Desipramine Ameliorates Cr(VI)-Induced Hepatocellular Apoptosis via the Inhibition of Ceramide Channel Formation and Mitochondrial PTP Opening

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Key Words
Cr(VI) • DES • ASMase • Ceramide • Apoptosis • Mitochondrial PTP

Abstract
Background: Hexavalent chromium (Cr(VI)) is a common environmental pollutant. Cr(VI) exposure can lead to severe damage in the liver, but the preventive measures to diminish Cr(VI)-induced hepatotoxicity need further study. Acid sphingomyelinase (ASMase) is responsible for the production of ceramide via the hydrolysis of sphingomyelin. The present study was designed to investigate effects of desipramine (DES), as an ASMase inhibitor, on Cr(VI)-induced hepatotoxicity. Methods: L-02 hepatocytes were incubated with different concentrations of Cr(VI) for 24h, and ASMase activities and ceramide levels were measured. Moreover, the study investigated the role of DES played in ASMase activities and ceramide levels. Finally, effects of DES on mRNA and protein expressions of the components of mitochondrial permeability transition pore (PTP) and PTP opening were detected. Results: The ASMase activities and ceramide contents increased in L-02 hepatocytes treated with Cr(VI). The results demonstrated that apoptosis rates, ASMase activities and ceramide content decreased in groups treated with the combination of DES and Cr(VI) compared to Cr(VI) groups. Furthermore, DES inhibited Cr(VI)-induced mitochondrial PTP opening by intervening the mRNA and protein expressions of the components of mitochondrial PTP. Conclusions: DES may exert protective effects on Cr(VI)-induced hepatocellular apoptosis probably by inhibiting ceramide channel formation and mitochondrial PTP opening.

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Introduction

Chromium is a silvery-white metal, which existed in the environment principally in two main valence states: trivalent (Cr(III)) and hexavalent (Cr(VI)) forms [1]. Compared with trivalent chromium (Cr(III)) that hardly penetrates cell membrane, hexavalent chromium (Cr(VI)) is highly toxic and has been widely used in industrial production, such as welding, metallurgy and leather tanning [2]. In the past, studies about Cr(VI) always focused on its respiratory damage, such as nasal perforation and bronchiogenic cancer, however, some studies suggested its ingestion carcinogenicity in animals and human [3, 4]. Recently, severe water pollution containing Cr(VI) has drawn researchers’ attention to preventive measures to control liver damage caused by Cr(VI) through digestive system.

Acid sphingomyelinase (ASMase) is one member of the enzyme family that cleaves the phosphodiester bond of sphingomyelin, generating phosphorylcholine and ceramide [5]. The resultant ceramide, as a key point of sphingolipid metabolism, is involved in several cellular responses, such as cell growth inhibition, apoptosis induction, senescence modulation and endoplasmic reticulum stress responses [6]. The relationship between liver injury and the ASMase/ceramide signaling pathway have drawn much attention from scientists [7]. A great number of researches have demonstrated that the liver is a major site for ASMase expression and deposition, and ASMase activation and subsequent ceramide generation play important roles in homeostatic mechanisms within the liver [8, 9]. Thus ASMase inhibitors were considered to be protective against hepatotoxicity induced by exogenous toxicants.

Desipramine (DES), as a tricyclic antidepressant, is one member of indirectly functional inhibitors of ASMase, and has been used in clinical applications for over 30 years [10]. DES efficiently reduced ASMase activities by interfering the binding of ASMase to the lipid bilayer, displacing the enzyme from its membrane-bound substrate, and thereby making ASMase susceptible to proteolytic degradation [11].

Although several studies had shown that blocking the ASMase/ceramide pathway by genetic or pharmacological approaches diminished liver injuries, the role of ASMase in Cr(VI)-induced hepatotoxicity has not been studied. The present study was undertaken to explore whether DES, as an ASMase inhibitor can modulate Cr(VI)-induced hepatotoxicity.

Materials and Methods

Materials

Potassium dichromate (K₂Cr₂O₇), desipramine (DES) and Igepal CA-630 were purchased from Sigma-Aldrich. RNA isolation and RT-PCR reagents were obtained from Invitrogen. BODIPY C₁₂-Sphingomyelin (B₁₂-Spm) and naphthalene-2,3-dicarboxyaldehyde (NDA) were from Molecular Probes. NBD C₁₂-Ceramide for acid sphingomyelinase assay and C₁₀-Ceramide for ceramide assay were purchased from Cayman Chemical and Avanti Polar Lipids respectively. All other chemicals and solvents were of analytical grade and obtained from Sigma-Aldrich except those specifically mentioned.

