

# Hexavalent chromium targets mitochondrial respiratory chain complex I to induce reactive oxygen species-dependent caspase-3 activation in L-02 hepatocytes

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**Abstract.** Hexavalent chromium [Cr(VI)], which is used for various industrial applications, such as leather tanning and chroming, can cause a number of human diseases including inflammation and cancer. Cr(VI) exposure leads to severe damage to the liver, but the mechanisms involved in Cr(VI)-mediated toxicity in the liver are unclear. The present study provides evidence that Cr(VI) enhances reactive oxygen species (ROS) accumulation by inhibiting the mitochondrial respiratory chain complex (MRCC) I. Cr(VI) did not affect the expression levels of antioxidative proteins such as superoxide dismutase (SOD), catalase and thioredoxin (Trx), indicating that the antioxidative system was not involved in Cr(VI)-induced ROS accumulation. We found that ROS mediated caspase-3 activation partially depends on the downregulation of the heat shock protein (HSP) 70 and 90. In order to confirm our hypothesis that ROS plays a key role in Cr(VI)-mediated cytotoxicity, we used N-acetylcysteine (NAC) to inhibit the accumulation of ROS. NAC successfully blocked the inhibition of HSP70 and HSP90 as well as the activation of caspase-3, suggesting that ROS is essential in Cr(VI)-induced caspase-3 activation. By applying different MRCC substrates as electron donors, we also confirmed that Cr(VI) could accept the electrons leaked from MRCC I and the reduction occurs at MRCC I. In conclusion, the present study demonstrates that Cr(VI) induces ROS-dependent caspase-3 activation by inhibiting MRCC I activity, and MRCC I has been identified as a new target and a new mechanism for the apoptosis-inducing activity displayed by Cr(VI).

## Introduction

Chromium (Cr) is a common industrial chemical used in diverse processes including metallurgy, electroplating, leather

tanning, and chroming (1). The most stable and common oxidation states of Cr are trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] (2). Cr(III) is required in tracing sugar amounts and in lipid metabolism (3). The exposure of Cr(III) is considered less toxic because of its tendency to form insoluble hydrated complexes which cannot cross cell membranes (4). Cr(VI) enters the body by inhalation, ingestion, or absorption through skin, and Cr(VI) enters into cells through an anion transport system (5). Once inside the cells, it can be reduced to its lower oxidation states, pentavalent chromium [Cr(V)] and tetravalent chromium [Cr(IV)] (6). The reduction process remains to be explored and fully understood. Cr(VI) is highly soluble and it exerts toxic effects in most living organisms. Occupational exposure to Cr(VI) is associated with several adverse effects of health, such as contact dermatitis, nasal perforation, and bronchogenic cancer (7). It has been reported that Cr(VI) could inhibit DNA, RNA and protein synthesis in hepatocytes and induce damage to liver structure and function thus causing toxic hepatitis (8,9).

Reactive oxygen species (ROS) are defined as oxygen-containing chemical species with reactive chemical properties including free radicals that contain unpaired electrons, such as superoxide ( $O_2^-$ ), hydroxyl radicals ( $HO^\bullet$ ), and non-radical molecules like hydrogen peroxide ( $H_2O_2$ ) (10). ROS are formed mainly by the interaction of oxygen molecules with electrons that escape from the mitochondrial respiratory chain (MRC) (11). MRC contains five multimeric protein complexes including reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase-ubiquinone oxidoreductase (MRCC I), succinate dehydrogenase-ubiquinone oxidoreductase (MRCC II), ubiquinone-cytochrome c oxidoreductase (MRCC III), cytochrome c oxidase (MRCC VI), and ATP synthase (MRCC V) (12). Inhibition of MRCC increases the electron leakage by blocking the electron transfer, thus enhancing ROS production (13). ROS can be scavenged by antioxidative proteins, including catalase, superoxide dismutase (SOD), and thioredoxin (Trx) (14,15). Although it is believed that ROS play a key role in the toxic effect of Cr(VI), the precise mechanisms by which Cr(VI) triggers apoptosis are not fully understood.

Apoptosis is a process controlled by a specific signaling pathway and is characterized by cellular shrinkage, nuclear condensation, and DNA fragmentation (16). Although

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numerous studies have suggested that mitochondria stress and caspase activation are the most typical events required for apoptotic cell death (17), the precise mechanisms by which Cr(VI) induces apoptosis in hepatocytes have not been elucidated. In the present study, we demonstrated that Cr(VI) induces ROS-dependent caspase-3 activation by inhibiting MRCC I activity. MRCC I is a site for ROS generation as well as Cr(VI) reduction. To the best of our knowledge, this is the first time that Cr(VI) is identified as an MRCC I inhibitor. These results present a new target and mechanism for Cr(VI)-induced cytotoxicity.

