Clusterin Protects Against Cr(VI)-Induced Oxidative Stress-Associated Hepatotoxicity By Mediating The Akt-Keap1-Nrf2 Signaling Pathway

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Research Article

Keywords: Cr(VI), Clusterin, oxidative stress, hepatotoxicity, Akt, Keap1-Nrf2.

Posted Date: September 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-665264/v1>

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Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on March 8th, 2022. See the published version at <https://doi.org/10.1007/s11356-022-19118-w>.

Abstract

Hexavalent chromium [Cr(VI)] is a serious environmental pollutant and threatens human health. Although it has been confirmed that oxidative stress is the main mechanism of liver injury caused by Cr(VI) exposure, the related toxic target and effective intervention measures have not been found. Clusterin (CLU) is an acute phase response protein with cytoprotective and apoptosis delaying effects, and its expression has been confirmed to increase significantly after exposure to Cr(VI). In this study, we demonstrate that CLU acts on the Protein Kinase B (PKB/Akt)-Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor E2-related factor 2 (Nrf2) signaling pathway to release Nrf2 into the nucleus. This to initiates the expression of a downstream protein, heme oxygenase 1 (HO-1), thereby attenuating the ubiquitination ability of Keap1 with Nrf2. We also demonstrated that CLU can affect oxidative stress through the Akt/Nrf2 pathway, which reduces the production of reactive oxygen species (ROS) induced by Cr(VI) and protects against Cr(VI)-induced oxidative stress-associated hepatotoxicity. This study demonstrates a the mechanism of Cr(VI)-induced hepatotoxicity, and indicates that CLU as an intervention target of oxidative stress can provide valuable experimental basis for the prevention and treatment of occupational diseases in Cr(VI)-exposed population.

1. Introduction

Chromium (Cr) exists in various oxidation states, among which hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)] are the most stable forms in both the environment and the workplace. Cr(VI) is considered to be the most toxic form of Cr because it can effectively penetrate the anion channels in the cell membrane. Cr(VI) salts, such as potassium dichromate ($K_2Cr_2O_7$) or chromic acid, are widely used in leather production, chromium plating and dye production(Bagchi et al., 2002). It is well known that occupational and environmental exposure to Cr(VI) has mutagenic and carcinogenic effects on organisms, which can seriously damage liver(Rafael et al., 2007), kidney(Linos et al., 2011), lung(Aw, 1997), skin(Lin et al., 2009) and other important organs. After the human body is exposed to heavy metals such as Cr(VI), the accumulation of Cr in some organs will cause functional damage. Exposure to Cr(VI) in rats results in progressive proteinuria, increased urea nitrogen and creatinine, elevated serum alanine aminotransferase activity and liver lipid peroxidation(Kim and Na, 1991). Oral exposure to Cr(VI) has also been shown to increase lipid peroxidation in liver mitochondria and microsomes, accompanied by increased excretion of lipid metabolites in urine(Bagchi et al., 1997). In addition, DNA strand breaks in peripheral blood lymphocytes and liver lipid peroxidation products in urine were observed in workers occupationally exposed to Cr(VI)(Gambelunghe et al., 2003; Goulart et al., 2005).

The liver is the most important target organ after Cr(VI) enters the human body, and the exact mechanism of Cr compounds on tissues has not been widely studied. Exposure to $K_2Cr_2O_7$ has been shown to cause severe liver toxicity, and its mechanism is related to the increase of reactive oxygen species (ROS), lipid peroxidation, inhibition of antioxidant enzymes and mitochondrial damage(Patlolla et al., 2009; Soudani et al., 2011). It has been reported that antioxidants can significantly improve or prevent Cr(VI)-induced hepatotoxicity. Excessive ROS production leads to oxidative stress, which can affect the functional

integrity of organs by damaging cell proteins, lipids and DNA(Kajarabille and Latunde-Dada, 2019). We previously have confirmed that Cr(VI) mainly targets and inhibits the mitochondrial respiratory chain complex I (MRCC I) and disrupts mitochondrial electron transport, resulting in a large increase in electron leakage and the intracellular ROS overload(Xiao et al., 2019).

