RESEARCH ARTICLE

Trivalent Chromium Induces Autophagy by Activating Sphingomyelin Phosphodiesterase 2 and Increasing Cellular Ceramide Levels in Renal HK2 Cells†

Running title: Cr(III), SMPD2 and autophagy

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Abbreviations: AIF, apoptosis-inducing factor; AM, autophagosome membrane; AO, acridine orange; ATAD3A, the ATPase family, AAA domain containing 3A; BPB, bromophenol blue; CERS, ceramide synthase; CI_{50}, half maximal inhibitory concentration; Cr(III), trivalent chromium; Cr(VI), hexavalent chromium; CERT, ceramide-transport protein; DDH, dihydrodiol dehydrogenase; DHE, dihydroethidium; DRP1, dynamin-related protein 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ER-EGFP, enhanced green fluorescent protein (EGFP)-conjugated ER-retention signal KDEL; ICM, immunofluorescence confocal microscopy; LBPA, lysobisphosphatidic acid; LC3, microtubule-associated protein 1 light chain 3; MAM, mitochondria-associated membrane; MP, bis(monoacylglyceryl) phosphate; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SMPD2, sphingomyelin phosphodiesterase 2; TLR, toll-like receptor

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Abstract

In this study, we examined the role of autophagy in the initiation of lipid increases in renal epithelial HK2 cells. We found that trivalent chromium [Cr(III)] induced autophagy by activating sphingomyelin phosphodiesterase 2 (SMPD2). SMPD2 increases levels of ceramide and other lipids. Confocal immunofluorescence microscopy showed that signals of ceramide overlapped with LC3, suggesting that ceramide might play an important role in the formation of autophagosome. In conclusion, our data indicate that Cr(III) induces autophagy via structural aberration of organelle membrane, in particular by the increase of lipid compositions in addition to autophagy-associated proteins. This article is protected by copyright. All rights reserved

**Key words:** sphingomyelin phosphodiesterase 2; ceramide; autophagy; reactive oxygen species; dihydrodiol dehydrogenase; ATAD3A
INTRODUCTION

In recent years, autophagy [1-4] has emerged as a major target for clinically valuable anticancer chemotherapeutics (e.g., intercalating agents) [5] and cardiac protection drugs (e.g., cardiac glycosides) [6]. In response to cell stress, part of cytoplasm as well as damaged and aging organelles, including mitochondria, are surrounded by a double membrane structure, an isolation membrane [1,7]. Subsequently, the activated autophagy-related gene 3 (ATG3) mediates conjugation of phosphatidylethanolamine (PE) to microtubule-associated protein 1A/1B-light chain 3 (LC3-I) to form LC3-II [1,8] complex. The open end of the growing autophagosome is then sealed by an enzyme complex, composed of ATG5, ATG12, and ATG16. The bent margins of double-layered lipids are separated into distinctive inner and outer membranes [1,2].

Because PE is synthesized in the mitochondria by phosphatidylserine (PS) decarboxylase (PSD) [9]. It has therefore been suggested that the source of autophagosome membranes could be derived from the mitochondria [3] or from the contact sites between the endoplasmic reticulum and mitochondria, i.e., the mitochondria-associated membranes (MAMs) [4]. The characteristics of the other membrane lipids in autophagosomes and the differential sensitivity of the inner membrane as well as the selective resistance of the outer membrane to lysosomal enzymes, however, have not been studied.

