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Disruption of lipid rafts impairs the production of nitric oxide in lipopolysaccharide-stimulated murine RAW264.7 macrophages

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Abstract—Upon stimulation with lipopolysaccharide, murine RAW264.7 macrophages generated a high amount of nitric oxide. Pretreatment of macrophages with methyl-β-cyclodextrin, which disrupted the lipid raft microdomains of the plasma membrane, inhibited the generation of nitric oxide by down-regulating the expression of inducible nitric oxide synthase. Methyl-β-cyclodextrin exposure significantly inhibited the degradation of IκB-α, blocked the translocation of p65/RelA into the nuclei and prevented the activation of the NF-κB signaling pathway. The results suggest that the expression of inducible nitric oxide synthase and the consequent production of nitric oxide depend on the integrity of the lipid rafts.

Keywords: RAW 264.7 macrophage; nitric oxide; lipopolysaccharide; iNOS; NF-κB; lipid raft; methyl-β-cyclodextrin.

INTRODUCTION

Nitric oxide (NO) is a poisonous, unstable free radical gas that has been known for years to be a constituent of air pollutants and is supposed to be involved in the depletion of the ozone layer. On the other hand, NO plays diverse biological roles including vascular regulation [1], host defense [2] and neuronal communication [3]. NO is synthesized from L-arginine by nitric oxide synthase (NOS) [4]. In activated macrophages, the transcriptionally expressed inducible nitric oxide synthase (iNOS) is responsible for the prolonged and profound production of NO [5]. The

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physiological generation of NO mediates the bactericidal and tumoricidal functions of macrophages. However, the excessive production of NO has been implicated in a number of pathological processes, such as the development of cardiovascular diseases, neurodegenerative disorders and cancer [6, 7]. Thus, understanding the molecular mechanisms of the induction of iNOS gene in macrophages will be beneficial for the prevention and therapy of inflammatory diseases associated with the over production of NO. Furthermore, the inhibition of NO production by blocking iNOS expression is an important target in the treatment of these diseases.

Lipopolysaccharide (LPS), the integral component of the outer membrane of Gram-negative bacteria, is a widely used agent for the study of macrophage activation. LPS consists of a hydrophilic polysaccharide and a highly conserved hydrophobic lipid A portion [8], which stimulates both innate and adaptive immune responses, particularly on macrophages and dendritic cells. Upon stimulation with LPS, which binds to a specific receptor (Toll-like receptor 4; TLR4) [9], macrophages can be activated to produce pro-inflammatory molecules including cytokines, chemokines, cell surface antigens and NO.

A large part of the signaling cascades involved in LPS signaling takes place in the plasma membrane. Recently, lipid rafts have been proposed to represent an important role in LPS signaling [10, 11]. Lipid rafts are detergent insoluble/resistant, cholesterol and sphingolipids-enriched membrane domains, which have been postulated as a platform for cell signaling in the immune system [12, 13]. LPS stimulation of the macrophage-like cell line RAW264.7 induced translocation of CD-14, ERK-2 and p38 to lipid rafts. However, whether lipid rafts are involved in cell signaling leading to the activation of macrophages and the production of NO remains to be elucidated. In the present investigation, the effect of methyl-β-cyclodextrin (M-β-CyD), which disrupts the lipid raft microdomains of the plasma membrane [14], on NO production in LPS-stimulated RAW264.7 macrophages was studied, and the underlying molecular mechanisms were discussed.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and cell-culture supplements were obtained from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS; from *E. coli* 055:B5) and methyl-β-cyclodextrin (M-β-CyD) were products of Sigma (St. Louis, MO, USA). Antibodies against iNOS, IκB-α and p65/RelA were from BD Transduction Laboratories (Lexington, KY, USA). Uniq-10^{TM} RNA purification kit was purchased from Sangon (Shanghai, China). Access Quick^{TM} RT-PCR kit was purchased from Promega (Shanghai, China). ECL^{Plus} Western blotting detection kit was from Amersham Biosciences (Hong Kong, China). Other reagents were made in China.

Cell culture and drug treatment

The RAW264.7 macrophage-like cell line was obtained from the Chinese Type Culture Collection (CTCC, Shanghai, China). RAW264.7 macrophages were seeded at a density of 4×10^5 cells/cm^2 into 25 cm^2 cell culture flasks. Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM Hepes buffer, 100 U/ml penicillin and 100 μg/ml streptomycin. RAW264.7 macrophages were incubated at 37°C under a humidified atmosphere containing 5% CO_2/95% air. After incubation for 24 h, culture medium was changed into serum-free RPMI 1640, and macrophages were cultured for additional 6 h. Then macrophages were treated with 1 μg/ml LPS in serum-free RPMI 1640 medium for the indicated amount of time. In some experiments macrophages were preincubated with methyl-β-cyclodextrin (M-β-CyD; 2.5 mM or 5 mM) for 3 h before the addition of LPS.