Clusterin (CLU) is a highly conserved heterodimer sulfated glycoprotein, which is ubiquitously expressed in the body. The main biological functions of CLU include tissue repair, lipid transport, and immune regulation. It has been confirmed that CLU is not only involved in a variety of biological processes, but also closely related to a variety of pathological states, such as neurodegenerative diseases, aging and cancer. Low concentration of hexavalent chromium can induce premature cell senescence, and during this process, the expression level of CLU increases continuously(Alexopoulos et al., 2008). We previously reported that CLU can play an anti-apoptotic role by acting on MRCC I subunit and inhibiting ROS production. Nuclear factor E2-related factor 2 (Nrf2) promotes the regulation of intracellular redox environment by inducing the coding of a series of antioxidant proteins, thus Nrf2 represents a very important regulator of cellular antioxidant stress. Kelch-like ECH-associated protein 1 (Keap1) is anchored to Actin in the cytoplasm. Under normal physiological conditions, Nrf2 binds to Keap1 and rapidly degrades in the cytoplasm; when oxides accumulate, Nrf2 is rapidly dissociated with Keap1 and then transferred to the nucleus to bind with antioxidant response elements (ARE) in the promoter region of detoxification phase II enzyme to activate the transcription of antioxidant enzymes, thus protecting cells from the damage of active substances such as ROS, or toxic substances such as carcinogens and drugs metabolic activation products. The ability of Nrf2 to up-regulate the expression of antioxidant genes suggests that increasing Nrf2 activation can provide a beneficial environment for the protection against oxidative damage. Our previous study has confirmed that CLU activated Akt pathway by directly binding to eukaryotic translation initiation factor I (EIF3I)(Zhang et al., 2019), but whether CLU affects oxidative stress through Akt/Nrf2 pathway is unclear.

Although it has been confirmed that oxidative stress is the main mechanism of liver injury caused by Cr(VI) exposure, the related toxic target and effective intervention measures have not been found. This study can provide valuable experimental basis for further elucidating the hepatotoxicity mechanism of Cr(VI), finding intervention targets related to oxidative stress, and preventing and treating occupational diseases in people exposed to Cr(VI).

2. Materials And Methods

2.1. Reagents and chemicals

Potassium dichromate ($K_2Cr_2O_7$) was purchased from Changsha Chemical Reagents Company (Changsha, China). RPMI-1640 medium and Fetal Bovine Serum (FBS) were obtained from Gibco (Gaithersburg, MD, USA). Trypsin-EDTA (0.25%) was obtained from Genviue (Beijing, China). Vitamin E (VitE) was purchased from Topscience (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Beijing

Dingguo Changsheng Biotech CO.LTD (Beijing, China). Uprosertib (Upr) (HY-15965) was obtained from MedChemExpress (Shanghai, China)

2.2. Animals

24 BALB/c mice (male, 8 week, 18-22g) purchased from Hunan SJA Laboratory Animal Co. Ltd. (SCXK (XIANG) 2019-0004) (Changsha, China), were randomly divided into a control group and three Cr(VI) groups (6 per group). Control group (0 day): mice were intragastrically given 0.9 % physiological saline (W/V). Cr(VI) treatment groups: mice were intragastrically given 200 mg/kg $K_2Cr_2O_7$ and were sacrificed one day (1 day), three days (3 day), and five days (5 day) after treatment. Animal experiments were carried out in accordance with the procedures approved by the Animal Care and Use Committee of Xiangya School of Public Health, Central South University.

2.3. Cell culture

L02 hepatocyte line was provided by China Center for Type Culture Collection of Wuhan University. The CLU over-expression (OE) and knockdown (sh) L02 hepatocytes were established as described previously. Cells transfected with empty vector (EV) and scrambled (Scr) sequence served as the controls (Zhang et al., 2019). L02 hepatocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin (50 units/ml)/streptomycin (50 μ g/ml) at 37°C in a humidified atmosphere containing 5% CO_2 . The culture medium was changed every other day.

