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Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes

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Abstract

Severe chronic liver disease results from the hepatic accumulation of copper nickel, cobalt or iron in humans and on the other hand cadmium, dichromate and arsenic may induce lung or kidney cancer. Acute or chronic $CdCl_2$, $HgCl_2$ or dichromate administration induces hepatic and nephrotoxicity in rodents. Oxidative stress is often cited as a possible cause but has not yet been measured. For the first time we have measured the reactive oxygen species (ROS) formation induced when cells are incubated with metals and determined its source. Hepatocytes incubated with 2′,7′-dichlorofluorescin diacetate resulted in its rapid uptake and deacetylation by intracellular esterases to form 2′,7′-dichlorofluorescin. A marked increase in ROS formation occurred with LD_{50} concentrations of cadmium [Cd(II)], Hg(II) or arsenite [As(III)] which was released by proton ionophores that uncouple oxidative phosphorylation. Uncouplers or oxidative phosphorylation also inhibited ROS formation induced by these metals, which suggests that mitochondria are major contributors to endogenous ROS formation. Glycolytic substrates also inhibited Cd(II)/Hg(II)/As(III)-induced ROS formation and confirms that mitochondria are the site of ROS formation. By contrast ROS formation by LD_{50} concentrations of Cu(II), Ni(II), Co(II) or dichromate [Cr(VI)] were not affected by uncouplers or glycolytic substrates. However they were inhibited by lysosomotropic agents or endogenous inhibitors [in contrast to Hg(II), Cd(II) or As(III)]. Furthermore Cu(II), Ni(II), Co(II) or Cr(VI) accumulated in the lysosomes and the ROS formed caused a loss of lysosomal membrane integrity. The release of lysosomal proteases and phospholipases also contributed to hepatocyte cytotoxicity. ROS formation and cytotoxicity induced by added H_2O_2 or generated by the intracellular redox cycling of nitrofurantoin was also inhibited by lysosomotropic agents and ferric chelators suggesting that lysosomal Fe(II) contributes to H_2O_2-induced cytotoxicity. In conclusion, lysosomes are sites of cytotoxic ROS formation with redox transition metals (CuII, CrVI, NiII, CoII) whereas mitochondria are the ROS sites for non-redox or poor redox cycling transition metals (CdII, HgII, AsIII).

1. Introduction

As a class of toxic agents, metals are a concern of the highest priority for human exposure. Metals have a vast array of remarkably adverse effects, including those of carcinogenicity and hepatotoxicity. Metals are also non-biodegradable and persist in the environment. Anthropogenic use has led to global dispersion of metals in the environment. Because of their wide distribution and extensive use in modern society, some human exposure to toxic metals is inevitable. Metals are also unique environmental pollutants in that they are neither created or destroyed by humans but are only transported and transformed into various products which in turn directly or indirectly affect the growth and longevity of aquatic or terrestrial animals. Defining the mechanisms of metal carcinogenecity has been problematic because of the intricate nature of the interactions of metals with living systems.

Various metals induced hepatocyte "ROS" formation before cytotoxicity ensued. The comparative effectiveness of metals (at a cytotoxic dose) for inducing "ROS" formation was $CuCl_2 > K_2Cr_2O_7 > HgCl_2 > CdCl_2$ (Pourahmad and O'Brien, 2000a; Pourahmad et al., 2001a; Pourahmad and O'Brien, 2001). Furthermore the cytotoxicity induced by these metals was prevented by the hydroxyl radical scavengers dimethyl sulfoxide or mannitol (Pourahmad and O'Brien, 2000a; Pourahmad et al., 2001a; Pourahmad and O'Brien, 2001). It was also demonstrated that lysosomal lipid peroxidation preceded $CuCl_2$ or $K_2Cr_2O_7$ induced hepatocyte cyto-

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toxicity (Pourahmad et al., 2001b; Pourahmad and O'Brien, 2001).

