2-Hydroxypropyl-β-cyclodextrin blocks autophagy flux and triggers caspase-8-mediated apoptotic cascades in HepG2 cells

HAI'DONG SUN, HUAI JIE ZONG and GANG WU

Department of General Surgery, Huashan Hospital, Fudan University, Shanghai 200040, P.R. China

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Abstract. The cyclodextrin derivative, 2-Hydroxypropyl-β-cyclodextrin (HPβCD), from the cyclodextrin family is widely used as a drug carrier and offers promising strategies for treating neurodegenerative diseases and atherosclerosis regression. However, its side effects are not fully understood. Therefore, the aim of the present study was to investigate the possible adverse effects of relatively high concentrations of HPβCD on hepatocytes. It was found that a high dose (20 mM) of HPβCD treatment significantly inhibited the AKT/mTOR pathway and disrupted infusion of autophagosomes and lysosomes, which rapidly led to massive autophagosome accumulation in HepG2 cells. The autophagosomal membrane serves as a platform for caspase-8 oligomerization, which is considered as the key step for its self-activation. Using flow cytometry and TUNEL assay, increased apoptosis of HepG2 cells treated with a high dose HPβCD (20 mM) for 48 h was observed. In addition, western blotting results demonstrated that the expression of cleaved-caspase-8 was positively associated with microtubule-associated protein 1 light chain 3 B II expression, which is an indicator of autophagosome level in the cytoplasm. Therefore, the present study provided novel evidence that HPβCD might be a potential risk contributing to the pathophysiological process of hepatic diseases, especially in an autophagy-deficient state.

Introduction

Autophagy is a well-conserved intracellular process involving the formation of double membrane structures and the engulfing biological macromolecules, organelles or lipid droplets for degradation (1-5). Moreover, autophagy is important for cell survival, clearance of impaired cellular compartments and lipid balances when challenged with various stressors, including starvation and cholesterol or triglyceride overloading (1-5). Impaired autophagic activities have been shown to increase lipid accumulation, and thus are accompanied by severe lipotoxicity in hepatocytes (4,6). Apoptosis, a major type of programmed cell death, is an evolutionarily conserved process (7). In addition, by activating the cascades of cysteine proteases, known as caspases, apoptosis plays a key role in deciding cell fate when confronted with extrinsic and intrinsic derived stressors (7). Furthermore, apoptosis contributes to the development of various disease states, including carcinogenesis (8), neurodegenerative disorders (9,10) and diabetes (11,12). In addition, p62 (13), Beclin1 and bcl2 (14,15), which are key molecules in autophagy, have been reported to act as a bridge between autophagy and apoptosis; however, the relationship between these factors is not fully understood.

2-Hydroxypropyl-β-cyclodextrin (HPβCD), a chemically-modified water-soluble cyclodextrin derivative, has been widely utilized as a drug delivery system (16-18), as well as an efficient therapeutic strategy for neurodegenerative diseases (19,20) and atherosclerosis regression (19,21), due to its distinctive capability of regulating cellular cholesterol transport and metabolism. Moreover, the main target organs of HPβCD are the kidney, liver, lungs and spleen (22). Approved by the Food and Drug Administration (FDA), HPβCD is recognized as a relatively innocuous therapeutic (18,22), but its adverse effects have rarely been investigated. However, it has been reported that a high dose HPβCD could impede autophagy flux in fibroblasts (23) and in a mouse model of Alzheimer's disease (AD) (24). Furthermore, HPβCD serves as a potent molecule for inducing apoptosis in human leukemic cell lines (25). Collectively, these findings suggest potential negative effects of long-term administration of HPβCD. Therefore, the present study examined the dose-response effects of HPβCD on autophagy and apoptosis in a liver cancer cell line (HepG2), which is widely used as a cellular model for normal hepatocytes to assess the potential of chemical hepatotoxicity (26). The present study aimed to determine whether high dose HPβCD could impair the autophagy flux and trigger apoptosis in HepG2 cells. Autophagy flux blockage leads to autophagosome accumulation, which serves as a platform for caspase-8 activation and may be responsible for the activation of apoptosis (27).
Materials and methods

Reagents and materials. HPβCD, chloroquine (CQ), DMSO, SC 79, 3-Methyladenine (3-MA), Z-1ETD-FMK and water-soluble cholesterol (in methyl-β-cyclodextrin) were purchased from Sigma-Aldrich (Merck KGaA) or APEXBio Technology LLC (Table SII). Details of the antibodies used in the present study are shown in Table SII.

