Original Paper

Cr(VI)-Induced Autophagy Protects L-02 Hepatocytes from Apoptosis Through the ROS-AKT-mTOR Pathway

Qi Liang^a Yuanyuan Xiao^b Kaihua Liu^b Caigao Zhong^b Ming Zeng^b
Fang Xiao^b

^aDepartment of Radiology, The Third Xiangya Hospital, Central South University, Changsha,

^bDepartment of Health Toxicology, Xiangya School of Public Health, Central South University, Changsha, China

Key Words

Cr(VI) • Autophagy • L-02 hepatocytes • Apoptosis • ROS • AKT-mTOR

Abstract

Background/Aims: Hexavalent chromium [Cr(VI)] pollution has become a global concern for both ecosystems and human health. Our previous study revealed Cr(VI) could induce both apoptosis and autophagy in L-02 hepatocytes. Here, we sought to explore the underlying mechanism of Cr(VI)-induced autophagy and its exact role in cell death. **Methods:** Autophagy ultrastructure was observed under transmission electron microscope (TEM), autophagy flux was measured with double-tagged mCherry-green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3) assay, long-lived protein degradation assay, and LC3II expression assay in the presence of lysosomal inhibitor, bafilomycin A1 (BafA1). Reactive oxygen species (ROS) level was determined using fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA). The expression levels of Beclin-1, LC3, p62/SQSTM1, and AKT-mammalian target of rapamycin (mTOR) pathway-related molecules including phosphorylation (p)-AKT, AKT, p-mTOR, and mTOR were examined using real-time polymerase chain reaction (RT-PCR) and western blotting. Apoptosis was determined using Annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. **Results:** Our results demonstrated Cr(VI) exposure activated autophagy in L-02 hepatocytes, as evidenced by the accumulation of autophagosomes, the increase of LC3-II and degradation of p62/SQSTM1, and the enhanced overall degradation of proteins. We also confirmed Cr(VI)-induced LC3-II elevation mainly came from autophagy induction rather than lysosomal degradation impairment. ROS-AKT-mTOR pathway was associated with Cr(VI)-induced autophagy, and ROS scavenger N-acetylcysteine (NAC) pretreatment inhibited Cr(VI)-induced autophagy by alleviating the inhibition of the AKT-mTOR pathway. Autophagy inhibitors 3-methyladenine

(3-MA) and chloroquine diphosphate (CDP) promoted Cr(VI)-induced apoptotic death. **Conclusion:** These findings indicated Cr(VI)-induced autophagy protected L-02 hepatocytes from apoptosis through the ROS-AKT-mTOR pathway.

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Introduction

Contamination with heavy metals such as chromium (Cr) represents a global concern for both ecosystems and human health [1]. Hexavalent chromium [Cr(VI)] and its various compounds are well-established human carcinogens that are widely used in various industrial processes including chrome plating, stainless steel production, and chrome pigment manufacturing [2]. With the aggravation of Cr(VI) pollution, events of drinking water contaminated with Cr(VI) have been frequently reported, with the focus and attention on Cr(VI) acting as a liver carcinogen. It has been suggested by numerous studies that heavy metals [3, 4] and other exogenous chemicals [5] can induce both apoptosis and autophagy, and autophagy has been confirmed as a prospective target for cancer therapy [6]. Previous studies reported conflicting results of the role autophagy plays in exogenous chemicals-induced cell death. Some studies revealed that it protected cells from apoptosis [7, 8], while others concluded that it promoted cell death [9]; thus it is necessary to clarify the exact role that autophagy plays in Cr(VI)-induced apoptotic death.

Autophagy, which can be induced by various forms of stress stimuli including hypoxia, growth factor withdrawal, or exogenous toxicants exposure, is a highly conserved metabolic process that is associated with the degradation and recycling of cellular constituents such as biomacromolecules and damaged organelles [10]. There are three types of autophagy that have been recognized in mammalian cells, macroautophagy (hereafter referred to as autophagy, macroautophagy begins with the formation of autophagosome precursors), microautophagy, and chaperone-mediated autophagy [11]. Autophagy begins with the formation of autophagosome precursors, and then the mature autophagosome fuses with lysosome to form autolysosome, resulting in the degradation and recycling of lysosomal damaged organelles and macromolecules. Under starvation conditions, hepatocyte autophagy plays a role in the maintenance of blood sugar concentration by degrading cellular metabolites, producing fatty acids, amino acids and other substances, and then participating in the processes of gluconeogenesis and β -oxidation of fatty acids [12]. In the process of infectious hepatitis, hepatitis B virus X protein (HBx) can enhance the activity of the Beclin-1-Vps34 complex and promote the formation of autophagosomes, thus leading to autophagy activation [13]. These results indicate that autophagy could help maintain normal liver physiological function and affect liver disease development.