The possible cellular sources of "ROS" production include plasma membrane NADPH oxidase and intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, mitochondrial electron transport components and lysosomal pool of Fe^{2+}/Cu^{+} which makes it susceptible for Haber–Weiss reaction with H_2O_2 generating agents. The two latter things are considered to be the major sources of "ROS" that have been implicated in a number of diseases and disorders (Skulachev, 1999).

The major objective of this study was to investigate the possible intracellular source of "ROS" formation for carcinogenic metals (CuII, CrVI, NiII, CoII, CdII, HgII, AsIII) which is likely one of their potential mechanisms at inducing carcinogenecity.

2. Materials and methods

2.1. Chemicals

Rhodamine 123 were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Collagenase (from Clostridium histolyticum) and Hepes were purchased from Roche (Montreal, Canada). Trypan blue, $CuCl_2$, $CdCl_2$, $HgCl_2$, $CoCl_2$, $NiCl_2$, potassium dichromate, sodium arsenite, d-mannitol, dimethyl sulfoxide, catalase, superoxide dismutase, chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, 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.

2.2. Isolation and incubation of hepatocytes

Male Sprague-Dawley rats (280–300 g) purchased from Institut Pasteur (Tehran, Iran), fed with a standard chow diet and water ad libitum, used for hepatocyte preparation. Isolation and incubation of hepatocytes were obtained by collagenase perfusion of the Sprague–Dawley rat liver. 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: 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 LD_{50} concentrations for the investigated metals in the isolated hepatocytes including; $CdCl_2$ (20 μM), $CuCl_2$ (50 μM), potassium dichromate (1 mM), $HgCl_2$ (20 μM), sodium atrsenite (50 μM), $NiCl_2$ (2 mM) and $CoCl_2$ (500 μM). The LD_{50} of a chemical in the hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period), is defined as the concentration which decreases the hepatocyte 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 LD_{50} was determined based on a regression plot of three different concentrations (data and curves not shown). To incubate each metal (all the mentioned 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 which 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). To measure cell viability, ROS or lipid peroxidation and also mitochondrial or lysosomal parameters, hepatocytes aliquots taken at different time points (as shown in all tables) during the 3 h incubation period. Our control flasks only contained hepatocyte suspension with no chemical incubation, and therefor the term "None" was used to show our controls in the tables. At least 80–90% of control cells were still viable after this 3 h.

2.3. Assessment of 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,b) in aliquots taken at different time points during the 3 h incubation period. At least 80–90% of control cells were still viable after this 3 h.

2.4. Determination of mitochondrial membrane potential decline ($\Delta\Psi_m$)

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.

2.5. Lysosomal membrane stability assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange (adapted from Brunk et al., 1995a,b). 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 twice 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.

2.6. 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. 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 carcinogenic metals at 37 °C for 30, 60 and 120 min. After centrifugation ($50\times g$ 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 μM 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 (LeBel et al., 1992).

2.7. 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 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). The concentration of thiobarbituric acid-reactive substances (TBARS) was then calculated using a molar extinction coefficient of 1.56×10^5/M per cm (Buege and Aust, 1978).

2.8. Statistical analysis

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

3. Results

As shown in Tables 1A and B however both redox active carcinogenic metals (CuII, CrVI, NiII, CoII) and non-redox active carcinogenic metals (CdII, HgII, AsIII) induced a rapid decline of mitochondrial membrane potential. But glycolytic ATP generators (Fructose / xylitol) or glutamine (a mitochondrial ATP generator) only prevented non-redox active carcinogenic metals (CdII, HgII, AsIII) induced cytotoxicity,"ROS" formation and the decline in membrane potential (Table 1A, Illustration 1).

Furthermore, uncouplers of oxidative phosphorylation (carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), or pentachlorophenol) inhibited non-redox active carcinogenic metals (CdII, HgII, AsIII) but not redox active carcinogenic metals (CuII, CrVI, NiII, CoII) induced cytotoxicity and "ROS" formation (Table 1A and B, Illustration 1).

