Inhibitory Effects of Novel Hydrophilic Cyclodextrin Derivatives on Nitric Oxide Production in Macrophages Stimulated with Lipopolysaccharide

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Purpose. The objective of this study is to examine the effects of cyclodextrins (CyDs) on nitric oxide (NO) production in macrophages stimulated with lipopolysaccharide (LPS).
Methods. RAW264.7 cells, a mouse macrophage-like cell, were used. Cytotoxicity of CyDs was evaluated by WST-1 method. Nitrite, iNOS, and *iNOS* mRNA were determined by Griess method, Western blotting, and reverse transcription-polymerase chain reaction (RT-PCR) analysis, respectively. The interaction of LPS with CyDs was evaluated by utilizing a competitive inclusion phenomenon. The binding of FITC-labeled LPS to the surface of RAW264.7 cells was measured by a flow cytometry.
Results. Of 15 CyDs, 2,6-di-*O*-methyl-α-CyD (DM-α-CyD), and 2,6-di-*O*-methyl-3-*O*- acetyl-β-cyclodextrin (DMA-β-CyD) had greater inhibitory activity than did the other CyDs against NO production in RAW264.7 cells stimulated with LPS, without showing any cytotoxicity. DM-α-CyD and DMA-β-CyD specifically inhibited the increase in iNOS and *iNOS* mRNA levels elicited by stimulation with LPS in RAW264.7 cells. DM-α-CyD and DMA-β-CyD suppressed the binding of FITC-labeled LPS to the surface of cells, probably resulting in inhibitory effects on iNOS expression and NO production. DM-α-CyD had a greater interaction with RAW264.7 cells than did DMA-β-CyD. The pretreatment of RAW264.7 cells with DM-α-CyD, not DMA-β-CyD, decreased the LPS binding to the cell surface. The results suggested that the inhibitory mechanism of the LPS binding to the cell surface is different between DM-α-CyD and DMA-β-CyD.
Conclusions. The present results suggest that DM-α-CyD and DMA-β-CyD attenuates NO production by inhibiting *iNOS* gene expression in RAW264.7 cells stimulated with LPS, probably due to the suppression of LPS binding to LPS receptors on the cells in the different way.

KEY WORDS: cyclodextrin; nitric oxide; lipopolysaccharide; macrophages; inducible nitric oxide synthase.

INTRODUCTION

Gram-negative bacterial lipopolysaccharides (LPS) are amphiphilic molecules composed of the following three parts: a negatively charged lipid A group with six or seven acyl chains; a core oligosaccharide covalently bound to the lipid A group; and the O-antigen polysaccharide chain (1,2). The lipid A moiety of the LPS molecule exerts endotoxic effects on cells (3), i.e., LPS activates monocytes and macrophages, which results in the induction and release of several inflammatory mediators, including nitric oxide (NO) and inflammatory cytokines. These mediators lead to a variety of pathophysiologic responses, including fever and septic shock in the host (4). The free-radical gas NO, generated by the NO synthase (NOS) family of enzymes, is a pleotropic signaling molecule that has been identified as a mediator of a wide range of physiologic and pathophysiologic events (5,6). Inducible NOS (iNOS) is expressed by transcriptional induction, and iNOS synthesizes high levels of NO at a constant rate for long periods. Sustained and excessive NO production from macrophages can be deleterious to the host, and NO and its metabolite peroxynitrite have been implicated in the development of DNA injury, atherosclerosis and the hypotension associated with septic shock (7). Therefore, it is crucial to regulate the excess NO production by iNOS when considering inflammatory reactions.

