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Role of 6-*O*-α-maltosyl-β-cyclodextrin in lysosomal cholesterol deprivation in *Npc1*-deficient Chinese hamster ovary cells

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ABSTRACT

We aimed to investigate whether 6-*O*-α-maltosyl-β-cyclodextrin (Mal-βCD) is incorporated into cells and lysosomes during the release of unesterified cholesterol in *Npc1*-deficient Chinese hamster ovary (CHO) cells (*Npc1 KO* cells) and CHO-JP17 cells (JP17 cells). Internalization of Mal-βCD in cells and lysosomes and extracellular release of lysosomal unesterified cholesterol were demonstrated by LC/MS/MS and LC/MS, respectively. Internalization of Mal-βCD was greater in *Npc1 KO* cells than in JP17 cells. The majority of internalized Mal-βCD in both cell types was metabolized by lysosomal α-glucosidase to 6-*O*-α-D-glucosyl-β-cyclodextrin (Glc-βCD). However, Mal-βCD did not directly enter the lysosomes prepared from cell homogenates. Mal-βCD-treated *Npc1 KO* cells and JP17 cells both released Mal-βCD and Glc-βCD, together with unesterified cholesterol, out of cells. The release of unesterified cholesterol by Mal-βCD in *Npc1 KO* cells was much greater than that in JP17 cells. This study is the first to report the influx of Mal-βCD into the lysosomes of *Npc1 KO* cells and JP17 cells, followed by generation of Glc-βCD, and the efflux of Mal-βCD/Glc-βCD and unesterified cholesterol to the extracellular fluid, based on the quantitative LC/MS analysis.

1. Introduction

Niemann-Pick Type C (NPC) disease is a lysosomal storage disease where endocytosed unesterified cholesterol becomes sequestered in the late endosomes/lysosomes (LE/LS). NPC disease is caused by defects in the NPC1 or NPC2 proteins, which play a role in unesterified cholesterol transport from lysosomes to the extracellular fluid or the endoplasmic reticulum. Potential therapies for NPC disease have focused on reduction of unesterified cholesterol storage in LE/LS. β cyclodextrin (βCD) derivatives have been proposed as therapy for NPC disease because they readily form inclusion complexes with unesterified cholesterol in *in vitro* experiments and reduce intracellular unesterified cholesterol levels in various cultured cells including NPC protein-deficient cells [1].

As one of the conventionally used βCDs, 2-hydroxypropyl-βCD (HP-βCD) has attracted increasing attention regarding improvement of viability and associated symptoms of NPC disease, such as hepatopathology and neuropathology [2–6]. Despite various studies reporting that HP-βCD is a more efficient depressor of

Abbreviations: βCD, β-cyclodextrin; CHO, Chinese hamster ovary; DAOS, (hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt; Glc-βCD, 6-*O*-α-D-glucosyl-βCD; HP-βCD, 2-hydroxypropyl-β-cyclodextrin; LDH, lactate dehydrogenase; Mal-βCD, 6-*O*-α-maltosyl-βCD; NPC, Niemann-Pick Type C.

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unesterified cholesterol levels, its safety for therapy for NPC disease is questionable. Moreover, Matsuo et al. reported a repeatedly occurring lung cloudiness, following HP-βCD infusion in one patient with NPC disease [7], and Vite et al. reported dose-dependent auditory toxicity of HP-βCD in cats with an *Npc1* missense mutation [8].

From our studies to date, 6-*O*-α-maltosyl-βCD (Mal-βCD), from the reaction products of maltose and βCD produced by the action of *Pseudomonas* isoamylase (EC 3.2.1.68), was shown to have a strong activity to reduce cellular unesterified cholesterol levels by forming inclusion complexes with less cytotoxicity in various cultured cells [9–11]. The negligible cytotoxicity of Mal-βCD was also reported in other studies [12,13]. Furthermore, we showed that Mal-βCD downregulated cholesterol-related ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1) mRNA and protein in mouse mastocytoma P-815 cells [14]. These results suggest that Mal-βCD may affect exclusion of unesterified cholesterol in NPC1 protein-deficient cells.

NPC1 protein-deficient cells are characterized by the accumulation of unesterified cholesterol in the lysosome, and βCD derivatives such as methyl-βCD and HP-βCD are known to reduce its unesterified cholesterol levels [15–17]. However, Li et al. indicated that the reported efficacy and potency of these βCD derivatives varied among different laboratories, which could have resulted from variations of the preparations from the manufacture processes [18]. In addition to heterogeneity of βCD derivatives used, the remaining issue is that the transition of βCD derivatives and unesterified cholesterol in the lysosome mainly resulted from qualitative analysis by fluorescence-staining and/or fluorescent-labeled βCD derivatives, but not by quantitative analysis of those molecules. Subsequently, it is unclear whether βCD molecules are actually present in the lysosome and are able to mediate extracellular efflux of lysosomal stored unesterified cholesterol by forming inclusion complexes, as shown *in vitro*. Hence, the current study aimed to determine the influx of abundance of Mal-βCD in the lysosomes in *Npc1*-deficient Chinese hamster ovary cells and the efflux of Mal-βCD and unesterified cholesterol from cells and lysosomes to investigate of the role of internalized Mal-βCD in the mobilization of stored unesterified cholesterol. For this purpose, LC/MS analyses were performed for a quantitative measurement of Mal-βCD, its metabolites, and unesterified cholesterol in the cell, in the lysosome, and in the extracellular fluid.