2.4. MTT assay

After treatment with different concentrations of Cr(VI) for 24 h, the L02 hepatocytes were incubated with MTT at 37°C for another 4 h. Subsequently, the culture medium was discarded and DMSO was used to dissolve the formed formazan crystals. The absorbance was measured using a microplate spectrophotometer at 492 nm. Cell viability (%) was measured using the following equation: cell viability (%) = (A treatment / A control) $\times$ 100%.

2.5. AST and ALT determination

After treatment, we used an aspartate aminotransferase (AST) assay kit and alanine aminotransferase (ALT) Assay Kit (Jiancheng Institute of Biological Products, Nanjing, China) to detect AST and ALT levels in the supernatants of treated hepatocytes and the serum of treated mice according to the manufacturer's instruction.

2.6. HE stain

Liver slices of 0.5 cm width were fixed by 4% paraformaldehyde, dehydrated, and then embedded in paraffin. The slices were cut into thin sections of 3 μ m, stained with hematoxylin and eosin (HE), and examined under a light microscope Leica (Cambridge, UK).

2.7. ROS measurement

ROS production was detected using flow cytometry. After treatment, cells were washed twice in PBS and loaded with 10 μ M DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate, Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C. Cells were then washed twice with PBS for 3 min each time and re-suspended in PBS (and kept out of light during handling). ROS production was detected by flow cytometry at 488/525 nm. The results were then analyzed using Flow jo software.

2.8. Western blotting

The hepatocytes and liver were harvested, washed with ice-cold PBS, and lysed with the RIPA buffer supplemented with protease and phosphatase inhibitors for 30 min. Then the proteins from different treatment groups were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with 5% non-fat milk, the membranes were incubated with the proper primary antibodies overnight at 4°C and the subsequent second antibodies for 1 h at room temperature. The protein bands were then visualized in the darkroom by enhanced chemiluminescence (ECL) detection reagents. The generation of ROS was measured by flow cytometry for the protein bands was obtained using Image-J software.

The primary antibodies for CLU (12289-1-AP), GAPDH (60004-1-Ig), Akt (10176-2-AP), Nrf2 (16396-1-AP), Keap1 (10503-2-AP) and HO-1 (27282-1-AP) were purchased from proteintech (Wuhan, China). The antibody for Phospho (p)-Akt (Ser473) (#4060) was obtained from Cell Signaling Technology (Danvers, MA, USA). The secondary antibody for HRP Goat Anti-Rabbit IgG (H + L) (AS014) was purchased from ABclonal (Wuhan, China).

2.9. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature after washed twice by PBS. The cells were treated with 0.5% Triton X-100 for 20 min and blocked with 5% Bovine Serum Albumin (BSA) (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at room temperature and then incubated with Nrf2 (1:200) overnight at 4°C. Then the cells were incubated with Cy3 Goat Anti-Rabbit IgG (H + L) at 37 °C for 1 h. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 5 min. The fluorescence was observed by using EVOS™ M7000 Imaging System (Thermo Scientific).

2.10. Quantitative real time polymerase chain reaction (qRT-PCR) for mRNA expressions

Cells were seeded onto 60 mm culture plates and treated with Cr(VI). After treatment, the total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cDNAs were synthesized using the HiScript® II Q RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, China). The qRT-PCR was conducted using ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, China). The amplification process data were analyzed using Ct ($2^{-\Delta\Delta C_t}$) method. The qRT-PCR reaction conditions were set according to manufacturer's protocol.

The sequences of the primers used in this study are as follows: 5'- ACGCTACTTCCTCCTCAA-3' (F), 5'- GACACCTCCATCTCTTCAG-3' (R) for *Akt*, 5'- GCATCACCAGAACACTCA-3' (F), 5'- CCAGGACTTACAGGCAATT-3' (R) for *Nrf2*, 5'-CACCAGGGCGTGATGGT-3' (F), 5'- CTCAAACATGATCTGGGTCAT-3' (R) for *ACTB*.

2.11. Statistical Analysis

All the results were expressed as mean $\pm$ standard deviation (SD). Data analysis was performed using the Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ was considered to be statistically significant.

