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Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide

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Abstract

Time course relationship between inductions of iNOS and HO-1 was evaluated in RAW264.7 macrophages stimulated with LPS. Expression of HO-1 mRNA increased in a biphasic pattern, but that of xCT (cystine transporter) and iNOS mRNA increased in a monophasic manner. HO-1 protein level increased also in a biphasic manner, at 1–2 h and again between 8 and 24 h. However, iNOS protein began to increase at 4 h, quickly reaching a high level in a monophasic induction pattern. Production of $NO^{\bullet}$ began to occur at 6 h and nitrite continued to accumulate in the culture medium. Total GSH level decreased markedly (50% of control) by 2 h, began to recover at 4 h, returned to control level by 6 h and increased above the control level during 10–24 h. Collectively, these results indicated that overproduced $O_2^{\bullet-}$ depletes GSH and triggers induction of xCT, HO-1, iNOS and HO-1 expression in sequence. Most notably, the second-phase induction of HO-1 was caused by overproduced $NO^{\bullet}$, resulting from LPS-derived iNOS induction. When this iNOS-derived delivery of $NO^{\bullet}$ was combined with prior depletion of GSH using buthioninesulfoximine, an inhibitor of GSH biosynthesis, induction of HO-1 was potentiated. Furthermore, upon such super-induction of HO-1, $NO^{\bullet}$ production was inhibited along with suppression of iNOS expression. Collectively, these results suggested that HO-1 is induced in a biphasic manner, sequentially by the overproduced $O_2^{\bullet-}$ and $NO^{\bullet}$, and the elevated HO-1 suppresses the production of these radicals in an auto-regulatory manner. This may allow the macrophages to survive from injuries that can be caused by concomitant oxidative and nitrosative stresses initiated by the LPS-driven oxidative burst.

Keywords: Heme oxygenase-1; Macrophages; Lipopolysaccharide; Glutathione; Inducible nitric oxide synthase; Nitric oxide

1. Introduction

Macrophage serves as the first-line defense against invading pathogens by overproducing $O_2^{\bullet-}$ and $NO^{\bullet}$ radicals. In macrophages, productions of $O_2^{\bullet-}$ and $NO^{\bullet}$ radicals are catalysed, respectively, by the heme-containing NADPH-oxidase and iNOS (EC 1.14.13.39). Expression and activity of these heme-containing enzymes increase in macrophages upon stimulation with LPS. These radicals cause damage not only to the invading pathogens but also to macrophages themselves and surrounding host cells. However, macrophages can protect themselves by enhancing the expression of HO-1 (EC 1.14.99.3), a heme-degrading enzyme. In the current study, we evaluated the time course relationship between inductions of iNOS and HO-1 expression in RAW264.7 macrophages stimulated with LPS.

The highly lipophilic heme molecule serves as the prosthetic catalytic center for electron transfer in many heme-containing enzyme proteins like hemoglobin, cytochromes, NADPH-oxidase, nitric oxide synthases, prostaglandin synthase, and guanylatecyclase, among others (reviewed in [1]). Heme exists only in association with protein and this association with protein limits free heme from getting involved in producing highly reactive hydroxyl radical ($HO^{\bullet}$) from H_2O_2 via Fenton chemistry. However, the protein-bound heme can be liberated by destabilization, fragmentation and proteasomal degradation, and these heme-liberating processes are promoted by $O_2^{\bullet-}$ and $NO^{\bullet}$ radicals produced by the heme-containing

Abbreviations: $O_2^{\bullet-}$, superoxide anion; $NO^{\bullet}$, nitric oxide; $ONOO^-$, peroxynitrite; CO, carbon monoxide; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; xCT, cystine/glutamate exchange transporter; GSH, reduced glutathione; GSSG, oxidized glutathione; LPS, lipopolysaccharide; BSO, DL-buthionine-[*S*,*R*]-sulfoximine; ROS, reactive oxygen species; L-NAME, N^G-nitro-L-arginine methyl ester.

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enzymes themselves (reviewed in [2]). Released free heme is toxic because it can catalyze the formation of $HO^{\bullet}$ from H_2O_2 and cause lipid peroxidation and oxidative damage to proteins. In aerobic cells, this ROS-generating toxic activity of free heme is counteracted by induction of HO-1, which catalyzes the oxidative degradation of heme (reviewed in [3]). HO-1 expression is enhanced not only by its substrate free heme but also by various other pro-inflammatory non-heme stimulants such as bacterial LPS, cytokines, heavy metals, hypoxia and other oxidants [4–9]. Most commonly, these HO-1 inducers cause oxidation of intracellular GSH and oxidative cell damage [10].

Heme oxygenases (HO) are the rate-limiting enzymes involved in catalyzing oxidative degradation of heme to generate Fe^{2+}, biliverdin, and carbon monoxide (CO) [11–13]. There are at least three iso-forms of HO in most mammalian cells: the oxidative stress- or heme-inducible HO-1 and the constitutively expressed non-inducible HO-2 and HO-3 [13]. In mammalian cells, biliverdin is converted subsequently to bilirubin by biliverdin reductase, a rapidly NADPH-consuming cytosolic enzyme [11–13]. Products generated from HO activity, namely CO and bilirubin, are known to have antioxidant and anti-inflammatory activities in vivo [14]. Thus, increased production of bilirubin and CO can protect cells against the toxicity of ROS by eliminating ROS and inhibiting further production of ROS, both to assist in preserving cellular redox homeostasis and promoting survival [15,16]. In support of this, elevated HO-1 expression has been associated with increased resistance to oxidative tissue injury in several clinical situations [17–19].

Small amounts of $O_2^{\bullet-}$ are generated even during the course of normal cellular metabolism by several heme-containing enzymes (i.e., NADPH-oxidase, mitochondrial cytochromes, P-450). Under normal conditions, they are sufficiently detoxified by existing antioxidant enzyme systems. However, upon excessive production and/or inadequate detoxification, cells undergo oxidative stress, most notably by oxidation of GSH, lowering thiol/disulfide ratio and depleting intracellular GSH [2]. In this connection, exposure of cells to LPS is known to cause overproduction of both $O_2^{\bullet-}$ and NO, which then combine to generate peroxynitrite ($ONOO^-$) and oxidize GSH causing nitrosative stress as well [20,21]. Surviving cells respond to these oxidative and nitrosative stresses in part by enhancing the expression of selected set of genes that encode antioxidant enzymes via activating several cytoplasmic redox-sensitive transcription factors like NF-κB, AP-1, and Nrf2 [4,7]. This leads to enhancement of the production of GSH and $NO^{\bullet}$ needed for rapid scavenging of ROS and also to enhance the synthesis of several antioxidant enzymes providing additional cytoprotective activities. In support, exposure to LPS is known to cause depletion of cellular GSH but to induce the expression of cystine transporter (xCT), iNOS and HO-1, among many antioxidant enzymes [22–24]. Once induced, xCT provides cystine needed for synthesis of GSH, iNOS provides abundant $NO^{\bullet}$ continuously to scavenge the overproduced $O_2^{\bullet-}$, and HO-1 degrades the toxic heme while generating bilirubin (antioxidant) and CO (binds avidly to heme-iron in heme enzymes and inhibits production of $O_2^{\bullet-}$ and $NO^{\bullet}$ by interfering electron transfer). Thus, initial production ROS triggers and enhances the synthesis of iNOS and HO-1 in sequence, respectively, to clear $O_2^{\bullet-}$ using $NO^{\bullet}$ and also to inhibit further production of $O_2^{\bullet-}$ and $NO^{\bullet}$ using CO, allowing the oxidatively stressed cells to survive [3,16].

