-(1-naphathyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid) for 10 min at room temperature. The absorbance at 546 nm was measured using a microplate reader and the nitrite concentration was determined by using a standard curve of sodium nitrite made up in DMEM free of phenol red.

2.8. Measurement of heme oxygenase activity

Heme oxygenase activity was determined both by the conventional bilirubin production assay and the GC-based CO production assay. Production of bilirubin from hemin was determined upon addition of rat-liver cytosol as the source of biliverdin reductase as described earlier [22]. Briefly, microsomal suspension obtained from harvested RAW 264.7 macrophages was added to the reaction mixture containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 μM hemin (substrate), 2 mg of rat-liver cytosol (source of biliverdin reductase), 100 mM potassium phosphate buffer, pH 7.4. Mixtures (1.0 ml total volume) were incubated at 37 °C for 1 h in the dark and then placed on ice for 2 min to terminate the reaction. Bilirubin was determined by calculation from the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 $mM^{-1} cm^{-1}$ for bilirubin). HO activity was expressed as nmoles of bilirubin formed per mg of microsomal protein per hour.

Alternatively, production of CO from hemin added to the lysed cell suspension was determined by the GC method as described by Vreman and Stevenson [35] with some modifications. Briefly, after counting the number of harvested cells, they were suspended in ice-cold lysis buffer (500 μl) and were lysed further by brief sonication. Hemin (20 μM) and NADPH (80 μM) were added to the lysate (0.9 ml total final volume). After capping the reaction vial (5 ml capacity), it was incubated at 37 °C for various times, generally for 30 min. Reaction was stopped at various times by injecting 100 μl of 50 mM 4-OH mercury benzoate through the septum. Subsequently, 1.0 ml of headspace gas was withdrawn and injected into a GC-based multi-gas analyzer (Trilyzer, Taiyo Instruments Inc., Osaka, Japan) equipped with a semiconductor detector, which can detect CO with 0.1 ppm sensitivity. Measuring the CO production from the lysates of cultured cells required highly sensitive semiconductor detector system (0.1 ppm), and it was not possible with thermal conductivity detector (10 ppm limit sensitivity) or with flame ionization detector (1.0 ppm). The software provided from Taiyo Instruments calculated the AUC of CO peak. After determining the protein concentration of the lysate in the vial, HO activity was expressed in nanomoles of CO produced per mg of total cellular protein per hour. Alternatively, the terminated reaction mixture remaining in the vial was used to determine the amount of bilirubin formed using the spectrophotometric method as described above. This was conducted to verify the CO production results. In this case, the HO activity was expressed in nmoles of bilirubin produced per mg of total cellular protein per hour.

2.9. Statistical analyses

Statistical analyses were performed using unpaired Student's *t*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. CO inhibits accumulation of ROS in LPS-stimulated macrophages without causing cytotoxicity and inhibits production of O_2^- in PMA-stimulated neutrophils

It is well known that LPS-stimulated RAW 264.7 macrophages undergo oxidative burst by activation of NADPH oxidase and overproduce O_2^-. This leads to accumulate hydrogen peroxide (H_2O_2), lipid peroxides and other reactive oxygen species (ROS). First, to determine the effect of CO on the overproduction of ROS stimulated by LPS, RAW 264.7 cells were pretreated with 50 μM CORM-2 for 1 h before stimulation with LPS and the ROS accumulated in the cell was determined using DCF fluorescence probe. Recently, it was reported that CORM-2 releases CO in the presence of DMSO, in a time and dose-dependent manner [29]. Using the CO-analyzer (Trilyzer, Taiyo Instr.), we confirmed that equimolar amount of CO is generated from the CORM-2 when it was dissolved in DMSO in a time and dose-dependent manner (data not shown). Results shown in Fig. 1 indicated that pretreatment of cells

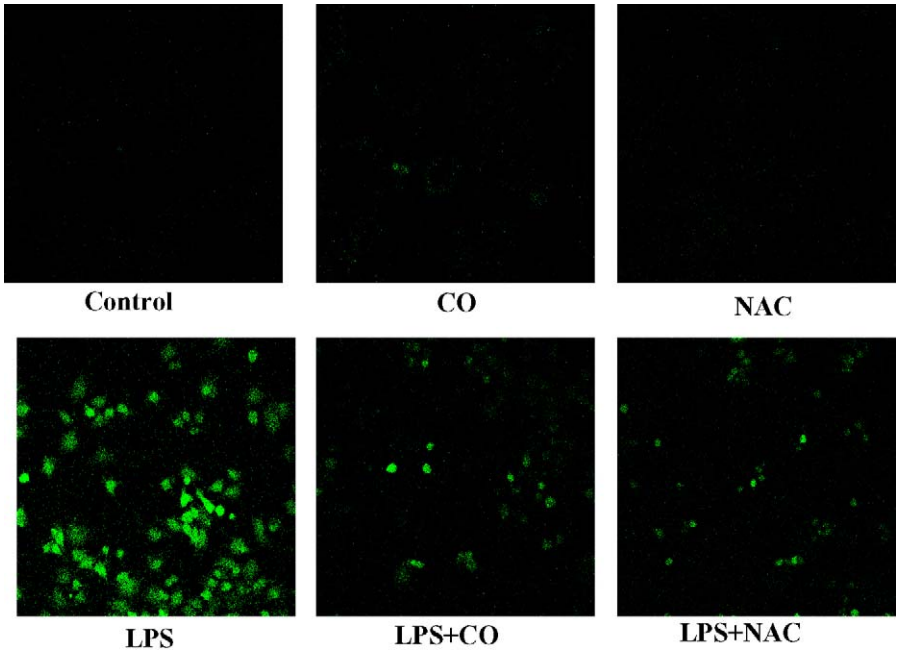

Fig. 1 – Effect of CORM-2 pretreatment on LPS-induced accumulation of ROS. RAW 264.7 cells were pretreated with CORM-2 or *N*-acetylcysteine (NAC) for 1 h before the LPS treatment. Representative confocal images obtained from three separate experiments conducted with cells exposed to the Kreb's Ringer solution containing DMSO (control), 50 μM CORM-2 dissolved in DMSO (CO), 10 mM NAC dissolved in Kreb's Ringer solution (NAC), 1 μg/ml LPS alone (LPS) or in combination with 50 μM CORM-2 (LPS + CO) or with 10 mM NAC (LPS + NAC) are presented.

with CORM-2 abolished the accumulation of ROS in macrophages stimulated with LPS.

