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Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages

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Abstract

Heme oxygenase-1 (HO-1) is induced under infectious diseases in macrophages. We performed experiments using various gene deficient mouse-derived macrophages to determine a detailed induction mechanism of HO-1 by lipopolysaccharide (LPS) and the functional role of HO-1 induction in macrophages. LPS (1 μg/mL) maximally induced inducible nitric oxide synthase (iNOS) and HO-1 mRNAs in wild-type (WT) macrophages at 6 h and 12 h after treatment, respectively, and liberated tumor necrosis factor α (TNFα) from WT macrophages. LPS also induced iNOS and HO-1 in TNFα(−/−) macrophages, but not in iNOS(−/−) macrophages. Interestingly, although LPS strongly induced iNOS, it failed to induce HO-1 almost completely in nuclear-factor erythroid 2-related factor 2 (Nrf2)(−/−) macrophages. The LPS-induced iNOS gene expression was suppressed by pretreatment with HO-1 inducers, hemin and Co-protoporphyrin (CoPP), but not with HO-1 inhibitor, Sn-protoporphyrin in WT macrophages. In the Nrf2(−/−) macrophages, the ability of CoPP to induce HO-1 and its inhibitory effect on the LPS-induced iNOS gene expression were lower than seen in WT macrophages. The present findings suggest that HO-1 is induced via NO-induced nuclear translocation of Nrf2, and the enzymatic function of HO-1 inhibits the overproduction of NO in macrophages.

Keywords: Heme oxygenase-1; Inducible nitric oxide synthase; Nuclear-factor erythroid 2-related factor 2; Lipopolysaccharide; Macrophage; Tumor necrosis factor α

1. Introduction

Macrophages serve as the first-line of defense against invading pathogens by undergoing immediate oxidative burst and nitric oxide (NO) production, and releasing various inflammatory cytokines which regulate immune function. The overproduced NO generates peroxynitrite, a strong oxidizing and cytotoxic product, through reaction with superoxide anion that can kill the invading pathogens directly (MacMicking et al., 1997). While the NO can kill invading pathogens, it can also influence macrophages themselves or surrounding tissues. On the other hand, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF) α are known as inflammatory cytokines and play important roles in the regulation of the immune system.

Abbreviations: HO-1, heme oxygenase-1; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; WT, wild-type; TNFα, tumor necrosis factor α; Nrf2, nuclear-factor erythroid 2-related factor 2; CoPP, Co-protoporphyrin; SnPP, Sn-protoporphyrin; IL, interleukin; CO, carbon monoxide; MAPK, mitogen-activated protein kinase; JNK, c-Jun *N*-terminal kinase; TLR4, toll-like receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ARE, antioxidant response element; EpRE, electrophile response element; NO, nitric oxide; NFκB, nuclear factor-kappa B; ANOVA, analysis of variance.

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Similar to NO, overliberation of inflammatory cytokines from macrophages causes oxidative stress, systemic inflammation and cell dysfunction. Therefore, macrophages are armed with various detoxify reactive oxygen species systems, such as Mn-superoxide dismutase, catalase, glutathione and heme oxygenase-1 (HO-1) (Campos et al., 2005; Ishii et al., 1999).

HO-1, a rate-limiting enzyme for heme degradation, breaks down heme into Fe^{2+}, carbon monoxide (CO) and biliverdin. Biliverdin is then rapidly converted into bilirubin by biliverdin reductase. HO-1 isozyme is normally expressed at low levels in almost all tissues and sensitively induced by heavy metals, glutathione depletors, immunostimulants and various stress and pathophysiological conditions (Bach, 2002; Oguro and Yoshida, 2004). Several studies have reported that the biological defense effect of HO-1 is provided by its enzymatic reaction products, CO and biliverdin which function as an anti-inflammatory agent and a radical scavenger, respectively (Camara and Soares, 2005; Kirkby and Adin, 2006; Ryter et al., 2006). Evidence is increasingly accumulating that the induction of HO-1 by stress agents, such as electrophile compounds and heavy metals, is regulated by nuclear-factor erithroid 2-related factor 2 (Nrf2) subcellular localization (Ishii et al., 2000). Additionally, we previously reported that TNFα plays a pivotal role in the lipopolysaccharide (LPS)-mediated induction of HO-1 gene expression, and its signal is conducted via p38 mitogen-activated protein kinase (MAPK) and/or c-Jun *N*-terminal kinase (JNK) in the mouse liver (Oguro et al., 2002). However, the contribution of Nrf2 in the HO-1 gene expression by LPS is not clear to date.

LPS is a major component of the outer membrane of Gram-negative bacteria. LPS acts as a prototypical endotoxin because it binds the CD14/toll-like receptor 4 (TLR4)/MD2 receptor complex, which promotes the secretion of inflammatory cytokines and induces the inducible nitric oxide synthase (iNOS) gene expression in immune cells in particular. LPS-released excessive inflammatory cytokines and/or NO lead to endotoxin shock. Several studies have reported that LPS induces HO-1 in various tissues and cells. A number of studies have elucidated the mechanisms of HO-1 induction by LPS in macrophages using the established cell line of a mouse macrophage (RAW 264.7) treated with chemical compounds, such as a competitive inhibitor of NOS activity (L-N^G-nitrosoarginine-methyester) (Srisook and Cha, 2004) or a NO-donor (spermine NONOate) (Srisook and Cha, 2005). However, there are some experimental problems in these studies; for example, we consider that these compounds evoke oxidative stress, and NO released from NO-donor does not reflect the intracellar NO produced via the induction of iNOS gene expression.

