Chemico-Biological Interactions 180 (2009) 202–210

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Cobalt protoporphyrin inhibition of lipopolysaccharide or lipoteichoic acid-induced nitric oxide production via blocking c-Jun N-terminal kinase activation and nitric oxide enzyme activity

Hui-Yi Lin[a,1], Shing-Chuan Shen[a,1], Cheng-Wei Lin[b], Ming-Shun Wu[c], Yen-Chou Chen[a,d,*]

[a] Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan
[b] Graduate Institute of Pharmacy, School of Pharmacy, Taipei Medical University, Taipei 110, Taiwan
[c] Department of Gastroenterology, Taipei Medical University Wan Fan Hospital, Taipei 110, Taiwan
[d] Cancer Research Center and Orthopedics Research Center, Taipei Medical University Hospital, Taipei 110, Taiwan

ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form
26 December 2008
Accepted 14 January 2009
Available online 22 January 2009

Keywords:
CoPP
HO-1
iNOS
LPS
LTA

ABSTRACT

In the present study, low doses (0.5, 1, and 2 μM) of cobalt protoporphyrin (CoPP), but not ferric protoporphyrin (FePP) or tin protoporphyrin (SnPP), significantly inhibited lipopolysaccharide (LPS) or lipoteichoic acid (LTA)-induced inducible nitric oxide (iNOS) and nitric oxide (NO) production with an increase in heme oxygenase 1 (HO-1) protein in RAW264.7 macrophages under serum-free conditions. IC_{50} values of CoPP inhibition of NO and iNOS protein individually induced by LPS and LTA were around 0.25 and 1.7 μM, respectively. This suggests that CoPP is more sensitive at inhibiting NO production than iNOS protein in response to separate LPS and LTA stimulation. NO inhibition and HO-1 induction by CoPP were blocked by the separate addition of fetal bovine serum (FBS) and bovine serum albumin (BSA). Decreasing iNOS/NO production and increasing HO-1 protein by CoPP were observed with CoPP pretreatment, CoPP co-treatment, and CoPP post-treatment with LPS and LTA stimulation. LPS- and LTA-induced NOS/NO productions were significantly suppressed by the JNK inhibitor, SP600125, but not by the ERK inhibitor, PD98059, through a reduction in JNK protein phosphorylation. Transfection of a dominant negative JNK plasmid inhibited LPS- and LTA-induced iNOS/NO production and JNK protein phosphorylation, suggesting that JNK activation is involved in LPS- and LTA-induced iNOS/NO production. Additionally, CoPP inhibition of LPS- and LTA-induced JNK, but not ERK, protein phosphorylation was identified in RAW264.7 cells. Furthermore, CoPP significantly reduced NO production in a cell-mediated, but not cell-free, iNOS enzyme activity assay accompanied by HO-1 induction. However, attenuation of HO-1 protein stimulated by CoPP via transfection of HO-1 siRNA did not affect NO's inhibition of CoPP against LPS stimulation. CoPP effectively suppressing LPS- and LTA-induced iNOS/NO production through blocking JNK activation and iNOS enzyme activity via a HO-1 independent manner is first demonstrated herein.

1. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme for breaking down heme into carbon monoxide, biliverdin, and free iron. Three HO isozymes including HO-1, HO-2, and HO-3 have been identified, among which HO-1 is an inducible enzyme, while HO-2 and HO-3 are constitutive ones. HO-1 was shown to have several biological effects including anti-inflammatory, antiapoptotic, and antiproliferative actions, and inhibits immune responses in organ transplantation and autoimmune diseases [1–3]. Studies indicated that the actions of HO-1 are mediated by increasing its metabolites such as CO and bilirubin production [4–6]; however, regulation of catalase and MnSOD by HO-1 indicates the possibility that HO-1 acts independently of its enzyme activity [7,8]. Therefore, identifying the contributions of protein and enzyme activities to the biological effects of HO-1 would be highly advantageous.

Nitric oxide (NO) has been identified as a neurotransmitter in the central nervous system and as a potent physiological vasorelaxant.

Abbreviations: HO-1, heme oxygenase-1; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; CO, carbon monoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DTT, dithiothreitol; L-NAME, N-nitro-L-arginine methyl ester; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; COX, cyclooxygenase; CoPP, cobalt protoporphyrin; SnPP, tin protoporphyrin.

* Corresponding author at: Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan. Tel.: +886 2 27361661x3421; fax: +886 2 23778620.
E-mail address: yc3270@tmu.edu.tw (Y.-C. Chen).
[1] These authors contributed equally to this work.

0009-2797/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2009.01.004

In large amounts, NO is considered a deleterious molecule in the process of inflammation and sepsis. After exposure to endogenous and exogenous stimulators, inducible NO synthase (iNOS) is quantitatively induced in various cells such as macrophages, smooth muscle cells, and hepatocytes to trigger several deleterious cellular responses [9–11]. Lipopolysaccharide (LPS) is a major component of gram-negative bacteria, and promotes the secretion of inflammatory cytokines and induces iNOS gene expression via binding to the CD14/Toll-like receptor (TLR) 4 in macrophages [11,12]. Instead of LPS, lipoteichoic acid (LTA) is in cell walls of gram-positive bacteria, and activation of iNOS and cytokine production by LTA was reported through binding with TLR2 [13,14]. Activation of ERKs and JNKs by LPS or LTA has been reported in different cell types. In smooth muscle cells, LTA-stimulated p42/p44 MAPK phosphorylation is mediated through a TLR2 receptor [15]. In macrophages, activation of ERKs and JNKs by LPS and LTA has been reported [16,17].

A number of studies have reported that HO-1 may contribute to NO inhibition elicited by inflammatory stimuli such as LPS [18–21]. Our studies demonstrated that HO-1 overexpression significantly inhibits individual LPS- and LTA-induced iNOS protein expression and NO production in RAW264.7 macrophages [22,23], and protects macrophages from oxidative stress-induced apoptosis [24]. Although HO-1 inhibition of NO production has been investigated, the contribution of the HO-1 protein or enzyme activity to its NO inhibitory effect is still unclear. Metalloprotoporphyrins including ferric protoporphyrin (FePP), cobalt protoporphyrin (CoPP), and tin protoporphyrin (SnPP) play roles as HO-1 protein inducers or HO-1 enzyme inhibitors. FePP is a substrate of HO, and HO-1 induction and NO inhibition by FePP have been identified [22]. CoPP and SnPP are analogs of FePP, and act as competitive inhibitors of HO enzyme via binding to the same regulatory sites as FePP [25,26]. However, several studies indicated that CoPP is a potent HO-1 inducer [27–29], and the effects of CoPP and SnPP on inflammatory iNOS protein expression and NO production are still undefined.

The aims of this study were to examine the differential effects of FePP, CoPP, and SnPP on iNOS/NO production elicited by LPS and LTA as related to HO-1 protein expression in conditions with and without FBS and BSA. Results suggest that CoPP is an effective inhibitor against LPS- and LTA-induced iNOS/NO production by stimulation of HO-1 protein expression. Evidence of HO-1-independent iNOS/NO inhibition via blocking JNK activation and iNOS enzyme activity by CoPP is provided.

2. Materials and methods

2.1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin) and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL) and maintained in a 37 °C humidified incubator containing 5% CO_2.

2.2. Agents

The chemical reagents of CoPP, cobalt chloride ($CoCl_2$), LPS, LTA, PD98059, and SP600125 were obtained from Sigma Chemical (St. Louis, MO). Antibodies of HO-1, iNOS, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies of pJNK and pERK were obtained from Cell Signaling Technology (Danvers, MA).

