Original Paper

Activation of Autophagy Protects Against ROS-Mediated Mitochondria-Dependent Apoptosis in L-02 Hepatocytes Induced by Cr(VI)

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Key Words

Autophagy • Hepatocytes • ROS • Mitochondria • Apoptosis

Abstract

Background: Hexavalent chromium (Cr(VI)) overdose causes hepatocellular injuries by inducing mitochondrial damage and subsequent apoptosis in animals and humans. Autophagy can selectively remove damaged organelles, especially impaired mitochondria, and in turn, protects against mitochondria-dependent cell death. The present study was designed to explore the effects of autophagy on the Cr(VI)-induced hepatotoxicity. **Methods:** L-02 hepatocytes were incubated with different concentrations of Cr(VI) for 24h and several indicators for evaluating mitochondrial damage and hepatocellular apoptosis were measured. Then effects of ROS scavenger NAC on ROS production and calcium overload during Cr(VI)-induced hepatotoxicity were examined. Finally, the study further investigated the role of autophagy played in repairing mitochondrial damage and subsequent hepatocyte injuries. **Results:** After exposed to different concentrations of Cr(VI) for 24h, cell viability, mitochondria membrane potential, ATP content were significantly decreased and caspase-3 activities and apoptosis rates increased in L-02 hepatocytes. The treatment of NAC reduced ROS formation and Ca^{2+} content, restored CRAC channel activities and further diminished mitochondrial injuries. Furthermore, autophagy inducer, rapamycin is beneficial for repairing mitochondrial function and limiting hepatocytes damage, and pharmacological inhibition of autophagy by 3-methyladenine further exacerbated Cr(VI)-induced hepatotoxicity. **Conclusions:** ROS production is a critical reason for Cr(VI)-induced mitochondria-dependent apoptosis. And activation of autophagy could repair mitochondria function to protect hepatocytes potentially by removing damaged mitochondria

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Introduction

Chromium is ubiquitous in the environment, principally in trivalent and hexavalent forms [1]. While Cr(III) is an essential micronutrient for human, highly toxic Cr(VI) is widely used in many different industries including welding, chrome pigmenting and leather tanning. Occupational exposure to Cr(VI) compounds is associated with several adverse effects on human health, such as nasal perforation and bronchiogenic cancer. Cr(VI) has long been recognized as an inhalation carcinogen, according to the research that the accident of lung cancer in human and animals increased as a result of the exposure to Cr(VI) [2]. However, recent studies suggested ingestion carcinogenicity in animals and human when exposed to Cr(VI) in drinking water [3, 4], which derives comprehensive attention on the human ingestion toxicity of Cr(VI).

The liver is a critical organ to metabolize and detoxify exogenous toxicants in mammals and humans, which is easily attacked. Apoptosis is a conserved cellular suicide mechanism in which multiple signaling pathways are implicated. There have been described two major pathways of apoptosis: mitochondria-dependent or -independent. Our previous studies have demonstrated the ROS formation and mitochondria stress were the most typical events required for Cr(VI)-induced apoptosis in L-02 hepatocytes [5]. When mitochondria are attacked by exogenous toxins, electron leaks from specific sites of mitochondrial electron transport chain (ETC) would be reduced to superoxide anion radical ($O_2^{\cdot-}$) [6]. $O_2^{\cdot-}$ was further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$ are referred as ROS, collectively. Accumulating oxidative stresses inhibit mitochondrial complexes and affect the efficiency of mitochondria, and further increase the rate of ROS production. This results in several harmful changes, such as membrane permeability transition, ATP depletion and mitochondria dysfunction. These signals that converge on mitochondria would trigger the release of caspase activators (such as cytochrome c and AIF), participation of pro- and antiapoptotic Bcl-2 family proteins and further give rise to apoptotic cell death. Therefore, it is credible that removal and reduction of damaged mitochondria would be beneficial for Cr(VI)-induced hepatotoxicity.