Cell culture

Human normal liver cell line (L-02) was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS at 37℃ in 5% CO₂.

Detection of ASMase activities

Activities of acid sphingomyelinase (ASMase) were measured by the modification of Xingxuan He’s protocol [12]. L-02 hepatocytes were harvested and re-suspended in ice-cold lysis buffer (20mM HEPES, 150mM NaCl, 0.2% Igepal CA-630, pH 7.5) for 30min. Then 3μL sample and 3μL assay buffer (200μM B₁₂-SPM, 0.1mM ZnCl₂, 0.6% Triton X-100, 0.1M sodium acetate buffer, pH 5.0) were mixed and incubated at 37℃ for 2h. After the reactions were completed, 5μL of assay mixtures was removed and pipetted into 95μL ethanol, mixed, and then centrifuged for 5 min for 12,000g. The supernatant was then transferred to a glass sampling
vial, and 10μL was autosampled on a High performance liquid chromatography (HPLC) equipped with a reverse-phase column and eluted with methanol: 13mM ammonium acetate (95:5), at a flow rate of 0.8 ml/min. The excitation and emission wavelengths of fluorescence detector were set to 505 and 540 nm respectively, and fluorescence was measured. The peak area of the product, B_{12}-Cer, was identified by comparing its retention time with a known standard, and the amount of product was calculated based on the B_{12}-Cer standard curve.

Quantification of ceramide
Ceramide content in hepatocytes treated with was quantitatively assessed according to Xingxuan He's protocol [13]. Cells were harvested and mixed with 6ml methanol: trichloromethane (1:2, v/v), and were broken by ultrasonication at 4°C. After centrifugation (5000g for 5min), the lower organic phase was collected and evaporated under vacuum drier. Then the residue was dissolved in 100μL methanol, and centrifuged (13,000g for 5min). The supernatant was then used for quantification of ceramide. Purified ceramide (3μl each of 0, 1, 5, 10, 20, and 100μM in 0.2% Igepal CA-630) and enzyme solution (0.2M citric phosphate buffer, pH 4.5, 0.3M NaCl, 0.2% Igepal CA-630, 10% FBS, 50ng/μL rhAC) were mixed and incubated at 37°C for 1 h. The reaction was stopped by adding ethanol (1:5) and centrifuged. Then 10μl of the supernatant (equivalent to 0.1, 0.5, 1.0, 2.0, and 10μM ceramide) was added into 20μl of the NDA derivatization reaction mixture (25mM borate buffer, pH 9.0, containing 1.25 mM each NDA and NaCN). The reaction mixture was diluted 1:4 with ethanol and centrifuged (13,000g for 5min) after being incubated at 50°C for 10min. After these treatment, ceramide was hydrolyzed to sphingosine, and the peak area of sphingosine derivative 1-Cyanobenz[f]isoindole (CBI) was then quantified by HPLC (Ex=252nm, Em=483nm), which could reflect the content of ceramide. The C_{12}-Ceramide was also used in the same procedure as standard substance.

Detection of PTP open rate
The open rate of mitochondrial permeability transition pore (PTP) was detected using GENMED mPTP assay kit according to the kit protocol. The L-02 hepatocytes were collected and rinsed twice with PBS. These cells were then washed with Reagent A from the kit again, incubated in Reagents B and C at 37˚C for 20min, and washed twice with Reagent A. The fluorescence intensity of the cell suspensions was measured by the fluorescence spectrophotometer, with a wavelength of 488 nm for the excitation and 505 nm for emission.

RNA isolation and quantitative RT-PCR analysis
Total RNA was isolated from cells using Trizol reagent and spectrophotometrically quantified at 260nm. 260/280 ratio was also determined to confirm its quality. First strand cDNA was synthesized from total RNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. The reaction was performed in Bio-Rad thermocycler at 42°C for 60 min, and 70°C for 5 min. Samples were thermocycled for PCR amplification using Roche Lightcycler Nano PCR instrument. Specific primers for VDAC1, ANT, CypD and β-Actin were designed using Primer 5.0 software and are shown in Table 1. The reaction mixture contained 5μL 100ng/μL cDNA, 10μL PCR master mix and 0.5μL of each primer (5μM) in a final volume of 20μL. Reaction conditions for PCR were: 10min at 95°C followed by 15s at 95°C, 20s at 55°C and 20s at 68°C. The optimum cycle number resulted to be 45 cycles.