A number of studies have reported that HO-1 is induced under infectious diseases and inflammatory diseases in macrophages and monocytes (Schaer et al., 2006; Yachie et al., 2003); however, the detailed induction mechanism of HO-1 and its function in macrophages have yet to be understood. Furthermore, since cytokines have many overlapping functions, few studies have focused on the role of each cytokine exerting the HO-1 gene expression in macrophages. By employing peritoneal macrophages collected from TNFα(−/−), iNOS(−/−) or Nrf2(−/−) mice, we investigated the mechanism of the HO-1 gene expression by LPS and the function of HO-1 using HO-1 inducers, hemin and cobalt-protoporphyrin (CoPP), or HO-1 inhibitor, tin-protoporphyrin (SnPP).

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* serotype (O111:B4) was purchased from Sigma–Aldrich Japan (Tokyo, Japan). The ExScritp™ RT reagent Kit and SYBR® *Premix Ex Taq*™ were obtained from Takara Bio (Tokyo, Japan). HO-1 antibody was from Stressgen Bioreagents (Victria, BC Canada). iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). IκB-α and phospho-IκB-α (Ser32) antibodies were from Cell Signaling Technology, Inc (Beverly, MA). All other reagents used were of the highest grade commercially available.

2.2. Animals

All mouse experiments were carried out under the control of the Committee Regulation of Animal Care and Welfare of Showa University. The TNFα(−/−) mice were established by Tagawa et al. (1997), the iNOS(−/−) mice were established by MacMicking et al. (1995) and Nrf2(−/−) mice were established by Itoh et al. (1997). C57BL/6 mice (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University (#27004).

2.3. Preparation of macrophages and cell culture

Four days after an intraperitoneal injection of 3 mL 3% thioglycolate medium, peritoneal exudate cells were obtained by lavage. The cells were separated by centrifugation, resuspended in an RPMI 1640 medium, plated at 1×10^6 cells/10-cm^2 culture dish in RPMI 1640 containing 20 mM Hepes, 10% fetal bovine serum, 10 unit/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C in 5% CO_2 in air. After 1 h the medium was replaced with a fresh medium in order to remove the nonadherent cells.

2.4. RNA extraction, reverse-transcription, and real-time polymerase chain reaction

Total RNA was isolated from macrophages using the acid guanidine thiocyanate–phenol–chloroform extraction method as previously described (Chomczynski and Sacchi, 1987), and first-stranded cDNA was synthesized with an ExScript™ RT reagent kit. The quantitative real-time PCR was performed according to the manufacturer's protocol using ABI PRISM® 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) and SYBR® *Premix Ex Taq*™. PCR was performed with a 10 s preincubation at 95 °C followed by 40 cycles of 5 s at 95 °C and 31 s at 60 °C. PCR products were subjected to melting curve analysis, using the ABI PRISM® 7000 Sequence Detection Sys-

tem, to exclude amplification of unspecific products. The mRNA levels were measured as the relative ratio to the GAPDH mRNA. The following primers were used:

- HO-1 forward: (5′-TCTCCAGTCGCCTCCAGAGTT-3′).
- HO-1 reverse: (5′-GCAGGCTCCACTCACTGGTT-3′).
- iNOS forward: (5′-AAACTGTGTGCCTGGAGGTTCTG-3′).
- iNOS reverse: (5′-CAAGGCCTCCAATCTCTGCCTA-3′).
- GAPDH forward: (5′-AAATGGTGAAGGTCGGTGTG-3′).
- GAPDH reverse: (5′-TGAAGGGGTCGTTGATGG-3′).

2.5. Western blot analysis

The macrophages were solubilized with 4% sodium dodecyl sulfate, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (HO-1, GAPDH and IκB; 5% stacking gel and 10% separating gel, iNOS; 5% stacking gel and 8% separating gel) according to the method of Laemmli (1970). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Japan Genetics Co., Tokyo, Japan) at 100 mA for 60 min. Western blots were performed using respective antibodies. A signal was detected using chemiluminescence (ECL; Amersham Biosciences Corp., Piscataway, NJ). Molecular weight was calculated with prestained protein marker (Bio-Rad) applied to the same gel run samples. The relative densities were analyzed using Scion Image software (Scion Image Beta 4.03).

2.6. Measurement of cytokine concentrations

IL-1β, IL-6 and TNFα concentrations of the culture medium were determined using their respective enzyme-linked immunosorbent assay kits (Mouse IL-1β, IL-6 and TNFα ELISA Kit; Pierce Endogen, Rockford, IL).

2.7. Statistical analysis

All data are expressed as mean ± S.E.M. and represent at least three independent experiments. The statistical analysis was performed using the one-way analysis of variance (ANOVA), with the Tukey–Kramer test for post hoc comparisons when significance was determined by ANOVA or Student's *t*-test at the analysis indicated in the figures. The accepted level of significance was set at $p < 0.05$.