Reactive oxygen species (ROS) include oxygen free radicals such as superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$), and nonradical oxidants such as hydrogen peroxide (H_2O_2) [14]. Under normal physiological conditions, the production and clearance of ROS are maintained at a stable level and regulated by oxidation and antioxidant systems in the organism. ROS can be triggered by various endogenous or exogenous stimuli and plays an important role in cell injury via enhancing oxidative stress [15]. The serine/threonine kinase AKT, also known as Protein Kinase B (PKB), plays a pivotal role in regulating cell survival, growth and proliferation by modulating several downstream molecules. Phosphatidylinositol-3-kinase (PI3K), which can be activated in response to growth factor or cytokine stimulation, facilitates the production of Phosphatidylinositol Triphosphate (PIP3), resulting in the membrane recruitment and subsequent phosphorylation of AKT-Thr308 and AKT-Ser473 by Phosphoinositide-Dependent Kinase (PDK) 1 and PDK 2, respectively [16]. Activated AKT can act through a variety of downstream molecules, of which the one most closely related to autophagy is mammalian target of rapamycin (mTOR). It is well known that autophagy is negatively regulated by mTOR kinase [17], and the AKT-mTOR pathway is closely associated with cell growth, autophagy, and malignant transformation as well as the development of hepatic fibrosis, acute hepatitis and liver cancers [18].

Our preliminary data revealed that exposure of hepatocytes to Cr(VI) caused ROS accumulation, lipid peroxidation, and mitochondrial injury, leading to cell cycle arrest, apoptosis, and other outcomes that contribute to the occurrence of liver disease [19, 20]. By exploring the role of ROS and the AKT-mTOR pathway in Cr(VI)-induced autophagy and its relationship with cell death, the present study aimed to uncover the effect of autophagy on Cr(VI)-induced liver injury and to provide experimental evidence for prevention and treatment of liver diseases for populations occupationally exposed to Cr(VI).

Materials and Methods

Reagents and antibodies

RPMI-1640 culture medium and 0.25% trypsin were purchased from HyClone (Logan, UT, USA). $K_2Cr_2O_7$ was obtained from Sigma (St Louis, MO, USA). Anti-AKT antibody was obtained from Santa Cruz Biotechnology (CA, USA). Anti-LC3A/B and anti-GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-mTOR, anti-phosphorylation (p)-mTOR (ser2248), anti-p-Akt (ser473), anti-PI3KCA, and anti-p62 were obtained from ABclonal Technology (Cambridge, MA, USA).

Cell culture and drug treatment

L-02 hepatocytes were cultured as previously described [21]. The stock solution of Cr(VI) was prepared with phosphate buffered solution (PBS). When indicated, N-acetylcysteine (NAC) (5 mM) was added 30 min before Cr(VI) administration, 3-methyladenine (3-MA) (2 mM) and chloroquine diphosphate (CDP) (50 μ M) were added to L-02 hepatocytes 2 h before Cr(VI) administration, and bafilomycin A1 (BafA1) (25 nM) was added 6 h after Cr(VI) treatment.

Cell viability and apoptosis measurement

Cell viability of L-02 hepatocytes was measured using a Cell Counting Kit-8 (CCK-8) (7sea Biotech, Shanghai, China) according to the manufacturer's protocol. CCK-8 allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays. The highly water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium), is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells. The optical density value of the formazan dye was measured at a wavelength of 450 nm. Briefly, L-02 hepatocytes were plated at a density of 0.8×10^4 cells per well and a volume of 100 μ l in 96-well plates. The cells were treated with different concentrations of Cr(VI) for 24 h. Then, 10 μ l of CCK8 assay solution was added to each well and incubated for 4 h at 37 °C. The wells containing the same volume of culture medium and CCK8 solution served as blank controls. Cell viability was calculated by measuring absorbance at a wavelength of 450 nm by using a spectrophotometer (PowerWave XS2, BioTek, Winooski, VT, USA). The experiments were performed in triplicate.

An Annexin V- fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Invitrogen, Carlsbad, CA, USA) was used for the detection of cellular apoptosis as described by us previously [22]. The early apoptotic cells (Annexin V-positive, propidium iodide (PI) -negative) and late apoptotic cells (positive for both Annexin V and PI) were examined.