Cyclodextrins (CyDs) form inclusion complexes with hydrophobic drugs, and improve their solubility, dissolution rate and bioavailability (8,9). CyDs have been reported to interact with membrane constituents such as cholesterol, phospholipids, and phosphatidylinositols (10,11), resulting in not only the induction of hemolysis of erythrocytes (12–14) but also disruption of the structures of rafts and caveolae (15–18). Rafts are microdomains formed by lateral assemblies of cholesterol and sphingolipids in the cell membranes (19) and caveolae are specialized raft domains that consist of 50–80-nm flask-shaped membrane invaginations thought to function in endocytosis and signal transduction (20). Membrane-bound CD14, a 55-kDa glycosyl phosphatidyl-inositol (GPI)-anchored protein that has been shown to be an LPS receptor, appears to exist in caveolae and rafts (21,22). Thus, CyDs may interact with LPS and the lipid constituents in caveolae and rafts on the surface of macrophages, leading to a change in signal transduction from LPS receptors. Therefore, we hypothesized that CyDs may affect NO production in macrophages stimulated with LPS. However, there have been no previous reports concerning the effects of CyDs on macrophage activation including NO production in monocytes or macrophages by LPS. Therefore, we studied the cytotoxic effects of 17 kinds of CyDs toward RAW264.7 cells, a mouse macrophage-like cell line, and then we compared the effects of 15 kinds of CyDs on NO production in the cells stimulated with LPS. Among these CyDs, 2,6-di-*O*-methyl-α-CyD (DM-α-CyD) (8) and 2,6-di-*O*-methyl-3-*O*-acetyl-β-cyclodextrin (DMA-β-CyD) (23) significantly inhibited NO production in RAW264.7 cells stimulated with LPS. To gain insight into the mechanism of this effect, the effects of CyDs on the induction of *iNOS* mRNA and iNOS in RAW264.7 cells as well as on LPS binding to the cell surface were studied. Moreover, we studied the interaction between LPS and CyDs and the pretreatment effects of RAW264.7 cells with CyDs on LPS binding to the cell surface.

MATERIALS AND METHODS

Materials

α-CyD, β-CyD, γ-CyD, 2-hydroxypropyl-α-CyD (HP-α-CyD), 2-hydroxypropyl-β-CyD (HP-β-CyD), 2-hydroxypropyl-γ-CyD (HP-γ-CyD), DM-α-CyD, and 2,6,-di-*O*-methyl-β-

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CyD (DM-β-CyD) were kind gifts from Nihon Shokuhin Kako (Tokyo, Japan). Maltosyl-α-CyD (G_2-α-CyD) and maltosyl-β-CyD (G_2-β-CyD) were kind gifts from Bio Research Corporation of Yokohama (Yokohama, Japan). Sulfobutyl ether-β-CyD (SBE7-β-CyD) was a gift from CyDex (Overland Park, KS). 6^A-Amino-6^A-deoxy-α-CyD (MA-α-CyD), 6^A-amino-6^A-deoxy-β-CyD (MA-β-CyD) and 6^A-amino-6^A-deoxy-γ-CyD (MA-γ-CyD), 6^A-(ω-aminoethylamino)-6^A-deoxy-amino-β-CyD (ED-β-CyD), 6^A-(ω-aminobutylamino)-6^A- deoxy-amino-β-CyD (BD-β-CyD) and DMA-β-CyD were prepared in our laboratory and purified according to the methods described previously (24–26). The chemical structures, abbreviations and the average degree of substitution of CyDs examined in this study are summarized in Table I. LPS from *Escherichia coli* (serotype O111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 culture medium and fetal calf serum (FCS) were obtained from Nissui Pharmaceutical (Tokyo, Japan) and JRH Biosciences (Renexa, KS), respectively. Rabbit polyclonal anti-mouse iNOS antibody and peroxidase-conjugated anti-rabbit IgG Fc antibody were obtained from Affinity Bioreagent (Neshanic Station, NJ) and Cappel (Durham, NC), respectively.

Deoxyribonuclease (DNase) and ribonuclease inhibitor (RNase inhibitor) were purchased from Nippon Gene (Toyama, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Reverse transcriptase (SuperScript II) and Taq polymerase (AmpliTaq Gold) were purchased from GibcoBRL (Gaithersburg, MD) and Applied Biosystems (Tokyo, Japan), respectively. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) was purchased from Funakoshi (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade.

Cell Culture

RAW264.7 cells (1×10^5 or 3×10^6 cells/well) were incubated with LPS at the indicated concentration for 1 h or 4 h in RPMI-1640 culture medium supplemented with 10% FCS. After this time, RAW264.7 cells were further incubated for 24 h or 4 h in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS to elicit the production of NO, iNOS and *iNOS* mRNA.

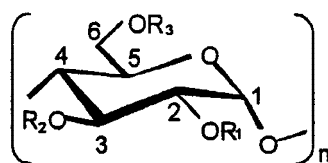
Cell Viability

Cell viability was assayed using a Cell Counting Kit (WST-1 method) from Wako Pure Chemical Industries (Osaka, Japan). RAW264.7 cells were seeded at 1×10^5 cells onto 96-well microplates (Iwaki, Tokyo, Japan) and incubated for 2 h in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were washed twice with phosphate buffered saline (PBS, pH 6.5) and then incubated for 1 h with 150 μL of RPMI-1640 culture medium supplemented with 10% FCS containing CyDs or Tween 20 at various concentrations in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After washing twice with PBS to remove CyDs, 100 μL of fresh HBSS (pH 7.4) and 10 μL of WST-1 reagent were added to the plates and incubated for 2 h at 37°C. The absorbance at 450 nm against a reference wavelength of 620 nm was measured with a miniplate reader (Nalge Nunc International NJ-2300, Rochester, NY).