CO gas, like the NO, is well known to be a strong poisonous gas and is known to block the mitochondrial oxidative phosphorylation by avid binding to respiratory cytochromes [36]. Thus, we determined the effect of CORM-2 on the mitochondrial reductase function by employing the MTT reducing cell survival assay. Results shown in Fig. 2 indicated that treating the cells with increasing doses of CORM-2 up to 50 μM either before or after the LPS-stimulation was not cytotoxic. Utilizing the MTT assay, 100 μM CORM-2 began to show minimal cytotoxicity in RAW 264.7 cells (data not shown) as was observed by Sawle et al. [37].

As the production and accumulation of ROS depends on the amount of O_2^- generated, and the production of O_2^- in LPS-stimulated macrophages is catalyzed primarily by the heme-containing NADPH oxidase, we attempted to determine the effect of CO on the LPS-driven activation of NADPH oxidase activity by determining the rate of O_2^- production. However, the amount of O_2^- produced by the LPS-stimulated RAW 264.7 cells was small and could not be measured readily by the SOD-inhibitable cytochrome c reduction assay (data not shown). Thus, we employed the PLB-985 neutrophil cell line to determine the inhibitory effect of CO on PMA-stimulated activation of NADPH oxidase activity because this system can produce O_2^- at the quantity that is readily measurable using the SOD-inhibitable cytochrome c reduction assay. The amount of CO released from 50 μM CORM-2 by 30 min pre-incubation was sufficient to demonstrate a marked inhibition of O_2^- production in PLB-985 cells stimulated with PMA (Fig. 3). Pre-incubation with 50 μM CORM-2 for 1 h produced severe inhibition and co-incubation with PMA produced a small but significant inhibition on O_2^- production (data not shown). Taille et al. [38] reported similar observation recently. This result suggested that CO might inhibit the NADPH oxidase

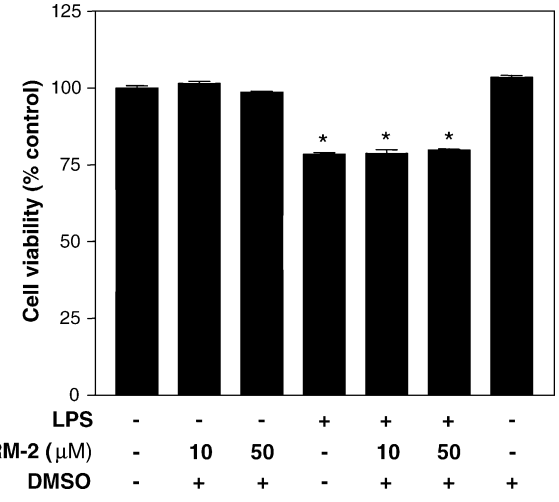

Fig. 2 – Effect of CORM-2 on cell viability before and after stimulation with LPS. RAW 264.7 cells were treated with CORM-2 before and after the LPS-stimulation and their viability was assessed using the MTT assay. Results from three separate experiments are presented as mean ± S.E.M. *Significant difference from untreated control at $p < 0.001$.

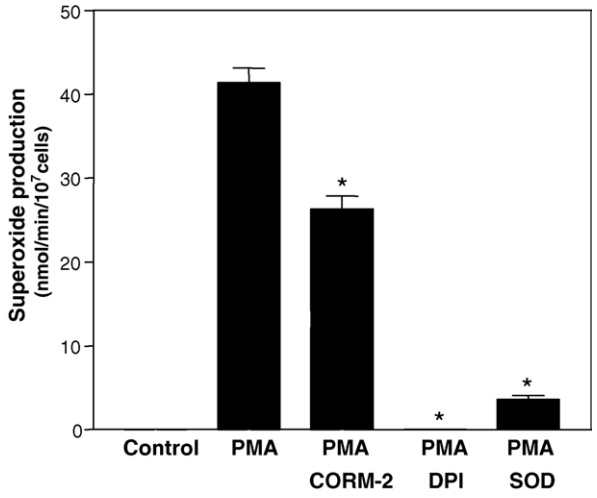

Fig. 3 – Effect of CORM-2 pretreatment on PMA-stimulated O_2^- production in neutrophils. PLB-985 neutrophils were pre-incubated for 30 min with 50 μM CORM-2 prior to stimulation with 200 ng/ml PMA and the rates of O_2^- production were monitored by determining the reduction rate of ferricytochrome c. A 5 μM DPI and SOD were used as respective positive controls for elimination and dissipation of the produced O_2^-. Data are expressed as mean ± S.E.M. obtained from $n = 6$. $^*p < 0.001$ compared to PMA-stimulated cells.

activity in LPS-stimulated RAW 264.7 macrophages as well. This suggested that CO limits O_2^- production in LPS-stimulated cells and may suppress both iNOS induction and NO overproduction, eventually causing smaller induction of HO-1 expression.

3.2. *CO inhibits NO production and decreases HO-1 expression in LPS-stimulated macrophages*

In macrophages pretreated with the CORM-2 at 1 h before LPS-stimulation, the LPS-derived increase of NO production was inhibited in a dose-dependent manner (Fig. 4A). This observation is very similar to the recent observation made by Sawle et al. [37]. This CO-derived inhibition of NO production may have been caused by the CO-dependent inhibition of NADPH oxidase activity (Fig. 3), limiting the production of O_2^- needed to cause oxidative stress, and therefore suppressing the NF-κB activation needed for induction of iNOS expression and NO overproduction. Alternatively, a direct binding of CO to the heme-center in iNOS and inhibition of iNOS activity may have been responsible also for the decreased NO production seen in LPS-stimulated macrophages as suggested also by Sawle et al. [37]. To determine which one of these two mechanisms is involved more importantly in the CO-derived inhibition of NO production in LPS-stimulated macrophages, effect of CO-pretreatment on LPS-derived upregulation of iNOS expression was determined. Results shown in Fig. 4B indicated that LPS-driven iNOS upregulation was suppressed in macrophages pretreated with CORM-2, again in a dose-dependent manner. This CO-dependent decrease in iNOS expression suggested that the limited production of O_2^- resulting from CO-derived inhibition of NADPH oxidase activity might be responsible for the indirect inhibition of NO production. In any case, this result observed in vitro with RAW 264.7 cells confirmed the in vivo observations made with lung macrophages obtained from LPS-stimulated mouse exposed to 250 ppm CO gas [17]. However, it was in contrast to the elevated iNOS expression observed in vivo in LPS-exposed mouse hepatocytes treated with the same dose of CO gas [39].