$NO^{\bullet}$ radical is a gaseous signal molecule involved in a wide variety of biological processes [25]. Small quantities of $NO^{\bullet}$ is produced immediately in stimulated cells by activating constitutively expressed NOSs, and this may be sufficient to scavenge the small amount of $O_2^{\bullet-}$ being produced normally. However, when production of $O_2^{\bullet-}$ becomes excessive, like when stimulated with LPS and causes oxidative stress by depleting GSH, it signals to activate redox-sensitive transcription factors and leads to increase the synthesis of iNOS. Once synthesized, iNOS produces abundant $NO^{\bullet}$ and this $NO^{\bullet}$ binds readily to the heme-iron contained in heme enzymes and to the –SH residues in proteins with high affinity. This causes nitrosative injury, enhancing destabilization, inactivation, and proteolytic degradation of heme- or thiol-containing proteins like the iNOS itself [2]. Thus, LPS appears to cause sequential oxidative and nitrosative stresses in macrophages. Binding of overproduced $NO^{\bullet}$ to iNOS can inhibit additional production of $NO^{\bullet}$ catalyzed by the iNOS itself and can also release heme from iNOS in a self-regulatory manner. Free heme released from iNOS can serve not only to enhance HO-1 expression but also to undergo degradation by the elevated HO-1 activity to generate bilirubin and CO [26]. In addition, studies investigating the influence of oxidative stress caused by LPS indicated that HO-1 expression is potentiated in a synergistic manner when delivery of $NO^{\bullet}$ occurs together with GSH depletion. Authors suggested that this $NO^{\bullet}$-derived induction of HO-1 expression is mediated by redox changes [24,27–29].

Thus, in the present study, by employing cultured murine peritoneal macrophage cells (RAW264.7 cell line) stimulated with LPS, we demonstrate that HO-1 expression increases in a biphasic manner, small initial induction by the $O_2^{\bullet-}$-derived oxidative stress and large secondary sustained induction by the $NO^{\bullet}$-derived nitrosative stress.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), LPS (*E. coli* serotype O111:B4), BSO and most other chemicals used in this study were purchased from Sigma. Fetal bovine serum (FBS) was purchased from HyClone. Polyclonal antibody of HO-1 was purchased from Santa Cruz

Biotechnology and monoclonal antibodies of iNOS as well as horseradish peroxidase-conjugated mouse- and rabbit-IgG antibodies were purchased from BD Biosciences-Transduction Laboratories. Oligonucleotide primers were bought from TaKaRa. Penicillin and streptomycin were obtained from Invitrogen/Gibco.

2.2. Cell culture

Murine macrophage cell line RAW264.7 was obtained from American-type culture collection (ATCC). Cells suspended in DMEM (free of phenol red) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal bovine serum (FBS) were cultured at 37 °C in a humidified air containing 5% CO_2. After overnight growth, cells were treated with LPS (1 μg/ml) or other chemicals at the indicated times.

2.3. Nitrite determination

Production of nitrite, a stable end product of $NO^{\bullet}$ oxidation, was used as a measure of NOS activity. Nitrite present in the conditioned media was determined by a spectro-photometric assay based on Griess reaction [30]. The absorbance at 546 nm was measured using a microplate reader, and nitrite concentration was determined by using the standard curve of sodium nitrite dilutions made up in DMEM.

2.4. Glutathione assay

RAW264.7 macrophage cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.4) and scraped after addition of 5% (w/v) 5-sulfosalicylic acid (5-SSA) and 0.2% (v/v) Triton X-100. Suspended cells were then freeze-thawed three times, sonicated and centrifuged. Total amount of GSH present in the acidic supernatant was determined by using the enzymatic recycling assay employing glutathione reductase (GR) as described by Anderson [31]. The rate of 5-thio-2-nitrobenzoic acid (TNB) formed from DTNB was followed spectrophotometrically at 405 nm for 6 min in a microplate reader kept at 30 °C. Results are expressed in nanomoles of glutathione per mg of cellular protein.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Isolation of total RNA was carried out using the TRI reagent (Molecular Research Center) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed using RNA PCR kit (AMV version 2.1) according to the manufacturer's protocol (TaKaRa Shuzo). The obtained complementary DNA (cDNA) was used in PCR. Table 1 shows the sequences of primer pairs used to amplify HO-1, iNOS, cystine transporter (xCT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. PCR was carried out as follows: one cycle of 1 min at 94 °C, 25 cycles (for HO-1 and xCT) or 30 cycles (iNOS and GAPDH) each for 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and one final cycle of 10 min at 72 °C. The amplified products were separated in a 1.5% agarose gel in TBE buffer and stained with ethidium bromide solution. Quantification of RT-PCR products was performed by densitometry using BIO-PROFIL software version 99.04 (Vilber Lourmat Biotechnology). The image densities of PCR products for HO-1, iNOS and xCT were normalized with that of GAPDH.

2.6. Western blot analysis

At the indicated times, macrophages were washed with ice-cold PBS and scraped in the presence of ice-cold lysis buffer. Collected cells were sonicated on ice and their protein concentrations were quantified by using the BCA protein assay kit. 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 non-specific bindings were blocked with 5% non-fat dried milk dissolved in TBS-T buffer. The membranes were then incubated for 1 h at room temperature with HO-1, iNOS and β-actin antibodies diluted respectively at 1:1000, 1:1000 and 1:5000. Subsequently, membranes were incubated with either goat anti-rabbit (1:5000 dilution) or goat anti-mouse (1:1000 dilution) IgG:horseradish peroxidase antibodies for 1 h at room temperature. Protein bands were visua-

Table 1
Primer sequences used in reverse transcriptase-polymerase chain reaction (RT-PCR)

Target	Primer	Sequences	Product size (bp)	Accession no.
iNOS	Sense	5′-AGA CTG GAT TTG GCT GGT CCC TCC-3′	527	NM010927
	Anti-sense	5′-AGA ACT GAG GGT ACA TGC TGG AGC C-3′		
HO-1	Sense	5′-TGA AGG AGG CCA CCA AGG AGG -3′	375	NM010442
	Anti-sense	5′-AGA GGT CAC CCA GGT AGC GGG-3′		
Xct	Sense	5′-TCC ATG GCT ATC ATC ACA GTG GGC- 3′	556	AB022345
	Anti-sense	5′-AGG TGA TAA GAA AAC CGA CCC CGG -3′		
GAPDH	Sense	5′-GTC GGT GTG AAC GGA TTT G -3′	386	XM123285
	Anti-sense	5′-ACA AAC ATG GGG GCA TCA G-3′		

lized on X-ray film activated by chemiluminescence using the Western blotting luminol reagent (Santa Cruz Biotechnology). Intensities of each band signal were determined by densitometry, and image densities of specific bands for iNOS and HO-1 at each time point were normalized with the density of respective β-actin band.

