Role of mitochondrial damage in Cr(VI)-induced endoplasmic reticulum stress in L-02 hepatocytes

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Abstract. Although it is well reported that mitochondrial damage and endoplasmic reticulum (ER) stress (ERS) are involved in heavy metal-induced cytotoxicity, the role of mitochondrial damage in hexavalent chromium [Cr(VI)]-induced ERS and the correlation between the two have not been described and remain to be elucidated. The present study evaluated the ability of Cr(VI) to induce ERS in L-02 hepatocytes, and subsequently examined the role of reactive oxygen species (ROS)-mediated mitochondrial damage in Cr(VI)-induced ERS. The findings demonstrated that Cr(VI) induced ERS, which was characterized by the upregulation of ERS-associated genes and the substantial release of Ca^{2+} from the ER. The Cr(VI)-induced mitochondrial production of ROS, by disturbing mitochondrial respiratory chain complexes I and II, may damage mitochondria directly by inducing mitochondrial permeability transition pore opening and mitochondrial membrane potential collapse. The results additionally demonstrated that Cr(VI) induced Ca^{2+} release from the ER through ROS/caveolin-1/protein kinase B/inositol 1,4,5-trisphosphate receptor signaling. The application of the ROS scavenger N-acetyl-cysteine confirmed the role of ROS in Cr(VI)-mediated mitochondrial damage, ERS and apoptotic cell death. The data obtained demonstrated the role of mitochondrial damage in Cr(VI)-induced ERS and provide novel insight into the elucidation of Cr(VI)-induced cytotoxicity.

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Abbreviations: ROS, reactive oxygen species; MRCC, mitochondrial respiratory chain complex; mPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential; AIF, apoptosis inducing factor; IP3, inositol 1,4,5-trisphosphate; CHOP/GADD153, CCAAT/enhancer-binding protein homologous protein; PERK, RNA-activated protein kinase-like ER kinase; IRE1/XBP-1, inositol-requiring enzyme-1/X-box-binding protein; ATF6, activating transcription factor 6

Key words: hexavalent chromium, mitochondria damage, endoplasmic reticulum stress, ROS, inositol 1,4,5-trisphosphate receptors, L-02 hepatocytes

Introduction

Industrial wastewater from diverse industrial processes, including steel manufacturing, electroplating, leather tanning and wood preservation, are responsible for the discharge of chromium (Cr) into the environment (1). Cr exists in a number of oxidation states in nature, of which the hexavalent [Cr(VI)] and trivalent [Cr(III)] states are the most stable forms with biological significance (2). Cr(III) is an essential trace nutrient that is necessary for lipid and glucose metabolism, whereas Cr(VI), a class IA human carcinogen that was recognized in 1990, is the most toxic form of Cr due to its rapid permeability through biological membranes and subsequent interactions with intracellular proteins, lipids, DNA and other biological macromolecules (3). Cr(VI) and its compounds have long been considered carcinogens, that primarily cause lung cancer via the inhalation route. In 2015, Karagiannis *et al* (4) conducted an epidemiological study in Greece which confirmed that exposure to Cr(VI) by drinking water elevated the incidence of primary liver cancer. Since then, general interest surrounding Cr(VI)-induced hepatotoxicity has consistently increased, with a shift in the prinicpal focus of investigation from lung cancer to liver cancer.

The ability of the mitochondria to produce reactive oxygen species (ROS) was first demonstrated in 1961 by Jensen (5), and it is certain that mitochondria are the primary source of cellular superoxide and hydrogen peroxide in the majority of cell types. The production of mitochondrial ROS, including oxygen free radicals, such as superoxide anion radicals ($O_2^{\cdot -}$) and hydroxyl radicals ($^{\cdot}OH$), and non-radical oxidants, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), is involved in the pathogenesis of various diseases and disorders, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis; this is due to its harmful effects on proteins, lipids and DNA that may cause cell damage and even death (6). A previous study demonstrated that mitochondrial respiratory chain complex (MRCC) I (NADH-ubiquinone oxidoreductase) was the principal source

of ROS as a consequence of electron leakage during respiration in mitochondria under pathological conditions; however, not under resting and healthy conditions; therefore, it is not unexpected that >40% of all mitochondrial-associated disorders are associated with mutations in subunits of MRCC I (7). MRCC III [cytochrome *c* (Cyt C) reductase] is additionally considered to be the primary site on the electron transfer chain to generate ROS (8); however, there remains a lack of exact mechanistic knowledge of the architecture of mitochondrial ROS-generation systems, including MRCC I and III, and of detailed insights into the molecular mechanisms controlling their expression or activities. The mitochondrial permeability transition pore (mPTP) is a voltage- and Ca^{2+}-dependent channel, the prolonged opening of which maintains the permeability of the inner mitochondrial membrane to solutes with a molecular weight <1,500 Da (9). It is widely recognized that brief mPTP openings have an essential physiological role in maintaining healthy mitochondrial homeostasis and functions. Mitochondrial membrane potential (MMP) disruption has been confirmed to be involved in a variety of apoptotic phenomena, including cytochrome *c* (Cyt C) release and caspase activation. ROS are key inducers of mPTP opening, which ultimately progress to MMP collapse, and initiate apoptotic pathways by promoting the release of Cyt C and apoptosis inducing factor (AIF) (10).

The endoplasmic reticulum (ER) is a complex, specialized organelle with functions including the synthesis and posttranslational modification of proteins, metabolism of lipids and carbohydrates, and homeostatic control of intracellular Ca^{2+} and the redox system. It has been confirmed that the principal Ca^{2+} release channels from the ER are ryanodine receptors in excitable cells and inositol 1,4,5-trisphosphate (IP3) receptors (IP3R) in non-excitable cells, including hepatocytes; ER Ca^{2+} release via IP3R is initiated by binding of the signaling molecule IP3. Glucose-regulated protein 78 (GRP78), an ER chaperone that is involved in protein processing and the provision of cellular protection, is used as a monitor of ER stress (ERS) (11). ERS may be alleviated by the unfolded protein response (UPR) in the early stage. A previous study identified that the apoptotic response is mediated through activation of the ERS-associated pro-apoptotic marker CCAAT/enhancer-binding protein homologous protein (CHOP/GADD153) primarily by three UPR signal pathways of the RNA-activated protein kinase-like ER kinase (PERK), inositol-requiring enzyme-1/X-box-binding protein (IRE1/XBP-1) and activating transcription factor 6 (ATF6) (12). Therefore, CHOP is considered to be the target gene of the UPR signal pathways and pro-apoptosis during ERS.