Ceramide is a member of sphingolipids, which constitute a part of membrane structures [10]. Three biochemical pathways (i.e., de novo biosynthesis, sphingomyelinase [SMase] hydrolysis, and the salvage pathway, involving the actions of ceramide synthases [CERS]) have been identified. Interestingly, ceramide is mostly synthesized in the inner leaflet of the ER and MAM by dihydroceramide (DHcer) synthase and DHcer desaturase [11-13]. The newly generated

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ceramide is then distributed to the other organelles. Hanada et al. showed that ceramide was transferred by a ceramide-transport protein (CERT) in an ATP-dependent manner from the ER to the Golgi apparatus [14]. Nonetheless, CERT mutation was not lethal to the cells. Funato and Riezman suggested that an ATP-independent vesicular passage might be present to supplement ceramide transport [15]. It is worth noting that ceramide as well as its derivatives, DHcer and sphingosine-1-phosphate (S-1-P) [16], induces autophagy. These facts implicate that the newly synthesized ceramide and its derivatives have to be protected [14,17-19] or rapidly concealed to prevent the cells from accidentally inducing autophagy prior to export of ceramide to the other organelles. However, the mechanism of ceramide transport has not been studied in detail.

Interestingly, while using trivalent chromium [Cr(III)] as an experiment control for hexavalent chromium [Cr(VI)] studies, we found that Cr(III), a supplement nutrient for type II diabetes, induced a substantial increase in staining intensity of acridine orange, a live dye of cellular autophagy, in kidney tubular epithelial (KTE) HK2 cells [20,21]. However, such effect and mechanism of Cr(III) on KTE cells has not been reported. In this study, we characterize the effect of Cr(III) on membrane changes and induction of autophagy in vitro. Moreover, we found that lipid conversion played a role in the initiation of autophagy, in particular the activation of sphingomyelin phosphodiesterase 2 (SMPD2) and the increase of ceramide on the organelle membrane.
MATERIALS AND METHODS

Materials

Acridine orange, bovine serum albumin, DMSO, glutaraldehyde, lead citrate, osmium tetroxide, paraformaldehyde, polybrene, protease inhibitor cocktail, shikonin, sodium deoxycholate, sodium orthovanadate, trivalent chromium [chromium(III) potassium sulphate dodecahydrate], uranyl acetate, and mouse anti-β-actin antibodies were purchased from Sigma Aldrich® (Basel, Switzerland). Antibodies to poly (ADP-ribose) polymerase (PARP) and vimentin were acquired from Cell Signalling Technology, Inc. (Danvers, MA). Antibodies to connexin-43 (CX-43) were from Merck Millipore. Agarose I™, PMSF, SDS, and sodium chloride were from Amresco. NP-40 and Tris-HCl were from USB Corporation. Sodium fluoride was from Fluka Biochemika. STS was from Calbiochem. Hoechst 33342, MitoTracker® Red CMXRos (M7512), and goat anti-mouse or anti-rabbit conjugated with Alexa Fluor 488 or Alexa Fluor 546 secondary antibodies were from Invitrogen. Mouse anti-ceramide primary antibodies and Cyto-ID® Green dye were obtained from Enzo Life Sciences. Mouse anti-LC3 was from Medical & Biological Laboratories. Rabbit anti-LC3 was from Cell Signalling Technology. Horseradish peroxidase-labelled (HRP) goat anti-mouse IgG or anti-rabbit IgG were purchased from Jackson Immuno Research Laboratories. LipidTOX™ and lyso-RFP (CellLight® Lysosomes-RFP, BacMam 2.0) were from ThermoFisher Scientific (Rockford, IL, USA).

Cell Culture

HK-2 cells (ATCC CRL®-2190™) were used for the in vitro effect evaluation of Cr(III). HK-2 is a human papilloma virus E6/E7-immortalized human kidney cell line derived from proximal tubules. HEK293 (CRL®-1573™), 293T (CRL®-3216™) and three human lung
adenocarcinoma (LADC) cell lines (H23, H838 and A549) [22,23], were also used in the study. Cells were maintained at 37°C as a monolayer in DMEM/F12, or RPMI-1640 for LADC cells supplemented with 10% foetal calf serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin in a humidified 5% CO$_2$ incubator.