2.7. Heme oxygenase activity assay

Heme oxygenase activity was determined using the method described by Yet et al. [32] with some modifications. RAW264.7 macrophages were scraped in the presence of 1 ml of cold homogenization buffer. Collected cells were sonicated, centrifuged and the obtained microsomal pellet was re-suspended in 100 mM potassium phosphate buffer (pH 7.4). This microsomal fraction 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, 100 mM potassium phosphate buffer, pH 7.4. Finally, 2 mg of rat liver cytosol was added as a source of biliverdin reductase. Mixtures were incubated for 1 h in the dark at 37 °C and placed on ice to terminate the reaction. Bilirubin formed was determined by calculating the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO activity was expressed as nanomoles of bilirubin formed per mg protein per hour.

2.8. Cell viability test by MTT assay

Cell viability was evaluated by determining mitochondrial function of living cells on the basis of their ability to reduce the yellow dye, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into blue formazan crystal mainly by the mitochondrial dehydrogenases. This method was performed as described by Kiemer et al. [33] with some modifications. Cells (1.5×10^5) were plated into 24-well plates and allowed to attach for 12 h and the test compounds were added to wells for an appropriate length of time. Ten microliters 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 product. The solubilized formazan solution was read in a microplate reader (PowerWaveX, Bio-Tek instrument, Inc.) and the absorbance of each well was measured at 550 nm. Percentage of cell viability was expressed as: (absorbance of treated well/absorbance of control well) × 100.

2.9. Statistical analysis

Statistical analyses were performed using the two-tailed Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Biphasic induction of HO-1 expression upon stimulation with LPS

Cultured murine macrophage cells (RAW264.7) were incubated with LPS (1 μg/ml) and cells were harvested at various times indicated and total RNAs were extracted and subjected to RT-PCR using HO-1 cDNA probe. Intensity of HO-1 mRNA signal obtained at each time point was corrected for the intensity of respective GAPDH mRNA signal (internal standard) and the LPS-derived increases of HO-1 mRNA contents were compared in a time course study. Interestingly, a biphasic induction of HO-1 mRNA expression was observed (Fig. 1A). There was a small initial increase of HO-1 mRNA expression, which occurred briefly at 1 h, and the level of HO-1 mRNA then decreased to the basal level by 2 h. Subsequently, a marked second-phase induction of HO-1 mRNA expression began to appear at 4 h, reached maximum at 10 h, and remained at high level throughout 24 h. We then examined the accumulation of HO-1 protein. The level of β-actin protein in each immuno-blotted sample was used as an internal standard to compare the accumulated levels of HO-1 protein at each time point. As expected, accumulated levels of HO-1 protein revealed a delayed biphasic induction pattern (Fig. 1B). Small early accumulation of HO-1 protein peaked at 1–2 h and decreased to background control level by 4–6 h. A marked second-phase accumulation of HO-1 protein began to be observed at 8 h and maximal increase was observed at 12 h and sustained up to 24 h. These results demonstrated that LPS enhances HO-1 expression in a biphasic manner in both its mRNA and protein in RAW264.7 macrophages.

3.2. Monophasic induction of iNOS in macrophages stimulated with LPS

Effect of LPS in enhancing the expression of iNOS mRNA was determined in the same cells that have been used to determine time-dependent biphasic induction of HO-1 mRNA expression. As shown in Fig. 1A, increased iNOS mRNA expression began to be detected at 2 h, reached peak at 4 h in a monophasic induction pattern and remained highly elevated for 24 h (Fig. 1A). Detectable accumulation of iNOS protein began to appear at 4 h and continued to increase even at 24 h, again in a monophasic induction pattern (Fig. 1B). In the non-stimulated cells, expression of iNOS mRNA and protein could not be detected. In accordance with this accumulation of iNOS protein, production of $NO^{\bullet}$, as determined by the concentration of nitrite accumulated in conditioned medium, began to increase at 6 h and continued to increase even at 24 h (Fig. 2). Thus, unlike the induction of HO-1 expression, which occurred in a biphasic manner, iNOS expression and $NO^{\bullet}$ production increased in a monophasic

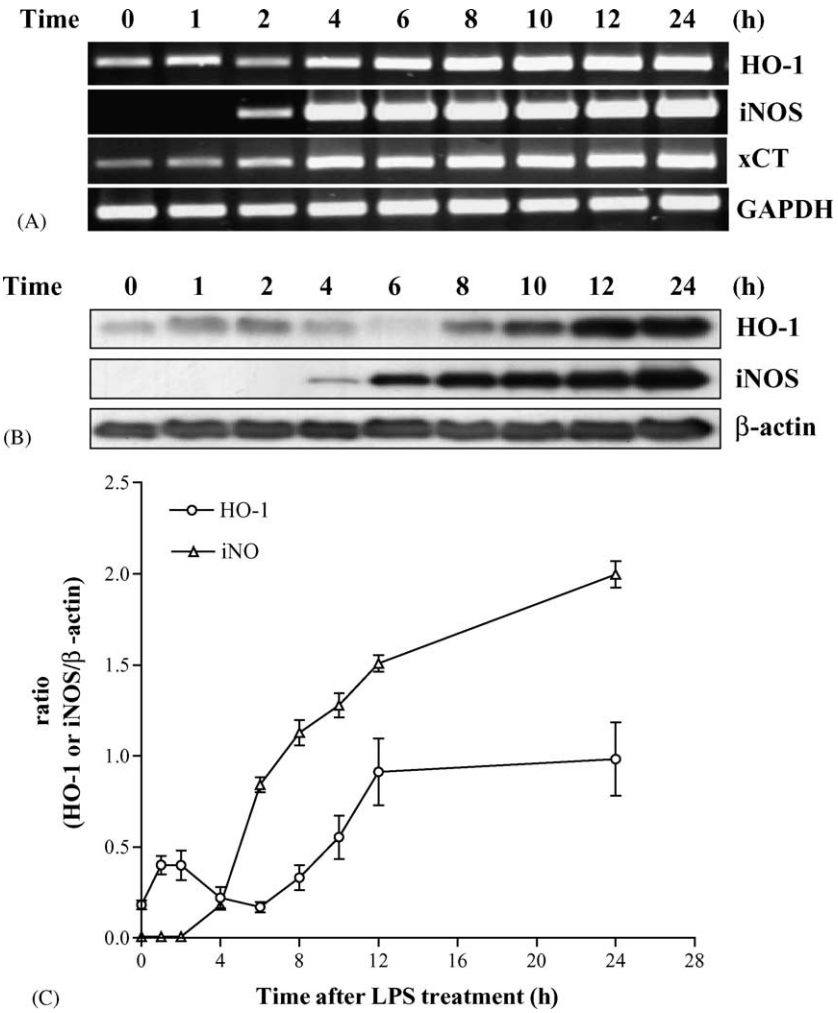


Fig. 1. Time-dependent inductions of HO-1, iNOS and xCT in LPS-stimulated macrophages. Cells were treated with LPS (1 μg/ml) and harvested at indicated time points. (A) Total RNA was extracted and subjected to RT-PCR. The gel picture shown is a representative of three separate experiments. (B) Harvested cells were homogenized and homogenates were analyzed to determine the contents of HO-1 and iNOS employing immuno-blot analysis. The immuno-blot picture shown is a representative of three separate experiments. (C) Graph shows the mean ± S.E.M. results of three densitometric analyses on the time-dependent accumulation of HO-1 and iNOS proteins, which have been normalized to β-actin content.

fashion. Most notably, this LPS-derived induction of iNOS began to occur shortly after the initial HO-1 expression has subsided, but shortly before the beginning of second-phase HO-1 induction (Fig. 1C). This suggested that $O_2^{\bullet-}$ derived initially from oxidative burst in LPS-stimulated cells is responsible for the early phase HO-1 expression and $NO^{\bullet}$ derived from elevated iNOS activity is responsible for the marked second-phase HO-1 expression.

