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A comparison of hepatocyte cytotoxic mechanisms for chromate and arsenite

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Abstract

In the following, we have compared the cytotoxic mechanisms of the chromate CrO_4^{2-} and arsenite AsO_2^-. Chromate (Cr (VI)) cytotoxicity was associated with reactive oxygen species (ROS) formation, lipid peroxidation and loss of mitochondrial membrane potential, which were prevented by catalase, antioxidants and ROS scavengers. Hepatocyte glutathione was also rapidly oxidized. Chromate reduction was inhibited in glutathione depleted hepatocytes, and glutathione depleted hepatocytes were also much more resistant to chromate induced cytotoxicity, ROS formation and lipid peroxidation. This suggests that chromate is reductively activated by glutathione. Chromate cytotoxicity also involved lysosomal injury and protease activation, which were prevented by lysosomotropic agents, endocytosis inhibitors, protease inhibitors and ROS scavengers. On the other hand, arsenite cytotoxicity was associated with much less oxidative stress, and lysosomal damage did not occur. However, arsenite cytotoxicity was also associated with loss of mitochondrial membrane potential, which in contrast to chromate cytotoxicity was inhibited by the ATP generators fructose, xylitol and glutamine. Arsenite induced cytotoxicity, mitochondrial membrane potential decline and also ROS formation were significantly increased by inactivating hepatocyte methionine synthase or hepatocyte methyl transferase. However, methyl donors such as betaine, methionine or folic acid prevented arsenite but not chromate cytotoxicity, and this suggests that arsenite is detoxified by reductive methylation. In conclusion, chromate induced cytotoxicity could be

Abbreviations: ANOVA, analysis of variance; BCNU, 1,3-bis(2-chloroethyl)-1nitrosourea; BHA, butylated hydroxyanisole; BHT, butylated hydroxy toluene; BSA, bovine serum albumin; DCF, dichlorofluorescein; DMSO, dimethyl sulfoxide; DPPD, N,N′-diphenyl-1,4-phenylenediamine; EGTA, ethyleneglycol-bis(*p*-aminoethylether)-N,NN′,N′-tetraacetic acid; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; rpm, rotations per minute; SE, standard error; SEM, standard error of mean; SOD, superoxide dismutase; TBARS, 2-thiobarbituric acid-reactive substances; TCA, trichloroacetic acid

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attributed to oxidative stress and lysosomal damage, whereas arsenite induced cytotoxicity could be attributed to mitochondrial toxicity and ATP depletion.
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Keywords: Hepatocyte; Arsenite; Chromate; Cytotoxicity; Methylation; Reduction; Mitochondria; Oxidative stress; Lysosomes

1. Introduction

The most toxic forms of arsenic and chromium in the environment are arsenite As (III) and chromate (hexavalent chromium Cr (VI)), respectively. In these forms, they are soluble and are transported into the cells. Chromate structurally resembles phosphate and sulfate, and can be transported into cells by the anion carrier (Alexander and Aaseth, 1995). As (III) in the form of arsenite can be uptaken into the cells by aquaglyceroporins. An aquaglyceroporin is a member of the aquaporin subfamily, which are multifunctional channels that transport neutral organic solutes such as glycerol and urea (Rosen, 2002). Inorganic arsenic as an environmental agent has been ranked highest in priority on a list of top 20 hazardous substances by the ATSDR and US-EPA, in part, because it has been classified as a known human carcinogen, and oral inorganic arsenic exposure can induce tumor formation in rodents (ATSDR, 1997; Waalkes et al., 2003). American Council on Science and Health report concluded that there is clear evidence that chronic exposure to inorganic arsenic at concentrations of at least several hundred micrograms per liter in drinking water may cause cancer of skin, bladder and lung (Brown and Ross, 2002).

Chromate on the other hand is believed to be an essential trace element required to regulate blood glucose levels (glucose tolerance) and act as a cofactor in the maintenance of normal lipid and carbohydrate metabolism. However, there is a 29-fold overall increase in respiratory cancer in workers involved in the production of chromium products, e.g. chrome plating, leather tanning and stainless steel industries. A dietary daily intake for chromium is recommended as marginal chromium deficiency may increase the risk for diabetes and possibly coronary heart disease (Merty, 1993). Chromate is also an environmental agent and the IARC have listed Cr (VI) but not Cr (III) as a human carcinogen (Group 1). The US-EPA estimates that most of total atmospheric Cr (VI) results from fossil fuel combustions and steel production. Cr (VI) salts can cause contact dermatitis and Cr (VI) species are also often carcinogenic in test animals (U.S. Public Health and Science, 1953).

Ingestion of arsenic contaminated drinking water is however the predominant source of significant environmental exposure globally with more than 200 million people at risk (Rojas et al., 1999). In clinical poisoning, an oral dose of 4 mg/kg Cr (VI) was associated with hepatic failure and acute renal tubular necrosis before death from cardiovascular shock occurred (Cohen et al., 1993). Nephrotoxicity and hepatotoxicity also occurred in rats administered 2 mg/kg Cr (VI) (Barceloux, 1999). The oral LD_{50} dose for Cr (VI) in rats was 54 mg/kg. Cr (VI) is likely much more toxic than Cr (III) because of its stronger oxidizing power and higher transport rate through the cell membrane. (Barceloux, 1999).

