Original Paper

Effect of Hexavalent Chromium on Electron Leakage of Respiratory Chain in Mitochondria Isolated from Rat Liver

Ying Xie Caigao Zhong Ming Zeng Lan Guan Lei Luo

Department of Health Toxicology, School of Public Health, Central South University, Changsha

Key Words

Hepatocyte • Mitochondria • Electron leak • GSH

Abstract

Background/Aims: In the present study, we explored reactive oxygen species (ROS) production in mitochondria, the mechanism of hexavalent chromium (Cr(VI)) hepatotoxicity, and the role of protection by GSH. **Methods:** Intact mitochondria were isolated from rat liver tissues and mitochondrial basal respiratory rates of NADH and FADH₂ respiratory chains were determined. Mitochondria were treated with Cr(VI), GSH and several complex inhibitors. Mitochondria energized by glutamate/malate were separately or jointly treated with Rotenone (Rot), diphenyleneiodonium (DPI) and antimycinA (Ant), while mitochondria energized by succinate were separately or jointly treated with Rot, DPI, thenoyltrifluoroacetone (TTFA) and Ant. **Results:** Cr(VI) concentration-dependently induced ROS production in the NADH and FADH₂ respiratory chain in liver mitochondria. Basal respiratory rate of the mitochondrial FADH₂ respiratory chain was significantly higher than that of NADH respiratory chain. Hepatic mitochondrial electron leakage induced by Cr(VI) from NADH respiratory chain were mainly from ubiquinone binding sites of complex I and complex III. **Conclusion:** Treatment with 50 μM Cr(VI) enhances forward movement of electrons through FADH₂ respiratory chain and leaking through the ubiquinone binding site of complex III. Moreover, the protective effect of GSH on liver mitochondria electron leakage is through removing excess H₂O₂ and reducing total ROS.

Copyright © 2013 S. Karger AG, Basel

Introduction

Hexavalent chromium (Cr(VI)) is a well-known highly toxic metal and environmental pollutant, and was categorized as human carcinogen by IARC (International Agency for

Research on Cancer) in 1990. Industrial sources of Cr(VI) include plating, electroplating, leather tanning, cooling tower blowdown, and anodizing baths, etc [1]. It has been reported that Cr(VI) would cause hepatocyte apoptosis, characterized by reactive oxygen species (ROS) generation [2]. Excess reactive oxygen species (ROS) production is the primary cause of mitochondrial function damage and energy metabolism disorder, leading to hepatocyte apoptosis.

Generally, the possible sources of ROS production is the mitochondrial electron transport chain in hepatocyte [3, 4]. When exogenous toxins attack the mitochondrial respiratory chain, they would selectively inhibit mitochondrial complexes, resulting in blockage of normal electron transport passway and electron accumulation at the ubiquinone site. This would cause electron leakage from specific sites [5]. O_2 would be reduced to superoxide anion radical ($O_2^{\cdot-}$) by electron leaked from mitochondrial respiratory chain, which was further reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (MnSOD) [4]. Then H_2O_2 would generate hydroxy radical ($\cdot OH$) by reduction reaction. $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$ are referred as ROS, collectively.

Mitochondria contain several ROS production sites. Determination of the production site is based on using inhibitors of electron transport. For example, if rotenone (complex I inhibitor) could increase ROS production in cells, endogenous ROS production would be from complex I. Moreover, it is well known that GSH in liver mitochondria (mtGSH) plays an important role in clearing away H_2O_2 and preventing hepatocyte mitochondria from impairment of respiratory function and dysfunction of energy metabolism [6, 7]. Mitochondria electron transport chain and the role of GSH in scavenging ROS are illustrated in Figure 1.

Previous studies strongly suggest that ROS produced in mitochondria plays a critical role in Cr(VI)-induced hepatocyte apoptosis. However, electron leakage of mitochondrial respiratory chain induced by Cr(VI) still remains unclear. In the present study, after treating isolated rat mitochondria with Cr(VI), we used glutamate/malate and succinate as substrates and a wide variety of complex inhibitors to explore the ROS production sites in mitochondria, the mechanism of Cr(VI) hepatotoxicity, and the role of GSH protection.

Materials and Methods

Materials

Rotenone (Rot), diphenyleneiodonium (DPI), thenoyltrifluoroacetone (TTFA), antimycinA (Ant), L-glutamine, L-Malic acid and sodium succinate were purchased from Sigma (St. Louis, MO, USA).

Mitochondrial isolation

Rats received anesthesia by chloral hydrate after an overnight fast. The rat liver was removed for immediate mitochondrial isolation. Liver mitochondria were isolated by standard differential centrifugation at 4°C as previously described with modifications [8]. Liver excised from rat were weighed and carefully homogenated in isolation buffer (70mM sucrose, 210mM mannitol, 5mM Hepes, 1mM EGTA, 1%BSA, PH=7.4). Tissue debris and nuclei were separated from liver homogenate by centrifugation at 1000×g for 10 min. The supernatant solution was collected and centrifuged at 9000×g for 10min. The mitochondria pellet was washed once with isolation buffer and re-suspended in respiratory buffer as mitochondria suspension (75mM sucrose, 50mM KCl, 30mM Tris-HCl, 2mM KH_2PO_4 , 2mM $MgCl_2$, 10μM EGTA, PH=7.4). Isolated mitochondria were used immediately for measurement of respiration rate and ROS and superoxide formation.