3. Results

3.1. The induction of HO-1 following iNOS induction by LPS in WT macrophages

LPS is a well-known endotoxin used as a model of bacterial infection. Macrophages recognize LPS via TLR4 and activate the immune system. It is known that the iNOS gene is strongly induced by LPS treatment. Consequently, we first performed time-course experiments to characterize the induction pattern of HO-1 and iNOS in WT macrophages. WT macrophages were cultured and incubated with LPS, and the mRNA and protein levels of HO-1 and iNOS were determined by real-time PCR and Western blot analysis, respectively (Fig. 1). LPS (1 μg/mL) induced iNOS mRNA from 3 h after treatment and reached a peak level at 6 h. The peak of HO-1 mRNA induction by LPS was observed 12 h after treatment (Fig. 1A). There was apparent time lag between iNOS and HO-1 induction for 6 h. Likewise, the iNOS protein reached a peak level at 12 h after LPS treat-

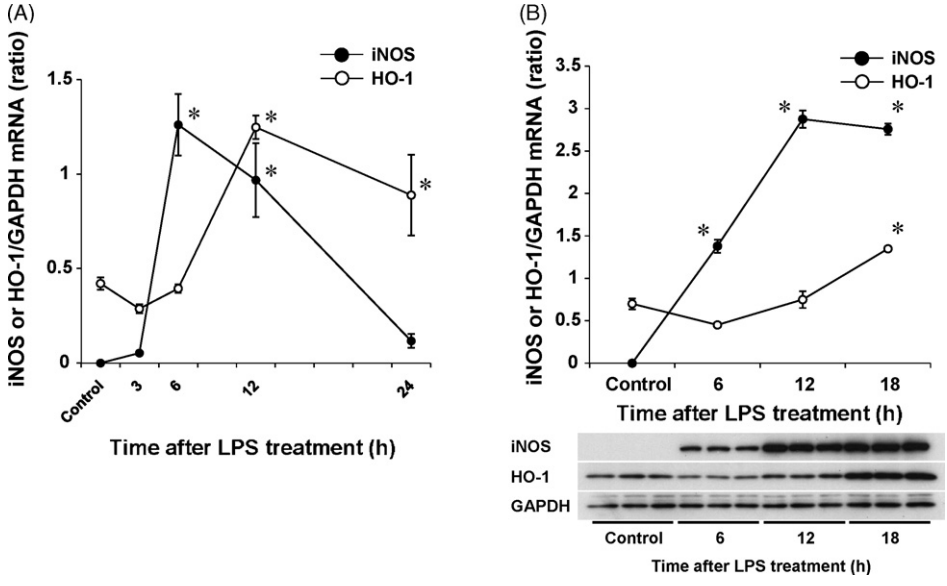

Fig. 1. Time-dependent inductions of iNOS and HO-1 in LPS-stimulated WT macrophages. The macrophages were treated with LPS (1 μg/mL) and harvested at the times indicated. (A) Total RNA was isolated from the macrophages, and real-time PCR was performed. iNOS and HO-1 mRNAs were semiquantified by normalizing with that for GAPDH mRNA. (B) The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of iNOS and HO-1 employing immune-blot analysis. Blots for iNOS and HO-1 proteins were semiquantified by normalizing with that for GAPDH protein. Values represent the mean ± S.E.M. ($n = 3$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ((*) significantly different from the controls at $p < 0.05$).

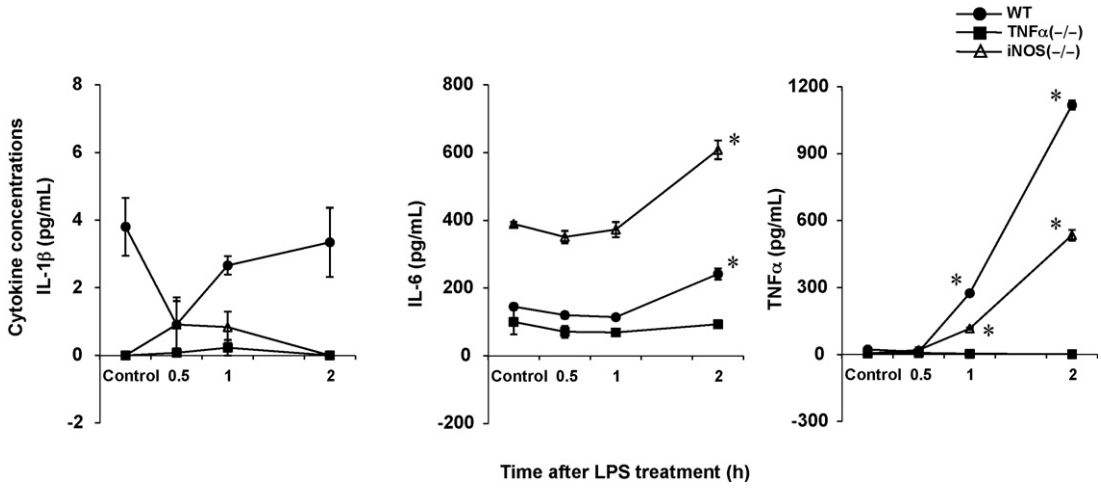

Fig. 2. Liberation of inflammatory cytokines from LPS-stimulated WT, TNFα(−/−) and iNOS(−/−) macrophages. Respective macrophages were treated with LPS (1 μg/mL), and the culture medium was collected at the times indicated. Cytokine concentrations in the culture medium were determined by respective ELISA kits. Values represent the mean ± S.E.M. ($n = 3$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ((*) significantly different from the controls at $p < 0.05$).

ment and followed the HO-1 protein peak level at 6 h later (Fig. 1B).

3.2. The release of inflammatory cytokines from WT, TNFα(−/−) and iNOS(−/−) macrophages by LPS

Inflammatory cytokines are released as a result of the immune response from macrophages. To investigate the pattern of LPS-released inflammatory cytokines from macrophages, we measured IL-1β, IL-6 and TNFα concentrations in all culture media of WT, iNOS(−/−) and TNFα(−/−) macrophages (Fig. 2A). LPS-treated WT and iNOS(−/−) macrophages resulted in a time-dependent release of large amounts of TNFα into the culture medium. TNFα concentrations of the LPS-treated WT macrophage culture medium (1120 ± 20 pg/mL) were higher than those of the LPS-treated iNOS(−/−) macrophage culture medium (530 ± 24 pg/mL). LPS-mediated release of IL-6 from WT and iNOS(−/−) macrophages was similar but different from that of TNFα(−/−) macrophages; however, the amount of liberated IL-6 was less extensive compared with the amount of TNFα liberation. On the other hand, IL-1β showed no release under this condition.