Detection of autophagosome formation

Autophagosome formation was observed as previously described [23]. L-02 hepatocytes treated with different drugs were pelleted and fixed in 2.5% phosphoric acid buffer solution of glutaraldehyde for 3 h, and then were rinsed with 0.1 M phosphate rinse solution three times for 15 min. Samples were then dehydrated with graded acetone (50%, 70%, 90% and 100%) (each concentration for 15 min, 2 times) and mixed with embedding solution. After soaking for 12 h, the samples were incubated at 37°C overnight and then at 60°C for 24 h. Ultrathin sections (100 nm) were cut on an ultramicrotome, counterstained with 0.3% uranium acetate and lead nitrate, and examined by a transmission electron microscope (TEM) (H7700,

Hitachi, Japan). The quantification of the results was obtained by counting the numbers of autophagosomes and autolysosomes in at least six cells.

Measurement of autophagy flux by adenovirus transduction

Autophagy flux was measured as previously described [24, 25] with slight modification. For electron microscope analysis, cells cultured on plastic coverslips in 24-well plates were transduced with double-tagged mCherry-green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3) adenovirus (BiYunTian Biotechnology Research Institute, Shanghai, China). The culture medium was discarded after 26 h and replaced with 500 μ l fresh medium. The transfected cells were treated with different concentrations of Cr(VI) for 24 h and then were fixed with 200 μ l 4% paraformaldehyde for 30 min. (We used 4% paraformaldehyde to fix the hepatocytes because the fixed cells showed more stable results, and the results are not difference for live cell imaging versus fixed cell imaging.) The coverslips were imaged with laser scanning confocal microscopy (LSM 510 Meta, Carl Zeiss, Germany). For each group, six cells were used for quantification.

Measurement of autophagy flux by long-lived protein degradation assay

The assay was performed according to the method described previously [26]. Long-lived protein degradation assay is a traditional and well-established method for evaluating autophagy flux. Briefly, long-lived proteins were labeled by removing the media, rinsing the cells twice with PBS and culturing in the presence of 1 ml leucine-free media containing 5 μ Ci/ml [3 H]-leucine for 48 h. After the labeling media was removed, short-lived proteins were depleted by culturing the labeled cells with 1 ml Opti-MEM containing 4% serum and 2 mM cold leucine for 24 h. At the end of the chase period, the hepatocytes were treated with different concentrations of Cr(VI) (0, 6, 12 μ M) for 24h. The autophagy inhibitor 3-MA (2 mM) was used to pretreat cells for 2 h to further ensure that the measured radioactivity is the result of degradation of proteins by the autophagic pathway.

Real-time polymerase chain reaction (RT-PCR) for mRNA expression

RT-PCR was done as previously described [27]. Total RNA of each treatment group was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). For first-strand cDNA synthesis, 1 μ g RNA was reverse transcribed with Primescript Reverse Transcriptase Enzyme Mix and 50 μ M Oligo DT Primer (Takara, Japan). Then, the cDNAs were subjected to quantitative RT-PCR analysis. The sequences of the primers used were as follows:

GAPDH, 5'-TCGGAGTCAACGGATTTGGT-3' (Forward), 5'-TGGAATTTGCCATGGGTGGA-3' (Reverse);

AKT1, 5'-GTCATCGAACGCACCTTCCAT-3' (Forward),

5'-AGTTCAGGTACTCAAACCTCGT-3' (Reverse);

PIK3CA, 5'-CCAGACCATCATCAGGTGAA-3' (Forward),

5'-CCTCACGGAGGCATTCTAAAGT-3' (Reverse);

mTOR, 5'-TCCGAGAGATGAGTCAAGAGG-3' (Forward), 5'-CACCTTCCACTCCTATGAGGC-3' (Reverse);

LC3B, 5'-AAGGCGCTTACAGCTCAATG-3' (Forward), 5'-CTGGGAGGCATAGACCATGT-3' (Reverse);

SQSTM1, 5'-GCACCCCAATGTGATCTGC-3' (Forward), 5'-CGCTACACAAGTCGTAGTCTGG-3' (Reverse);

BECN1, 5'-CCATGCAGGTGAGCTTCGT-3' (Forward), 5'-GAATCTGCGAGAGACCATC-3' (Reverse).

Real-time PCR was performed using a LightCycler®96 Sequence Detection System (Roche, Basel, Switzerland) in a 20 μ l reaction containing 10 μ M of each primer, 1 μ l template cDNA, 10 μ l SYBR Premix EX Taq (SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, Takara, Japan), and 0.25 μ l 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. GAPDH was used as the endogenous control. Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method.

Western blot analysis for protein levels

Hepatocytes were treated, harvested, washed with ice-cold PBS, and subjected to western blot analysis, as previously described [21]. Briefly, approximately 50 μ g protein of each different treatment group was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was then blocked with 5% milk for 2h, rinsed, incubated with the primary antibodies overnight at 4°C, followed by the secondary antibodies for 1 h at room temperature. The proteins were then visualized and exposed to X-ray film. The quantitative data of the bands were obtained using Image-pro plus 6.0. software.