Mitochondrial protein measurements

Mitochondrial protein concentrations were measured using a BCA protein quantification kit (Abcam, SF, USA) with bovine serum albumin used as concentration standards. Standards and the samples were measured in duplicates and the mean values were used to calculate the protein concentrations.

Fig. 1. Electron transport illustration chart of respiratory chain of hepatocyte mitochondria.

Measurement of mitochondrial purity and functionality

Mitochondria re-suspended in respiratory buffer were stored on the ice for 2.5 hours, then we measured purity and functionality of mitochondria isolated with our method. We detected mitochondrial purity by detection of succinate dehydrogenase (SDH) activity in liver homogenate, supernatant solution and mitochondria suspension, respectively with SDH kit (BIOTANG Inc., Waltham, MA 02452 USA). The mitochondria suspension were fixed overnight with 2.5% glutaraldehyde, postfixed with 2% osmid acid, dehydrated, and embedded with epoxy resin. Thin sections were observed with a Hitachi H-600 transmission electron microscope. Mitochondrial ultra-structure was observed by electron microscope (magnification $\times 10,000$ and $\times 30000$, respectively). Then respiratory function of mitochondria was measured with Clark-type oxygen electrode. One mg of mitochondria protein was incubated in 2 ml of respiratory buffer preheated to 25°C. After 2 min later, 5 mm succinate was added as substrate. Then 3 min later, state 3 respiration was initiated by adding 2 μ M ADP, and oxygen consumption was measured as the slope of oxygen consumption curves. The respiratory control ratio (RCR) were calculated as the rate of ADP-stimulated oxygen consumption (State 3 respiratory) divided by the rate of oxygen consumption (state 4 respiratory) when all the ADP has been phosphorylated.

Measurement of mitochondrial basal respiratory rate

Mitochondrial basal respiratory rate of the NADH and FADH₂ respiratory chain were measured using substrate-stimulated O₂ consumption recorded with Clark-type oxygen electrode. Mitochondrial suspensions (1 mg of protein) were diluted to a total volume of 2 ml in reaction buffer preheated to 25°C. Then 10 mM glutamate/5 mM malate or 5 mM succinate were used as substrate 2 min later, respectively. Respiration rate was measured by the slope of oxygen consumption curves after substrates were added as nmol O₂/min/mg prot.

Measurement of ROS formation

Mitochondria (0.5 mg/ml) were energized with complex I (10 mM glutamate/ 5 mM malate) and complex II (5 mM succinate) substrates respectively. Mitochondria suspension were treated with Cr(VI) at 0 μ M, 12.5 μ M, 25 μ M, 50 μ M, and 100 μ M. Liver mitochondria exhibited a concentration-dependent increase in ROS production. Considering feasible mitochondria activity and distinct ROS production, we choose 50 μ M Cr(VI) as the final experimental concentration. ROS production in mitochondria co-incubated with 50 μ M Cr(VI) was measured with addition of specific inhibitors of respiratory complexes and 200 μ M glutathione as the following: Complex I inhibitors, 6.35 μ M Rot, 10 μ M DPI; Complex II inhibitor, 20 μ M TTFA; Complex III inhibitor, 3.75 μ M Ant. ROS production was assessed with 10 μ M 2',7'-dichlorfluorescein-diacetate

Fig. 2. Mitochondria purity and functionality. (A) Liver homogenate, supernatant solution and mitochondria suspension were analyzed for succinate dehydrogenase (SDH) activity. #P<0.05 vs liver homogenate group, n=6. Mitochondrial ultra-structure was observed by electron microscope at magnification x10000 (B) and x30000 (C), respectively. (D) Mitochondrial oxygen consumption curves. RCR is the ratio between the oxygen consumption in state 3 respiration (in the presence of ADP) and state 4 respiration (when all the ADP has been phosphorylated).

(DCFH-DA) fluorescent probes using VarioskanFlash 4.00.52 multifunctional microplate reader (excitation/emission wavelengths: 488nm/525nm).

Measurement of $O_2^{\cdot -}$ production and rate of electron leakage

Mitochondria were used under similar experimental conditions as mentioned above to measure $O_2^{\cdot -}$ production using 30 μ M dihydroethidium (DHE) fluorescent probes. $O_2^{\cdot -}$ -mediated formation of fluorescent ethidium was recorded at Ex_{498nm}/Em_{598nm}. The rate of electron leakage could be calculated as the rate of $O_2^{\cdot -}$ production / 2 × rate of O_2 consumption [9].

Statistical analysis

All data was expressed as mean ± SEM. Statistical comparisons of basal respiratory rates of the mitochondrial respiratory chain were done using t test. Comparisons among multiple treatments were done with analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

Mitochondrial purity and functionality

Succinate hydrogenase is tightly bound to the inner mitochondrial membrane and easily precipitated along with mitochondria. The activities of succinate dehydrogenase as marker enzymes of mitochondria, in mitochondria suspension were significantly higher than that in liver homogenate (Fig. 2A), which revealed that mitochondria were relatively pure.