Assay of nitric oxide

NO produced from activated RAW264.7 macrophages was detected directly by Electron Paramagnetic Resonance (EPR) spin trapping [15]. Briefly, RAW264.7 macrophages cultured in 25 cm^2 cell culture flasks were incubated with or without certain concentrations of M-β-CyD for 3 h and stimulated with 1 μg/ml LPS in RPMI 1640 medium for 12 h. Then the spin-trapping agent containing 1 mM $FeSO_4$, 5 mM diethyldithiocarbamate sodium salt (DETC) and 5 mM $Na_2S_2O_3$ were added into activated macrophages, which were incubated at 37°C for additional 3 h. The paramagnetic [ON-Fe^{2+}(DETC)$_2$] complex was enriched by extraction with 200 μl of ethyl acetate and detected by Bruker ER-200 D-SRC EPR spectrometer with conditions described as follows: X-band; sweep width 400 G; microwave power 20 mW; 100 kHz modulation with amplitude 3.2 G; time constant 0.128 s.

Detection of iNOS mRNA

For the analysis of iNOS gene expression, iNOS mRNA levels in RAW264.7 macrophages were detected by RT-PCR. After M-βCyD treatment, macrophages were stimulated with LPS for 6 h. Total RNA was isolated with Uniq-10^{TM} RNA purification kit according to manufacturer's instructions. RT-PCR was carried out using the AccessQuick^{TM} RT-PCR system with primers specific for iNOS and GAPDH (house-keeping gene). For iNOS, the primers were 5′-GTGTTCCACCAGGAGATGTTG-3′/5′-CTCCTGCCCACTGAGTTCGTC-3′, the length of the PCR product was 576 bp; For GAPDH, the primers were 5′-GAAGGGTGGGGCCAAAAG-3′/5′-GGATGCAGGGATGATGTTCT-3′, the length of the PCR product was 295 bp. PCR products were visualized by electrophoresis through a 1.5% agarose gel stained with ethidium bromide.

Detection of iNOS and NF-κB proteins

The levels of iNOS and NF-κB proteins in macrophages were determined by Western blot. Following M-β-CyD treatment and LPS stimulation, RAW264.7 macrophages were detached from the cell-culture flasks with rubber policemen, and lysed with 300 μl of buffer A (10 mM HEPES, 10 mM KCl, 100 μM EDTA, 100 μM EGTA, 2 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin) at 4°C for 15 min. After centrifugation at 12 000 × *g* for 15 min, the supernatant containing cytosolic proteins was collected and used for the assay of iNOS and IκB-α proteins. The pellet was washed once with buffer A and then suspended in 100 μl of buffer B (20 mM HEPES, 390 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM PMSF). After centrifugation at 12 000 × *g* for 15 min, the supernatant was collected and used as nuclear proteins for the assay of p65/RelA. Each sample containing 40 μg of cytosolic or nuclear proteins was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was incubated with antibodies against iNOS, IκB-α and p65/RelA proteins, respectively, and then incubated with proper horseradish peroxidase-conjugated second antibodies. The target proteins were detected by the ECL^{Plus} Western blotting detection kit and exposed to Kodak X-ray autoradiography films.

Statistical analysis

Each experiment was performed at least three times and the results were presented as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA). A level of $P < 0.05$ was considered significant.

RESULTS

Disruption of lipid rafts impairs the production of nitric oxide

A three-line EPR spectrum corresponding to the [ON-$Fe^{2+}$$(DETC)_2$] complex at $g = 2.035$ was observed in RAW264.7 macrophages treated with LPS for 12 h (Fig. 1A), suggesting that activated macrophages generate NO. Pretreatment of RAW264.7 macrophages with M-β-CyD, which disrupts the lipid raft microdomains of the plasma membrane, suppresses the EPR signal intensity dose-dependently, as shown in Fig. 1B and 1C. In macrophages pretreated with 2.5 mM M-β-CyD, the EPR signal intensity of [ON-$Fe^{2+}$$(DETC)_2$] complex decreased to 33±6.5% of control macrophages. In macrophages pretreated with 5 mM M-β-CyD, the EPR signal intensity of [ON-$Fe^{2+}$$(DETC)_2$] complex decreased to 7.1±3.3% of control macrophages. No apparent EPR signal of [ON-$Fe^{2+}$$(DETC)_2$] was observed in RAW264.7 macrophages without LPS stimulation (Fig. 1D).