ROS level detection

Intracellular ROS levels were detected by monitoring the conversion of cell membrane permeable dichloro-dihydrofluorescein diacetate (DCFH-DA) (BiYunTian Biotechnology Research Institute, Shanghai, China) to fluorescent dichlorofluorescein (DCF) [28]. Briefly, L-02 hepatocytes were seeded in 6-well plates and treated with different drugs. Hydrogen peroxide served as a positive control. After washing with PBS two times, the treated hepatocytes were incubated with DCFH-DA at 37°C for 30 min in the dark, digested with trypsin, and then collected after centrifugation. DCF fluorescence was detected by flow cytometry (BD Biosciences, USA) at an excitation wavelength (488 nm) and emission wavelength (525 nm).

Kinase activity detection

AKT kinase activity was measured using AKT Kinase Activity Assay Kit (Wuhan Amyjet Scientific Co., Ltd., Wuhan, China), using glycogen synthase kinase-3 α (GSK-3 α) as substrate, according to the manufacturer's instructions. Briefly, the hepatocytes were lysed by lysis buffer after Cr(VI) treatment, then co-immunoprecipitation was done after the adding of anti-AKT antibody. The acquired immune complex was lysed, and the expression of phosphorylated GSK-3 α protein was detected by immunoblotting using the specific antibody. GAPDH was used as a reference.

mTOR kinase activity was measured using K-LISA™ mTOR Activity Kit (Merck-Calbiochem, Germany). The enzymelinked immunosorbent assay (ELISA)-based K-LISA mTOR assay uses p70S6K-GST fusion protein as the mTOR substrate. p70S6K-GST is first bound to a glutathione-coated 96-well plate, and then mTOR-containing samples are incubated with ATP in the wells where active mTOR phosphorylates p70S6K at Thr389 (T389). The assay was carried out according to the protocol.

Caspase-3 activity detection

Caspase 3 activity of the treated hepatocytes was detected using a commercial assay kit (BiYunTian Biotechnology Research Institute, Shanghai, China) [29]. Caspase-3 can catalyze its substrate Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) to generate yellow p-nitroaniline (pNA), which has strong absorption near 405nm. Thus the activity of caspase-3 can be detected by the determination of absorbance of pNA at 405nm. Briefly, protein was isolated from the different treatment groups using the lysis buffer supplied with the kit, and then was incubated in assay buffer for 2 hours at 37°C. The results representing caspase-3 activity were detected using a microplate reader at an absorbance of 405 nm.

Statistical analysis

All of the experimental data are expressed as the mean $\pm$ standard deviation (SD), and each experiment was performed at least three times. The statistical analysis for the difference between different treatment groups was performed by one-way analysis of variance (ANOVA) using SPSS 19.0, and the difference within the groups was analyzed by a SNK-q test. $p < 0.05$ was considered statistically significant.

Results

Cr(VI) induced autophagy in L-02 hepatocytes

L-02 hepatocytes were treated with different concentrations of Cr(VI) (0, 1, 2, 4, 6, 8, 16, 32, and 64 μ M) for 24 h and then were collected for cell viability determination using the CCK8 method. As shown in Fig. 1A, the cell viability of L-02 hepatocytes was decreased as the Cr(VI) concentration increased. We chose 6 μ M and 12 μ M of Cr(VI) for the following experiments. To investigate whether Cr(VI) could induce autophagy in L-02 hepatocytes, their autophagic activity was observed by measuring autophagosomes formation under TEM, which is considered as one of the most widely accepted methods for monitoring autophagy [30]. The formation of membrane-bound autophagic vacuoles, which is indicative of autophagy induction, was frequently observed in the cytoplasm of Cr(VI)-treated L-02 hepatocytes, but rarely in control cells, indicating the induction of autophagy after Cr(VI) exposure (Fig. 1B). The maturing autophagosome can be converted into a degradative organelle (autolysosome) and becomes acidified. Autolysosome, which contains degraded cargo including internal membranes originating from the capture, has a single delimiting

Fig. 1. Cr(VI) induced autophagy in L-02 hepatocytes. (A) The effect of different concentrations of Cr(VI) on cell viability was determined using the CCK8 method. (B) Representative transmission electron micrographs demonstrating the ultrastructure of Cr(VI)-treated L-02 hepatocytes. The yellow arrow indicated the autophagosomes with double membrane structures while the red arrow indicated the autolysosomes with single membrane structures. The quantifications of autophagosomes and autolysosomes were also shown. (C) Autophagy flux was observed in the hepatocytes transduced with double-tagged mCherry-GFP-LC3 adenovirus. Yellow puncta represent the autophagosomes whereas the red puncta represent autolysosomes. (D) Quantitative results of (C). * $p < 0.05$, ** $p < 0.01$, compared with the control group. # $p < 0.05$, compared with the control group in overall dots.

