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Inhibitory effects of dimethylacetyl-β-cyclodextrin on lipopolysaccharide-induced macrophage activation and endotoxin shock in mice

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Abstract

The potential use of hydrophilic cyclodextrins (CyDs) as an inhibitor for lipopolysaccharide (LPS) was examined. Of the five CyDs used in this study, dimethylacetyl-β-cyclodextrin (DMA7-β-CyD) had greater inhibitory activity than other CyDs against the production of nitric oxide (NO) and various proinflammatory cytokines including tumor necrosis factor-α (TNF-α) in murine macrophages stimulated with two serotypes of LPS and lipid A. The inhibitory effect of DMA7-β-CyD on NO production was also observed in macrophages stimulated with lipoteichoic acid (LTA), but not peptidoglycan (PGN), polyinosinic–polycytidylic acid (poly I:C) or CpG oligonucleotide (CpG-ODN). Several studies have suggested that the inhibitory effects of DMA7-β-CyD could be ascribed to the interaction with LPS. Simultaneous administration of DMA7-β-CyD not only intraperitoneally but also intravenously and intraperitoneal injection of aqueous solution containing LPS and D-galactosamine in murine endotoxin shock model suppressed fatality. Also, DMA7-β-CyD decreased blood level of TNF-α as well as serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) in mice. In conclusion, DMA7-β-CyD may have promise as a new therapeutic agent for endotoxin shock induced by LPS.

Keywords: Cyclodextrin; Dimethylacetyl-β-cyclodextrin; Lipopolysaccharide; Sepsis; Macrophages; Antagonist

1. Introduction

Sepsis is a serious, life-threatening disorder that occurs when an overwhelming infection and immune response lead to low blood pressure and blood flow, resulting in tissue damage, multiple organ failure and death [1]. Sepsis has a high death rate of 30–70%, depending on the type of organism causing the infection and the degree of organ failure [2]. Several strategies for treating sepsis induced by LPS have been proposed, e.g. anti-endotoxin antibodies, an endotoxin antagonists and the hemofiltration [3–5]. However, a number of clinical trials using an antibody-induced blockade of LPS during sepsis did not show enough substantial benefits [6]. Additionally, monoclonal antibodies to the lipid A of murine LPS (E5) or humanized (HA-1A) are, unfortunately, reported to be unable to block LPS-induced cytokine production in human monocytes in vitro [7–9]. Thus, a new paradigm is needed for the future regimen.

Cyclodextrins (CyDs) and their hydrophilic derivatives form inclusion complexes with hydrophobic molecules. CyDs can improve solubility, dissolution rate and bioavailability of drugs, and so the widespread use of CyDs is well known in the pharmaceutical field [10,11]. Some CyDs including methyl-β-CyD (M-β-CyD) and dimethyl-β-CyD (DM-β-CyD) have been reported to interact strongly with membrane constituents, such as cholesterol and phospholipids, resulting in the induction of hemolysis of erythrocytes [12–14], disruption of the structures of lipid rafts [15–17] and inhibition of P-glycoprotein function [18]. Recently, we reported that 2,6-di-*O*-methyl-3-*O*-acetyl-β-

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cyclodextrin with a degree of the substitution (DS) of the acetyl group of 7 (DMA7-β-CyD) inhibited NO production in RAW264.7 cells, a mouse macrophage-like cell line, stimulated with LPS from *Escherichia coli* (serotype O111:B4) [19]. In the subsequent study, we demonstrated that DM-α-CyD decreased NO and tumor necrosis factor-α (TNF-α) production in murine macrophages stimulated with LPS via release of CD14 from the cell surface [20]. However, the mechanism by which DMA7-β-CyD suppresses NO production in murine macrophages stimulated with LPS still remains unknown. In the view of the in vitro data, one intriguing question is whether DMA7-β-CyD protects mice from endotoxin shock. In the present study, we extended our previous studies and examined whether β-CyDs inhibit the production of NO and proinflammatory cytokines in various murine macrophages stimulated with LPS from *E. coli* (serotype O111:B4 and O55:B5), lipid A and the other toll-like receptor (TLR) ligands as well as whether DMA7-β-CyD interacts with LPS in solution and with membrane constituents such as cholesterol, lipid rafts and LPS receptors on macrophage surface. In addition, we investigated whether intraperitoneal and intravenous administrations of β-CyDs suppresses fatality, hepatic inflammation and plasma levels of TNF-α as well as serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) in endotoxin shock mice inducted by intraperitoneal injection of aqueous solution containing LPS and D-galactosamine.

2. Materials and methods

2.1. Materials

CyDs used in this study are depicted in Table 1. β-CyD and 2-hydroxypropyl-β-CyD (HP-β-CyD) were gifts from Nihon Shokuhin Kako (Tokyo, Japan). DMA7-β-CyD was prepared in our laboratory and purified according to the methods described previously [21]. The purity of synthetic DMA7-β-CyD used in this study was more than 98%, which was determined by the ^{1}H NMR and FAB mass spectra. LPS from *E. coli* (serotype O111:B4 and O55:B5), fluorescein isothiocyanate (FITC)-conjugated LPS (O111:B4), lipid A (monophosphoryl-lipid A from *E. coli* F583), D-galactosamine, lipoteichoic acid (LTA), peptidoglycan (PGN), polyinosinic–polycytidylic acid (poly I:C) and M-β-CyD were purchased from Sigma (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. CpG phosphorothioate oligonucleotide (CpG-ODN, 5′-TCCATGACGTTCCTGACGTT-3′) was obtained from Hokkaido System Science (Sapporo, Japan). Deoxyribonuclease I (DNase I) 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 Invitrogen (Carlsbad, CA) and Applied Biosystems (Tokyo, Japan), respectively. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) was purchased from Funakoshi (Tokyo, Japan). Thioglycollate was obtained from Difco Laboratories (Detroit, MI). All other chemicals and solvents were of analytical reagent grade.

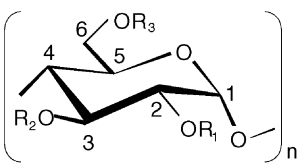
2.2. Cell viability and nitrite determination

Cell viability was assayed using a Cell Counting Kit (WST-1 method) [22,23] from Wako Pure Chemical Industries (Osaka, Japan) and the nitrite level in culture medium was determined using Griess reagent [24] as reported previously [19].