Western Blot Analysis

Western blot was performed following the previously described methods [22,24]. Briefly, total cell lysate was prepared by resuspending $5 \times 10^7$ cells in 100 µl PBS, and then mixing with equal volume of 2 × NP-40 lysis buffer [40 mM Tris-HCl, pH 7.6, 2 mM EDTA, 300 mM NaCl, 2 mM phenylmethylsulfonylfluoride (PMSF2%), and NP-40]. Loading buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM PMSF, 1 mM Na$_2$EDTA, 10% glycerol, 5% β-mercaptoethanol, 0.01% BPB and 1% SDS), 1/20 of volume, was added to cell lysate prior to electrophoresis, which was carried out in a 10% polyacrylamide gel with 4.5% stacking. Following electrophoresis, proteins on the gel were transferred to a nitrocellulose membrane. The membrane was probed with specific antibodies. The signal was amplified by biotin-labelled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. The protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY) with enhanced chemiluminescent reagent (NEN, Boston, MA, USA). Antibodies for β-actin were obtained from Chemicon International (Temecula, CA, USA). The digital images on X-Omat film were processed using Adobe Photoshop 7.0 (http://www.adobe.com/). Intensity of each immunoblotting band was analysed and quantified using the image-J software (NIH, Bethesda, MD, USA). The blots were stripped using Restore Western Blot Stripping Buffer (ThermoFisher Scientific, Rockford, IL, USA) before incubation with other antibodies.

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Cr(III)-Sensitivity Assay

Cr(III) sensitivity was measured by a WST-1 assay [20]. Cells were seeded at 100, 1,000, and 5,000 cells/96-well plate 18 hr prior to Cr(III) challenge. Cells were continuously incubated with various concentrations (ranging from 0.1 to 10 μM) of hexavalent chromium. The negative control cells were treated with the phosphate-buffered saline, a solvent for the Cr(III). Total survival of the cells was determined 72 hr following Cr(III) challenge, and percent survival was estimated by dividing optical absorbance resulted from each test group with that of the control group. Each experiment was done in triplicates, and the optical absorbance was measured by a change of colourless WST-1 to bright yellow colour of oxidized WST-1 (BioVision, Mountain View, CA, USA). Oxidation of WST-1 was catalysed by mitochondrial dehydrogenase. The line graph drawing of cytotoxicity was performed using GraphPad Prism6 statistics software (San Diego, CA, USA).

For determining the effect of Cr(III) on inducing autophagy, the HK-2 cells were exposed to a serial dilution of trivalent chromium potassium sulphate dodecahydrate (ranging from 0.25 to 10 mM) for 1 to 72 hr. Culture medium was removed, and the cells were rinsed once with phosphate buffered saline (PBS) before addition of acridine orange (AO) solution to 20 mg/ml PBS. The reaction was carried out at room temperature for 15 minutes. The AO solution was removed and the cells were rinsed once with PBS before observing under a fluorescence microscope [20].

Immunofluorescence Microscopy

The method for our immunofluorescence microscopy had been previously described [22,24].
Concisely, the HK-2 cells, which were grown on aminopropyl silane-coated slides, were fixed with 4% paraformaldehyde at room temperature for 15 minutes and permeabilised with 0.1% Triton X-100 prior to staining with antibodies to ceramide, apoptosis-inducing factor (AIF) or CX-43. After washing off the primary antibodies, the slide was incubated with fluorescein- or Alexa 488-conjugated secondary antibodies (Invitrogen™, Life Technologies, Grand Island, NY, USA). Nuclei of the cells were stained with 4’, 6-diamidino-2-phenylindole (DAPI) and the slides were examined under a fluorescence microscope (Olympus BX51, Tokyo, Japan). Fluorescence was activated using X-Cite® 120 Fluorescence Microscope Illumination System (EXFO, Quebec, Canada). For confocal immunofluorescence microscopic examination, images were captured by using Laser Scanning Confocal Microscopy, LSM510 (Carl Zeiss, New York, USA). Images were generated by using Adobe Photoshop.

