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Hydroxyl Radical Formation and Lipid Peroxidation Enhancement by Chromium

In Vitro Study

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ABSTRACT

Chromium VI compounds have been shown to be carcinogenic in occupationally exposed humans, and to be genotoxic, mutagenic, and carcinogenic in a variety of experimental systems. In contrast, most chromium III compounds are relatively nontoxic, noncarcinogenic, and nonmutagenic. Reduction of Cr^{6+} leads to reactive intermediates, such as Cr^{5+}, Cr^{4+}, or other radical species. The molecular mechanism for the intracellular Cr^{6+} reduction has been the focus of recent studies, but the details are still not understood.

Our study was initiated to compare the effect of Cr^{6+}-hydroxyl radical formation and Cr^{6+}-induced lipid peroxidation vs those of Cr^{3+}. Electron spin resonance measurements provide evidence for the formation of long-lived Cr^{5+} intermediates in the reduction of Cr^{6+} by glutathione reductase in the presence of NADPH and for the hydroxyl radical formation during the glutathione reductase catalyzed reduction of Cr^{6+}. Hydrogen peroxide suppresses Cr^{5+} and

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enhances the formation of hydroxyl radical. Thus, Cr^{5+} intermediates catalyze generation of hydroxyl radicals from hydrogen peroxide through a Fenton-like reaction.

Comparative effects of Cr^{6+} and Cr^{3+} on the development of lipid peroxidation were studied by using rat heart homogenate. Heart homogenate was incubated with different concentrations of Cr^{6+} compounds at 22°C for 60 min. Lipid peroxidation was determined as thiobarbituric acid reacting materiels (TBA-RM). The results confirm that Cr^{6+} induces lipid peroxidation in the rat heart homogenate. These observations might suggest a possible causative role of lipid peroxidation in Cr^{6+} toxicity. This enhancement of lipid peroxidation is modified by the addition of some metal chelators and antioxidants. Thus, strategies for combating Cr^{6+} toxicity should take into account the role of the hydroxy radicals, and hence, steps for blocking its chain propagation and preventing the formation of lipid peroxides.

Index Entries: Electron spin resonance; hydroxyl radical; lipid peroxidation; rat heart homogenate; chromium; scavengers; metal chelators.

Abbreviations: Cr, chromium; Desferal, Deferoxamine; DMPO, 5,5′-dimethyl-1-pyroline-*N*-oxide; DMSO, Dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance, GRx, glutathione reductase; H_2O_2, hydrogen peroxide; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; °OH, hydroxyl radical; TBA-RM, thiobarbituric acid reacting materials.

INTRODUCTION

Chromium (Cr), in traces, is known as an essential factor for the maintenance of life as well as growth of animals (*1*). Chromium (Cr^{6+}) compounds have been found to have serious toxic and carcinogenic effects on humans and animals (*2*). In contrast, most chromium (Cr^{3+}) compounds are relatively nontoxic, noncarcinogenic, and nonmutagenic.

Earlier studies concerning the Cr^{6+} reduction include those on the reduction of Cr^{6+} by glutathione (GSH), ascorbic acid, glucose, and microsomal and mitochondrial electron transport chain complexes (*3*). Redox active cellular proteins capable of reducing Cr^{6+} include the soluble cytoplasmic proteins DT-diaphorase (*4*) and aldehyde oxidase (*5*). The microsomal membrane-associated cytochrome P-450 has also been shown to reduce Cr^{6+} to Cr^{3+} and produce transient chromium (Cr^{5+}) intermediates (*6*). These studies have shown that the Cr^{6+} reduction involves the formation of Cr^{5+}-containing species that are often considered to be the toxic form of Cr.

Electron spin resonance (ESR) measurements on solutions provide direct evidence for the involvement of Cr^{5+} species in the reduction of Cr^{6+} by NADPH in presence of glutathione reductase (GRx). GRx and NADPH are chosen as a model reductant because of their ubiquitous

existence inside the cells. We have investigated the reactivity of Cr^{5+} and found that it catalyzed generation of hydroxyl radical (°OH) through a Fenton-like reaction. On the other hand, lipid peroxidation in vivo results in membrane damage and plays an important role in tissue injuries by chemicals. Indeed, some metal ions have been reported to show either stimulative or inhibitory action on lipid peroxidation in tissues. Yonaha et al. (1980) (*7*) reported an inhibitory action of Cr^{6+} and Cr^{3+} at low concentrations on lipid peroxidation in rat liver microsomes and a prooxidant activity of Cr^{6+} at higher concentration. Comparative effects of Cr^{6+} and Cr^{3+} on the development of lipid peroxidation were studied in this article using rat heart homogenate.