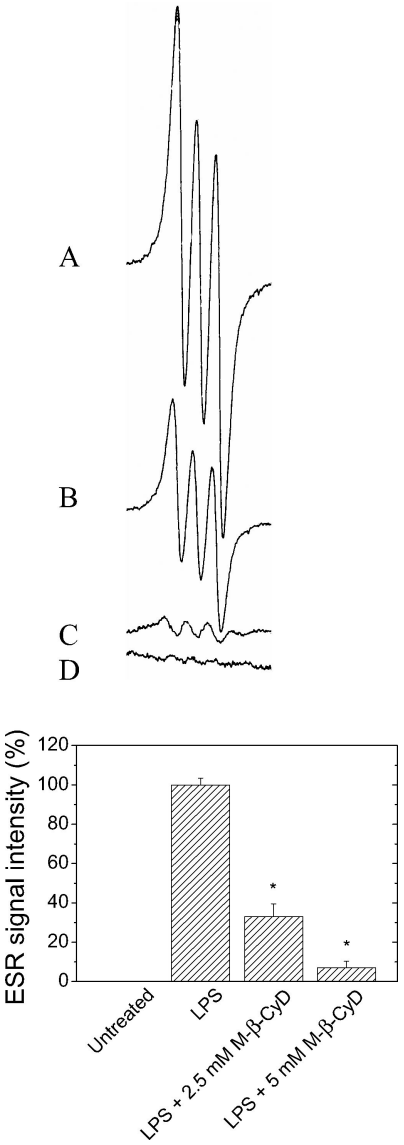

Figure 1. Inhibition of NO production by M-β-CyD in LPS-treated RAW264.7 macrophages. RAW264.7 macrophages were pretreated with different concentrations of M-β-CyD for 3 h and stimulated with 1 μg/ml LPS for 12 h. The production of NO was trapped with $[Fe^{2+}(DETC)_2]$ and the EPR spectrum was recorded with a Bruker ER-200 D-SRC EPR spectrometer. (A) RAW264.7 macrophages without M-β-CyD pretreatment; (B) RAW264.7 macrophages pretreated with 2.5 mM M-β-CyD; (C) RAW264.7 macrophages pretreated with 5 mM M-β-CyD; (D) RAW264.7 macrophages without M-β-CyD and LPS treatment. The graph at the bottom presents the quantitative determination of the inhibition of NO production by M-β-CyD in LPS-stimulated RAW264.7 macrophages. Data presented in the graph at the bottom are mean $\pm$ SD, $n = 6$. $^*P < 0.05$ in comparison with control macrophages.

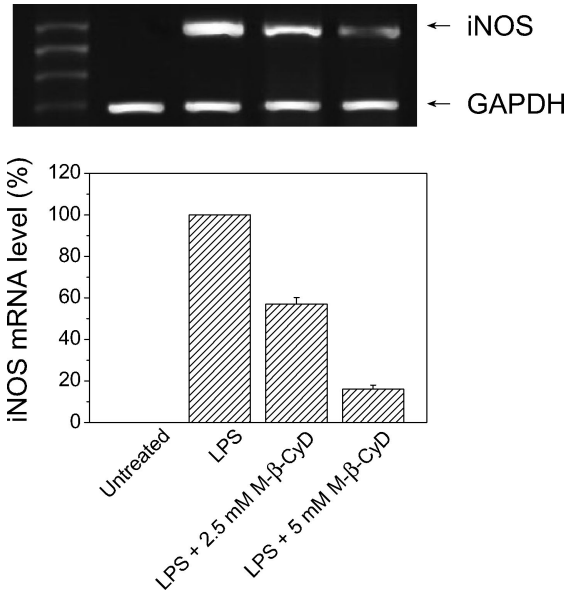

Figure 2. Effects of M-β-CyD on iNOS mRNA levels in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with 2.5 mM or 5 mM of M-β-CyD for 3 h and then stimulated with 1 μg/ml LPS for 6 h. The levels of iNOS mRNA were determined by RT-PCR.