## Material and methods

**Materials.** The L-02 hepatocyte line was provided by the China Center for Type Culture Collection of Wuhan University. The MRCC substrates (glutamate/malate, succinate, coenzyme Q, vitamin C), rotenone (ROT) and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 culture medium, fetal bovine serum (FBS), and trypsin-EDTA (0.25%) were obtained from Gibco (Gaithersburg, MD, USA). Potassium dichromate ( $K_2Cr_2O_7$ ) was obtained from Changsha Chemical Reagents Co. (Changsha, China).

**Cell culture and chemicals treatment.** L-02 hepatocytes were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and antibiotics (50 U/ml penicillin and 50  $\mu$ g/ml streptomycin) at 37°C under a humidified atmosphere of 5%  $CO_2$ . The medium was changed every other day.

**Measurement for cell viability.** MTT assay was performed to evaluate cell viability as previously described (18). The cells in exponential growth were seeded in 96-well plates with 100  $\mu$ l of medium containing  $10^4$  cells.  $K_2Cr_2O_7$  solution of indicated final concentrations (0, 2, 8, 32, 128 and 512  $\mu$ M) was added. Control cells and medium controls without cells received DMSO without  $K_2Cr_2O_7$ . After incubation at 37°C in a 5%  $CO_2$  saturated atmosphere for an indicated time period, the cells were treated with 5  $\mu$ l 5 mg/ml of MTT solution for an additional 4 h at 37°C, and then lysed in phosphate-buffered saline (PBS, pH 7.4) containing 20% sodium dodecyl sulfate (SDS) and 50% N, N-dimethylformamide (pH 4.5). The absorbance was read on a multiwell ELISA reader Versamax (Molecular Devices, Sunnyvale, CA, USA) at 570 nm for each well.

**Measurement of ROS production.** Intracellular ROS production was determined by detecting the fluorescent intensity of 2',7'-dichlorofluorescein (DCF), the oxidized product of the fluoroprobe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes, USA). Briefly,  $2 \times 10^6$  cells were collected by centrifugation and were then incubated with 10  $\mu$ M CM-H2DCFDA in PBS for 40 min at 37°C in the dark. After incubation, the cells were split into two parts. One part was checked with a fluorescence microscope equipped with a Leica DC 100 digital camera. The other part was measured with a flow cytometer with excitation at 488 nm and emission at 535 nm. The amount of ROS production was considered directly proportional to the fluorescence intensity.

**Western blotting for protein levels determination.** L-02 hepatocytes were lysed using a Mammalian Cell Lysis kit from Sigma-Aldrich. Western blotting was performed with the WesternBreeze Chemiluminescent Immunodetection protocol (Invitrogen, Carlsbad, CA, USA). Proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and were then transferred to polyvinylidene fluoride (PVDF) membranes by electroelution. The membranes were incubated with a primary antibody overnight at 4°C following blocking with 4% non-fat milk. Membranes were then incubated for 1 h at room temperature with secondary antibodies, developed with a detection system and then exposed onto films.

The primary antibodies for MRCC including Complex I subunit NDUFS3 (MS110), Complex II subunit 70 kDa Fp (MS204), Complex III subunit core 2 (MS304), Complex IV subunit II (MS405), and ATP synthase subunit  $\alpha$  (MS502) were purchased from MitoScience (Eugene, OR, USA). Antibodies for SOD 1 (#2770), catalase (#8841), Trx (C63C6) (#2429), caspase-3 (#9662), heat shock protein (HSP)70 (#4872), HSP90 (E289) (#4877), and  $\beta$ -actin (#4967) were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Measurement of activities of respiratory chain complexes (MRCC) I-IV.** The mitochondria were isolated as previously described with slight modifications (19). Cells were washed twice with cold PBS, and resuspended with 5 ml 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, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5). Cells were homogenized and centrifuged twice at 750 x g for 10 min. Mitochondria pellets were obtained after centrifugation at 10,000 x g for 15 min.