Macroautophagy (hereafter referred to as autophagy), is a cellular pathway by which cytoplasmic material and damaged organelles are degraded and recycled back as amino acid, nucleotide, fatty acid, and so on [7]. Autophagy occurs at low basal levels in all eukaryotes as a critical survival mechanism to maintain cellular homeostasis and energy balance as response to various death stimuli [8]. Under the regulation of series of autophagy-related genes (Atg), autophagy begins with the newly formation of double-membrane autophagosome, which enclose cellular content including lipid droplet and damaged organelles, thus degraded by the fusion with lysosomes via lysosomal hydrolase [9].

Although autophagy is usually activated as a survival mechanism in response to the pathogenesis of liver disease [10, 11], it is still unknown whether autophagy can modulate Cr(VI)-induced hepatotoxicity. The present study were undertaken to explore the mechanism of Cr(VI)-induced hepatotoxicity and evaluate the potentially protective effect of autophagy by the removal of damaged mitochondria.

Materials and Methods

Materials

Potassium dichromate ($K_2Cr_2O_7$), N-acetylcysteine (NAC), Chloroquine(CQ), Rapamycin (Rap) and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNA isolation and RT-PCR reagents were obtained from Invitrogen (Carlsbad ,CA, USA). All other chemicals and solvents were of analytical grade and obtained from Sigma-Aldrich except those specifically mentioned.

Cell culture and protein measurements

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% (vol/

vol) FBS at 37°C in 5% CO₂. Protein concentrations were measured using a BCA protein quantification kit (Abcam, SF, USA) with bovine serum albumin used as concentration standards.

Measurements of cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate viability of cells. L-02 hepatocytes were treated with Cr(VI) of different concentrations (0–8 μM) for 24 h. Then 10 μl of a MTT solution (5 mg/ml in PBS as stock solution) was added into each well of 96-well plates, and incubated for additional 4 h at 37 °C. Cell viability was measured by treating with dimethylsulfoxide (DMSO) prior to reading at 492 nm with microplate reader (Powerwave XS2, Biotek, Vermont).

Detection of mitochondria membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was monitored using 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a lipophilic cationic fluorescence dye. JC-1 is capable of selectively entering mitochondria, where it forms monomers, and emits green fluorescence when $\Delta\Psi_m$ is relatively low. At a high $\Delta\Psi_m$, JC-1 aggregates and gives red fluorescence. Thus the red and green fluorescence of JC-1 reflect the change of $\Delta\Psi_m$ of the mitochondrial membrane. Briefly, cells were trypsinized, washed in ice-cold PBS, and incubated with JC-1 at 37°C for 20 min. Finally, cells were washed twice with PBS and analyzed by FACScalibur flow cytometry (Becton Dickinson, NY, USA), measuring the fluorescence emission at 530 nm and excitation at 488 nm.

ATP content assay

Cellular ATP content was assessed using the ATP assay kit (Abcam, Cambridge, UK) which utilizes the phosphorylation of glycerol to generate a product that is quantified by colorimetric methods. Cells were disposed under assay protocol and ATP content was measured under 570 nm.

Caspase 3 activity assay

According to Abcam caspase-3 assay kit, caspase-3 activity assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA), after its cleavage from the labeled substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). The activities of caspase-3 were described as the cleavage of the colorimetric substrate by measuring the absorbance at 405 nm.

Determination of apoptosis rate

Apoptosis rates were detected with Invitrogen Annexin V/PI cell apoptosis kit. Harvest the cells after the incubation period and re-suspend the cells in Annexin-binding buffer. Add 5 μL FITC-labeled Annexin V and 1 μL 100 μg/mL PI working solution to each 100 μL of cell suspension. Then the apoptosis rates were calculated by the percentage of stained cells using FACScalibur flow cytometry, measuring the fluorescence emission at 530 nm and excitation at 488 nm.