membrane since the inner of the two membranes is dissolved. The yellow arrow indicated the autophagosomes with double membrane structures while the red arrow indicated the autolysosomes with single membrane structures. The quantifications of autophagosomes and autolysosomes were also shown. Both autophagosomes and autolysosomes were increased after Cr(VI) exposure. We observed autophagic flux to evaluate Cr(VI)-induced autophagosomal maturation. The GFP signal is sensitive to the acidic conditions, and a low

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Fig. 2. Cr(VI) induced autophagy in L-02 hepatocytes. (A) Long-lived protein degradation assay was performed to evaluate autophagy flux. (B) Expression of mRNA levels of BECN1, LC3B, and p62. (C) Expression of protein levels of Beclin1, LC3A/B and p62, and the quantitative results. (D) The hepatocytes were exposed to 6 μ M Cr(VI) for 24 h with or without the post-treatment of the lysosomal inhibitor BafA1 (25 nM, 6 h), and the protein expression level of LC3 A/B-I and -II was determined using western blotting. All pictures are representative of three independent experiments with similar results. * $p < 0.05$, compared with the control group.

pH inside the lysosome quenches the GFP fluorescent signal; thus, the delivery of GFP-LC3 to lysosomes cannot be observed. The vesicular compartment that has not fused with lysosomes such as phagophores or autophagosomes can be stained by the colocalization of both mCherry and GFP fluorescence signals and is showed as yellow puncta. The mCherry vesicle without GFP fluorescence emission represented the autolysosome and is showed as red puncta [24]. Therefore, we used a novel mCherry-GFP-LC3 tandem construct to morphologically trace the autophagic flux. Both red and yellow puncta increase when autophagic flux is activated. The L-02 hepatocytes were transfected with mCherry-GFP-LC3. As shown in Fig. 1C&D, when the pictures were merged, yellow puncta represent autophagosomes whereas the red puncta represent autolysosomes. The number of autophagosomes and autolysosomes are few in the control group but obviously increased in the Cr(VI) treatment groups. The statistical analysis result also revealed that the numbers of autophagosomes, autolysosomes and the sum of the two were all increased when compared with the control group. We also performed long-lived protein degradation assay, the additional autophagic flux assay to evaluate autophagic flux. As shown in Fig. 2A, Cr(VI) treatment significantly enhanced the overall degradation of proteins in L-02 hepatocytes in a concentration-dependent manner. However, 3-MA pretreatment alleviated Cr(VI)-induced increase of protein degradation rate, confirming that the measured radioactivity is the result of degradation of proteins by the autophagic pathway. We further detected the expression levels of Beclin-1, which is active at the initial stage of autophagy; LC3A/B, which is a marker of mature autophagy [12]; and p62/SQSTM1, which serves as a cargo receptor for autophagic degradation of ubiquitinated targets. The RT-PCR results shown in Fig. 2B revealed that compared with the control, after Cr(VI) treatment of 6 and 12 μ M for 24 h, BECN1 and LC3-II mRNA expression levels were increased while the p62 level was decreased. The protein expression levels of LC3A/B and p62 in Fig. 2C show similar results as Fig. 1F. The results suggested that Cr(VI) enhanced autophagy in L-02 hepatocytes by regulating the key genes/proteins at the initial, mature, and late stages of autophagy. To clarify if the Cr(VI)-induced LC3-II elevation was resulting from autophagosome formation increase or degradation impairment, we examined the change of LC3-II level in the presence of a lysosomal inhibitor, bafilomycin A1 (BafA1). For the alone treatment, Cr(VI) (6 μ M) was given to the hepatocytes for 24 h, and for the combination treatment, BafA1 (25 nM) was

Fig. 3. The ROS-AKT-mTOR pathway is associated with Cr(VI)-induced autophagy. (A) Intracellular ROS level in L-02 hepatocytes was detected using the fluorescent probe DCFH-DA. (B) Quantitative results of (A). (C) Protein expression levels of p-AKT (ser473) and p-mTOR(ser2248). Expression of mRNA levels (D) and protein levels (E) of AKT, PI3K, and mTOR. (F) The relative protein ratios of p-AKT/AKT and p-mTOR/mTOR. AKT kinase activity assay (G) and K-LISA mTOR assay (H) were performed using the commercial kits. * $p < 0.05$, compared with the control group.

added 6 h after Cr(VI) treatment. As shown in Fig. 2D, western blotting analysis showed that treatment with Cr(VI) did not further increase the LC3-II level in BafA1 co-treated cells, implying that the Cr(VI)-induced LC3-II elevation mainly came from autophagy induction rather than lysosomal degradation impairment.