Cell culture. RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin (100 IU/ml penicillin and 100 mg/ml streptomycin) was used as cell culture medium for HepG2 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences). For further experiments, such as flow cytometry or western blot analysis, HepG2 cells were plated at a density of 5x10³ cells/well in six-well plates. When cells reached ~50% confluence, the medium was replaced and HepG2 cells were treated with HPβCD with a combination of either cholesterol, SC 79 (Akt activator), CQ (lysosomal inhibitor), 3-MA (Class III PI3K inhibitor), Z-1ETD-FMK (selective caspase-8 inhibitor) for either 48 h to induce apoptosis or 24 h to detect autophagy flux at 37°C. DMSO (0.5%) was added to cultures as solvent control. After treatment, each well was washed twice with cold PBS and cells were harvested.

To investigate the association between the expression levels of phosphorylated (p)-AKT or p-mTOR and membrane cholesterol levels, the cells were divided into four groups and treated with either 0, 10, 20 or 20 mM HPβCD at 37°C for 12 h. Then, the culture medium was removed, and the cells were washed with PBS twice at room temperature. New culture medium was subsequently added to each group and the fourth group of cells treated with 20 mM HPβCD were further treated with 5 µg/ml free cholesterol at 37°C for another 12 h.

Cell viability assay. A Cell Counting Kit-8 (CCK-8) colorimetric assay (Dojindo Molecular Technologies, Inc.) was used to examine cell viability, according to the manufacturer’s protocol. Prior to the CCK-8 colorimetric assay, HepG2 cells were plated in 96-well plates at 2.5x10⁵ cells/well in a volume of 80 µl cell culture medium and treated with 0.2, 2 or 20 mM HPβCD for 0, 24, 48 or 72 h, respectively. Cells in 6-replicated wells were treated with 8 µl CCK-8 and incubated at 37°C for 2 h. Absorbance was measured at a wavelength of 450 nm using a microtiter plate reader (Tecan Safire 2; Tecan Group, Ltd.). The specific formula used to calculate cell viability was described previously (28).

Monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3) adenovirus transduction. mRFP-GFP-LC3 adenovirus, expressing a tandem RFP-GFP-LC3B fusion protein, was provided by Hanbio Biotechnology Co., Ltd. For mRFP-GFP-LC3 adenovirus transduction, HepG2 cells were plated at a density of 5x10³ cells/well in confocal dishes. Upon reaching 30% confluence, 1 µl adenoviral particles were added into each well, which were used to infect cells at ~10 multiplicity of infection at 37°C for 16 h. After the transduction process, infected cells were treated with 20 mM HPβCD at 37°C for 24 h. Furthermore, 50 µg/ml CQ was utilized as a positive control of autophagosomes accumulation. Then, cells were fixed with 4% paraformaldehyde at room temperature for 30 min and subjected to Zeiss LSM-710 confocal microscopy (magnification, x630) to observe GFP and mRFP staining.

Western blot analysis. HepG2 cell lysates were obtained by RIPA lysis buffer (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions, and protein concentrations were determined using a standard bicinchoninic acid method. Then, 20 µg protein/lane was separated by 12.5% SDS-PAGE. After blocking by 5% BSA (Sigma-Aldrich; Merck KGaA) in TBS-0.1% Tween-20 at room temperature for 2 h, PVDF (EMD Millipore) membranes were cut into different strips according to the size of target proteins and the strips were further incubated with appropriate primary antibodies (1:2,000; Table SII) for ≥12 h overnight at 4°C. The next day, the membranes were incubated with either mouse or rabbit secondary antibodies (1:5,000; Table SII) for 2 h at room temperature. Protein bands were visualized using High-sig ECL western blotting substrate (Tanon Science and Technology, Co., Ltd.) and GAPDH was used as the internal reference protein. Acquired bands were quantified by ImageJ 1.52 software (National Institutes of Health). Each independent experiment was replicated 3 times.