Although it is well demonstrated that mitochondrial damage and ERS are involved in heavy metal-induced cytotoxicity (13), the role of mitochondrial damage in Cr(VI)-induced ERS, and the correlation between the two, have not been described and remain to be elucidated. In the present study, the ability of Cr(VI) to induce ERS in L-02 hepatocytes was first evaluated by describing mitochondrial damage and the associated mechanism, following which the role of ROS-mediated mitochondria damage in Cr(VI)-induced ERS was investigated. The present data indicate the role of mitochondrial damage in ERS and provide novel insight into the elucidation of Cr(VI)-induced cytotoxicity.

Materials and methods

Reagents. RPMI-1640 medium, trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were obtained from Gibco (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Potassium dichromate was obtained from Changsha Chemical Reagents Company (Changsha, China). Antibodies specific for CHOP (L63F7; cat. no. 2895), PERK (D11A8; cat. no. 5683), IRE1α (14C10; cat. no. 3294), GRP78/BiP (C50B12; cat. no. 3177) and GAPDH (D16H11; cat. no. 5174) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies specific for IP3R2 (C-20; cat. no. sc-7278), AIF (B-9; cat. no. sc-55519), Cyt C (6H2; cat. no. sc-13561), caveolin-1 (Cav-1; 4H312; cat. no. sc-70516), phosphoinositide 3-kinase (PI3K; 4F3; cat. no. sc-293172), protein kinase B (AKT) 1 (cat. no. sc-135829), phospho-AKT (p-AKT; Ser 473; cat. no. sc-7985-R), goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP; cat. no. sc-2004), rabbit anti-goat IgG-HRP (cat. no. sc-2768) and goat anti-mouse IgG-HRP (cat. no. sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Caspase-3 antibody (cat. no. 19677-1-AP) was obtained from ProteinTech Group, Inc. (Wuhan, China). The primary antibodies for the MRCCs, including Complex I subunit NDUFS3 (cat. no. MS110), Complex II (succinate dehydrogenase) subunit 70 kDa Fp (cat. no. MS204), Complex III subunit Core 2 (cat. no. MS304), Complex IV (Cyt C oxidase) subunit II (cat. no. MS405), and complex V (ATP synthase) subunit α (cat. no. MS502) were purchased from MitoSciences, Inc. (Eugene, OR, USA). All other chemicals and solvents were of analytical grade or superior pharmaceutical grade.

Cell culture. The immortalized human L-02 hepatocyte cell line was provided by The China Center for Type Culture Collection of Wuhan University (Wuhan, China). The cells were maintained in RPMI-1640 with 10% (v/v) FBS, 2 mM L-glutamine, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) in a 5% CO_2 environment at 37˚C, as previously described (14).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for gene expression analysis. RT-qPCR analysis was performed as previously described (15). Total cellular RNA of L-02 hepatocytes was prepared using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Total cDNA was synthesized using a PrimeScript RT reagents kit (Takara Biotechnology, Co., Ltd., Dalian, China). qPCR analysis was performed using a LightCycler® 96 Sequence Detection System (Roche Diagnostics, Basel, Switzerland) in a 20 μl reaction containing 10 μM of each primer, 1 μl template cDNA, 10 μl SYBR Premix EX Taq (SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Inc., Otsu, Japan), and 0.25 μl ROX reference dye. The PCR was run at 95˚C for 30 sec followed by 45 cycles of 95˚C for 5 sec and 60˚C for 34 sec (15). ACTB was used as a control. Gene expression was calculated using the comparative threshold cycle $2^{-\Delta\Delta Cq}$ method (16). The data were collected from

three separate experiments. The forward (F) and reverse (R) primer sequences were as follows: CHOP, (F) 5'-TTCTCG GGCAGGGCGTACTGA-3' and (R) 5'-TGGTGCCCTTCT TCCTTCCC-3'; PERK, (F) 5'-TCAGCCTTCACCTTAGGC CGA-3' and (R) 5'-AAGCCTCTGCTCCCTTTCCTAC-3'; IRE1, (F) 5'-TGCATTAGGACATATGCGCCCTAA-3' and (R) 5'-CTAAGGCTGCTCCACGTGCA-3' (R); GRP78, (F) 5'-CATACCCCGTATCCTGTCG-3' and (R) 5'-CGAATC AGATGCCGTTCGCT-3'; ACTB, (F) 5'-CACGACGGCGTG TAGGT-3' and (R) 5'-CTCCAAAATATGCTGGGTCAT-3'.

Immunoblot for protein expression. The hepatocytes were lysed using a Mammalian Cell Lysis kit obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The protein concentrations of the mitochondrial suspensions were determined using the Bradford Coomassie blue protein-binding (17). Protein samples containing 50 μg protein were separated by 12% SDS-PAGE and subsequently transferred onto a polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with 4% non-fat milk and immunostained with primary antibodies (1:1,000) overnight at 4˚C. The membranes were incubated with the appropriate secondary antibodies (1:1,000) for 1 h at room temperature, developed with the Super Enhanced Chemiluminescence Detection kit (Applygen Technologies, Inc., Beijing, China) and subsequently exposed onto films.

Flow cytometry analysis for apoptotic cells. For the determination of apoptotic cell death, the L-02 hepatocytes were stained with Annexin V-Fluorescein Isothiocyanate (FITC; 0.5 μg/ml final concentration) and propidium iodide (PI; 1 μg/ml final concentration), analyzed on a flow cytometer equipped with a 488 nm argon laser light source, and evaluated using CellQuest software version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA). Apoptotic cell death was determined by quantifying the population of Annexin V-FITC-positive cells (early apoptotic) and Annexin V-FITC/PI-positive cells (late apoptotic).