3.3. Changes of GSH levels in LPS-stimulated macrophages

Time-dependent changes of intracellular GSH level were determined in LPS-stimulated macrophages. Acidic protein-free supernatants were prepared from harvested cells at 0, 1, 2, 4, 6, 8, 10, 12 and 24 h after exposing the cells to LPS. Intracellular level of total GSH did not change initially at 1 h, but it decreased drastically after 2 h (down to 50% of control level). Subsequently, the depleted GSH level began to recover at 4 h, reached near the control level by 6 h, and increased further above control level during 10–24 h (Fig. 3).

In macrophages, major amino acid transporter responsible for supplying cysteine to permit GSH biosynthesis is the Xc^- system, which is involved in the exchange transport of extracellular cystine with intracellular glutamate [22,34]. Once transported inside, cystine is reduced to cysteine and used as a precursor of GSH biosynthesis catalyzed by γ-glutamylcysteine synthetase, a cytoplasmic enzyme also known to undergo rapid induction by oxidative stress. Increased rate of cystine uptake is crucial for enhanced GSH biosynthesis, and thus enhanced xCT (one of the protein components in Xc^- system) expression is readily observed in LPS-treated or oxidatively stressed cells with depleted GSH [22]. Thus, we determined the expression of xCT mRNA in the same LPS-exposed cells in a time course study. Enhanced expression of xCT mRNA was observed already at 1 h and increased quickly to the

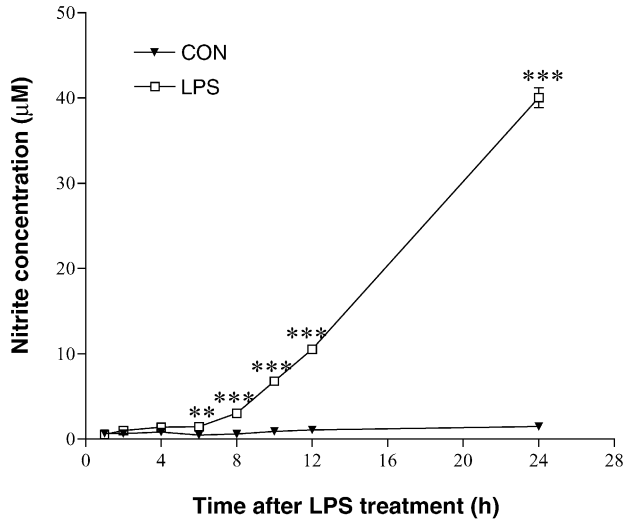


Fig. 2. Time-dependent accumulation of nitrite in culture medium of LPS-stimulated macrophages. Accumulated concentrations of nitrite present in the conditioned medium of LPS-stimulated cells were determined at indicated sampling times. Values and bars in the graph indicate mean ± S.E.M. obtained from three independent experiments with three determinations at each time point. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the levels in control cell media sampled at various times.

peak level in a monophasic pattern and remained highly elevated even at 24 h (Fig. 1A), the time point when intracellular level of total GSH was elevated already above the control level (Fig. 3).

3.4. Suppression of HO-1 induction by inhibiting NO• production with L-NAME

To determine whether the NO• overproduced by up-regulated iNOS is involved in enhancing HO-1 expression, as was reported by Motterlini et al. [8], we attempted to

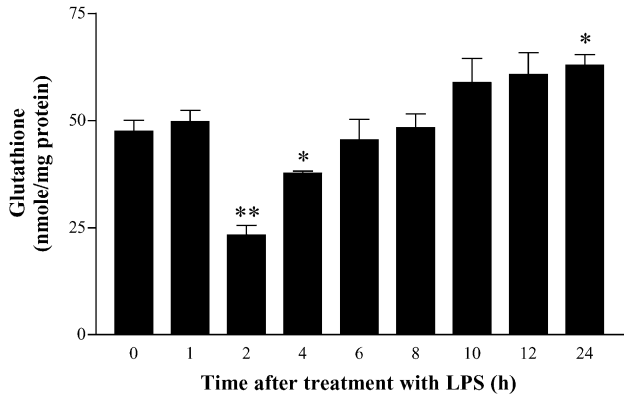


Fig. 3. Time-dependent changes of total glutathione level in LPS-stimulated macrophages. Cells suspended in DMEM containing 10% FBS were treated with LPS (1 μg/ml) and harvested at indicated times. Harvested cells were rinsed, treated with 5-SSA, and solubilized with Triton X-100. Acidic supernatants were analyzed to determine the total glutathione (GSH + GSSG) concentration. Graph shows the mean ± S.E.M. obtained from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the total glutathione level at 0 h.

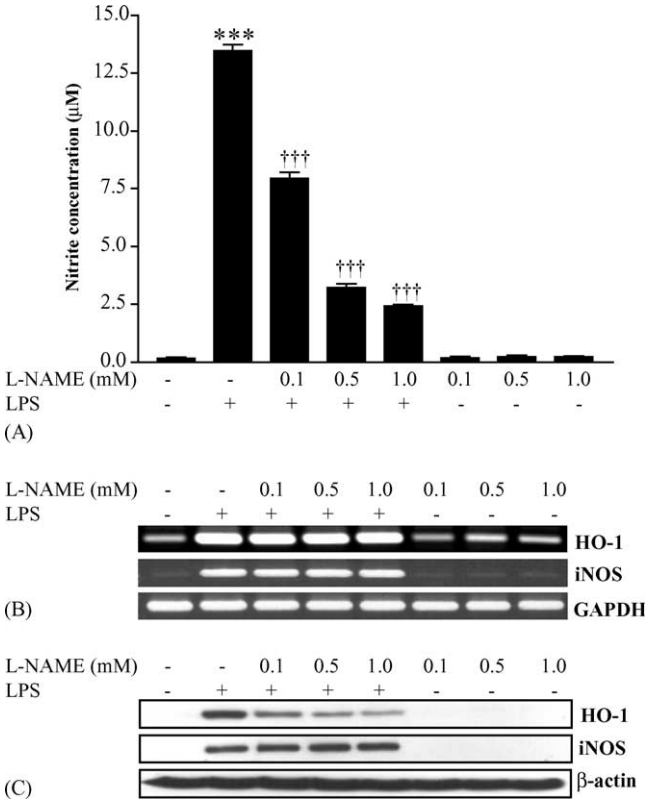


Fig. 4. Effect of L-NAME on LPS-derived induction of HO-1 and iNOS and nitrite production in LPS-stimulated macrophages. Cells were pretreated with varying doses of L-NAME (0.1, 0.5 and 1.0 mM) at 30 min prior to addition of LPS (1 μg/ml). Cells were harvested at 12 h after addition of LPS. (A) Accumulated nitrite concentrations present in conditioned media were determined. Bar graph indicates mean ± S.E.M. obtained from six separate experiments. $^{***}P < 0.01$ versus control media obtained without additions of L-NAME or LPS. $^{\dagger\dagger\dagger}P < 0.001$ versus LPS-conditioned medium. (B) Total RNA was extracted and subjected to RT-PCR. The gel picture shown is a representative of three separate experiments. (C) Contents of HO-1 and iNOS proteins in homogenates were determined by employing immuno-blot analysis. The blot picture shown is a representative of three separate experiments.