Flow cytometry for detecting apoptotic cells. Flow cytometry was performed using a method described previously (27). Both cell culture medium and attached cells, were collected for centrifugation at 1,332 x g at 25°C for 5 min. Then, cells were washed twice with cold PBS, suspended in 100 µl PBS and incubated with 3 µl Annexin V-FITC and 5 µl Propidium Iodide (PI) for 15 min in the dark at room temperature. Following incubation, 300 µl Annexin V binding buffer was added to each tube and detected by flow cytometry (FACScalibur; BD Biosciences). All Annexin V+ cells were considered as apoptotic cells and data were analyzed by CellQuest software (version 7.5.3; BD Biosciences).

TUNEL assay. After treatment with 20 mM HPβCD at 37°C for 48 h, 200 HepG2 cells/mm² were fixed with 4% paraformaldehyde at 25°C for 15 min. Then, an in-situ cell death detection kit, Fluorescein (Roche Applied Science) was used according to the manufacturer’s instructions. Subsequently, cell nuclei were counterstained with 0.2 µg/ml DAPI at room temperature for 10 min and mounted with glycerol gelatin (Sigma-Aldrich; Merck KGaA). An Olympus IX-71 fluorescence microscope (magnification, x40) was used to acquire the images in ≥3 randomly selected fields of view.

Statistical analysis. Data are presented as the mean ± SEM of ≥3 experimental repeats. Comparisons among multiple groups were performed using one-way ANOVA with Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 21.0 software (IBM Corp.).

Results

HPβCD leads to apoptosis in HepG2 cells. Cell viability was examined to determine whether HPβCD treatment exerted...
any adverse effects on HepG2 cells. It was found that 20 mM HPβCD significantly inhibited cell viability compared with the 0.2 or 2 mM groups at 24, 48 and 72 h time points (Fig. S1). Then, flow cytometry was used to identify Annexin V-FITC/PI stained apoptotic cells. Annexin V+PI− cells were considered as early apoptotic and Annexin V+PI+ cells were considered as late apoptotic (28). The results indicated that high concentration (20 mM) of HPβCD treatment increased the proportion of apoptotic cells compared with control and 2 mM HPβCD-treated groups (Fig. 1a and c), which is in line with the results of TUNEL assay (Fig. S2). Furthermore, HepG2 cells were treated with a high dosage (20 mM) HPβCD for different durations. It was found that the apoptotic rate was significantly higher after 48-h treatment compared with 24-h
Collectively, the results suggested that apoptosis caused by HPβCD treatment occurred in a dose- and time-dependent manner.

Subsequently, these results were assessed using western blotting. Compared with the 2 mM HPβCD treatment group, it was demonstrated that cleaved-poly ADP-ribose polymerase (PARP), a major target of cleaved-caspase-3, was significantly upregulated by 20 mM HPβCD treatment (Fig. 1E). Since apoptosis is induced mainly via extrinsic and intrinsic pathways (7,8), cleaved-caspase-8 and cleaved-caspase-9 expression levels in HepG2 cells with different concentrations of HPβCD treatment were detected. However, the results indicated that there was no significant difference in cleaved-caspase-9 expression, while there was increased expression of cleaved-caspase-8 at higher concentrations of HPβCD, which suggested that the HPβCD-induced HepG2 cell apoptosis did not occur through the intrinsic pathway (Fig. 1E).

**HPβCD blocks autophagy flux in HepG2 cells.** The expression levels of two important autophagy protein markers, LC3 and p62, were detected in HepG2 cells treated with different concentrations of HPβCD. Using the lysosomal inhibitor CQ as a control, it was found that LC3B-II and p62 protein expression levels were significantly upregulated at a high dose of HPβCD compared with controls and low doses of HPβCD-treated cells (Fig. 2A), which suggested that HPβCD blocked autophagy flux in HepG2 cells in a dose-dependent manner. Then, cells were transfected with mRFP-GFP-LC3 adenovirus expressing a tandem RFP-GFP-LC3 fusion protein. As GFP signal is quenched in acidic conditions after fusing with lysosomes, merged red puncta were considered as autolysosomes. ^P<0.01 vs. control. HPβCD, 2-hydroxypropyl-β-cyclodextrin; LC3, microtubule-associated protein 1 light chain 3; CQ, chloroquine; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; HPβCD 0.2, cells treated with 0.2 mM HPβCD; HPβCD 20, cells treated with 20 mM HPβCD; HPβCD 2, cells treated with 2 mM HPβCD; HPβCD 0.2, cells treated with 0.2 mM HPβCD; HPβCD 20, cells treated with 20 mM HPβCD; A.U., arbitrary units.
and yellow dots represent autolysosomes and autophagosomes, respectively (29). Using CQ as a positive control, an obvious cluster of red dots was observed in negative control cells, while HPβcd treatment almost completely inhibited the autophagy flux, as shown by collections of yellow dots (Fig. 2B).

