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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

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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.

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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.