inhibit NO• production in LPS-stimulated cells by employing L-NAME, a well-known inhibitor of NOS activity. L-NAME inhibited NO• production in a dose-dependent manner in LPS-stimulated cells (Fig. 4A) and suppressed the expression of HO-1 mRNA and protein, again in a dose-dependent manner (Fig. 4B and C). This L-NAME dependent depression of HO-1 expression was not complete and suppressed HO-1 expression only down to certain extent (60% reduction) without affecting the magnitude of initial HO-1 induction (data not shown). These results supported the observations of Motterlini et al. [8] and suggested that the NO• overproduced endogenously by up-regulated iNOS was causing the L-NAME inhibitable second-phase induction of HO-1 expression. However, these results suggested further that L-NAME un-inhibitable initial HO-1 induction and remaining 40% secondary HO-1 induction may have been caused by $O_2^{\bullet-}$, which is overproduced by oxidative burst in LPS-stimulated cells but not sufficiently scavenged by NO•, due to inhibition of its production by L-NAME.

3.5. Super-induction of HO-1 in cells pretreated with BSO and stimulated with LPS

Expecting that early induction of HO-1 expression may have been caused by oxidation or depletion of GSH initiated by LPS-stimulated oxidative burst, HO-1 induction was determined in macrophages pretreated with BSO to deplete intracellular level of GSH, in a similar manner as reported by Rizzardini et al. [24]. Subsequently, these BSO-pretreated cells were exposed to LPS, and then time course of increasing HO-1 and iNOS expressions were followed (Fig. 5). Compared to the un-stimulated control cells (Con 24 column, 24 h normal condition without BSO), the BSO-treated control cells (BSO 24 column) had increased levels of HO-1 mRNA expressed even without exposure to LPS (Fig. 5A). This indicated that depletion of GSH alone can enhance HO-1 mRNA expression, supporting the results of Rizzardini et al. [24]. When the cells were pretreated with BSO for 4 h and then exposed to LPS, an immediate and almost steady monophasic increase of HO-1 mRNA expression was observed and it continued to increase even at 24 h (Fig. 5A). In accordance, the content of HO-1 protein began to increase immediately and continued to increase almost steadily even at 24 h in a nearly monophasic induction pattern (Fig. 5B). Accumulated HO-1 protein was much greater than that induced in cells treated with LPS without BSO-pretreatment (Fig. 5C), again supporting the results of Rizzardini et al. [24]. Most notably however, decreased HO-1 expression normally observed at 4–6 h in cells treated with LPS alone did not occur in these BSO-pretreated cells exposed to LPS. Furthermore, significant HO-1 induction occurred before any significant $NO^{\bullet}$ production could be detected, which began at 6 h (Fig. 5D). Collectively, these results suggested that $O_2^{\bullet -}$ produced immediately upon LPS stimulation was responsible for the observed early phase induction of HO-1 expression and perhaps this is mediated by oxidation and/or depletion of GSH (undetectable at 1 h).

Exposing cells to BSO alone either for 4 h or 24 h did not appear to cause any significant accumulation of iNOS protein (Fig. 5B, BSO 0 and BSO 24 columns). Upon exposing these 4-h BSO-pretreated GSH-depleted cells to LPS, expression of iNOS protein began to increase at 4 h (Fig. 5B), the same time point observed in cells treated with LPS without prior BSO treatment (Fig. 1B). Comparing the levels of iNOS protein determined in these LPS-stimulated cells (24 h) with and without prior BSO treatment, accumulated level of iNOS protein in cells with prior depletion of GSH (BSO-pretreated) was decreased markedly by more than 50% (Fig. 5C). In support of this reduced accumulation of iNOS protein, nitrite accumulated at 24 h in culture medium was decreased significantly (Fig. 5D and Fig. 6B). This observation appeared to support the results of Zamura et al. [35] and Hothersall et al. [36], who reported that nitrite production was decreased in J774 murine macrophages treated with BSO and LPS together.

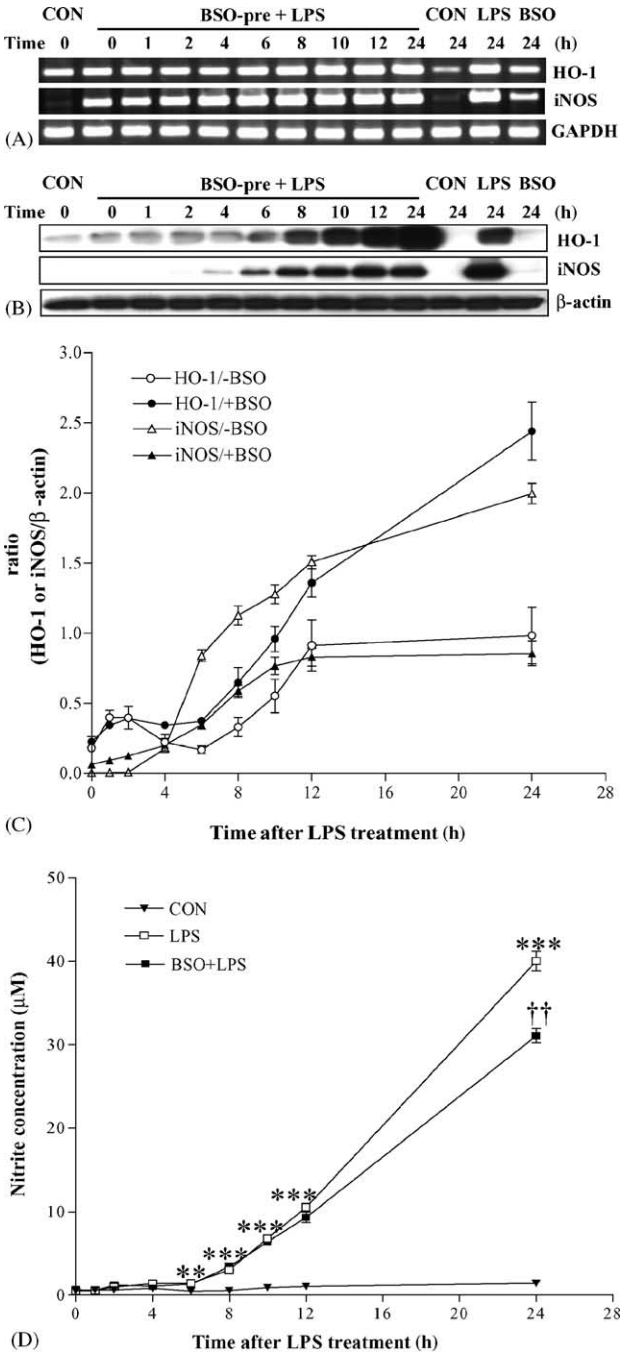
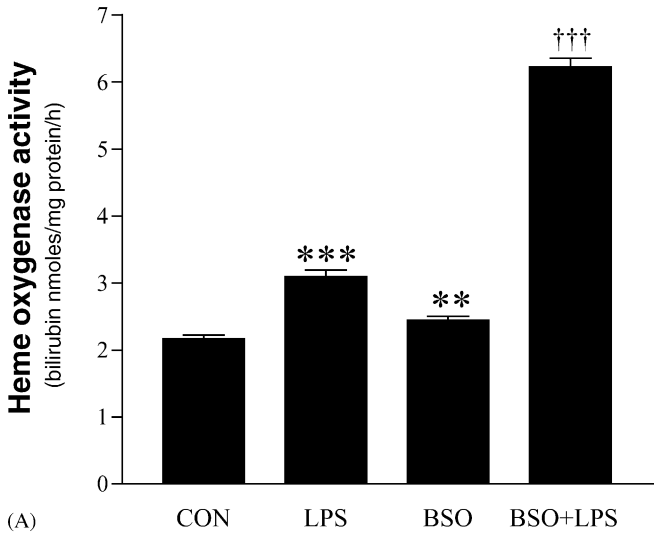