Fig. 3. Basal respiratory curves of mitochondrial NADH and FADH₂ respiratory chain. (A) Basal respiratory curves of mitochondrial NADH respiratory chain; (B) Basal respiratory curves of mitochondrial FADH₂ respiratory chain; (C) Comparison of basal respiratory rates between mitochondrial NADH and FADH₂ respiratory chain. #P<0.05 vs NADH respiratory chain, n=6.

Table 1. Basal respiratory rate of mitochondrial NADH and FADH₂ respiratory chain. Note: #P<0.05 vs NADH respiratory chain

Groups	N	Basal respiratory rate (nmol O ₂ /min/mgprot)	t	P
NADH respiratory chain	6	0.935±0.170		
FADH ₂ respiratory chain	6	15.350±0.689#	-0.642	0.001

Fig. 4. ROS content of isolated rat mitochondria exposed to Cr(VI). (A) ROS production of mitochondrial NADH respiratory chain induced by Cr(VI); (B) ROS production of mitochondrial FADH₂ respiratory chain induced by Cr(VI). #P<0.05 vs 0 μm Cr(VI) group, n=6.

Mitochondrial ultra-structure observation showed that mitochondria were relatively pure and morphologically intact and dense with no signs of swelling and damage (Fig. 2B, 2C). Mitochondrial respiratory control ratios (RCR) were higher than 3, which was indicative of tightly coupled mitochondria (Fig. 2D) [10].

Fig. 5. Effect of complex I inhibitors and complex III inhibitors on NADH respiratory chain in liver mitochondria. (A)(B) Effect of complex I inhibitors (ROT and DPI) on ROS production and electron leak rate from mitochondrial NADH respiratory chain induced by Cr(VI); (C)(D) Effect of complex III inhibitors (Ant) on ROS production and electron leak rate from mitochondrial NADH respiratory chain induced by Cr(VI). The concentrations of Cr(VI), Rot, DPI and Ant were 50 μ M, 6.35 μ M, 10 μ M and 3.75 μ M, respectively. #P<0.05, n=6.

Groups	N	ROS content (FU/mgprot)	O ₂ ^{•-} content (FU/mgprot)	O ₂ ^{•-} content (nmol/min/mgprot)	electron leak rate (‰)
control	6	1.252±0.047	0.431±0.097	0.137±0.035	7.352±1.891
Rot	6	1.709±0.095 #	0.623±0.191 #	0.208±0.070 #	11.100±3.749 #
DPI	6	0.991±0.054 #	0.379±0.084	0.118±0.031	6.320±1.643
Rot+DPI	6	1.627±0.057 #	0.591±0.129	0.196±0.047	10.486±2.521
Cr(VI)	6	4.452±0.119	0.885±0.315	0.304±0.115	16.232±6.169
Cr(VI)+Rot	6	7.237±0.525*	1.236±0.094*	0.432±0.034*	23.114±1.843*
Cr(VI)+DPI	6	5.205±0.192*	0.955±0.045	0.329±0.016	17.603±0.873
Cr(VI)+Rot+DPI	6	6.195±0.078*	1.121±0.137*	0.390±0.050*	20.868±2.690*

Table 2. Effect of complex I inhibitors on NADH respiratory chain in liver mitochondria. Note: #P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI were 50 μ M, 6.35 μ M and 10 μ M, respectively

Basal respiration rate of NADH and FADH₂ respiratory chain

O₂ consumption of glutamate/malate-energized and succinate-energized liver mitochondria was measured as basal respiratory rate of NADH and FADH₂ respiratory chain (Fig. 3A, 3B). The basal respiratory rate of FADH₂ respiratory chain was much greater than that of NADH respiratory chain (Fig. 3C, Table 1).

ROS production of mitochondria induced by Cr(VI) of different concentrations

Cr(VI) could induce the increase of ROS production in NADH and FADH₂ respiratory chain in liver mitochondria in a concentration-dependent manner (Fig. 4).

Groups	N	ROS content (FU/mgprot)	O ₂ ^{•-} content (FU/mgprot)	O ₂ ^{•-} production rate (nmol/min/mgprot)	electron leak rate (%)
control	6	1.252±0.047	0.431±0.097	0.137±0.035	7.352±1.891
Ant	6	2.002±0.111 [#]	0.519±0.506	0.169±0.019	9.062±0.991
Rot+Ant	6	2.053±0.135 [#]	0.605±0.087 [#]	0.201±0.032 [#]	10.760±1.713 [#]
DPI+Ant	6	3.378±0.049 [#]	0.819±0.111 [#]	0.279±0.041 [#]	14.939±2.166 [#]
Cr(VI)	6	4.452±0.119	0.885±0.315	0.304±0.115	16.232±6.169
Cr(VI)+Ant	6	5.684±0.192 [*]	1.172±0.101 [*]	0.409±0.037 [*]	21.860±1.975 [*]
Cr(VI)+Rot+Ant	6	7.580±0.272 [*]	1.508±0.119 [*]	0.532±0.043 [*]	28.442±2.324 [*]
Cr(VI)+DPI+Ant	6	6.298±0.184 [*]	1.124±0.115 [*]	0.391±0.042 [*]	20.920±2.249 [*]

Table 3. Effect of complex III inhibitors on NADH respiratory chain in liver mitochondria. Note: [#]*P*<0.05 vs the control group; ^{*}*P*<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI and Ant were 50μM, 6.35μM, 10μM and 3.75μM, respectively.