There are few cellular studies in the literature about the molecular cytotoxic mechanisms of chromium (Ueno et al., 1989; Sugiyama and Tsuzuki, 1994; Blankenship et al., 1994; Susa et al., 1997) and arsenic (Hei et al., 1998; Larochette et al., 1999). In the following, we have compared the cytotoxic mechanisms of chromate (Cr (VI)) and arsenite (As (III)), and have shown that there are significant differences in molecular aspects (e.g. reactive oxygen species (ROS) generation) and subcellular targets (e.g. mitochondria/lysosomes) between these two known environmental poisons.

2. Materials and methods

2.1. Chemicals

1-Bromoheptane, NN′-diphenyl-1,4-phenylenediamine (DPPD), rhodamine 123 and hydrazine monohydrate were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Collagenase (from *Clostridium histolyticum*), bovine serum albumin (BSA) and Hepes (4-(2-hydroxyethyl)-1-piperazine-

ethanesulfonic acid) were purchased from Boehringer-Mannheim (Montreal, Canada). Trypan blue, sodium arsenite, potassium dichromate, d-mannitol, dimethyl sulfoxide (DMSO), catalase, superoxide dismutase (SOD), chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, thiobarbituric acid (TBA), ethyleneglycol-bis(*p*-aminoethyl ether)-N,NN′,N′-tetra acetic acid (EGTA), trichloroacetic acid(TCA), cyclosporine, betaine, methionine, trifluoprazine, folic acid, sodium selenite, fructose, sodium pentobarbital and heparin were obtained from Sigma (St. Louis, MO, USA). Acridine orange and dichlorofluorescin diacetate were purchased from Molecular Probes (Eugene, Ore, USA). Desferoxamine was a gift from Ciba-Geigy Canada Ltd. (Toronto, ON, Canada). Carnitine and xylitol were obtained from ICN Biomedicals (St. Thuringen, Eschwege, Germany). All chemicals were of the highest commercial grade available.

2.2. Animals

Male Sprague–Dawley rats (280–300 g), fed on a standard chow diet and given water ad libitum, were used in all experiments.

2.3. Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Pourahmad and O'Brien (2000a). Approximately 85–90% of the hepatocytes excluded trypan blue. Cells were suspended at a density of 10^6 cells/ml in round bottomed flasks rotating in a water bath maintained at 37 °C in Krebs–Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes under an atmosphere of 10% O_2, 85% N_2 and 5% CO_2. Each flask contained 10 ml of hepatocyte suspension. Hepatocytes were preincubated for 30 min prior to addition of chemicals. Stock solutions of all chemicals (×100 concentrated for the water solutions or ×1000 concentrated for the methanolic solutions) were prepared fresh prior to use. To avoid either non-toxic or very toxic conditions in this study, we used ED_{50} concentrations for the investigated metals in the isolated hepatocytes including Cr (VI) and As (III). The ED_{50} of a chemical in hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period) is defined as the concentration, which decreases the hepatocye viability down to 50% following the 2 h of incubation (Galati et al., 2000). In order to determine this value for the investigated metals, dose-response curves were plotted and then ED_{50} was determined based on a regression plot of three different concentrations (data and curves not shown). To incubate each metal (both of the metal salts easily dissolved in water) with the required concentration, we added 100 μl sample of its concentrated stock solution (×100 concentrated) to one rotating flask containing 10 ml hepatocyte suspension. For the chemicals, each dissolved in methanol, we prepared methanolic stock solutions (×1000 concentrated), and to achieve the required concentration in the hepatocytes, we added 10 μl samples of the stock solution to the 10 ml cell suspension. Ten microlitres of methanol did not affect the hepatocyte viability after 3 h incubation (data not shown). Glutathiones (reduced form) (GSH) depleted hepatocytes were prepared by preincubation of hepatocytes with 200 μM 1-bromoheptane for 30 min as described by Khan and O'Brien, 1991. Methionine synthase-inactivated hepatocytes were prepared by preincubation of hepatocytes with 8 mM hydrazine monohydrate as described by Kenyon et al. (1999). Methyl trasferase of the hepatocytes were inactivated by preincubation of hepatocytes with 4 μM sodium selenite as described by Styblo and Thomas, 2001.

2.4. Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% (w/v)) exclusion test (Pourahmad and O'Brien, 2000a). Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period. At least 80–90% of the control cells were still viable after 3 h.

2.5. Determination of reactive oxygen species "ROS"

To determine the rate of hepatocyte "ROS" generation induced by the metals, dichlorofluorescin diacetate was added to the hepatocyte incubate as it penetrates hepatocytes and becomes hydrolysed to non-fluorescent dichlorofluorescin (DCF). The latter then reacts with "ROS" to form the highly fluorescent dichlorofluorescein which effluxes the cell. Hepatocytes (1×10^6 cells/ml) were suspended in 10 ml mod-

ified Hank's balanced salt solution (HBS), adjusted to pH 7.4 with 10 mM Hepes (HBSH), and were incubated with As^{3+} or Cr^{6+} at 37 °C for 30, 60 and 120 min. After centrifugation (50 × *g*, 1 min), the cells were resuspended in HBS adjusted to pH 7.4 with 50 mM Tris–HCl, and loaded with dichlorofluorescin by incubating with 1.6 μl dichlorofluorescin diacetate for 2 min at 37 °C. The fluorescence intensity of the "ROS" product was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Shen et al., 1996).