3. Results

1. Cr(VI) induced acute liver injury *in vivo* and *in vitro*

In order to determine the toxic dose of Cr(VI), we used different concentrations of Cr(VI) (0, 1, 2, 4, 8, 16, 32, 64 μ M) to treat the L02 hepatocytes for 24 h. As shown in Fig. 1A, Cr(VI) significantly inhibited the viability of L02 hepatocytes and showed a significant dose-response relationship. To further investigate the acute injury to hepatocytes by Cr(VI), L02 hepatocytes were treated with 8 and 16 μ M Cr(VI) for 24 h and the cell supernatant was taken to measure the levels of aminotransferases. Both AST and ALT were found to be elevated (Fig. 1B), suggesting that Cr(VI) could cause acute injury to L02 hepatocytes. We used 200 mg/kg Cr(VI) to treat mice by gavage to study the effect of Cr(VI) on liver tissue damage *in vivo*, and the results showed that 1 day after treatment with Cr(VI), there were a few scattered bleeding points in the liver tissue. 3 days after Cr(VI) treatment, a large number of haemorrhagic spots appeared in the liver tissue; while 5 days after treatment, the liver tissue structure was completely destroyed, suggesting that Cr(VI) induced serious damage to the liver (Fig. 1C). The detection of AST and ALT content in serum of mice also confirmed that Cr(VI) caused acute damage to liver tissue (Fig. 1D).

2. Cr(VI)-induced acute liver injury was oxidative stress-dependent

It has been reported that Cr(VI) can produce cellular oxidative stress and multi-organ system dysfunction including liver damage (Yu et al., 2020). Thus, we measured intracellular ROS formation in Cr(VI)-treated cells using flow cytometry. As shown in Fig. 2A, Fig. S1A, the level of intracellular ROS rose gradually with the increasing concentrations of Cr(VI) treatment. To further confirm that Cr(VI) can cause liver dysfunction by triggering oxidative stress, the liver was homogenized after Cr(VI) treatment and was processed to measure intracellular ROS. As shown in Fig. 2B, Fig. S1B. Cr(VI) treatment induced a clear time-dependent manner increase of ROS level in liver tissues.

To further identify whether increased ROS production may play a role in Cr(VI)-induced acute liver injury, we used Vitamin E (VitE), a ROS scavenger (50 μ M) to pre-treat L02 hepatocytes for 1 h before Cr(VI) exposure. As shown in Fig. 2C, Fig. S1C, and Fig. 2D, pre-treatment with VitE markedly inhibited ROS production and reduced AST and ALT levels after Cr(VI) exposure, thereby alleviating the degree of

damage to L02 hepatocytes. Based on this evidence, the Cr(VI)-induced acute liver injury was oxidative stress-dependent.

3. CLU expression is up-regulated in vivo and in vitro after Cr(VI) treatment

As mentioned above, CLU can increase MRCC I activity and protect against Cr(VI)-induced cytotoxicity in L-02 hepatocytes. Therefore, we further investigated whether CLU played a role in acute liver injury. We detected CLU expression *in vivo* and *in vitro* after Cr(VI) treatment and found that CLU expression was up-regulated (Fig. 3A-3B). To test the functional role of CLU in Cr(VI)-induced oxidative stress-associated hepatotoxicity, we employed the L02 CLU OE and sh hepatocytes. As shown in Fig. 3C, Fig. S2A, CLU OE significantly reduced Cr(VI)-induced elevated intracellular ROS levels, while interfering CLU by shRNA obviously aggravated Cr(VI)-induced elevated intracellular ROS levels. These results revealed that the elevated CLU intended to inhibit the intracellular ROS production. Meanwhile, we found that under the cytotoxic effect of Cr(VI), CLU OE reduced the aminotransferases levels to a certain extent compared to the EV group; CLU knockdown increased the aminotransferases levels when compared to the Scr group (Fig. 3D and Fig. S2B).