Nitrite Determination

NO production was estimated by measurement of nitrite in the culture supernatant using Griess reagent as described

Table I. Chemical Structures of CyDs Used in this Study



Compound	Abbreviation	n	R_1	R_2	R_3	D.S.[a]
α-cyclodextrin	α-CyD	6				–
β-cyclodextrin	β-CyD	7				–
γ-cyclodextrin	γ-CyD	8				–
2-hydroxypropyl-α-cyclodextrin	HP-α-CyD	6		–H or $-CH_2CH(CH_3)OH$		4.1
2-hydroxypropyl-β-cyclodextrin	HP-β-CyD	7		–H or $-CH_2CH(CH_3)OH$		4.8
2-hydroxypropyl-γ-cyclodextrin	HP-γ-CyD	8		–H or $-CH_2CH(CH_3)OH$		4.2
2,6-di-*O*-methyl-α-cyclodextrin	DM-α-CyD	6	$-CH_3$	–H	$-CH_3$	12
2,6-di-*O*-methyl-β-cyclodextrin	DM-β-CyD	7	$-CH_3$	–H	$-CH_3$	14
maltosyl-α-cyclodextrin	G_2-α-CyD	6	–H	–H	–H or –maltose	1
maltosyl-β-cyclodextrin	G_2-β-CyD	7	–H	–H	–H or –maltose	1
sulfobutyl ether-β-cyclodextrin	SBE7-β-CyD	7		–H or $-(CH_2)_4SO_3Na$		6.2
6^A-amino-6^A-deoxy-amino-α-cyclodextrin	MA-α-CyD	6	–H	–H	–H or $-NH_2$	1
6^A-amino-6^A-deoxy-amino-β-cyclodextrin	MA-β-CyD	7	–H	–H	–H or $-NH_2$	1
6^A-amino-6^A-deoxy-amino-γ-cyclodextrin	MA-γ-CyD	8	–H	–H	–H or $-NH_2$	1
6^A(ω-aminoethylamino)-6^A-deoxy-amino-β-cyclodextrin	ED-β-CyD	7	–H	–H	–H or $-H(CH_2)_2NH_2$	1
6^A(ω-aminobutylamino)-6^A-deoxy-amino-β-cyclodextrin	BD-β-CyD	7	–H	–H	–H or $-NH(CH_2)_4NH_2$	1
2,6-di-*O*-methyl-3-acetyl-β-cyclodextrin	DMA-β-CyD	7	$-CH_3$	$-COCH_3$	$-CH_3$	7[b]

[a] Average degree of substitution.
[b] Acetyl group.

by Stuehr and Nathan (27). Briefly, RAW264.7 cells (1×10^5 cells/well) were treated with LPS at the indicated concentrations for 1 h in the absence or presence of CyDs, then the cells were incubated for 24 h in culture medium. Then, 90-μL aliquots of culture supernatants were mixed with 90 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-[1-naphthyl] ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 490 nm was measured with a miniplate reader described above. Nitrite levels were determined using $NaNO_2$ as a standard.

Detection of iNOS by Western Blotting Analysis

iNOS in the RAW264.7 cells was detected by Western blotting. Briefly, RAW264.7 cells (3×10^6 cells/dish) were stimulated with 10 μg/mL of LPS for 4 h in the absence or presence of CyDs, then washed with culture medium, and incubated for 4 h in culture medium. Then, the cells were scraped and lysed with buffer consisting of 3% sodium dodecylsulfate, 2 mM dithiothreitol, 73 μM pepstatin A, 0.1 mM leupeptin, and 1 mM phenylmethylsulfonylfluoride at 4°C for 1 h. After determining protein concentrations using the bicinchoninic acid reagent from Pierce Chemical (Rockford, IL) with bovine serum albumin (BSA) as a standard, samples (20 μg as protein) were separated by 7.5% SDS-PAGE and transferred onto Immobilon P membranes (Nihon Millipore, Tokyo, Japan). The membranes were blocked with 2% BSA in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and incubated with rabbit anti-mouse iNOS antibody for 2 h. After washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit antibody and washed with PBS-T. Specific bands were detected using an ECL Western blotting analysis kit (Amersham-Pharmacia Biotech, Tokyo, Japan).