2.6. Lipid peroxidation assay

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU®-7 spectrophotometer after treating 1.0 ml aliquots of hepatocyte suspension (10^6 cells/ml) with trichloroacetic acid (70% (w/v)) and boiling the suspension with thiobarbituric acid (0.8% (w/v)) for 20 min (Smith et al., 1982).

2.7. Mitochondrial membrane potential assay

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential (Andersson et al., 1987). Aliquots of the cell suspension (0.5 ml) were separated from the incubation medium by centrifugation at 1000 rpm for 1 min. The cell pellet was then resuspended in 2 ml of fresh incubation medium containing 1.5 μM rhodamine 123, and incubated at 37 °C in a thermostatic bath for 10 min with gentle shaking. Hepatocytes were then separated by centrifugation, and the amount of rhodamine 123 remaining in the incubation medium was measured fluorimeterically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence.

2.8. Lysosomal membrane stability assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange (Pourahmad et al., 2001a–c). Aliquots of the cell suspension (0.5 ml), that were previously stained with acridine orange 5 μM, were separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out for two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths.

2.9. Statistical analysis

The statistical significance of differences between the control and treatment groups in these studies was determined using a one-way analysis of variance (ANOVA) and the Bartlett's test for homogeneity of variances. Results represent the mean ± standard error of the mean (SEM) of triplicate samples. The minimal level of significance chosen was $P < 0.001$.

3. Results

As shown in Table 1, arsenite was more effective than chromate in causing hepatocyte membrane lysis as determined by trypan blue uptake. The ED_{50} concentrations found for arsenite and chromate (i.e., 50% membrane lysis in 2 h) were 50 μM and 1 mM, respectively. However, when hepatocytes were incubated with arsenite or chromate at these ED_{50} concentrations, "ROS" formation as determined by the oxidation of dichlorofluorescin diacetate to dichlorofluorescein was increased by chromate much more than by arsenite (Table 1). Furthermore, chromate induced "ROS" formation was prevented by the hydroxyl radical scavengers dimethylsulfoxide or mannitol as well as by catalase (Pourahmad and O'Brien, 2000a,b; Siraki et al., 2002). All of these agents did not show any toxic effect on hepatocytes at concentrations used (data not shown).

Table 1
Preventing metal induced hepatocyte necrosis by antioxidants and "ROS" scavengers

Addition	Cytotoxicity (%) 3 h	"DCF" 3 h	"TBARS" 3 h
None	20 ± 2	79 ± 5	0.41 ± 0.05
Dichromate (1 mM)	76 ± 8[a]	508 ± 6[a]	20.62 ± 2.28[a]
+Catalase (200 u/ml)	35 ± 5[b]	129 ± 5[b]	4.35 ± 1.15[b]
+Dimethyl sulfoxide (150 μM)	45 ± 4[b]	137 ± 8[b]	4.45 ± 1.14[b]
+Mannitol (50 mM)	35 ± 5[b]	109 ± 5[b]	3.35 ± 0.65[b]
+BHA (50 μM)	37 ± 4[b]	105 ± 6[b]	4.37 ± 1.26[b]
+BHT (50 μM)	35 ± 5[b]	111 ± 5[b]	4.35 ± 0.85[b]
+DPPD (1 μM)	33 ± 3[b]	81 ± 6[b]	2.18 ± 1.98[b]
+Phenylimidazole (300 μM)	37 ± 2[b]	210 ± 9[b]	6.85 ± 1.26[b]
+Diphenyliodonium chloride (50 μM)	74 ± 5	207 ± 5[b]	6.92 ± 1.05[b]
+BCNU (50 μM)	73 ± 6	280 ± 8[b]	9.83 ± 1.34[b]
+Dicumarol (30 μM)	76 ± 8	500 ± 8	17.4 ± 2.07
Arsenite (50 μM)	76 ± 8[a]	165 ± 6[a]	2.62 ± 0.98[a]
+Catalase (200 u/ml)	75 ± 5	129 ± 5[b]	2.35 ± 1.15
+Dimethyl sulfoxide (150 μM)	75 ± 4	137 ± 8[b]	2.45 ± 1.14
+Mannitol (50 mM)	73 ± 5	109 ± 5[b]	2.35 ± 0.65
+BHA (50 μM)	75 ± 5	129 ± 5[b]	2.35 ± 1.15
+BHT (50 μM)	75 ± 4	137 ± 8[b]	2.45 ± 1.14
+DPPD (1 μM)	73 ± 5	109 ± 5[b]	2.35 ± 0.65
+Phenylimidazole (300 μM)	75 ± 4	155 ± 6	2.48 ± 1.26
+Diphenyliodonium chloride (50 μM)	74 ± 5	158 ± 5	2.31 ± 0.85
+BCNU (50 μM)	73 ± 6	161 ± 6	2.18 ± 0.18
+Dicumarol (30 μM)	76 ± 8	165 ± 6	2.42 ± 0.17
GSH depleted hepatocytes	26 ± 3	88 ± 8	0.73 ± 0.08
+*Dichromate* (1 mM)	37 ± 4[b]	135 ± 4[b]	6.37 ± 1.94[b]
+*Arsenite* (50 μM)	87 ± 4[b]	235 ± 4[b]	3.37 ± 1.94