Fig. 5. Effect of BSO-pretreatment on LPS-derived inductions of HO-1 and iNOS and nitrite production. Cells were pretreated with BSO (100 μM) for 4 h prior to addition of LPS (1 μg/ml) and harvested at indicated times. As controls, cells without any treatment (Con 24), treated with LPS alone (LPS 24) or BSO alone (BSO 24) were used. (A) Total RNA was extracted and subjected to RT-PCR. The gel picture shown is a representative of three separate experiments. (B) Harvested cells were homogenized and analyzed to determine the contents of HO-1 and iNOS proteins by employing immuno-blot analysis. The immuno-blot picture shown is a representative of three separate experiments. (C) Graph shows the mean ± S.E.M. results obtained from three sets of densitometric analyses on time-dependent accumulations of HO-1 and iNOS, which have been normalized to β-actin contents at each time point. (D) Graph shows nitrite concentrations present in conditioned media determined at indicated times. Values indicate mean ± S.E.M. obtained from three sets of paired independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control cell media sampled at various times. $^{\dagger\dagger}P < 0.01$ compared to the nitrite level in conditioned media of LPS-treated cells.



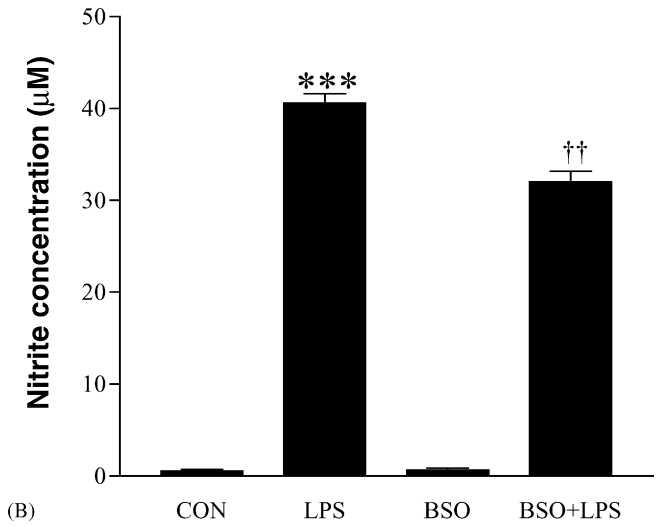


Fig. 6. Effect of BSO-pretreatment on LPS-derived enhancements of HO activity and nitrite production. Cells were pretreated with BSO (100 μM) for 4 h prior to addition of LPS (1 μg/ml) and incubated for 24 h before harvesting. (A) Harvested cells were sonicated and centrifuged to prepare microsomal fractions. Microsomal HO activity was determined by production of bilirubin from hemin. Bar graph shows mean ± S.E.M. values obtained from six separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control cells not treated with BSO or LPS. $^{\dagger\dagger\dagger}P < 0.001$ compared with LPS treated cells. (B) Graph shows nitrite concentrations present in conditioned media. Values indicate mean ± S.E.M. obtained from four separate experiments. $^{***}P < 0.001$ compared to the conditioned medium of control cells. $^{\dagger\dagger}P < 0.01$ compared to the conditioned medium of LPS treated cells.

Conversely, level of HO-1 protein accumulated in cells with prior depletion of GSH and determined at 24 h was 2.4 fold higher (Fig. 5C). This indicated that $NO^{\bullet}$ delivery combined with GSH depletion potentiates HO-1 expression in a synergistic or additive manner, again, supporting the results of previous reports [24,29]. In support of this markedly higher accumulation of HO-1 protein, HO activity determined in these cells was nearly 2-fold higher (Fig. 6A). These increases of HO activity occurring in response to LPS-stimulation in cells with or without prior BSO

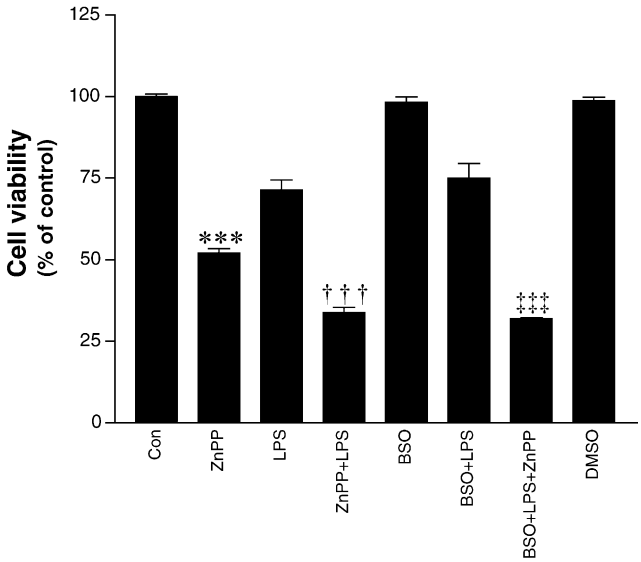


Fig. 7. Effect of inhibiting HO activity with ZnPP on viability of cells treated with LPS with or without BSO pretreatment. LPS (1 μg/ml) was added to cells pre-incubated with BSO (100 μM) for 4 h and/or ZnPP (10 μM) for 30 min. Viability of cells harvested at 24 h after LPS addition was determined using the MTT test. Bar graph shows results obtained from six separate experiments and values indicate mean ± S.E.M. $^{***}P < 0.001$ compared to the viability of control cells. $^{\dagger\dagger\dagger}P < 0.001$ compared with cells treated with LPS alone. $^{\ddagger\ddagger\ddagger}P < 0.001$ compared with cells treated with BSO plus LPS.

treatment were observed mostly in surviving cells (Fig. 7). However, upon inhibition of HO activity with zinc protoporphyrin IX (ZnPP), most of these LPS-treated cells (with and without BSO pretreatment) containing highly elevated HO-1 protein did not survive (Fig. 7).