The activities of MRCC were determined using Mitochondrial Respiratory Chain Complexes Activity Assay kits (Genmed Scientifics, Inc., Shanghai, China). All assays were performed in a final volume of 1 ml using a UV-9100 spectrophotometer. To establish the optimum conditions for the release of complexes, the mitochondria were freeze-thawed three times at 20/-20°C in hypotonic media (25 mM potassium phosphate, 5 mM  $MgCl_2$ , pH 7.2) before the determination. The activity of MRCC I [nicotinamide adenine dinucleotide (NADH) coenzyme Q (CoQ) oxidoreductase, expressed as nmol oxidized NADH/min/mg protein] was measured following the oxidation of NADH at 340 nm. The activity of MRCC II (succinate: 2,6-dichlorophenolindophenol (DCIP) oxidoreductase, expressed as nmol reduced DCIP/min/mg protein) was measured following the reduction of DCIP at 600 nm. The activity of MRCC III (ubiquinol: cytochrome c (Cyt c) reductase, expressed as nmol reduced Cyt c/min/mg protein) was measured following the reduction of Cyt c at 550 nm. The activity of MRCC IV (Cyt c oxidase, expressed as nmol oxidized Cyt c/min/mg protein) was measured following the oxidation of Cyt c at 550 nm. All measurements were performed in triplicate.

**Measurement of the Cr(VI) reduction rate in hepatocytes mitochondria.** Cr(VI) reduction was determined colorimetrically with a spectrophotometer using the S-diphenylcarbazide (DPC) (Nacalai Tesque, Inc., Japan) method (20). The mitochondria isolated from hepatocytes were pretreated with the

MRCC I substrates 10 mM glutamate/10 mM malate (Glu/Mal), MRCC II substrate 10 mM succinate (Suc), MRCC III substrate 5  $\mu$ M CoQ, or MRCC IV substrate 2 mM vitamin C (Vit C) for 10 min prior to 32  $\mu$ M Cr(VI) treatment. Cr(VI) reduction rate was measured at different time points (5, 30 and 60 min). After 3 freeze and thaw cycles, the mitochondria treatment suspensions (2 ml) were centrifuged for 5 min at 15,000  $\times$  g. The supernatant was added with 20  $\mu$ l H<sub>2</sub>SO<sub>4</sub> and 20  $\mu$ l H<sub>3</sub>PO<sub>4</sub>, mixed and then added with 80  $\mu$ l DPC (0.076  $\times$  g DPC previously dissolved in 20 ml of 95% ethanol). Twenty minutes later, the absorbance of the color produced was measured at 540 nm. Cr(VI) concentration in the sample was calculated from a standard curve using K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> as a standard.

**Measurement of caspase-3 activity.** Caspase-3 activity was detected using a Caspase-3 colorimetric assay kit (Millipore, Billerica, MA, USA). Briefly, L-02 hepatocytes were harvested and washed twice with ice-cold PBS. Then cell pellets were incubated with lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid, pH 7.4) for 30 min on ice. After centrifugation at 13,000  $\times$  g at 4°C for 5 min, the supernatants were collected and added with caspase-3 substrate Ac-DEVD-pNA to the final concentration of 100  $\mu$ M. The samples were incubated at 37°C for 1 h and the alternative activity of caspase-3 was described as the cleavage of the colorimetric substrate by measuring the absorbance at 405 nm.

**Flow cytometry analysis for apoptotic cells.** L-02 hepatocytes were harvested by trypsinization and washed with PBS. Washed cells were treated with FITC-conjugated Annexin V (0.5  $\mu$ g/ml final concentration) and propidium iodide (PI, 1  $\mu$ g/ml final concentration). After incubation for 20 min at room temperature, the apoptosis was determined by flow cytometry and analyzed by the CellQuest software. For each measurement, 20,000 cells were analyzed. PI was added to a sample to distinguish early apoptotic cells (Annexin V-positive, PI-negative) and late apoptotic cells (positive for both Annexin V and PI).

**Statistical analysis.** Statistical analysis was performed using SPSS 15.0 one-way analysis of variance (ANOVA) to assess the significance of differences between groups. The acceptance level of significance was  $P < 0.05$ . Results are expressed as mean  $\pm$  SD.

## Results

**Cr(VI) induces a concentration-dependent loss of cell viability in L-02 hepatocytes.** L-02 hepatocytes exposed to varying doses of Cr(VI) (2-512  $\mu$ M) over a 24 h period and a concentration-dependent loss of cell viability was observed in Fig. 1. The Cr(VI) concentration that required for 50% inhibition of cell viability (IC<sub>50</sub>) was 38.97  $\mu$ M. Therefore, we chose two concentrations of Cr(VI) (16, 32  $\mu$ M) for the following experiments.