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of potassium dichromate and sodium arsenite. Cytotoxicity was determined as the percentage of cells that take up trypan blue (Pourahmad and O'Brien, 2000a). DCF formation was expressed as fluorescent intensity units (Shen et al., 1996). GSH depleted hepatocytes were prepared as described by Khan and O'Brien, 1991. TBARS formation was expressed as μM concentrations (Smith et al., 1982). Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

The involvement of "ROS" in the cytotoxic mechanism was also studied and as shown in Table 1, chromate induced cytotoxicity was inhibited by the "ROS" scavengers catalase, dimethyl sulfoxide or mannitol; however, arsenite induced cytotoxicity was not inhibited by the "ROS" scavengers. A significant amount of thiobarbituric acid-reactive substances was formed, which markedly increased in the third hour for chromate, but not for arsenite-treated hepatocytes (Table 1).

Chromate reduction was required for oxygen activation. However, inhibiting DT-diaphorase with dicumarol (Sood et al., 1997) or glutathione reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Gunaratnam and Grant, 2001) did not show any significant effect on Cr (VI) induced cytotoxic alterations, nevertheless, depleting hepatocyte GSH beforehand or inhibiting P450 reductase with diphenyliodonium chloride (DPI) (Pourahmad et al., 2001a–c; Siraki et al.,2002) prevented chromate induced cytotoxicity as well as "ROS" formation and lipid peroxidation. Inhibiting CYP2E1 with phenylimidazole (Pourahmad et al., 2001a–c; Siraki et al., 2002) also protected the hepatocyte against Cr (VI) induced cytotoxicity and lipid peroxidation. All of these mentioned enzyme inhibitors did not show any toxic effect on hepatocytes at concentrations used (data not shown).

On the other hand, arsenite was detoxified by methylation as arsenite, but not chromate induced cytotoxicity. "ROS" formation and lipid peroxidation were prevented by methyl donors methionine, betaine, folic acid and methylcobalamine (Chu et al., 1993; DeLong et al., 2002; Friso and Choi, 2002) (Table 1). However, phenylimidazole, DPI, dicumarol or BCNU did not affect arsenite induced cytotoxicity (Table 1). All of these agents including methyl donors and enzyme inhibitors did not show any toxic effect on hepatocytes at concentrations used (data not shown).

As shown in Table 2, however, both arsenite and chromate induced a rapid decline of mitochondrial membrane potential. Fructose/xylitol (glycolytic ATP generators) (Khan and O'Brien, 1995; Kim et al., 2003) or glutamine (a mitochondrial ATP generator) (Sood and O'Brien, 1994; Markley et al., 2002; Pourahmad et al., 2003) only prevented arsenite induced cytotoxicity (Table 3A) and the mitochondrial membrane potential decrease (Table 2). All of these ATP generators did not show any toxic effect on hepatocytes at concentrations used (data not shown).

Furthermore, permeability transition pore sealing agents (Strakov et al., 1994; Lemasters et al., 1999; Pourahmad et al., 2001a–c) carnitine, cyclosporine and trifluoprazine prevented both chromate and arsenite induced cytotoxicity, "ROS" formation and lipid peroxidation (Table 3A). All of these pore sealing agents did not show any toxic effect on hepatocytes at concentrations used (data not shown).

However, the chromate but not arsenite induced decline of mitochondrial membrane potential was prevented by the "ROS" scavengers dimethyl sulfoxide or mannitol, which indicates that chromate induced mitochondrial membrane potential decrease was a consequence of "ROS" formation and lipid peroxidation. The arsenite but not chromate induced decline of mitochondrial membrane potential was prevented by GSH or the methyl donors methionine or betaine (Table 3A). As shown in Table 3A, fructose and xylitol (glycolytic ATP generators) or glutamine (a mitochondrial ATP generator) only delayed the chromate induced cytotoxicity, "ROS" formation and lipid peroxidation while they completely protected hepatocytes against arsenite. The metal chelator dimercaptosuccinic acid (Aaseth et al., 1981; Chattopadhyay et al., 2002) also prevented both chromate and arsenite induced cytotoxicity, "ROS" formation and lipid peroxidation (Table 3A). The dithiol

Table 2
Mitochondrial membrane potential changes during chromate vs. arsenite induced hepatocyte injury