2.3. Nitrite assay

RAW264.7 cells were plated at a density of 5×10^5 cells/ml in 24-well plates for 12 h, followed by treatment with LPS (50 ng/ml) and indicated compounds for a further 12 h. The amount of NO production in the medium was detected by the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid). The absorbance of the mixture at 530 nm was determined with an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA), and the nitrite concentration was determined using a dilution of sodium nitrite as a standard.

2.4. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used as an indicator of cell viability as determined by its mitochondrion-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 12 h, followed by treatment with the indicated compound for a further 12 h. Cells were washed with PBS three times, and MTT (0.5 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed, and formazone crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was read at 600 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories).

2.5. Western blotting

Total cellular extracts were prepared according to our previous papers, separated on 8–12% sodium dodecylsulfate (SDS)–polyacrylamide minigels, and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were incubated with 1% bovine serum albumin (BSA) and then incubated with specific antibodies overnight at 4 °C. Expression of protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.6. Cell-free iNOS enzyme activity assay

RAW264.7 cells were treated with LPS (100 ng/ml) or LTA (1 μg/ml) for 12 h, and LPS or was washed out with ice-cold PBS. LPS-treated cells were resuspended in 40 mM Tris–HCl (pH 8.0), pepstatin A (5 μg/ml), chyncostatin (1 μg/ml), aprotinin (5 μg/ml), and PMSF (100 μM). Total cell lysates (100–200 μg) were incubated with CoPP or L-NAME in 20 mM Tris-OH (pH 7.4) containing L-arginine (1 mM), tetrahydrobiopterin (TTBP; 4 μM), riboflavin 5′-adenosine diphosphate (FAD; 4 μM), NADPH (2 mM), and DTT (3 mM) for 24 h in 37 °C. The amount of NO produced in the medium was detected by the Griess reaction.

2.7. Cell-mediated iNOS enzyme activity assay

RAW264.7 cells were treated with LPS (100 ng/ml) or LTA (1 μg/ml) for 12 h. LPS or LTA was washed out with ice-cold PBS, and cells (2×10^5) were sub-cultured into incubated in to 24 well followed by treating with or without different doses of CoPP for 12 h, and the expressions of iNOS, HO-1 protein, and α-tubulin were analyzed by Western blotting. The amount of NO production in the medium was detected by the Griess reaction.

2.8. Transient HO-1 SiRNA to RAW264.7 macrophages

RAW264.7 macrophages were transfected with the control siRNA and HO-1 siRNA using the Lipofectamin^{2000} transfection reagent (Invitrogen) in serum and antibiotic-free DMEM. After 24 h of incubation, cells were treated with CoPP or LPS for an additional 12 h, and the expressions of HO-1 protein and NO production were respectively analyzed by Western blotting and the Griess reaction.

2.9. Establishment of DN-JNK RAW264.7 macrophages

pcDNA and DN-JNK were transfected into RAW264.7 cells using the Lipofectamin^{2000} transfection reagent (Invitrogen) in serum and antibiotic-free DMEM. After 24 h, cells were replaced in DMEM with 10% FBS, and G418 (400 μg/ml) was added. G418-resistant cells were selected and expanded.

2.10. Statistical analysis

Values are expressed as the mean ± S.E. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A *P* value <0.05 or 0.01 was regarded as indicating a significant difference.

3. Results

3.1. Low doses of CoPP inhibit iNOS/NO production and induction of HO-1 protein expression in LPS- and LTA-treated macrophages

Effects of CoPP on LPS- and LTA-induced iNOS protein expression in a serum-free (SF) condition were examined by Western blotting using an anti-iNOS antibody. As shown in Fig. 1A, CoPP at the doses of 0.5, 1, and 2 μM dose-dependently inhibited LPS- and LTA-induced iNOS protein expression, accompanied by induction of HO-1 protein expression. iNOS protein inhibition by CoPP was observed at the doses of 1 and 2 μM, and the IC_{50} value of CoPP against LPS-induced iNOS protein expression was 1.75 μM. In the same part of the experiment, induction of iNOS protein was detected at 8 and 12 h after separate LPS and LTA stimulation, and it was blocked by the addition of CoPP (2 μM) with increasing HO-1 protein expression (Fig. 1B and C). Quantification of NO in the medium examined by the Griess reaction showed that CoPP, but not its analogs FePP or SnPP, at a dose of 2 μM, significantly inhib-