On the other hand lysosomotropic agents (chloroquine, methylamine, monensin and 3-methyl adenine) prevented redox active carcinogenic metals (CuII, CrVI, NiII, CoII) but not non-redox active carcinogenic metals (CdII, HgII, AsIII) induced hepatocyte toxicity, "ROS" and lipid peroxidation (Table 2A and B, Illustration 1).

Illustration 1
Summary of effects of different cytoprotectants on cytotoxicity and "ROS" formation induced by carcinogenic metals

Cytoprotectants	Redox active metals	Non-redox metals
	(Cu, Cr, Ni, Co)	(Hg, Cd, As)
Uncouplers of mitochondrial respiration	—	+
ATP generators	–	+
Lysosomotropic agents	+	–
Ferric chelator	+	–

Table 1

Addition	"ROS"	% $\Delta\Psi_m$	Cytotoxicity	
Incubation time	15 min	15 min	1 h	3 h
a. Effect of uncouplers and ATP generators on non-redox active metal induced hepatocyte lysis, "ROS" formation and collapse of the mitochondrial membrane potential				
Control hepatocytes	78±5	3±3	18±2	20±2
+ $HgC1_2$ (20 μM)	322±11[a]	55±5[a]	48±5[a]	82±8[a]
+ CCCP (2 μM)	55±6[b]	14±2[b]	37±4[b]	48±4[b]
+ pentachlorophenol (10 μM)	65±8[b]	12±2[b]	35±3[b]	43±4[b]
+ Fructose (10 mM)	68±5[b]	13±3[b]	28±2[b]	40±4[b]
+ Xylitol (10 mM)	78±5[b]	11±3[b]	32±2[b]	42±3[b]
+ L-glutamine (1 mM)	66±5[b]	8±1[b]	28±2[b]	35±3[b]
+ $CdC1_2$ (20 μM)	225±7[a]	54±5[a]	42±4[a]	79±8[a]
+ CCCP (2 μM)	59±6[b]	14±2[b]	34±4[b]	38±4[b]
+ pentachlorophenol (10 μM)	62±8[b]	12±2[b]	35±3[b]	38±4[b]
+ Fructose (10 mM)	65±5[b]	12±2[b]	31±2[b]	39±2[b]
+ Xylitol (10 mM)	71±5[b]	13±2[b]	28±2[b]	42±4[b]
+ L-glutamine (1 mM)	73±5[b]	10±2[b]	31±2[b]	40±3[b]
+ *Arsenite* (50 μM)	165±6[a]	62±5[a]	43±4[a]	76±8[a]
+ CCCP (2 μM)	60±6[b]	14±2[b]	34±4[b]	48±4[b]
+ pentachlorophenol (10 μM)	66±8[b]	12±2[b]	31±3[b]	43±4[b]
+ Fructose (10 mM)	72±5[b]	13±1[b]	31±3[b]	41±3[b]
+ Xylitol (10 mM)	68±5[b]	11±2[b]	28±2[b]	40±4[b]
+ M-glutamine (1 mM)	67±5[b]	13±2[b]	31±3[b]	44±4[b]
b. Effect of uncouplers and ATP generators on redox active metal induced hepatocyte lysis, "ROS" formation and collapse of the mitochondrial membrane potential				
Control hepatocytes	78±5	3±3	18±2	20±2
+ *Copper chloride (50 μM)*	402±11[a]	55±5[a]	48±5[a]	82±8[a]
+ CCCP (2 μM)	395±6	12±2[b]	44±4	88±7
+ pentachlorophenol (10 μM)	385±8	12±2[b]	45±3	83±8
+ Fructose (10 mM)	377±5	13±2[b]	48±5	80±7
+ Xylitol (10 mM)	385±5	16±2[b]	45±4	78±8
+ L-glutamine (1 mM)	398±5	14±2[b]	48±4	81±7
+ *Dichromate (1 mM)*	508±7[a]	52±5[a]	43±4[a]	75±7[a]
+ CCCP (2 μM)	485±6	14±2[b]	37±4	78±6
+ pentachlorophenol (10 μM)	465±8	15±2[b]	35±3	73±7
+ Fructose (10 mM)	477±5	13±2[b]	38±3	80±8
+ Xylitol (10 mM)	478±5	13±2[b]	39±3	79±7
+ L-glutamine (1 mM)	498±5	13±2[b]	40±3	80±7
+ *Cobalt chloride* (500 μM)	565±6[a]	42±5[a]	43±4[a]	76±5[a]
+ CCCP (2 μM)	555±6	14±2[b]	47±4	78±4
+ pentachlorophenol (10 μM)	565±8	21±2[b]	45±4	73±5
+ Fructose (10 mM)	578±5	17±2[b]	38±4	80±6
+ Xylitol (10 mM)	573±5	18±2[b]	38±4	74±5
+ L-glutamine (1 mM)	562±5	17±2[b]	39±4	75±6
+ *Nickel chloride (1 mM)*	425±7[a]	54±5[a]	42±4[a]	79±5[a]
+ CCCP (2 μM)	385±6	17±2[b]	42±4	88±5
+ pentachlorophenol (10 μM)	365±8	19±2[b]	45±3	83±5
+ Fructose (10 mM)	378±5	18±2[b]	39±3	76±4
+ Xylitol (10 mM)	396±5	21±2[b]	38±3	81±4
+ L-glutamine (1 mM)	408±5	23±2[b]	38±3	80±5