The expression of iNOS gene depends on the integrity of lipid rafts

To understand the mechanisms of the inhibition of NO production by M-β-CyD, the effect of M-β-CyD on the expression of iNOS gene in LPS-stimulated RAW264.7 macrophages was studied. An immunoblot with antibody against murine macrophage iNOS and RT-PCR analysis of iNOS mRNA clearly showed that disruption of lipid rafts by M-β-CyD inhibited the induction of iNOS gene at both the protein and the mRNA level, as shown in Figs 2 and 3.

Lipid rafts mediates LPS-activated NF-κB signaling

To further understand the modulation mechanisms of iNOS gene expression by M-β-CyD, effects of M-β-CyD on the nuclear factor-κB (NF-κB) transcription factor signaling pathway were studied. The activation of NF-κB requires the phosphorylation, ubiquitination and degradation of its inhibitor protein IκB, and the consequent translocation of Rel proteins (p65/RelA, p50, c-Rel, RelB and p52) into the nuclei. In the present investigation, the effects of M-β-CyD on the degradation of IκB-α and the translocation of p65/RelA into the nuclei were studied by Western blot. As shown in Fig. 4, the cytosolic IκB-α level decreased significantly 1 h after exposure to LPS; and the nuclear p65/RelA level increased markedly, suggesting the degradation of IκB-α and the translocation of p65/RelA into nuclei after LPS treatment. Pretreating RAW264.7 macrophages with M-β-CyD significantly prevented the degradation of IκB-α and inhibited the translocation of p65/RelA into

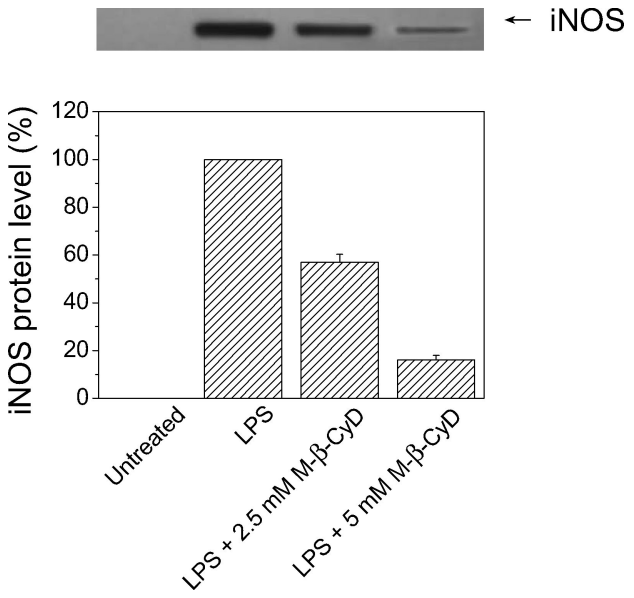

Figure 3. Effects of M-β-CyD on iNOS protein levels in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with 2.5 mM or 5 mM of M-β-CyD for 3 h and then stimulated with 1 μg/ml LPS for 12 h. The levels of iNOS protein were determined by Western blot.

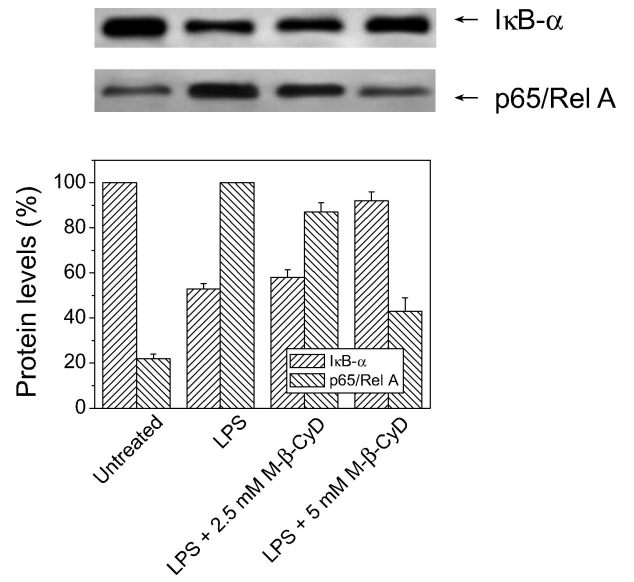

Figure 4. Effects of M-β-CyD on the degradation of IκB-α and the translocation of p65/Rel A into the nuclei in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with 2.5 mM or 5 mM of M-β-CyD for 3 h and then stimulated with 1 μg/ml LPS for 1 h. The levels of cytosolic IκB-α and nuclear p65/Rel A were determined by Western blot.

the nuclei in a dose-dependent manner. In RAW264.7 macrophages pretreated with 5 mM M-β-CyD for 3 h, the degradation of IκB-α and the translocation of p65/RelA into the nuclei was blocked, suggesting that LPS/IFN-γ-induced activation of NF-κB signaling pathway depended on the integrity of the lipid rafts.