Electron Microscopy
Cells were fixed in situ on culture dish with 2.5% glutaraldehyde (Sigma, Missouri, USA) in 100 mM PBS (pH 7.2) at 4°C overnight. Cells were washed with PB before post-fixation with 1% osmium tetroxide in PB for 1 hour. After washing with distilled water, the cells were suspended in 2% agarose, and the agarose blocks were trimmed and dehydrated in a serial dilution of ethanol for 15 min each. The blocks were further dehydrated with 100% ethanol for 15 min three times, and infiltrated with 100% ethanol/LR white (1:1) mixture overnight. The blocks were changed to the LR white (Agar Scientific Ltd., Essex, UK) for continuous infiltration at 4°C for 24 hours before transferred to a capsule filled with LR white. LR white were polymerized and solidified at 60°C for 48 hours. The resin blocks were trimmed and cut with ultramicrotome (Leica Ultracut R, Vienna, Austria). The thin sections were transferred to 200 mesh copper grids, and stained with
2% uranyl acetate for 15 min, and 2.66% lead citrate (pH 12.0) for 15 min, before observation with JEM1400 electron microscope (JEOL USA, Inc., Massachusetts, USA) at 100-120 kV.

Lentivirus Production and Infection

Lentiviral vector carrying shRNA was prepared by a three-plasmid transfection method. Briefly, the pMD.G containing vesicular stomatitis virus glycoprotein, and pCMV-Δ 8.91 carrying HIV-based packaging plasmid were co-transfected with shRNA-expressing lentiviral vector into 293T cells. The medium was changed to fresh DMEM containing 10 mg/mL BSA 24 hours post-transfection. The supernatant containing lentivirus was collected at 48 hour and 72 hour post-transfection, respectively, and was used to infect HEK293 cells in the presence of 8 μg/ml polybrene. After infection, the cells were selected by 1 μg/ml puromycin.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells by using an SNAP RNA column (Invitrogen, San Diego, CA, USA). After measuring RNA yield, cDNA was synthesized by AMV reverse transcriptase using oligo random primers. An aliquot of cDNA was then subjected to 35 cycles of PCR. The reaction mixture contained 50 mM Tris (pH 7.2), 1.5 mM MgCl₂, 2 μM dNTP, 0.25 μM of 5’ and 3’ primers respectively (Primer sequences were listed in Table 1), 0.5 U of Taq DNA polymerase (Protech Technology, Taipei, Taiwan), and 2 μl of cDNA (1 μg). PCR was carried out according to the standard procedures: denaturing at 94°C for 30 seconds, hybridizing at 55°C for 45 seconds, and elongating at 72°C for 2 minutes. The amplified PCR products were analysed in 1% agarose gel, and visualized by ethidium bromide staining [22,23].
RESULTS

Cr(III) Increases Massive Acridine Orange Staining in Renal Epithelial HK-2 Cells

Addition of Cr(III) increased substantial acridine orange (AO) staining, a hallmark of autophagy that appeared as crimson intracytoplasmic vesicles [21], in a dose-dependent manner in HK-2 cells (Figure 1A). Unlike Cr(VI), of which the half maximal inhibitory concentration (IC$_{50}$) of cell growth was 1.21 µM, IC$_{50}$ of Cr(III) was higher than 10.0 mM (Figure 1B). Difference of IC$_{50}$ between Cr(VI) and Cr(III) was more than 10,000 folds. Cr(VI) was much more inhibitory and toxic. Induction of autophagy was confirmed by the appearance of LC3B-II in a Western blot analysis (Figure 1C). Formation of autophagic vesicles (AV) surrounded by green fluorescence protein-conjugated LC3B (Figure 1D) and an appearance of autophagic vesicles in electron micrographs (Figure 1E) further supported our findings. Interestingly, no mitochondria, but invaginated membranous structures were detected in Cr(III)-induced AV in electron micrographs. Cr(III) decreased protein level of dihydrodiol dehydrogenase (DDH (also named as aldo-keto reductase 1C2), but not that of ATPase family AAA domain-containing 3A (ATAD3A). ATAD3A is essential for intracellular material transport from the endoplasmic reticulum (ER) to the mitochondria [22]. Moreover, addition of Cr(III) did not reduce mitochondrial staining (MitoTracker®), an enzyme marker for mitochondrial damage (Figure 1F). Neither did Cr(III) increase cleavage of Poly (ADP-ribose) polymerase (PARP), or nuclear translocation of apoptosis-inducing factor (AIF) (Figure 1G), an apoptotic signal of mitochondrial depolarization [22,23].