Groups	N	ROS content (FU/mgprot)	O ₂ ^{•-} content (FU/mgprot)	O ₂ ^{•-} production rate (nmol/min/mgprot)	electron leak rate (%)
control	6	22.905±0.501	0.699±0.021	0.235±0.008	0.767±0.025
Rot	6	10.665±0.660 [#]	0.338±0.098 [#]	0.104±0.036 [#]	0.337±0.118 [#]
DPI	6	16.048±0.594 [#]	0.448±0.046 [#]	0.144±0.017 [#]	0.469±0.054 [#]
Rot+DPI	6	16.305±0.921 [#]	0.469±0.069 [#]	0.152±0.025 [#]	0.496±0.082 [#]
Cr(VI)	6	36.326±0.357	1.006±0.065	0.348±0.024	1.134±0.078
Cr(VI)+Rot	6	37.237±2.818	1.059±0.107	0.368±0.039	1.197±0.128
Cr(VI)+DPI	6	35.409±0.501	1.083±0.051	0.376±0.019	1.225±0.061
Cr(VI)+Rot+DPI	6	35.435±1.856	1.104±0.095	0.384±0.035	1.251±0.113

Table 4. Effect of complex I inhibitors on FADH₂ respiratory chain in liver mitochondria. Note: [#]*P*<0.05 vs the control group; ^{*}*P*<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI were 50μM, 6.35μM and 10μM, respectively.

Groups	N	ROS content (FU/mgprot)	O ₂ ^{•-} content (FU/mgprot)	O ₂ ^{•-} production rate (nmol/min/mgprot)	electron leak rate (%)
control	6	22.905±0.501	0.699±0.021	0.235±0.008	0.767±0.025
TTFA	6	35.369±2.205 [#]	1.155±0.079 [#]	0.403±0.029 [#]	1.312±0.094 [#]
Rot+TTFA	6	20.580±1.576 [#]	0.713±0.800	0.241±0.029	0.786±0.095
DPI+TTFA	6	16.134±0.447 [#]	0.430±0.044 [#]	0.137±0.016 [#]	0.446±0.053 [#]
Cr(VI)	6	36.326±0.357	1.006±0.065	0.348±0.024	1.134±0.078
Cr(VI)+TTFA	6	42.653±0.978 [*]	1.456±0.128 [*]	0.513±0.047 [*]	1.671±0.153 [*]
Cr(VI)+Rot+TTFA	6	38.130±2.058 [*]	1.215±0.064 [*]	0.425±0.023 [*]	1.382±0.076 [*]
Cr(VI)+DPI+TTFA	6	28.630±0.332 [*]	0.787±0.061 [*]	0.268±0.022 [*]	0.873±0.072 [*]

Table 5. Effect of complex II inhibitors on FADH₂ respiratory chain in liver mitochondria. Note: [#]*P*<0.05 vs the control group; ^{*}*P*<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI and TTFA were 50μM, 6.35μM, 10μM and 20μM, respectively.

Effect of complex inhibitors on NADH respiratory chain in liver mitochondria treated with Cr(VI)

In glutamate/malate-energized mitochondria, ROS production increased in the Rot group and the Rot+DPI group and decreased in the DPI group. In mitochondria treated with Cr(VI), ROS production increased significantly in the Cr(VI)+Rot group, the Cr(VI)+Rot+DPI group, and the Cr(VI)+DPI group (Fig. 5A, 5B), Table 2. Ant could significantly increase ROS production and electron leakage in mitochondria treated with or without Cr(VI) (Fig. 5C, 5D), Table 3.

Fig. 6. Effect of complex I inhibitors and complex III inhibitors on FADH_2 respiratory chain in liver mitochondria. (A) (B) Effect of complex I inhibitors (ROT and DPI) on ROS production and electron leak rate from mitochondrial FADH_2 respiratory chain induced by Cr(VI); (C)(D) Effect of complex II inhibitors (TTFA) on ROS production and electron leak rate from mitochondrial FADH_2 respiratory chain induced by Cr(VI); (E)(F) Effect of complex III inhibitors (Ant) on ROS production and electron leak rate from mitochondrial FADH_2 respiratory chain induced by Cr(VI). The concentration of Cr(VI), Rot, DPI, TTFA and Ant were 50 μM , 6.35 μM , 10 μM , 20 μM and 3.75 μM , respectively. # $P < 0.05$, $n = 6$.