Measurement of ROS production

The fluorescent probe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probers, USA) are specific dyes used for ROS content. L-02 hepatocytes were seeded onto a glass coverslip in the bottom of a 6-well plate overnight. The cells were incubated with CM-H₂DCFDA (5 μM final) for 30 min. Cells were washed with PBS, mounted, and observed under Motican 3000 fluorescence microscope (Motic, Xiamen, China). In addition, for quantitative examination, cells were directly seeded into 60 mm culture dishes and treated with CM-H₂DCFDA as described above. The fluorescence intensity was assessed by VarioskanFlash fluorescence microplate reader (Thermo Scientific, MA, USA) with excitation wavelength at 488 nm and emission wavelength at 525 nm.

Determination of Ca²⁺ content and Ca²⁺ channel activity

L-02 hepatocytes were incubated at 5 μM Fluo-3 AM (Beyotime, Nanjing, China) at 37°C for 30 min. Then cells were harvested, washed three times with PBS and then re-suspended with RPMI-1640 medium. The fluorescence intensity was assessed by VarioskanFlash fluorescence microplate reader with excitation wavelength at 488 nm and emission wavelength at 525 nm. Currents of calcium release activated channel (CRAC) were recorded at room temperature using a computer-based patch-clamp amplifier in whole-

Table 1. Primers used for semi-quantitative RT-PCR assays

Gene	Primer	Product size
Beclin1	Forward 5'- CCGCAAGATAGTGGCAGAA-3'	264 bp
	Reverse 5'- CGACCCAGCCTGAAGTTAT-3'	
β -actin	Forward 5'- AGGGGCCGGACTCGTCATACT-3'	202 bp
	Reverse 5'- GGCGGCACCATGTACCCT-3'	

cell patch clamping mode. Patch-pipettes were pulled (Narishige PC10) and fire-polished to 2-3M Ω (Narishige MF830). Recordings of membrane currents were made using a Multiclamp 700B amplifier (Axon Instruments) and a CV-70 headstage. The bath solution contained 140mM NaCl, 2.8mM KCl, 10mM CaCl₂, 0.5mM MgCl₂, 11mM glucose, 10mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 140mM K-glutamate, 8mM NaCl, 1mM MgCl₂, 0.5mM Mg-ATP, 10mM EGTA, 10mM HEPES, adjusted to pH 7.2 with NaOH. To monitor changes in the amplitude of membrane currents, voltage ramps between -100mV and -80mV were applied every 2s, starting immediately after achieving the whole-cell configuration. Acquired currents were sampled at 10 kHz and filtered at 2 kHz. Pipette and cell capacitance were compensated. Voltage-clamp commands were generated using Clampex 10.2 (Axon Instruments). Data were recorded via a Digidata 1440A interface (Axon Instruments).

RNA isolation and RT-PCR analysis

We assessed the induction of autophagy by determination the mRNA expression of Beclin 1. Specific primers for Beclin 1 and β -Actin were designed using Primer 5.0 software and are shown in Table 1. 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 RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, NY, USA). The reaction was performed in thermocycler (Bio-Rad, CA, USA) at 42°C for 60 min, and 70°C for 5 min. Samples were thermocycled for PCR amplification (Roche, Mannheim, Germany). The reaction mixture contained 1 μ L cDNA, 10 μ L 2 $\times$ SYBR Green qPCR Mix and 0.5 μ L of each primer (5 μ M) in a final volume of 20 μ L. Reaction conditions for PCR were: 3min at 95°C followed by 10s at 95°C and 30s at 58°C. The optimum cycle number resulted to be 35 cycles for Beclin 1 and β -Actin.

Western blot assay

Cells were washed in PBS, and cell pellets were suspended in lysis buffer and centrifuged. Then 30 μ g of protein were separated by SDS-PAGE on 12% or 15% polyacrylamide gels 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-LC3 (1:1000 dilution; No. 128025; Abcam, SF, USA), anti-phospho-4EBP1 (1:1000 dilution; No. 236B4; Cell Signaling, BSN, USA) and anti- β -Actin (1:3000; No. 4967; Cell Signaling, BSN, USA). Then membranes were incubated with anti-rabbit secondary antibodies and visualized using a chemiluminescent substrate (Thermo Scientific, MA, USA). All experiments were performed in at least triplicate.

