Journal of Inclusion Phenomena and Macrocyclic Chemistry (2006) 56: 75–79
DOI 10.1007/s10847-006-9064-7

Inhibitory Effects of 2,6-Di-*O*-methyl-3-*O*-acetyl-β-cyclodextrins with Various Degrees of Substitution of Acetyl Group on Macrophage Activation and Endotoxin Shock Induced by Lipopolysaccharide

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(Received: 26 August 2005; in final form 25 September 2005)

Key words: degree of substitution, dimethylacetyl-β-cyclodextrin, endotoxin shock, lipopolysaccharide, nitric oxide

Abstract

The effects of 2,6-di-*O*-methyl-3-*O*-acetyl-β-cyclodextrins (DMA-β-CyD) with various degrees of substitution (DS) of an acetyl group of 1.5, 3.8, 6.3 and 7, which are abbreviated to DMA2-β-CyD, DMA4-β-CyD, DMA6-β-CyD and DMA7-β-CyD, respectively, on murine macrophage activation and endotoxin shock induced by lipopolysaccharide (LPS) were examined. Of four DMA-β-CyDs used in the present study, cytotoxicity of DMA-β-CyDs in RAW264.7 cells, a murine macrophage-like cell line, decreased with an increase in the DS values of DMA-β-CyD, and DMA7-β-CyD had no cytotoxicity on RAW264.7 cells up to 100 mM. DMA2-β-CyD and DMA7-β-CyD at the concentration of 5 mM had greater inhibitory effects on nitric oxide (NO) production in RAW264.7 cells stimulated with LPS than DMA4-β-CyD and DMA6-β-CyD. In addition, these inhibitory effects of DMA2-β-CyD and DMA7-β-CyD were concentration-dependent. In the *in vivo* study, all of the mice died within 12 h after intraperitoneal administration of the solution containing LPS and D-galactosamine. When 100 mM DMA7-β-CyD was concomitantly administered with both LPS and D-galactosamine intraperitoneally in mice, the survival rate significantly increased, but DMA4-β-CyD and DMA6-β-CyD did not. In conclusion, we revealed that DS values of DMA-β-CyDs strikingly affect not only the cytotoxic activity but also the inhibitory effects of LPS-induced NO production in RAW264.7 cells and fatality of endotoxin shock mice induced by LPS and D-galactosamine. These results suggest the potential use of DMA7-β-CyD as an antagonist of LPS-induced endotoxin shock.

Abbreviation: CyDs: cyclodextrins; DMA-β-CyD: 2,6-di-*O*-methyl-3-*O*-acetyl-β-cyclodextrin; DM-β-CyD: 2,6-di-*O*-methyl-β-CyD; DM-α-CyD: 2,6-di-*O*-methyl-α-CyD; DS: degree of the substitution; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; NO: nitric oxide; RT-PCR: reverse transcriptase-polymerase chain reaction; TLR-4: Toll-like receptor-4; TNF-α: tumor necrosis factor-α

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]. So far several targets for the treatment of sepsis have been reported, especially lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacterial, is well known to be the most momentous [3]. Several strategies for treating sepsis induced by LPS have been proposed, e.g. the use of anti-endotoxin antibodies, that of an endotoxin antagonist and the elimination of LPS (hemofiltration) [4–6]. However, a number of clinical trials using an antibody-induced blockade of LPS during sepsis showed no substantial benefits [7]. Additionally, monoclonal antibodies to the lipid A of mouse LPS (E5) or humanized (HA-1A) are, unfortunately, reported to be unable to block LPS-induced cytokine production in human monocytes *in vitro* [8, 9]. Thus, a new strategy is needed for the future regimen.