**Cr(VI) causes ROS accumulation.** ROS play a critical role in mediating the cytotoxicity induced by Cr(VI), but the targets by which Cr(VI) induces ROS accumulation are unknown. To identify the targets for Cr(VI)-induced ROS accumulation, we first measured ROS levels. L-02 hepatocytes exposed

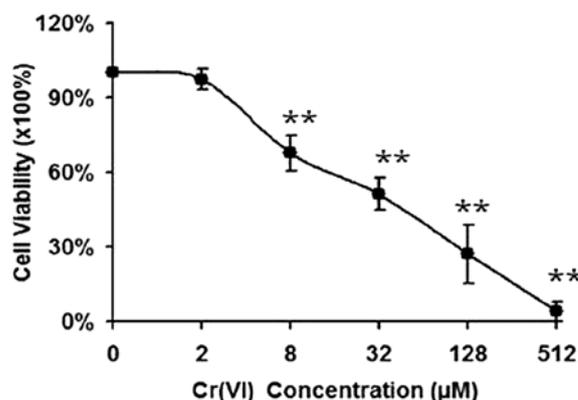


Figure 1. Cr(VI) induces a concentration-dependent loss of cell viability in L-02 hepatocytes. L-02 hepatocytes were incubated with different concentrations of Cr(VI) (0, 2, 8, 32, 128, 512  $\mu$ M) for 24 h and then processed for MTT assay to detect cell viability. The Cr(VI) concentration required for 50% inhibition of cell viability (IC<sub>50</sub>) was 38.97  $\mu$ M. \* $P < 0.05$ , \*\* $P < 0.01$  compared with the control group.

to 16 or 32  $\mu$ M Cr(VI) for 24 h were analyzed for ROS production utilizing the oxidant-sensitive fluorogenic probe CM-H<sub>2</sub>DCFDA. Cr(VI) stimulation induced higher levels of fluorescence signal in a dose-dependent manner, indicating the generation of a large amount of intracellular ROS in the Cr(VI) treatment groups (Fig. 2A). Quantitative analysis by flow cytometry showed that the ROS levels were about 3-fold higher in the 16  $\mu$ M Cr(VI) treatment group, and were about 5-fold higher in the 32  $\mu$ M Cr(VI) treatment group compared with the control group (Fig. 2B). It has been reported that downregulation of antioxidative proteins results in ROS accumulation (15). Therefore, we investigated the effect of Cr(VI) on the expression of antioxidative proteins such as SOD, catalase and Trx. It was revealed that Cr(VI) had no significant effect on the three proteins (Fig. 2C). Therefore, we reached the conclusion that Cr(VI) does not cause ROS overproduction by downregulating the antioxidative proteins.

**Cr(VI) induces ROS accumulation by inhibiting the activity of MRCC I.** Inhibiting the activity of MRCC promotes ROS accumulation (13). Thus we speculated that Cr(VI) may induce ROS accumulation by inhibiting MRCC activity. We measured the effect of Cr(VI) (16, 32  $\mu$ M) on different MRCC activities. Cr(VI) significantly inhibited MRCC I and slightly inhibited MRCC II at a dose of 32  $\mu$ M, but had no effect on MRCC III and IV (Fig. 3A), indicating that MRCC I as well as MRCC II may be targets of Cr(VI) in mediating ROS accumulation. This was further explored by measuring the activities of MRCC I and II at different time points after 32  $\mu$ M Cr(VI) exposure. MRCC I activity was significantly inhibited as early as 30 min after Cr(VI) treatment (Fig. 3B). In contrast, the activity of MRCC II was not altered until 120 min after Cr(VI) exposure, indicating that MRCC I is the target of Cr(VI) in promoting ROS production, and the decrease in MRCC II activity may be viewed as the result of inhibited MRCC I activity. The data were further confirmed by western blotting. Cr(VI) significantly decreased the expression of MRCC I (subunit NDUFS3), but had no effect on the expression levels of the left three complexes (Fig. 3C).