MATERIALS AND METHODS

Materials

Glutathione reductase and NADPH were purchased from Sigma and used as received. Spin trap 5.5′-dimethyl-1-pyroline-*N*-oxide (DMPO) was purchased from Sigma and used after purification by distillation. Deferoxamine (Desferal) was purchased from Ciba (Ruei-Malmaison, France). All other chemicals and reagents employed were of commercial reagent-grade quality.

ESR Recording

ESR spectra were obtained at X-band (9.7 GHz) using a Bruker ER 100 D ESR spectrometer. Spectrometer settings were: receiver gain 5×10^5, modulation amplitude 1.6 G, scan time 100 s, field 3480 ± 50 G, time constant 0.5 s, and hyperfrequence power 10 dB.

Preparation of Rat Heart Homogenate

Male rats of the Wistar stain were maintained on rat diet chow. Rats were anesthetized by pentobarbital and heparinized. Then the heart was removed, perfused, and homogenized in 9 vol of 0.10*M* Tris-HCl, containing 1 m*M* diethylenetriaminepentaacetic acid (DTPA), pH 7.4. Proteins were determined by the method of Lowry et al. (1951) (*8*).

Lipid Peroxidation

The incubation mixture contained 500 μL of homogenate and 100 μL of 10 m*M* tested chromium solutions (Cr^{6+} or Cr^{3+}) with other cofactors or scavengers in a final vol of 1 mL. Incubations were carried out aerobically at room temperature for 60 min. Lipid peroxides were measured by the thiobarbituric acid method described by Ohkawa et al. (*9*) and modified in our laboratory: The incubation mixture was treated with 100 μL of sodium dodecyl sulfate (SDS) 0.8%, 1 mL of acetic acid 20% (pH

3.5), and 1 mL of thiobarbituric acid 0.8% (pH 3.5). The whole was heated for 60 min in a boiling-water bath. The mixture was then cooled, and the colored pigment extracted with 4 mL of *n*-butanol. The OD of the organic phase was determined at 532 nm using an Uvikon-680 spectrophotometer.

The concentrations given in the figure legends are final concentrations. All experiments were carried out at room temperature.

RESULTS

ESR Studies

An aqueous chromium (Cr^{6+}) solution at pH 7.4 containing 2 m*M* NADPH, 2 m*M* H_2O_2, 12 IU Grx/mL, and 500 m*M* DMPO gives an important ESR signal corresponding to the DMPO—OH spectrum (Fig. 1a). The absence of one of the mixture constituents results in an evident modification of this signal quantitatively or qualitatively. Thus, the removal of Cr^{6+} abolishes completely the signal (Fig. 1b), whereas an important diminution in the signal size is noted in the absence of NADPH or GRx (Fig. 1c, d). Finally, the removal of H_2O_2 results in qualitative modification: a decrease in the DMPO—OH signal and apparition of a new signal corresponding to the Cr^{5+} spectrum (Fig. 2). These spectra obtained here are similar to those reported earlier by Shi and Dalal (1989) (*10*).

Additional support for the °OH radical identification was obtained by using two °OH scavengers: thiourea and DMSO (100 and 10 m*M*, respectively). The spectrum shown in Fig. 3 indicates the results obtained with or without scavenger additions. Thiourea decreases significantly the DMPO—OH signal (70%) and DMSO decreases it only by 40%. On the other hand, the use of ascorbic acid results in a complete disappearance of signal. Ascorbate is known to destroy the nitroxide signal of DMPO. Furthermore, when we use Desferal, the DMPO—OH signal disappears and a new signal forms (Fig. 2).

LIPID PEROXIDATION MEDIATED BY CR^{6+}/ NADPH/GRx/H_2O_2 SYSTEM

The importance of each reagent was studied in the induction of lipid peroxidation in rat heart homogenate. Figure 4 shows clearly that only Cr^{6+} is responsible of triggering off lipid peroxidation. The removal of NADPH, GRx, or even H_2O_2 does not affect this Cr^{6+}-induced lipid peroxidation. Only the removal of Cr^{6+} or its substitution by Cr^{3+} can prevent the process of lipid peroxidation.

