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INVOLVEMENT OF METABOLIC REACTIVE INTERMEDIATE Cr (IV) IN CHROMIUM (VI) CYTOTOXIC EFFECTS

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ABSTRACT

Addition of Cr VI (dichromate) to isolated rat hepatocytes results in rapid glutathione oxidation, reactive oxygen species (ROS) formation, lipid peroxidation, decreased mitochondrial membrane potential and lysosomal membrane rupture before hepatocyte lysis occurred. Cytotoxicity was prevented by ROS scavengers, antioxidants, and glutamine (ATP generator). Hepatocyte dichlorofluorescin oxidation to dichlorofluorescein (DCF) to determine ROS formation was inhibited by mannitol (a hydroxyl radical scavenger) or butylated hydroxyanisole and butylated hydroxytoluene (antioxidants). The Cr VI reductive mechanism required for toxicity is not known. Cytochrome P450 inhibitors, particularly CYP 2E1 inhibitors, but not inhibitors of DT diaphorase or glutathione reductase also prevented cytotoxicity. This suggests that P450 reductase and/or reduced cytochrome P450 contributes to Cr VI reduction to Cr IV. Glutathione depleted hepatocytes were resistant to Cr (VI) toxicity and much less dichlorofluorescin oxidation occurred. 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). Cr VI induced cytotoxicity and ROS formation was also inhibited by Mn II, which suggests that, Cr IV and Cr IV GSH mediate "ROS" formation in isolated hepatocytes. In conclusion Cr VI cytotoxicity is associated with mitochondrial/lysosomal toxicity by the metabolic reactive intermediate Cr IV and "ROS".

Key words: Chromium, Hepatocytes, Reactive intermediate, Cytotoxicity

Abbreviations: ANOVA, analysis of variance; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazine- ethanesulfonic acid; MDA, malondialdehyde; "ROS", Reactive oxygen species; rpm, Rotations per minute; SD, Standard deviation; SOD, Superoxide dismutase.

INTRODUCTION

Hexavalent chromium is recognized as an environmental and occupational carcinogen and causes DNA damage both in vivo and in vitro (1). Airborne exposure to chromate occurs in the chromate production industry, during leather tanning, chrome plating and welding and leads to increased incidence of lung cancer (1). Dermal exposure to chromate occurs in workers who handle paints, dyes, inks and detergents, and contamination of land by disposal of industrial chromate waste has increased exposure of the general public. The recognition that chromium is carcinogenic, has led to concerns about the risk associated with the leaching of chromate from the alloys used in orthopedic implants eg. joint replacement.

Human Cr VI intoxication is also associated with hepatotoxicity, nephrotoxicity and cardiotoxicity whereas systemic Cr VI exposure of animals causes toxicity to the liver, kidneys, myocardium, testes and blood vessels. The liver is the main organ for chromium VI metabolic reduction and therefore one of the most important sites for its toxicity (2), but the intracellular reductive pathway or the biological reactive chromium or oxygen intermediates responsible are not known.

MATERIALS AND METHODS

Chemicals:

1-Bromoheptane and rhodamine 123 were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Collagenase (from Clostridium histolyticum) and Hepes were purchased from Boehringer-Mannheim (Montreal, Canada). Trypan blue, potassium dichromate, d-mannitol, dimethyl sulfoxide, catalase, superoxide dismutase, chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, carnitine, cyclosporine, trifluprazine,

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magnesium chloride, diphenyliodonium chloride, dicumarol, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), butylated hydroxyanisole, butylated hydroxytoluene, thiobarbituric acid, trichloroacetic acid (TCA), sodium pentobarbital and heparin were obtained from Sigma (St. Louis, MO, USA). Acridine orange and dichlorofluorescin diacetate was purchased from Molecular Probes (Eugene, Ore, USA). All chemicals were of the highest commercial grade available.

Isolation of Hepatocytes:
Male Sprague-Dawley rats (280-300g), fed a standard chow diet and given water ad libitum, were used in all experiments. Hepatocytes were obtained by collagenase perfusion of the liver and their viability was assessed by the trypan blue (0.2% w/v) exclusion test as described previously (3). 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% 0_2: 85% N_2: 5% $C0_2$.

Mitochondrial membrane potential assay:
The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential (4). Aliquots of the cell suspension (0.5 ml) were separated from the incubation medium by centrifugation at 1000 rpm for 1 minute. 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.