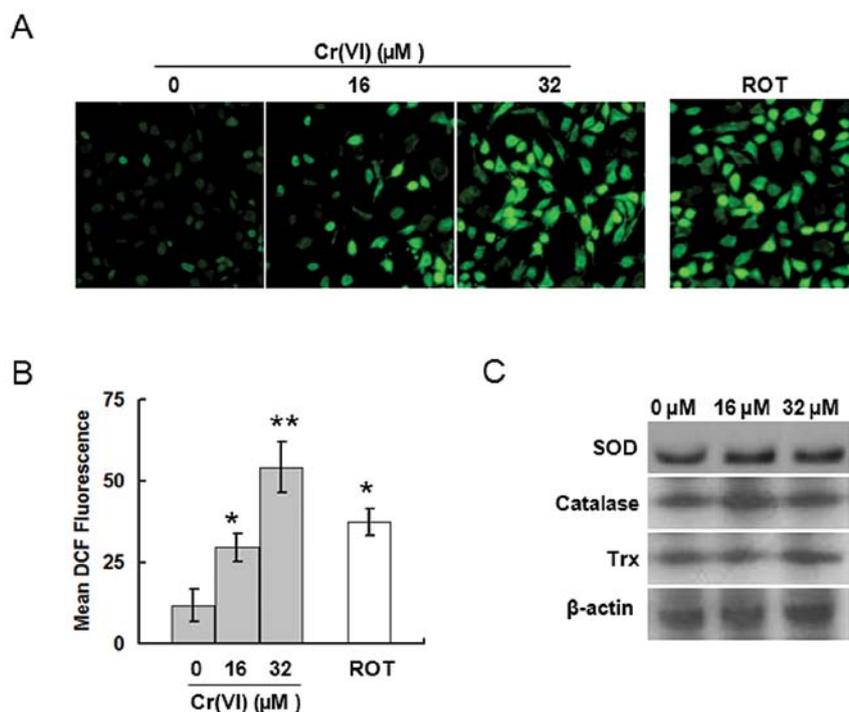


Figure 2. Cr(VI) enhances ROS accumulation in L-02 hepatocytes. (A and B) Cr(VI) induces ROS accumulation. The hepatocytes were treated with PBS or 16, 32  $\mu\text{M}$  Cr(VI) for 24 h. The MRCC I inhibitor, ROT (5  $\mu\text{M}$ , 24 h) was used as a positive treatment control. Treated cells were incubated with the oxidant-sensitive fluorogenic probe CM-H<sub>2</sub>DCFDA. (A) Fluorescence was detected by fluorescence microscope. (B) The amount of ROS production, which was considered to be directly proportional to the fluorescence intensity, was also quantitated by flow cytometer. The values were expressed as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to the control. (C) The alteration of antioxidative proteins induced by Cr(VI) is not involved in promoting ROS accumulation. Whole cell lysates were analyzed by western blotting to examine the expression levels of SOD, catalase and Trx. Representative images of at least three independent experiments are shown.

Based on the previous results, we inferred that Cr(VI) accepted the electrons and was reduced at the MRCC I. Mitochondria can reduce Cr(VI) using MRCC substrates as electron donors (21). The mitochondria isolated from hepatocytes were pretreated with the MRCC I substrates glutamate/malate (Glu/Mal), the MRCC II substrate succinate (Suc), the MRCC III substrate CoQ, or the MRCC IV substrate Vit C prior to Cr(VI) treatment. We then measured the Cr(VI) reduction rate in mitochondria at different time points. Cr(VI) reduction rate significantly increased in the mitochondria treated with Glu/Mal, indicating that Cr(VI) reduction occurs at the MRCC I (Fig. 3D).

**Cr(VI) activates caspase-3.** Our results suggested that Cr(VI) acts as an MRCC I inhibitor. Thus we used the specific MRCC I inhibitor rotenone (ROT) as a positive control to confirm the functional role of Cr(VI). ROT at 5  $\mu\text{M}$  for 24 h resulted in the accumulation of 3.6-fold higher ROS compared with control (Fig. 2A and B). Caspase-3 activity was determined after different doses of Cr(VI) exposure in L-02 hepatocytes. Similar to ROT, Cr(VI) significantly increased caspase-3 activity in a dose-dependent manner (Fig. 4A), again confirming that Cr(VI) targets MRCC I to induce the activation of caspase-3. Western blotting results also revealed that Cr(VI) exposure induced the increased expression of cleaved caspase-3 (17/19 kDa) and decreased expressions of full length caspase-3 (35 kDa), thus leading to the activation of caspase-3 (Fig. 4B). Occurrence of apoptosis was assayed by flow cytometry. The results of Annexin V-FITC and PI staining showed that Cr(VI) induced apoptosis in a dose-dependent manner

(Fig. 4C). The percentages of both early apoptotic cells (LR, as reflected in the lower-right-hand quadrant, Annexin V positive) and late apoptotic cells (UR, depicted in the upper-right-hand quadrant, positive for both Annexin V and PI) were significantly increased in the Cr(VI) treatment groups compared with control (LR, 5%; UR, 6%).

HSP has been identified as caspase-3 negative regulator and mediates the prevention of apoptosis (22,23). Thus, we determined whether Cr(VI) also mediated the inhibition of HSP to induce caspase-3 activation. The levels of HSP70, HSP90 were decreased following Cr(VI) treatment in a dose-dependent manner (Fig. 4D).