Observation of autophagosome

To directly observe autophagosomes, hepatocytes were harvested and cell deposit were fixed overnight with 2.5% glutaraldehyde for 30 min at 4°C, then post-fixed with 2% osmic acid, dehydrated, and embedded with epoxy resin. Thin sections were observed with an H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Mitochondria isolation

Cells were harvested and resuspended in an ice-cold lysis buffer (250mM sucrose, 20mM HEPES-KOH, 10mM KCl, 1.5mM MgCl₂, 1 mM EGTA, 1mM EDTA, 1mM DTT and 0.1mM PMSE, pH 7.5). The cellular suspension is homogenized with a Teflon-glass homogenizer with 20 up and down passes of the pestle. The homogenate is then centrifuged at 750 $\times$ g for 10min, and the supernatants were further centrifuged at 10,000g for 15 min at 4 °C. The mitochondria pellet was washed once with lysis buffer and re-suspended

Fig. 1. Cr(VI) induces mitochondria-dependent apoptosis in L-02 hepatocytes. (A) L-02 hepatocytes were incubated with indicated concentrations for 24h and then were subjected to a MTT assays for evaluating the percentage of viable cells. (B-C) After exposed to Cr(VI) with increasing concentrations (0-4μM) for 24h, mitochondrial membrane potential and ATP production were assessed in L-02 hepatocytes. (D-E) Cells were treated with (0-4μM) Cr(VI) for 24h, then caspase-3 activities and apoptosis rates of hepatocytes were detected with microplate reader and flow cytometry. The results are presented as mean±SEM (n=5). #P<0.05 compared to 0μM Cr(VI) group.

in lysis buffer. Isolated mitochondria were used immediately for measurement of mitochondrial respiration function.

Measurement of mitochondrial respiration function

Mitochondrial respiratory function was measured using a Clark oxygen electrode (Hansatech Instruments, Norfolk, UA) in a thermostat controlled chamber. Briefly, mitochondrial suspension (1mg protein) were added to 1mL of respiratory medium preheated to 25°C (300mM mannitol, 10mM NaH₂PO₄, 5mM MgCl₂, 10mM KCl, PH=7.2). Mitochondrial respiration was initiated by addition of the complex II substrate succinate (2.5mM final), and state 4 respiration was first measured in the absence of ADP for 2mins. Subsequently, state 3 respiration was initiated by the addition of ADP (7.5mM final) to determine the maximal rate of coupled ATP synthesis. Respiration rate was measured by the slope of oxygen consumption curves and respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration rate.

Statistical analysis

All the data are expressed as mean±SEM. Comparisons among groups were 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.

Fig. 2. ROS formation is a critical reason for mitochondrial damage in L-02 hepatocytes exposed to Cr(VI). (A) After L-02 hepatocytes treated with PBS and 4μM Cr(VI) with or without 5mM NAC for 24h, ROS generation was detected with the fluorescence probe CM-H₂DCFDA. (B) Ca^{2+} contents were assessed with probe Fluo-3 AM using fluorescence microplate reader. (C-D) CRAC channel currents were detected with Multiclamp 700B patch-clamp amplifier for evaluating CRAC channel activities. (E) Mitochondrial membrane potential was detected with JC-1 staining. The results are shown as mean±SEM (n=5). [#]*P*<0.05 compared with 0μM Cr(VI) group. ^{*}*P*<0.05 compared with 4μM Cr(VI) group.

Results

Cr(VI) induces mitochondria-dependent apoptosis in L-02 hepatocytes in a dose-dependent manner

To determine the cytotoxic effects of Cr(VI) on L-02 hepatocytes, MTT assays were performed. As shown in Fig. 1A, the percentage of viable cells significantly diminished after treated with 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8μM Cr(VI) for 24h. Then cells were exposed to Cr(VI) of increasing concentrations (1-4μM) for 24h, and mitochondrial membrane potential and ATP production decreased in a concentration-dependent manner in Fig. 1B and C. Moreover, Cr(VI) also increased caspase-3 activities and apoptosis rates increased significantly in Fig. 1D and E.