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°.

Cytotoxicity was determined as the percentage of cells that take up trypan blue.

"ROS" formation was expressed as fluorescent intensity units by following dichlorofluorescin oxidation (Shen et al., 1996). $\Delta\Psi_m$ was determined as the difference in rhodamine 123 uptake by control and treated cells and expressed as fluorescence intensity unit (Andersson et al., 1987). 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; ($P<0.001$).

Lysosomal protease inhibitors (leupeptin and pepstatin) also prevented redox active carcinogenic metals (CuII, CrVI, NiII, CoII) but not non-redox active carcinogenic metals (CdII, HgII, AsIII) induced hepatocyte lysis (Table 2A and B).

Ferric chelator desferoxamine significantly inhibited redox active carcinogenic metals (CuII, CrVI, NiII, CoII) induced hepatocyte toxicity, ''ROS'' formation and lipid peroxidation, nevertheless it only narrowly delayed non-redox metals lipid peroxidation (Table 2A and B).

When hepatocyte lysosomes were loaded with acridine orange, a release of acridine orange into the cytosolic fraction ensued within 60 minutes if the loaded hepatocytes were treated with redox active carcinogenic metals (CuII, CrVI, NiII, CoII) but not non-redox active carcinogenic metals (CdII, HgII, AsIII) (Table 3). The redox active carcinogenic metals (CuII, CrVI, NiII, CoII) induced acridine orange release was prevented by inhibitors of oxidative stress including; ''ROS'' scavengers (dimethylsulfoxide, mannitol), catalase or superoxide dismutase and the ferric chelator desferoxamine (Table 3).

Dimethylsulfoxide, mannitol, catalase or superoxide dismutase or deferal did not cause any acridine orange release in the absence of redox active carcinogenic metals (CuII, CrVI, NiII, CoII) in control hepatocytes (data not shown).