4. CLU targeted Akt and Nrf2

We have shown that in the acute toxicity of Cr(VI), the increase in CLU leads to a reduction the intracellular ROS production, thus mitigating the hepatotoxicity; and we hypothesize that CLU reduced ROS production is via the Akt/Nrf2 signaling pathway. Therefore, we performed western blotting to confirm that CLU could regulate the expression of p-Akt, Keap1 and Nrf2 (Fig. 4A). To further explore the effect of CLU on Nrf2 signaling, we assessed cytoplasmic and nuclear Nrf2 protein expression in L02 hepatocytes by immunofluorescence. CLU over-expression enhanced the cytoplasmic localization of Nrf2 in L02 hepatocytes (Fig. 4B). The qRT-PCR result also revealed that CLU targeted Akt and Nrf2 at mRNA level (Fig. 4C). We used Upr (15 μ M), an Akt inhibitor, to confirm that Akt was the up-stream regulator of Nrf2, and CLU acted through Akt /Keap1/Nrf2 signaling (Fig. 4D).

5. CLU inhibited ROS formation via Akt/Nrf2 signaling

After demonstrating that CLU can regulate the Akt/Keap1/Nrf2 signaling, and combined with previous results(Zhang et al., 2019), it can be reasonably inferred that CLU may inhibit the elevated intracellular ROS caused by Cr(VI) through this pathway and thus protect against Cr(VI)-induced oxidative stress-associated hepatotoxicity. As shown in Fig. 5A and Fig. S2C, intracellular ROS increased significantly in response to the toxic effects of Cr(VI), but decreased in CLU OE hepatocytes compared to controls. Moreover, in the presence of Akt inhibitor, intracellular ROS was further increased, but ROS in CLU OE hepatocytes still remained lower than in the control group. HO-1 is an important antioxidant enzyme that catalyzes the decomposition of hemoglobin into ferrous iron, carbon monoxide and biliverdin. Nuclear translocated Nrf2 binds to ARE located in the promoter region of a cytoprotective gene and initiates transcriptional activation of the antioxidant gene HO-1(Bajpai et al., 2017; Zhang et al., 2017). Our results show that over-expression of CLU can lead to increased Nrf2 expression accompanied by an increase in

HO-1 expression induced by Cr(VI). This phenomenon was achieved through CLU regulation of the Akt/Nrf2 signaling pathway (Fig. 5B). The Fig. 5C proved that CLU can reduce AST and ALT levels in hepatocytes under Cr(VI)-induced hepatotoxicity by mediating the Akt/Nrf2 signaling pathway. The combination of these results confirms that CLU protected against Cr(VI)-induced oxidative stress-associated hepatotoxicity by mediating the Akt-Keap1-Nrf2 signaling pathway.

Discussion

The liver is the main organ involved in exogenous metabolism, and is especially vulnerable to injury. Many reports have shown that chromium is a hepatotoxin (Rao et al., 2006). Our data clearly indicate that over-expression of CLU can effectively inhibit the hepatotoxicity induced by Cr(VI), and this protective effect is related to the prevention of oxidative damage. The liver toxicity induced by Cr(VI) was mainly manifested by the increase of plasma ALT, AST, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities, as well as the histopathological changes. There is also evidence that Cr(VI) can lead to the reduction of weight gain, liver weight and liver/body ratio (Dey et al., 2003). The increased activity of ALT and AST in plasma indicates that hepatocytes are damaged because of the rupture of plasma membrane and the leakage of intracellular enzymes into the blood stream (Hassan et al., 2012). Cr(VI) treatment significantly enhanced the activities of these enzymes, and histopathological abnormalities were observed in the liver of rats treated with Cr(VI), which were consistent with the increase of plasma enzyme activities. Redox changes induced by oxidants such as Cr(VI) compounds have been confirmed to cause apoptosis and necrosis of hepatocytes (Han et al., 2006), while we demonstrate in the present study that CLU prevented the structural damage caused by Cr(VI), maintained the normal structure of liver tissue and protected hepatocytes from ROS attack.