Detection of iNOS mRNA by RT-PCR Analysis

To isolate total RNA from RAW264.7 cells, the cells (3×10^6 cells/dish) were scraped and lysed using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and the samples were treated with DNase I (1 unit) and RNase inhibitor (35 unit) for 30 min at 37. cDNA was synthesized by reverse transcription using mouse *iNOS* gene reverse primer (5′-CATGCAAGGAAGGGAACTCTTC-3′) or mouse β-*actin* gene reverse primer (5′-TTGGCATAGAGGTCTTTACGGA-3′) and reverse transcriptase (SuperScript II). Approximately, 2.5 μM mouse *iNOS* or β-*actin* gene reverse primer was annealed to 1 μg of total RNA and extended with reverse transcriptase (200 units) in a buffer containing 2 μL of PCR buffer, 5 mM $MgCl_2$, and 1 mM deoxynucleotide triphosphates for 50 min at 42°C.

PCR amplification was carried out in a Takara PCR Thermal Cycler (Tokyo, Japan). PCR was conducted in a total volume of 25 μL with 0.5 μL of cDNA, 0.75 μM primers, 0.5 mM deoxynucleotide triphosphates, and 2.5 units of Taq DNA polymerase. PCR for the mouse *iNOS* and β-*actin* genes was performed using the forward primers 5′-ACAGGGAAGTCTGAAGCACTAG-3′ and 5′-GCACCACACCTTCTACAATGAG-3′, respectively. PCR was performed for 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 120 s, and extension at 72°C for 180 s. The amplified products were analyzed on 1.5% agarose gels containing 0.1 μg/mL of ethidium bromide.

Cellular Binding of LPS

RAW264.7 cells (2×10^6 cells/well) were incubated with 1000 ng/mL of FITC-labeled LPS in the presence or absence of 10 mM CyDs at 4°C for 30 min. The cells were washed with HBSS and then fluorescence was quantified using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). In the pretreatment study with CyDs, RAW264.7 cells (2×10^6 cells/well) were incubated with 10 mM CyDs at 4°C for 30 min, then washed with HBSS to remove CyDs, and incubated with 1000 ng/mL of FITC-labeled LPS at 4°C for 30 min. The following procedure was performed as described above.

Interaction Between LPS and CyDs

Fluorescence spectra were recorded with a Hitachi F-4500 spectrofluorometer at 25°C. The concentrations of TNS and CyDs were 11.4 μM and 10 mM, respectively. The concentrations of LPS were in the range of 100 μg/mL to 500 μg/mL in 10 mM PBS. The excitation wavelength was 333 nm and the fluorescence spectra were measured in the range of 300–550 nm.

RESULTS AND DISCUSSION

First, we examined the effects of 17 kinds of CyDs (Table I) on viability of RAW264.7 cells using the WST-1 method. In the absence of LPS, almost all of CyDs showed no cytotoxicity up to 10 mM, although DM-β-CyD and ED-β-CyD decreased cell viability to about 40% of the control value at 10 mM (Fig. 1). In the presence of 1000 ng/mL of LPS, similar results were observed (data not shown). Therefore, in the following experiments to analyze the effects of CyDs on NO production, the 15 CyD derivatives other than DM-β-CyD and ED-β-CyD were used in the range of 0–10 mM. Figure 2A shows the effects of CyDs on NO production in RAW264.7 cells stimulated with LPS (1000 ng/mL). In the present study, RAW264.7 cells were pre-treated with CyDs in the presence of LPS for 1 h to prevent the effects of CyDs on the Griess reaction. In the absence of LPS as a control, CyDs themselves did not affect NO production (data not shown). In the presence of 10 mM CyDs, HP-α-CyD and G_2-α-CyD had no effect on NO production and other CyDs slightly decreased the nitrite levels. On the other hand, when 10 mM DM-α-CyD or DMA-β-CyD was added, NO production elicited by LPS was markedly reduced to about 40 or 50%, respectively, of the control value (LPS alone). As shown in Fig. 2B, DM-α-CyD and DMA-β-CyD significantly inhibited NO production in the range of 1-1000 ng/mL of LPS. In addition, the inhibitory effects of DM-α-CyD and DMA-β-CyD were dose-dependent to 1000 ng/mL of LPS (Fig. 2C). These results indicated that DM-α-CyD and DMA-β-CyD have greater inhibitory activity than do the other CyDs against NO production in RAW264.7 cells stimulated with LPS.