Cyclodextrins (CyDs) and their hydrophilic derivatives form inclusion complexes with hydrophobic molecules. CyDs can improve the solubility, dissolution

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rate and bioavailability of the drugs, and so the widespread use of CyDs is well-known in the pharmaceutical field [10, 11]. CyDs have also been reported to interact with membrane constituents such as cholesterol, phospholipids and phosphatidylinositols, resulting in not only the induction of hemolysis of erythrocytes [12, 13] but also disruption of the structures of lipid rafts [14–16], lipid microdomains formed by lateral assemblies of cholesterol and sphingolipids in the cell membrane [17]. Recently, we reported that 2,6-di-*O*-methyl-α-cyclodextrin (DM-α-CyD) and 2,6-di-*O*-methyl-3-*O*-acetyl-β-cyclodextrin (DMA-β-CyD) with a degree of the substitution (DS) of the acetyl group of 7 (DMA7-β-CyD) inhibited NO production in RAW264.7 cells, a murine macrophage-like cell line, stimulated with LPS from *Escherichia coli* (serotype O111:B4) [18, 19]. In addition, we revealed that DMA7-β-CyD, but not DM-α-CyD, significantly lowered fatality of endotoxin shock mice induced by intraperitoneal injection of LPS and D-galactosamine [20]. Meanwhile, controlling the DS value is important in balancing water solubility and complexing capability of CyD derivatives. For example, in the case of 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD), the higher the degree of hydroxypropyl substitution the poorer the drug binding was observed [21, 22]. Additionally, aqueous solubility of monosubstituted HP-β-CyD is lower than β-CyD, although that of HP-β-CyD with DS values more than 2 is higher than β-CyD [23]. Likewise, we reported that hemolytic activity of DMA4-β-CyD with DS 3.8 of the acetyl group is higher than that of DMA7-β-CyD with DS 7 of the acetyl group [20]. In the present study, we examined whether DMA-β-CyDs with various DS values of an acetyl group have a different cytotoxic activity and an inhibitory effect on NO production in RAW264.7 cells stimulated with LPS *in vitro*. In addition, we investigated whether DMA-β-CyDs have a different inhibitory effect on fatality of endotoxin shock mice induced by LPS and D-galactosamine.

Experimental

Materials

DMA-β-CyDs with different DS values (DS = 1.5, 3.8, 6.3 and 7.0) were prepared in our laboratory and purified according to the methods described previously (Table 1) [24]. These DMA-β-CyDs are abbreviated to DMA2-β-CyD, DMA4-β-CyD, DMA6-β-CyD and DMA7-β-CyD, respectively. LPS from *Escherichia coli* (serotype O111:B4) and D-galactosamine 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. All other chemicals and solvents were of analytical reagent grade.

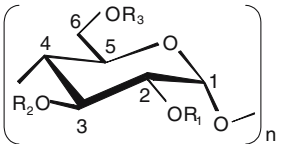
Cell viability

Cell viability was assayed using a Cell Counting Kit (WST-1 method) [25, 26] from Wako Pure Chemical Industries (Osaka, Japan). RAW264.7 cells (1×10^5 cells/well) were incubated for 1 h with 150 μL of RPMI-1640 culture medium supplemented with 10% FCS containing DMA-β-CyDs at various concentrations in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After washing twice, 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 the Griess reagent as described by Stuehr and Nathan [27]. Briefly, RAW264.7 cells (1×10^5 cells/well) were stimulated with LPS (1–1000 ng/mL) for 1 h in the absence and presence

Table 1. Chemical structures of CyDs used in this study



Compound	Abbreviation	*n*	R_1	R_2	R_3	DS[a]
2,6-di-*O*-dimethyl-β-cyclodextrin	DM-β-CyD	7	$-CH_3$	–H	$-CH_3$	14
2,6-di-*O*-dimethyl-3-*O*-acetyl-β-cyclodextrin	DMA2-β-CyD	7	$-CH_3$	$-COCH_3$ or –H	$-CH_3$	1.5[b]
2,6-di-*O*-dimethyl-3-*O*-acetyl-β-cyclodextrin	DMA4-β-CyD	7	$-CH_3$	$-COCH_3$ or –H	$-CH_3$	3.8[b]
2,6-di-*O*-dimethyl-3-*O*-acetyl-β-cyclodextrin	DMA6-β-CyD	7	$-CH_3$	$-COCH_3$ or –H	$-CH_3$	6.3[b]
2,6-di-*O*-dimethyl-3-*O*-acetyl-β-cyclodextrin	DMA7-β-CyD	7	$-CH_3$	$-COCH_3$	$-CH_3$	7.0[b]