4. Discussion

The present study demonstrated for the first time that LPS induces expression of HO-1 in macrophages in a biphasic manner (Fig. 1C). In macrophages stimulated with LPS, marked depletion of intracellular GSH level was observed initially at 2 h and this may be caused by the small amount of $ONOO^{-}$ generated from combination of $O_2^{\bullet -}$ (oxidative burst) and $NO^{\bullet}$ (eNOS activation) [37]. LPS-driven $O_2^{\bullet -}$ production also appeared to serve as the triggering signal for a coordinated sequential increase in the expression of xCT, HO-1, iNOS, and again HO-1 (Fig. 1A). Induction of these enzymes may have been caused by activation of several cytoplasmic redox-sensitive transcription factors like AP-1, NF-κB and Nrf2 [4,38,39], which occurs in response to oxidation and depletion of intracellular GSH level [40]. Subsequently, along with sustained over-expression of xCT, the depleted GSH level recovered and even elevated to a higher level by 10 h and remained elevated up to 24 h (Fig. 3). However, sustained iNOS induction and second-phase HO-1 induction occurred even when xCT expression has been enhanced in a sustained

manner and when the depleted GSH level has already been restored or even elevated.

During early periods after LPS stimulation, although increased expression of iNOS is not yet detected by RT-PCR or immunoblot assays (Fig. 1), small amounts of $NO^{\bullet}$ not detectable with Griess reaction as yet (Fig. 2) can be produced by LPS-driven activation of eNOS [37] and this $NO^{\bullet}$ can react rapidly with the overproduced $O_2^{\bullet-}$ in a diffusion-limited rate [2]. Such a reaction would scavenge and clear $O_2^{\bullet-}$, but generate $ONOO^-$. This $ONOO^-$ can oxidize GSH rapidly to GSSG, lowering GSH/GSSG ratio and depleting GSH [21,40]. Such a GSH-oxidizing $ONOO^-$ stress, named as ''GSH-protected peroxynitrite stress,'' may have been responsible for the marked GSH depletion observed initially at 2 h after LPS exposure (Fig. 3) [41]. Thus, when there is an ample reserve of intracellular GSH or when GSH is replenished rapidly, cells would be protected from the toxic effects caused by small amounts of $ONOO^-$ generated. During this ''GSH-protected $ONOO^-$ stress'' period and also while the reserve of intracellular GSH level can be maintained, redox-sensitive transcription factors like Nrf2 involved in the induction of HO-1 may not be activated and thus up-regulation of HO-1 expression may not occur [41]. This is evidenced by results indicating that, when initial production of NO is prevented by pre-exposure to L-NAME, early depletion of GSH could not be detected (data not shown). Thus, the GSH present endogenously may have been responsible for the temporary decrease of HO-1 expression observed at 4–6 h in the LPS-stimulated cells (Fig. 1C). During this ''GSH-protected $ONOO^-$ stress'' period, however, cells may be protected from damages that can be caused directly either by $O_2^{\bullet-}$ or $NO^{\bullet}$, perhaps by converting the relatively GSH-unreactive $O_2^{\bullet-}$ and $NO^{\bullet}$ to the rapidly GSH-oxidizing $ONOO^-$.

Although undetectable, early oxidation of GSH caused by $ONOO^-$ produced from LPS-driven activation of eNOS may have been responsible also for the rapid up-regulation of xCT mRNA expression observed immediately after LPS stimulation. Alternatively, sustained production of $ONOO^-$ resulting from LPS-driven induction of iNOS and overproduction of NO may have been responsible for the sustained up-regulation of xCT mRNA expression observed even at 24 h, the time point when cellular GSH level has already been elevated above control level (Figs. 1 and 3). In support of this, in macrophages exposed to an exogenous $NO^{\bullet}$-donor, spermine NONOate, GSH level was depleted initially and expression of xCT mRNA was also increased in a sustained manner. Eventually, GSH level became elevated markedly by the $NO^{\bullet}$-donor (data not shown). This enhanced expression of xCT mRNA and eventual elevation of GSH content in cells treated with exogenous $NO^{\bullet}$-donor was observed even in the absence of iNOS expression (data not shown). Collectively, these results suggested that $ONOO^-$ being generated continuously from the $NO^{\bullet}$ overproduced by accumulated iNOS can also support sustained up-regulation of xCT even in the presence of elevated total GSH level.

In the LPS-treated cells co-exposed to varying doses of L-NAME, $NO^{\bullet}$ production was diminished in a dose-dependent manner and HO-1 expression was suppressed markedly, again in the L-NAME dose-dependent manner. This supported the observations of Motterlini et al. [8] and indicated that overproduced $NO^{\bullet}$ derived from up-regulated iNOS was responsible for the marked second-phase up-regulation of HO-1 expression. Although this NO-derived induction of HO-1 in cells co-exposed to a high dose of L-NAME was suppressed markedly (60%), the small initial phase HO-1 induction or the remaining 40% of second-phase HO-1 induction could not be abolished, however. Thus, both the initial and the remaining 40% of second-phase HO-1 induction may have been caused by the $O_2^{\bullet-}$, which is not sufficiently scavenged by $NO^{\bullet}$, whose production was inhibited with high-dose L-NAME. Collectively, these results indicated that cells co-exposed to LPS and L-NAME are still under moderate oxidative stress and HO-1 is induced even in the absence of NO. This suggested further that initial induction of HO-1 observed immediately after the LPS stimulation may have been caused by the $O_2^{\bullet-}$ overproduced from oxidative burst. This raises a question as to why HO-1 is induced so rapidly in LPS-stimulated macrophages undergoing oxidative burst. This may occur perhaps to increase CO production and to inhibit further production of $O_2^{\bullet-}$ catalyzed by the heme-containing NADPH-oxidase. Early induction of HO-1 expression leads to enhance CO production and this CO can bind readily to the heme-iron contained in many heme enzymes like NADPH-oxidase [14]. Therefore, the CO produced initially by an early induction of HO-1 expression can contribute toward abolishing further production of $O_2^{\bullet-}$ and this remains to be tested.

Early increase of GSH biosynthesis supported by enhanced supply of cystine (rapid induction of Xc^- system) may be sufficient to detoxify the minute amount of $ONOO^-$ produced initially. Such rapid synthesis of GSH may have also contributed toward the decreased HO-1 expressions observed at 4–6 h and have allowed the HO-1 level to return back to basal level (Fig. 1). However, when iNOS expression becomes up-regulated and produces abundant $NO^{\bullet}$ to generate excessive $ONOO^-$, the enhanced rate of GSH biosynthesis supported by increased supply of cystine may become overpowered. As the result, even in presence of elevated total GSH level, intracellular GSH/GSSG ratio may become lowered by the abundant $ONOO^-$ being produced (unpublished observation). This may cause a secondary activation of Nrf2 and trigger the second-phase induction of HO-1 expression. Such expression of HO-1 was sustained as long as the delivery of $NO^{\bullet}$ caused either by iNOS or NO-donor continued.

Alternatively, in cells treated with BSO, an inhibitor of GSH biosynthesis, GSH level was decreased and, in response, HO-1 expression was elevated even without

exposure to LPS, as was observed originally by Rizzardini et al. [24]. As was reported, the accumulated level of HO-1 protein in the BSO-LPS co-incubated cells was much higher than that observed in cells treated with LPS alone (Fig. 5B). However, in contrast to HO-1, the level of iNOS protein accumulated in these BSO-LPS co-incubated cells was decreased to 50% and did not appear to increase further beyond 12 h. This was in contrast to the high level of iNOS protein being accumulated in control cells treated with LPS alone, which continued to increase even at 24 h (Fig. 5C). More interestingly, and as was reported by Zamura et al. [35] and Hothersall et al. [36], the amount of $NO^{\bullet}$ produced or the nitrite accumulated in the conditioned medium was significantly lower than that accumulated by cells exposed to LPS alone (Fig. 5D, Fig. 6B). Collectively, these results suggested that higher accumulation of HO-1 protein is causatively associated with lower iNOS protein accumulation and lower NO production. Such a causative auto-inhibitory relationship between increased HO-1 expression (greater HO activity) and decreased iNOS protein synthesis (lower $NO^{\bullet}$ production) was observed previously [42,43] and the underlying reason proposed for this reciprocal relationship is presented schematically in Fig. 8.