Addition of Cr(III) Markedly Increases Contents of Lipid, Including Ceramide, in HK2 Cells

Cr(VI) has chemically higher electric potential, therefore it is compulsorily reduced to
Cr(III) intracellularly [25]. Besides formation of mutagenic Cr(III)-DNA adducts, a large scale of Cr(VI) reduction might generate a substantial reactive oxygen species (ROS) and cause oxygen depletion [26]. Our results showed that Cr(III) alone also increased intracellular ROS levels (Figure 2A), as measured by dihydroethidium (DHE) staining. However, Cr(III) did not affect expression of vimentin, a marker of mesenchymal cells [27-29], or connexin-43 (CX-43), a structural protein of gap junction which served as a typical non-stem cell surface marker for mature human epithelial cells (Figures 2B and 2C) [30]. Neither Cr(III) affect cell morphology (Figure 2D). Dramatic differences among measures of AO staining, ROS production and changes of protein levels, however, suggested that Cr(III) might affect other major cellular constituents. As shown by electron micrographs, increase of vesicular formation indicated that Cr(III) could influence intracellular lipid composition. Immunofluorescence microscopy demonstrated that intracellular levels of ceramide, cholesterol and bis(monoacylglyceryl) phosphate (BMP), were all elevated (Figure 2E). In addition, level of polar lipids (stained by ReZolve-L1) (Figure 2F) and phospholipids (stained by LipidTox) (Figure 2G) was markedly increased as well. In these experiments, shikonin was used as a positive control for autophagy induction (Figure S1).

Silencing of SMPD2 Reduces Ceramide Levels and Inhibits Cr(III)-induced Autophagy

As noted previously, addition of Cr(III) increased cellular ceramide levels [14] and addition of ceramide alone induced autophagy [17-19]. Unlike Cr(III), the acridine orange (AO) staining was mostly crimson fluorescence (acidified AO), ceramide-induced AO staining was a mixture of amber (neutral AO) and crimson fluorescence (Figure 3A), implying that Cr(III) could activate lysosomal fusion. Ceramide, on the other hand, had less effect on acidification of acridine orange. Previous studies had shown that ceramide could be synthesized by three established pathways,
namely, (A) a *de novo* pathway, which involves dihydroceramide (DHcer) synthase and DHcer desaturase; or (B) a salvage pathway, which involve 6 types of ceramide synthases (CERS); and (C) hydrolysis of sphingomyelin by four different types of sphingomyelinase (SMase). To determine the gene that is crucial for ceramide synthesis during initiation of autophagy, the respective gene was silenced [levels of mRNA were determined by RT-PCR (Figure 3B)] before cells challenged with Cr(III) or shikonin. Results showed that knockdown of sphingomyelin phosphodiesterase 2 (SMPD2) clearly inhibited autophagy-associated increase of ceramide levels (Figures 3C and 3D). Silencing of CERS 5 had lesser effect.