ER Ca^{2+} concentration determination. Ca^{2+} concentration in the ER was detected using the Intracellular Ca^{2+} Concentration in Cell Endoplasmic Reticulum Detection kit (Genmed Scientifics, Inc., Shanghai, China). All procedures were performed according to the manufacturer's protocol. The result was quantified using a fluorescence spectrophotometer with excitation at 490 nm and emission at 525 nm.

MMP assay. Variations in MMP were assessed using the fluorescent cationic dye Rhodamine 123 (Rh123), which is a cationic membrane-permeant fluorescent probe that accumulates in mitochondria and is released upon membrane depolarization. The treated hepatocytes were harvested, washed twice with PBS and stained with Rh123 (2 μg/ml) for 30 min in the dark. The fluorescence intensity was analyzed with a fluorescence spectrometer at an excitation wavelength of 495 nm and an emission wave length of 535 nm.

Measurement of mPTP opening. The treated L-02 hepatocytes were collected and processed for mitochondrial isolation. The pellets of the treated hepatocytes were washed twice with ice-cold PBS and resuspended with five volumes of buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride; pH 7.5). They were left on ice for 2 min and subsequently homogenized with a syringe (20-30 times, confirmed ~90% cells breakage occurred). The homogenates were centrifuged twice at 1,500 x g for 15 min at 4˚C. The supernatant obtained was centrifuged at 10,000 x g for 15 min at 4˚C. The resulting obtained mitochondrial pellets were resuspended in buffer A. The protein content in the mitochondrial suspensions was determined using Coomassie Brilliant Blue (G-250) by the Bradford method (17). The opening of the PTPs was determined using a mitochondrial permeability transition pore detection kit (Genmed Scientifics, Inc.) according to the manufacturer's protocol. The results were evaluated using a fluorescence spectrophotometer, with an excitation wavelength of 488 nm and an emission wave length of 505 nm.

Measurement of the activity of MRCC I-V. The activities of MRCC I-V were determined with Mitochondrial Respiratory Chain Complexes Activity Assay kits (Genmed Scientifics, Inc.). All experiments were performed according to the protocol provided by the manufacturer. All measurements were performed in triplicate.

Measurement of ROS production. The hepatocytes were exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h with or without the pretreatment with 10 mM N-acetyl-cysteine (NAC) for 1 h at 37˚C. The production of ROS was measured using hydroethidine (HE; Molecular Probes; Thermo Fisher Scientific, Inc.) in the hepatocytes. HE is a non-fluorescent compound that is able to diffuse through cell membranes and may be rapidly oxidized to ethidium under the action of $O_2^{\cdot-}$. The hepatocytes were treated with 2 μM HE for 15 min at 37˚C, and were subsequently analyzed by flow cytometry. For each sample, 10^4 cells were analyzed.

Statistical analysis. For all quantitative data collected, all values are expressed as the mean ± standard deviation of at least three independent experiments. Statistical significance was determined by one-way analysis of variance followed by Dunnett's post-hoc test. All statistical analyses were performed using SPSS 19.0 (IBM Corp, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cr(VI) exposure induces ERS in hepatocytes. The L-02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h and processed for mRNA expression determination of ERS-associated genes, *CHOP*, *PERK*, *IRE1* and *GRP78*. As presented in Fig. 1A, Cr(VI) upregulated the mRNA expression levels of all the detected genes in a concentration-dependent manner. The western blot results, presented in Fig. 1B, confirmed that Cr(VI) additionally upregulated the relative protein expression levels. A previous study suggested that ER stress is pivotal in cellular apoptosis (18), the present study examined whether Cr(VI)-induced ERS was accompanied by apoptotic cell