Addition	"$\Delta\Psi_m$ (%)"		
	Incubation time		
	5 min	15 min	60 min
None	2 ± 1	3 ± 1	4 ± 1
Dichromate (1 mM)	44 ± 4[a]	52 ± 5[a]	65 ± 7[a]
+Catalase (200 u/ml)	11 ± 1[b]	8 ± 1[b]	9 ± 1[b]
+Mannitol (50 mM)	12 ± 2[b]	8 ± 1[b]	6 ± 1[b]
+DMSO (150 μM)	11 ± 2[b]	12 ± 2[b]	10 ± 1[b]
+Carnitine (2 mM)	31 ± 3[b]	30 ± 3[b]	39 ± 4[b]
+Cyclosporine (2 μM)	32 ± 3[b]	32 ± 3[b]	36 ± 3[b]
+Trifluoprazine (15 μM)	31 ± 2[b]	32 ± 2[b]	31 ± 2[b]
+GSH (2 mM)	42 ± 3	46 ± 4	54 ± 4
+Methionine (1 mM)	41 ± 3	48 ± 5	58 ± 5
+Betaine (2 mM)	41 ± 4	48 ± 5	58 ± 5
+Fructose (10 mM)	41 ± 4	48 ± 5	62 ± 5
+Xylitol (10 mM)	41 ± 3	51 ± 5	58 ± 5
+L-glutamine (1 mM)	42 ± 3	44 ± 4	52 ± 4
Arsenite (50 μM)	51 ± 4[a]	62 ± 5[a]	77 ± 7[a]
+Catalase (200 u/ml)	45 ± 4	58 ± 1	72 ± 1
+Mannitol (50 mM)	46 ± 5	58 ± 1	66 ± 1
+DMSO (150 μM)	44 ± 4	57 ± 2	70 ± 1
+Carnitine (2 mM)	11 ± 1[b]	10 ± 1[b]	9 ± 1[b]
+Cyclosporine (2 μM)	12 ± 2[b]	12 ± 2[b]	6 ± 1[b]
+Trifluoprazine (15 μM)	11 ± 2[b]	12 ± 2[b]	11 ± 2[b]
+GSH (2 mM)	12 ± 1[b]	13 ± 1[b]	14 ± 1[b]
+Methionine (1 mM)	11 ± 2[b]	14 ± 1[b]	18 ± 2[b]
+Betaine (2 mM)	10 ± 2[b]	14 ± 1[b]	16 ± 2[b]
+Folic acid (100 μM)	9 ± 1[b]	12 ± 2[b]	17 ± 2[b]
+Methylcobalamine (4 μM)	11 ± 1[b]	12 ± 1[b]	16 ± 2[b]
+Fructose (10 mM)	11 ± 2[b]	13 ± 1[b]	15 ± 2[b]
+Xylitol (10 mM)	11 ± 1[b]	11 ± 1[b]	18 ± 2[b]
+L-glutamine (1 mM)	8 ± 1[b]	11 ± 1[b]	12 ± 1[b]
+Dithiothreitol (1 mM)	10 ± 1[b]	12 ± 1[b]	15 ± 2[b]
GSH depleted hepatocytes	6 ± 1	6 ± 1	7 ± 1
+*Arsenite* (50 μM)	61 ± 2[a]	78 ± 1[a]	88 ± 2[a]
+*Dichromate* (1 mM)	11 ± 2	18 ± 1	18 ± 2

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C. Mitochondrial membrane potential was determined as the difference in mitochondrial uptake of the rhodamine 123 between control and treated cells and expressed as fluorescence intensity unit (Andersson et al., 1987). GSH depleted hepatocytes were prepared as described by Khan and O'Brien, 1991.Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Table 3A
Preventing metal induced hepatocyte necrosis by ATP generators and MPT pore sealing agents and methyl donors

Addition	Cytotoxicity (%) 3 h	"DCF" 3 h	"TBARS" 3 h
None	20 ± 2	79 ± 5	0.41 ± 0.05
Dichromate (1 mM)	76 ± 8[a]	508 ± 6[a]	20.62 ± 2.28[a]
+Fructose (10 mM)	55 ± 5[b]	389 ± 5[b]	12.35 ± 1.25[b]
+Xylitol (10 mM)	55 ± 5[b]	409 ± 5[b]	13.35 ± 0.65[b]
+L-glutamine (1 mM)	51 ± 5[b]	348 ± 5[b]	10.35 ± 1.15[b]
+Dimercaptosuccinic acid (100 μM)	47 ± 4[b]	135 ± 6[b]	6.37 ± 1.26[b]
+Dithiothreitol (1 mM)	65 ± 5[b]	429 ± 5[b]	16.35 ± 0.85[b]
+Methionine (1 mM)	75 ± 8	515 ± 6	20.82 ± 1.05
+Betaine (2 mM)	77 ± 8	510 ± 6	20.66 ± 0.84
+Carnitine (2 mM)	43 ± 6[b]	371 ± 6[b]	15.18 ± 1.98[b]
+Cyclosporine (2 μM)	46 ± 8[b]	405 ± 6[b]	16.42 ± 1.47[b]
+Trifluoprazine (15 μM)	45 ± 5[b]	429 ± 5[b]	16.35 ± 1.15[b]
Arsenite (50 μM)	76 ± 8[a]	165 ± 6[a]	2.62 ± 0.98[a]
+Fructose (10 mM)	35 ± 5[b]	129 ± 5[b]	1.35 ± 1.15[b]
+Xylitol (10 mM)	35 ± 5[b]	109 ± 5[b]	1.35 ± 0.65[b]
+L-glutamine (1 mM)	33 ± 5[b]	101 ± 3[b]	1.15 ± 0.11[b]
+Dimercaptosuccinic acid (100 μM)	37 ± 4[b]	105 ± 6[b]	1.37 ± 1.26[b]
+Dithiothreitol (1 mM)	35 ± 5[b]	129 ± 5[b]	2.35 ± 0.85[b]
+Methionine (1 mM)	33 ± 3[b]	99 ± 5[b]	1.25 ± 0.15[b]
+Betaine (2 mM)	37 ± 4[b]	103 ± 5[b]	1.31 ± 0.09[b]
+Folic acid (100 μM)	36 ± 2[b]	104 ± 5[b]	1.17 ± 0.11[b]
+Methylcobalamine (4 μM)	36 ± 3[b]	101 ± 5[b]	1.25 ± 0.16[b]
+Carnitine (2 mM)	33 ± 6[b]	111 ± 6[b]	1.18 ± 1.08[b]
+Cyclosporin(2 μM)	36 ± 8[b]	105 ± 6[b]	1.42 ± 1.07[b]
+Trifluoprazine (15 μM)	35 ± 5[b]	109 ± 5[b]	1.35 ± 1.05[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of potassium dichromate and sodium arsenite. Cytotoxicity was determined as the percentage of cells that take up trypan blue (Pourahmad and O'Brien, 2000a). DCF formation was expressed as fluorescent intensity units (Shen et al., 1996). GSH depleted hepatocytes were prepared as described by Khan and O'Brien, 1991. TBARS formation was expressed as μM concentrations (Smith et al., 1982). Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