2.3. Western blot and enzyme-linked immunosorbent assay (ELISA)

Inducible NO synthase (iNOS) in the RAW264.7 cells and TNF-α in cell-free supernatant were detected by Western blot and ELISA, respectively, as reported previously [20]. Low-density lipid rafts-enriched domains

Table 1
Chemical structures of CyDs used in this study



Compound	Abbreviation	*n*	R_1	R_2	R_3	D.S.[a]
β-Cyclodextrin	β-CyD	7	-H	-H	-H	–
2-Hydroxypropyl-β-cyclodextrin	HP-β-CyD	7		-H or -$CH_2CH(CH_3)OH$		4.8
Methyl-β-cyclodextrin	M-β-CyD	7		-H or -CH_3		12.6
2,6-Di-*O*-methyl-3-*O*-acetyl-β-cyclodextrin	DMA7-β-CyD	7	-CH_3	-$COCH_3$	-CH_3	7[b]
2,6-Di-*O*-methyl-α-cyclodextrin	DM-α-CyD	6		-H or -CH_3		12.0

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

were isolated by a carbonate-based fractionation method without detergents [25]. CD14 and TLR4-MD-2 complex in low-density lipid rafts were detected by Western blot. Rat anti-mouse CD14 monoclonal Ab (Pharmingen, San Diego, CA) and HRP-goat anti-rat IgG Ab (Amersham Bioscience, Tokyo, Japan) were used for this assay.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The mRNA levels of *iNOS* and various cytokines in RAW264.7 cells were assayed by semiquantitative RT-PCR method as reported previously [20]. Total RNA was isolated from RAW264.7 cells (3×10^6 cells/dish) following manufacturer's instructions. cDNA was synthesized using a reverse primer and SuperScript II. The sequences of the forward and reverse primer pairs used were the following: 5′-TTGGCATAGAGGTCTTTACGGA-3′ and 5′-GCACCACACCTTCTACAATGAG-3′ for mouse β-*actin*; 5′-CTCCTGGTATGAGATAGCAAA-3′ and 5′-CAAAGGGATGAGAAGTTCCCAA-3′ for mouse *TNF-α*; 5′-ACAGGGAAGTCTGAAGCACTAG-3′ and 5′-CATGCAAGGAAGGGAACTCTTC-3′ for *iNOS*; 5′-CTCTGAATCAGAAATCCTTC-3′ and 5′-TAGGCATA-CATGTCAAATTT-3′ for mouse *IL-1α*; 5′-TTCCTCT-CTGCAAGAGACT-3′ and 5′-TGTATCTCTCTGAAG-GACT-3′ for mouse *interleukin-6* (*IL-6*); 5′-GGACAACA-TACTGCTAACCGACTC-3′ and 5′-AAAATCACTCTT-CACCTGCTCCAC-3′ for mouse *interleukin-10* (*IL-10*).

2.5. NF-κB activation

NF-κB activation was assayed as described previously [20]. In brief, RAW264.7 cells (2×10^6 cells/dish) were stimulated with LPS (1000 ng/ml, O111:B4) with or without β-CyDs for 15 min. After LPS treatment, the cells were fixed in methanol, incubated with mouse anti-NF-κB p65 mAb for 1 h. Primary antibody was detected with FITC-conjugated goat anti-mouse IgG2a. Samples were viewed and photographed using a confocal fluorescence microscopic system (Olympus FV300-BX, Tokyo) with an argon ion laser (excitation wavelength, 488 nm).

2.6. Flow cytometry

RAW264.7 cells (2×10^6 cells/dish) were incubated with or without 10 mM β-CyDs at 4 °C for 30 min. The cells were washed with HBSS and incubated with FITC-conjugated anti-CD14 or anti-TLR4-MD-2 complex antibody (MTS510) at 4 °C for 30 min. In the latter case, the cells were washed with PBS twice and incubated with FITC-conjugated anti-IgG2a at 4 °C for 30 min. The stained cells were resuspended in HBSS and were quantified using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA).

2.7. Determination of cholesterol and proteins released in culture supernatants

The releases of cholesterol and proteins were evaluated as reported previously [20]. Briefly, RAW264.7 cells (3×10^6 cells/dish) were incubated for 10 h in growth medium supplemented with 10% FCS containing [^{3}H]cholesterol (5 μCi/ml of serum). Prior to experiments, the cells were incubated in HBSS containing various concentrations of β-CyDs. The total amount of [^{3}H]cholesterol in the cells was detected by the same method after being lysed in 1N NaOH. The concentrations of [^{3}H]cholesterol in HBSS were determined with an Aloka LSC-3500 liquid scintillation counter. The concentrations of proteins released from the cells in the buffer were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

2.8. Interaction between LPS and CyDs

Fluorescence spectra were recorded with a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) at 25 °C. The concentrations of TNS and β-CyDs were 11.4 μM and 10 mM in 10 mM PBS (pH 5.4), respectively. The concentrations of LPS (O111:B4) were 100–500 μg/ml in the PBS. The excitation and emission wavelengths were 333 and 436 nm, respectively. Ultraviolet spectra were recorded with a Hitachi U-2000A spectrophotometer (Tokyo, Japan) at 25 °C. The concentrations of LPS and β-CyDs in the PBS were 100 μg/ml and 0–10 mM β-CyDs, respectively.

2.9. In vivo study

Male C57BL/6 mice (4 weeks old, Nihon SLC, Shizuoka, Japan) were used. Murine endotoxic shock was induced by intraperitoneal injection of 500 μl of aqueous solution containing LPS (100 ng/mouse) and D-galactosamine (25 mg/mouse). The effects of β-CyDs on the survival rate of endotoxin shock mice were estimated by the intraperitoneal administration of 500 μl of the mixed aqueous solution containing LPS (O111:B4), D-galactosamine and β-CyDs (0–100 mM) or by the intravenous administration of 250 μl of the aqueous solution containing β-CyDs (0–200 mM) after intraperitoneal administration of 250 μl of LPS and D-galactosamine solution at the same dose described above. The survival rate was monitored over the next 60 h. The plasma TNF-α levels were assayed by ELISA described above. The serum levels of AST and ALT in the mice were measured using diagnostic kits as reported previously [26]. The liver was excised and fixed in 4% paraformaldehyde, embedded in paraffin, and tissue sections were stained with hematoxylin-eosin. Apoptotic cells of hepatocytes were determined by terminal deoxytransferase-mediated dUTP nick-end labeling method according to the manufacturer's protocols. Animal use and relevant experimental procedures were approved

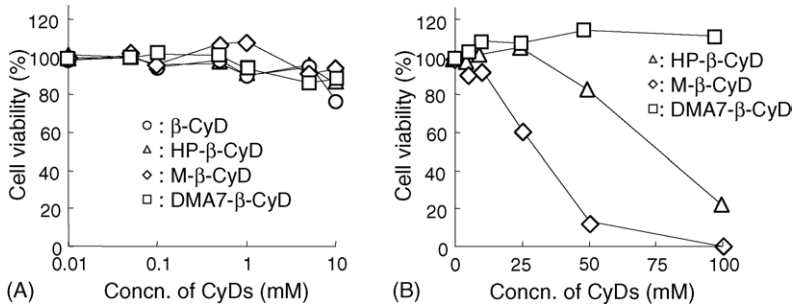


Fig. 1. Cytotoxicty of β-CyDs in RAW264.7 cells. RAW264.7 cells (1×10^5) were incubated for 1 h with 150 μl of RPMI-1640 culture medium supplemented with 10% FCS containing CyDs at the designated concentrations. The cell viability was assayed using a Cell Counting Kit. The concentrations of β-CyDs were up to 10 mM (A) and 100 mM (B), respectively. Each point represents the mean ± S.E.M. of three experiments.

by the Kumamoto University Committee on Animal Care and Use of Laboratory Animals.

2.10. Statistical analysis

Data are given as the mean ± S.E.M. Statistical significance of means for the studies was determined by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

3. Results

3.1. DMA7-β-CyD was nontoxic in murine macrophages up to 100 mM tested

We examined the effects of β-CyDs on the viability of murine macrophages by the WST-1 method. In the absence of LPS, almost all of β-CyDs showed no cytotoxicity up to 10 mM (Fig. 1A). In the range of high concentrations, the viability was decreased by adding β-CyDs and LD_{50} values lowered in the order of DMA7-β-CyD > HP-β-CyD > M-β-CyD (Fig. 1B). These results indicate that DMA7-β-CyD has cytotoxicity lower than the other β-CyDs. Similar cytotoxic effects of β-CyDs were observed on the other murine macrophages such as PU5-18, J774.1 and peritoneal exudate cells (PEC, data not shown).