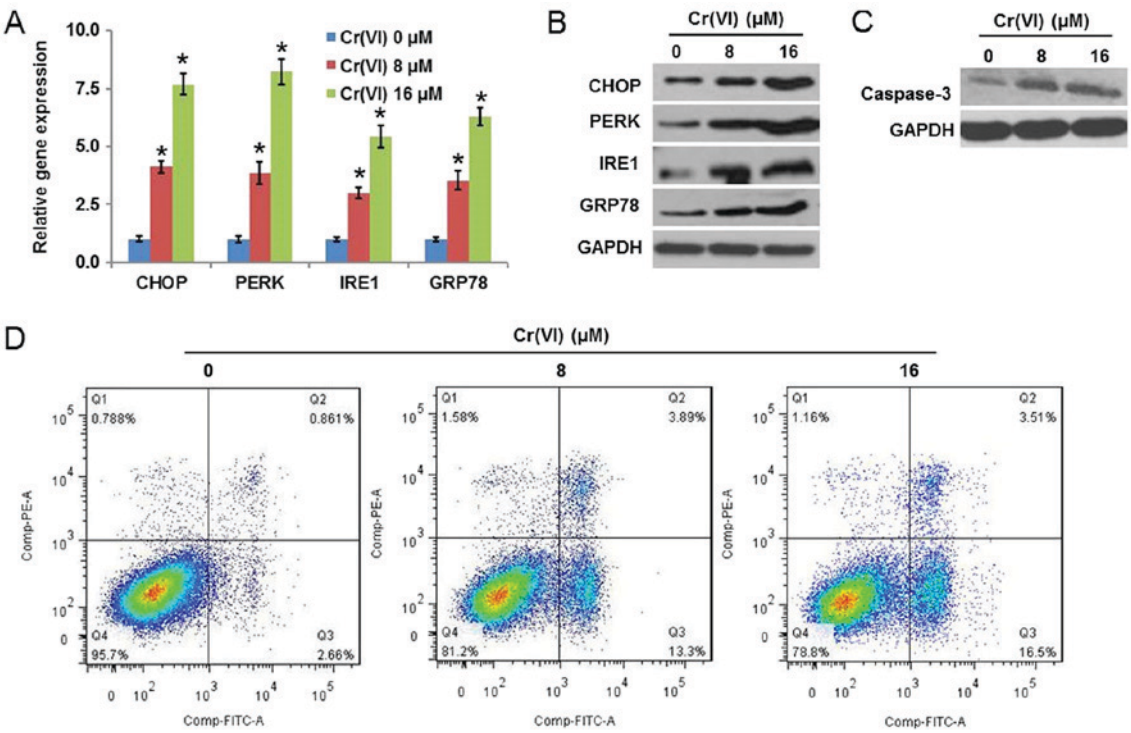

Figure 1. Cr(VI) exposure induces ERS and apoptosis in hepatocytes. The L-02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h. The mRNA and protein expression levels of ERS-associated genes, *CHOP*, *PERK*, *IRE1* and *GRP78* were determined using (A) reverse transcription-quantitative polymerase chain reaction analysis and (B) western blotting, respectively. (C) Expression of caspase-3 was examined by western blotting. (D) Population of early apoptotic cells [Annexin V-FITC (+)] and late apoptotic cells [(Annexin V-FITC (+)/PI (+)] was determined by flow cytometry. All values are expressed as the mean ± standard deviation and each experiment was repeated at least three times. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; ERS, endoplasmic reticulum stress; CHOP, CCAAT/enhancer-binding protein homologous protein; PERK, RNA-activated protein kinase-like ER kinase; IRE1, inositol-requiring enzyme-1; GRP78, glucose-regulated protein 78; FITC, fluorescein isothiocyanate; PI, propidium iodide.

death. Caspase-3 is the primary executioner caspase in apoptosis, and it was identified that Cr(VI) increased the expression of caspase-3 in a concentration-dependent manner (Fig. 1C). The flow cytometry results (Fig. 1D) demonstrated that Cr(VI) increased the population of early [Annexin V-FITC (+)] and late [(Annexin V-FITC (+)/PI (+)] apoptotic cells.

It is known that the disturbance of ER Ca^{2+} homeostasis may lead to ER stress (19). As the concentration of Ca^{2+} in the ER varies with time during ERS, the present study determined ER Ca^{2+} concentration following treatment with different concentrations (0, 8 and 16 μM) and treatment durations (12, 24 and 36 h) of Cr(VI). As presented in Fig. 2A, Cr(VI) decreased ER Ca^{2+} concentration in a concentration-dependent manner when the treatment times were 12 and 24 h, with the decrease more marked at 24 h. Cr(VI) increased ER Ca^{2+} concentration when the treatment time was 36 h. As the principal Ca^{2+} release channels from the ER are IP3R, the expression of IP3R in Cr(VI)-treated hepatocytes was detected. It was identified that following exposure to different concentrations of Cr(VI), the mRNA expression level of IP3R was upregulated at 12 h, was downregulated at 36 h, and demonstrated no obvious alteration at 24 h compared with the control (Fig. 2B). The protein expression levels, presented in Fig. 2C, demonstrated that the expression of IP3R was increased at 12 and 24 h, with the increase being more marked at 24 h, and decreased at 36 h following Cr(VI) exposure; this suggested that Ca^{2+} release from the ER was increased at 12 and 24 h, and decreased at 36 h, which explains the result in Fig. 2A.

Cr(VI) induces mitochondrial damage. Mitochondrial damage is frequently accompanied by a decrease of MMP and an increase of mPTP opening. As demonstrated in Fig. 3A, Cr(VI) increased the fluorescence intensity in a concentration-dependent manner, indicating the collapse of MMP. It was additionally identified that Cr(VI) increased the mPTP opening rate (Fig. 3B). As the release of AIF and Cyt C is associated with the loss of MMP and the increase in mPTP opening (20), the expression of these proteins was examined and it was observed that the protein expression levels of AIF and Cyt C were increased in a concentration-dependent manner (Fig. 3C). These results suggested that Cr(VI) induced a mitochondrial-mediated apoptotic pathway in human L-02 hepatocytes.

Cr(VI)-induced mitochondria damage is associated with ROS. To elucidate the source of ROS, the activity of MRCCs was examined (Fig. 4A), and it was identified that Cr(VI) significantly inhibited the activity of MRCC I, marginally inhibited the activity of MRCC II in a concentration-dependent manner, and demonstrated no regulatory effect on MRCC III, IV or V. The protein expression levels of MRCCs (Fig. 4B) demonstrated similar results. Increasing evidence suggests that ROS are essential in various cytotoxic mechanisms induced by exogenous toxicant exposure. ROS production was measured by HE staining and the result was quantified by flow cytometry using the mean intensity of fluorescence. As presented in Fig. 4C, Cr(VI) treatment