Hepatocytes are usually rich in mitochondria, which are the target of metal toxicity and are related to oxidative stress and mitochondrial dysfunction in many cases (Roy et al., 2009). Oxidative stress leads to the dysfunction of mitochondria, the reduction of oxygen consumption and ATP generation, the oxidation of DNA, proteins and lipids, the changes in calcium homeostasis, the opening of PTP, and the alteration of antioxidant enzyme expression (Oliveira et al., 2006). ROS, mainly generated in the mitochondria, are by-products of molecular oxygen consumption in the electron transport chain (ETC), and we have previously confirmed that MRCC I located on the ETC is the main site for ROS generation after Cr(VI) exposure. Mitochondrial ETC is one of the main intracellular sources of destructive free radicals, and unpaired electrons escaping from MRCC I can cause the generation of $O_2^{\cdot-}$ through the interaction with O_2 . ROS includes hydrogen peroxide (H_2O_2), superoxide anion radical $O_2^{\cdot-}$, and highly active hydroxyl radical ($\cdot OH$). Cr(VI) could cause the imbalance between oxidation and antioxidant systems by enhancing ROS production, resulting in irreversible damage of genomic DNA and destructive oxidative degradation of proteins and lipids. The *CLU* gene has two sheddors that translate into two interrelated proteins, the secretory Clusterin (sCLU) and the nuclear clusterin (nCLU) (Leskov et al., 2003). The protein precursor translated by the CLU gene is driven from the original signal peptide to the endoplasmic reticulum, where it is cleaved into two distinct peptides (α and β), held together by five disulfide bonds. The product is then

glycosylated to secrete mature isodiglycan protein, which is named sCLU (Jones and Jomary, 2002; Yang et al., 2000). nCLU is synthesized by the AUG codon located in the second box at bit 152. AUG₁₅₂ acts through alternative splicing of CLU mRNA or initiation of alternative translation. Thus, nCLU lacking the leader peptide does not enter the endoplasmic reticulum and thus bypasses α/β lysis and glycosylation and is primarily located in the nucleus (Reddy et al., 1996). sCLU is expressed in all tissues and body fluids, and is the predominant form of CLU in the physiological state, and in this paper, CLU refers to sCLU. Following exposure to various cytotoxic agents such as ionising radiation, it is reported that the DNA damage response (DDR) activates Insulin-like growth factor 1 (IGF-1) and increases CLU expression through the IGF-1/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)/early growth response protein-1 (EGR-1) to enhance cell survival. At the same time, as a regulator of DDR, ataxia telangiectasia-mutated kinase (ATM) can also regulate IGF-1 to activate the elevated CLU expression after the onset of DDR (Goetz et al., 2011; Luo et al., 2014). Thus, we reached the conclusion that Cr(VI) led to the massive production of ROS in L02 hepatocytes, which in turn induced DDR and activated ATM kinase and its downstream executor IGF-1, leading to increased expression of CLU.

In this study, we found that the over-expression of CLU can prevent the oxidative damage induced by Cr(VI), and affect the expression of antioxidant enzymes by activating Akt and promoting the dissociation of Nrf2 and Keap1, and finally scavenge ROS. As is well-known that Akt/Nrf2 is important to antioxidant signaling, and evidence confirms that Akt/Nrf2 signaling protects neurons from oxidative stress injury (Liu et al., 2019). Under Cr(VI)-induced oxidative stress, with the increase of CLU in L02 hepatocytes, CLU activates the Akt pathway by directly binding to EIF3l, which in turn promotes the rapid separation of Nrf2 from Keap1 and its transfer to the nucleus to exert antioxidant effects. Alexopoulos, E.C., et al. demonstrated that the CLU gene responds to oxidative stress induced by Cr(VI) in occupational workers, and our work provides laboratory evidence for this phenomenon (Alexopoulos et al., 2008). The possible mechanism is that Cr(VI)-induced ROS directly promotes the separation of Keap1 from Nrf2 or activates Nrf2 through phosphorylation of Nrf2 by various kinases such as Akt, ERK, p38 mitogen-activated protein kinase (P38), and protein kinase C (PKC) (Cheung et al., 2013; Huang et al., 2015; Rizvi et al., 2014).