3.2. DMA7-β-CyD suppressed NO, iNOS and cytokine expression in macrophages stimulated with LPS

To reveal whether DMA7-β-CyD inhibits NO production in RAW264.7 cells stimulated with two LPS (serotype O111:B4 and O55:B5) and lipid A, we assayed nitrite levels in the culture medium by the Griess method [19]. DMA7-β-CyD had a superior inhibitory effect on NO production in RAW264.7 cells stimulated with two LPS and lipid A to β-CyD and HP-β-CyD (Fig. 2A). Almost the same inhibitory effects of DMA7-β-CyD were elicited in PU5-18, J774.1 and PEC (data not shown). These inhibitory effects of DMA7-β-CyD were concentration-dependent at various LPS concentrations (Fig. 2B) and were observed in PEC as well (data not shown). Furthermore, DMA7-β-CyD lowered NO production stimulated with LTA and LPS, but not PGN, poly I:C or CpG-ODN (Fig. 2C). Here, we actually confirmed no contamination of LPS in the commercial preparation of LTA used in this study. These results indicate that the inhibitory effects of DMA7-β-CyD depend on the nature of TLR ligand. In subsequent experiments, we used LPS (serotype O111:B4) as a stimulant because it is possible that the relative lipid A content of *E. coli* O111:B4 LPS is proportionally higher than *E. coli* O55:B5 LPS [27].

To test whether DMA7-β-CyD suppresses *iNOS* and various cytokines expression in RAW264.7 cells, their mRNA levels in cells 4 h following stimulation of LPS were detected by RT-PCR. The induction of *iNOS*, *TNF*-α, *IL-1*α, *IL-6* and *IL-10* mRNA was inhibited by the addition of DMA7-β-CyD, but not β-CyD or HP-β-CyD (Fig. 3A). In addition, these inhibitory effects of DMA7-β-CyD on *iNOS* and various cytokine mRNA expression were concentration-dependent (Fig. 3B). However, neither DMA7-β-CyD nor the other β-CyDs used here altered the control β-*actin* mRNA levels up to the concentration of 10 mM (Fig. 3A and B).

To examine whether DMA7-β-CyD suppresses the protein expression of iNOS and TNF-α after stimulation of RAW264.7 cells with LPS, iNOS in the cells and TNF-α in cell-free supernatant 4 h after stimulation with LPS were determined by Western blot and ELISA, respectively. The induction of iNOS expression upon LPS stimulation was inhibited by DMA7-β-CyD, but not β-CyD or HP-β-CyD (Fig. 3C, upper). The inhibitory effects of DMA7-β-CyD on iNOS expression were concentration-dependent (Fig. 3C, lower). Likewise, DMA7-β-CyD significantly inhibited TNF-α production in RAW264.7 cells, but the other β-CyDs did not (Fig. 3D). Taken together, it is evident that DMA7-β-CyD suppresses LPS-activated NO and proinflammatory cytokines expression at the pre-transcription level.

3.3. DMA7-β-CyD suppressed LPS-induced NF-κB localization into nucleus

LPS signaling via myeloid differentiation factor 88 (MyD88) requires NF-κB activation and involves the

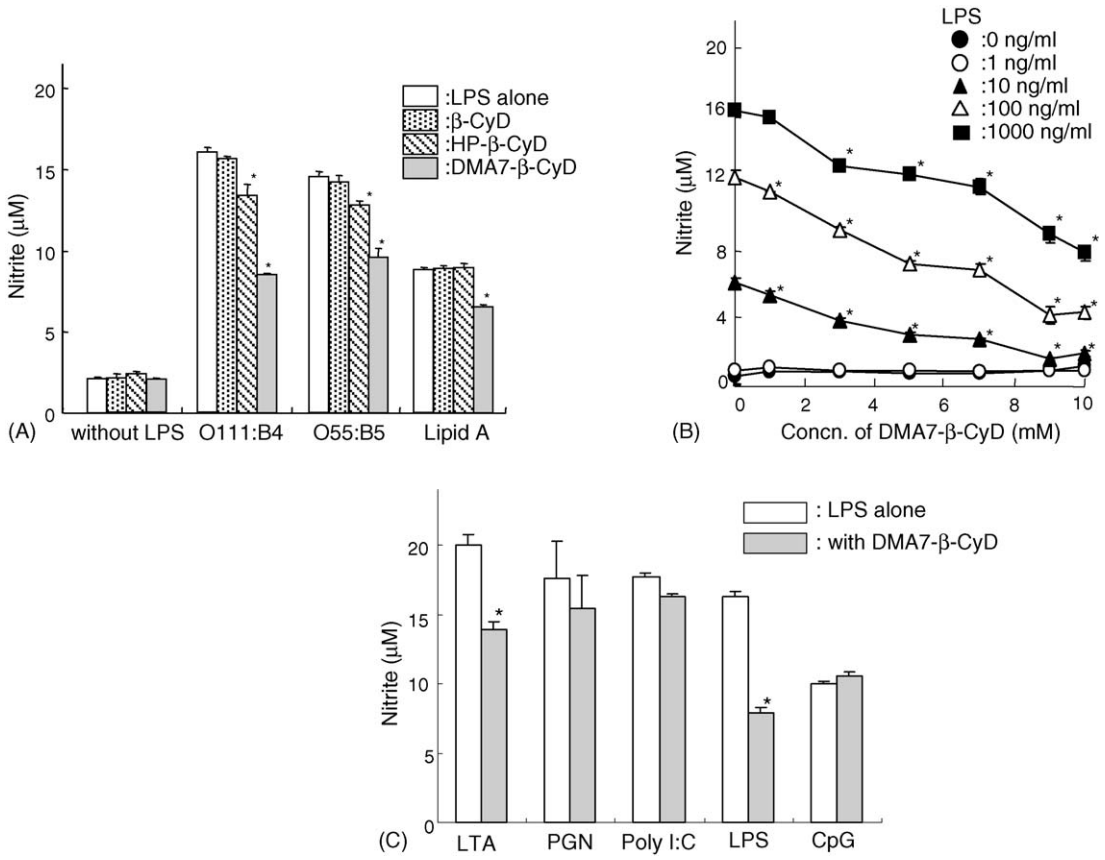


Fig. 2. DMA7-β-CyD inhibits NO production in RAW264.7 cells stimulated with LPS. Macrophages (1×10^5) were incubated for 1 h with 150 μl of RPMI-1640 culture medium supplemented with 10% FCS containing LPS or lipid A with or without β-CyDs. After washes with PBS to remove their ligands and β-CyDs, the cells were incubated for 24 h. The nitrite level in the culture supernatant was assayed by the Griess method. (A) The concentrations of LPS (O111:B4), LPS (O55:B5) and lipid A were 1000 ng/ml and those of β-CyDs were 10 mM. (B) DMA7-β-CyD inhibits NO production in a concentration-dependent manner. The concentrations of LPS (O111:B4) and DMA7-β-CyD were in the range of 1–1000 ng/ml and 1–10 mM, respectively. Each value represents the mean ± S.E.M. of three experiments. $^{*}p < 0.05$, compared to LPS alone. (C) DMA7-β-CyD inhibits NO production in a ligand-specific manner. The concentrations of LTA, PGN, poly I:C, LPS, CpG-ODN and DMA7-β-CyD were 3 μg/ml, 30 μg/ml, 50 μg/ml, 1000 ng/ml, 10 μg/ml and 10 mM, respectively. The open and closed columns represent without and with DMA7-β-CyD, respectively. Each value represents the mean ± S.E.M. of three to five experiments. $^{*}p < 0.05$, compared to TLR ligand alone.

production of NO and proinflammatory cytokines. Then, we investigated whether DMA7-β-CyD prevents NF-κB activation after stimulation with LPS in RAW264.7 cells. Activation of NF-κB after LPS stimulation was estimated by immunofluorescence staining in the absence and presence of β-CyDs. LPS treatment elicited NF-κB activation as is obvious from NF-κB nuclear translocation (Fig. 4A and B). The same activation was observed when treated with HP-β-CyD (Fig. 4C). Meanwhile, DMA7-β-CyD inhibited NF-κB activation upon LPS stimulation (Fig. 4D). Collectively, the inhibitory effects of DMA7-β-CyD on NO and TNF-α production induced by LPS may be due to suppression of NF-κB activation.