2. Materials and methods

2.1. Reagents

Mal-βCD was produced from maltose and βCD by the action of *Pseudomonas* isoamylase (EC 3.2.1.68), and purified in accordance with a previously reported procedure [9]. It comprises βCD and one maltose side-chain attached to the βCD ring by an α-(1 → 6)-linkage. Ham's F-12 and DMEM were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA), and fetal bovine serum (FBS) from Biowest (Nuaillé, France). All other reagents were special grade or LC/MS-grade.

2.2. Cell culture and cytotoxicity measurement

Parental Chinese hamster ovary (CHO)-JP17 cells (JP17 cells) and *Npc1* gene-deficient CHO cells (*Npc1 KO* cells) were generated in accordance with a previously reported procedure [19]. Both cell types were grown in a culture medium consisting of a 1:1 mixture of Ham's F-12 and DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL); they were maintained at 37 °C under a saturated-humidity atmosphere of 95% air and 5% CO_2. The evaluation of the cytotoxicity of Mal-βCD was performed using a lactate dehydrogenase (LDH) activity assay kit (Cytotoxicity Detection Kit^{PLUS}, Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. In addition, Trypan Blue staining was performed to determine the extent of cell death. Mal-βCD at doses below 10 mM did not show cytotoxic effect in *Npc1 KO* and JP17 cells (data not shown).

2.3. Quantification of unesterified and esterified cholesterol, Mal-βCD, and Glc-βCD

Cells were incubated in 10-cm plates (5×10^5 cells/mL) in FBS-containing culture medium, overnight, at 37 °C. The cells were then washed with FBS-free medium, and treated with various doses of Mal-βCD (0–10 mM) for 0.5 h at 37 °C. After treatment, the extracellular medium samples were subjected to LC/MS to quantify uneserified cholesterol levels. The cells were collected by centrifugation at 2300 × *g* for 5 min at 4 °C and washed twice with cold PBS, and the cell samples were divided into two portions. One portion was homogenized in 2-propanol using a SONIFIER (BRANSON, Kanagawa, Japan) to determine cellular unesterified cholesterol levels via LC/MS (see below) and to determine total cholesterol and unesterified cholesterol levels, using a Cholesterol E test Wako kit (Wako, Osaka, Japan) and Free Cholesterol E test Wako kit (Wako) (cholesterol oxidase-DAOS method). The amount of esterified cholesterol was calculated after subtracting the amount of unesterified cholesterol from that of total cholesterol. The other portion was homogenized in PBS, using the SONIFIER, and filtered using a Nanosep 10 K device (Pall Life Sciences, Port Washington, NY, USA); Mal-βCD and Glc-βCD were quantified using LC/MS/MS (see below).

2.4. Preparation of lysosomes and determination of unesterified cholesterol, Mal-βCD, and Glc-βCD contents

Cells (5×10^5 cells/mL) were incubated in FBS-containing culture medium overnight at 37 °C. Thereafter, the cells were washed with the FBS-free medium and incubated in FBS-free medium in the presence or absence of 5 mM Mal-βCD for 0.5, 1.5 or 3 h, at 37 °C. Following the incubation, the cells were collected by centrifugation at 2300 × *g* for 5 min at 4 °C and washed twice with cold PBS. The lysosome-enriched fraction (referred to afterwards as lysosomes) was prepared using the Thermo Fisher Scientific lysosome enrichment kit for tissue and cultured cells in accordance with the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). The activities of lysosomal enzymes acid phosphatase [20] and α-glucosidase [21] were determined using *p*-nitrophenyl phosphate and *p*-nitrophenyl α-D-glucoside as substrates, respectively. The lysosomes were suspended in ice-cold PBS and was divided into two portions: one portion was homogenized in 2-propanol, using the SONIFIER, to determine lysosomal unesterified cholesterol levels by LC/MS (see below); the other portion was homogenized in PBS, using the SONIFIER and filtered using a Nanosep 10 K device to determine βCDs by LC/MS/MS (see below). The amount of unesterified cholesterol and βCDs in the lysosome fraction was expressed in nmol per 100 μg of protein. Protein concentration was determined using Bradford protein assay reagent (Thermo Fisher Scientific).

2.5. Quantification of Mal-βCD, Glc-βCD, and unesterified cholesterol in cell pellets and in the extracellular fluid

Cells were treated with 5 mM Mal-βCD for 0.5 h as described above. Mal-βCD-treated cells were then washed with cold-PBS twice, and the cells (5×10^6 cells in 0.3 mL per each treatment)