Induction of HO-1 is likely to inhibit $NO^{\bullet}$ production in many ways as suggested by Maines [3] and several of these inhibitory mechanisms may reflect the fact that iNOS is a hemoprotein. These include: (1) Elevated HO-1 activity would accelerate the degradation of available heme and would impair de novo synthesis of functional iNOS by limiting its incorporation into newly synthesized apo-iNOS protein (active site of functional iNOS requires two heme molecules) [44]. (2) Heme can be liberated from existing iNOS when the $NO^{\bullet}$ produced by iNOS itself binds to the heme-iron contained in iNOS. Upon binding, iNOS activity is inhibited and iNOS undergoes destabilization and becomes subject to enhanced proteolytic digestion by proteasome and releases heme [2,41]. This liberated heme is degraded by the elevated HO-1 activity to produce more CO. (3) This CO can move freely within the cell and bind to the heme-iron present in the remaining iNOS, which may still be functioning to produce $NO^{\bullet}$. This CO-binding can inhibit additional production of $NO^{\bullet}$ by inhibiting the electron transfer within iNOS. In fact, CO has been reported to bind iNOS and inhibit $NO^{\bullet}$ production [45]. (4) Iron released from the degraded heme is also known to inhibit additional transcription of iNOS gene and to suppress additional synthesis of iNOS protein [46]. Thus, when HO-1 is induced and is actively degrading heme to release iron and CO, both the synthesis of new iNOS protein and the production of $NO^{\bullet}$ by existing iNOS may become inhibited. In support of this reciprocal relationship between HO-1 and iNOS inductions [42,43], exposure of LPS-treated cells to ZnPP, an inhibitor of HO activity, permitted higher level of iNOS to be expressed and also greater amount of $NO^{\bullet}$ to be produced (unpublished observation), and produced increased cytotoxicity ([Fig. 7].

In conclusion, when aerobic macrophage is stimulated with LPS, sequential and coordinated homeostatic survival responses (hormesis) appear to occur, as presented schematically in Fig. 9. LPS initiates the overproduction of $O_2^{\bullet-}$ by oxidative burst catalyzed by the heme-containing NADPH-oxidase. Along with overproduction of $O_2^{\bullet-}$, GSH is oxidized and intracellular ratio of thiol/disulfide

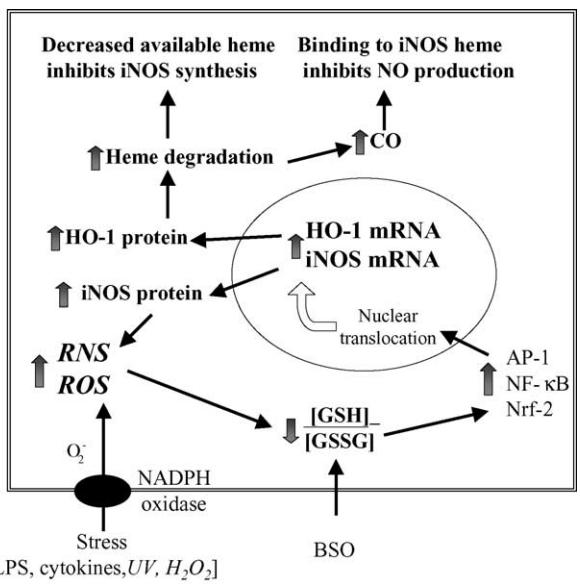


Fig. 8. Schematic diagram showing the pathway leading to inductions of HO-1 and iNOS initiated by external stress stimuli and the role of HO-1 induction in inhibiting iNOS synthesis and $NO^{\bullet}$ production.

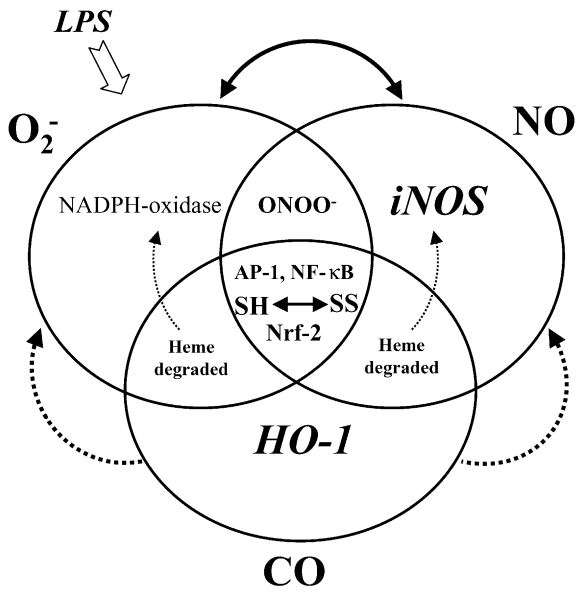


Fig. 9. Schematic diagram showing the pathway leading to biphasic induction of HO-1 and thiol/disulfide mediated "cross-talks" between $O_2^{\bullet-}$, $NO^{\bullet}$ and CO leading to survival of LPS-stimulated macrophages. Dotted lines indicate inhibitory pathways.

is lowered. In response, several cytoplasmic redox-sensitive transcription factors like AP-1, NF-κB, and Nrf2 may become activated, transported into nucleus, and enhance the expressions of xCT, iNOS and HO-1. This will lead to increased synthesis of GSH, overproduction of $NO^{\bullet}$ and CO, respectively, in a sequential and coordinated manner. Upon induction of iNOS, abundant $NO^{\bullet}$ is produced continuously and this $NO^{\bullet}$ would scavenge $O_2^{\bullet -}$ and generate large amounts of $ONOO^-$. This $ONOO^-$ will then oxidize GSH rapidly and, in response, Nrf2 may become activated again, triggering the second-phase induction of HO-1 expression. As long as abundant $NO^{\bullet}$ is being produced by the iNOS, marked induction of HO-1 expression is sustained. Highly elevated HO-1 activity would then degrade the heme rapidly and will limit the heme available for incorporation into apo-iNOS and apo-NADPH-oxidase, inhibiting additional synthesis of these heme enzymes catalyzing the production $NO^{\bullet}$ and $O_2^{\bullet -}$, respectively. Furthermore, the CO being produced rapidly from degradation of heme catalyzed by the highly accumulated HO-1 can bind to the heme-iron contained in existing heme enzymes and will also inhibit additional productions of $NO^{\bullet}$ and $O_2^{\bullet -}$. Such inter-regulatory ''cross-talks'' between the overproduced $O_2^{\bullet -}$, $NO^{\bullet}$, and CO, which are all initiated by LPS-driven oxidative burst and mediated by lowered thiol/disulfide ratio, may then allow the LPS-stimulated macrophages to survive from damages that can be caused by concomitant oxidative and nitrosative stresses.

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