agent dithiothreitol (Nakagawa et al., 1992) only protected hepatocytes against arsenite but not chromate induced cytotoxicity (Table 3A), suggesting that thiol activity of arsenite may be responsible for thiol groups complexation in the mitochondrial membrane pore region and consequent pore opening associated with outer membrane potential decrease. Dithiothreitol did not show any toxic effect on hepatocytes at concentrations used (data not shown).

On the other hand, as shown in Table 3B, depleting hepatocyte methyl groups beforehand by hypomethylators such as azacytidine and butyric acid (Rojas et al., 1999), and also inactivating hepatocyte methionine synthase or methyl transferase (both enzymes are involved in biomethylation) by hydrazine monohydrate and sodium selenite, respectively, (Kenyon et al., 1999; Styblo and Thomas, 2001) significantly increased arsenite induced cytotoxicity, "ROS" formation and collapse of mitochondrial membrane potential. All of these agents including hypomethylators and enzyme inhibitors did not show any toxic effect on hepatocytes at concentrations used (data not shown).

Endocytosis inhibitors (Brunk et al., 1995; Luiken et al., 1996; Gaynor et al., 1998) chloroquine, methylamine and monensin prevented chromate but not arsenite induced hepatocyte toxicity, "ROS" and TBARS generation (Table 4). All of these agents did not show any toxic effect on hepatocytes at concentrations used (data not shown).

Table 3B
Effect of methylation on arsenite induced hepatocyte toxicity

Addition	Cytotoxicity (%) 3 h	"DCF" 3 h	"$\Delta\Psi_m$ (%)" 1 h
None	20 ± 2	79 ± 5	4 ± 1
Arsenite (50 μM)	76 ± 8[a]	165 ± 6[a]	77 ± 7[a]
+Azacytidine (2.5 μM)	93 ± 3[b]	220 ± 5[b]	96 ± 5[b]
+Butyric acid (1 mM)	90 ± 4[b]	212 ± 5[b]	92 ± 5[b]
+Sodium selenite(4 μM)	97 ± 6[b]	221 ± 7[b]	100[b]
+Hydrazine (8 mM)	100[b]	230 ± 7[b]	100[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of sodium arsenite. Cytotoxicity was determined as the percentage of cells that take up trypan blue (Pourahmad and O'Brien, 2000a). Mitochondrial membrane potential was determined as the difference in mitochondrial uptake of the rhodamine 123 between control and treated cells and expressed as fluorescence intensity unit (Andersson et al., 1987). DCF formation was expressed as fluorescent intensity units (Shen et al., 1996). Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Table 4
Preventing chromate but not arsenite induced hepatocyte necrosis by endocytosis inhibitors

Addition	Cytotoxicity (%) 3 h	"DCF" 3 h	"TBARS" 3 h
None	20 ± 2	79 ± 5	0.41 ± 0.05
Dichromate (1 mM)	76 ± 8[a]	508 ± 6[a]	20.62 ± 2.28[a]
+Monensin (10 μM)	35 ± 5[b]	129 ± 5[b]	4.35 ± 1.15[b]
+Methylamine (30 mM)	35 ± 5[b]	125 ± 7[b]	4.31 ± 1.85[b]
+Chloroquine (100 μM)	45 ± 4[b]	137 ± 8[b]	4.45 ± 1.14[b]
Arsenite (50 μM)	76 ± 8[a]	165 ± 6[a]	2.62 ± 0.98[a]
+Monensin (10 μM)	75 ± 5	169 ± 5	2.35 ± 1.15
+Methylamine (30 mM)	75 ± 5	165 ± 7	2.31 ± 1.85
+Chloroquine (100 μM)	75 ± 4	167 ± 8	2.45 ± 1.14

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of potassium dichromate and sodium arsenite. Cytotoxicity was determined as the percentage of cells that take up trypan blue (Pourahmad and O'Brien, 2000a). DCF formation was expressed as fluorescent intensity units (Shen et al., 1996). GSH depleted hepatocytes were prepared as described by Khan and O'Brien, 1991. TBARS formation was expressed as μM concentrations (Smith et al., 1982). Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Furthermore, when hepatocyte lysosomes were loaded with acridine orange, a significant release of acridine orange into the cytosolic fraction ensued within 60 min if the loaded hepatocytes were treated with chromate but not with arsenite (Table 5). The chromate induced acridine orange release was prevented by "ROS" scavengers dimethylsulfoxide, mannitol, catalase or superoxide dismutase as well as autophagy inhibitor 3-methyladenine (Ege et al., 1984; Pourahmad et al., 2003) (Table 5).