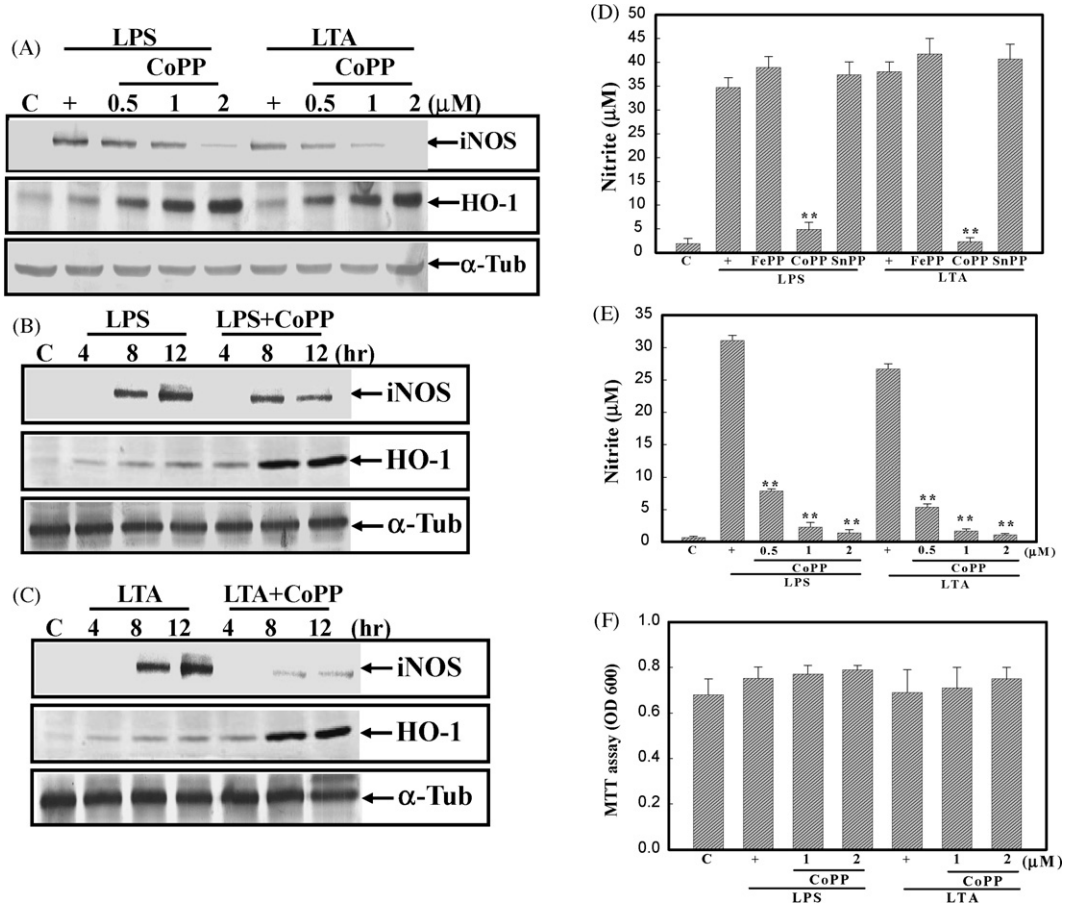

Fig. 1. CoPP inhibition of iNOS/NO production and induction of HO-1 protein expression in RAW264.7 cells under individual LPS and LTA stimulation. (A) Macrophages were treated with different doses (0.5, 1, and 2 μM) of CoPP for 30 min followed by LPS (100 ng/ml) and LTA (1 μg/ml) stimulation individually for an additional 12 h. The expressions of iNOS, HO-1, and α-tubulin protein were detected by Western blotting using specific antibodies. (B, C) In the same part of (A), macrophages were treated with CoPP (2 μM) followed by the separate addition of LPS and LTA for different times (4, 8, and 12 h), and the expression of the indicated protein was examined. (D) CoPP, but not FePP or SnPP, reduced LPS- and LTA-induced NO production in macrophages. Cells were treated with the indicated compounds (2 μM) for 30 min followed by separate LPS and LTA stimulation for 12 h. The amount of NO in the medium was examined by the Griess reaction. (E) As described in (A), the amount of NO in the medium under different treatments was detected. (F) No cytotoxicity of CoPP toward macrophages was evident. Cells were treated with CoPP (1 and 2 μM) for 30 min followed by separate LPS and LTA addition for an additional 12 h. The viability of cells under different treatments was examined by the MTT assay. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments. $^{**}P < 0.01$, indicates a significant difference from the respective LPS- and LTA-treated groups or indicated groups as analyzed by Student's *t*-test.

ited LPS- and LTA-induced NO production (Fig. 1D). NO production individually elicited by LPS and LTA significantly decreased with CoPP treatment at doses of 0.5, 1, and 2 μM (Fig. 1E), and the IC_{50} values of CoPP inhibition of LPS- and LTA-induced NO production were both <0.25 μM. No cytotoxicity of CoPP at the doses of 1 and 2 μM was detected by the MTT assay (Fig. 1F). In the presence of the NO donor, sodium nitroprusside (SNP), CoPP was unable to directly scavenge NO-derived SNP, and FePP or SnPP shows no effect on iNOS protein expression elicited by LPS or LTA (data not shown). These data indicate that CoPP possesses the ability to effectively inhibit iNOS/NO production elicited by LPS and LTA, and the IC_{50} value of NO inhibition was far less than that of iNOS protein inhibition by CoPP.

3.2. Inhibition of iNOS/NO production and induction of HO-1 protein expression by CoPP was blocked by the addition of FBS

We further examined if the addition of FBS affected iNOS/NO's inhibition of CoPP in macrophages. As shown in Fig. 2A (upper panel), CoPP inhibition of LPS-induced iNOS protein expression and induction of HO-1 protein expression was detected in the SF condition, and it was blocked by adding 5% FBS. Inhibition of NO production by CoPP was blocked by adding FBS (Fig. 2A; lower panel). In the same part of the experiment, CoPP inhibition of LTA-induced iNOS/NO production with induction of HO-1 protein expression was prevented by the addition of FBS (Fig. 2B). Decreases in iNOS protein and NO production elicited by LPS and LTA accompanied by HO-1 protein induction were detected in CoPP- but not $CoCl_2$-treated macrophages in the SF condition (Fig. 2C). These data indicate that the addition of FBS may diminish iNOS/NO inhibition and HO-1 induction of CoPP in response to stimulation by LPS and LTA.

3.3. Characterization of CoPP inhibition of iNOS/NO inhibition and HO-1 protein induction in macrophages

Three experimental conditions including individual LPS and LTA pre-treatment for 30 min followed by CoPP treatment for a further 12 h (Pre-t), individual LPS and LTA treatment with CoPP (Co-t), and CoPP pretreatment for 30 min followed by separate LPS and LTA addition for a further 12 h (Post-t) were applied to investigate if CoPP's effect is dependent on LPS- and LTA-induced signal activation. As shown in Fig. 3A, CoPP inhibited iNOS and induction of HO-1 protein under these conditions. Quantification of NO production by Griess reaction showed that CoPP inhibition of NO production was identified in the condition before and after LPS and LTA stimulation (Fig. 3B). These data indicate that CoPP may effectively inhibit iNOS/NO production before and after LPS and LTA stimulation. Albumin is a major component of FBS, and binding of CoPP and albumin has been identified in our previous study [37]. Data of Coomassie blue staining showed that the percentage content of BSA in FBS was around 0.2% (w/v) (data not shown). Therefore, we further investigated if FBS's suppression of CoPP-inhibited iNOS/NO production and -induced HO-1 protein was mediated by BSA. As shown in Fig. 3C and D, CoPP-inhibited iNOS protein was elicited by both LPS and LTA, and the increase in HO-1 protein was reversed by adding different doses (0.1% and 0.2%) of BSA. Analysis of NO production indicated that inhibition of LPS- and LTA-induced NO production by

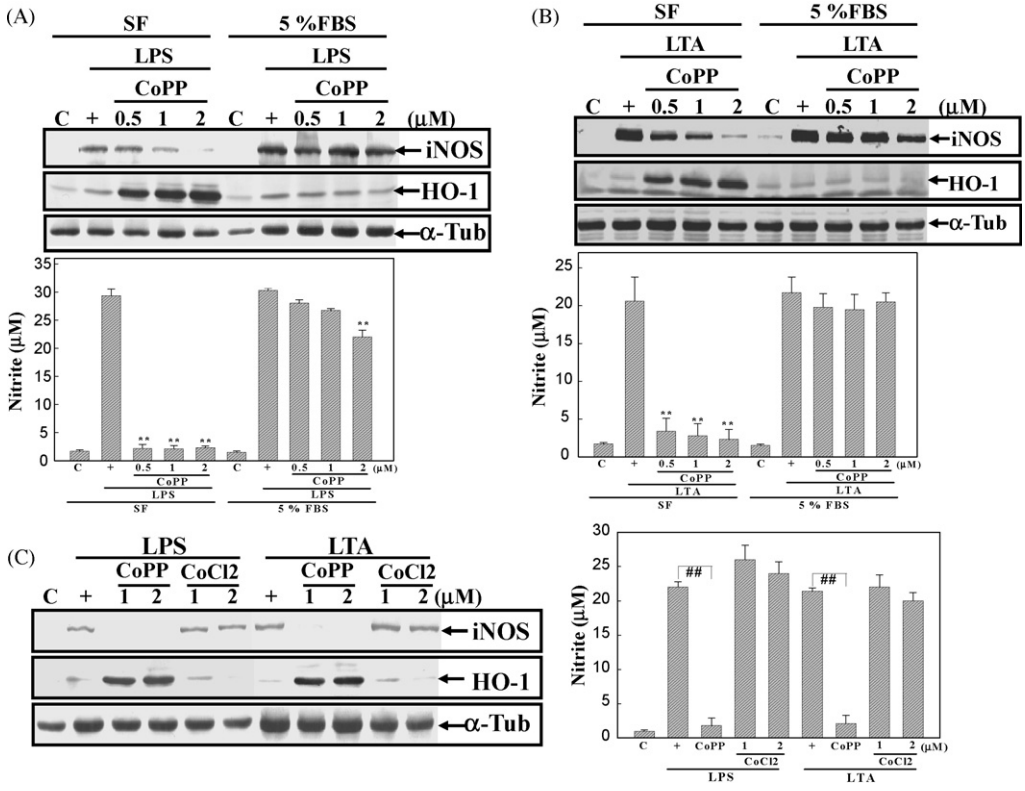

Fig. 2. FBS attenuated CoPP inhibition of separate LPS- and LTA-induced iNOS/NO production and induction of HO-1 protein expression in macrophages. RAW264.7 cells were treated with different doses (0.5, 1, and 2 μM) of CoPP for 30 min followed by separate LPS and LTA stimulation for an additional 12 h in a condition with or without 5% FBS. Upper panel: Expressions of iNOS, HO-1, and α-tubulin protein were detected by Western blotting. Lower panel: The amount of NO produced in each group was measured by the Griess reaction. (C) CoPP, but not $CoCl_2$, inhibited LPS- and LTA-induced iNOS/NO production by inducing HO-1 proteins in macrophages. Cells were treated with different doses (1 and 2 μM) of CoPP or $CoCl_2$ for 30 min followed by individual LPS and LTA stimulation for 12 h. Left panel: protein expressions of iNOS, HO-1, and α-tubulin were evaluated. Right panel: NO production was measured by the Griess reaction. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments. ** $P < 0.01$, indicates a significant difference from the respective LPS- and LTA-treated groups; ## $P < 0.01$, indicates a significant difference between the indicated groups as analyzed by Student's *t*-test.