Microscopic determination of lysosomal membrane integrity:
Hepatocytes were attached to glass coverslips and stained with 5 μg/ml acridine orange (AO) for 15 min at 37°C. The hepatocytes were then rinsed with buffer and Cu^{2+} (50 μM) or Cd^{2+} (20 μM) were added. The AO-induced granular red (lysosomal) fluorescence from 50 cells in each experimental group was then measured at 10 minute time intervals on a Nikon microphote-SA fluorescence microscope (Nikon, Tokyo, Japan) using green excitation light (Nikon G-cube) in combination with an extra 630 nm barrier filter (5).

Lysosomal membrane stability assay:
Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange [adapted from 5]. 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 minute 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.

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 hydrolyzed to non-fluorescent dichlorofluorescin. The latter then reacts with "ROS" to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. Hepatocytes (1×10^6 cells/ml) were suspended in 10 ml modified Hank's balanced salt solution (HBS), adjusted to pH 7.4 with 10 mM Hepes (HBSH) and were incubated with Cu^{2+} or Cd^{2+} 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 1.6μM 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 500nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (6).

Lipid peroxidation:
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 532nm 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 (7).

Statistical analysis:

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

RESULTS

Dichromate (Cr VI) induced cytotoxicity toward isolated rat hepatocytes increased with time and was dose dependent with approximately 50% cell lysis occurring in 2 hours at a concentration of 1 mM. As shown in table 1, dichromate toxicity was associated with the generation of a large amount of reactive oxygen species "ROS" and malondialdehyde (MDA). Cr VI induced cytotoxicity; MDA and "ROS" generation was prevented by the antioxidants butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA). Hydroxyl radical scavengers, mannitol and DMSO, and the Cr IV reductant, Mn II also protected the hepatocytes.

Dichromate reduction is required for oxygen activation but the intracellular reductive system has not yet been identified. Table 1 shows that the DT-diaphorase inhibitor, dicumarol or the glutathione reductase inhibitor, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) did not show any significant effect on Cr VI induced cytotoxic alterations. However, the CYP2E1 inhibitor, phenylimidazole and the P450 reductase inhibitor, diphenyliodonium chloride (DPI) protected the hepatocyte against Cr VI induced cytotoxicity and lipid peroxidation although they both significantly increased DCF formation. 1 mM Cr VI oxidized 85% hepatocyte GSH in about 5 mins and the GSSG formed effluxed the cell (data not shown). Dichromate reduction by GSH or cysteine in vitro was accompanied by oxygen uptake and ROS formation and both were inhibited by Mn II (data not shown). Depletion of hepatocyte GSH beforehand prevented Cr VI induced hepatocyte toxicity, "ROS" and MDA generation (table 1).

As demonstrated in table 2, Cr VI decreased the hepatocyte mitochondrial membrane potential by 44% within 5 mins of Cr VI addition and further decreased by 65% in 1 hr. This was prevented by addition of catalase, mannitol or dimethyl sulfoxide or the Cr IV reductant Mn II. Mitochondrial permeability transition (MPT) inhibitors, carnitine, cyclosporin and trifluoperazine also prevented the Cr VI induced decrease in mitochondrial membrane potential. No significant decrease in hepatocyte mitochondrial membrane potential was observed when 1 mM Cr VI was incubated with GSH depleted hepatocytes. When hepatocyte lysosomes were loaded with acridine orange, a massive release of acridine orange into the cytosolic fraction ensued within 60 minutes when the loaded hepatocytes were treated with Cr VI indicating leakiness of the lysosomal membranes (table 3). The Cr VI induced acridine orange release was prevented by the "ROS" scavengers dimethylsulfoxide, mannitol or catalase, superoxide dismutase or when Cr VI was incubated with GSH depleted hepatocytes. Endocytosis inhibitors including lysosomotropic agents, chloroquine and methylamine, Na^+ ionophore, monensin and the endocytosis inhibitor, 3-methyladenine also protected the hepatocytes (table 1).

DISCUSSION

Intracellular reduction appears to be critical for the expression of DNA damage and toxicity (8). Enzymatic reduction by glutathione reductase (9), DT-diaphorase (10), NADPH-cytochrome c reductase, cytochrome P450 dependent systems or GSH and cysteine have been proposed (11). The results presented here suggest that in intact hepatocytes, Cr VI is mostly reduced by CYP2E1 and/or P450 reductase. GSH was depleted rapidly during the metabolism of Cr VI. Of particular interest is that even though GSH is an intracellular antioxidant which prevents intracellular "ROS" formation and lipid peroxidation, depleting GSH beforehand protected the cells against Cr VI induced cytotoxicity and "ROS" or MDA formation. GSH could be required for cytotoxicity as Liu et al., 1997 (12) have reported the formation of "ROS" arising from Cr IV-GSH formation when GSH is added to dichromate (Fig. 1).