**Cr(VI)-induced activation of caspase-3 is mediated by ROS.** In order to confirm that Cr(VI)-induces caspase-3 activation is dependent on ROS function, we used NAC to inhibit ROS. The hepatocytes were exposed to Cr(VI) (16, 32  $\mu\text{M}$ ) in the presence of 10 mM NAC for 24 h. The production of ROS was blocked in each group, which confirmed the specificity of NAC (Fig. 5A). Western blot analysis revealed that NAC blocked the activation of caspase-3 and the inhibition of HSP70 and HSP90 (Fig. 5B), suggesting that Cr(VI) activated caspase-3 by inducing ROS-dependent decrease of HSP70 and HSP90. ROS is essential in Cr(VI)-induced caspase-3 activation.

## Discussion

Cr(VI) displays significant apoptosis-inducing activity *in vivo* and *in vitro* (24,25). Although it is believed that ROS, DNA

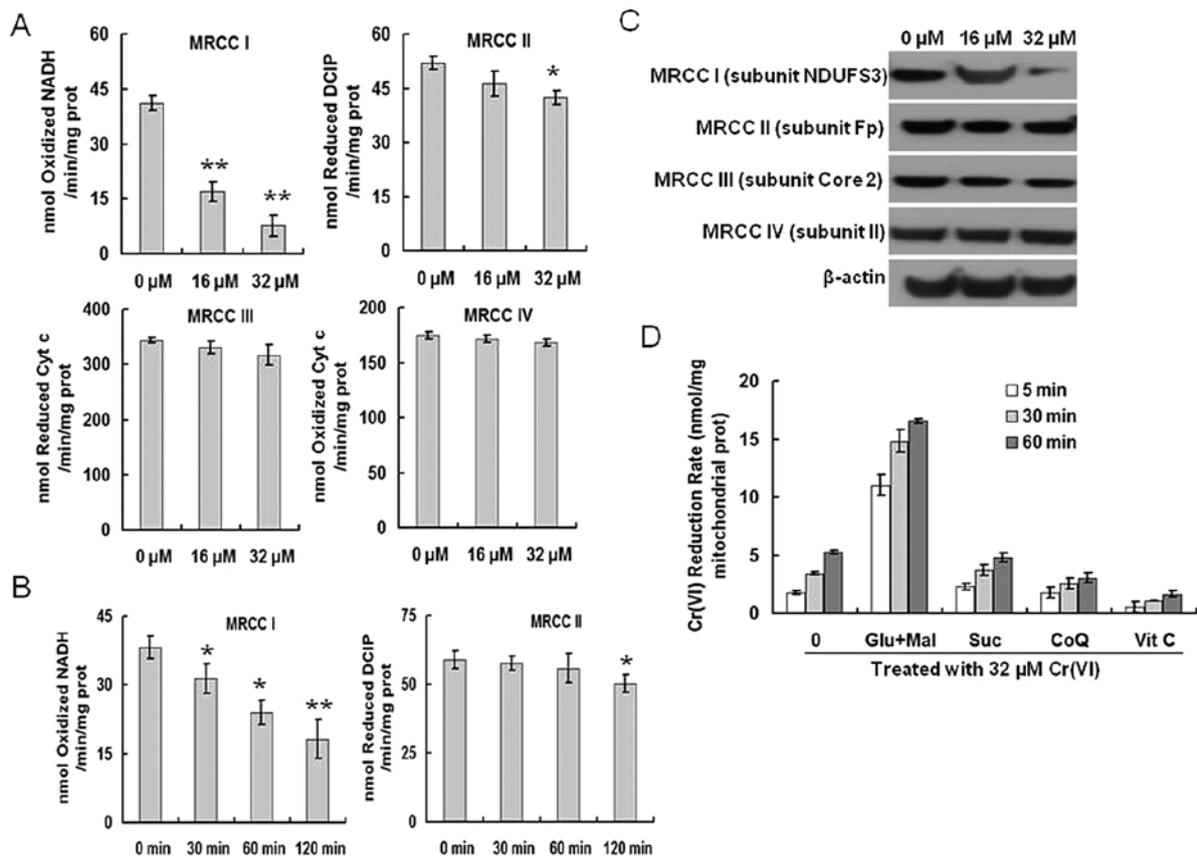


Figure 3. Cr(VI) targets and inhibits MRCC I. (A) Cr(VI) significantly inhibits MRCC I activity. L-02 hepatocytes were treated with Cr(VI) (16, 32  $\mu\text{M}$ ) for 24 h. The activities of MRCC I, II, III and IV were measured with the Mitochondrial Respiratory Chain Complex Enzyme Activity kits. All columns display the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to control. (B) Inhibition of MRCC I activity is involved in mediating ROS accumulation induced by Cr(VI). L-02 hepatocytes were treated with 32  $\mu\text{M}$  Cr(VI) for the indicated time (30, 60, 120 min). The activities of MRCC I and II were measured by Mitochondrial Respiratory Chain Complex Enzyme Activity kit. All columns display the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to control. (C) Cr(VI) decreases the protein expression levels of MRCC I. The hepatocytes were exposed to Cr(VI) (16, 32  $\mu\text{M}$ ) for 24 h and then were processed for western blotting analysis to examine the protein levels of MRCC I-IV. (D) Cr(VI) reduction occurs at MRCC I. The mitochondria isolated from hepatocytes were pretreated with MRCC I substrates 10 mM glutamate/10 mM malate (Glu/Mal), MRCC II substrate 10 mM succinate (Suc), MRCC III substrate 5  $\mu\text{M}$  coenzyme Q (CoQ), or MRCC IV substrate 2 mM vitamin C (Vit C) for 10 min prior to 32  $\mu\text{M}$  Cr(VI) treatment. The Cr(VI) reduction rate was measured using a spectrophotometer at different time points (5, 30, 60 min).

damage, and p53 activation played important roles in Cr(VI)-induced apoptosis, the precise targets and mechanisms remain to be fully understood.