Results shown in Fig. 4B indicated that the CORM-2 also suppressed the LPS-driven upregulation of HO-1 in macrophages, again in a dose-dependent manner. This occurred along with suppression of LPS-driven increase in HO activity (bilirubin and CO production) (Fig. 4C and D). Combined, these results indicated that 50 ppm CO generated from CORM-2 was not cytotoxic to both control and LPS-stimulated macrophages (Fig. 2) and yet, inhibited the LPS-driven activation of NADPH oxidase activity suppressing the overproduction of O_2^-. Results indicated further that limited production of O_2^- was responsible for the decreased induction of iNOS and NO production, leading to a decreased (NO-dependent) upregulation of HO-1 expression.

3.3. *Inhibiting HO activity enhances NO production and cell death not reversed by CORM-2*

Unstimulated macrophages have some CO-producing HO activity without any induction of HO-1 expression (Fig. 4C and D) and this may be derived from the constitutively expressed HO-2. Inhibition of this constitutive CO-producing HO activity in the unstimulated macrophages with ZnPP caused significant cell death (ZnPP column in Fig. 5B). This indicated the importance of HO activity in cell survival even under normal condition. Treating macrophages with 1 μg/ml LPS caused marked increase in iNOS expression (Fig. 4B) and NO production (Figs. 4A and 5A) along with marked increases in HO-1 expression and HO activity (Fig. 4B–D), but caused some cell death (LPS column in Fig. 5B). However, when the HO activity (both basal and enhanced) in these LPS-stimulated cells was inhibited by ZnPP treatment, to our surprise, a small but significant increase of NO production was observed (Fig. 5A; LPS + ZnPP column) along with severe increase in cell death (Fig. 5B). The cells pretreated with CORM-2, when compared with those not pretreated, produced slightly decreased amount of NO without any effect on cell viability (Fig. 5A and B; CORM-2 + LPS column). When the HO activity in these cells treated with LPS was inhibited by addition of ZnPP, regardless of CORM-2 pretreatment, there was a small but significant increase in NO production along with severe increase in cell death (Fig. 5A and B; CORM-2 + LPS + ZnPP column). This indicated that giving exogenous CO in the absence of heme degradation could not provide cytoprotection. The small but significant increase of NO production observed in these ZnPP-treated cells undergoing death (Fig. 5A) suggested that it may have been caused by relieving the CO-derived inhibition on iNOS activity that was present in cells with intact HO activity (basal and enhanced). Furthermore, the increased cell death observed in ZnPP-treated cells regardless of the absence or presence of CO may have been caused by the increased lipid peroxidation resulting from the

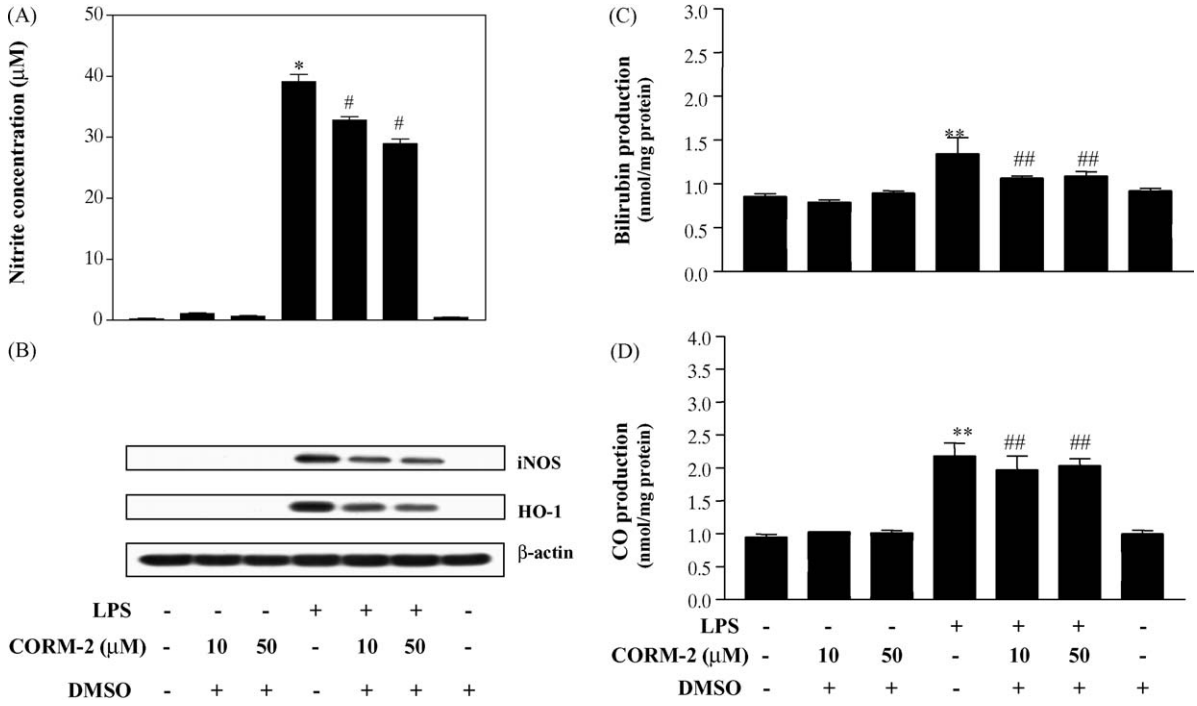

Fig. 4 – Effect of CORM-2 pretreatment on LPS-inducible production of NO, upregulations of iNOS and HO-1 expression. RAW 264.7 cells were pre-incubated with 10 or 50 μM CORM-2 dissolved in DMSO for 1 h before treatment with 1 μg/ml LPS and incubated for 24 h. (A) The bar graph shows nitrite concentrations present in the conditioned media of LPS-treated cells with and without CORM-2. Data represent the mean ± S.E.M. obtained from $n = 3$. $^{*}p < 0.001$ compared to control cells. $^{\#}p < 0.001$ compared to LPS-treated cells. (B) Cellular contents of iNOS and HO-1 protein were determined and compared with β-actin. The presented immuno-blot shows a representative result obtained from three separate experiments. (C) and (D) The bar graphs show HO activity measured using bilirubin (C) and CO production (D). Data are expressed as mean ± S.E.M. obtained from $n = 3$. $^{**}p < 0.001$ compared to control cells, and $^{\#\#}p < 0.05$ compared to LPS-treated cells.

uninhibited Fenton reaction with increased availability of free heme (Fig. 5B). Combined, these results indicated that CO alone does not provide survival advantage when the HO activity is inhibited. These results suggested further that CO could protect cells only when the toxic heme is eliminated along with production of cytoprotective bile pigment antioxidants.