3.3. Changes of iNOS and HO-1 inductions by LPS in WT, TNFα(−/−) and iNOS(−/−) macrophages

Since LPS strongly induced iNOS and released TNFα in WT macrophages, we next investigated the induction of iNOS and HO-1 by LPS using TNFα(−/−) and iNOS(−/−) macrophages (Fig. 3). LPS strongly increased the iNOS protein in TNFα(−/−) macrophages as well as in WT macrophages at 18 h after treatment (Fig. 3A). LPS also significantly induced HO-1 mRNA in TNFα(−/−) macrophages (230% of control) and WT macrophages (250% of control). Interestingly, LPS failed to induce HO-1 mRNA in iNOS(−/−) macrophages (Fig. 3B). The HO-1 protein levels were also changed in a similar manner to that seen in the mRNA levels (Fig. 3C).

3.4. Phosphorylation of IκB-α by LPS in WT and iNOS(−/−) macrophages

The nuclear factor-kappa B (NF-κB) transcription factor is present in the cytosol in an inactive state complexed with the IκB-α protein. Activation occurs via phosphorylation of IκB-α followed by proteasome-mediated degradation, resulting in the release and nuclear translocation of active NF-κB. To investigate the reason for the lack of HO-1 mRNA induction by LPS in iNOS(−/−)-derived macrophages, we examined LPS-induced phosphorylation of IκB-α using WT and iNOS(−/−) macrophages (Fig. 4). Phosphorylation of IκB-α was similarly observed in macrophages derived from either WT or iNOS(−/−) mice from 0.5 h after LPS treatment.

3.5. Changes of iNOS and HO-1 expressions by LPS in WT and Nrf2(−/−) macrophages

It has been reported that Nrf2 is a major transcriptional regulator of HO-1. Although there are many reports on HO-1 induction via Nrf2 nuclear translocation by electrophilic compounds, so far there are no reports concerning HO-1 induction by LPS in Nrf2(−/−) macrophages. Accordingly, we investigated iNOS and HO-1 induction by LPS using Nrf2(−/−) macrophages (Fig. 5). HO-1 mRNA of the control level in Nrf2(−/−) macrophages was lower than that of the WT macrophages (65% of WT macrophages) (Fig. 5C). Similar to HO-1 mRNA, the HO-1 protein of the control level in Nrf2(−/−) macrophages was also lower than that of the WT macrophages (Fig. 5A and B). As shown in Fig. 5A, LPS strongly induced the iNOS protein in Nrf2(−/−) macrophages. However, LPS failed to induce HO-1 in Nrf2(−/−) macrophages (Fig. 5A–C).