ROS are defined as oxygen-containing chemical species with reactive chemical properties. MRC is the most important source of ROS within most cells, and ROS produced from the inhibition of MRCC are of pathological importance in a wide variety of degenerative diseases and cancer (26). SOD, catalase and Trx are main antioxidative proteins involved in ROS clearance. It is reported that some chemotherapeutic agents cause ROS-dependent cytotoxicity by downregulating the expression of the antioxidative proteins to facilitate ROS overproduction (27,28). However, in the present study we observed that Cr(VI) did not affect the expression levels of the antioxidative proteins, indicating that the antioxidative system was not involved in Cr(VI)-induced ROS accumulation. ROT is a cytotoxic agent that has been shown to induce ROS-dependent cytotoxicity by specifically targeting MRCC I (29). We found that after Cr(VI) exposure, MRCC I and II were inhibited, especially the former. By measuring the activities of MRCC I and II at different time points after 32  $\mu\text{M}$  Cr(VI) exposure, we found that the inhibition of MRCC I activity occurred at least

90 min earlier than the inhibition of MRCC II, indicating that MRCC I is the target of Cr(VI) in promoting ROS production, and the decrease in MRCC II activity may be viewed as the result of inhibited MRCC I activity. By comparing with ROT which can also induce the activation of caspase-3, we identified Cr(VI) as a novel MRCC I inhibitor. However, whether Cr(VI) can directly inhibit MRCC I activity required further study. Isolated rat liver mitochondria are also capable of reducing Cr(VI) (20), and the reduction of Cr(VI) has been suggested to occur at the expense of MRCC I, interfering with the electron flow and inducing the generation of hydroxyl radicals ( $\text{HO}^{\bullet}$ ) via the Fenton-mechanism (30). In the present study, by applying different MRCC substrates as electron donors, we also confirmed that Cr(VI) could accept the electrons leaked from MRCC I and that the reduction occurs at MRCC I.

Caspase-3 is an executioner caspase that has virtually no activity until it is cleaved after apoptotic signaling events have occurred (31). It has been suggested that ROS-dependent caspase-3 activation is achieved by Cyt c, which can be released after the disturbance of MRC (32). p53 can also activate caspase-3 (33). The caspase-3 activation that was observed in this study was not p53-dependent, as Cr(VI) can still activate