4. Discussion

In this study we found that the ATP generators fructose and xylitol and L-glutamine (a mitochondrial ATP generator) prevented non-redox active metals induced cytotoxicity and ''ROS'' formation (Table 1A, Illustration 1) which indicates that the cell death may be a consequence of mitochondrial MPT pore opening and

Table 2

Addition	%Cytotoxicity	''ROS''	TBARS
Incubation time	2h	2h	2h
a. Preventing redox active redox active metal induced hepatocyte oxidative stress and cell lysis with lysosomotropic/endocytosis agents or lysosomal protease inhibitors			
None	19±2	90±5	0.541±0.006
Copper chloride (50 μM)	56±6[a]	412±9[a]	10.447±0.011[a]
+Monensin (10μM)	26±3[b]	91±8[b]	0.715±0.006[b]
+Methylamine (30 mM)	25±3[b]	112±5[b]	0.772±0.008[b]
+Chloroquine (100 μM)	24±3[b]	88±8[b]	0.592±0.009[b]
+3-Methyladenine (5 mM)	25±3[b]	98±6[b]	0.637±0.006[b]
+Leupeptin (100 μM)	37±4[b]	408±5	7.046±0.006
+Pepstatin (100 μM)	35±3[b]	405±4	7.418±0.005
+Desferoxamine (200 μM)	28±3[b]	93±7[b]	0.062±0.006[b]
Dichromate (1 mM)	54±5[a]	502±6[a]	16.948±0.008[a]
+Monensin (10 μM)	26±3[b]	131±8[b]	0.997±0.009[b]
+Methylamine (30 mM)	25±3[b]	140±5[b]	0.956±0.006[b]
+Chloroquine (100 μM)	22±3[b]	148±8[b]	0.988±0.006[b]
+3-Methyladenine (5 mM)	23±3[b]	121±6[b]	0.908±0.007[b]
+Leupeptin (100 μM)	31±3[b]	488±5	14.950±0.006
+Pepstatin (100 μM)	30±3[b]	495±4	15.948±0.005
+Desferoxamine (200 μM)	27±3[b]	93±6[b]	0.068±0.006[b]
Cobalt chloride (500 μM)	52±5[a]	621±9[a]	13.948±0.008[a]
+Monensin (10 μM)	36±3[b]	231±8[b]	1.107±0.009[b]
+Methylamine (30 mM)	35±3[b]	240±5[b]	1.346±0.006[b]
+Chloroquine (100 μM)	32±3[b]	248±8[b]	1.438±0.006[b]
+3-Methyladenine (5 mM)	33±3[b]	221±6[b]	1.908±0.007[b]
+Leupeptin (100 μM)	37±4[b]	578±5	12.156±0.006
+Pepstatin (100 μM)	35±3[b]	585±4	12.911±0.005
+Desferoxamine (200 μM)	26±3[b]	97±6[b]	0.085±0.011[b]
Nickel chloride (2 mM)	53±5[a]	473±8[a]	11.108±0.008[a]
+Monensin (10 μM)	34±3[b]	111±6[b]	0.937±0.011[b]
+Methylamine (30 mM)	33±3[b]	220±5[b]	0.915±0.016[b]
+Chloroquine (100 μM)	32±3[b]	138±4[b]	0.932±0.016[b]
+3-Methyladenine (5 mM)	31±3[b]	141±5[b]	0.951±0.007[b]
+Leupeptin (100 μM)	32±4[b]	448±5	9.750±0.016
+Pepstatin (100 μM)	34±3[b]	445±4	9.648±0.015
+Desferoxamine (200 μM)	25±3[b]	90±7[b]	0.076±0.009[b]

(*continued on next page*)

Table 2 (*continued*)