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

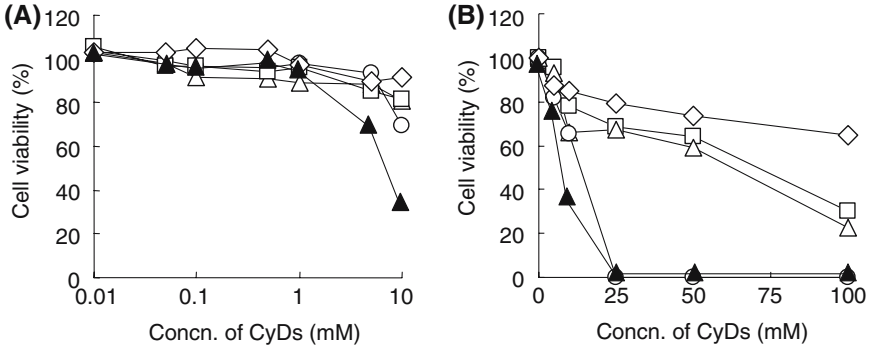


Figure 1. Cytotoxicity of CyDs for RAW264.7 cells. Cytotoxicity was assayed by WST-1 method. RAW264.7 cells (1×10^5 cells) were incubated for 1 h with 150 μL of RPMI-1640 culture medium supplemented with 10% FCS containing CyDs at various concentrations in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After washing twice, 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. The symbols of CyDs represent DM-β-CyD (closed triangles), DMA2-β-CyD (open circles), DMA4-β-CyD (open triangles), DMA6-β-CyD (open squares) and DMA7-β-CyD (open diamonds). (A), the concentration up to 10 mM; (B), the concentration up to 100 mM. Each point represents the mean ± SEM of three experiments.

of 5 mM DMA-β-CyDs, then the cells were incubated for 24 h in culture medium. Likewise, RAW264.7 cells (1×10^5 cells/well) were stimulated with LPS (100 ng/mL) for 1 h in the absence and presence of DMA-β-CyDs at the concentrations of 2.5, 5 and 10 mM, then the cells were incubated for 24 h in culture medium. Then, 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.

In vivo *study*

Male C57/BL6 mice (4 weeks old, Nihon SLC, Shizuoka, Japan) were used. Murine endotoxin shock was induced by intraperitoneal injection of the 500 μL of solution containing LPS (500 ng/mouse) and D-galactosamine (25 mg/mouse). DMA-β-CyDs (100 mM) were added to the solution containing LPS and D-galactosamine, and then 500 μL of the mixed solution was injected intraperitoneally. The survival rate was monitored over the next 60 h. Animal use and relevant experimental procedures were approved by the Kumamoto University Committee on Animal Care and Use of Laboratory Animals.

Statistical analysis

Data are given as the mean ± SEM. 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.

Results and discussion

Effects of DS value of DMA-β-CyDs on cell viability

We examined the effects of DMA-β-CyDs on the viability of RAW264.7 cells using the WST-1 method. Any DMA-β-CyDs did not show cytotoxicity in RAW264.7 cells up to 10 mM, but DM-β-CyD decreased cell viability to about 40% of the control value at 10 mM (Figure 1A). In the range of high concentrations, cytotoxic activity of DMA-β-CyDs with low DS values was observed, but it decreased as the DS value increased (Figure 1B). Furthermore, the order of the magnitude of cytotoxic activity of DMA-β-CyDs on RAW264.7 cells was consistent with that of hemolytic activity of DMA-β-CyDs (data not shown). These results suggest that the interaction between DMA-β-CyDs and cells impaired with an increase in the number of the acetyl group introduced in the molecule.