ROS formation is a critical reason for mitochondria damage in L-02 hepatocytes exposed to Cr(VI)

ROS scavenger, 5mM NAC could effectively limit ROS production in hepatocytes treated with 4μM Cr(VI) in Fig. 2A. To assess whether or not NAC may mitigate Ca^{2+} overload, we detected the Ca^{2+} contents and CRAC channel activities. Results in Fig. 2B-D showed that 4μM Cr(VI) increased Ca^{2+} contents and decreased CRAC channel currents compared to control group, and the combination treatment of NAC and Cr(VI) inhibited Ca^{2+} overload and restored CRAC channel activities. After application of 4μM Cr(VI) and 5mM NAC for

Fig. 3. Cr(VI) induces autophagy in L-02 hepatocytes. (A) Cells were treated with Cr(VI) with increasing concentrations (0–4 μM) for 24 h, then Beclin 1 mRNA expressions were assessed with RT-PCR. (B) Cells were treated with PBS, Cr(VI) (4 μM), CQ (20 μM) alone or Cr(VI) plus CQ for 24 h. Total lysates were subjected to immunoblotting assay for LC3. (C–D) L-02 hepatocytes were exposed to PBS, 4 μM Cr(VI), 1.5 μM Rap or 4 μM Cr(VI)+1.5 μM Rap respectively. The phosphorylation level of mTOR downstream protein, translational initiation factor 4E binding protein-1 (4EBP-1) was detected by western blot assay. And cells were processed for EM analysis and representative images of autophagosomes were observed. Arrows denote autophagosomes containing damaged mitochondria. The results are presented as mean±SEM (n=5). [#]P<0.05 compared with control group.

24 h, mitochondria membrane potential increased compared to 4 μM Cr(VI) group in Fig. 2E. Therefore, these data revealed that NAC exerted a protective effect on Cr(VI)-induced mitochondrial damage.

Cr(VI) induces autophagy in L-02 hepatocytes

As shown in Fig. 3A, Cr(VI) induced the increase of Beclin 1 mRNA expression compared to control group in a concentration-dependent manner. Autophagy flux was also assessed by using a combination of lysosomal inhibitor CQ in Fig. 3B. Cr(VI) treatment increased endogenous LC3-II levels and combined CQ and Cr(VI) treatment further increased the level of LC3-II protein. The phosphorylation level of mTOR downstream protein, translational initiation factor 4E binding protein-1 (4EBP-1) decreased in Cr(VI)-induced hepatocytes in Fig. 3C. And combined Cr(VI) and mTOR inhibitor rapamycin treatment further decreased the phospho-4EBP1 protein levels. EM analysis indicated an increased accumulation of autophagosomes after 4 μM Cr(VI) and/or 1.5 μM Rap treatment in Fig. 3D. It is noteworthy that the double-membrane autophagosomes had enveloped injured mitochondria, suggesting that autophagy may help remove the damaged mitochondria induced by Cr(VI).

Activation of autophagy protects against Cr(VI)-induced apoptosis

Cells were incubated with PBS, 4 μM Cr(VI) only, or co-treated with 1.5 μM Rap or 5 mM 3-MA for 24 h. As shown in Fig. 4A, 1.5 μM Rap ameliorated the decrease of mitochondria membrane potential caused by Cr(VI), which is deteriorated by autophagy inhibitor 3-MA (5 mM). The mitochondria isolated from L-02 hepatocytes co-treated with Rap exhibited higher RCR compared to 4 μM Cr(VI) group, while 5 mM 3-MA aggravated mitochondria

Fig. 4. Activation of autophagy protects against Cr(VI)-induced hepatocellular apoptosis. Administration of autophagy inducer Rap and autophagy inhibitor 3-MA modulated Cr(VI)-induced apoptosis in L-02 he-

patocytes. L-02 hepatocytes were divided into 6 groups and treated with PBS, 4 μ M Cr(VI) only, or co-treated with 1.5 μ M Rap or 5mM 3-MA for 24h. (A) Mitochondria membrane potential was detected with JC-1 staining. (B-C) Mitochondria respiratory curves were recorded by Clark oxygen electrode and respiratory control ratio (RCR) were calculated. (D-E) The caspase-3 activities and apoptosis rates of hepatocytes were detected with microplate reader and flow cytometry. The results are shown as mean $\pm$ SEM (n=5). * P <0.05 compared with control group. * P <0.05 compared with 4 μ M Cr(VI) group.