3.4. *Preconditioning with BSO causes super-induction of HO-1 and down-regulation of iNOS expression in LPS-stimulated macrophages*

As reported elsewhere, in macrophages and tissues with depleted GSH level, the increases of iNOS expression and NO production induced by exposure to LPS are suppressed [40,41]. In support, the LPS-derived increase of NO production in macrophages pretreated with BSO was reduced significantly (Fig. 6A). This was associated with a marked increase in the LPS-derived enhancement of HO activity (Fig. 6B) and induction of HO-1 expression (Fig. 6C). This indicated that in cells with super-induction of HO-1 and enhanced HO activity the LPS-dependent upregulation of iNOS expression and overproduction of NO are diminished. This explained the underlying causes for the suppressed iNOS expression and NO production in LPS-stimulated tissues with depleted GSH level [23,40,41].

3.5. *Pretreatment with hemin also causes superinduction of HO-1 expression but suppresses iNOS expression in LPS-stimulated macrophages*

Hemin (oxidized heme) is well known to serve not only as the native substrate of HO activity but also as an inducer of HO-1 expression. Thus, when macrophages were treated with increasing doses of hemin alone, HO-1 expression increased without any induction of iNOS expression (Fig. 7A) as was reported recently by Sawle et al. [37]. However, when the cells were treated with LPS together with increasing doses of hemin, there was a dose-dependent super-induction of HO-1, much above that induced by LPS alone (Fig. 7A). While hemin alone had no effect on iNOS expression, when combined with LPS treatment, there was a dose-dependent suppression on the LPS-derived iNOS upregulation. The suppression of iNOS expression was inversely associated with the superinduction of HO-1 expression (Fig. 7A). Also, along with hemin-derived super-induction of HO-1 expression in LPS-stimulated macrophages, HO activity increased and the CO-producing HO activity increased to the same extent as the bilirubin-producing HO activity (Fig. 7B and C). In the LPS-stimulated cells co-treated with hemin, however, the NO-producing ability was not diminished even with superinduction of HO-1 expression and overproduction of endogenous CO, but appeared to increase (Fig. 7D). This unexpected increase in NO

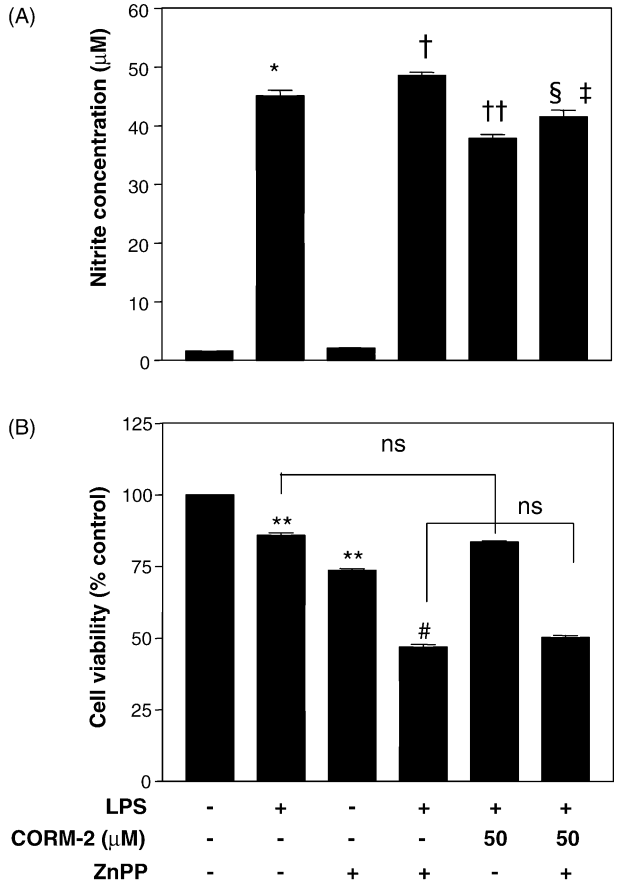

Fig. 5 – Effect of CORM-2 on NO production and cell survival in LPS-stimulated macrophages treated with and without ZnPP. RAW 264.7 cells were pre-incubated with 50 μM CORM-2 for 1 h and 10 μM ZnPP for 30 min in the presence or absence of CORM-2 before the LPS-stimulation. (A) Nitrite concentrations were determined after 24 h incubation. Data represent the mean ± S.E.M. from three separate experiments. $^{*}p < 0.001$ compared to control cells. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to LPS alone. $^{\ddagger}p < 0.01$ compared to LPS plus CORM-2 treated cells, and $^{\S}p < 0.01$ compared to LPS plus ZnPP-treated cells, respectively. (B) Viability of cells at 24 h after the addition of LPS. Data are expressed as the mean ± S.E.M. obtained from $n = 3$. $^{**}p < 0.001$ compared to control cells. $^{\#}p < 0.001$ compared to LPS alone, and ns indicates no significant difference.