The ROS-AKT-mTOR pathway is associated with Cr(VI)-induced autophagy

The fluorescence pictures in Fig. 3A and the quantitative results in Fig. 3B revealed that different concentrations of Cr(VI) treatments induced higher fluorescence intensity than that of the control group, indicating that Cr(VI) exposure could increase ROS accumulation in L-02 hepatocytes. The AKT/mTOR pathway is directly associated with the cell cycle, apoptosis, cell homeostasis and other cellular outcomes, and plays an important role in the occurrence

Fig. 4. NAC pretreatment inhibited Cr(VI)-induced autophagy in L-02 hepatocytes. Intracellular ROS level determination (A) and the quantitative results (B). (C) Expression of mRNA levels of AKT and mTOR. (D) Expression of protein levels of p-AKT, p-mTOR, AKT and mTOR. (E) The quantitative data. The relative protein ratios of p-AKT/AKT and p-mTOR/mTOR were also calculated. * $p < 0.05$, compared with the control group. # $p < 0.05$, compared with the Cr(VI) alone treatment group.

and development of hepatocarcinoma, lung cancer, lymphoma and other cancers, but the role this pathway plays in Cr(VI)-induced cytotoxicity has not yet been well elucidated.

We also examined the effect of Cr(VI) on the gene and protein expression of members of the AKT-mTOR pathway. As shown in Fig. 3C-E, Cr(VI) exposure decreased the protein levels of p-AKT (ser473) and p-mTOR(ser2248) and both the mRNA and protein expression of PI3K, AKT1, and mTOR in a dose-dependent manner. Since Cr(VI) altered both transcriptional profiles and total protein levels of AKT/mTOR pathway, we also calculated the ratios of p-AKT/AKT and p-mTOR/mTOR to better interpret the corresponding phosphorylation of AKT and mTOR. As shown in Fig. 3F, the ratios of both p-AKT/AKT and p-mTOR/mTOR were decreased in a dose-dependent manner. We also detected the activities of AKT and mTOR directly using the commercial kits. Fig. 3G revealed that the expression of p-GSK-3 α was decreased in a concentration-dependent manner after Cr(VI) treatment, indicating that the inhibition of AKT kinase activity. Fig. 3H revealed that Cr(VI) inhibited mTOR kinase activity in a concentration-dependent manner. These results together suggested the inhibitory effect of Cr(VI) on AKT/mTOR pathway.

NAC pretreatment inhibited Cr(VI)-induced autophagy in L-02 hepatocytes

Our previous data revealed that a burst generation of cellular ROS could account for a series of damage and repair events after Cr(VI) exposure. We used the ROS scavenger NAC to confirm the involvement of the ROS-AKT-mTOR pathway in Cr(VI)-induced autophagy. Fig. 4A and B show that after incubation with fluorescent probe DCFH-DA, the ROS level in the 5 mM NAC alone treatment group was not significantly different from that in the control group. Compared with the control, pretreatment with NAC for 30 min significantly decreased 6 μ M Cr(VI)-induced ROS accumulation, confirming the effective role of NAC. We further

examined whether NAC pretreatment affected Cr(VI)-induced inhibition of the AKT-mTOR pathway. As displayed in Fig. 4C-E, NAC pretreatment alleviated the inhibition of expression levels of p-AKT (ser473), p-mTOR (ser2248), AKT, and mTOR caused by Cr(VI) exposure, and also increased the relative protein ratios of p-AKT/AKT and p-mTOR/mTOR, indicating that ROS played an important role in Cr(VI)-induced inhibition of AKT-mTOR pathway. Autophagosome formation under TEM at a magnification of 5,000 showed that pretreatment with NAC obviously decreased the number of autophagosomes and autolysosomes after 6 μ M Cr(VI) exposure (Fig. 5A), suggesting NAC pretreatment inhibited Cr(VI)-induced autophagy in L-02 hepatocytes. This result can be confirmed by the western blot results in Fig. 5B that NAC alleviated the increase in LC3A/B expression and the decrease in p62 expression caused by Cr(VI) treatment. We also performed the long-lived protein degradation assay to better reflect autophagic activity. As shown in Fig. 5C, both NAC and 3-MA pretreatments significantly inhibited Cr(VI)-induced increase of the overall degradation of proteins.