respiratory function in Fig. 4B. Due to that mitochondria normal respiratory function is essential for ATP production, Cr(VI)-induced the reduction of ATP generation could be recovered to a higher level after co-treated with Rap, while 3-MA aggravated mitochondrial imbalance of energy metabolism in Fig. 4C. Finally, caspase-3 activities and apoptosis rates decreased in 4 μ M Cr(VI)+1.5 μ M Rap group compared to 4 μ M Cr(VI) group, and in contrast, caspase-3 activities and apoptosis rates in 5mM 3-MA+4 μ M Cr(VI) group were significantly higher than that in 4 μ M Cr(VI) group in Fig. 4D and E.

Discussion

Mitochondria are critical for cellular energy metabolism and cellular homeostasis. The study found that Cr(VI) induced the decrease of mitochondrial membrane potential in a concentration-dependent manner. Mitochondria permeability transition (MPT) would cause the release of cytochrome c (Cyt c). Cyt c, as a water-soluble electron carrier is indispensable for oxidative phosphorylation. Cyt c passes electron to cytochrome c oxidase, which uses the electrons and hydrogen ions to reduce molecular oxygen to water. So Cyt c release aggravated the blockage of mitochondria electron transport chain and diminished mitochondrial ATP production. And the release of cytochrome c from mitochondria to cytoplasm brought about caspase activation, which is an important step in programmed cell death. Our study has demonstrated that Cr(VI) induced cell death of L-02 hepatocytes through mitochondria-mediated and caspase-dependent pathways.

Considering that excess ROS generation would be upstream signaling of mitochondria damage, NAC as an antioxidant were used to assess the role of ROS in mitochondria injury induced by Cr(VI). Mitochondria are the main sources of ROS, and therefore are vulnerable to ROS damage. ROS directly attacks mitochondrial respiratory chain enzymes, or by the induction of mutations of the mitochondrial genome, leading to the inhibition of mitochondrial respiratory chain enzymes and the dysfunction of oxidative phosphorylation. The results have demonstrated that NAC diminished ROS formation to maintain mitochondria homeostasis.

ROS production is also related with calcium overload in hepatocytes, which could exacerbate mitochondria damage [12]. Ca^{2+} can enhance cytochrome c dislocation from the mitochondrial inner membrane, either by competing for cardiolipin binding sites or by inducing MPT, which results in an effective blockage of electron transport and enhancement of ROS generation [13]. Conversely, cytochrome c can bind to the endoplasmic reticulum inositol 1,4,5-trisphosphate receptor (IP3R), resulting in enhanced ER Ca^{2+} release and further Ca^{2+} overload [14]. According to our results, NAC inhibits the increase of Ca^{2+} content in L-02 hepatocytes exposed to Cr(VI). Calcium release activated channels (CRAC) are specialized plasma membrane Ca^{2+} ion channels in hepatocytes [15]. Cr(VI) decreased the current amplitude of CRAC, while co-treatment with NAC could restore CRAC activities. Therefore, NAC, as a widely used antioxidant, repaired mitochondrial dysfunction by the clearance of ROS generation and the suppression of calcium overload.

Many mitochondrial toxins regulate autophagic process [16]. Both mitochondrial superoxide and MPT are reported to influence autophagic flux [17, 18]. Beclin 1, mammalian homolog of Atg6, is reported to be essential for autophagosome formation [19]. We found that Cr(VI) increased the mRNA expression of pro-autophagic protein Beclin 1 in L-02