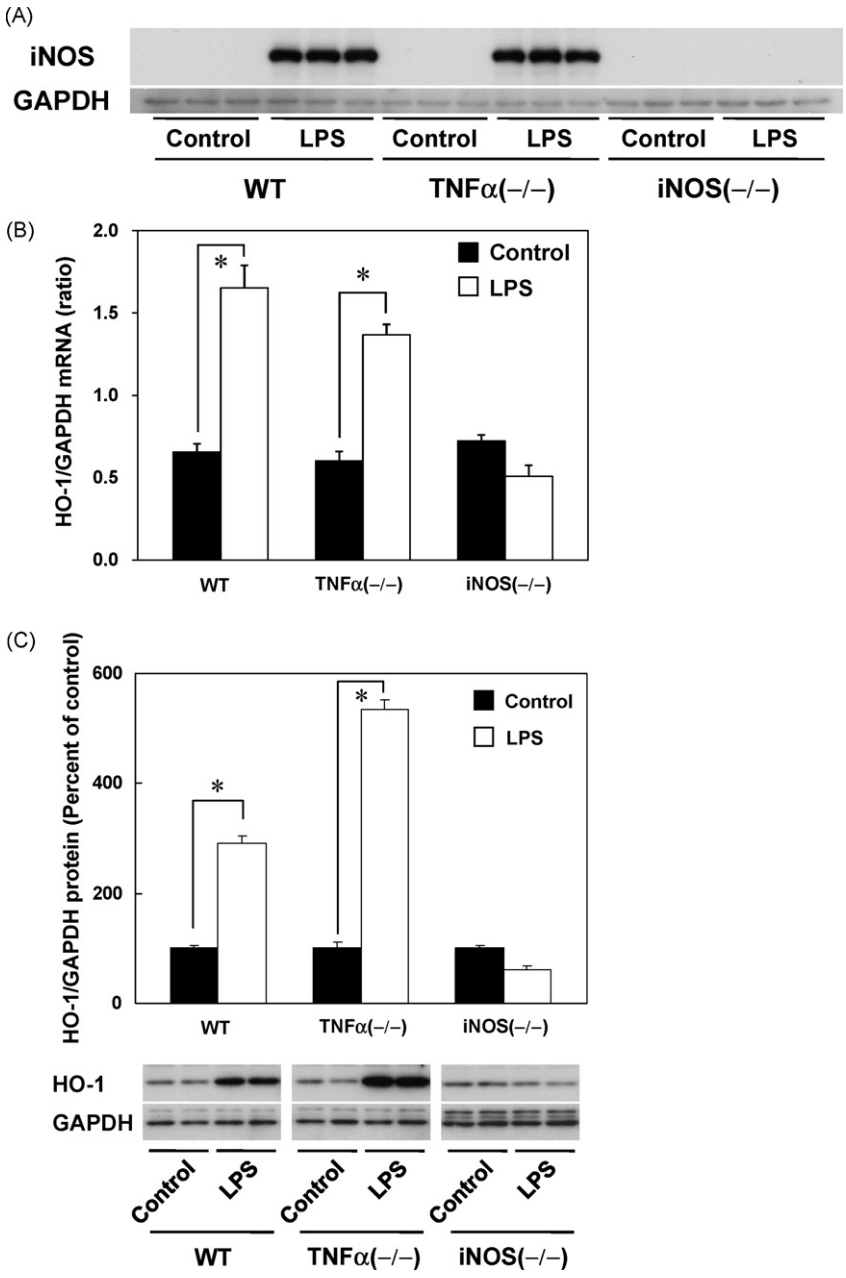

Fig. 3. Induction of iNOS and HO-1 by LPS in WT, TNFα(−/−) and iNOS(−/−) macrophages. (A and C) The respective macrophages were treated with LPS (1 μg/mL) and harvested at 18 h after treatment. The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of iNOS and HO-1 employing immune-blot analysis. The blot for the HO-1 protein was semiquantified by normalizing with that for GAPDH protein. (B) The respective macrophages were treated with LPS (1 μg/mL) and harvested at 12 h after treatment. Total RNA was isolated from the macrophages, and real-time PCR was performed. HO-1 mRNA was semiquantified by normalizing with that for GAPDH mRNA. Values represent the mean ± S.E.M. ($n = 4$). The significance of difference was assessed by the Student's t-test ($^{*}p < 0.05$).

3.6. Inhibition of LPS-mediated induction of iNOS by pretreatment with HO-1 inducers

It is well known that HO-1 is induced by infectious agents in macrophages. However, the function of infectious disease-induced HO-1 has yet to be clarified. NO is known as one of the major factors causing endotoxin shock. Therefore, we investigated the relationship between HO-1 and iNOS by pretreatment with HO-1 inducers, hemin and CoPP, or HO-1 activity inhibitor, SnPP (Fig. 6). Pretreatment with hemin (20 μM) and CoPP (20 μM) significantly inhibited LPS-mediated induction of the iNOS gene expression in WT macrophages approximately 80% and 95%, respectively. Although SnPP (20 μM) induced HO-1 protein in WT macrophages the same as hemin or CoPP (data not shown), pretreatment with SnPP did not have the inhibitory effect of LPS-mediated induction of iNOS because HO-1 activity was probably inhibited by SnPP.

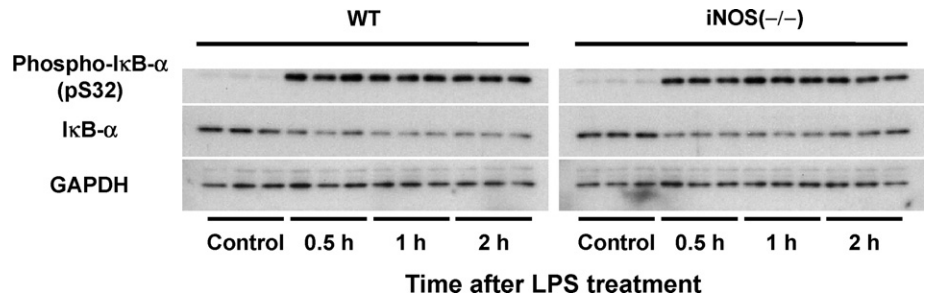

Fig. 4. Phosphorylation of IκB-α by LPS in WT and iNOS(−/−) macrophages. The respective macrophages were treated with LPS (1 μg/mL) and harvested at the times indicated. The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of IκB-α and its phosphorylated forms employing immune-blot analysis.

3.7. Change of inhibitory effect of CoPP on LPS-mediated induction of iNOS in Nrf2(−/−) macrophages

LPS failed to induce HO-1 in Nrf2(−/−) macrophages (Fig. 5). The increased HO-1 led to the inhibition of LPS-mediated induction of iNOS (Fig. 6). Subsequently, we examined whether inhibition of LPS-mediated induction of iNOS by CoPP required HO-1 induction. We investigated this by comparing the effect of CoPP in WT and Nrf2(−/−) macrophages. The ability of Nrf2(−/−)-derived macrophages to induce HO-1 by CoPP was markedly lower than that of WT macrophages (Fig. 7A). The effect of CoPP on LPS-mediated induction of iNOS in Nrf2(−/−) macrophages (61% suppression) was significantly weaker as compared to WT macrophages (92% suppression) (Fig. 7B).

3.8. Decrease of LPS-mediated phosphorylation of IκB-α by pretreatment with CoPP

We found that the increase in HO-1 resulted in the inhibition of LPS-mediated induction of iNOS in macrophages (Figs. 6 and 7). Therefore, we investigated the effect of pretreatment with CoPP or SnPP on phosphorylation of IκB-α by LPS in macrophages (Fig. 8). Pretreatment with CoPP significantly suppressed LPS-mediated phosphorylation of IκB-α in macrophages, and pretreatment with SnPP did not have the suppressive effect of LPS-mediated phosphorylation of IκB-α.