Groups	N	ROS content (FU/mgprot)	$\text{O}_2^{\bullet -}$ content (FU/mgprot)	$\text{O}_2^{\bullet -}$ production rate (nmol/min/mgprot)	electron leak rate (%)
control	6	22.905 $\pm$ 0.501	0.699 $\pm$ 0.021	0.235 $\pm$ 0.008	0.767 $\pm$ 0.025
Ant	6	29.240 $\pm$ 2.519 #	1.003 $\pm$ 0.081 #	0.347 $\pm$ 0.030 #	1.130 $\pm$ 0.096 #
Rot+Ant	6	30.497 $\pm$ 1.463 #	1.074 $\pm$ 0.197 #	0.374 $\pm$ 0.072 #	1.217 $\pm$ 0.235 #
DPI+Ant	6	28.991 $\pm$ 1.388 #	0.781 $\pm$ 0.102	0.266 $\pm$ 0.038	0.866 $\pm$ 0.122
TTFA+Ant	6	37.030 $\pm$ 0.888 #	1.126 $\pm$ 0.150 #	0.392 $\pm$ 0.055 #	1.277 $\pm$ 0.179 #
Cr(VI)	6	36.326 $\pm$ 0.357	1.006 $\pm$ 0.065 *	0.348 $\pm$ 0.024	1.134 $\pm$ 0.078
Cr(VI)+Ant	6	53.031 $\pm$ 2.265 *	1.165 $\pm$ 0.055 *	0.407 $\pm$ 0.020 *	1.325 $\pm$ 0.065 *
Cr(VI)+Rot+Ant	6	42.818 $\pm$ 0.694 *	1.557 $\pm$ 0.061 *	0.550 $\pm$ 0.022 *	1.791 $\pm$ 0.072 *
Cr(VI)+DPI+Ant	6	41.486 $\pm$ 3.265 *	1.059 $\pm$ 0.082	0.368 $\pm$ 0.030	1.197 $\pm$ 0.098
Cr(VI)+TTFA+Ant	6	47.715 $\pm$ 2.015 *	1.451 $\pm$ 0.061 *	0.511 $\pm$ 0.025 *	1.665 $\pm$ 0.082 *

Table 6. Effect of complex III inhibitors on FADH_2 respiratory chain in liver mitochondria. Note: # $P < 0.05$ vs the control group; * $P < 0.05$ vs the Cr(VI) group. The concentration of Cr(VI), Rot, DPI, TTFA and Ant were 50 μM , 6.35 μM , 10 μM , 20 μM and 3.75 μM , respectively.

Effect of complex inhibitors on FADH_2 respiratory chain in liver mitochondria treated with Cr(VI)

In succinate-energized liver mitochondria, ROS production and electron leakage decreased significantly in the Rot group, the DPI group and the Rot+DPI group. However, in mitochondria treated with Cr(VI), no significant difference was observed among the

Fig. 7. Effect of GSH on electron leakage from mitochondrial NADH and FADH₂ respiratory chain induced by Cr(VI). (A)(B) Effect of GSH on ROS production and electron leakage from mitochondrial NADH respiratory chain induced by Cr(VI); (C)(D) Effect of GSH on ROS production and electron leakage from mitochondrial FADH₂ respiratory chain induced by Cr(VI). The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50μM, 200μM, 6.35μM, 10μM, 20μM and 3.75μM, respectively. *P<0.05, n=6.

Groups	N	ROS content (FU/mgprot)		electron leak rate (‰)	
		Cr(VI)	Cr(VI)+GSH	Cr(VI)	Cr(VI)+GSH
control	6	4.452±0.119	1.712±0.060	16.232±6.169	14.900±8.440
Rot	6	7.237±0.525	5.057±0.072 #	23.114±1.843	22.748±2.137
DPI	6	5.205±0.192	4.143±0.131 #	17.603±0.873	18.269±2.582
Ant	6	5.684±0.192	4.285±0.122 #	21.860±1.975	22.540±1.324
Rot+DPI	6	6.195±0.078	4.025±0.092 #	20.868±2.691	21.051±2.593
Rot+Ant	6	7.580±0.272	5.866±0.163 #	28.442±2.324	27.502±2.554
DPI+Ant	6	6.298±0.184	4.053±0.142 #	20.920±2.249	20.528±2.742

Table 7. Effect of GSH on electron leakage from mitochondrial NADH respiratory chain induced by Cr(VI). Note: *P<0.05 vs the Cr(VI) group. The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50μM, 200μM, 6.35μM, 10μM, 20μM and 3.75μM, respectively.

Cr(VI)+Rot group, the Cr(VI)+DPI group and the Cr(VI)+Rot+DPI group (Fig. 6A, 6B, Table 4). Then we investigated the effect of TTFA, specific inhibitor of complex II, on FADH₂ respiratory chain. TTFA could increase ROS production and electron leakage in mitochondria treated with Cr(VI) with or without Rot, which was reversed by DPI (Fig. 6C, 6D, Table 5). The effect of Ant on FADH₂ respiratory chain was similar to that on NADH respiratory chain. ROS production and electron leakage in groups treated with Ant were higher than those not treated with Ant (Fig. 6E, 6F, Table 6).