Table 1: Preventing metal induced hepatocyte necrosis by inhibiting "ROS" formation and lipid peroxidation

Addition	%Cytotoxicity 3h	"DCF" 3h	MDA 3h
None	20±2	79±5	0.41±0.05
Dichromate (1 mM)	76±8 [a]	508±6 [a]	20.62±2.28 [a]
+Butylated hydroxyanisole (50 μM)	35±5 [b]	129±5 [b]	4.35±1.15 [b]
+Butylated hydroxytoluene (50 μM)	35±3 [b]	125±7 [b]	5.31±1.85 [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]
+Mn (II) (2 mM)	35±3 [b]	129±7 [b]	4.35±0.85 [b]
+Phenylimidazole (300 μM)	37±4 [b]	635±6 [b]	4.37±1.26 [b]
+Diphenyliodonium Chloride (50 μM)	35±5	629±5 [b]	4.35±0.85 [b]
+BCNU (50 μM)	73±6	511±6	20.18±1.98
+Dicumarol (30 μM)	76±8	505±6	20.42±1.47
+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]
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]

Hepatocytes (10^9 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37C for 3.0 hrs following the addition of potassium dichromate. Cytotoxicity was determined as the percentage of cells that take up trypan blue (3). DCF formation was expressed as fluorescent intensity units (6). GSH depleted hepatocytes were prepared as described by Khan and O'Brien, 1991 (19). MDA formation was expressed as μM concentrations (7). Values are expressed as means of three separate experiments (SD). a: Significant difference in comparison with control hepatocytes ($P < 0.001$). b: Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Table 2: Mitochondrial membrane potential changes during chromate induced hepatocyte injury

Addition (Incubation Time)	(% of decrease) 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]
+Dimethyl sulfoxide (150 μM)	11±2 [b]	12±2 [b]	10±1 [b]
+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]
+Mn (II) (2 mM)	10±1 [b]	9±1 [b]	8±1 [b]
GSH depleted hepatocytes	2±1	3±1	4±1
+Dichromate (1 mM)	11±2	8±1	8±2

Hepatocytes (106 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37C. 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 (4). 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 ($P < 0.001$)

Reduction of dichromate by glutathione in vitro was accompanied by oxygen uptake and ROS formation and both were inhibited by Mn II. Mn II has been shown to be a Cr IV reductant (13).

As shown in Fig. 1, the oxygen uptake was due to Cr IV or Cr IV.GSH reacting with O_2 to form "ROS" (14, 12). Cr VI induced cytotoxicity and ROS formation, lipid peroxidation, mitochondrial membrane potential collapse and lysosomal membrane damage were also inhibited by Mn II which suggests that Cr IV or Cr IV.GSH mediated ROS formation and lipid peroxidation contributed to mitochondrial and lysosomal damage.

Table 3: Preventing chromate induced hepatocyte lysosomal membrane damage by inhibitors of oxidative stress and autophagy

Addition	(Acridine orange redistribution)		
	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]
+3-Methyladenine (5mM)	5±1[b]	8±1[b]	40±5[b]
+Mn (2 mM)	6±[b]	7±1[b]	38±4[b]
GSH depleted hepatocytes	3±1	4±1	15±3
+Dichromate (1 mM)	9±4[b]	9±6[b]	41±8[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°. Lysosomal membrane damage was determined as intensity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosomes (Adapted from 5). Values are expressed as means of three separate experiments (SD). a: Significant difference in comparison with control hepatocytes ($P < 0.001$). b: Significant difference in comparison with metal treated hepatocytes ($P < 0.001$).

Phenylimidazole or P450 reductase inhibitors prevented Cr VI induced cytotoxicity and lipid peroxidation but not DCF oxidation. However GSH oxidation and mitochondrial toxicity occurred more rapidly than DCF oxidation or lipid peroxidation. One explanation is that DCF is oxidized by Cr V (15) or lipid peroxidation rather than by "ROS". CYP2E1 or P450 reductase therefore reduces Cr V to Cr IV not to Cr III and finally inhibitors of CYP2E1 or P450 reductase would increase the concentration of Cr V. Cr VI induced a rapid decline of mitochondrial membrane potential (within 5 mins), which was prevented by catalase, dimethyl sulfoxide, mannitol or by prior depletion of hepatocyte GSH which indicates that the decline of mitochondrial membrane potential was a consequence of "ROS" formation and reductive activation of Cr VI. Collapse of the mitochondrial membrane potential results in opening of the mitochondrial permeability transition pore and causes severe mitochondrial ATP depletion. Lack of mitochondrial ATP results in intracellular acidosis and osmotic injury, which leads to plasma membrane lysis (3). Glutamine (a mitochondrial ATP generator) protected the cells against Cr VI induced cytotoxicity (data not shown).