4. Discussion

To our knowledge, this is the first report demonstrating that LPS-mediated induction of iNOS is inhibited in a feedback manner under HO-1 induction due to Nrf2 nuclear translocation using the iNOS(−/−) and Nrf2(−/−) primary macrophages. There have been reports on HO-1 induction by NO using RAW 264.7 with NO-donor and/or iNOS inhibitor (Srisook and Cha, 2004; Srisook and Cha, 2005). However, there has been no report concerned with the involvement of NO in HO-1 induction by LPS using iNOS(−/−) primary macrophages. We also found that LPS-released TNFα is not involved in HO-1 induction. We suggest that to explain these phenomena using normal peritoneal macrophages and gene deficient macrophages is of particular importance because these cells can avoid the impact of stress by chemical compounds and the transformation of the murin leukemia virus.

HO-1 is induced by various compounds, such as heavy metals, glutathione depletors and electrophiles (Ishii et al., 2000; Oguro and Yoshida, 2004), and various pathological conditions, such as infectious diseases and inflammation (Bach, 2002), which induce oxidative stress. Genomic analysis of HO-1 revealed an antioxidant response element (ARE) (Rushmore et al., 1990; Rushmore et al., 1991) and electrophile response element (EpRE) (Friling et al., 1990) that were identified as *cis*-acting elements required for gene expression induced by antioxidants and electrophiles, respectively. The oxidative stress liberates Nrf2 from the hold cytoplasmic compartment by Kelch-like ECH-associated protein 1 into the nucleus. Nrf2 then forms a heterodimer with a small Maf protein, interacts with ARE/EpRE, and induces transcription of HO-1 (Numazawa and Yoshida, 2004). In this study, we demonstrated that LPS did not induce HO-1 in iNOS(−/−) macrophages (Fig. 3); however, CoPP and hemin induced HO-1 in iNOS(−/−) macrophages (data not shown) and the LPS-mediated increase in NO did not induce HO-1 in Nrf2(−/−) macrophages (Fig. 5). These results suggest that NO causes oxidative stress and changes the redox condition, and induces nuclear transport of Nrf2.

LPS is recognized by TLR4, and induces degradation of IκB by phosphorylation following activation of IκB kinase. IκB-associated NF-κB is transferred to the nucleus followed by degradation of IκB enhancing transcriptional activation of target genes (Guha and Mackman, 2001). In this study, LPS mediated phosphorylation of IκB-α in iNOS(−/−) macrophages, as well as in WT macrophages (Fig. 4). Consequently, we suggest that iNOS(−/−) macrophages can normally recognize LPS.

There are also reports showing that MAPKs, such as p44/42 MAPK, JNK and p38 MAPK, are involved in the induction of HO-1 (Alam et al., 2000; Otterbein et al., 2000; Zipper and Mulcahy, 2000). However, it is still not clear how MAPKs induce ARE/EpRE-dependent transcription because there is no direct evidence indicating that activation of the kinase affects subcellular localization of Nrf2. Though MAPKs were phosphorylated by LPS treatment in iNOS(−/−) macrophages the same as WT macrophages (data not shown), LPS did not induce HO-1 in iNOS macrophages. This result suggests that Nrf2 is not activated by MAPKs. Other reports show that protein kinase C inhibitors significantly suppress the ARE/EpRE-mediated gene

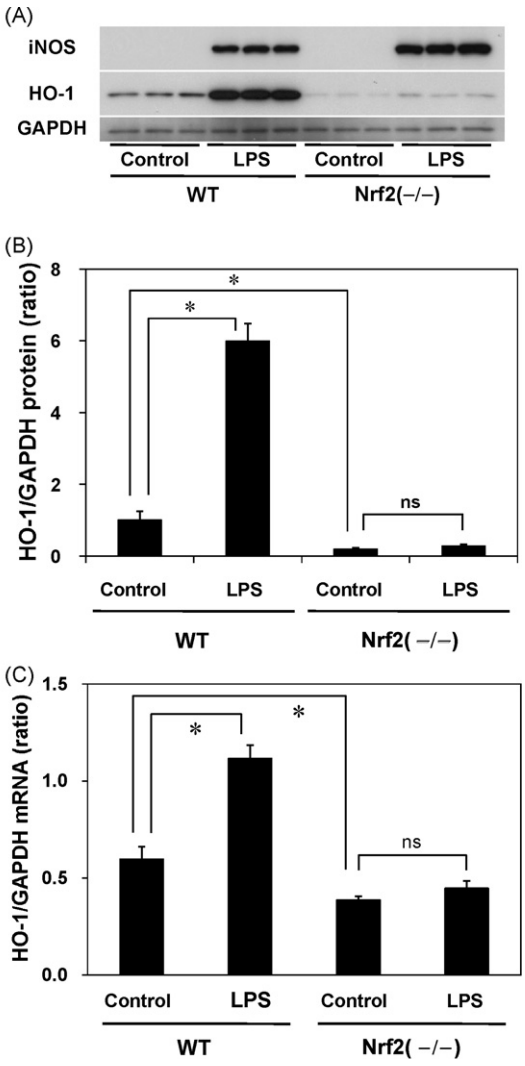

Fig. 5. Induction of iNOS and HO-1 by LPS in WT and Nrf2(−/−) macrophages. (A and B) The respective macrophages were treated with LPS (1 μg/mL) and harvested at 18 h after treatment. The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of iNOS and HO-1 employing immune-blot analysis. (A) Western blot analysis was performed. (B) Blots for HO-1 protein were semiquantified by normalizing with that for GAPDH protein. Values represent the mean ± S.E.M. ($n = 3$). (C) The respective macrophages were treated with LPS (1 μg/mL) and harvested at 12 h after treatment. Total RNA was isolated from the macrophages, and real-time PCR was performed. HO-1 mRNA was semiquantified by normalizing with that for GAPDH mRNA. Values represent the mean ± S.E.M. ($n = 4$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ($^{*}p < 0.05$ and ns, indicating no significant difference).