Groups	N	ROS content (FU/mgprot)		electron leak rate (%)	
		Cr(VI)	Cr(VI)+GSH	Cr(VI)	Cr(VI)+GSH
control	6	36.326±0.357	27.288±1.487 #	1.134±0.078	1.204±0.033
Rot	6	37.237±2.818	30.909±0.695 #	1.197±0.128	1.214±0.122
DPI	6	35.409±0.501	23.204±1.459 #	1.225±0.061	1.299±0.173
TTFA	6	42.653±0.978	30.948±1.118 #	1.671±0.153	1.708±0.068
Ant	6	43.031±2.265	17.411±0.473 #	1.325±0.065	1.329±0.121
Rot+DPI	6	35.435±1.856	24.297±0.800 #	1.250±0.113	1.336±0.166
Rot+TTFA	6	38.130±2.058	19.277±0.673 #	1.382±0.076	1.388±0.096
Rot+Ant	6	42.818±0.694	32.980±1.119 #	1.791±0.072	1.876±0.121
DPI+TTFA	6	28.630±0.332	16.709±1.089 #	0.873±0.072	0.917±0.052
DPI+Ant	6	41.486±3.265	35.683±0.440 #	1.197±0.098	1.222±0.118
TTFA+Ant	6	47.715±2.015	28.617±0.520 #	1.665±0.082	1.609±0.082

Table 8. Effect of GSH on electron leakage from mitochondrial FADH₂ respiratory chain induced by Cr(VI). Note: **P*<0.05 vs the Cr(VI) group. The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50μM, 200μM, 6.35μM, 10μM, 20μM and 3.75μM, respectively.

Effect of GSH on electron leakage from liver mitochondrial respiratory chain

Effect of GSH on the NADH and FADH₂ respiratory chains in liver mitochondria were shown in Figure 7. ROS production in the Cr(VI) treatment groups were significantly higher than that in groups treated with Cr(VI)+GSH (*P*<0.05) (Table 7). However, no significant difference in electron leakage was observed between groups treated with Cr (VI) alone and groups treated with Cr (VI)+GSH (Table 8).

Discussion

Mitochondria, rich in hepatocytes, provide the main source of cytosolic ATP via the oxidative phosphorylation system. As is well known, Cr(VI) could move into hepatocyte by non-specific ion channels and produce large amounts of ROS, which would damage mitochondrial membrane structure and energy metabolism, and further induce cellular apoptosis [11]. Generally, the quantity of ROS generated varies under different sites, substrates and mitochondrial metabolic conditions caused by different chemicals [12]. Many studies reported that critical sites for electron leakage were the ubiquinone binding sites of complex I and complex III, and that of complex II were negligible [13, 14]. Some studies also suggest that ROS would be produced from complex II and complex IV [15, 16]. However, the mechanism of how excess ROS is produced in hepatic mitochondria exposed to Cr(VI) has not been elaborated clearly.

Above all, we separated mitochondria from the rat liver by differential centrifugation and ensured mitochondria could maintain integrity and functionality at a suitable level during experiments. Then we used several inhibitors of complexes to characterize the mechanism of enhanced ROS production in hepatic mitochondria treated with Cr(VI). Rot, which is highly lipophilic, could easily penetrate the membrane and selectively block electrons transferring from iron-sulfur center of complex I to ubiquinone [17]. DPI, as FMN site-specific inhibitor of complex I, could combine flavin mononucleotide to reduce electron leakage from FMN site of complex I [18]. The main effect of TTFA is to inhibit electron transfer from complex II ubiquinone binding site to complex III [19]. Ant, the complex III inhibitor, acts on complex III and inhibit electron driving from cytochrome b (Cyt bH) to ubiquinone, which could increase electron leakage and ROS production [20, 21].

ROS production in succinate-energized hepatic mitochondria treated with Cr(VI) were much higher than that in glutamate/malate-energized mitochondria, which may due to

higher basal respiratory rate of FADH_2 respiratory chain. In glutamate/malate-energized mitochondria, ROS production increased in the Rot group. It is suggested that in mitochondria under normal physiological conditions, Rot inhibit electron driving from iron-sulfur center of complex I to ubiquinone and enhance their leakage from NADH respiratory chain [11]. ROS generation decreased in the DPI group, indicated that DPI would act on FMN site and block electron moving into the reduction center of complex I and then decrease electron leakage. These results were similar with others' studies [22]. However, when mitochondria were treated with Cr(VI), the results were different. ROS content and electron leakage increased in the Rot+Cr(VI) group, but not changed in the DPI+Cr(VI) group. The results suggested that the ubiquinone binding site, but not the FMN site, is the main site of ROS production in NADH respiratory chain. Moreover, Ant could increase ROS production and electron leakage in mitochondria treated with Cr(VI), which suggests that Ant could inhibit electron moving from Cyt bH to ubiquinone, accumulate semiquinone and increase electron leakage [23, 24].