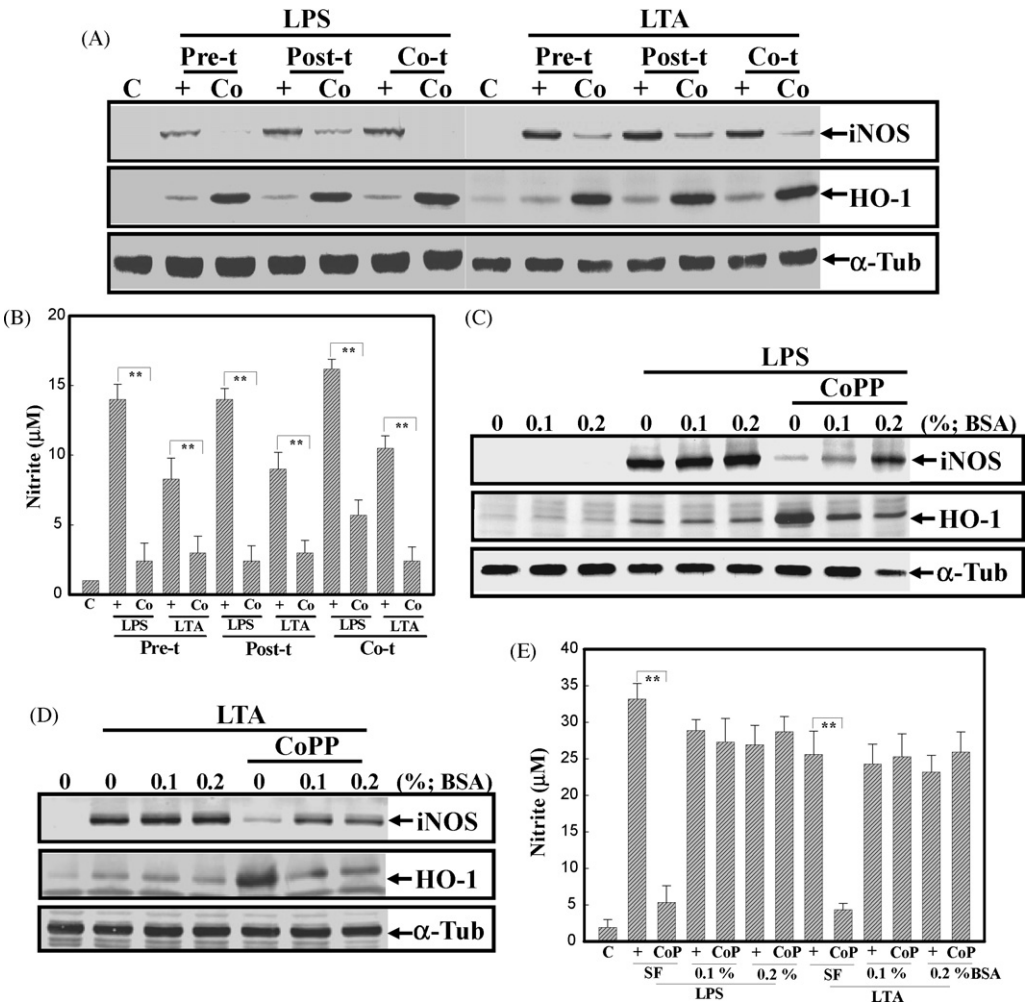

Fig. 3. Characterization of CoPP inhibition of iNOS/NO production and induction of HO-1 protein expression in macrophages. (A) Macrophages were separately treated with LPS and LTA for 30 min followed by CoPP (Co; 2 μM) treatment for an additional 12 h (Pre-t), LPS and LTA with CoPP for 12 h (Co-t), or CoPP for 30 min followed by the addition of LPS and LTA for an additional 12 h (Post-t). The expressions of iNOS, HO-1, and α-tubulin protein were examined by Western blotting (A), and the amount of NO was detected by the Griess reaction (B). (C, D) RAW264.7 cells were treated with CoPP (2 μM) for 30 min followed by the separate addition of LPS and LTA for 12 h in a condition with or without BSA (0.1% and 0.2%). Expressions of iNOS, HO-1, and α-tubulin proteins were detected by Western blotting. (E) The amount of NO produced in each groups in (C, D) was determined by the Griess reaction. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments. $^{**}P < 0.01$, indicates a significant difference between indicated groups as analyzed by Student's *t*-test.

CoPP was reversed by the addition of BSA according to the Griess reaction (Fig. 3E). This indicates that BSA in FBS may block the effects of CoPP such as inhibiting LPS- and LTA-induced iNOS/NO production and inducing HO-1 protein expression in macrophages.

3.4. Involvement of JNK activation in LPS- and LTA-induced iNOS/NO production which was blocked by CoPP in macrophages

We further investigated if CoPP inhibition of separate LPS- and LTA-induced iNOS/NO production occurs through suppression of MAPK activation. Pharmacological studies using the ERK inhibitor, PD98059, and the JNK inhibitor, SP600125, showed that SP600125, but not PD98059, inhibited separate LPS- and LTA-induced iNOS/NO production in macrophages by reducing LPS- and LTA-induced JNK protein phosphorylation in macrophages (Fig. 4A–D). Transfection of macrophages with dominant negative JNK plasmids significantly inhibited LPS- and LTA-induced iNOS/NO production by reducing JNK protein phosphorylation (Fig. 4E and F). This indicates that JNK activation is involved in LPS- and LTA-induced iNOS/NO production in macrophages. Therefore, the effects of CoPP on individual LPS-induced ERK and JNK protein phosphorylation were investigated. As shown in Fig. 4G, CoPP alone exhibited no effect on the phosphorylation status of ERK and JNK proteins in cells, and LPS time-dependently induced ERK and JNK protein phosphorylation. In the presence of CoPP treatment, JNK, but not ERK, protein phosphorylation stimulated by LPS was significantly suppressed. These data indicate that activation of JNKs participates in LPS- and LTA-induced iNOS/NO production, and that it was blocked by the addition of CoPP.