3.4. DMA7-β-CyD interacted with LPS

We previously reported that DMA7-β-CyD inhibits the cellular binding of FITC-labeled LPS in RAW264.7 cells in a concentration-dependent manner [19]. Then, we formulated a working hypothesis that the inhibitory effects of DMA7-β-CyD on NO and proinflammatory cytokine production result from the interaction of DMA7-β-CyD with LPS and/or LPS receptors on cell surface. First, we studied the interaction between LPS and DMA7-β-CyD by utilizing a competitive inclusion phenomenon using TNS (Fig. 5A). Here a 100% indicates the fluorescence intensity of the solution containing CyD and TNS without LPS. Adding LPS to the solution, the relative fluorescent intensity lowered in the DMA7-β-CyD-containing solution in a concentration-dependent manner, but not in other CyD-containing solutions. These results suggest that the interaction of LPS with DMA7-β-CyD is much stronger than that with other CyDs.

Next, UV spectra of LPS with or without β-CyDs were measured at 210 nm. Here, we used a spectral subtraction technique so that changes in UV absorbance of LPS itself in the absence and presence of β-CyDs at each concentration can be measured. The intensity of UV absorbance of LPS at approximately 210 nm was decreased by adding DMA7-β-CyD, but not the other β-CyDs (Fig. 5B). The lowering

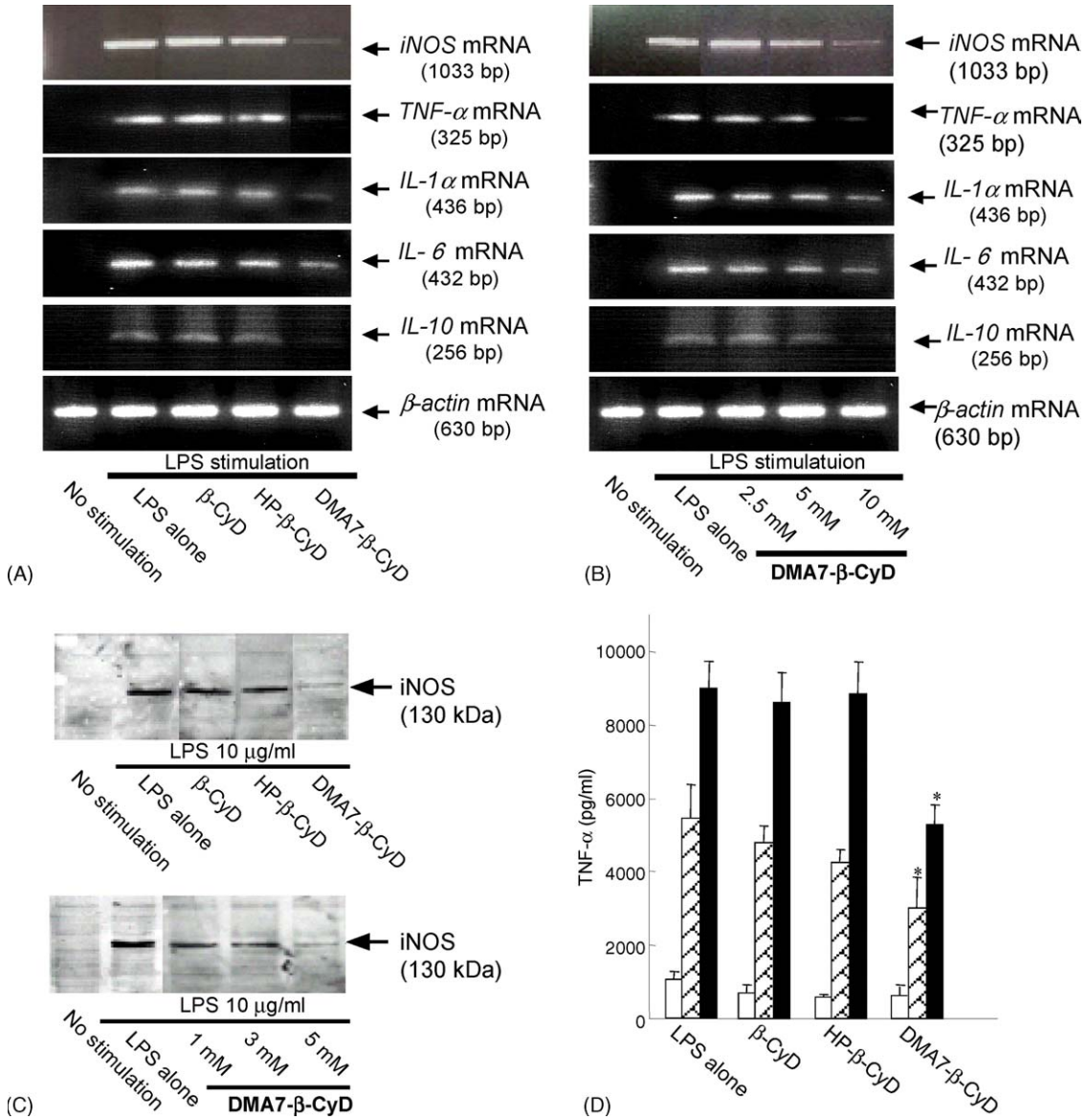


Fig. 3. DMA7-β-CyD lowers the *iNOS* and cytokine mRNA levels in RAW264.7 cells stimulated with LPS. RAW264.7 cells (3×10^6) were stimulated with 10 μg/ml of LPS for 4 h with or without β-CyDs, and then washed with culture medium, and further incubated for 4 h. (A and B) *iNOS* and cytokines mRNA in the cells were determined by RT-PCR. The concentrations of LPS for iNOS, TNF-α and the other cytokines assay were 10 μg/ml, 10 ng/ml and 1 μg/ml, respectively. The concentrations of β-CyDs and DMA7-β-CyD were (A) 10 mM and (B) 2.5–10 mM, respectively. (C) iNOS in the cells and (D) TNF-α in the cell supernatant were assayed by Western blot and ELISA, respectively. (C, upper and D) The concentrations of LPS and β-CyDs were 10 μg/ml and 10 mM, respectively. (C, lower) The concentrations of DMA7-β-CyD were 1–5 mM. (A–C) These figures show representative data for three experiments. (D) Each value represents the mean ± S.E.M. of three experiments. $^{*}p < 0.05$, compared to LPS alone.

effect of DMA7-β-CyD was in a concentration-dependent manner (Fig. 5C). These results suggest much greater interaction of LPS with DMA7-β-CyD in solution compared with the other β-CyDs used here.