With regard to mitochondrial FADH_2 respiratory chain, it is generally acknowledged that complex II could not release electron by itself and electron could leak from complex I when they move backward from complex II to complex I [25], or leak from the ubiquinone binding site of complex III if they move forward along respiratory chain [26]. In succinate-energized mitochondria without Cr(VI) treatment, ROS production and electron leakage decreased in the Rot group and DPI group, and increased in the TTFA group and the Ant group. It is suggested that when electron generated from succinate moves backward from complex II to complex I, Rot would inhibit this process and DPI protects the FMN site of complex I to reduce electron leakage [18]. On the contrary, TTFA could block electron moving backward from complex II to ubiquinone, while Ant acts on the ubiquinone binding site of complex III, which leads to electron leakage and excess ROS generation.

As far as succinate-energized mitochondria treated with Cr(VI) are concerned, compared with the Cr(VI) group, ROS content and electron leakage in the Rot+Cr(VI) group and the DPI+Cr(VI) group showed no significant difference. ROS generation in Cr(VI)-treated mitochondria was irrelevant with electron moving backward from complex II to complex I. Then ROS content and electron leakage increased in the Cr(VI)+TTFA group compared with the Cr(VI) group. It is seemingly found that co-treatment of Cr(VI) and TTFA could promote electron leaked from the ubiquinone binding site of complex II, however, ROS content and electron leakage in the Cr(VI)+Rot+TTFA group and the Cr(VI)+DPI+TTFA group were significantly lower than those in the Cr(VI)+TTFA group. It is rational to postulate that the increase of electron leakage in the Cr(VI)+TTFA group was actually due to the backflow of electron from complex II to complex I. Furthermore, after Ant inhibits the ubiquinone binding site of complex III in mitochondria exposed to Cr(VI), ROS content and electron leakage increased significantly. The result above suggested that Cr(VI) could induce electron leakage from FADH_2 respiratory chain in liver mitochondria through the ubiquinone binding site of complex III, other than enhancing electron backflow from complex II to complex I or leaking from the ubiquinone binding site of complex II.

GSH, a critical endogenous antioxidant in mitochondria, could transform H_2O_2 into H_2O under the effect of glutathione peroxidase and transform itself into oxidized glutathione disulfide (GSSG), which could then transform into glutathione by glutathione reductase. Excess H_2O_2 could be cleared during this cycle [27]. Cederbaum et al. found that exogenous S-adenosyl-L-methionine (SAM) supplement, as a precursor of GSH synthesis, could protect liver mitochondria from oxidative damage [28, 29]. In our study, protective effects of exogenous GSH supplement on NADH and FADH_2 respiratory chain of liver mitochondria were similar. GSH could clear over excess ROS production in liver mitochondria induced by Cr(VI) in both NADH and FADH_2 respiratory chains, but no significant protective effect could be observed on electron leakage. It is indicated that GSH do exhibit protective effects on liver mitochondria by the elimination of excess H_2O_2 and ROS, but could not decrease electron leakage from mitochondrial respiratory chain.

In summary, our study demonstrated the sites of Cr(VI)-induced ROS production and the protective effect of GSH in mitochondrial NADH and FADH_2 respiratory chain. Cr(VI)

induced the increase of electron leakage from ubiquinone binding sites of complex I and complex III in NADH respiratory chain. While in succinate-energized mitochondria, Cr(VI) induced electron leakage from the ubiquinone binding site of complex III when the electron moved forward in the respiratory chain. GSH can alleviate Cr(VI)-induced liver mitochondrial oxidative damage through effective removal of excess H_2O_2 and total ROS content, rather than the improvement of mitochondrial respiration function to reduce electron leakage.

Acknowledgements

We are very grateful for the generous assistance of Physiology Department and Preventive Medicine Experimental Center of Central South University, Changsha, China. This work was supported by the National Natural Science Foundation of China (No. 30972511). The authors declare no conflict of interest.