3.5. CoPP may affect iNOS enzyme activity in a cell-mediated, but not cell-free, iNOS enzyme activity assay

Because the IC_{50} value of CoPP against NO production was far lower than that against iNOS protein in the presence of LPS and LTA stimulation, the effect of CoPP on iNOS enzyme activity was examined. Both cell-mediated and cell-free iNOS activity assays were performed in the present study. In the cell-mediated NOS enzyme activity assay, macrophages were individually treated with LPS (Fig. 5A) and LTA (Fig. 5B) for 12 h followed by LPS and LTA removal, and cells were subcultured in 24-well plates in the presence of CoPP for an additional 12 h. Data in Fig. 5A and B shows that

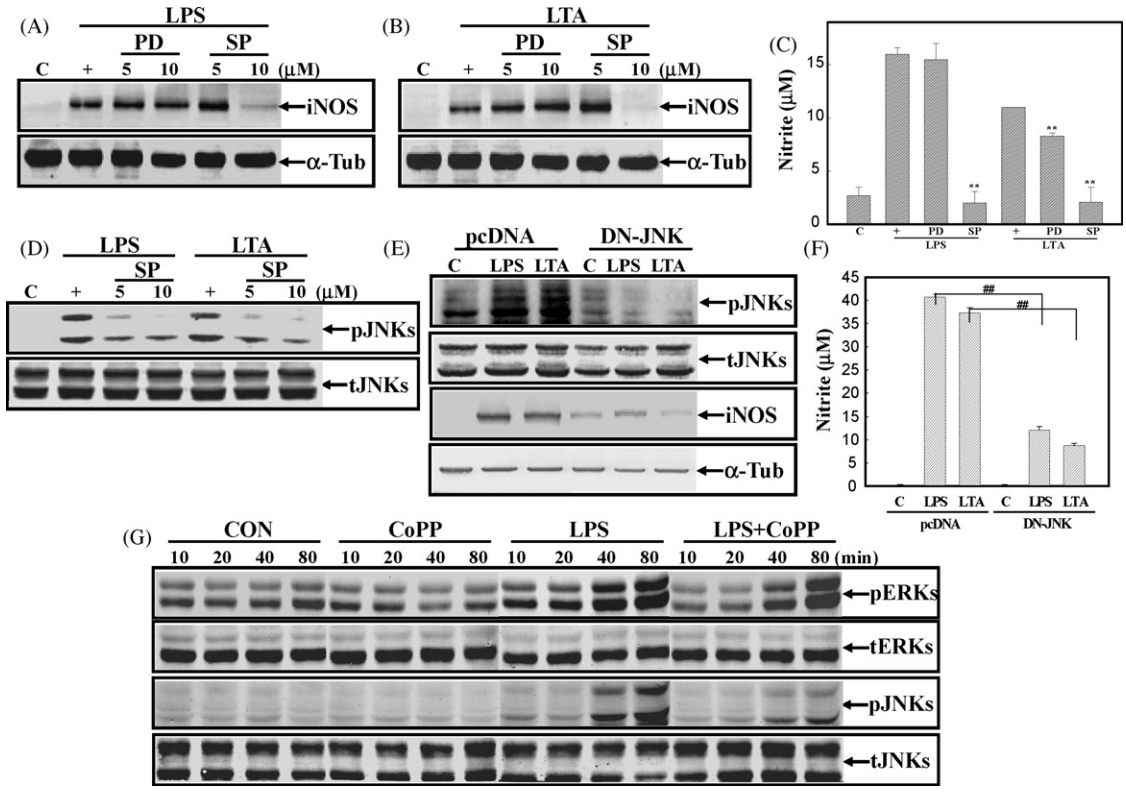

Fig. 4. CoPP inhibition of separate LPS- and LTA-induced iNOS/NO production via suppression of JNKs activation. Cells were treated with PD98059 (PD; 5 and 10 μM) and SP600125 (SP; 5 and 10 μM) for 30 min followed by separate LPS and LTA incubation for 12 h. Expressions of iNOS and α-tubulin proteins were detected by Western blotting. (C) The amounts of NO produced in PD and SP (10 μM)-treated cells in response to LPS and LTA stimulation were detected by the Griess reaction. (D) SP600125 inhibition of LPS- and LTA-induced JNK protein phosphorylation. Cells were treated with SP600125 (SP; 5 and 10 μM) for 30 min followed by separate LPS and LTA stimulation for an additional 40 min. Expressions of phosphorylated (p-JNKs) and total JNKs (t-JNKs) were detected by Western blotting. (E) DN-JNKs but not pcDNA neo-control inhibited LPS- and LTA-induced iNOS/NO production through inhibiting JNK protein phosphorylation. Both DN-JNK- and pcDNA-transfected macrophages were treated separately with LPS and LTA for 40 min (p-JNKs and t-JNKs) and 12 h (iNOS and α-tubulin), and expressions of the indicated proteins were examined by Western blotting. Amounts of NO in each group after individual LPS and LTA treatment for 12 h were examined by the Griess reaction (F). (G) CoPP inhibition of LPS-induced pJNKs, but not pERKs, in macrophages. Cells were treated CoPP (2 μM) for 30 min followed by LPS and LTA for different times (10, 20, and 40 min), and expressions of the indicated proteins were determined by Western blotting. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments. $^{**}P < 0.01$ indicates a significant difference from the respective LPS- and LTA-treated groups; $^{\#\#}P < 0.01$ indicates a significant difference between the indicated groups as analyzed by Student's *t*-test.

CoPP treatment did not significantly affect iNOS protein, but significantly reduced the amount of NO, production. In the cell-free NOS enzyme activity assay, total proteins derived from separate LPS- and LTA-treated RAW264.7 macrophages were used for *in vitro* iNOS enzyme activity with or without CoPP. As illustrated in Fig. 5C, CoPP addition was unable to reduce NO production in the cell-free iNOS enzyme activity assay. The addition of the NOS enzyme activity inhibitor, N-nitro-L-arginine methyl ester (L-NAM), significantly reduced NO production in the cell-free iNOS enzyme activity assay as a positive control. This suggests that CoPP may interfere with iNOS enzyme activity in a cell-mediated manner.

3.6. Induction of HO-1 protein might not contribute to iNOS/NO inhibition of CoPP

We further examined if HO-1 protein induction contributes to CoPP's inhibition of LPS-induced NO production. Macrophages were transfected with control or HO-1 siRNA for 12 h, followed by LPS stimulation. As illustrated in Fig. 6A, HO-1 protein induced by CoPP was significantly reduced by the transfection of HO-1 siRNA, but not control siRNA. However, there was no difference in NO inhibition by CoPP among parental, control siRNA-transfected (Con-siRNA), and HO-1 siRNA-transfected (HO-1 siRNA) RAW264.7 macrophages (Fig. 6B). These data indicate that HO-1 induction might not contribute to CoPP inhibition of NO production elicited by LPS.

4. Discussion

The results of the present study support CoPP effectively inhibiting macrophages from inflammatory insults such as LPS and LTA by reducing iNOS/NO production, and the IC_{50} value of iNOS protein inhibition by CoPP was much higher than that of NO inhibition. Inhibition of JNK, but not ERK, protein phosphorylation by CoPP was identified, and CoPP inhibited NOS enzyme activity in a cell-mediated, but not a cell-free, iNOS enzyme activity assay. Induction of HO-1 protein by CoPP was detected; however a reduction in HO-1 protein expression by HO-1 siRNA showed no effect on NO inhibition of CoPP in LPS-treated macrophages. HO-1-independent inhibition of LPS- and LTA-induced iNOS/NO production by CoPP via blocking JNK activation and iNOS enzyme activity was illustrated.