Cr VI induced cytotoxicity as well as hepatocyte "ROS" formation and lipid peroxidation were prevented by the hepatocyte lysosomotropic agents methylamine (16), chloroquine (17), monensin, (a Na^+ ionophore that inhibits hepatocyte endocytosis and endosomal acidification (5) or 3-methyladenine, an inhibitor of hepatocyte autophagy (18). These results show that Cr VI overload in rat hepatocytes increases lysosomal fragility and leakiness likely as a result of lysosomal lipid peroxidation or "ROS" formation, which contributes to cell death.

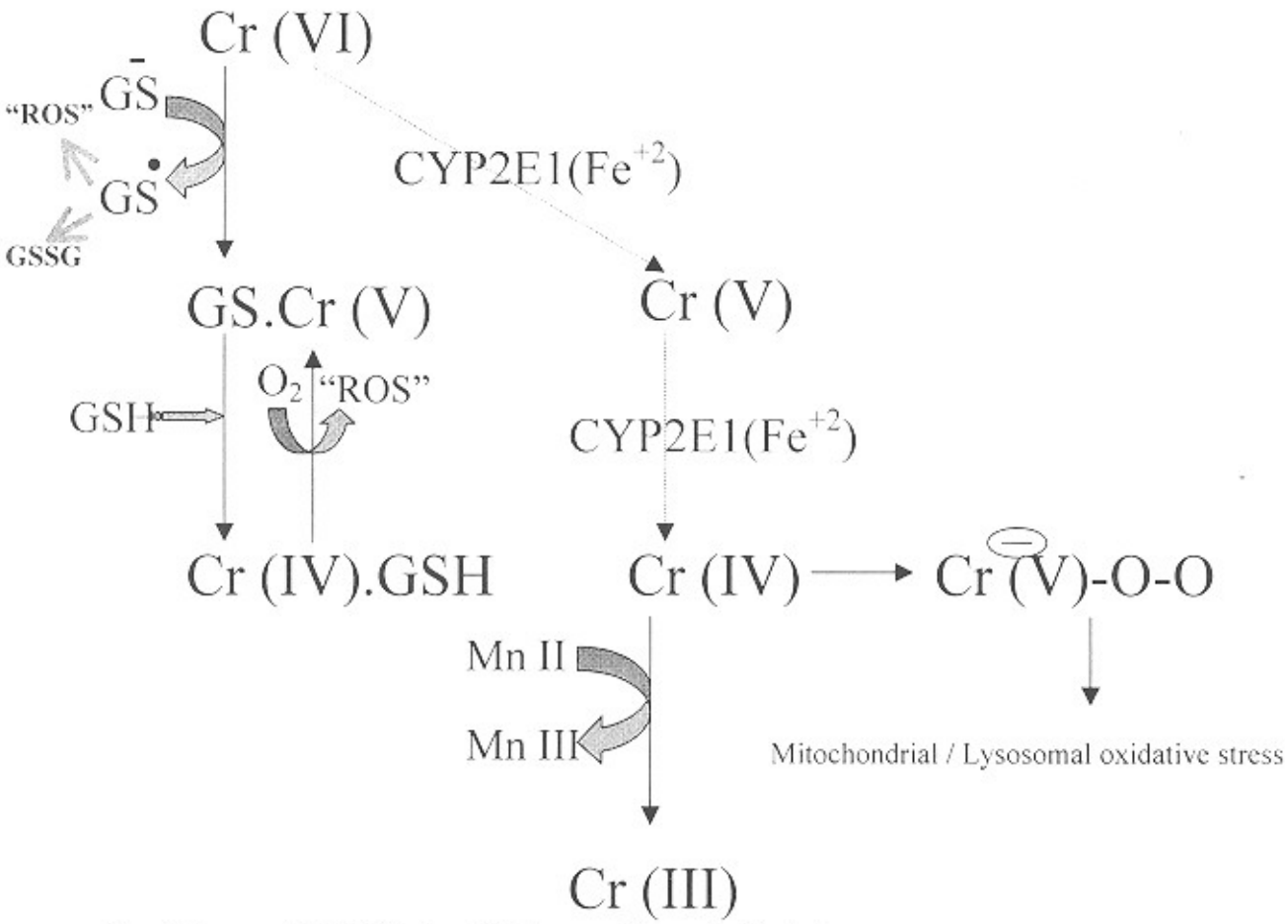

Fig. 1. Proposed "ROS" Mediated Dichromate Cytotoxic Mechanism

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