To gain insight into the mechanism of this effect, we examined the effects of CyDs on iNOS and *iNOS* mRNA expression by immunoblotting and RT-PCR analysis, respectively. In the study regarding the effects of CyDs on NO production as described above, the concentration up to 1000

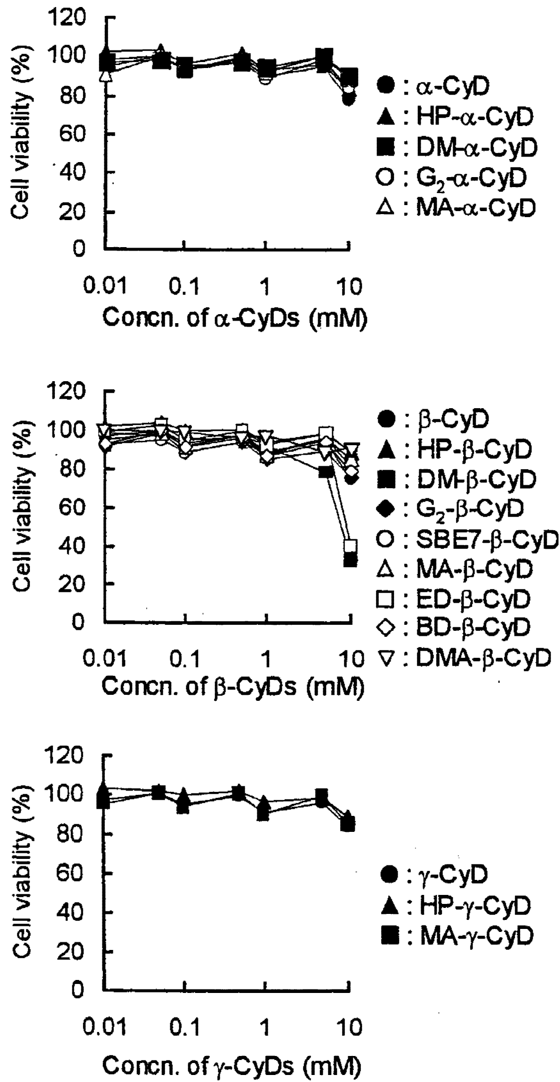


Fig. 1. Cell viability in the presence of CyDs. The cell viability was assayed using a cell counting kit. Each point represents the mean ± SEM of three experiments.