Metalloporphyrins can induce HO-1 gene expression while paradoxically acting as competitive inhibitors. FePP is an HO substrate, and induction of HO-1 gene expression by FePP has been identified in several cells such as macrophages, hepatocytes, and monocytes [30–32]. CoPP and SnPP are synthetic metalloporphyrins which can act as inhibitors of HO. However, CoPP, albeit an inhibitor of HO activity *in vitro*, has been shown to be a potent inducer of HO-1 gene expression and activity *in vivo* [33]. Therefore, the HO-1 gene is induced, yet HO enzyme activity is inhibited in CoPP- and SnPP-treated cells. The effects of metalloporphyrins on inflammatory iNOS/NO production are still unclear. Ashino et al. indicated that LPS-induced iNOS gene expression was suppressed by the HO-

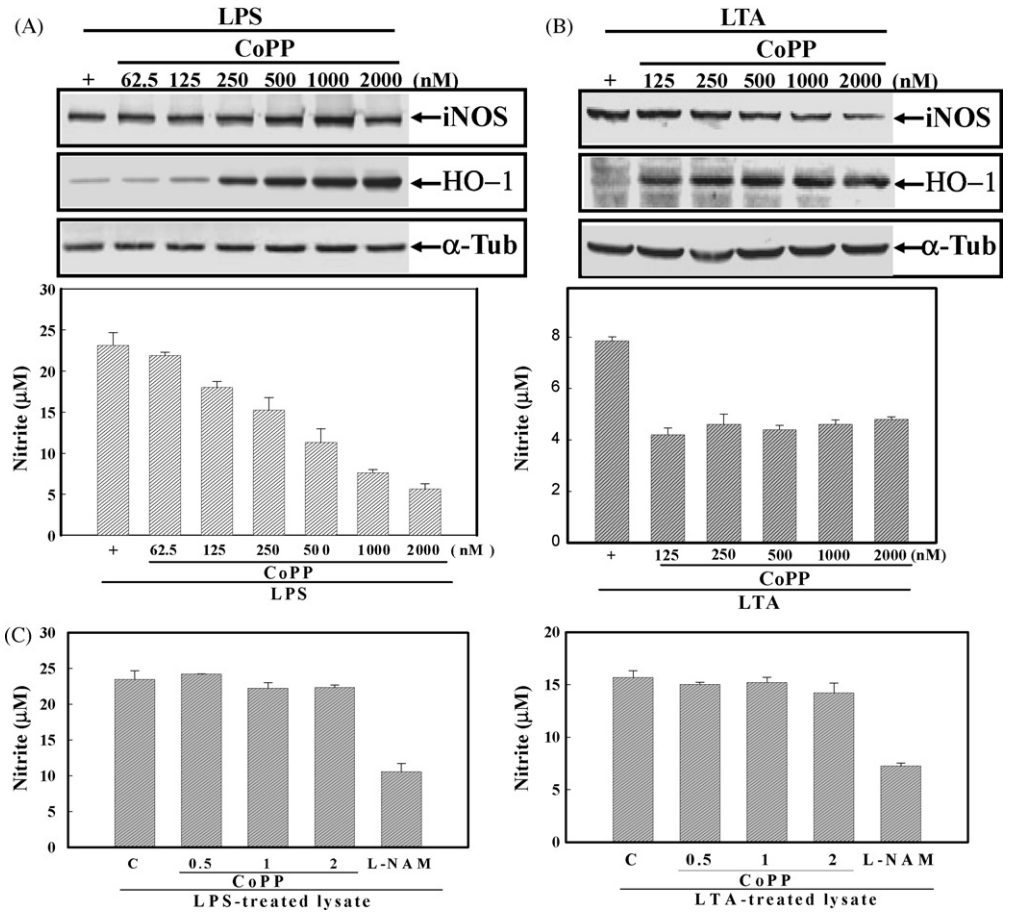

Fig. 5. CoPP inhibited iNOS enzyme activity in a cell-mediated, but not cell-free, iNOS activity assay. (A, B) CoPP inhibited NO production in a cell-mediated iNOS enzyme activity assay. RAW264.7 cells were individually treated with LPS (A) and LTA (B) for 12 h, followed by a cell-mediated iNOS enzyme activity assay as described in Section 2. (Upper panel) Expressions of iNOS, HO-1 protein, and α-tubulin were detected by Western blotting. Lower panel: the amount of NO produced in the medium was detected by the Griess reaction. (C) CoPP did not affect NO production by the cell-free iNOS enzyme activity assay. Macrophages were separately treated with LPS (left panel) and LTA (right panel) for 12 h, followed by the iNOS enzyme activity assay as described in Section 2. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments.

1 inducers, FePP and CoPP, but not SnPP, in macrophages [34]. Our previous study demonstrated that HO-1 induced by FePP significantly inhibited LPS-induced iNOS/NO production in macrophages [22,23]. Stimulation of HO-1 enzyme activity catalyzes the degradation of heme to byproducts including CO, biliverdin, bilirubin, and iron. Bilirubin can reduce iNOS/NO induction by endotoxin via inhibition of NAD(P)H oxidase [35]. Cepinskas et al. indicated that CO might attenuate inflammation in the liver of septic mice [36]. However, HO-1 protein was shown to directly interact with cellular defense proteins such as MnSOD and catalase [7,8]. Therefore, whether protein or enzyme activities contribute to iNOS/NO inhibition of HO-1 is still undefined. The present study first reports that CoPP dose-dependently inhibited LPS- and LTA-induced iNOS/NO production with increasing HO-1 protein expression in the SF condition. Suppression of HO-1 protein expression via transfection of HO-1 siRNA showed no effect on CoPP-inhibited NO production elicited by LPS. This suggests that HO-1 induction might not participate in iNOS/NO's inhibition of CoPP.

We further found that iNOS/NO inhibited by CoPP in the SF condition, but not in a condition with FBS or BSA. Plasma protein albumin can bind FePP with medium affinity to prevent the toxic effects of FePP in blood cells and vascular endothelium. Our previous study demonstrated that metal protoporphyrins such as FePP, CoPP, and SnPP are able to bind with albumin in FBS [37]. Data of the present study show that the addition of BSA inhibited CoPP-induced HO-1 protein and inhibited LPS- and LTA-induced iNOS/NO production in RAW264.7 macrophages. This suggests that FBS's abolishment of CoPP-induced anti-inflammatory effects in response to LPS or LTA insults occurs through binding with BSA.

Regulation of iNOS, as indicated by its name (i.e., inducible), has long thought to be transcriptional or translational rather than catalytic. Indeed, many modulators participate in regulating iNOS catalytic activities, including NADPH, tetrahydrobiopterin, FAD, and flavin mononucleotide. Metalloprotoporphyrins have been shown to bind with the heme-binding pocket of various proteins such as myoglobin, hemoglobin, and cytochrome P-450 [38,39]. It was shown that CoPP, but not FePP, SnPP, or MnPP, can reconstitute neuronal (n)NOS from apo-nNOS to affect nNOS enzyme activity *in vitro* [40,41]. Li et al. indicated that CoPP decreases nNOS enzyme activity *in vivo* [42]. However, whether or not CoPP directly or indirectly inhibits iNOS enzyme activity and the mechanism of its interaction with iNOS enzyme are unknown. In the present study, CoPP was more sensitive to blocking NO production than decreasing iNOS protein expression in response to inflammatory stimuli such as LPS and LTA. The IC_{50} values of CoPP against NO production and iNOS protein expression elicited by LPS were < 0.5 and 1.75 ± 0.36 μM, respectively. Both cell-mediated and cell-free iNOS enzyme activities were evaluated, and CoPP significantly inhibited NO production in a cell-mediated, but not a cell-free, condition. This suggests that CoPP possesses the ability to inhibit iNOS enzyme activity in a cell-dependent manner. The reason why CoPP inhibited iNOS enzyme activity via a cell-mediated manner is still unclear. Modulators