3.5. DMA7-β-CyD did not cause a release of membrane constituents from cell membranes or alter membrane location or expression of LPS receptors

It is acknowledged that M-β-CyD extracts cholesterol from cell surface, resulting in disruption of lipid rafts in various cells [17]. We previously reported that DM-α-CyD attenuates NO production in RAW264.7 cells stimulated with LPS through the release of CD14 from lipid rafts [20]. To reveal whether DMA7-β-CyD allows the release of cholesterol and proteins from RAW264.7 cell surface to cell-free supernatant, we determined [^{3}H]cholesterol and total proteins released from cell membranes in cell-free supernatant by a liquid scintillation counter and protein assay kit, respectively. As shown in Fig. 6A and B, the treatment of the cells with DMA7-β-CyD released neither [^{3}H]cholesterol nor proteins from the RAW264.7 cells,

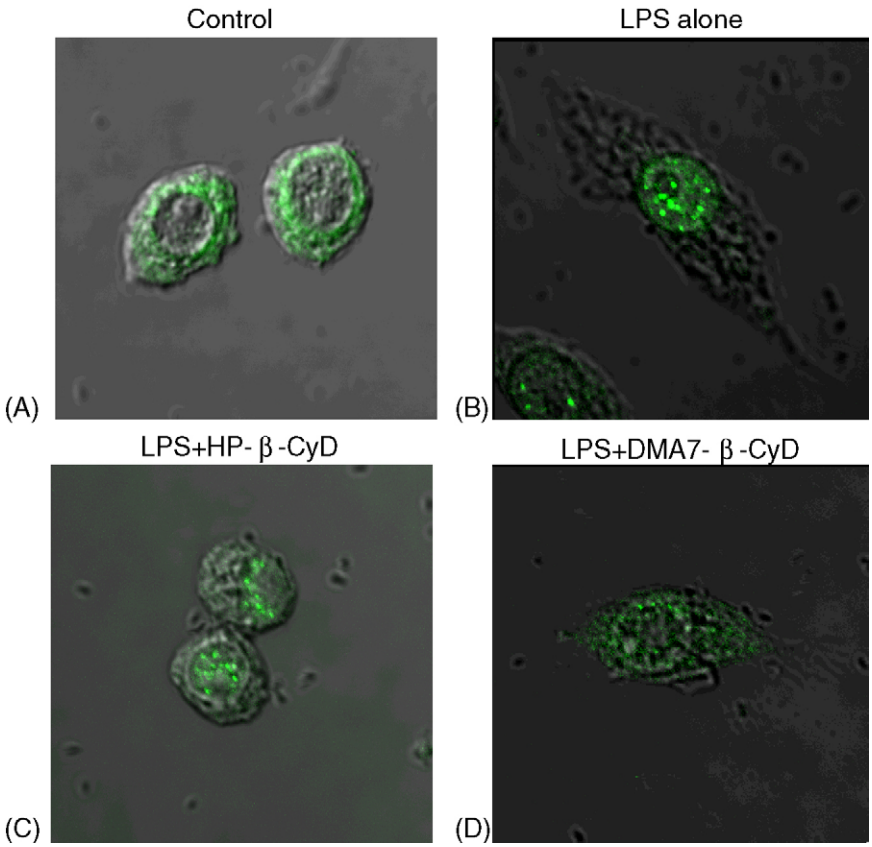


Fig. 4. DMA7-β-CyD inhibits the translocation of NF-κB to nucleus in RAW264.7 cells. RAW264.7 cells (2×10^6 cells) were stimulated with LPS (1000 ng/ml) for 15 min before fixation by methanol for 5 min. The mouse anti-NF-κB p65 was treated overnight at 4 °C. The goat anti-mouse IgG-FITC was added to the cells and incubated for 1 h at 37 °C. Slides were analyzed under a confocal fluorescence microscopic system. These figures show representative data for three experiments.

although M-β-CyD and DM-α-CyD released proteins and/or [^{3}H]cholesterol (Fig. 6A and B). Next, we examined the effects of β-CyDs on CD14 and TLR4-MD-2 complex expression on cell surface using a flow cytometer. DMA7-β-CyD did not shift the curve corresponding to CD14 or TLR4-MD2 complex to the left-hand side. However, a positive control DM-α-CyD shifted the curve corresponding to CD14 (Fig. 6C), but not TLR4-MD-2 complex (Fig. 6D). Meanwhile, negative control β-CyD and HP-β-CyD shifted both curves to a much lesser extent (data not shown).

It is well known that membrane localization of various proteins alters between lipid rafts and non-raft domain in response to raft inhibitors [28]. Then, we examined

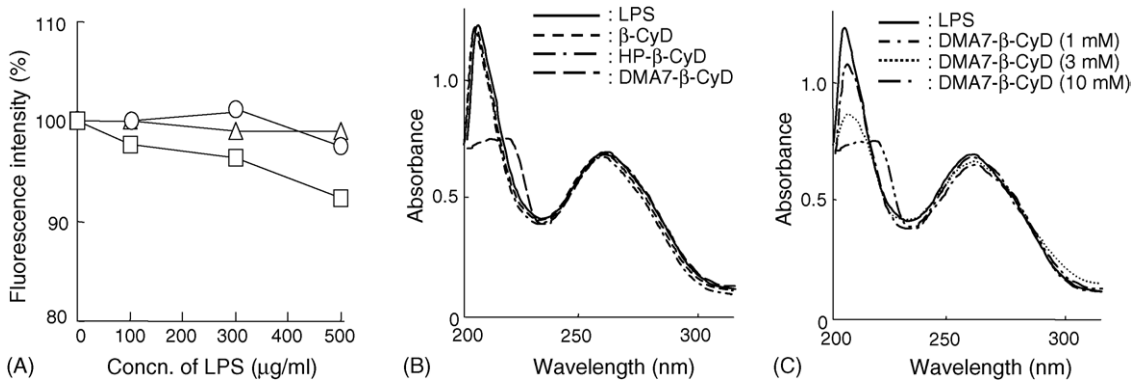


Fig. 5. DMA7-β-CyD interacts with LPS. (A) The fluorescence intensity of TNS in the absence and presence of CyDs and LPS was measured. The concentrations of TNS and CyDs were 11.4 μM and 10 mM, respectively, and those of LPS were in the range of 0–500 μg/ml in 10 mM phosphate buffer (pH 5.4). The experiments were performed at 25 °C and the excitation wavelength was 333 nm. Open triangle, β-CyD; open circle, HP-β-CyD; open square, DMA7-β-CyD. (B and C) The UV spectra of LPS in the absence and presence of β-CyDs. (B) The concentrations of LPS and β-CyDs in PBS (pH 6.5) were 100 μg/ml and 10 mM, respectively. (C) The concentrations of DMA7-β-CyD in PBS (pH 6.5) were 0, 1, 3 and 10 mM. These experiments were performed at 25 °C.