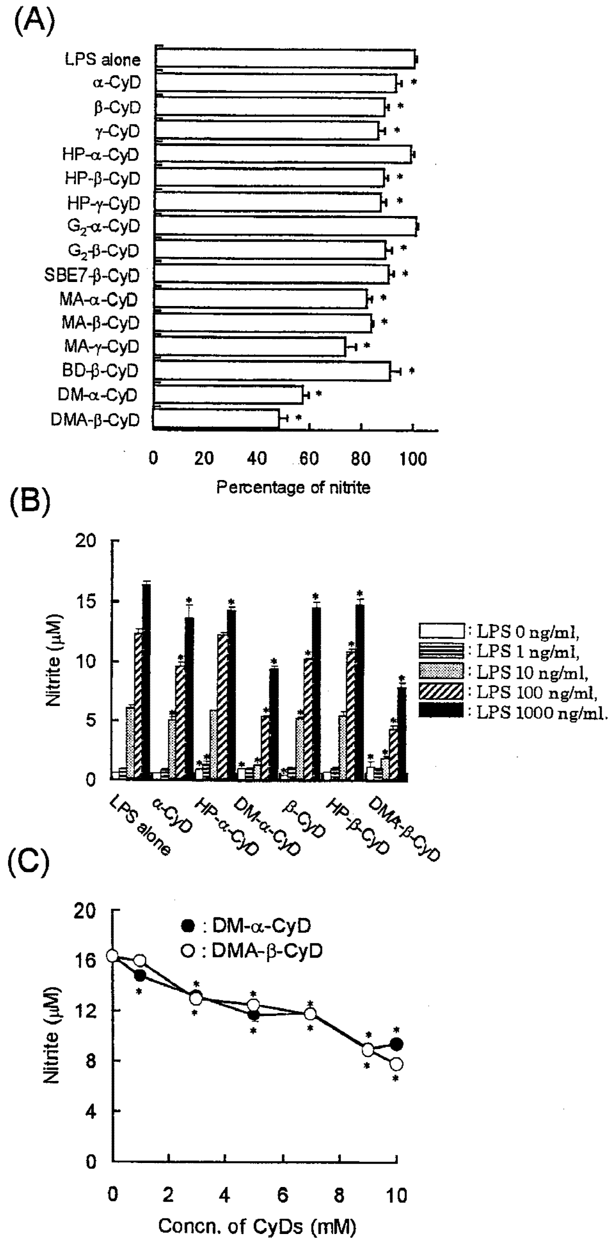


Fig. 2. (A) Effects of CyDs on NO production in RAW264.7 cells stimulated with LPS. The concentrations of LPS and CyDs were 1000 ng/mL and 10 mM, respectively. Each value represents the mean ± SEM of three experiments. $*P < 0.05$, compared to LPS alone. (B) Effects of CyDs on NO production in RAW264.7 cells stimulated with LPS at various concentrations. The concentration of CyDs was 10 mM. Each value represents the mean ± SEM of three experiments. $*P < 0.05$, compared to LPS alone. (C) Effects of CyDs concentration on NO production in RAW264.7 cells stimulated with LPS. The concentration of LPS was 1000 ng/mL. Each value represents the mean ± SEM of three experiments. $*P < 0.05$, compared to LPS alone.

ng/mL of LPS were used. However, we could not observe the obvious bands corresponding to iNOS and *iNOS* mRNA under the present experimental conditions of immunoblotting and RT-PCR analysis, respectively. Thus, we used 10 μg/mL of LPS in these studies. Figure 3A shows the results of immunoblotting analysis. An immunoreactive band at 130 kDa corresponding to the molecular weight of iNOS was observed in the extract of the cells stimulated with LPS. On the other hand, only a faint band was observed in extracts of cells treated with 5 mM DM-α-CyD or DMA-β-CyD. However, when other CyDs were added, the band densities almost same as that of control were observed. Figure 3B shows the *iNOS* mRNA levels determined by RT-PCR of total RNA in RAW264.7 cells. Under these experimental conditions, we checked that amplification of *iNOS* and β-*actin* cDNA did not reach saturation. DM-α-CyD and DMA-β-CyD caused a substantial reduction in the density of the 1033 bp band derived from *iNOS* mRNA but not in that of the 630 bp band of control β-*actin* mRNA. Under the experimental conditions of immunoblotting and RT-PCR studies, no cytotoxicity was observed. In addition, DM-α-CyD and DMA-β-CyD inhibited the NO production to the same extent (data not shown). These results indicated that DM-α-CyD and DMA-β-CyD specifically inhibited the increase in *iNOS* mRNA level elicited by stimulation with LPS, probably leading to a decrease in the iNOS level in RAW264.7 cells.

Next, we examined whether DM-α-CyD and DMA-β-CyD inhibited the cellular binding of LPS. Figure 4 shows the

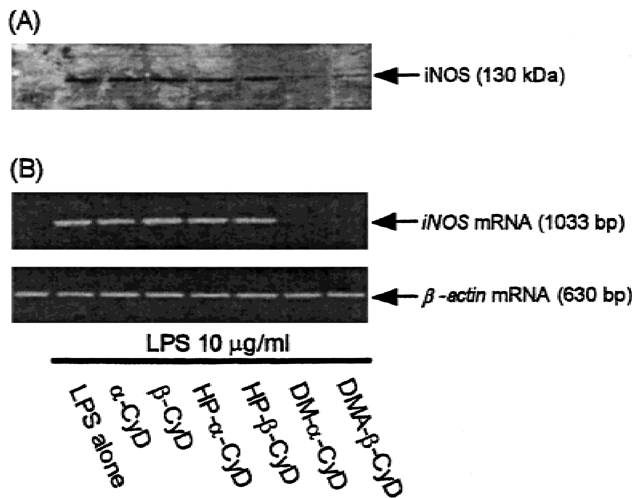


Fig. 3. Effects of CyDs on expression of iNOS (A) and *iNOS* mRNA (B) in RAW264.7 cells stimulated with LPS. The concentrations of LPS and CyDs were 10 μg/mL and 5 mM, respectively.