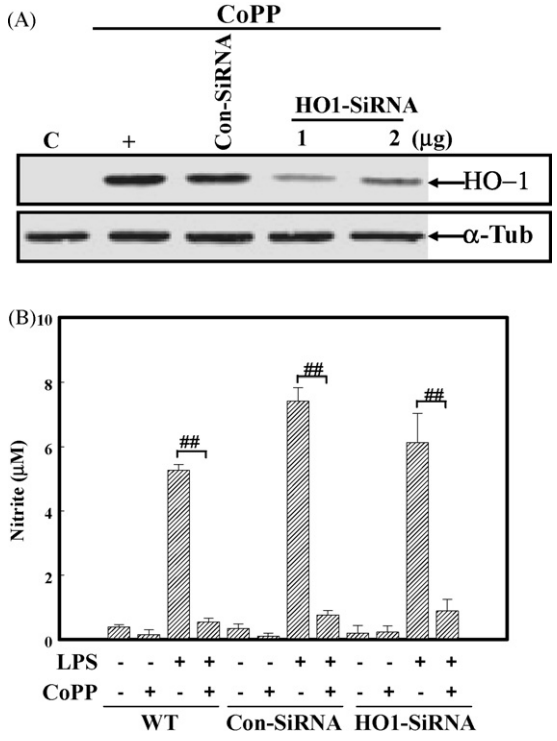

Fig. 6. CoPP inhibition of LPS-induced iNOS/NO protein was independent of HO-1 induction. (A) HO-1 siRNA inhibited CoPP-induced HO-1 protein expression. Cells were transfected with control or HO-1 siRNA for 24 h followed by CoPP treatment for 12 h. Expressions of HO-1 and α-tubulin (α-Tub) were examined by Western blotting. (B) HO-1 siRNA showed no effect on CoPP-inhibited NO production elicited by LPS. Parental (WT), control-siRNA (Con-siRNA), and HO-1 siRNA-transfected (HO-1-siRNA) macrophages were treated with CoPP (2 μM) for 30 min followed by LPS stimulation for 12 h. The amount of NO was detected by the Griess reaction. Data of Western blotting were repeated at least three times, and similar results were obtained. Each value is presented as the mean ± S.E. of three independent experiments. $^{\#\#}P < 0.01$ indicates a significant difference between the indicated groups as analyzed by Student's *t*-test.

including L-arginine, tetrahydrobiopterin, FAD, and flavin mononucleotide have been identified as participating in iNOS catalytic reactions to produce NO. NADPH is an important factor in modulating iNOS enzyme activity, and Jellinck and Galbraith indicated that CoPP inhibited NADPH-dependent elimination of dihydrotestosterone *in vitro* [43]. There is no report related to the effect of CoPP on L-arginine, tetrahydrobiopterin, FAD, or the flavin mononucleotide. CoPP inhibition of iNOS enzyme activity may be mediated by reducing intracellular NADPH. Identification of the mechanism of iNOS enzyme activity inhibited by CoPP deserves further study.

In summary, we explored the effect of CoPP on the regulation of iNOS/NO production in RAW264.7 macrophages individually stimulated by LPS and LTA by increasing HO-1 protein expression in a condition with or without FBS and BSA. It is clear the CoPP significantly inhibited iNOS/NO production in an SF condition through blocking JNK activation elicited by LPS and LTA. Blocking HO-1 protein did not affect iNOS/NO's inhibition of CoPP, and CoPP inhibition of iNOS enzyme activity was identified in a cell-mediated iNOS enzyme activity assay. This suggests that CoPP may inhibit JNK activation and iNOS enzyme activity, leading to the inhibition of iNOS/NO production in response to inflammatory stimulators in a HO-1-independent manner.

Acknowledgements

This study was supported by the National Science Council of Taiwan (NSC95-2320-B-038-029-MY2 and NSC96-2320-B-038-031-MY3) and DOH-TD-B-111-002 (Center of Excellence for Clinical Trial and Research in Neurology and Neurosurgery Taiwan).

References

[1] P. Ewing, G.C. Hildebrandt, S. Planke, R. Andreesen, E. Holler, A. Gerbitz, Cobalt protoporphyrine IX-mediated heme oxygenase-I induction alters the inflammatory cytokine response, but not antigen presentation after experimental allogeneic bone marrow transplantation, Int. J. Mol. Med. 20 (2007) 301–308.
[2] T. Chang, L. Wu, R. Wang, Inhibition of vascular smooth muscle cell proliferation by chronic hemin treatment, Am. J. Physiol. Heart Circ. Physiol. 295 (2008) H999–H1007.
[3] A.A. Chora, P. Fontoura, A. Cunha, T.F. Pais, S. Cardoso, P.P. Ho, L.Y. Lee, R.A. Sobel, L. Steinman, M.P. Soares, Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation, J. Clin. Invest. 117 (2007) 438–447.
[4] H. Matsumoto, K. Ishikawa, H. Itabe, Y. Maruyama, Carbon monoxide and bilirubin from heme oxygenase-1 suppresses reactive oxygen species generation and plasminogen activator inhibitor-1 induction, Mol. Cell. Biochem. 291 (2006) 21–28.
[5] C.W. Lin, S.C. Shen, W.C. Hou, L.Y. Yang, Y.C. Chen, Heme oxygenase-1 inhibits breast cancer invasion via suppressing the expression of matrix metalloproteinase-9, Mol. Cancer Ther. 7 (2008) 1195–1206.
[6] A. Nakao, D.J. Kaczorowski, R. Sugimoto, T.R. Billiar, K.R. McCurry, Application of heme oxygenase-1, carbon monoxide and biliverdin for the prevention of intestinal ischemia/reperfusion injury, J. Clin. Biochem. Nutr. 42 (2008) 78–88.
[7] D. Frankel, K. Mehindate, H.M. Schipper, Role of heme oxygenase-1 in the regulation of manganese superoxide dismutase gene expression in oxidatively-challenged astroglia, J. Cell. Physiol. 185 (2000) 80–86.
[8] S. Turkseven, A. Kruger, C.J. Mingone, M. Inaba, L.F. Rodella, S. Ikehara, M.S. Wolin, N.G. Abraham, Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes, Am. J. Physiol. Heart Circ. Physiol. 289 (2005) H701–H707.
[9] P.F. Austin, A.J. Casale, M.P. Cain, R.C. Rink, S.J. Weintraub, Lipopolysaccharide and inflammatory cytokines cause an inducible nitric oxide synthase-dependent bladder smooth muscle fibrotic response, J. Urol. 170 (2003) 645–648.
[10] K. Habara, Y. Hamada, M. Yamada, K. Tokuhara, H. Tanaka, M. Kaibori, Y. Kamiyama, M. Nishizawa, S. Ito, T. Okumura, Pitavastatin up-regulates the induction of iNOS through enhanced stabilization of its mRNA in pro-inflammatory cytokine-stimulated hepatocytes, Nitric Oxide 18 (2008) 19–27.
[11] T. Roger, J. David, M.P. Glauser, T. Calandra, MIF regulates innate immune responses through modulation of Toll-like receptor 4, Nature 414 (2001) 920–924.
[12] G. Zhang, S. Ghosh, Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors, J. Endotoxin Res. 6 (2000) 453–457, Review.
[13] O. Takeuchi, K. Hoshino, S. Akira, Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection, J. Immunol. 165 (2000) 5392–5396.
[14] H. He, K.J. Genovese, D.J. Nisbet, M.H. Kogut, Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes, Mol. Immunol. 43 (2006) 783–789.
[15] C.W. Lee, C.S. Chien, C.M. Yang, Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-like receptor 2 in tracheal smooth muscle cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 286 (2004) L921–L930.
[16] H.F. Chien, K.Y. Yeh, Y.F. Jiang-Shieh, I.H. Wei, C.Y. Chang, M.L. Chang, C.H. Wu, Signal transduction pathways of nitric oxide release in primary microglial culture challenged with gram-positive bacterial constituent, lipoteichoic acid, Neuroscience 133 (2005) 423–436.
[17] B. Jawan, Y.H. Kao, S. Goto, M.C. Pan, Y.C. Lin, L.W. Hsu, T. Nakano, C.Y. Lai, C.K. Sun, Y.F. Cheng, M.H. Tai, H.L. Eng, C.S. Wang, C.J. Huang, C.R. Lin, C.L. Chen, Propofol pretreatment attenuates LPS-induced granulocyte-macrophage colony-stimulating factor production in cultured hepatocytes by suppressing MAPK/ERK activity and NF-kappaB translocation, Toxicol. Appl. Pharmacol. 229 (2008) 362–373.
[18] W. Lin, R.T. Wu, T. Wu, T.O. Khor, H. Wang, A.N. Kong, Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway, Biochem. Pharmacol. 76 (2008) 967–973.
[19] G.S. Oh, H.O. Pae, B.S. Lee, B.N. Kim, J.M. Kim, H.R. Kim, S.B. Jeon, W.K. Jeon, H.J. Chae, H.T. Chung, Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide, Free Radic. Biol. Med. 41 (2006) 106–119.
[20] K. Tsoyi, H.J. Kim, J.S. Shin, D.H. Kim, H.J. Cho, S.S. Lee, S.K. Ahn, H.S. Yun-Choi, J.H. Lee, H.G. Seo, K.C. Chang, HO-1 and JAK-2/STAT-1 signals are involved in preferential inhibition of iNOS over COX-2 gene expression by newly synthesized tetrahydroisoquinoline alkaloid, CKD712, in cells activated with lipopolysaccharide, Cell. Signal. 20 (2008) 1839–1847.
[21] H.Y. Hong, W.K. Jeon, B.C. Kim, Up-regulation of heme oxygenase-1 expression through the Rac1/NADPH oxidase/ROS/p38 signaling cascade mediates the anti-inflammatory effect of 15-deoxy-delta 12,14-prostaglandin J2 in murine macrophages, FEBS Lett. 582 (2008) 861–868.
[22] H.Y. Lin, S.H. Juan, S.C. Shen, F.L. Hsu, Y.C. Chen, Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1, Biochem. Pharmacol. 66 (2003) 1821–1832.