**Silencing of SMPD2 Reduces Rapamycin-Induced Formation of Autophagosome**

Knockdown of SMPD2 (Figure 4A1) or CERS5 (Figure 4A2) did not evidently affect LC3 conversion during rapamycin, an autophagy inducer, treatment (Figure 4A3). Silencing of SMPD2, however, reduced rapamycin-induced formation of autophagosome (Figure 4B). When formation of autophagosome was examined in a transfectant of lyso-RFP (CellLight® Lysosomes-RFP, BacMam 2.0, ThermoFisher Scientific), which labelled lysosome, the fluorescence intensity of lyso-RFP was markedly reduced in SMPD2\textsuperscript{KD} cells (Figure 4C).
DISCUSSION

Results presented above show that Cr(III) induces autophagy. Cr(III) inhibits DDH to increase massive amount of ROS, and activates SMPD2 to induce formation of autophagosomes, which contain mainly cytoplasmic membranes. These data suggested that in addition to PE [8], ceramide, cholesterol and BMP could be constituent lipids of autophagosome membrane (AM). Moreover, the presence of ceramide in AM provides an explanation for the selective sensitivity of autophagosomal inner membranes to lysosomal enzymes.

A study by Holopainen et al. showed that ceramide-containing domains on the membrane could spontaneously form a negative curvature, (i.e., a concave membrane) [31]. Cooke and Deserno, using a coarse-grained lipid model, found that PE-containing regions of the membrane produced a positive curvature, (i.e., a convex bilayer) [32]. Silencing of DRP1 expression inhibits budding-off of transport vesicles from MAM, leading to accumulation of materials that were scheduled for shipping to mitochondria in MAM, and causing the MAM to swell [24]. Accumulated products, including sphingomyelinases, could be aberrantly activated to generate ceramide and to induce invagination of the expanded membrane. The concave curvature of ceramide-containing membrane on the inside gradually approached the convex curvature of PE-containing lipid bilayer on the outside and formed a bowl-shaped double membrane structure (the pre-autophagosome or, the isolation membrane) [3,4] (As shown in Figure S2, the hypothetical formation of isolation membrane, megasome, and autophagosomes). Our study model suggested that besides autophagy-related proteins, formation of autophagosomes could be directed by an asymmetrical distribution of phospholipid subtypes in the local membrane. Our model also suggested that some specific shape of organelle membrane could be governed by a sheer physical force embedded in intermolecular lipid reaction with parametric protein factors.
It is worth noting that although phosphatidylethanolamine (PE) is not readily conjugated to ATG8 (LC3-I) in Atg5\(^{-/-}\) or Atg7\(^{-/-}\) cells, the autophagosome is formed despite the lack of LC3-II and ATG5-ATG12-ATG16 protein complex assembly, indicating that other mechanisms may be involved in the initiation of autophagy [33-35]. Composition of different lipid subtypes may be as imperative as protein lipidation in the formation of intracellular phagocytic vacuoles. In fact, two types of intracellular vacuole have been identified. Vacuoles, which are originated extracellularly by phagocytosis or endocytosis, are induced by microbes binding to toll-like receptor (TLR), or ligand-receptor binding, e.g., epidermal growth factor (EGF) to EGF receptor (EGFR) [36]. Cholesterol- and ceramide-enriched membranes facilitate invagination of plasma membranes during phagosomal or endosomal formation [36,37]. Membranes of this type of vacuoles measured approximately 9-10 nm and are coated with signal transduction-associated proteins, such as clathrin [37].

Vacuoles that originated intracellularly, by contrast, such as those derived from autophagy [7,33] or intracytoplasmic phagocytosis reacting to the escaped microbes (e.g., *Listeria monocytogenes*, in innate immunity) [38], their membranes measured around 6-7 nm and were coated with LC3-II. The intracytoplasmic phagocytic vacuoles were formed around mitochondria, which might already have certain levels of membrane damages [7,38,39]. Our results supported their findings, showing that the enlarged MAMs were juxta-positioned to the mitochondria. Outer membranes of those mitochondria were evidently abraded or had disappeared, indicating that analogous to intracellular microbes, the injured mitochondria might trigger signals to segregate the impaired organelles to confine cellular injuries.