Autophagy inhibitors pretreatments promoted Cr(VI)-induced apoptotic death

The above results have confirmed that Cr(VI) exposure induced autophagy in L-02 hepatocytes by increasing ROS accumulation. To further explore the role of autophagy played in Cr(VI)-induced hepatocytes injury, the present study applied 3-MA and CDP. 3-MA is the inhibitor for both autophagosome biogenesis and maturation. CDP damages the structure and function of lysosomes and alters the autophagy-lysosome fusion process to inhibit autophagy [31]. The western blot results in Fig. 6A show 1mM 3-MA alone treatment

Fig. 5. NAC pretreatment inhibited Cr(VI)-induced autophagy in L-02 hepatocytes. (A) Detection of autophagosome formation under TEM. The yellow arrow indicated the autophagosomes with double membrane structures while the red arrow indicated the autolysosomes with single membrane structures. The quantifications of autophagosomes and autolysosomes were also shown. (B) Protein expression of LC3A/B and p62. (C) Long-lived protein degradation assay was performed to evaluate autophagy flux. * $p < 0.05$, compared with the control group. # $p < 0.05$, compared with the Cr(VI) alone treatment group.

Fig. 6. Autophagy inhibitors pretreatments promote Cr(VI)-induced apoptotic death. (A) Protein expression of LC3A/B and p62. (B) Cellular apoptosis was examined by Annexin V-FITC double-stain. (C) Quantitative results of apoptotic cells were calculated. (D) Caspase-3 activity determination. * $p < 0.05$, compared with the control group. # $p < 0.05$, compared with the Cr(VI) alone treatment group.

decreased the protein expression of LC3A/B, but did not affect p62 expression. Pretreatment with 3-MA for 2 h alleviated Cr(VI)-induced increased expression of LC3A/B and decreased expression of p62, indicating that 3-MA could effectively inhibit Cr(VI)-induced autophagy. We next examined the role of autophagy in Cr(VI)-induced apoptosis. As shown in Fig. 6B, compared with the control, both 3-MA and CDP treatment had no effect on apoptosis rate. Compared with Cr(VI) alone treatment, 3-MA and CDP did not appear to increase double-positive Cr(VI) cells, indicating that 3-MA and CDP treatment mainly aggravated Cr(VI)-induced early apoptosis not the late apoptosis. We also applied Rapamycin (Rap) to explore if inducing autophagy will rescue Cr(VI)-induced cell death. Rap (100 nM) was added 4 h before Cr(VI) administration. Rap pretreatment rescued Cr(VI)-induced cell death. For each FACS experiment, we calculated the percentages of early apoptotic cells, the late apoptotic cells, and the apoptotic cells (apoptotic cells plus late apoptotic cells). The quantitative results of apoptotic cells were shown in Fig. 6C. The results of caspase-3 activity determination in

Fig. 6D revealed that both 3-MA and CDP pretreatments amplified Cr(VI)-induced caspase-3 activation while Rap pretreatment revealed the opposite result. Since inhibitors such as 3-MA and CDP have off target effects [32], more specific inhibitor should be applied or additional experiments should be done to demonstrate role of autophagy plays in Cr(VI)-induced cell death.

Discussion

In recent years, numerous studies have demonstrated the association between the AKT-mTOR pathway and autophagy. As the key protein in the AKT-mTOR pathway, AKT can not only directly phosphorylate apoptosis protein BAD at the ser136 site and inhibit apoptosis by blocking caspase-9 activity [33], but also enhance mTOR expression via phosphorylating tuberous sclerosis complex 2 (TSC2) at the ser939 site [34]. The present study found that Cr(VI) treatment inhibited the AKT-mTOR pathway by down-regulating the expression levels of key mediators including PI3K, AKT, and mTOR, and decreasing the phosphorylation of AKT at ser473 and mTOR at ser2448. It has been reported that the signals of hypoxia and carbon dioxide retention could accelerate the phosphorylation of PI3K and inhibit the AKT-mTOR pathway, thereby activating autophagy in brain neurons [35]. Although it is reasonable to conclude that Cr(VI)-induced autophagy in L-02 hepatocytes is closely related to inhibition of the AKT-mTOR pathway, further experimental confirmation is still needed.