Western blot assay
Cells were washed in PBS, and cell pellets were suspended in lysis buffer and centrifuged. Then sample proteins (30μg) were separated by electrophoresis on SDA polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF). The membranes were incubated with 5% non-fat dried milk for 1h and incubated overnight at 4°C with anti-VDAC (1:500 dilution; No. sc32063; Santa Cruz), anti-
ANT (1:3000 dilution; No. sc9300; Santa Cruz), anti-CypD (1:1000 dilution; No. ab167513; Abcam) and anti-β-Actin (1:3000; No. 4967; Cell Signaling Technology). Then membranes were incubated with secondary antibodies and visualized using a Thermo Scientific chemiluminescent substrate.

**Statistical analysis**

All the data was expressed as mean±SEM and were calculated from quantitative data obtained from at least three replicate experiments. Statistical analysis was performed with one-way ANOVA using SPSS 17.0 software or, where appropriate, by two-way ANOVA, followed by LSD test. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

*Cr(VI) induced ASMase activation and ceramide increase in L-02 hepatocytes*

ASMase activities and ceramide content were measured with specific fluorescent substances, BODIPY C12-sphingomyelin and naphthalene-2,3-dicarboxyaldehyde (NDA) respectively. BODIPY C12-sphingomyelin was hydrolyzed to BODIPY C12-ceramide (B_{12}-Cer) under the effect of ASMase, so the content of B_{12}-Cer could indirectly reflect ASMase activity. (B) Cellular ceramide was hydrolyzed to sphingosine, and the sphingosine derivative 1-Cyanobenz[f]isoindole (CBI) was quantified, which could reflect ceramide content. The results are shown as mean±SEM (n=5). *\( P < 0.05 \) compared with 0μM Cr(VI) group.

![Fig. 1. Cr(VI) induced ASMase activation and ceramide increase in L-02 hepatocytes](image)

Cr(VI) induced ASMase activation and ceramide increase in L-02 hepatocytes. After L-02 hepatocytes treated with Cr(VI) (0-4μM) for 24h, acid sphingomyelinase (ASMase) activity and ceramide content were measured by HPLC. (A) BODIPY C12-sphingomyelin was hydrolyzed to BODIPY C12-ceramide (B_{12}-Cer) under the effect of ASMase, so the content of B_{12}-Cer could indirectly reflect ASMase activity. (B) Cellular ceramide was hydrolyzed to sphingosine, and the sphingosine derivative 1-Cyanobenz[f]isoindole (CBI) was quantified, which could reflect ceramide content. The results are shown as mean±SEM (n=5). *\( P < 0.05 \) compared with 0μM Cr(VI) group.
of ceramide. Ceramide was hydrolyzed to sphingosine, and sphingosine combined with NDA to generate 1-Cyanobenz[f]isoindole (CBI), which was then detected to evaluate ceramide contents. The level of ceramide increased in hepatocytes treated with Cr(VI) in Fig. 1B.

To investigate effects of DES on Cr(VI)-induced hepatotoxicity, cell viabilities were assessed by MTT assays to select an appropriate concentration of DES for subsequent experiments. DES significantly inhibited Cr(VI)-induced apoptosis.

DES decreased Cr(VI)-induced ASMase activation and ceramide increase

We assessed ASMase activities and ceramide content in L-02 hepatocytes after treated with Cr(VI) and/or DES. Compared with 2μM Cr(VI) groups, ASMase activities and ceramide levels decreased significantly in 2μM Cr(VI)+2.5μM DES groups in Fig. 3A and Fig. 3B.

DES inhibited Cr(VI)-induced mitochondrial PTP opening

Effects of DES on Cr(VI)-induced mitochondrial PTP opening were further studied. Both Cr(VI) and DES did not show apparent effect on ANT1 mRNA expression and the changes of protein expression of three components of mitochondrial PTP are similar with
Discussion

The liver is a critical organ to metabolize and detoxify exogenous toxicants in mammals and humans, which is easily attacked. Our results demonstrated that Cr(VI) induced the decrease of cell viability in a dose-dependent manner. Cr(VI) primarily enters into hepatocytes via non-specific ion channels and undergoes a reductive metabolism to stable trivalent species Cr(III) [14]. The reduction of Cr(VI) to Cr(III) is accomplished by the generation of reactive oxygen species (ROS) and caused oxidative damage and DNA mutants to hepatocytes [15, 16]. In our study, L-02 hepatocytes were treated with Cr(VI) of different concentrations (0-128μM) for 24h and MTT assay was conducted to evaluate cell viability. Our previous study had demonstrated that Cr(VI) induced the increase of ROS production and DNA mutations, and finally, apoptosis rates significantly raised in L-02 hepatocytes [17].