results of flow cytometric analysis of cellular binding of FITC-labeled LPS at 4°C. When FITC-labeled LPS was added to RAW264.7 cells in the presence of 10% FCS, the control curves were shifted to the right, indicating that LPS bound to the cell surface. On the other hand, when DM-α-CyD and DMA-β-CyD were added, the curve shifted to the left, but neither HP-α-CyD nor HP-β-CyD had any effect. We confirmed that there was no interaction of FITC with CyDs under the present experimental conditions (data not shown). These results suggested that DM-α-CyD and DMA-β-CyD suppress the binding of FITC-labeled LPS to the cells, probably resulting in inhibitory effects on iNOS expression and NO production. To clarify the reason why DM-α-CyD and DMA-β-CyD have significant inhibitory effects on LPS binding on the surface of RAW264.7 cells, we studied the interaction between LPS and CyDs by utilizing a competitive inclusion phenomenon using TNS (28,29), because we could not detect the interaction between LPS and CyDs directly in our preliminary study. As shown in Fig. 5, the fluorescence of TNS was markedly enhanced by the addition of DM-α-CyD or DMA-β-CyD, being consistent with the results of the complexation of TNS with parent CyDs (28,29). The stability constants of the TNS/DM-α-CyD and TNS/DMA-β-CyD complexes were determined by analyzing the CyD concentration dependence of the fluorescence changes, and were 420(±5) M^{-1} and 750(±5) M^{-1}, respectively. These results indicate that TNS interacts more strongly with DMA-β-CyD than with DM-α-CyD. The enhanced fluorescence intensity of TNS by the binding to DMA-β-CyD was decreased by the addition of LPS in the concentration dependent manner (Fig. 5B), in spite of the larger stability constant of the TNS/DMA-β-CyD complex. On the other hand, the enhanced fluorescence intensity of the TNS/DM-α-CyD system was hardly affected by the addition of LPS (Fig. 5A), in spite of the small stability constant of the TNS/DM-α-CyD complex. Similarly, the fluorescence intensity corresponding to TNS/HP-α-CyD complex or TNS/HP-β-CyD complex was not affected by the addition of LPS (data not shown). Unfortunately, the stability constant of LPS/CyD complexes could not be determined because the accurate molecular weight of LPA is unknown. However, the results on the competitive inclusion clearly indicate that the interaction of LPS with DMA-β-CyD is much stronger than that with DM-α-CyD. Therefore, it is likely that DMA-β-CyD inhibits the cellular binding of LPS through the complex

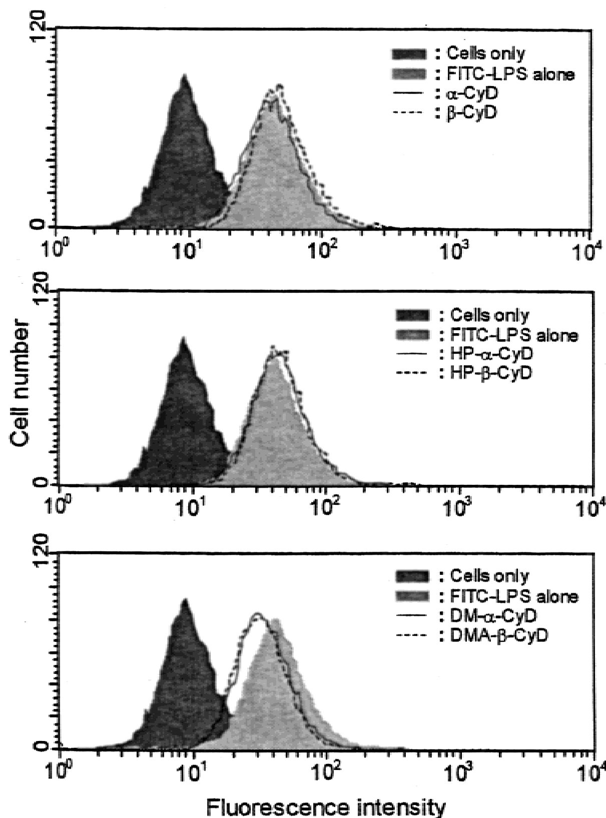


Fig. 4. Flow cytometric analysis of the binding of FITC-labeled LPS to the surface of RAW264.7 cells in the absence and presence of CyDs. The concentrations of FITC-labeled LPS and CyDs were 1000 ng/mL and 10 mM, respectively. The experiment was performed at 4°C.