hepatocytes. Light chain 3 (LC3), a mammalian homolog of Atg8, is a critical protein in autophagic process. The phosphatidylethanolamine-conjugated form of LC3-II translocates to the autophagosomal membrane and promotes the formation of a double-membrane autophagosome, so the increase of LC3-II levels is a landmark change to verify autophagic flux. We treated hepatocytes with Cr(VI) in the absence or presence of lysosomal inhibitor chloroquine (CQ). CQ, a commonly used antimalarial drug, increases lysosomal pH and blocks the fusion of autophagosome with lysosomes, and then autophagic protein LC3-II degradation was inhibited [20]. The results showed that Cr(VI) induced the increase of LC3-II levels and combined CQ and Cr(VI) treatment further increase the level of LC3-II protein. In addition, under stress conditions, suppression of mammalian target of rapamycin (mTOR) is a central molecular signaling pathway leading to autophagy induction [10], and we further found that Cr(VI) treatment would decrease the level of phosphorylated 4EBP1, a downstream target protein of mTOR in L-02 hepatocytes. Another criterion for autophagy is to monitor the formation of autophagosome [21]. The study confirmed autophagic degradation of damaged mitochondria by observing double-membrane mitochondria-containing vacuolar structures using transmission electron microscopy [22]. All these results supported that Cr(VI) induced autophagy in hepatocytes. It could be suggested that autophagy act as a survival mechanism in trying to rescue cells from apoptosis, but cell fate may be finally as a result of a balance between cell rescue and cell damage. Recent studies suggested that autophagy reduces alcohol-induced liver injury by the removal of damaged mitochondria [23]. When Cr(VI)-activated autophagy by itself could not resist cellular damage, selective autophagic elimination of depolarized mitochondria might be a feasible way to eliminate defective mitochondria and augment energy production.

Mitochondria are common targets for various toxicants-induced hepatotoxicities, and maintaining a healthy population of mitochondria is necessary for the well-being of hepatocytes. Numerous studies have demonstrated that autophagy could mitigate hepatocytes injuries by selectively removing damaged mitochondria [24]. Several signaling pathways are involved to regulate autophagy in mammalian cells. Rapamycin, as an mTOR inhibitor, could be used as a standard inducer for autophagy as stated above [25]. Another critical regulator of autophagy is the class III PI3k Vps34, which is necessary for the formation of autophagosomes, so pharmacological inhibition of autophagy by 3-MA leads to inhibition of autophagosome formation [26]. We investigated the effects of rapamycin or 3-MA on L-02 hepatocytes cell death caused by Cr(VI).

Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in hepatocytes. Eliminate damaged mitochondria to avoid vicious cycle of excess ROS generation and impaired respiratory function is beneficial to stimulate ATP synthesis and maintain mitochondrial homeostasis [27]. Our studies demonstrated that pretreatment with rapamycin prevented the reduction of the mitochondrial membrane potential, and then restoring mitochondrial respiration function and ATP content. Rapamycin also results in lower levels of cytochrome c release into the cytosol by the induction of Bcl-2 protein levels [28]. Apoptosis is an energy-dependent process that is regulated by a cascade activation of caspase proteins. Several studies found that autophagy inhibitors increased DNA fragmentation, caspase-3 activation and apoptosis [29]. Consistent with these findings, pharmacological suppression of autophagy by 3-MA accelerated apoptotic cell death in L-02 hepatocytes exposed to Cr(VI). Co-treatment Cr(VI) with 3-MA markedly increased mitochondria damage, caspase activation and ensuing apoptotic cell death in our experiments. Therefore, the activation of autophagy was beneficial for restoring mitochondria biogenesis and maintaining mitochondrial cellular homeostasis probably by selective elimination of mitochondria [30].

In conclusion, we have demonstrated that Cr(VI) induces ROS-mediated mitochondria-dependent apoptosis in L-02 hepatocytes. NAC exhibited a protective role against ROS accumulation and mitochondria damage. Moreover, the present study for the first time demonstrated the activation of autophagy apparently attenuated Cr(VI)-induced hepatotoxicity and the protection may be mediated by the removal of damaged mitochondria.

Water contaminated with Cr(VI) has been suggested as a severe health threat in humans through oral route. These finding implied that induction of autophagy could be a novel therapeutic approach to mitigate Cr(VI)-induced hepatotoxicity and liver injury and protect human health.

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