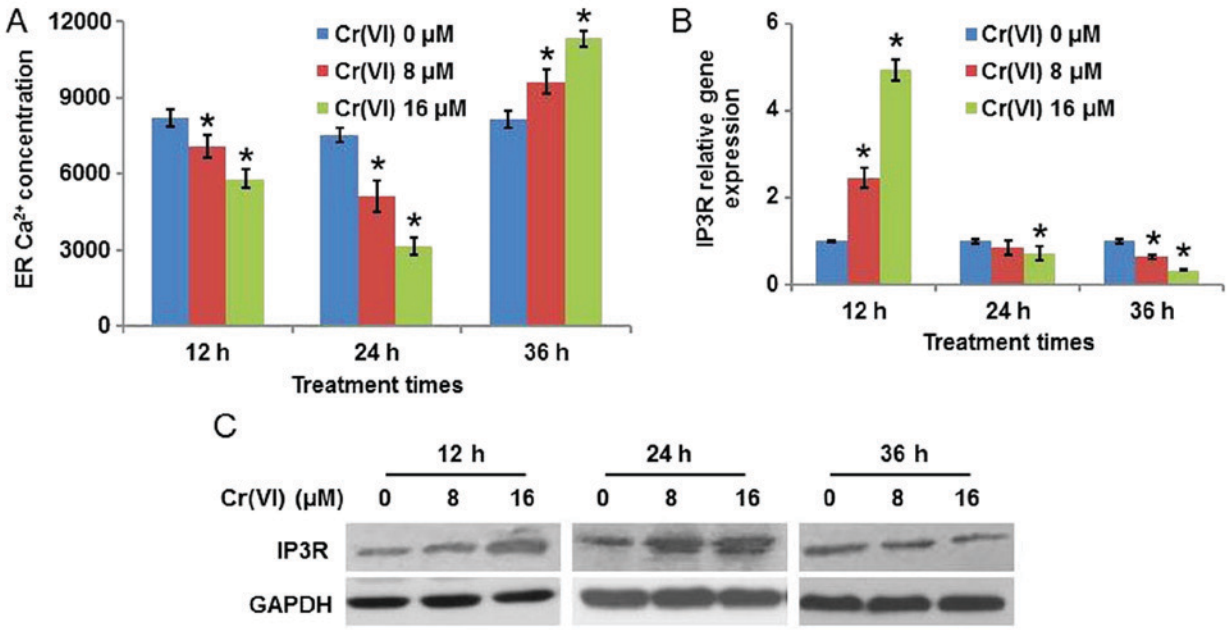

Figure 2. Cr(VI) induces IP3R-associated alteration of ER Ca^{2+}. The L-02 hepatocytes were exposed to different concentrations (0, 8 and 16 μM) of Cr(VI) for different treatment durations (12, 24 and 36 h). (A) Ca^{2+} concentration in the ER was detected using a commercial kit. (B) Gene expression of IP3R was evaluated using reverse transcription-quantitative polymerase chain reaction analysis. (C) Protein expression of IP3R was determined by western blotting. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; ER, endoplasmic reticulum; IP3R, inositol 1,4,5-trisphosphate.

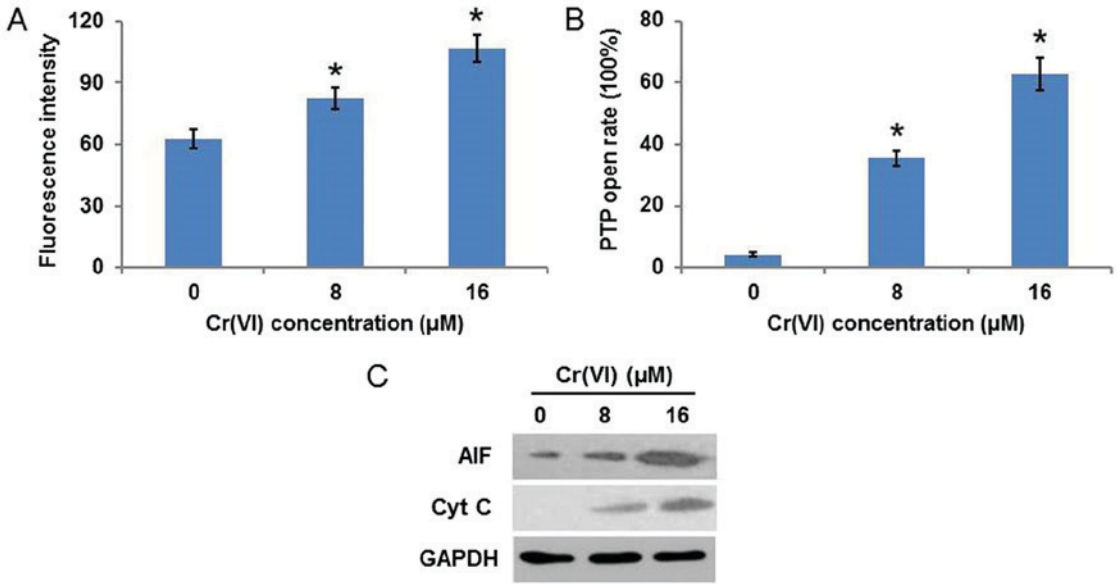

Figure 3. Cr(VI) induces mitochondria damage. The L-02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h. (A) Variations of mitochondrial membrane potential were assessed using the fluorescent cationic dye Rh123. (B) Opening of the mPTP was determined using a commercial mPTP detection kit. (C) Protein expression of AIF and Cyt C was determined using western blotting. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; mPTP, mitochondrial permeability transition pore; AIF, apoptosis inducing factor; Cyt C, cytochrome *c*.

increased ROS production in a concentration-dependent manner, indicating the generation of a large quantity of intracellular ROS.

As it was inferred that ROS are key in Cr(VI)-induced mitochondrial damage, the ROS scavenger, NAC, was used to inhibit ROS. The hepatocytes were exposed to different concentrations of Cr(VI) following pretreatment with 10 mM NAC for 1 h. As presented in Fig. 5A, ROS production was inhibited following Cr(VI) exposure, which confirmed the specificity of NAC. Notably, NAC application alleviated the Cr(VI)-induced collapse of MMP (Fig. 5B), decreased the susceptibility of the hepatocytes to mPTP opening (Fig. 5C), and inhibited the release of AIF and Cyt C from mitochondria (Fig. 5D).

Role of ROS-mediated mitochondria damage in Cr(VI)-induced ERS. The effect of NAC application on the mRNA expression levels of ERS-associated genes following Cr(VI)