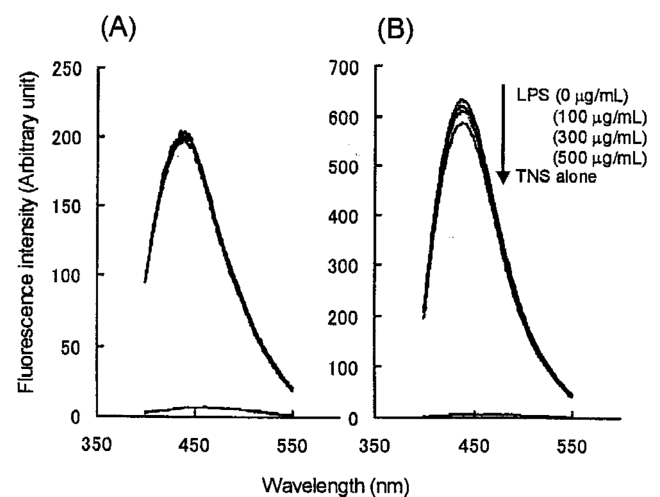


Fig. 5. Fluorescence spectra of TNS in the absence and presence of DM-α-CyD (A) or DMA-β-CyD (B) and LPS. The concentrations of TNF and CyDs were 11.4 μM and 10 mM, respectively, and those of LPS were in the range of 0 to 500 μg/mL in 10 mM phosphate buffer (pH 5.4). The experiments were performed at 25°C, and the excitation wavelength was 333 nm.

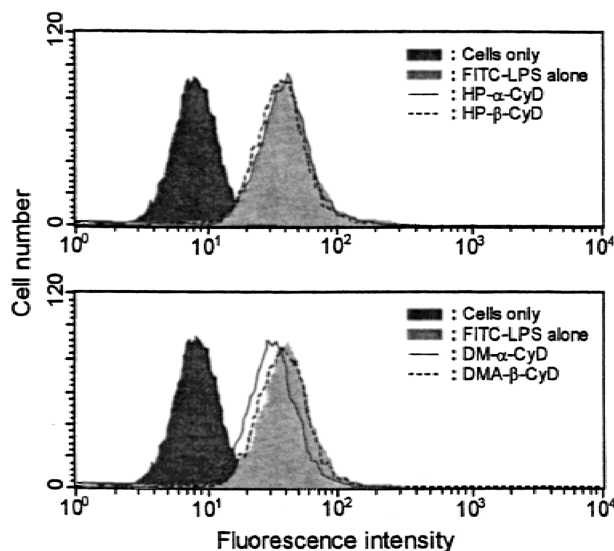


Fig. 6. Pretreatment effect of RAW264.7 cells with CyDs on the binding of FITC-labeled LPS to the cell surface. RAW264.7 cells (2×10^6 cells/well) were incubated with 10 mM CyDs at 4°C for 30 min. After washing with HBSS, the cells were incubated with 1000 ng/mL of FITC-labeled LPS at 4°C for 30 min. The concentrations of FITC-labeled LPS and CyDs were 1000 ng/ml and 10 mM, respectively. The experiment was performed at 4°C.

formation with LPS, because various CyD complexes have been reported to bind to the cellular surface only very slightly (9,13).

It remains a question how DM-α-CyD inhibits the cellular binding of LPS. To address the question, we studied the pretreatment effects of RAW264.7 cells with CyDs on the cellular binding of LPS at 4°C (Fig. 6). Interestingly, DM-α-CyD shifted the curve corresponding to the LPS binding to the cell surface to the left, but DMA-β-CyD, HP-α-CyD, and HP-β-CyD had no effect. These results suggested that the inhibitory mechanism of the LPS binding to the cell surface is different between DM-α-CyD and DMA-β-CyD. CyDs are known to induce hemolysis to erythrocytes in high concentrations. The hemolytic activity of CyDs correlates with the inclusion ability of CyDs with lipids such as phospholipids and cholesterol (13). We have reported that the hemolytic activity of methylated CyDs is relatively higher, whereas that of DMA-β-CyD extremely low (13,23). These results suggest that DM-α-CyD has a greater interaction with RAW264.7 cells than does DMA-β-CyD, which may support the difference in the inhibitory mechanism between DM-α-CyD and DMA-β-CyD on LPS binding to RAW264.7 cells, i.e., the interaction with the cell surface for the former CyD and the interaction with LPS for the latter CyD. Further studies on the mechanisms of these effects are currently underway in our laboratory by investigating interactions between CyDs and CD14 and Toll-like receptor 4/MD-2 complex, a receptor for LPS signaling (30).

In conclusion, the present results suggest that DM-α-CyD and DMA-β-CyD attenuated NO production by inhibiting *iNOS* gene expression in RAW264.7 cells stimulated with LPS, probably due to the suppression of LPS binding to LPS receptors on the cells in the different way. We are planning the study whether DM-α-CyD and DMA-β-CyD can also suppress the development of septic shock *in vivo*.

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