It has been reported that Nrf2 can protect cells against both Cr(VI)-induced ROS burst generation and apoptosis by regulating the induction of cytoprotective genes, NADPH quinone oxidoreductase 1 (NQO1) and HO-1 (He et al., 2007). Specifically, Nrf2 and Keap1 are ubiquitinated in the cytoplasm, and when they were translocated into the nucleus in association with each other, these two proteins were deubiquitinated upon nuclear translocation. Keap1 contains many cysteine residues that are sensitive to oxidation, such as Cys151, Cys273 and Cys288 (Quinti et al., 2017). When oxidative stress occurs, the sensitive cysteine residues at the terminus of Keap1 like Cys273 and Cys288 were modified by the REDOX reaction to produce efficient molecules, resulting in conformational changes (Hu et al., 2011). Thus, Nrf2 was shed from the ubiquitin binding region and enters the nucleus, where it forms a heterodimer with its partner proteins (such as small Maf transcription factors, sMafs), which then transcriptionally activates a set of genes encoding cell-protective molecules, including HO-1, NQO1, and other antioxidant proteins (Taguchi et al., 2011). HO-1 can be induced by a variety of stress conditions, such as oxidative stress and

inflammatory signals(Wu et al., 2019). Therefore, the induction of HO-1 may be in the antioxidant defense of cells and regulated by Nrf2(Li et al., 2021). Under various stress conditions, HO-1 expression is up-regulated, degrading heme, a potentially toxic substance, and producing three powerful products: bilirubin, Fe²⁺ and carbon monoxide (CO). In this study, we did observe that CLU increased HO-1 expression via Akt-Keap1-Nrf2 signaling pathway. The increase of HO-1 can reduce the excessive production of ROS and the continuous increase of transaminases in L02 liver cells caused by Cr(VI), thus achieving the protective effect of liver injury. The related mechanism is that HO-1 catalyzes the conversion of heme to bilirubin, which not only consumes molecular oxygen, but also reduces the oxygen radical-producing heme. At the same time, the products of HO-1, bilirubin and Fe²⁺, also have the function of consuming oxygen molecules. CO reduces oxidative damage by improving microcirculation(Katori et al., 2002). Hayashi, et al. also revealed that HO-1 mainly plays an inflammatory role by regulating the adhesion factors and chemokines on the surface of endothelial cells and by inhibiting the chemotaxis of neutrophils. Both CO and biliverdin, the downstream products of HO-1 metabolism, have powerful anti-inflammatory functions(Hayashi et al., 1999). Based on this evidence, we can confirm that CLU has a protective effect against Cr(VI)-induced oxidative stress-related hepatotoxicity by mediating the Akt-Keap1-Nrf2 signaling pathway, especially through Nrf2/HO-1 pathway to reduce the increase of intracellular ROS and transaminases.

In summary, we demonstrated in this study that CLU protected against acute hepatotoxicity caused by Cr(VI) via regulating the Akt-Keap1-Nrf2 signaling pathway. Specifically, under the state of Cr(VI)-induced oxidative stress, CLU induced a change in Keap1 conformation through phosphorylation Akt or activation of highly reactive oxides directly driving Nrf2 phosphorylation, leading to Nrf2 dissociation from Keap1. Activated Nrf2 entered the nucleus and formed the next step in the chain reaction, thus binding to the structure of the antioxidant response element ARE, which activated the downstream antioxidant protein HO-1, resulting in the decrease in intracellular ROS and the inhibition of transaminase elevation through its anti-inflammatory effect, thereby protecting hepatocytes as well as liver tissues (Fig. 6). The review of Nigam, et al. comprehensively demonstrated the current toxicity mechanisms of hexavalent chromium, and cited the toxic effects of Cr(VI) in BEAS-2B, BJ-HERT and PMBC cells(Nigam et al., 2014). Our confirmation of the mechanism of toxicity of Cr(VI) in L02 hepatocytes expands the laboratory evidence of the toxic effects of Cr(VI), and identified that CLU as an intervention target of oxidative stress can provide valuable experimental basis for future methods for the prevention and treatment of occupational diseases in Cr(VI)-exposed populations.