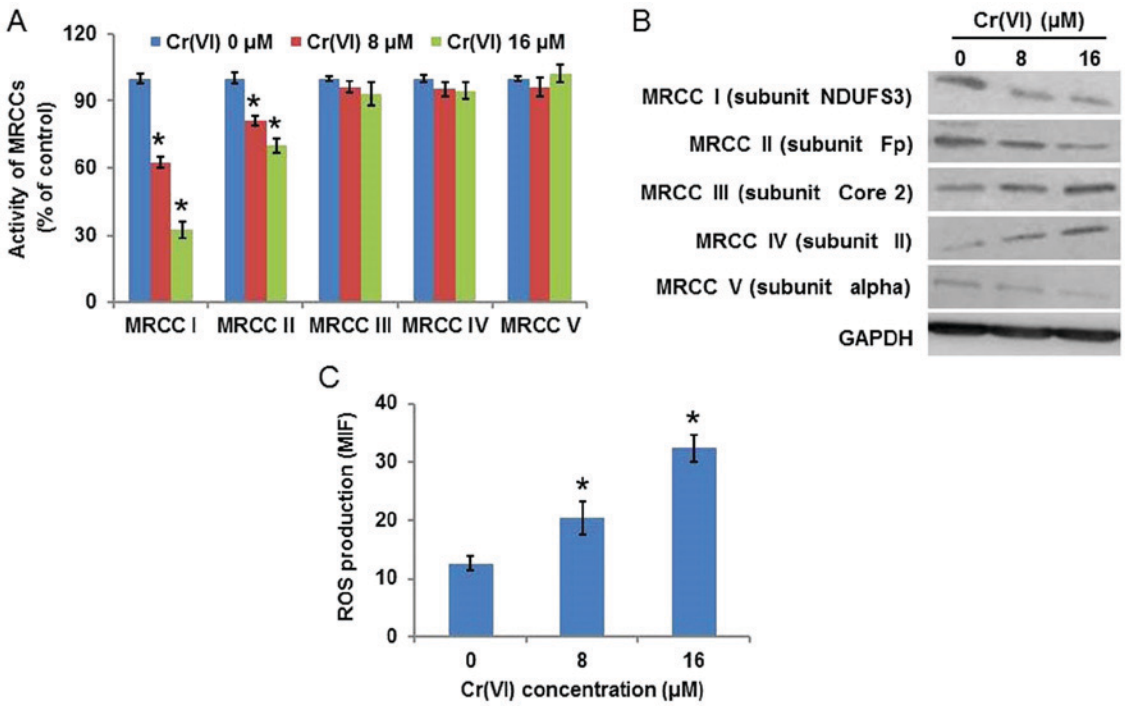

Figure 4. Cr(VI) induced MRCC-associated ROS production. The L-02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h. (A) Activities of MRCC I-V were determined with the commercial MRCC Activity Assay kits. (B) Protein expression of MRCC I-V was examined by western blotting. (C) Intracellular ROS production was determined using hematoxylin and eosin. The result was quantified by flow cytometry using the mean intensity of fluorescence. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; MRCCs, mitochondrial respiratory chain complexes; ROS, reactive oxygen species.

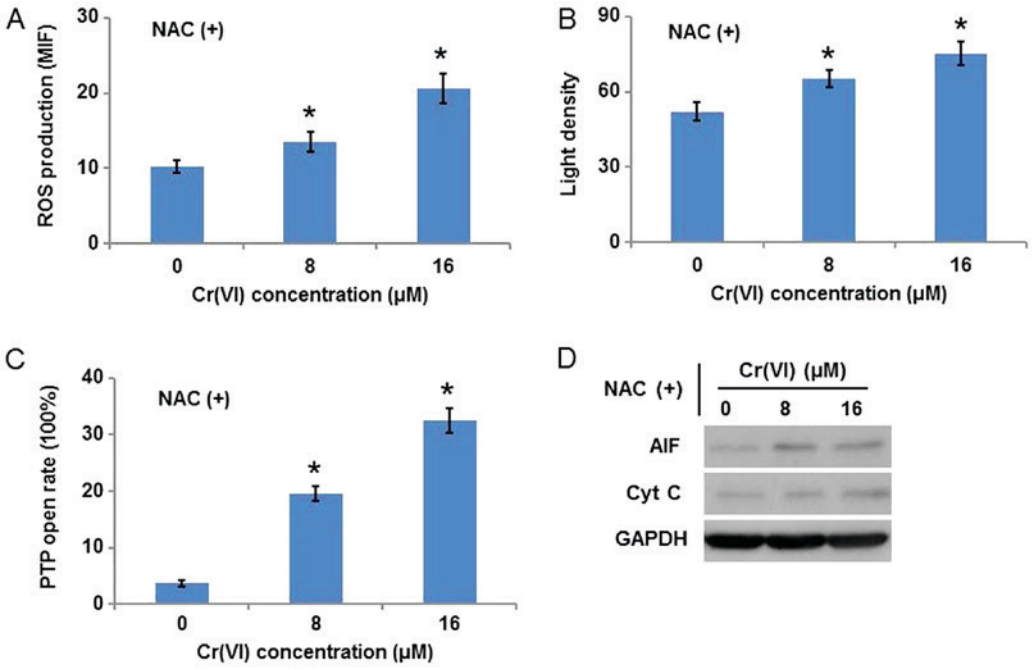

Figure 5. Cr(VI)-induced mitochondria damage is associated with ROS. The hepatocytes were pretreated with 10 mM NAC for 1 h and were subsequently exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h. (A) ROS production was determined using the fluoroprobe CM-H2DCFDA by flow cytometry. (B) Mitochondrial membrane potential was assessed using the fluorescent dye Rhodamine 123. (C) Opening of mPTPs was determined using a commercial kit. (D) Protein expression of AIF and Cyt C was examined by western blotting. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; NAC, N-acetyl-cysteine; AIF, apoptosis inducing factor; Cyt C, cytochrome *c*.

exposure were examined. The results (Fig. 6A) demonstrated that NAC alleviated the Cr(VI)-induced increase of the mRNA expression levels of *CHOP*, *PERK*, *IRE1* and *GRP78*. The protein expression levels (Fig. 6B) exhibited similar results. It was additionally identified that NAC decreased the population of early and late apoptotic cells following Cr(VI) exposure,