This study found that Cr(VI) exposure increased the number of cellular autophagosomes as well as vacuoles in L-02 hepatocytes. The accumulated vacuoles could be autolysosomes whose contents were completely degraded, or the swelling and vacuolated mitochondria caused by Cr(VI) treatment. By transducing double-tagged mCherry-GFP-LC3 adenovirus in L-02 hepatocytes, we confirmed Cr(VI) induced autophagy. Beclin-1, LC3B and p62 are important proteins in the process of mammalian autophagy. Beclin-1 can participate in the formation of autophagosomes at the initial stage of autophagy. During the mature stage of autophagy, LC3 plays a key role in the formation of autophagic vacuoles by conjugating with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE) via a ubiquitination-like enzymatic reaction. LC3-I protein is dissociated in the cytoplasm under physiological conditions, and transformed to LC3-II by binding to PE on the surface of the autophagic membrane when autophagy is activated by internal and external stimuli, which promotes the extension of the autophagic membrane [36]. LC3-II is localized on the autophagic membranes; thus, detecting the expression of LC3-II protein may serve as a common indicator of autophagy. At the late stage of autophagy, the polyubiquitin binding protein p62/SQSTM1 plays a central role in degradation of the contents in autolysosome by directly binding to LC3. We showed in this study that Cr(VI) treatment increased LC3 expression while decreasing p62 expression. However, it is worth noting that the expression of Beclin-1 was increased at the mRNA level but had no obvious change at the protein level after Cr(VI) exposure, and this may be because Cr(VI) could induce the ubiquitination of Beclin-1 and enhance its degradation process, or Cr(VI) may act through modifying the phosphorylation and acetylation of Beclin-1 without altering its total protein level. Chen et al. reported that whereas p62 levels fluctuated in a time-dependent manner during autophagy, inhibition or knockdown of Cdk9/cyclin T1 transcriptionally down-regulated p62 but did not affect autophagic flux, while short hairpin RNA (shRNA) directly targeting p62 resulted in cargo loading failure and inefficient autophagy [37]. Thus we think both p62 mRNA and protein levels dropped in Cr(VI)-treated L-02 hepatocytes invalidates using p62 as a marker (substrate) for autophagy in this cell line under the experimental conditions utilized. Similarly, the increase in both LC3A/B mRNA and protein levels suggested transcriptional up-regulation of LC3A/B, rather than indicating autophagic flux. This is why we also performed additional autophagic flux assay such as long-lived protein degradation assay and LC3II expression assay in the presence of lysosomal inhibitor.

Recent studies have also found that ROS has a close relationship with autophagy. Accumulation of ROS in neurocytes induced membrane lipid peroxidation and mitochondrial dysfunction, resulting in autophagic cell death [38]. Shiomi et al. also revealed that a burst generation of ROS triggered by sevoflurane in the isolated heart of a guinea pig could activate autophagy by inhibiting AMPK-associated mTOR signaling [39]. We have previously confirmed that Cr(VI) mainly targeted the mitochondrial respiratory chain complex (MRCC) I to increase electron leakage and induce ROS accumulation [22], which could further attack the respiratory chain and mitochondrial DNA (mtDNA) to induce apoptosis. Our results showed that NAC pretreatment alleviated Cr(VI)-induced inhibition of the AKT-mTOR pathway, thus inhibiting autophagy in the hepatocytes.

Cell death, the state of irreversible injury, can occur through three mechanisms including apoptosis, autophagy, and necrosis [40]. The programmed cell death apoptosis, which results in controlled cell shrinkage and nuclear fragmentation via caspases activation, plays an important role in cell survival, proliferation, differentiation and the maintenance of a relatively stable internal environment in the body. At present, controversies regarding the relationship between autophagy and apoptotic cell death still remain. One kind of conventional viewpoint is that as a protective mechanism after stress, autophagy could maintain energy homeostasis and promote cell survival by removing cytoplasmic constituents, damaged subcellular organelles, and denatured proteins [41]. With developments in research, investigators have found that the role autophagy plays in cell death varies greatly in different cell types and disease states. In glioma cells, ceramide can induce autophagy via the JNK-c-Jun pathway, causing autophagic cell death [42]. Liu et al. reported an autophagy dependent but apoptotic independent cell death characterized by cell matrix adhesion and endoplasmic reticulum expansion, named autosis [43]. These views allow researchers to have a new understanding of the cellular functional roles of autophagy. Currently, researches on the role of autophagy in cell death are mainly focused on the interactions between the two. It has been reported by Wang et al. that autophagy could mediate CoCrMo particle-induced peri-implant osteolysis by promoting osteoblast apoptosis [44]. Another study established the prominence of autophagy in mediating delayed neuronal death in mouse models of brain ischemia and demonstrated that post-ischemic pharmacological inhibition of autophagy can provide protection against death [45]. Existing research results have shown that the apoptosis-associated Bcl-2 protein could regulate autophagy by interacting with nutrient-deprivation autophagy factor-1 (NAF-1) [46] and the BH3 domain of Beclin-1 [47], and some genes responsible for autophagy such as Atg5 [48] and Atg12 [49] also play a role in the process of apoptosis. Our results showed that the applications of autophagy inhibitors aggravated Cr(VI)-induced cellular apoptosis and death. The molecular mechanisms involved in the antagonism of autophagy to apoptosis after Cr(VI) exposure, direct association with the apoptosis pathway or indirect action by providing energy for cells, should be further explored.

Conclusion

Cr(VI) exposure induced autophagy via the ROS-AKT-mTOR pathway, and the activation of autophagy protected L-02 hepatocytes from apoptosis.

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Disclosure Statement

The authors have no conflicts of interest to declare in relation to this article.

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