Hepatocyte proteolysis as determined by the release of the amino acid tyrosine into the extracellular medium over 120 min was markedly increased when hepatocytes were incubated with chromate but not arsenite (Table 6). The chromate induced tyrosine release was completely prevented by the lysosomal protease inhibitors (Fengsrud et al., 1995; Olav et al., 1998) leupeptin and pepstatin, dimethylsulfoxide, mannitol, catalase or superoxide dismutase, deferoxamine. Phenylimidazole, diphenyliodonium chloride, 3-methyladenine, methylamine and chloroquine also inhibited chromate induced tyrosine release (Table 6). All of these agents did not show any toxic effect on hepatocytes at concentrations used (data not shown).

4. Discussion

We have previously shown that addition of Cr (VI) to isolated rat hepatocytes results in rapid glutathione oxidation, reactive oxygen species formation, lipid peroxidation, decreased mitochondrial membrane potential and lysosomal membrane rupture before hepatocyte lysis occurred (Pourahmad and O'Brien, 2001; Pourahmad et al. 2001b). Reduction of dichromate by glutathione or cysteine in vitro was also accompanied by oxygen uptake and was inhibited by Mn II (a Cr (IV) reductant) (Pourahmad and O'Brien, 2001; Pourahmad

Table 5
Lysosomal membrane integrity changes during chromate vs. arsenite induced hepatocyte injury

Addition	(Acridine orange redistribution)		
	Incubation time		
	15 min	30 min	60 min
None	3 ± 1	4 ± 1	4 ± 1
Dichromate (1 mM)	39 ± 4[a]	68 ± 7[a]	395 ± 8[a]
+Catalase (200 u/ml)	3 ± 1[b]	3 ± 1[b]	35 ± 4[b]
+SOD (100 u/ml)	6 ± 1[b]	7 ± 1[b]	71 ± 6[b]
+Dimethyl sulfoxide (150 μM)	3 ± 1[b]	4 ± 1[b]	47 ± 4[b]
+Mannitol (50 mM)	6 ± 1[b]	5 ± 1[b]	28 ± 4[b]
+*Arsenite* (50 μM)	3 ± 1	6 ± 1	7 ± 1
+Catalase (200 u/ml)	3 ± 1	3 ± 1	5 ± 1
+SOD (100 u/ml)	4 ± 1	4 ± 1	6 ± 1
+Dimethyl sulfoxide (150 μM)	3 ± 1	4 ± 1	7 ± 1
+Mannitol (50 mM)	3 ± 1	5 ± 1	6 ± 1

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C. Lysosomal membrane damage was determined as intensity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosomes (adapted from Brunk et al., 1995). Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Table 6
Preventing chromate induced hepatocyte proteolysis with inhibitors of oxidative stress lysosomotropic agents and lysosomal protease inhibitors

Addition	Hepatocyte tyrosine release (μM)		
	30 min	60 min	120 min
None	13 ± 1	15 ± 1	21 ± 2
Arsenite (50 μM)	12 ± 2	14 ± 2	21 ± 2
Dichromate (1 mM)	55 ± 4[a]	78 ± 6[a]	115 ± 7[a]
+Catalase (200 u/ml)	15 ± 2[b]	17 ± 2[b]	27 ± 3[b]
+SOD (100 u/ml)	14 ± 2[b]	17 ± 2[b]	23 ± 2[b]
+Dimethyl sulfoxide (150 mM)	15 ± 2[b]	19 ± 2[b]	28 ± 3[b]
+Mannitol (50 mM)	14 ± 2[b]	16 ± 2[b]	20 ± 2[b]
+Desferal (200 μM)	12 ± 2[b]	14 ± 2[b]	20 ± 2[b]
+Monensin (10 μM)	7 ± 1[b]	14 ± 2[b]	15 ± 1[b]
+Methylamine (30 mM)	10 ± 1[b]	15 ± 3[b]	16 ± 2[b]
+Chloroquine (100 μM)	11 ± 1[b]	18 ± 5[b]	19 ± 7[b]
+3-Methyladenine (30 mM)	12 ± 2[b]	12 ± 2[b]	10 ± 2[b]
+Leupeptin (100 μM)	12 ± 2[b]	12 ± 2[b]	17 ± 2[b]
+Pepstatin (100 μM)	12 ± 2[b]	13 ± 2[b]	18 ± 2[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C. Lysosomal induced proteolysis was determined by measuring the cellular release of tyrosine into the media. Values are expressed as means of three separate experiments (S.D.).

[a] Significant difference in comparison with control hepatocytes ($P < 0.001$).

[b] Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

et al. 2001b). It was proposed that Cr (VI) and Cr (IV.GSH) mediated ROS formation in isolated hepatocytes as Cr (VI) induced cytotoxicity and ROS formation was inhibited by Mn II (Pourahmad and O'Brien, 2001).