Effects of DS value of DMA-β-CyDs on NO production in RAW264.7 cells stimulated with LPS

We previously reported that DMA7-β-CyD suppressed NO production from macrophages stimulated with LPS [18]. Then, we examined the effect of the DS value of DMA-β-CyDs on NO production in RAW264.7 cells stimulated with LPS. We assayed nitrite levels in the culture supernatant 24 h after stimulation with LPS by the Griess method [27]. As shown in Figure 2A, these inhibitory effects of DMA-β-CyDs on NO production showed a biphasic pattern: DMA2-β-CyD and DMA7-β-CyD had greater inhibitory activities than DMA4-β-CyD and DMA6-β-CyD. Similar results were observed in the other murine macrophage cell lines such as J774.1 and PU5-18 cells. In addition, the inhibitory effects of DMA2-β-CyD and DMA7-β-CyD on LPS-stimulated NO production augmented with an increase in the concentrations (Figure 2B). These results indicate that the DS value of DMA-β-CyDs markedly affect the inhibitory effect on LPS-induced NO production in RAW264.7 cells. As described before, cytotoxicity between DMA2-β-CyD and DMA7-β-CyD was totally different (Figure 1). Taken together, these results suggest that the inhibitory mechanism of DMA2-β-CyD and DMA7-β-CyD may be different.

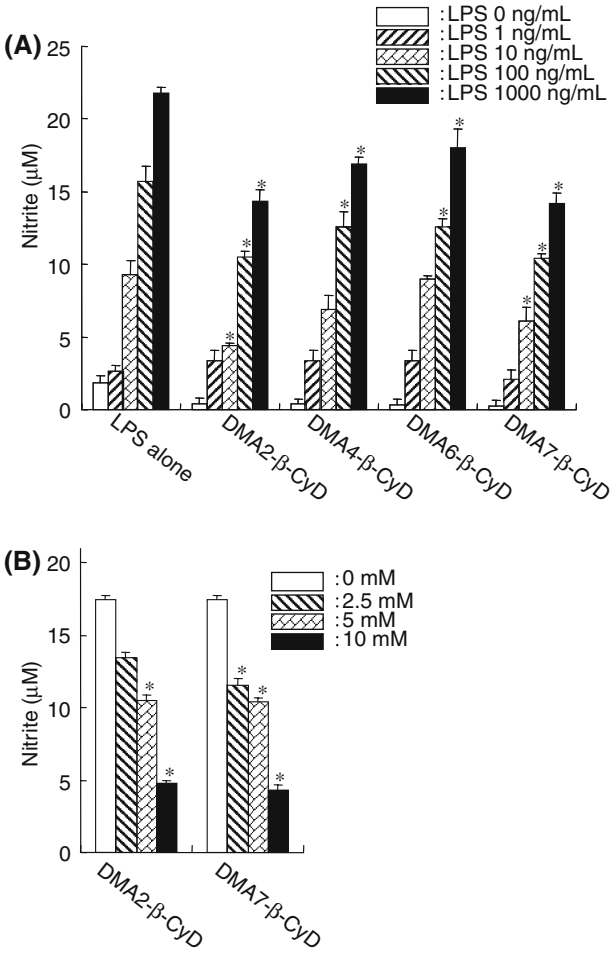


Figure 2. Effects of DS values of DMA-β-CyDs on NO production from macrophage stimulated with LPS. NO production was estimated by measurement of nitrite in the culture supernatant using the Griess method. (A) RAW264.7 cells (1×10^5 cells/well) were stimulated with LPS (1–1000 ng/mL) for 1 h in the absence and presence of DMA-β-CyDs (5 mM), then the cells were incubated for 24 h in culture medium. (B) RAW264.7 cells (1×10^5 cells/well) were stimulated with LPS (100 ng/mL) for 1 h in the absence and presence of DMA-β-CyDs at the designated concentrations, then the cells were incubated for 24 h in culture medium. Then, 90 μL of Griess reagent was added and incubated at room temperature for 10 min. The absorbance at 490 nm was measured with a miniplate reader. Nitrite levels were determined using $NaNO_2$ as a standard. Each value represents the mean ± SEM of three experiments. *$p < 0.05$, compared to LPS alone.