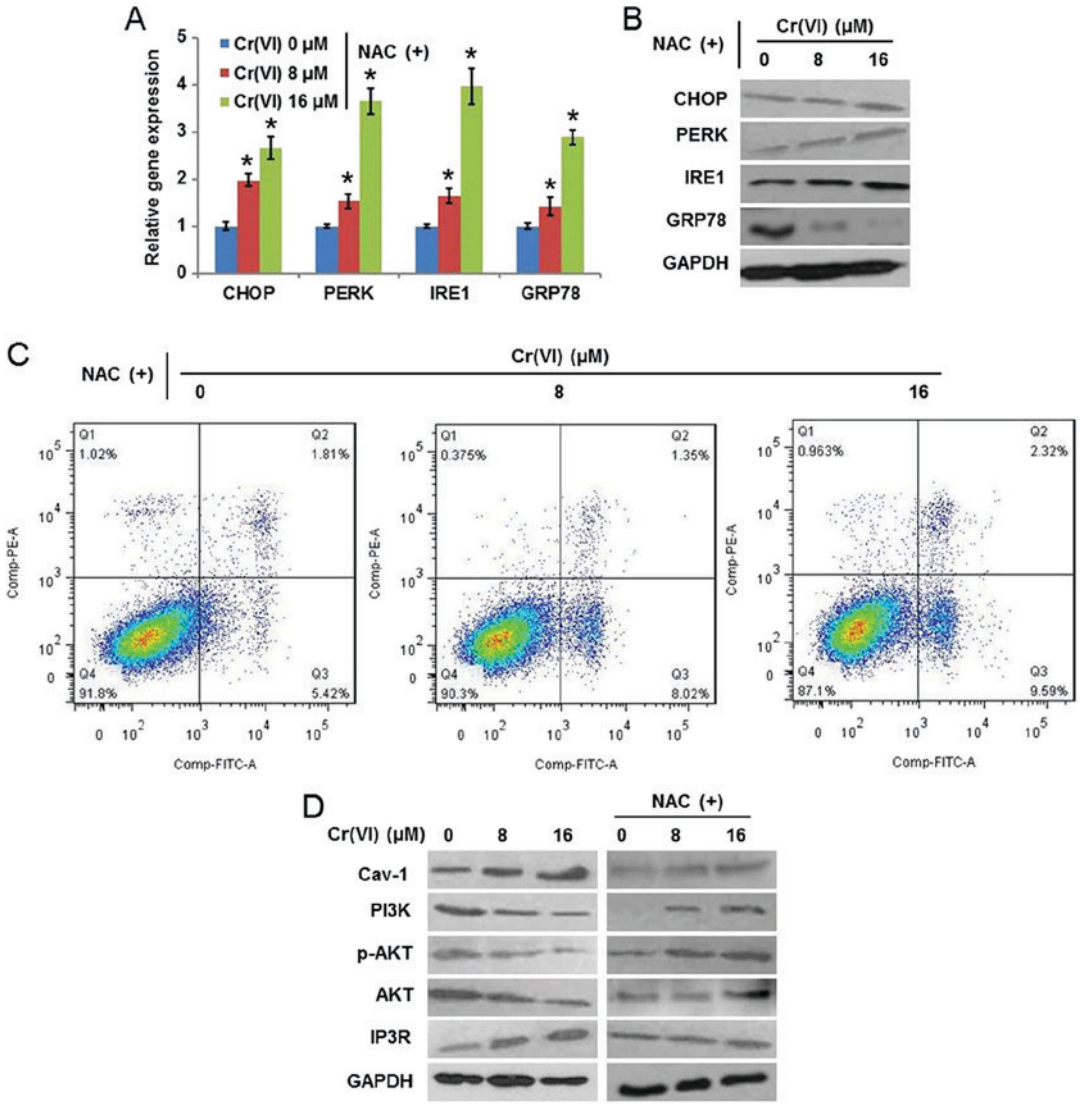

Figure 6. Role of reactive oxygen species-mediated mitochondrial damage in Cr(VI)-induced ERS. The hepatocytes were pretreated with 10 mM NAC for 1 h and then exposed to different concentrations of Cr(VI) (0, 8 and 16 μM) for 24 h. The (A) mRNA and (B) protein expression of ERS-associated genes were determined using reverse transcription-quantitative polymerase chain reaction analysis and western blotting, respectively. (C) Apoptotic cell death was examined by flow cytometry using Annexin V-FITC/PI double staining. (D) Protein expression of Cav-1, PI3K, p-AKT, AKT and IP3R was determined by western blotting. *P<0.05 vs. the control. Cr(VI); hexavalent chromium; NAC, N-acetyl-cysteine; CHOP, CCAAT/enhancer-binding protein homologous protein; PERK, RNA-activated protein kinase-like ER kinase; IRE1, inositol-requiring enzyme-1; GRP78, glucose-regulated protein 78; FITC, fluorescein isothiocyanate; PI, propidium iodide; Cav-1, caveolin-1; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; p-AKT, phospho-AKT; IP3R, inositol 1,4,5-trisphosphate.

indicating the inhibition of apoptotic cell death (Fig. 6C). Cav-1 is the most important component of caveolae that has been demonstrated to regulate intracellular Ca^{2+} signals (21) and the PI3K/AKT pathway (22), and AKT is reported to regulate IP3R (23). As presented in Fig. 6D, Cr(VI) treatment increased the protein expression of Cav-1 and IP3R; however, decreased the protein expression of PI3K, p-AKT (Ser 473) and AKT, indicating the regulatory effect of the Cav-1/PI3K/AKT pathway on the expression of IP3R. NAC application alleviated the Cr(VI)-induced alterations of these proteins. These results suggested that ROS activated Cav-1, which further abrogated the inhibitory effect of AKT on IP3R. These findings suggest a cross-talk of apoptotic signaling between the mitochondria and the ER, which is involved in Cr(VI)-induced apoptosis.

Discussion

In the present study, it was demonstrated that Cr(VI) upregulated ERS-associated genes, *CHOP*, *PERK*, *IRE1* and *GRP78*, indicating that Cr(VI) was capable of inducing ERS in L-02 hepatocytes. It was additionally identified that the concentration of Ca^{2+} in the ER varied with time during Cr(VI)-induced ERS. Cr(VI) decreased the ER Ca^{2+} concentration when the treatment durations were 12 and 24 h, and increased the ER Ca^{2+} concentration when the treatment lasted for 36 h. These were consistent with the alterations in the protein expression of IP3R, which was increased at 12 and 24 h, and decreased at 36 h. The mRNA and protein expression levels of IP3R were decreased at 36 h post-Cr(VI) exposure, indicating the existence of cellular

self-protection mechanisms which alleviate the marked Cr(VI)-induced release of Ca^{2+} from the ER. Mitochondrial damage may be characterized by the collapse of MMP and the increase of mPTP opening. To elucidate the source of ROS, the activities and protein expression of MRCC I-V were detected. It was identified that, contrary to a previous study that suggested that MRCC I and III in mitochondria are important sites of ROS generation in mammalian cells (24), MRCC I and II were inhibited, whereas ,other complexes were not affected by Cr(VI) exposure. It was confirmed that Cr(VI) induced burst generation of ROS in the hepatocytes. As redox homeostasis appears to be a critical factor for the maintenance of normal functioning of mitochondria, a high level of intracellular ROS (oxidative stress) is deleterious and apparently had a causative effect on mitochondrial damage (25). Therefore, the balance between ROS formation and removal allows for normal cellular function, whereas, the imbalance causes oxidative stress and results in pathobiological consequences (25). mPTP opening and MMP collapse are additionally viewed as the mitochondrial response to various oxidative stresses resulting in the amplified ROS signal (25). In the present study, the rapid loss of MMP, the increase of mPTP opening, the release of Cyt C and AIF, and the activation of caspase-3 were observed in hepatocytes following Cr(VI) exposure.