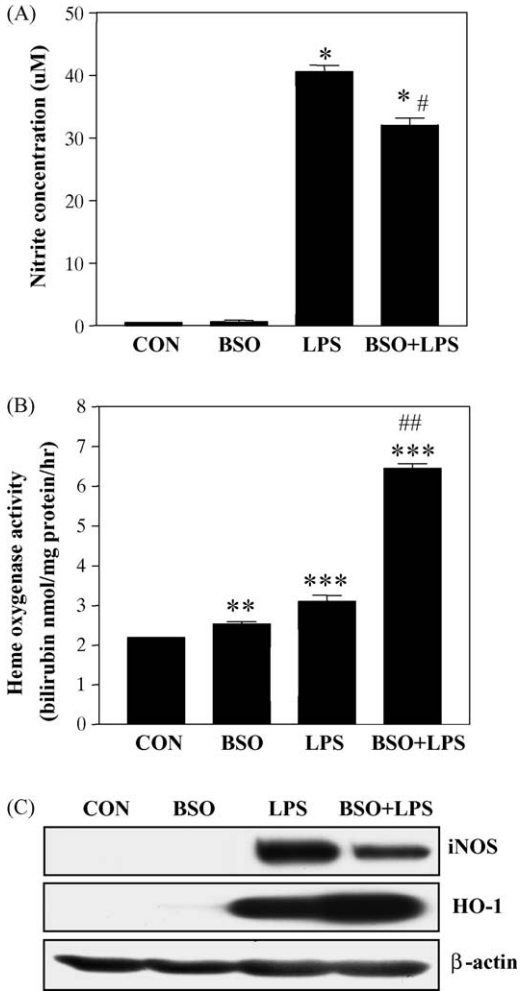

Fig. 6 – Effect of BSO pretreatment on LPS-stimulated increases of NO production, HO activity and iNOS and HO-1 expression. RAW 264.7 cells were pretreated with BSO (100 μM) for 4 h before stimulation with LPS (1 μg/ml) and incubated for additional 24 h. (A) Nitrite accumulation was determined. Data represent the mean ± S.E.M. obtained from $n = 3$. $^{*}p < 0.001$ compared to control cells, and $^{\#}p < 0.001$ compared to LPS-treated cells. (B) HO activity was determined using the bilirubin production assay. Data represent the mean ± S.E.M. obtained from $n = 3$. $^{**}p < 0.05$ and $^{***}p < 0.001$ compared to control cells. $^{\#\#}p < 0.001$ compared to LPS-treated cells. (C) Total protein was extracted and analyzed for the contents of iNOS and HO-1. The presented immuno-blot is a representative of three separate experiments.

production in the presence of decreased iNOS expression in LPS-stimulated macrophages co-treated with hemin is similar to the increased O_2^- production observed in HO-1 induced macrophages pretreated with CoPP, a well-known inducer of HO-1 expression [6]. Taille et al. showed that expression of $gp91^{phox}$, the heme-containing catalytic subunit of NADPH oxidase, was suppressed in macrophages pretreated with CoPP. When these cells were stimulated with LPS, smaller amount of O_2^- was produced. However, when hemin was added to these macrophages, they produced increased amount of O_2^- [6]. The increased NO production observed in the hemin-LPS treated macrophages with reduced iNOS expression suggested that the added hemin might incorporate into the pre-existing apo-iNOS induced by LPS-stimulation and enhance their functional assembly for increased NO production. Currently, we are investigating this possibility (data not shown).

4. Discussion

This study with exogenous CO-releasing molecule ($[Ru(CO)_3Cl_2]_2$, CORM-2) shows that CO inhibits NADPH