ASMase is a major regulator of sphingolipid metabolism, which hydrolyzes sphingomyelin to ceramide [18]. Ceramide is an essential structural component of cell membranes, as well as a bioactive lipid and second messenger that regulates the proliferation, survival, and
death of cells [19]. The ASMase/ceramide pathway is a key component of the oxidative stress response, and activation of ASMase and ceramide generation have been observed in a variety of human diseases [20, 21]. Ichi et al. observed that oxidative stress, activation of neutral sphingomyelinase, and accumulation of ceramide in liver after CCl₄ administration, suggesting that ceramide is a biologically active lipid causing apoptosis during CCl₄-induced liver injury [22]. Lang et al. found that Cu²⁺ triggered hepatocyte apoptosis through activation of ASMase and release of ceramide, and the inhibition of ASMase prevented Cu²⁺-induced hepatocyte apoptosis [23]. Some studies have shown that ASMase activation may occur through the production of reactive oxygen species (ROS), and ASMase activation, in turn, may create excess or abnormally distributed ceramides, which could lead to liver injuries [24, 25]. In the present study, Cr(VI) induced the increase of ASMase activities and ceramide levels in hepatocytes, and DES, a frequently employed inhibitor of acid sphingomyelinase (ASMase) [26], effectively inhibited ASMase activation and ceramide generation, and protected against Cr(VI)-induced hepatocellular apoptosis.

In general, it is the release of proapoptotic proteins that is responsible for the activation of caspases and DNases that are responsible for the execution of apoptosis. Excess ROS production attacks mitochondrial membrane and induces the opening of mitochondrial permeability transition pore (PTP), which leads to apoptosis-inducing factors release and p53-caspase cascade effect, and finally mitochondria-dependent apoptosis [27]. Ceramide has been shown to form large protein permeable channels in planar phospholipid and mitochondrial outer membranes [28]. Ceramide channels are good candidates for the release of proapoptotic proteins from mitochondria to the cytoplasm [29]. Therefore, the reduction of total ceramide concentration by inhibiting ASMase activation with DES, was beneficial for limiting liver injuries [30].

Additionally, the present study found that DES influenced Cr(VI)-induced mitochondrial PTP opening. The major components of PTP includes the voltage-dependent anion channel (VDAC) localized in the outer mitochondrial membrane, the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane and the cyclophillin-D (CypD), a matrix cis-trans...
isomerase [31]. Although DES treatments alone did not significantly change mRNA and protein expression of PTP components, however, DES influenced the changes of mRNA and protein expression of VDAC1 and CypD induced by Cr(VI), and was beneficial for the inhibition of Cr(VI)-induced mitochondrial PTP opening, therefore, inhibited mitochondria-dependent apoptosis. Ceramide induced membrane permeability increases in isolated mitochondria via ceramide channel formation [32]. Although the relationship between mitochondrial PTP and ceramide channel still needed further studies, it is possible that DES partly influenced mitochondrial PTP function by decreasing ceramide content.

**Conclusion**

The present study indicated that in addition to overmuch ROS production and DNA damage which were well known, ASMase activation and the following ceramide generation were involved in Cr(VI)-induced hepatocyte apoptosis. Moreover, for the first time the present study indicated that DES may exert protective effects on Cr(VI)-induced hepatotoxicity as an inhibitor of ASMase. DES ameliorated Cr(VI)-induced liver injury probably by inhibiting ceramide channel formation and mitochondrial PTP opening. According to our present date, strategies aimed to antagonize ASMase activities or reduce ceramide levels may prevent or diminish the susceptibility of the liver to Cr(VI) exposure.

**Abbreviations**

Cr(VI) (Hexavalent chromium); ASMase (Acid sphingomyelinase); DES (desipramine); ROS (reactive oxygen species); PTP (permeability transition pore); HPLC (High performance liquid chromatography); VDAC (voltage-dependent anion channel); ANT (adenine nucleotide translocator); CypD (cycophilin-D).

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