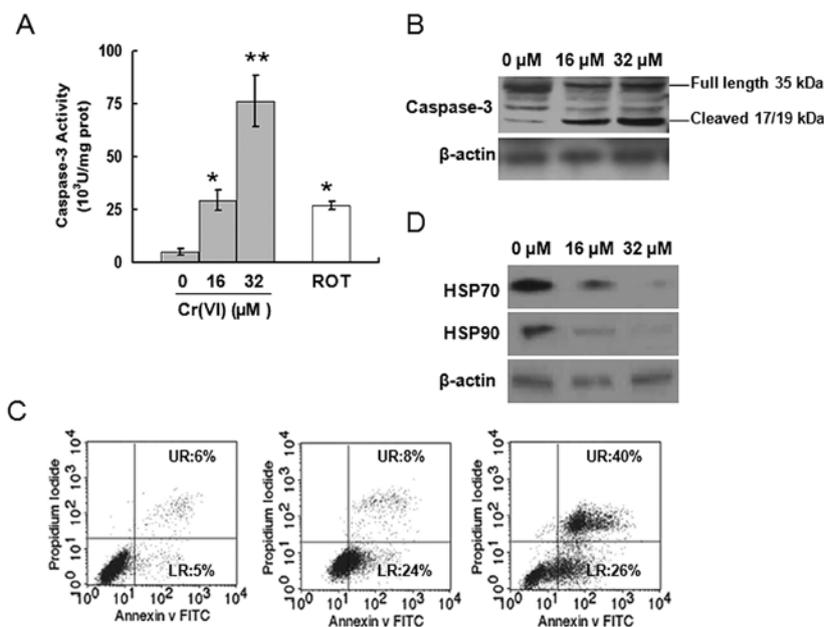


Figure 4. Cr(VI) induces caspase-3 activation. (A) Cr(VI) increases caspase-3 activity. L-02 hepatocytes were treated with Cr(VI) (16, 32  $\mu$ M) for 24 h and then harvested and lysed with lysis buffer. The samples were incubated with specific colorimetric substrate Ac-DEVD-pNA and the alternative activity of caspase-3 was described as the cleavage of the colorimetric substrate by measuring the absorbance at 405 nm. ROT (5  $\mu$ M, 24 h) was used to interpret that Cr(VI) function as MRCC I inhibitor. (B) Cr(VI) upregulates the expression of cleaved caspase-3. Whole cell lysates were analyzed by western blotting to detect the expression of full-length and cleaved caspase-3. (C) Cr(VI) induces apoptosis in a dose-dependent manner. After Cr(VI) treatment, L-02 hepatocytes were harvested and stained with Annexin V and PI, and analyzed with flow cytometry. The horizontal and vertical axes represent labeling with Annexin V and PI, respectively. LR represents early apoptotic cells (Annexin V-positive, as reflected in the lower-right-hand quadrant), UR represents late apoptotic cells (positive for both Annexin V and PI, depicted in the upper-right-hand quadrant). (D) Cr(VI) decreases the expression of heat shock protein. Western blotting was performed to determine the expression levels of HSP70 and HSP90.

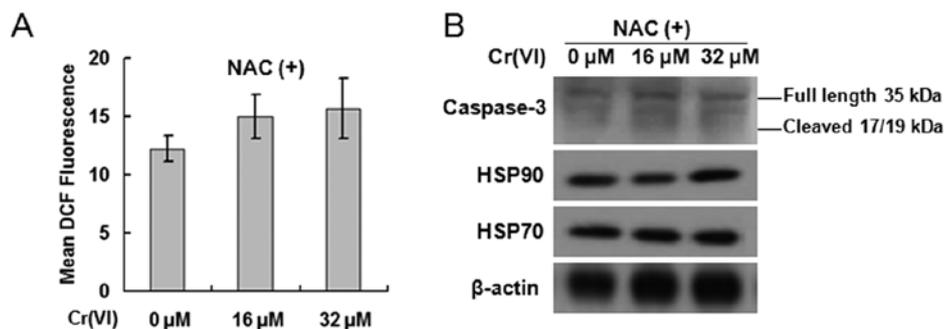


Figure 5. Cr(VI)-induced caspase-3 activation is dependent on ROS. (A) The specificity of NAC was confirmed. The hepatocytes were exposed to Cr(VI) (16, 32  $\mu$ M) in the presence of 10 mM NAC for 24 h. The amount of ROS production was quantitated by flow cytometer. (B) ROS mediate the decrease of HSP and the activation of caspase-3 induced by Cr(VI). The whole cell lysates were analyzed by western blotting to examine the protein levels of caspase-3 (full-length and cleaved), HSP70, and HSP90.

caspase-3 when p53 was blocked by Pifithrin- $\alpha$  (PFT- $\alpha$ ) (data not shown). HSPs are a class of functionally related proteins, the expression of which will be upregulated when the cells are exposed to elevated temperatures or other factors (34). HSP has been shown to antagonize apoptosis-inducing factors, such as caspases-9 and -3 (35). The present study provided the evidence that Cr(VI) can activate caspase-3 by inducing ROS-dependent decrease of HSP70 and HSP90. In order to confirm our hypothesis that ROS play a key role in Cr(VI)-mediated cytotoxicity, we used NAC to inhibit the accumulation of ROS. NAC successfully blocked the activation of caspase-3 and the inhibition of HSP70 and HSP90, suggesting that ROS is essential in Cr(VI)-induced caspase-3 activation.

In conclusion, we demonstrated that Cr(VI) targets MRCC I and induces ROS accumulation, and the accumulated ROS act as the key intermediate that downregulates HSP70, HSP90 to induces caspase-3 activation. Therefore, in the present study, MRCC I has been identified as a new target and a new mechanism for the apoptosis-inducing activity displayed by Cr(VI).

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