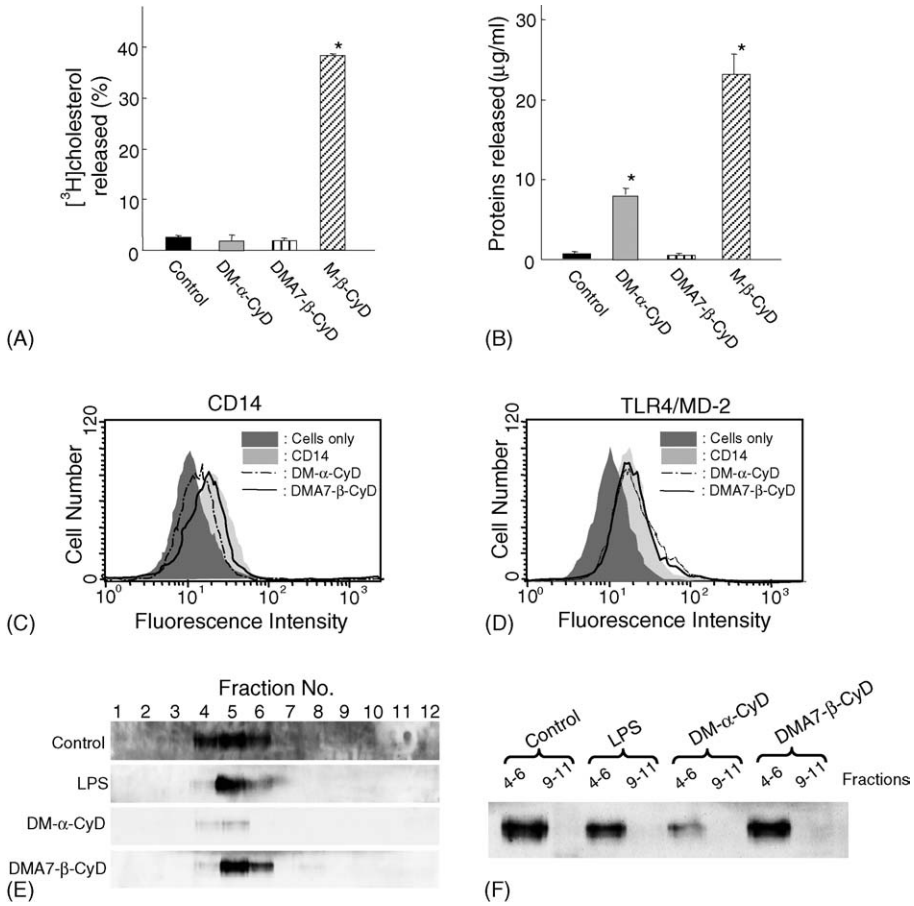


Fig. 6. DMA7-β-CyD interacts with cell surface only very slightly. (A, B) β-CyDs, but not DMA7-β-CyD, release [^{3}H]cholesterol and proteins from RAW264.7 cells. (A and B) The cells (3×10^6) were labeled with [^{3}H]cholesterol and then treated with 10 mM β-CyDs for 60 min at 37 °C. The extent of [^{3}H]cholesterol and total proteins released from cells into cell supernatant was determined by liquid scintillation counter and the bicinchoninic acid reagent with BSA as a standard. Each value represents the mean ± S.E.M. of three to four experiments. $^*p < 0.05$, compared to control. (C, D) DMA7-β-CyD affects on expression of neither CD14 nor TLR4-MD-2 complex. RAW264.7 cells (2×10^6) were incubated with FITC-conjugated anti-CD14 (C) or anti-TLR4-MD-2 complex antibody (D) at 4 °C for 30 min. In the latter case, the cells were washed with PBS twice and incubated with FITC-conjugated anti-IgG2a at 4 °C for 30 min. The stained cells were resuspended in HBSS and quantified using a flow cytometer. (E and F) DMA7-β-CyD does not release CD14 from lipid rafts of RAW264.7 cells into culture medium. RAW264.7 cells (3×10^6) were treated with 1000 ng/ml of LPS or 10 mM CyDs for 1 h at 37 °C. (E) After isolation of lipid rafts, the CD14 levels in all of fractions were assayed by immunoblot. (F) CD14 levels in lipid rafts were assayed by immunoblot after adjusting the total protein content (5 and 10 μg proteins/lane in lipid rafts and non-rafts, respectively). These figures show representative data for three experiments.

whether DMA7-β-CyD alters localization of LPS receptors in lipid rafts by a Western blot. Consistent with the finding reported by Pfeiffer et al. [29], CD14 resided in alkaline-resistant and low-density fractions (Fraction No. 4–6), showing the localization of CD14 in lipid rafts (Fig. 6E). The treatment of RAW264.7 cells with DMA7-β-CyD, however, changed neither the localization of CD14 (Fig. 6E) nor CD14 expression in lipid rafts (Fig. 6F). Besides, we confirmed that DMA7-β-CyD did not affect the TLR4-MD2 complex levels (data not shown). Meanwhile, a positive control DM-α-CyD impaired CD14 levels in lipid rafts and released it from cell surface to culture medium (Fig. 6E and F) as reported previously [20], but β-CyD or HP-β-CyD did not (data not shown). These results strongly suggest that there is very little interaction of DMA7-β-CyD with membrane lipids and LPS receptors on the cell surface.

3.6. DMA7-β-CyD suppressed LPS-induced endotoxin shock in mice

To examine the inhibitory effects of β-CyDs on endotoxin shock induced by LPS and D-galactosamine in C57BL/6 mice, we examined the survival rate of endotoxin shock mice. In the absence of β-CyDs, all of the mice died within 12 h after administration of the aqueous solution containing 100 ng of LPS and 25 mg of D-galactosamine (Fig. 7A). When 100 mM DMA7-β-CyD was concomitantly administered with both LPS and D-galactosamine intraperitoneally in mice, the rate of survival was significantly improved in a concentration-dependent manner (Fig. 7B). Interestingly, DMA7-β-CyD increased the survival rate of endotoxin shock mice even when DMA7-β-CyD was administered intravenously and LPS and D-galactosamine were intraperitoneally administered, sug-