By studying the transportation of apoptosis-inducing factor (AIF) and cytochrome C (cyt C), the two apoptosis-provoking proteins in mitochondrial intermembrane space, we serendipitously
found an alternate mitochondrial import pathway, which showed that AIF and cyt C were synthesized in the ER and transported to the mitochondria through MAM by a vesicular passage [21]. In this way, both AIF and cyt C were concealed away from caspase 9 and human homolog of rad23A (hHR23A) to avoid inadvertent initiation of apoptosis [40]. Likewise, ceramide and its derivatives, dihydroceramide and sphingosine-1-phosphate (S-1-P), had been shown to induce autophagy. Therefore, the newly synthesized ceramide and its derivatives must be protected or rapidly covered to prevent cells from accidental induction of autophagy prior to be properly exported to the target organelles [14,17-19]. A study by Hanada et al. showed that ceramide was transferred by a ceramide-transport protein (CERT) in an ATP-dependent manner from the ER to the Golgi apparatus [4]. However, because CERT mutation was not lethal, Funato and Riezman suggested that an ATP-independent vesicular passage could be involved in shipping the required ceramide to the organelles [15].

In fact, in addition to soluble neutral sphingomyelinase (nSMase, AF069740), other types of SMase and CERS, including DHcer desaturase, are all membrane-bound (Figures S3A and S3B). The active sites of sphingomyelin phosphodiesterase 1 [SMPD1, or acid sphingomyelinase (aSMase), X63600], CERS2, and CERS6 are facing the lumen of the ER. The active sites of SMPD2, SMPD3, CERS1, CERS3, CERS4, and CERS5, on the other hand, are facing the cytoplasm. Our data suggested that SMPD2 was the major enzyme involved in autophagosome formation. CERS5 has a lesser role.

In conclusion, our data showed that Cr(III) inhibited DDH expression to increase intracellular ROS levels and to induce autophagy by activating SMPD2 and increasing cellular ceramide levels. The effect of SMPD2 on autophagosomal formation was confirmed by addition of rapamycin, an autophagy inducer. Overlapping of images with LC3 suggested that ceramide...
could be a constituent of autophagosome membranes, which were originated from abnormally enlarged MAMs to corral the aged proteins and the damaged mitochondria for the purpose of recycling building block material. Furthermore, our findings could shed some light onto the recent histological observations in accumulated trivalent chromium [Cr(III)]-related fatty lesions in mouse liver and nephrotic tubules [41].
REFERENCES


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AUTHOR CONTRIBUTIONS

C.L.Y., experiment manipulations, data analysis and manuscript preparation; S.H.C., K.C.C., experiment manipulations and data analysis; C.L.Y. W.C.T. and N.A.J., RT-PCR, ELISA and raise of monoclonal antibodies; K.C.C. perception of the idea, experimental design, guidance, manuscript preparation and writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.
Figure 1. Cr(III) induces autophagy, but not evident cytotoxicity of renal epithelial HK-2 cells. (A) Addition of Cr(III) increased substantial acridine orange (AO) staining, an autophagy hallmark that appeared as yellow to crimson intracytoplasmic vesicles, in a dose-dependent manner in HK-2 cells (The quantitative difference of AO staining is shown in Figure S4A). (B) IC$_{50}$ of Cr(VI) in HK-2 cells was estimated to be around 1.21 µM, and that of Cr(III), however, was higher than 10.0 mM. (C) Induction of autophagy by Cr(III) was confirmed by the appearance of LC3B-II in a Western blotting analysis. Concurrently, Cr(III) inhibited expression levels of DDH, but not ATAD3A. The quantitative difference of protein band intensity is shown beneath each respective Figure. (D) Cr(III) induced autophagic vesicle formation, which was shown by green fluorescent LC3-II. (E) Cr(III)-induced autophagosome formation was visualized by electron microscopy. No mitochondria, but vesicular membrane structures were identified. (F) Cr(III) did not mediate mitochondrial damage, which was determined by an appearance of cleaved Poly (ADP-ribose) polymerase (PARP). (G) Cr(III) did not activate cell apoptosis, which was determined by a decrease of mitochondrial enzyme staining (MitoTracker®) or the nuclear translocation of apoptosis-inducing factor (AIF), a sign of mitochondrial depolarization and the initiation of irreversible apoptosis.