AKT/mTOR axis contributes to autophagosome accumulation and apoptosis in HPβCD-treated HepG2 cells. The protein expression levels of several proteins, including ERK, AMP-activated protein kinase, AKT, mTOR and Beclin-1, were then detected in HepG2 cells with HPβCD treatment. The results suggested that only the AKT/mTOR axis, a master pathway regulating autophagy (30,31), was downregulated, which was reversed via free cholesterol (Fc) replenishment (Figs. 3A and S3). Inhibition of the AKT/mTOR axis caused substantial formation of new autophagosomes, which serve as platforms for caspase-8 activation and subsequent apoptotic cascades (27,32). It was found that SC 79, a potent AKT activator, significantly reduced the expression of LC3B-II protein, which is an indicator for the level of...
autophagosomes (Fig. 3C and E) (29). Furthermore, compared with 20 mM HPβCD-treated cells, significantly decreased cleaved-caspase-8 and PARP protein expression levels (Fig. 3C and E), as well as decreased levels of apoptotic cells, were observed (Fig. 3B and D) after SC 79 treatment.

**Autophagosome accumulation is positively associated with caspase-8 activation.** To further assess the results, the concentration of HPβCD was increased to a higher level, and cells were also incubated with 3-MA, CQ or Z-IETD-FMK. Flow cytometry results demonstrated that higher concentration of HPβCD treatment significantly increased the proportion of apoptotic cells compared with controls and 20 mM HPβCD-treated cells (Fig. 4A and C). Moreover, when incubated with CQ, the percentage of 20 mM HPβCD-treated apoptotic cells significantly increased and the protein expression...
Caspase-8 activation.

The present results suggested that high dosage of HPβCD significantly upregulated compared with cells treated with 20 mM HPβCD alone (Fig. 4A-D). However, after incubation with 3-MA, a Class III PI3K inhibitor, apoptotic cells were significantly reduced and the protein expression levels of LC3B-II, cleaved-PARP and cleaved-caspase-8 were significantly downregulated compared with 20 mM HPβCD-treated cells (Fig. 4A-D). Therefore, the results suggested that HPβCD-induced apoptosis was positively associated with LC3B-II. In addition, it was found that Z-IETD-FMK, a selective caspase-8 inhibitor, almost completely blocked caspase-8 expression and apoptosis was significantly reduced compared with 20 mM HPβCD-treated cells (Fig. 4A-D), which may serve as evidence for autophagosomal membrane-mediated caspase-8 activation.

Discussion

The present results suggested that high dosage of HPβCD inactivated the AKT/mTOR pathway, which facilitated the initiation of autophagy and blocked autophagy flux in the late stage, and thus led to massive autophagosomes accumulation in HepG2 cells. Therefore, it was speculated that the membrane of gradually stacked autophagosomes may serve as platforms for the cleavage of caspase-8, which lead to downstream caspase-cascades and ultimately apoptosis.

Due to its ability to enhance efflux from cell membranes and endo-lysosomal trafficking, HPβCD is a promising therapeutic strategy for treating cholesterol metabolism-related diseases such as Niemann-Pick type C (19) and atherosclerosis regression (21). As a derivative of the cyclodextrin family, HPβCD can directly alter the properties of lipid bilayers by extracting lipids, including cholesterol and phospholipids from biological membranes (33). Lipid rafts, highly ordered membrane domains that are enriched in cholesterol and gangliosides (34), are essential for the transduction of various cell signaling cascades, such as the activation of the PI3K/AKT and Fas/CD95 signaling pathways. In addition, the amount of cellular lipid rafts is reported to be positively associated with cholesterol levels, because cholesterol promotes the formation of lipid rafts (35,36). For instance, increasing membrane cholesterol by adding free cholesterol to prostate cancer cells increased the amount of cellular lipid rafts and, thus, upregulated the phosphorylation of AKT (35), while reducing the membrane cholesterol of glioblastoma cells by inhibiting sterol carrier protein 2, which is one of the main cholesterol transporters targeting the plasma membrane (PM), reduced cellular lipid rafts and subsequently, downregulated the phosphorylation levels of AKT (36).