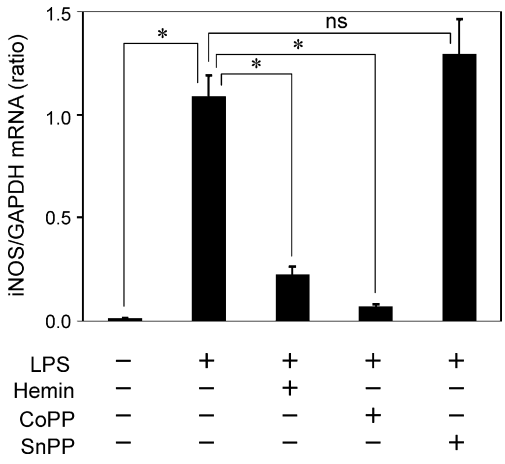

Fig. 6. Change of LPS-mediated induction of iNOS by pretreatment with HO-1 inducers or inhibitor in WT macrophages. WT macrophages were pretreated with hemin (20 μM), CoPP (20 μM) or SnPP (20 μM). After 1 h, the macrophages were treated with LPS (1 μg/mL) and harvested at 12 h after treatment. Total RNA was isolated from the macrophages, and real-time PCR was performed. HO-1 mRNA was semiquantified by normalizing with that for GAPDH mRNA. Values represent the mean ± S.E.M. ($n = 4$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ($^{*}p < 0.05$ and ns, indicating no significant difference).

expression (Huang et al., 2000; Rushworth et al., 2005). There is a probability that NO-mediated PKC activation is involved in phosphorylation dependent Nrf2 activation.

NO plays important antibacterial and antiviral roles (MacMicking et al., 1997). However, its continuous overproduction induces cell dysfunction. It has been reported that lethality of LPS-induced endotoxin shock is partially decreased in iNOS(−/−) mice (MacMicking et al., 1995). Thus, it is conceivable that the accurate regulatory mechanisms of the iNOS gene expression exist in cells. The present study demonstrated that LPS-mediated induction of HO-1 was elicited following the iNOS gene expression in primary macrophages (Fig. 1). This result complements those reported by Srisook and Cha (2004) who found LPS-derived induction of HO-1 began to occur shortly after the iNOS gene expression in RAW264.7 cells. In this study, we expanded findings that macrophages lacking the iNOS gene almost lost HO-1 inducibility, and LPS-mediated induction of iNOS was inhibited by pretreatment with the HO-1 inducers (Figs. 3 and 6). Furthermore, our study was the first to characterize the critical importance of Nrf2 in HO-1 induction by NO (Fig. 5). It is reported that disruption of Nrf2 causes a drastic increase in lethality during LPS-induced septic shock (Thimmulappa et al., 2006). Our present findings suggest that the iNOS gene expression is regulated in a negative feedback manner under induction of HO-1 via Nrf2.

Numerous studies have shown that HO-1 is induced by treatment with inflammatory cytokines, such as IL-1β, IL-6 and TNFα. TNFα is known as a cytotoxic cytokine which causes inflammation. In this study we demonstrated that LPS liberated TNFα, in particular from WT macrophages (Fig. 2) and induced HO-1 in TNFα(−/−) macrophages, as well as in WT macrophages (Fig. 3). These results clearly showed that LPS-mediated release of TNFα from macrophages themselves is not involved in HO-1 induction. Furthermore, it was reported that transfection of human monocyte leukemia THP-1 cells with the dominant negative Nrf2 mutant resulted in no change in LPS-induced TNFα expression (Rushworth et al., 2005), suggesting that Nrf2 does not play a role in the LPS-induced TNFα expression in these cells.

The present report demonstrated that the abilities of CoPP to induce HO-1 and to inhibit LPS-mediated induction of iNOS were attenuated in Nrf2(−/−) macrophages (Fig. 7). These results suggest that the induction of HO-1 via Nrf2 by CoPP suppresses the iNOS gene expression by LPS. Little is known