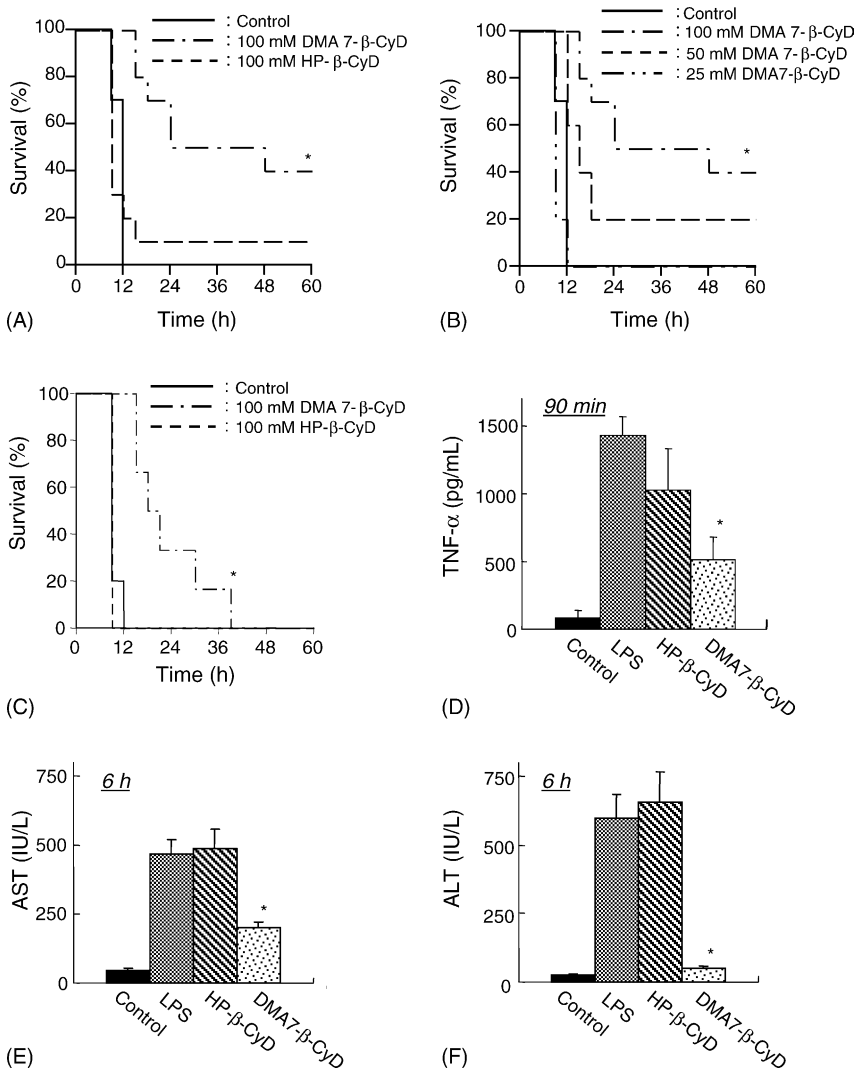


Fig. 7. DMA7-β-CyD improves the survival rate of endotoxin shock mice induced by LPS and D-galactosamine through, at least in part, impairment of hepatic inflammation. (A) C57BL/6 mice were administered intraperitoneally with a solution containing 100 ng of LPS and 25 mg of D-galactosamine (LPS treatment) with or without 100 mM CyDs. Each line represents the survival of 10 mice. $^{*}p < 0.05$, compared to alone. (B) Effects of the DMA7-β-CyD concentration on lethality in endotoxin shock mice. Each line represents the survival of 10 mice. $^{*}p < 0.05$, compared to LPS treatment. (C) Effects of intravenous administration of the DMA7-β-CyD-containing solution on the survival rate of septic mice. Mice were injected intraperitoneally with the solution containing 500 ng of LPS and 25 mg of D-galactosamine and concomitantly with the 200 mM DMA7-β-CyD or 200 mM HP-β-CyD-containing solution into the tail vein. Each line represents the survival of 5 mice. $^{*}p < 0.05$, compared to alone. (D) Effect of β-CyDs on plasma TNF-α levels in endotoxin shock mice. The plasma was collected 90 min after intraperitoneal administration of the aqueous solution containing 100 ng of LPS and 25 mg of D-galactosamine with or without 100 mM β-CyDs. The TNF-α level was determined by ELISA. Each value represents the mean ± S.E.M. of three to four experiments. $^{*}p < 0.05$, compared to LPS treatment. (E and F) Effect of β-CyDs on serum AST (E) and ALT (F) levels in endotoxin shock mice at 6 h after intraperitoneal administration of LPS and D-galactosamine. Each value represents the mean ± S.E.M. of three to four experiments. $^{*}p < 0.05$, compared to LPS.

gesting the interaction of LPS with DMA7-β-CyD not only in the intraperitoneal cavity but also in blood and/or tissues of mice (Fig. 7C). In addition, the plasma TNF-α levels at 90 min after concomitantly stimulation with LPS and D-galactosamine significantly increased compared to that of control, but were decreased by the co-administration with DMA7-β-CyD intraperitoneally (Fig. 7D). Upon stimulation with LPS and D-galactosamine, the serum levels of AST and ALT markedly augmented, but DMA7-β-CyD strikingly inhibited the increase in these levels (Fig. 7E and F). However, co-administration with a negative control HP-β-CyD did not alter the TNF-α, AST or ALT level (Fig. 7D–F). Besides, macroscopic and microscopic observations demonstrated that severe liver inflammation took place in LPS/D-galactosamine-treated mice, i.e. macroscopic observation, hematoxylin-eosin stain and TUNEL assay showed a hemorrhage in liver (Fig. 8A), strong infiltration of neutrophils (Fig. 8B) and apoptosis of hepatocytes (Fig. 8C), respectively. Almost all of the same severe inflammation was elicited in mice treated with the HP-β-CyD system, whereas these inflammatory symptoms in liver were strikingly reduced in mice administered

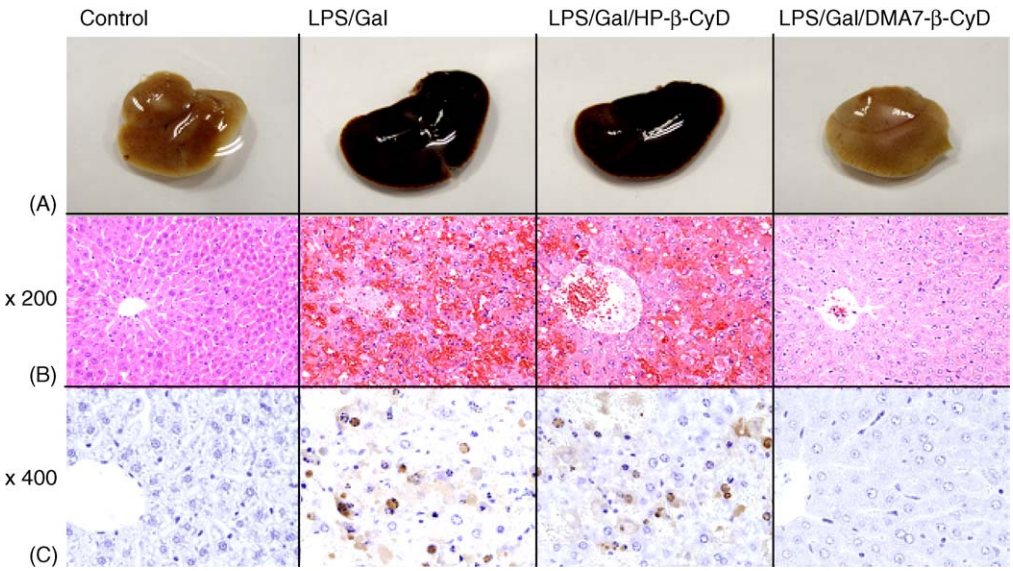


Fig. 8. DMA7-β-CyD suppressed hepatic inflammation in endotoxin shock mice at 6 h after intraperitoneal administration. (A) macroscopic appearance of the liver; (B) hematoxylin-eosin staining of the liver; (C) hepatocyte apoptosis assayed by TUNEL method. These figures show representative data for five experiments.

DMA7-β-CyD intraperitoneally (Fig. 8A and B). These results suggest that DMA7-β-CyD significantly suppresses lethality in endotoxin shock mice induced by LPS and D-galactosamine, probably resulting from the reduction of hepatic inflammation.

4. Discussion

We revealed that DMA7-β-CyD suppressed the production of NO and various proinflammatory cytokines in murine macrophages stimulated with LPS in vitro and suppressed mortality of endotoxin shock mice induced by LPS and D-galactosamine. Therefore, DMA7-β-CyD may be a novel applicant as an inhibitor of endotoxin shock.