References

- 1 Mohan D, Pittman CU Jr: Activated carbons and low cost adsorbents for remediation of tri- and hexavalent chromium from water. *J Hazard Mater* 2006;137:762-811.
- 2 Wang XF, Xing ML, Shen Y, Zhu X, Xu LH: Oral administration of Cr (VI) induced oxidative stress, DNA damage and apoptotic cell death in mice. *Toxicology* 2006;228:16-23.
- 3 Hoffman DL, Brookes PS: Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions. *J Biol Chem* 2009;284:16236-16245.
- 4 Li J, Xu Z, Tan M, Su W, Gong XG: 3-(4-(Benzo[d] thiazol-2-yl) -1-phenyl- 1H- pyrazol-3-yl) phenyl acetate induced Hep G2 cell apoptosis through a ROS-mediated pathway. *Chem Biol Interact* 2010;183:341-348.
- 5 Murphy MP: How mitochondria produce reactive oxygen species. *Biochem J* 2009;417:1-13.
- 6 Gopi S, Setty OH: Protective effect of Phyllanthus fraternus against bromobenzene induced mitochondrial dysfunction in rat liver mitochondria. *Food Chem Toxicol* 2010;48:2170-2175.
- 7 Fernandez-Checa JC, Kaplowitz N: Hepatic mitochondrial glutathione: transport and role in disease and toxicity. *Toxicol Appl Pharmacol* 2005;204:263-273.
- 8 Sailaja R, Setty OH: Protective effect of Phyllanthus fraternus against allyl alcohol-induced oxidative stress in liver mitochondria. *J Ethnopharmacol* 2006;105:201-209.
- 9 Wen JJ, Garg NJ: Mitochondrial generation of reactive oxygen species is enhanced at the Q(o) site of the complex III in the myocardium of Trypanosoma cruzi-infected mice: beneficial effects of an antioxidant. *J Bioenerg Biomembr* 2008;40:587-598.
- 10 Fernández-Vizarra E, Ferrín G, Pérez-Martos A, Fernández-Silva P, Zeviani M, Enríquez JA: Isolation of mitochondria for biogenetical studies: An update. *Mitochondrion* 2010;10:253-262.
- 11 Panieri E, Gogvadze V, Norberg E, Venkatesh R, Orrenius S, Zhivotovsky B: Reactive oxygen species generated in different compartments induce cell death, survival, or senescence. *Free Radic Biol Med* DOI: 10.1016/j.freeradbiomed.2012.12.024.
- 12 Hoffman DL, Brookes PS: Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions. *J Biol Chem* 2009;284:16236-16245.
- 13 Viola HM, Hool LC: Qo site of mitochondrial complex III is the source of increased superoxide after transient exposure to hydrogen peroxide. *J Mol Cell Cardiol* 2010;49:875-885.
- 14 Brand MD: The sites and topology of mitochondrial superoxide production. *Exp Gerontol* 2010;45:466-472.
- 15 Zhang JG, Tirmenstein MA, Nicholls-Grzemeski FA, Fariss MW: Mitochondrial electron transport inhibitors cause lipid peroxidation-dependent and independent cell death: protective role of antioxidants. *Arch Biochem Biophys* 2002;393:87-96.

- 16 Moreno-Sánchez R, Hernández-Esquivel L, Rivero-Segura NA, Marín-Hernández A, Neuzil J, Ralph SJ, Rodríguez-Enríquez S: Reactive oxygen species are generated by the respiratory complex II - evidence for lack of contribution of the reverse electron flow in complex I. *FEBS J* 2013;280:927-938.
- 17 Fato R, Bergamini C, Leoni S, Lenaz G: Mitochondrial production of reactive oxygen species: role of complex I and quinone analogues. *Biofactors* 2008;32:31-39.
- 18 Lambert AJ, Buckingham JA, Boysen HM, Brand MD: Diphenyleneiodonium acutely inhibits reactive oxygen species production by mitochondrial complex I during reverse, but not forward electron transport. *Biochim Biophys Acta* 2008;1777:397-403.
- 19 Zhang JG, Nicholls-Grzemeski FA, Tirmenstein MA, Fariss MW: Vitamin E succinate protects hepatocytes against the toxic effect of reactive oxygen species generated at mitochondrial complexes I and III by alkylating agents. *Chem Biol Interact* 2001;138:267-284.
- 20 Liang B, Wang L, He T, Liu W, Li Q, Li M: In vitro reactive oxygen species production by mitochondria from the rabbitfish *Siganus fuscus* livers and the effects of Irgarol-1051. *Environ Toxicol Pharmacol* 2012;35:154-160.
- 21 Shiryaeva A, Arkadyeva A, Emelyanova L, Sakuta G, Morozov V: Superoxide anion production by the mitochondrial respiratory chain of hepatocytes of rats with experimental toxic hepatitis. *J Bioenerg Biomembr* 2009;41:379-385.
- 22 Fato R, Bergamini C, Bortolus M, Maniero AL, Leoni S, Ohnishi T, Lenaz G: Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species. *Biochim Biophys Acta* 2009;1787:384-392.
- 23 Fang J, Beattie DS: Rotenone-insensitive NADH-dehydrogenase is a potential source of superoxide in procyclic *Trypanosoma brucei* mitochondria. *Mol Biochem Parasitol* 2002;123:135-142.
- 24 Dröse S, Hanley PJ, Brandt U: Ambivalent effect of diazoxide on mitochondrial ROS production at respiratory chain complexes I and III. *Biochim Biophys Acta* 2009;1790:558-565.
- 25 Han D, Canali R, Rettori D, Kaplowitz N: Effect of Glutathione Depletion on Sites and Topology of Superoxide and Hydrogen Peroxide Production in Mitochondria. *Mol Pharmacol* 2003;64:1136-1144.
- 26 Liu Y, Fiskum G, Schubert D: Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* 2002;80:780-787.
- 27 Yuan L, Kaplowitz N: Glutathione in liver diseases and hepatotoxicity. *Mol Aspects Med* 2009;30:29-41.
- 28 Cederbaum AI: Hepatoprotective effects of S-adenosyl-L-methionine against alcohol- and cytochrome P450 2E1-induced liver injury. *World J Gastroenterol* 2010;16:1366-1376.
- 29 Wang X, Cederbaum AI: S-adenosyl-L-methionine decreases the elevated Fas agonistic antibody plus acute ethanol pretreatment in mice. *Arch Biochem Biophys* 2008;477:1-11.