The present results suggested that HPβCD treatment directly inhibited the AKT/mTOR pathway in HepG2 cells, which was significantly restored after the replenishment of extra FC. Moreover, these results were in line with the results from studies by Oh et al (37,38). Direct alterations of the cholesterol levels of the biological membrane by HPβCD may also cause disruption in the fusion of autophagosomes and lysosomes, thus hindering the autophagy flux in the late stage (23). In the brain tissue of AD model mice or C57BL wild-type mice treated with HPβCD for 2 weeks, a large number of immature autophagosomes are observed (24). In addition, it has been shown that high dose of HPβCD treatment blocked the autophagy flux in fibroblasts (23). Moreover, these findings were identified in the present study, as indicated by increased protein expression levels of LC3B-II and p62, and the accumulation of merged yellow puncta in HepG2 cells. Collectively, due to its modulation of lipid rafts in the PM and disruption of basal lysosome membrane lipid properties, HPβCD inhibited the AKT/mTOR pathway that potentiated the formation of autophagosomes, while the downstream infusion with lysosomes was blocked, thus leading to massive accumulation of autophagosomes in cytoplasm, which may be a risk factor for the development of various diseases, especially in the long-term (39-41).

Caspase-8 plays a vital role in the activation of caspase-cascades for extrinsic signaling pathways (42,43), and oligomerization is a crucial step for caspase-8 activation (44). It has been reported that the autophagosomal membrane acts as a platform for the oligomerization of caspase-8 (32,27). Furthermore, either knockdown of p62 (27) or LC3 (32) is shown to significantly reduce the activity of apoptosis. In addition, pharmacologically inhibiting autophagosome formation using 3-MA, a Class III PI3K inhibitor, attenuated caspase-8 activation in 293T cells (45). However, induction of autophagy by SKI-1, a pansphingosine kinase inhibitor, facilitated the activation of caspase-8 and subsequent caspase-cascades, which is further enhanced when treated with lysosomal inhibitors, including bafilomycin A1, CQ and ammonium chloride (27). In the present study, it was identified that SC 79 and 3-MA significantly mitigated HPβCD-induced autophagosome accumulation and caspase-8 activation in HepG2 cells.
which was via the activation of Class I PI3K and inhibition of the Class III PI3K pathway. However, it was demonstrated that when incubated with CQ, HPβCD further facilitated the activation of caspase-8, which may be due to the effect of CQ alkalizing the acidic condition of lysosomes, and that HPβCD disrupting the lysosomal membrane property. Caspase-8 is the initiator caspase-of the extrinsic apoptotic pathway and is commonly activated by cell surface death receptors (42,46,47).

The present results suggested that Z-IETD-FMK, a selective inhibitor for caspase-8, inhibited the apoptotic activities induced by HPβCD treatment, thus suggesting that autophagosomal membrane-mediated caspase-8 self-activation may be the pivotal mechanism for HPβCD-induced programmed cell death in HepG2 cells.

Moreover, Song et al (48) reported that HPβCD could promote the nuclear translocation of Transcription Factor EB (TFEB), the main regulator of lysosomal function and autophagy, in fibroblasts with a lysosomal storage disorder, which restored its lysosome-autophagy system and enhanced autophagy, in fibroblasts with a lysosomal storage disorder, especially when administrated at high doses or long-term use regarding autophagy and apoptosis should be considered, our knowledge, the present study was the first to demonstrate that, accompanied by the downregulation of the AKT/mTOR pathway, a master pro-survival and autophagy regulating signaling axis, high doses of HPβCD treatment impaired autophagy flux and induced autophagosome caspase-8-mediated apoptosis in hepatocytes; the specific mechanism of which is depicted in Fig. 5. While HPβCD has been extensively studied and approved by the FDA, its potential adverse effects regarding autophagy and apoptosis should be considered, especially when administrated at high doses or long-term use for patients with disfunctions of kidney, which is the main organ for the clearance of HPβCD (18,22). The present study provided novel evidence of the significance of the side effects of HPβCD administration with regard to particular conditions. For instance, HPβCD may worsen the status of patients with non-alcoholic fatty liver disease or impede the recovery of patients who have undergone liver transplantation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ and GW conceived the study, carried out the experimental design and data interpretation, and prepared and revised the manuscript. HS performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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