DMA7-β-CyD is likely to suppress macrophage activation by LPS through interaction with LPS in vitro. CyDs are known to possess the ability of inclusion complexation with various hydrophobic drugs and cell membrane constituents [11,30]. We reported that of the various β-CyDs, DMA7-β-CyD prefers an inclusion of small molecules with alkyl or acyl chain into the cavity to bulky molecules [21]. This intriguing finding allowed us to formulate a working hypothesis that DMA7-β-CyD interacts with LPS, which has several acyl chains in the molecules, resulting in attenuation of excess macrophage activation in response to LPS. Indeed, several lines of evidence may indicate the relevance of the hypothesis: first, spectrophotometric studies clarified the direct interaction of DMA7-β-CyD with LPS (Fig. 5). Second, DMA7-β-CyD inhibited macrophage activation upon stimulation with LPS from two sources and lipid A as well as LTA, which have acyl chains in these molecules, but not with PGN, poly I:C or CpG-ODN, which have no acyl chain (Fig. 2A and C). Apparently, DMA7-β-CyD works at the level of delivery of LPS by CD14 to the receptor (TLR4-MD2 complex). These findings strongly suggest that DMA7-β-CyD is likely to include an acyl chain of lipid A moiety of LPS molecule. However, it still remains obscure whether DMA7-β-CyD binds a single molecule of LPS or an aggregated LPS in the present study, but DMA7-β-CyD may include an acyl chain in the cavity because of restricted cavity size. Further studies needed to reveal this interaction mode.

Intracellular signals activated by LPS-CD14-TLR4-MD-2 complex are divided into MyD88-dependent and –independent pathways, which occur early and later, respectively. LPS signaling leads to the early activation of NF-κB, interferon response factor 3 (IRF3) and mitogen-activated protein kinase (MAPK) pathways, which is mediated by the adapters MyD88 and Mal. As a later response to LPS, TLR4 gives rise to the activation of TRAF6 and TBK1, an event mediated by the adapters TRIF and TRAM [31]. In the present study, DMA7-β-CyD was found to suppress NF-κB activation (Fig. 5) followed by inhibition of NO and various proinflammatory cytokine production (Figs. 2–4), although the effects of DMA7-β-CyD on the other LPS signaling such as IRF3 and MAPK still remain unclear. However, we previously reported that DMA7-β-CyD lowered cellular binding of LPS in RAW264.7 cells when they exist together in culture medium [19]. Therefore, it is possible that DMA7-β-CyD impairs not only NF-κB signaling but also IRF3 and MAPK signaling because of an interference of the initial step of LPS signaling by reducing the number of LPS available for binding from CD14 to the receptor.

DMA7-β-CyD is highly unlikely to suppress macrophage activation by LPS through interaction with cell

surface. It is well known that β-CyDs such as M-β-CyD and DM-β-CyD induce hemolysis and disruption of lipid rafts through extraction of cholesterol from cells [13,20,32,33]. However, hemolytic activity of β-CyDs apparently depends on the CyD type, and the intensity actually increased in the order of DMA7-β-CyD < HP-β-CyD < β-CyD < M-β-CyD < DM-β-CyD among β-CyDs [13,21], consistent with the order of their cytotoxic activity (Fig. 1). This fact allows us to speculate that DMA7-β-CyD interacts with cell membranes only very slightly. In accordance with the hypothesis, the ability of DMA7-β-CyD to release cholesterol and proteins from RAW264.7 cells was found to be lowest of these β-CyDs (Fig. 6). Additionally, DMA7-β-CyD affected neither CD14 nor TLR4-MD-2 complex levels on cell surface, and exerted no change in their membrane localization in RAW264.7 cells (Fig. 6C–F). However, the reason why DMA7-β-CyD is capable of interacting with LPS, but not with membrane lipids such as phosphatidylcholines in macrophage still remains unclear. Given the difference in the molecular structure between LPS and phospholipids, the number of acyl chain may be critical for the interaction intensity, since biologically active and/or antagonistic lipid A can have from 4 to 7 fatty acyl groups. Thus, it remains obscure how DMA7-β-CyD strictly distinguishes between LPS and phospholipids. Moreover, we cannot rule out the possibility that the amphiphilic DMA7-β-CyD penetrates into the plasma membrane of macrophages to inhibit LPS signaling, although this possibility may be only slight. Additional studies are still needed to identify these mechanisms.

It is evident that DMA7-β-CyD suppresses endotoxin shock in mice treated with LPS and D-galactosamine in vivo, most likely through impairment of hepatic inflammation. In this study, we clarified that DMA7-β-CyD improved the fatal rate and lowered the plasma levels of TNF-α and serum levels of AST and ALT when concomitantly administered LPS, D-galactosamine and DMA7-β-CyD to mice (Fig. 7C–E). In addition, the gross and histological observation showed that the treatment with DMA7-β-CyD suppressed hemorrhage, infiltration of neutrophils and the number of apoptotic hepatocytes in liver in endotoxin shock mice (Fig. 8). These results suggest that DMA7-β-CyD is capable of inhibiting hepatic inflammation in endotoxin shock mice, leading to attenuation of proinflammatory cytokine production from hepatocytes and Kupffer cells. Thereafter, elaborate studies regarding the effects of DMA7-β-CyD on pharmacokinetic properties of LPS in vivo should be, however, required.

It remains unknown whether the direct interaction between LPS and DMA7-β-CyD causes improving fatality of endotoxin shock mice. Our preliminary study demonstrated that (1) when DMA7-β-CyD was intraperitoneally administered together with LPS, the blood LPS levels significantly changed, (2) intravenous administration of DMA7-β-CyD to normal mice elicited no increase in blood levels of proinflammatory cytokines, AST or ALT (data not shown), (3) DMA7-β-CyD increased the survival rate of endotoxin shock mice when DMA7-β-CyD was administered intraperitoneally 5 min after intraperitoneal injection of LPS and D-galactosamine (data not shown) as well as even when DMA7-β-CyD was intravenously administered as shown in Fig. 7C. Collectively, these results therefore suggest the interaction of LPS with DMA7-β-CyD not only in the intraperitoneal cavity but also in blood and/or tissues of mice. Studies are underway to examine the interaction and pharmacokinetics of LPS and DMA7-β-CyD in vivo.

The cause of sepsis may affect the outcome of an intervention. For instance, LPS may contribute to Gram-positive organisms such as *S. aureus* [34–36]. Strikingly, we revealed that the similar treatment of macrophages with DMA7-β-CyD suppressed macrophage activation by LTA from *S. aureus*, although did not suppress macrophage activation stimulated with PGN, poly I:C or CpG-ODN (Fig. 2C). Hence, it is likely that DMA7-β-CyD would be effective against sepsis caused by LPS from Gram-negative bacteria or LTA from Gram-positive bacteria, although it would not be effective against other ligands of the innate immunity system.

It is noteworthy that DMA7-β-CyD is highly water soluble (>50% in water) and causes little local irritation since it exhibits low hemolytic activity and low muscular irritancy [21]. Furthermore, DMA7-β-CyD is a pure synthetic compound with low molecular weight (1624 Da) compared to antibodies and LPS antagonists such as polymyxin B, Eritoran (E5564), HA-1A, E5 and surfactant protein A [4,37–40]. Therefore, DMA7-β-CyD has potential as a novel excipient for injections.

In conclusion, the present study suggests that DMA7-β-CyD lowered NO and various proinflammatory cytokines' production in murine macrophages stimulated with LPS and lipid A, probably by suppressing the binding of LPS to its LPS receptors on the cells. In addition, DMA7-β-CyD possesses less cytotoxicity and sufficient physicochemical and biopharmaceutical properties for injections. Therefore, a new strategy is proposed involving the intravenous injection of DMA7-β-CyD for prophylaxis or treating endotoxin shock.

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