On the other hand, metabolic reduction is likely a detoxification process for arsenite as dimethylarsinic acid, the major metabolite, was more readily excreted in the urine and less bound to tissue proteins than inorganic arsenate. Dimethylarsinic acid was also much less effective than arsenate as a promotor of hepatocarcinogenesis (Zakharian et al., 1996), mutagen (Moore et al., 1997) or a clastogen (Oya-Ohta et al., 1996).

The liver is a major site for arsenite methylation, and this capacity varies as much as 10-fold among individuals (Styblo et al., 1999). The results presented here suggest that in intact hepatocytes, arsenite is mostly reduced by GSH and methyl donors (e.g. methionine, betaine and folic acid).

Both arsenite and chromate induced a rapid decline of mitochondrial membrane potential (within 5 min). The Cr (VI) induced decline of mitochondrial membrane potential was prevented by catalase, dimethyl sulfoxide, mannitol or by prior depletion of hepatocyte GSH, which indicates that the chromate induced decline of mitochondrial membrane potential was a consequence of “ROS” formation and reductive activation of Cr (VI). Furthermore, the ATP generators fructose, xylitol and L-glutamine (a mitochondrial ATP generator) or dithiol agent dithiothreitol prevented arsenite induced mitochondrial membrane potential decrease (Table 2), which indicates that the collapse may be a consequence of mitochondrial permeability transition (MPT) pore opening and ATP depletion following the thiol cross linking of the pore region. Lack of mitochondrial ATP results in intracellular acidosis and

osmotic injury, which leads to plasma membrane lysis (Pourahmad and O'Brien, 2000b).

We have already demonstrated that chromate was reduced by GSH (Pourahmad and O'Brien, 2001). In this process, thiyl radicals were formed (Liu et al., 1997) and Cr (IV)–GSH complex reacted with O_2 to form hydroxyl radicals. (Pourahmad and O'Brien, 2001). Incubating isolated rat hepatocytes with 1 mM Cr (VI) inactivated more than 40% GSH reductase in an hour (Ueno et al., 1989), and oxidized more than 85% GSH in just 5 min (Pourahmad and O'Brien, 2001). Interestingly, the rapid sequestration and reduction of intracellular Cr (V1) by GSH may increase the entry of Cr (VI) into the cells as GSH depleted cells accumulated less Cr (VI) (Sugiyama and Tsuzuki, 1994).

GSH also forms a (GS)3As(III) complex with arsenite which is involved in the biliary excretion of arsenic (Gyurasics et al., 1991), but can donate As (III) to dithiol containing molecules (Zakharyan et al., 1999). In the cell, GSH may play an important role in the mono- and bimethylation of inorganic arsenic by methylvitamin B_{12} (Zakharyan and Aposhian, 1999) or by arsenic methyltransferases and S-adenosylmethionine. (GS)3As(III) is also a substrate for the latter methylation system (Ghosh et al., 1999). In our study, arsenite depleted about 38% of hepatocyte GSH in 1 h and 45% in 2 h without showing any significant change in hepatocyte GSSG (oxidized form of GSH) content (data not shown).

The results presented here showed that GSH depleted hepatocytes were much more resistant to Cr (VI) toxicity than control hepatocytes with normal GSH levels, and much less dichlorofluorescin oxidation and mitochondrial membrane potential decrease occurred. Nevertheless, GSH depleted hepatocytes were highly sensitive to arsenite toxicity, and much higher dichlorofluorescin oxidation and mitochondrial membrane potential decrease occurred.

Chromate but not arsenite induced cytotoxicity as well as hepatocyte "ROS" formation and lipid peroxidation were prevented by the hepatocyte lysosomotropic agents methylamine, chloroquine or monensin, a Na^+ ionophore that inhibits hepatocyte endocytosis and endosomal acidification (Table 4). In addition, when hepatocyte lysosomes were loaded with acridine orange (a lysosomotropic flourescent probe), a significant release of acridine orange into the cytosolic fraction ensued within 60 min if the loaded hepatocytes were treated with chromate but not arsenite, suggesting a lysosomal membrane damage caused by chromate (Table 5). We have also already shown that chromate overload in rat hepatocytes increased lysosomal fragility and leakiness likely as a result of formation of intracellular H_2O_2 that reacts with lysosomal Fe^{3+} to form "ROS" which damages the lysosomal membrane (Pourahmad et al., 2003). The hepatocyte lysosomal protease inhibitors leupeptin or pepstatin prevented chromate induced cytotoxicity and hepatocyte proteolysis. This finding is further evidence that chromate induced hepatocyte injury involves lysosomal membrane damage and release of proteolytic enzymes.

In conclusion, although arsenite has poor redox properties and oxidizing effects, it is more cytotoxic than chromate likely because arsenite is more effective than chromate at decreasing hepatocyte mitochondrial membrane potential even though it induces much less "ROS" formation. Considering our findings that arsenite induced cytotoxicity, mitochondrial membrane potential decline and also ROS formation were significantly increased by inactivating hepatocyte methionine synthase or hepatocyte methyl transferase, but were prevented by methyl donors such as methionine or betaine, we suggest that arsenite detoxification pathway in mammalian cells is surely metabolic methylation. The results presented also suggest that chromate causes the leakiness of the lysosomal membrane that may lead to the release of lysosomal proteases/phospholipases. The release of this deadly digestive lysosomal enzyme could then be the ultimate cause of the induction of cell death process.

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