Effects of DS value of DMA-β-CyDs on fatality of endotoxin shock mice induced by intraperitoneal injection of LPS and D*-galactosamine*

Recently, we revealed that intraperitoneal administration of DMA7-β-CyD to endotoxin shock mice induced by LPS and D-galactosamine lowered fatality as well as blood levels of TNF-α, aspartate transaminase (AST) and alanine transaminase (ALT), most likely through the impairment of hepatitis in endotoxin shock mice [20]. Then, we examined whether other DMA-β-CyDs with different DS values improve fatality of endotoxin shock mice induced by intraperitoneal injection of LPS and D-galactosamine.

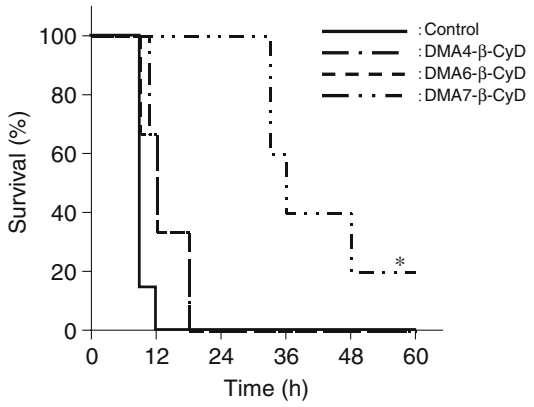


Figure 3. Effects of DS values of DMA-β-CyDs on endotoxin shock induced by LPS and D-galactosamine in mice. C57/BL6 mice were intraperitoneally injected the solution containing LPS and D-galactosamine (500 ng and 25 mg/mouse, respectively) with or without DMA-β-CyDs (100 mM) in distilled water. Each line represents the survival of 3–7 mice. *$p < 0.05$ compared to control.

Of four DMA-β-CyDs used in this study, the intraperitoneal administration of the solution containing 100 mM DMA2-β-CyD unfortunately killed mice, possibly due to strong interaction with various cells (data not shown). Therefore, we declined the use of DMA2-β-CyD in this study. In the absence of DMA-β-CyD, all of the mice died within 12 h after administration of the solution containing LPS and D-galactosamine (Figure 3). When 100 mM DMA7-β-CyD was concomitantly administered with both LPS and D-galactosamine intraperitoneally in mice, the survival rate was significantly improved. However, DMA4-β-CyD and DMA6-β-CyD had no protective effects on endotoxin shock, reflecting the *in vitro* results.

Unfortunately, the mechanism by which DMA7-β-CyD lowered fatality of endotoxin shock mice induced by LPS and D-galactosamine is still unknown. We previously proposed that DMA7-β-CyD inhibits NO production in murine macrophages through complex formation of LPS with DMA7-β-CyD out of cells [18, 20]. However, it is acknowledged that CyDs change no pharmacokinetic parameters after muscular and intravenous administration of the solution containing a hydrophobic drug [28, 29]. Thereby, it may be hardly expected that DMA7-β-CyD changes pharmacokinetic and/or pharmacodynamic properties of LPS in mice. However, Anton *et al*. [30] recently reported that post-treatment of a hydrophilic CyD derivative (Org 25969) changes the pharmacokinetic parameter of rocuronium bromide, a steroidal neuromuscular blocker, *in vivo*, because the derivative has a high affinity to the drug (stability constant $> 10^7\ M^{-1}$). Based on these facts, DMA7-β-CyD may suppress endotoxin shock through the strong interaction with LPS locally and/or systemically in mice, and may act as a scavenger of LPS *in vivo*. The effects of DMA7-β-CyD on pharmacokinetic and pharmacodynamic properties of LPS after intraperitoneal injection in mice are currently under investigation.

In conclusion, we revealed that DS values of DMA-β-CyDs strikingly affect not only the cytotoxicity but also the inhibitory effects of LPS-induced NO production in RAW264.7 cells and fatality of endotoxin shock mice induced by LPS and D-galactosamine. These results suggest the potential use of DMA7-β-CyD as an antagonist of LPS-induced endotoxin shock.

Acknowledgements

This work was partially supported by a Grant-in-aid of Sagawa Foundation for Promotion of Frontier Science and Grant-in-aid of Japan Science and Technology Agency.

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