Figure 2. Cr(III) treatment significantly increases lipid contents in HK2 cells. (A) Cr(III) increased intracellular levels of reactive oxygen species (ROS), as measured by staining with dihydroethidium (DHE). (B) Cr(III) did not significantly affect expression of vimentin or that of connexin-43 (CX-43) as determined by a fluorescence microscopy. (C) Cr(III) did not clearly
affect vimentin or CX-43 expression as determined by Western blotting analysis. (D) Cr(III) did not markedly influence HK2 cell morphology (arrows indicate putative EMT cells). (E) Cr(III) increased intracellular concentration of bis(monoacylglyceryl) phosphate (BMP), ceramide (stained by immunofluorescence monoclonal antibodies) and cholesterol (stained by filipin). (F) Cr(III) induced autophagy as measured by an increase of Cyto-ID® Green stain or immunofluorescence stain of LAMP1. (G) Cr(III) increased levels of phospholipids in cells (stained by LipidTox). Chloroquine raises pH in autophagolysosome and inhibits lysosomal enzyme activity. Shikonin was used as a positive control of autophagy induction (Figure S1). The quantitative difference of cell staining as measured by a fluorescence microscopy is shown in Figure S4B-S4G.

Figure 3. Expression of SMPD2 is associated with initiation of autophagy. (A) Addition of either Cr(III) or ceramide induced autophagy, and substantially increased acridine orange staining. In Cr(III)-treated cells, the fluorescence of AO staining is a dark crimson colour (acidified AO). In ceramide-treated cells fluorescence of AO staining was a mixture of amber (a blending of neutral and acidified AO) and crimson colours. (B) Measurement of the mRNA level of the respectively silenced genes by reverse transcription-polymerase chain reaction (RT-PCR), which are essential for ceramide synthesis. (C) Knockdown of SMPD2 inhibited shikonin- and Cr(III)-induced increase of ceramide level as determined by fluorescence microscopy. (D) The change of ceramide levels and appearance of autophagosomes in the respective gene knockdown cells. □, Fluorescence intensity; ■, number of vesicles; The results are shown as the means ± S.D. of three independent experiments. Number of vesicles was measured from 10 randomly selected cells following Cr(III)- or shikonin-treatment. Silencing of CERS 5 had lesser effect.
Figure 4. Silencing of SMPD2 expression reduces rapamycin-induced autophagy. (A) The effect of the respective gene knockdown on LC3 conversion during rapamycin treatment. (A1) Using siRNA to knockdown gene expression reduced about 75% protein level of SMPD2 as determined by film scanning. (A2) Using siRNA to silence gene expression decreased about 70% protein contents of CERS 5 as determined by film scanning. (A3) Rapamycin-induced conversion of LC3-II from LC3-I was not evidently affected in SMPD2\textsuperscript{KD} or CERS 5\textsuperscript{KD} cells. (B) To consolidate the role of SMPD2 in the initiation of autophagy, rapamycin was respectively added to the wildtype and SMPD2\textsuperscript{KD} cells. Irrefutably, silencing of SMPD2 reduced rapamycin-induced formation of autophagosome as measured by the intensity of ceramide and the presence of ceramide-positive intracellular vesicles. (C) When formation of autophagosome was examined in lyso-RFP-transfected cells, fluorescence intensity of lyso-RFP, which labelled lysosomes, was markedly decreased in SMPD2\textsuperscript{KD} cells.
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<td>R: CAGCTTCACCCTCCAGTTT</td>
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F and R indicate the forward and reverse primers respectively. F, forward; R, reverse.
Figure 1
Figure 2
Figure 3
Figure 4

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