Addition	%Cytotoxicity	"ROS"	TBARS
Incubation time	2h	2h	2h
b. Effect of lysosomotropic/endocytosis agents or lysosomal protease inhibitors on non redox active metal induced hepatocyte oxidative stress and cell lysis			
None	19±2	90±5	0.541±0.006
$HgCl_2$ (20 μM)	56±6[a]	322±11[a]	3.447±0.011[a]
+Monensin (10μM)	56±3	301±8	3.715±0.006
+Methylamine (30 mM)	55±3	312±5	3.772±0.008
+Chloroquine (100 μM)	54±3	308±8	3.592±0.009
+3-Methyladenine (5 mM)	55±3	298±6	3.637±0.006
+Leupeptin (100 μM)	57±4	308±5	3.046±0.006
+Pepstatin (100 μM)	55±3	305±4	3.418±0.005
+Desferoxamine (200 μM)	47±4[b]	273±6[b]	1.983±0.012[b]
$CdCl_2$ (20 μM)	52±5[a]	225±7[a]	2.948±0.308[a]
+Monensin (10 μM)	56±3	231±8	2.997±0.009
+Methylamine (30 mM)	55±3	240±5	2.956±0.006
+Chloroquine (100 μM)	52±3	248±8	2.988±0.006
+3-Methyladenine (5 mM)	53±3	221±6	2.908±0.007
+Leupeptin (100 μM)	57±4	248±5	2.950±0.006
+Pepstatin (100 μM)	55±3	245±4	2.948±0.005
+Desferoxamine (200 μM)	46±3[b]	193±6[b]	1.019±0.008[b]
Arsenite (50 μM)	52±5[a]	165±9[a]	2.648±0.248[a]
+Monensin (10 μM)	48±3	141±8	2.297±0.109
+Methylamine (30 mM)	55±3	150±5	2.056±0.106
+Chloroquine (100 μM)	54±3	158±8	2.308±0.026
+3-Methyladenine (5 mM)	53±3	161±6	2.138±0.107
+Leupeptin (100 μM)	47±4	158±5	2.150±0.036
+Pepstatin (100 μM)	49±3	155±4	2.048±0.015
+Desferoxamine (200 μM)	48±3[b]	143±6[b]	1.597±0.008[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°.
Cytotoxicity was determined as the percentage of cells that take up trypan blue.
"ROS" formation was expressed as fluorescent intensity units.
TBARS formation was expressed as μM concentrations.
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 ($P<0.001$).

consequent ATP depletion. Lack of mitochondrial ATP results in intracellular acidosis and osmotic injury which leads to plasma membrane lysis (Pourahmad and O'Brien, 2000b).

Mitochondrial respiration consumes most of the oxygen used by cells and therefore some of the endogenous "ROS" formation of the hepatocytes may be attributed to autoxidation of the reduced components of the respiratory electron transport chain. Previously hepatocyte "ROS" formation was found to markedly be increased by rotenone, an inhibitor of NADH dehydrogenase (Complex I) and Complex I was suggested to be an important mitochondrial site of "ROS" generation (Siraki et al., 2002). Previously antimycin A, an inhibitor of the ubiquinone-cytochrome b reductase (Complex III), was also found to stimulate hepatocyte "ROS" formation which was attributed to reaction of O_2 with reduced cytochrome b566 and/or ubisemiquinone (Siraki et al., 2002). Hepatocyte "ROS" formation was reported to be increased by keeping the hepatocytes under a nitrogen atmosphere for 90 min before restoring oxygen and adding DCFH (Moridani et al., 2003). similar results were previously reported using luminol chemiluminescence to follow "ROS" formation (Niknahad et al., 1995).

Recently, mitochondrial uncouplers (e.g. CCCP and pentachlorophenol) were reported to strongly inhibit H_2O_2 generation by isolated heart muscle mitochondria as well as inhibit reverse electron transfer from succinate to NAD^+ (Korshunov et al., 1998). It was suggested that mitochondrial reverse electron transfer to complex I produces H_2O_2 (Siraki et al., 2002).

Of particular interest in our findings was the strong inhibition of non-redox active metals (CdII, HgII, AsIII) induced "ROS" formation on addition of non-toxic concentrations of the uncouplers CCCP and pentachlorophenol (Table 1A,Iillustration 1). We therefore conclude that mitochondria are likely a major contributer for "ROS" formation by non-redox active carcinogenic metals.