Abbreviations

Abbreviations	Full Name
Cr(VI)	Hexavalent chromium
CLU	Clusterin
AKT	AKT serine/threonine kinase
Keap1	Kelch-like ECH-associated protein 1
Nrf2	Nuclear factor E2-related factor 2
HO-1	Heme oxygenase 1
ROS	Reactive oxygen species
Cr(III)	Trivalent chromium
K ₂ Cr ₂ O ₇	Potassium dichromate
MRCC I	Mitochondrial respiratory chain complex I
ARE	Antioxidant response elements
EIF3I	Eukaryotic translation initiation factor I
FBS	Fetal Bovine Serum
VitE	Vitamin E
DMSO	Dimethyl sulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Upr	Uprosertib
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactate dehydrogenase
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
mPTP	Mitochondrial permeability transition pore
ETC	Electron transport chain
DDR	DNA damage response
IGF-1	Insulin-like growth factor 1
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase

Abbreviations	Full Name
EGR-1	Early growth response protein-1
ATM	Ataxia telangiectasia-mutated kinase
p38	p38 mitogen-activated protein kinase
PKC	Protein kinase C
NQO1	NADPH quinone oxidoreductase 1
sMafs	small Maf transcription factors
CO	Carbon Monoxide

Declarations

Data availability

All data generated or analyzed during this study are included in this article.

Funding

The current work was supported by the National Natural Science Foundation of China (NO. 81773478) and Natural Science Foundation of Hunan Province, China (NO. 2019JJ40402).

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Ethics declarations

The mice were adaptively fed for 1 week before the final experiment that was conducted according to the European Community Council Directive on November 24, 1986 (86/609/EEC). All protocols of animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Central South (No.2019sydw0027).

Consent to Publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors Contributions

Yu Ma: Investigation, Formal analysis, Writing-original draft. Siwen Li: Software, Investigation. Sixuan Tang and Shuzi Ye: Visualization, Investigation. Ningjuan Liang, Ying Su and Yuehui Liang: Software, Supervision. Fang Xiao: Conceptualization, Resources, Validation, Writing- review & editing.

Acknowledgements

We thank all of the individuals in this laboratory for their valuable suggestions.

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Figures

Figure 1

Cr(VI) induced acute liver injury in vivo and in vitro. (A) L02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 1, 2, 4, 8, 16, 32, 64 μM) for 24 h. MTT assay for cell viability. (B) AST and ALT concentrations were detected in the supernatant of L02 hepatocytes treated with Cr(VI) (0, 8, 16 μM) for 24h. (C) The H&E stain of liver sections (magnification: 20 $\times$). (D) AST and ALT levels in the serum of mice

exposed to 200 mg/kg Cr(VI) and sacrificed at different time points. ***p < 0.001, compared with the control.

Figure 2

Cr(VI)-induced acute liver injury was oxidative stress-dependent. (A-C) The generation of ROS was measured by flow cytometry and the results were analyzed using Flow jo software. (D) AST and ALT levels were examined using the commercial kits. ***p < 0.001, compared with the control.

Figure 3

CLU expression is up-regulated in vivo and in vitro after Cr(VI) treatment. (A) The CLU protein in L02 hepatocytes treated with Cr(VI) was examined using western blot analysis. (B) The CLU protein in liver tissue treated with Cr(VI) was examined using western blot analysis. (C-D) ROS levels determination. (E) AST and ALT levels examination. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control.

Figure 4

Figure 4

CLU targeted Akt and Nrf2. (A) p-Akt, Akt, Keap1 and Nrf2 protein were examined using western blot analysis, and the quantification was determined by densitometric analysis using Image J software. (B) Immunofluorescence staining of Nrf2 in the CLU OE and sh L02 hepatocytes. (C) mRNA level of Akt and Nrf2 in L-02 CLU OE and CLU sh cells was detected using qPCR. (D) The levels of proteins Keap1 and

Nrf2 in CLU OE L02 hepatocytes pre-treated with 15 μ M Upr were assayed using western blotting. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control.

Figure 5

Figure 5

CLU inhibited ROS formation via Akt/Nrf2 signaling. (A) ROS levels of L02 EV and CLU OE cells after treatment with 16 μ M Cr(VI) and pre-treatment with 15 μ M Upr. (B) The levels of proteins associated with

Nrf2/HO-1 signaling pathway were assayed using western blotting. (C) AST and ALT concentrations were assayed using the commercial kits. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control.

Figure 6

Schematic diagram showing CLU protects against Cr(VI)-induced oxidative stress-associated hepatotoxicity by mediating the Akt-Keap1-Nrf2 signaling pathway.