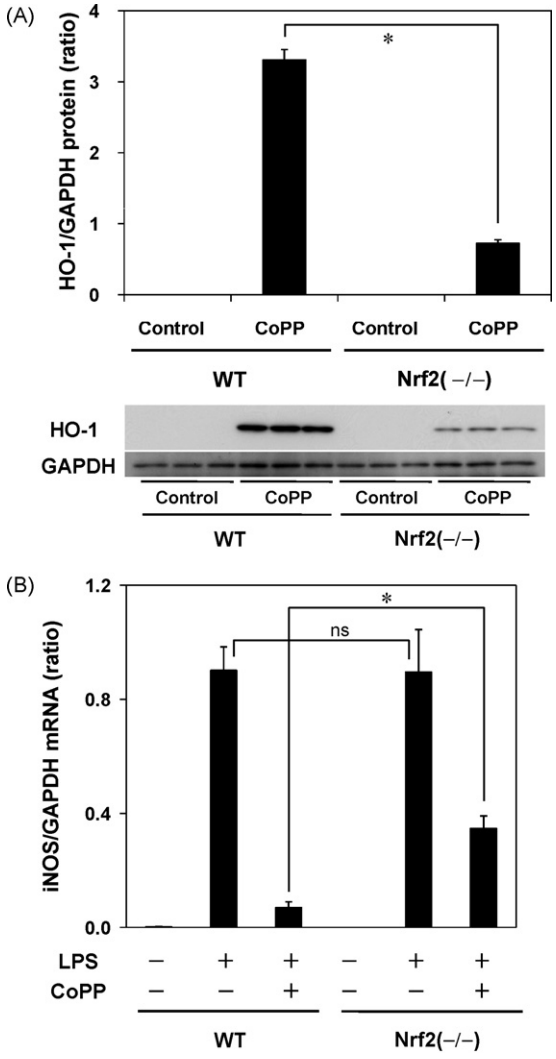

Fig. 7. Change of inhibitory effect of CoPP on LPS-mediated induction of iNOS in Nrf2(−/−) macrophages. (A) WT and Nrf2(−/−) macrophages were treated with CoPP (20 μM) and harvested at 18 h after treatment. The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of HO-1 employing immune-blot analysis. (B) WT and Nrf2(−/−) macrophages were pretreated with CoPP (20 μM). After 1 h, the macrophages were treated with LPS (1 μg/mL) and harvested at 12 h after treatment. Total RNA was isolated from the macrophages, and real-time PCR was performed. iNOS mRNA was semiquantified by normalizing with that for GAPDH mRNA. Values represent the mean ± S.E.M. ($n = 4$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ($^{*}p < 0.05$ and ns, indicating no significant difference).

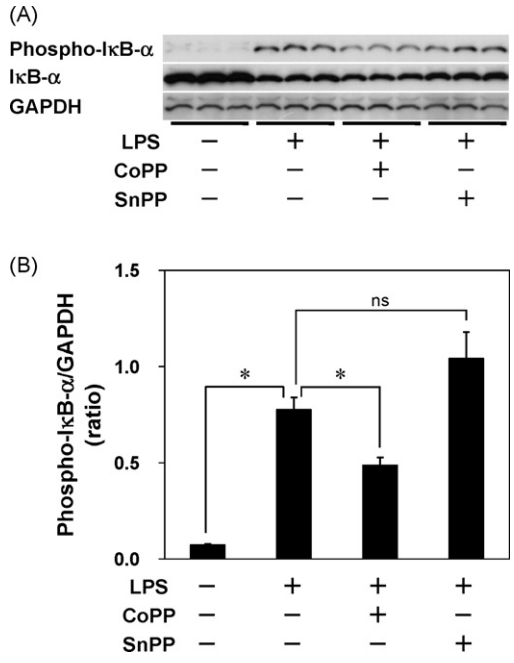

Fig. 8. Change of LPS-mediated phosphorylation of IκB-α by pretreatment with CoPP or SnPP in WT macrophages. (A and B) WT macrophages were pretreated with CoPP (20 μM) or SnPP (20 μM). After 1 h, the macrophages were treated with LPS (1 μg/mL) and harvested at 2 h after treatment. The macrophages were solubilized with 4% SDS, and were analyzed to determine the content of IκB-α and phosphor-IκB-α employing immune-blot analysis. (A) Western blot analysis was performed. (B) Blots for phosphor-IκB-α protein were semiquantified by normalizing with that for GAPDH protein. Values represent the mean ± S.E.M. ($n = 3$). The significance of difference was assessed by one-way ANOVA, followed by the Tukey–Kramer test ($^{*}p < 0.05$ and ns, indicating no significant difference).

about the suppressive mechanism of the iNOS gene expression. Several studies have reported that the defense effects of HO-1 on oxidative stress and others depend on biliverdin or CO which are degradation products of heme catabolizing by HO-1. Biliverdin functions as radical scavengers. However, no report is available on the inhibition of the iNOS gene expression by biliverdin or bilirubin. It is known that upregulation of the iNOS expression is promoted by activation of NF-κB (Guha and Mackman, 2001). It was reported that $[Ru(CO)_3Cl_2]$, a CO-releasing molecule, inhibits induction of iNOS by LPS (Srisook et al., 2006), and hydrogen sulfide-induced HO-1 prevents LPS-induced NF-κB activation in RAW 264.7 cells (Oh et al., 2006). In this study, we demonstrated that the pretreatment with CoPP, a HO-1 inducer, significantly suppressed LPS-mediated IκB-α phosphorylation, but not SnPP, a HO-1 inhibitor (Fig. 8). This result suggests that LPS-mediated the inhibition of iNOS gene expression by HO-1 induction partially involves prevention of LPS-mediated IκB phosphorylation. Taken together, these results support the notion that CO from heme degradation by HO-1 inhibits the iNOS gene expression by prevention of LPS-mediated NF-κB activation, which probably leads to its anti-inflammatory effect.

In summary, we explored the effect of HO-1 and the importance of its transcription factor Nrf2 on negative regulation of the iNOS gene expression in mouse primary macrophages stimulated with LPS. LPS induced the HO-1 expression through the Nrf2-dependent pathway via NO production during the increased iNOS expression. Here we extended the idea of mutual regulation iNOS and HO-1 gene expressions induced by LPS. Specifically, the LPS-induced iNOS expression was inhibited by the HO-1 inducer through the Nrf2 pathway. It is clear that Nrf2 is involved in the induction of biological defense proteins such as antioxidant proteins, associated proteins of glutathione synthesis and/or phase II detoxification enzymes. Nrf2(−/−) mice are much more susceptible to hepatic toxicities of acetaminophen (Chan et al., 2001; Enomoto et al.,

2001), pulmonary toxicities of hyperoxia (Cho et al., 2002) and stomach carcinogenesis by benzo[*a*]pyrene (Ramos-Gomez et al., 2001). Therefore, Nrf2 plays an important role in the regulation of the biological defense system. We conclude that HO-1 helps to protect from cell dysfunction by persistence of NO production and to maintain homeostasis. Further studies are needed in order to elucidate the functional roles of HO-1 *in vivo*.

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