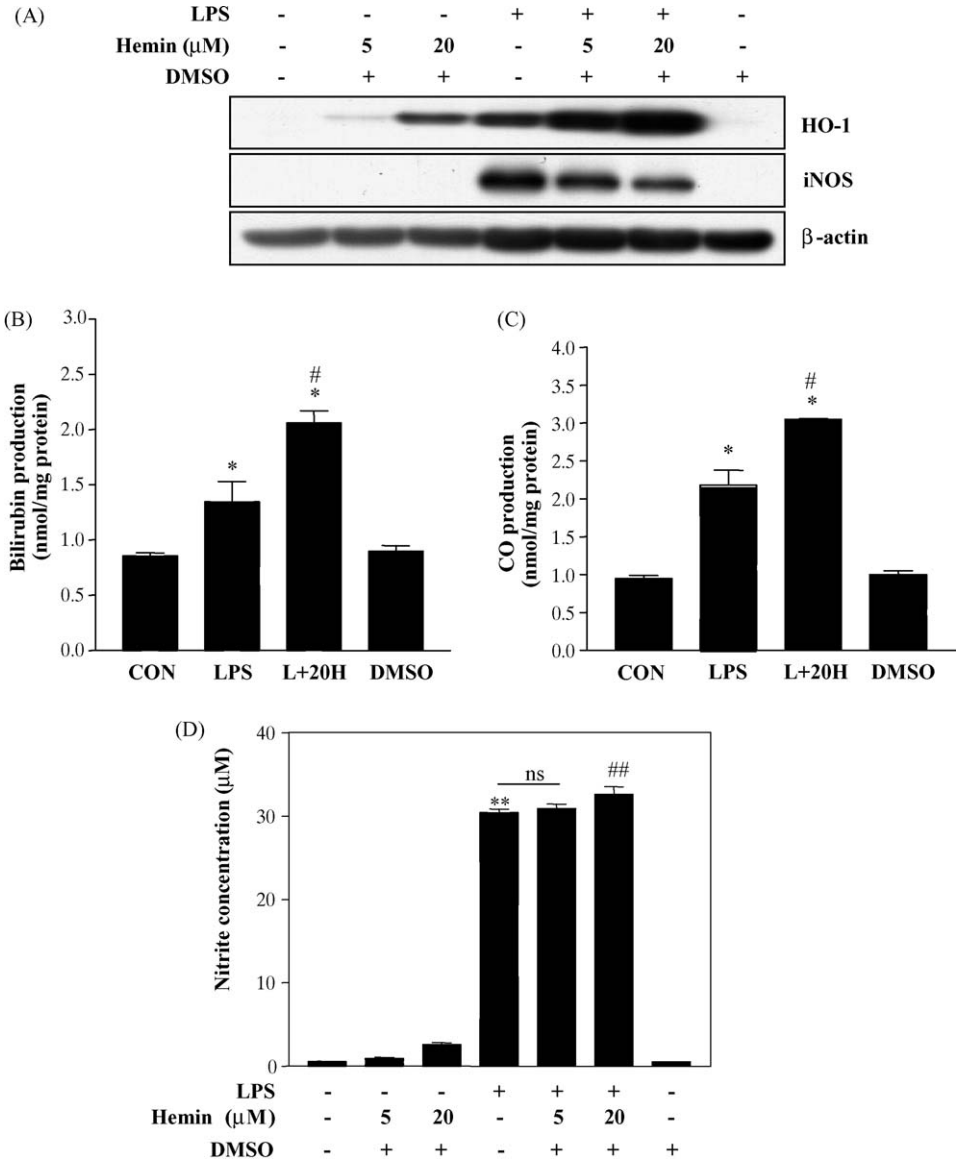

Fig. 7 – Effect of hemin on LPS-stimulated increases HO-1 and iNOS expression, HO activity and NO production. RAW 264.7 cells were treated with 1 μg/ml LPS in the presence and absence of 5 or 20 μM hemin for 24 h. (A) The expression of iNOS and HO-1 was determined. The presented immuno-blot is a representative of three separate experiments. (B) and (C) HO activity determined using bilirubin and CO production, respectively. Data represent the mean ± S.E.M. obtained from $n = 3$ (note that the specific activity of HO is decreased by about 50% when compared with that obtained using isolated microsomes). $^{*}p < 0.001$ compared to control cells and $^{\#}p < 0.001$ compared to LPS-treated cells, respectively. (D) Nitrite concentration was determined. Data indicate the mean ± S.E.M. obtained from $n = 3$. $^{**}p < 0.001$ compared to control cells, ns indicates no significant difference, and $^{\#\#}p < 0.05$ compared to LPS-treated cells, respectively.

oxidase activity and suppresses the overproduction of O_2^- in the PMA-stimulated neutrophils, a phagocytic cell line which kills invading pathogens by overproducing O_2^- in a similar manner with macrophages. As the O_2^- production is catalyzed by the heme-containing NADPH oxidase in both macrophages and neutrophils, it is expected that the exogenously delivered CO may bind to the heme-center and inhibits the oxidase activity, suppressing the overproduction of O_2^- (Fig. 3). Recently published report by Taille et al. supported this hypothesis [38]. Supporting the CO-derived inhibition of O_2^- overproduction, ROS accumulation in LPS-stimulated macrophages was ablated by pretreatment of CORM-2 (Fig. 1). Along with such CO-derived inhibition on the overproduction of O_2^-, upregulation of iNOS was suppressed and overproduction of NO was decreased in the LPS-stimulated macrophages (Fig. 4A and B). It is well known that upregulation of iNOS expression is promoted by activation of NF-κB, a redox-sensitive transcription factor activated by the oxidative stress resulting from ROS accumulation. Thus, inhibition of O_2^- production and ablation of ROS accumulation in the CO pretreated macrophages was responsible for this suppression on the LPS-dependent upregulation of iNOS expression and overproduction of NO.

Alternatively, as the iNOS is also a heme-containing enzyme, decreased NO production observed in the CORM-2 treated cells may have resulted from the CO-dependent inhibition on iNOS activity as well [37]. Whether caused by suppression of iNOS induction or inhibition of the enhanced iNOS activity, our observation supports the in vivo results reported by Sarady et al. [17]. They demonstrated that the LPS-stimulated induction of iNOS expression and NO overproduction in the mouse lung and alveolar macrophages were ablated by exogenous delivery of 250 ppm CO gas. Along with such CO-dependent inhibition of O_2^- and NO overproduction in LPS-stimulated macrophages, less of the $ONOO^-$ may have been produced and less of intracellular GSH was depleted (data not shown and manuscript in preparation). In support, the CO derived from 50 μM CORM-2 dissolved in DMSO (generating 50 ppm CO) was not cytotoxic by itself and did not enhance or reduce the cytotoxicity of LPS (Fig. 2) as was observed by Sawle et al. [37]. Combined, the exogenously delivered CO appears to suppress its own production in LPS-stimulated macrophages, eventually by decreasing HO-1 expression and HO activity, but without affecting the viability of CO-treated cells. Thus, induction of HO-1 seen in the LPS-stimulated inflammatory macrophages, removing the potentially toxic free heme while producing CO, appears to function as if ''an alerted fireman (macrophage) arrives to remove the flammable sources (induction of HO-1 and removal of free heme) and to turn the firehydrant on to let out the water (CO) and puts the fire (inflammation) out'' by inhibiting the NADPH oxidase activity and down-regulating the iNOS expression, respectively inhibiting additional productions of both O_2^- and NO, the inflammatory mediators.

In recent years, there have been extensive studies on the physiological and beneficial effects of HO-1 induction in a wide variety of pulmonary, cardiovascular and neurological diseases (reviewed in [26]). Among the products of heme degradation catalyzed by HO, bile pigments and CO have been shown to have antioxidant and anti-inflammatory functions. In particular, CO received most attention and has been documented to have wide physiological functions mimicking the beneficial effects of HO-1 induction (reviewed in [27]). Thus, as with NO, administering CO gas in low dose was able to produce the anti-inflammatory and anti-apoptotic effects and could replace the protective effects of HO-1 induction [24]. However, when HO-1 is induced before the inflammatory response, while the overproduced CO may allow the macrophages and tissues to survive, it will make the cells unresponsive to stimulation by limiting the production of inflammatory mediators (O_2^- and NO). Although not tested, this may make the macrophages unable to kill the phagocytosed pathogens and make the phagocytes unable to conduct their normal physiological function. Thus the induction of HO-1, which occurs only during the resolution phase of inflammation in nature [42], can protect host tissues from inflammatory damages initiated by invading pathogens, but not when it occurs before the pathogen invasion.

As mentioned earlier, both the O_2^--producing NADPH oxidase and the NO-producing iNOS contain two molecules of heme in their functional form and these enzymes require ready supply of free heme for their functional assembly of the upregulated apo-enzyme proteins. Under conditions of markedly increased HO-1 expression (Fig. 6C) and elevated HO activity (Fig. 6B), such as those in the BSO-pretreated macrophages stimulated with LPS, intracellular pool of free heme may be depleted. This may have limited the assembly of functional iNOS (Fig. 6C) and thus, may have decreased NO production (Fig. 6A). In support, recent studies by Taille et al. [6] have shown that in macrophages pretreated with CoPP and with upregulated HO-1, the expression of $gp91^{phox}$ was decreased and O_2^- production was suppressed. Alternatively, we also reasoned that the CO overproduced from markedly increased HO activity (Figs. 6B and 7B and C) might bind to the heme in the NADPH oxidase and inhibit O_2^- production (Fig. 3) [38]. This may prevent the macrophages to accumulate ROS (Fig. 1), lowering the NF-κB activation and causing smaller upregulation of iNOS expression and leading to decrease NO production. In addition, as was suggested by Sawle et al. [37], the overproduced CO from superinduced HO-1 may also bind to the heme contained in iNOS and inhibit the overproduction of NO. This may explain the previous studies showing that both the iNOS expression and NO production were inhibited in the LPS-treated macrophages overexpressing HO-1 [9,23]. In any case, the down-regulation of iNOS expression caused by CORM-2 observed in this study coincided with the decreased iNOS expression and GM-CSF production seen in the LPS-stimulated lung macrophages treated with 250 ppm CO gas [17]. They reported that this was mediated by the CO-dependent inhibition of NF-κB activation and prevention of IκB phosphorylation. Based on these results, Sarady et al. suggested that the anti-inflammatory effect of CO was provided by suppression of NF-κB pathway. Furthermore, Otterbein et al. [43] and Brouard et al. [44] suggested that the cytoprotective anti-apoptotic and anti-inflammatory functions of CO could occur through selective activation of mitogen-activated protein kinase (MAPK) signaling pathway.