[23] H.Y. Lin, S.C. Shen, Y.C. Chen, Anti-inflammatory effect of heme oxygenase 1: glycosylation and nitric oxide inhibition in macrophages, J. Cell. Physiol. 202 (2005) 579–590.
[24] H.Y. Lin, S.C. Shen, C.W. Lin, L.Y. Yang, Y.C. Chen, Baicalein inhibition of hydrogen peroxide-induced apoptosis via ROS-dependent heme oxygenase 1 gene expression, Biochem. Biophys. Acta 1773 (2007) 1073–1086.
[25] T. Yoshinaga, S. Sassa, A. Kappas, Purification and properties of bovine spleen heme oxygenase Amino acid composition and sites of action of inhibitors of heme oxidation, J. Biol. Chem. 257 (1982) 7778–7785.
[26] M.K. Sardana, A. Kappas, Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 2464–2468.
[27] L. Brasile, R. Buelow, B.M. Stubenitsky, G. Kootstra, Induction of heme oxygenase-1 in kidneys during ex vivo warm perfusion, Transplantation 76 (2003) 1145–1149.
[28] M. Benallaoua, M. François, F. Batteux, N. Thelier, J.Y. Shyy, C. Fitting, L. Tsagris, J. Boczkowski, J.F. Savouret, M.T. Corvol, S. Poiraudeau, F. Rannou, Pharmacologic induction of heme oxygenase 1 reduces acute inflammatory arthritis in mice, Arthritis Rheum. 56 (2007) 2585–2594.
[29] T. Iwai, K. Kitamoto, K. Teramoto, Y. Machida, S. Tamada, T. Yukimura, H. Iwao, T. Nakatani, K. Miura, Cobalt protoporphyrin attenuates rat obstructive nephropathy: role of cellular infiltration, Urology 72 (2007) 432–438.
[30] H. Chiu, J.A. Brittingham, D.L. Laskin, Differential induction of heme oxygenase-1 in macrophages and hepatocytes during acetaminophen-induced hepatotoxicity in the rat: effects of hemin and biliverdin, Toxicol. Appl. Pharmacol. 181 (2002) 106–115.
[31] D. Lang, S. Reuter, T. Buzescu, C. August, S. Heidenreich, Heme-induced heme oxygenase-1 (HO-1) in human monocytes inhibits apoptosis despite caspase-3 up-regulation, Int. Immunol. 17 (2005) 155–165.
[32] J.L. Ma, P.Y. Yang, Y.C. Rui, L. Lu, H. Kang, J. Zhang, Hemin modulates cytokine expressions in macrophage-derived foam cells via heme oxygenase-1 induction, J. Pharmacol. Sci. 103 (2007) 261–266.
[33] G.S. Drummond, A. Kappas, The cytochrome p-450 depleted animal: an experimental model for *in vivo* studies in chemical biology, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 2384–2388.
[34] T. Ashino, R. Yamanaka, M. Yamamoto, H. Shimokawa, K. Sekikawa, Y. Iwakura, S. Shioda, S. Numazawa, T. Yoshida, Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages, Mol. Immunol. 45 (2008) 2106–2115.
[35] S. Lanone, S. Bloc, R. Foresti, A. Almolki, C. Taillé, J. Callebert, M. Conti, D. Goven, M. Aubier, B. Dureuil, J. El-Benna, R. Motterlini, J. Boczkowski, Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats, FASEB J. 19 (2005) 1890–1892.
[36] G. Cepinskas, K. Katada, A. Bihari, R.F. Potter, Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice, Am. J. Physiol. Gastrointest. Liver Physiol. 294 (2008) G184–G191.
[37] J.M. Chow, G.C. Huang, H.Y. Lin, S.C. Shen, L.Y. Yang, Y.C. Chen, Cytotoxic effects of metal protoporphyrins in glioblastoma cells: roles of albumin, reactive oxygen species, and heme oxygenase-1, Toxicol. Lett. 177 (2008) 97–107.
[38] O. Daltrop, S.J. Ferguson, In vitro studies on thioether bond formation between Hydrogenobacter thermophilus apocytochrome c(552) with metalloprotoporphyrin derivatives, J. Biol. Chem. 279 (2004) 45347–45353.
[39] L.M. Bornheim, D.W. Parish, K.M. Smith, D.A. Litman, M.A. Correia, The influence of side chain modifications of the heme moiety on prosthetic acceptance and function of rat hepatic cytochrome P-450 and tryptophan pyrrolase, Arch. Biochem. Biophys. 246 (1986) 63–74.
[40] A.T. Bender, M. Nakatsuka, Y. Osawa, Heme insertion, assembly, and activation of apo-neuronal nitric-oxide synthase *in vitro*, J. Biol. Chem. 275 (2000) 26018–26023.
[41] A.T. Bender, Y. Kamada, P.A. Kleaveland, Y. Osawa, Assembly and activation of heme-deficient neuronal NO synthase with various porphyrins, J. Inorg. Biochem. 91 (2002) 625–634.
[42] M. Li, M.A. Vizzard, D.M. Jaworski, R.A. Galbraith, The weight loss elicited by cobalt protoporphyrin is related to decreased activity of nitric oxide synthase in the hypothalamus, J. Appl. Physiol. 100 (2006) 1983–1991.
[43] P.H. Jellinck, R.A. Galbraith, Suppression of 5 alpha-androstane-3 beta,17 beta-diol hydroxylase activity in rat pituitary by cobalt protoporphyrin: implications for testosterone homeostasis, J. Steroid Biochem. Mol. Biol. 39 (1991) 947–951.