DISCUSSION

RAW264.7 cells, initially derived from Balb/c mice infected with Abelson leukemia virus, are extremely sensitive to LPS [16]. Many of the responses of activated macrophages to LPS have been observed in cultured RAW264.7 cells [17]. In this regard, RAW264.7 macrophage-like cells are ideal model cells for the study of LPS signaling and macrophage activation.

Cyclodextrins (CyDs) are host molecules that form inclusion complexes with lipophilic drugs as guests and, thus, have been utilized for improving their water solubility and dissolution rates [18]. CyDs have also been reported to interact with lipid molecules such as cholesterol, phospholipids and phosphatidylinositols, depending on their cavity sizes, resulting in the disruption of the structures of lipid rafts, the lipid microdomains formed by lateral assemblies of cholesterol and sphingolipids in the plasma membrane [11, 19–21]. Recently, Cuschieri *et al.* reported that cholesterol depletion with M-β-CyD in THP-1 cells is associated with a significant attenuation of LPS-mediated mitogen-activated protein kinase activation [22]. Arima *et al.* reported that 2,6-di-O-methyl-α-CyD (DM-α-CyD) inhibited NO production in RAW264.7 cells stimulated with LPS [23, 24]. However, the underlying mechanisms remain to be further elucidated.

In the present study, the effects of M-β-CyD on the induction of iNOS gene and the consequent production of NO in LPS-activated RAW264.7 macrophages were investigated. Upon stimulation with 1 μg/ml LPS, RAW264.7 macrophages generated large amount of NO, which could be spin trapped by the $[Fe^{2+}(DETC)_2]$ complex and enriched by extraction with ethyl acetate. The three-line spectrum corresponding to the $[ON\text{-}Fe^{2+}(DETC)_2]$ complex at $g = 2.035$ was observed by EPR. Pretreatment with M-β-CyD for 3 h significantly decreased the EPR signal intensity of $[ON\text{-}Fe^{2+}(DETC)_2]$ in RAW264.7 macrophages stimulated with LPS in a dose-dependent manner. Because M-β-CyD showed no direct scavenging effect on authentic NO (data not shown), the decrease of EPR signal intensity was due to the inhibition of NO production. Western blotting and RT-PCR analysis revealed that M-β-CyD treatment decreased the expression of iNOS at both the protein and the mRNA level.

Considering that the NF-κB signaling pathway is one of the dominant signaling pathways in the regulation of iNOS expression, the effect of M-β-CyD on the LPS-stimulated NF-κB activation was studied. NF-κB is a sequence-specific DNA binding protein complex that regulates immune and inflammatory responses by increasing the expression of certain genes, including some cytokines, chemokines, and inducible enzymes such as iNOS [25]. The phosphorylation and degradation

of cytoplasmic IκB-α, and the translocation of p50/p65 from the cytoplasm into the nucleus, are two key steps in NF-κB activation. As shown in Fig. 4, M-β-CyD inhibited the degradation of IκB-α and the translocation of p65/RelA in a dose-dependent manner, suggesting that disruption of lipid rafts by M-β-CyD prevented the LPS-induced activation of the NF-κB signaling pathway.

Recently, it was accepted that LPS is rapidly delivered from the plasma membrane to an intracellular site and then rapidly encounters intracellular TLR4, and downstream signaling is triggered. These lines of evidence make it tempting to speculate that molecules that can interact with the plasma membrane might be modulators of LPS signaling. M-β-CyD, a non-toxic, widely-used host molecule, has been reported to disrupt the lipid raft microdomains of the plasma membrane by interacting with cholesterol and sphingolipids. Results of the present investigation clearly showed that M-β-CyD effectively inhibited the activation of RAW264.7 macrophages and decreased the production of NO *via* down-regulating the NF-κB signaling pathways. The results suggested that M-β-CyD might be used as an effective preventive and therapeutic agent for inflammatory diseases associated with the over-production of NO.

CONCLUSIONS

M-β-CyD impaired NO production in murine RAW264.7 macrophages stimulated with LPS. The inhibitory effect of M-β-CyD on NO production could be attributed to the inhibition of iNOS gene expression *via* NF-κB-mediated, lipid raft-dependent signaling pathways.

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