In several experimental models, the CO produced endogenously by the enhanced HO activity resulting from upregulation of HO-1 expression has been demonstrated to protect cells against the damages caused by oxidative and nitrosative stresses (reviewed in [27]). In support, exposing cells and tissues to low-dose CO gas (250 ppm) has been shown to mimic the protective effects of HO-1 induction, preventing cell death caused by overproduction of NO [17]. Similarly, Motterlini et al. have demonstrated in vivo that CO delivered from CORM-2 was not toxic by itself but protects against the vaso-constriction and development of acute hypertension caused by injection of H_2O_2 or L-NAME (an inhibitor of NO production causing un-opposed O_2^- toxicity) [29]. The CORM-2 given to macrophages at 50 μM did not inhibit the mitochondrial reductase activity (MTT assay) and did not enhance cell death both in the unstimulated and LPS-stimulated macrophages (Fig. 2). However, CO is known to bind the heme-iron contained in mitochondrial respiratory cytochromes and to inhibit electron transfer causing the leakage of O_2^- from mitochondria and the secondary oxidative stress [36]. Thus, it was possible that delivery of 250 ppm CO gas may have caused secondary oxidative stress in hepatocytes, activating NF-κB and inducing iNOS expression [39]. However, delivery of the same 250 ppm CO gas inhibited NF-κB activation and iNOS expression in lung macrophages [17]. This opposing effect of CO produced in hepatocytes and lung macrophages may be

due to differences in the primary mechanism of O_2^- overproduction in these cell types. With many mitochondria in hepatocytes and leaking much more O_2^- when CO inhibits the electron transfer in respiratory cytochromes [36], it was understandable that NF-κB is activated and iNOS expression is enhanced in hepatocytes [39]. Conversely, with fewer mitochondria and the overproduction of O_2^- being dependent mainly on the plasma membrane NADPH oxidase activity in lung macrophages, activation of NF-κB and induction of iNOS may be prevented when CO inhibits the overproduction of O_2^- via inhibition of the heme-containing NADPH oxidase activity [37].

Inhibition of the HO activity, originating either from the constitutively expressed HO-2 or from the LPS-induced expression of HO-1, caused significant increase in cell death (Fig. 5B). Once the HO activity is inhibited, the free heme arising either from de novo synthesis [5] or released from destruction of heme-proteins promoted by ROS (O_2^-, H_2O_2) and RNS (NO, $ONOO^-$) [45] cannot be eliminated. Such free heme may catalyze the production of highly reactive HO^* from H_2O_2 via Fenton reaction and cause lipid peroxidation and protein degradation. This may have been responsible for the observed increase in cell death observed in the ZnPP-treated macrophages. Thus, providing CO under this condition did not revive the mitochondrial reductase activity and could not substitute for the cytoprotective effect of elevated HO activity (Fig. 5B). However, in cases of organ graft rejection [20], lung injury of hyperoxia [24], and ischemia/reperfusion injury of hepatocytes [19], delivering low-dose CO gas in vivo has provided protective effects. The discrepancy may have been caused by differences in the parameters used to determine the cytotoxicity. In future studies, we need to determine the cytotoxicity by including other parameters of apoptosis or necrosis and compare the results obtained with the MTT assay. This may provide additional and more precise information on the mechanisms involved in the cytoprotective effect of CO-delivery and/or HO-1 induction.

In conclusion, the heme-degrading and CO-producing HO activity was enhanced markedly in LPS-stimulated macrophages preconditioned by prior depletion of cellular GSH level (BSO pretreatment). In association with such increase of CO-exposure, whether produced endogenously from super-induction of HO-1 or delivered exogenously via CORM-2, the LPS-derived upregulation of iNOS expression and NO overproduction was decreased. In addition to inhibiting the NO production, the CO also inhibited NADPH oxidase activity blocking overproduction of O_2^-. Following such decreases of O_2^- and NO overproduction caused by the CO, the LPS-stimulated macrophages could survive. However, when the HO activity was inhibited with ZnPP, the LPS-stimulated cells (with undegraded free heme and lack of CO-derived cytoprotection) died extensively and the exogenously supplied CO did not prevent the cell death. Thus, oxidative degradation and elimination of free heme along with overproduction of CO and bile pigment by the enhanced HO activity appears necessary in the survival of LPS-stimulated macrophages. In the LPS-stimulated macrophages, continuous cross talk between overproductions of O_2^- (oxidative burst) and NO (iNOS induction) modulates the redox state of the cell and upregulates HO-1 expression. Conversely, the CO produced from the elevated HO activity modulates this cross talk between NADPH oxidase and iNOS. Thus, enhancing HO activity and overproducing CO may, in turn, play a central role leading to preservation of cellular redox homeostasis and survival.

Acknowledgements

INHA University Research Grant supported this work. This work was conducted in partial fulfillments for a Ph.D. degree for KS and for a M.S. degree for SSH. We wish to take this opportunity to thank Mr. Dong-bo Suh, President of Taiyo Instruments Inc. for his enthusiasm and technical support on the use of Trilyzer, the sensitive CO analyzer.