It has been demonstrated that mitochondrial ROS production increases ER Ca^{2+} release and mitochondrial Ca^{2+} loading (26). In our previous study, it was observed that Cr(VI) additionally induced Ca^{2+} overload in the mitochondria, and the increased levels of mitochondrial Ca^{2+} additionally stimulated mitochondrial ROS production (27). The increased mitochondrial ROS demonstrated damaging effects on mitochondrial membrane lipids, which consequently resulted in the disruption of MMP and the pathological opening of mPTPs; the increased mPTP opening additionally led to further collapse of the MMP (28). This vicious circle may damage mitochondria and result in rupture of the outer mitochondrial membrane, with the consequent release of Cyt C and AIF from the mitochondria into the cytosol, which ultimately activates the caspase cascade and induces apoptosis (29). Additionally, it has been confirmed that ROS-damaged mitochondria tend to produce more ROS in order to activate mitochondrial-mediated apoptotic or necrotic pathways (30).

Previous studies suggested that mitochondrial ROS generation-induced altered redox homeostasis in the cell is sufficient to initiate ERS, which may in turn induce the production of ROS in the ER and mitochondria (31,32). In the present study, it was demonstrated that mitochondrial ROS generation initiates a destructive cycle involving mitochondrial damage and ERS, which further increases ROS production and leads to apoptotic cell death. Extensive evidence is accumulating that burst generation of ROS can disturb ER protein folding and thus induce ERS, which may activate the UPR to resolve this protein-folding defect (33). ERS is known to trigger three principal branches of the UPR, including the PERK, IRE1 and ATF6 pathways, which serve as proximal sensors of the protein folding status in the ER (34). Increasing literature suggests the mitochondria and ER build a dynamic network where they cooperate in the generation of Ca^{2+} signals (35). In addition, ROS accumulation affects Ca^{2+} homeostasis in the ER, which is followed by the activation of ER chaperone gene GRP78 to prevent intracellular Ca^{2+} overload-induced cytotoxicity (36). Therefore, when ERS and ER dysfunction occur beyond possibility of restoration, the activation of pro-apoptotic signaling pathways can be viewed as the mechanism to protect the organism by eliminating damaged cells. To further confirm the functional role of ROS in Cr(VI)-mediated mitochondrial damage and ERS in the hepatocytes, the ROS scavenger NAC was used in the present study.

Cav-1, an oncoprotein and tumor suppressor, is known to be the essential structural protein component of the plasma membrane microdomains called caveolae and is associated with various membranous structures, including the ER (37). Numerous ion channels and key proteins involved in Ca^{2+} signals are located in caveolae, indicating that caveolae may be important in the regulation of Ca^{2+} signals (38). Cav-1 is a marker protein and the most important structural component of caveolae. It has been reported that ROS generation can promote the phosphorylation of Cav-1 on tyrosine-14 and then result in the activation of Cav-1 (39). Previous evidence suggested that Cav-1 positively regulates the PI3K/Akt pathway (40), whereas others demonstrated the opposite result that Cav-1 negatively regulates the PI3K/AKT pathway (41). It has additionally been confirmed that AKT phosphorylates IP3R and subseuqently suppresses the pro-apoptotic Ca^{2+}-release function (42), indicating that AKT may negatively regulate IP3R. Therefore, it was suggested that Cr(VI) induced IP3R-mediated Ca^{2+} release from the ER through ROS/Cav-1/AKT signaling. It was confirmed in the present study that Cr(VI) increased the protein expression levels of Cav-1 and IP3R; however, decreased the protein expression levels of PI3K, p-AKT (Ser 473) and AKT, indicating that Cav-1 was activated, whereas the AKT pathway was inhibited following ROS accumulation induced by Cr(VI). Therefore, it was demonstrated in the present study that Cr(VI) induced Ca^{2+} release from the ER through ROS/Cav-1/AKT/IP3R signaling. As it has additionally been reported that Cav-1 may directly interact with IP3R (43), further investigations are required to elucidate the possible molecular mechanism involved in Cr(VI)-induced IP3R-mediated Ca^{2+} release from the ER.

In conclusion, the present findings demonstrated that Cr(VI) induced ERS in L-02 hepatocytes, which was characterized by the upregulation of ERS-associated CHOP, PERK, IRE1 and GRP78, and by the mass release of Ca^{2+} from the ER. The Cr(VI)-induced mitochondrial production of ROS by disturbing MRCC I and II was the key inducer of a destructive cycle of mitochondrial damage involving mPTP opening and MMP collapse, which further increased ROS production. These results additionally provide detailed mechanistic information on how Cr(VI)-induced ROS exerts its regulatory effects on principal Ca^{2+} release channels from the ER, IP3R (activation of Cav-1 and inhbition of the PI3K/AKT pathway), which confirmed that Cr(VI) induced the release of Ca^{2+} from the ER through ROS/Cav-1/AKT/IP3R signaling. These data demonstrated the role of mitochondrial damage in Cr(VI)-induced ERS and provide novel insight into the elucidation of Cr(VI)-induced cytotoxicity.

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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

FX designed the experiments, supervised the project and wrote the manuscript. QL, YX and MH performed the experiments and analyzed the data. YZ contributed to the conception of the study and interpreted the data.

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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