Table 3
Preventing redox active metal induced hepatocyte lysosomal membrane damage by inhibitors of oxidative stress or endocytosis

Addition	(Acridine orange redistribution)		
Incubation time	15 min	30 min	60 min
None	3±1	4±1	4±1
Mercuric chloride (20μM)	4±2	7±2	8±2
Cadmium chloride (20μM)	4±2	5±2	7±2
Arsenite (50 μM)	4±2	6±2	6±2
Copper chloride (50μM)	39±4[a]	68±7[a]	95±8[a]
+Catalase (200 μ/ml)	3±1[b]	3±1[b]	5±1[b]
+SOD (100 μ/ml)	6±1[b]	7±1[b]	11±2[b]
+Dimethyl sulfoxide (150 mM)	3±1[b]	4±1[b]	7±1[b]
+Mannitol (50 mM)	6±1[b]	5±1[b]	8±2[b]
+Desferal (200μM)	3±1[b]	3±1[b]	3±1[b]
Dichromate (1 mM)	39±4[a]	72±6[b]	395±8[a]
+Catalase (200 μ/ml)	3±1[b]	3±1[b]	35±4[b]
+SOD (100 μ/ml)	6±1[b]	7±1[b]	71±5[b]
+Dimethyl sulfoxide (150 mM)	3±1[b]	4±1[b]	47±4[b]
+Mannitol (50 mM)	6±1[b]	5±1[b]	28±3[b]
+Desferal (200μM)	3±1[b]	3±1[b]	23±2[b]
Cobalt chloride (500μM)	42±4[a]	77±7[a]	268±8[a]
+Catalase (200 μ/ml)	3±1[b]	4±1[b]	24±1[b]
+SOD (100 μ/ml)	5±1[b]	9±1[b]	21±2[b]
+Dimethyl sulfoxide (150 mM)	3±1[b]	8±1[b]	27±1[b]
+Mannitol (50 mM)	4±1[b]	9±1[b]	23±2[b]
+Desferal (200μM)	4±1[b]	4±1[b]	19±1[b]
Nickel chloride (2 mM)	36±3[a]	65±6[a]	165±4[a]
+Catalase (200 μ/ml)	3±1[b]	7±1[b]	33±1[b]
+SOD (100 μ/ml)	6±1[b]	6±1[b]	41±2[b]
+Dimethyl sulfoxide (150 mM)	3±1[b]	5±1[b]	37±1[b]
+Mannitol (50 mM)	7±1[b]	6±1[b]	38±2[b]
+Desferal (200μM)	3±1[b]	3±1[b]	23±1[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.

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 ($P<0.001$).

On the other hand our results showed that redox active metals induce lysosomal membrane disruption. Our findings also showed that redox active metals induced cytotoxicity as well as hepatocyte "ROS" formation and lipid peroxidation were prevented by the hepatocyte lysosomotropic agents methylamine, chloroquine, monensin or 3-methyladenine. 3-Methyladenine also prevented the redox active metals induced lysosomal disruption. These findings suggest that redox active carcinogenic metals (CuII, CrVI, NiII, CoII) induced hepatocyte membrane lysis and lipid peroxidation involves lysosomal "ROS" formation.

We also found that the hepatocyte lysosomal protease inhibitors leupeptin or pepstatin prevented redox active metals induced cytotoxicity, These findings are further evidence that redox active carcinogenic metals induced hepatocyte injury involves oxidative injury to hepatocyte lysosomal membrane and the release of lysosomal proteolytic enzymes.

Our results also showed that lysosomal membrane disruption induced by redox active carcinogenic metals was also prevented by the ferric chelator desferoxamine (Table 3). Ferric chelator desferoxamine also prevented hepatocyte cytotoxicity induced by nirofurantoin and other intracellular hydrogen peroxide generator glucose/glucose oxidase (Pourahmad et al., 2001c; Starke et al., 1985).

These findings suggest that free Fe^{2+} ions in the lysosomes undergo redox cycling and form "ROS" from H_2O_2 (Haber-weiss reaction) which diffuses into the lysosomes after formation by the redox cycling of (CuII, CrVI, NiII, CoII) in the cytoplasm. Therefore we conclude that lysosomes are the "ROS" generating sites for redox cycling carcinogenic metals.

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