REFERENCES

[1] Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun 1993;18:195–9.
[2] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–54.
[3] Durante W, Kroll MH, Christodoulides N, Peyton KJ, Schaefer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. Circ Res 1997;80:565–74.
[4] Foresti R, Clark JE, Green CJ, Motterlini R. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. J Biol Chem 1997;272:18411–7.
[5] Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci USA 1968;61:748–55.
[6] Taille C, El-Benna J, Lanone S, Dang MC, Ogier-Denis E, Aubier M, et al. Induction of heme oxygenase-1 inhibits NAD(P)H oxidase activity by down-regulating cytochrome b558 expression via the reduction of heme availability. J Biol Chem 2004;279:28681–8.
[7] Haider A, Olszanecki R, Gryglewski R, Schwartzman ML, Lianos E, Kappas A, et al. Regulation of cyclooxygenase by the heme–heme oxygenase system in microvessel endothelial cells. J Pharmacol Exp Ther 2002;300:188–94.
[8] Li Volti G, Seta F, Schwartzman ML, Nasjletti A, Abraham NG. Heme oxygenase attenuates angiotensin II-mediated increase in cyclooxygenase-2 activity in human femoral endothelial cells. Hypertension 2003;41:715–9.
[9] Turcanu V, Dhouib M, Poindron P. Nitric oxide synthase inhibition by heme oxygenase decreases macrophage nitric-oxide-dependent cytotoxicity: a negative feedback mechanism for the regulation of nitric oxide production. Res Immunol 1998;149:741–4.
[10] Kappas A, Drummond GS. Control of heme metabolism with synthetic metalloporphyrins. J Clin Invest 1986;77:335–9.
[11] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043–6.
[12] Minetti M, Mallozzi C, Di Stasi AM, Pietraforte D. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. Arch Biochem Biophys 1998;352:165–74.
[13] Mancuso C, Bonsignore A, Di Stasio E, Mordente A, Motterlini R. Bilirubin and S-nitrosothiols interaction:

evidence for a possible role of bilirubin as a scavenger of nitric oxide. Biochem Pharmacol 2003;66:2355–63.
[14] Samb A, Taille C, Almolki A, Megret J, Staddon JM, Aubier M, et al. Heme oxygenase modulates oxidant-signaled airway smooth muscle contractility: role of bilirubin. Am J Physiol Lung Cell Mol Physiol 2002;283:L596–603.
[15] Tulis DA, Durante W, Peyton KJ, Evans AJ, Schafer AI. Heme oxygenase-1 attenuates vascular remodeling following balloon injury in rat carotid arteries. Atherosclerosis 2001;155:113–22.
[16] Wagener FA, da Silva JL, Farley T, de Witte T, Kappas A, Abraham NG. Differential effects of heme oxygenase isoforms on heme mediation of endothelial intracellular adhesion molecule 1 expression. J Pharmacol Exp Ther 1999;291:416–23.
[17] Sarady JK, Otterbein SL, Liu F, Otterbein LE, Choi AM. Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages. Am J Respir Cell Mol Biol 2002;27:739–45.
[18] Tamion F, Richard V, Lacoume Y, Thuillez C. Intestinal preconditioning prevents systemic inflammatory response in hemorrhagic shock. Role of HO-1. Am J Physiol Gastrointest Liver Physiol 2002;283:G408–14.
[19] Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999;104:1631–9.
[20] Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, et al. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998;4:1073–7.
[21] Srisook K, Jung NH, Kim BR, Cha SH, Kim HS, Cha YN. Heme oxygenase-1-mediated partial cytoprotective effect by NO on cadmium-induced cytotoxicity in C6 rat glioma cells. Toxicol In Vitro 2005;19:31–9.
[22] Srisook K, Cha YN. Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide. Biochem Pharmacol 2004;68:1709–20.
[23] Srisook K, Cha YN. Superinduction of HO-1 in macrophages stimulated with lipopolysaccharide by prior deletion of glutathione decreases iNOS expression and NO production. Nitric Oxide 2005;12:70–9.
[24] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000;6:422–8.
[25] Otterbein LE. Carbon monoxide: innovative anti-inflammatory properties of an age-old gas molecule. Antioxid Redox Signal 2002;4:309–19.
[26] Morse D, Choi AM. Heme oxygenase-1: the ''emerging molecule'' has arrived. Am J Respir Cell Mol Biol 2002;27:8–16.
[27] Ryter SW, Otterbein LE. Carbon monoxide in biology and medicine. Bioessays 2004;26:270–80.
[28] Scharte M, Bone HG, Van Aken H, Meyer J. Increased carbon monoxide in exhaled air of critically ill patients. Biochem Biophys Res Commun 2000;267:423–6.
[29] Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res 2002;90:E17–24.
[30] Zhen L, King AA, Xiao Y, Chanock SJ, Orkin SH, Dinauer MC. Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91^{phox}. Proc Natl Acad Sci USA 1993;90:9832–6.
[31] Kim C, Dinauer MC. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. J Immunol 2001;166:1223–32.
[32] Kim C, Marchal CC, Penninger J, Dinauer MC. The hemopoietic Rho/Rac guanine nucleotide exchange factor Vav1 regulates N-formyl-methionyl-leucyl-phenylalanine-activated neutrophil functions. J Immunol 2003;171:4425–30.
[33] Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 1993;303:474–82.
[34] Titheradge MA. The enzymatic measurement of nitrate and nitrite. Meth Mol Biol 1998;100:83–91.
[35] Vreman HJ, Stevenson DK. Heme oxygenase activity as measured by carbon monoxide production. Anal Biochem 1988;168:31–8.
[36] Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch Biochem Biophys 1996;328:5–92.
[37] Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, Motterlini R. Carbon monoxide-releasing molecules (CORMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW 264.7 murine macrophages. Br J Pharmacol 2005;145:800–10.
[38] Taille C, El-Benna J, Lanone S, Borzkowski J, Motterlini R. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. J Biol Chem 2005;280:25350–6.
[39] Zuckerbraun BS, Billiar TR, Otterbein SL, Kim PK, Liu F, Choi AM, et al. Carbon monoxide protects against liver failure through nitric oxide-induced heme oxygenase-1. J Exp Med 2003;198:1707–16.
[40] Kang KW, Pak YM, Kim ND. Diethylmaleate and buthionine sulfoximine, glutathione-depleting agents differentially inhibit expression of inducible nitric oxide synthase in endotoxemic mice. Nitric Oxide 1999;3:265–71.
[41] Wang F, Wang LY, Wright D, Parmely MJ. Redox imbalance differentially inhibits lipopolysaccharide-induced macrophage activation in the mouse liver. Infect Immun 1999;67:5409–16.
[42] Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med 1996;2:87–90.
[43] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000;6:422–8.
[44] Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, et al. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 2000;192:1015–26.
[45] Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.