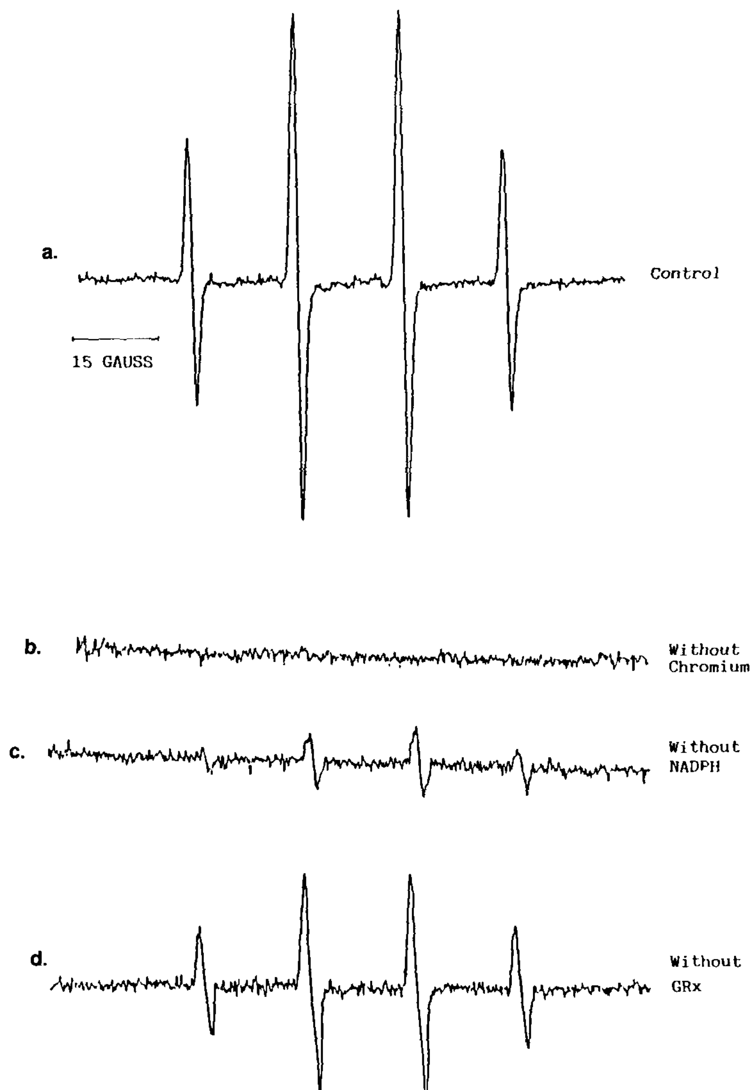

Fig. 1. ESR spectra recorded 1 min after mixing in a Tris buffer solution (pH 7.4), of (a) 2 m*M* Cr^{6+}, 2 m*M* NADPH, 2 m*M* H_2O_2, 12 U/mL GRx, and 500 m*M* DMPO. (b) Same as (a) without Cr. (c) Same as (a) without NADPH. (d) Same as (a) without GRx. Spectrometer settings were: receiver gain 5×10^5, modulation amplitude 1.6 G, scan time 100 s, field 3480 ± 50 G, time constant 0.5 s, and Hyper frequence power 10 dB.

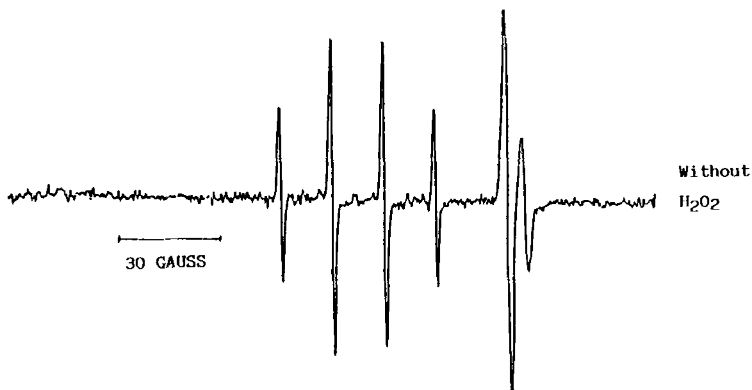

Fig. 2. ESR spectra recorded 1 min after mixing in a Tris buffer solution (pH 7.4), of 2 m*M* Cr^{6+}, 2 m*M* NADPH, 12 U/mL GRx, and 500 m*M* DMPO (without H_2O_2). Spectrometer settings were same as Fig. 1, but field 3480 ± 100 G.

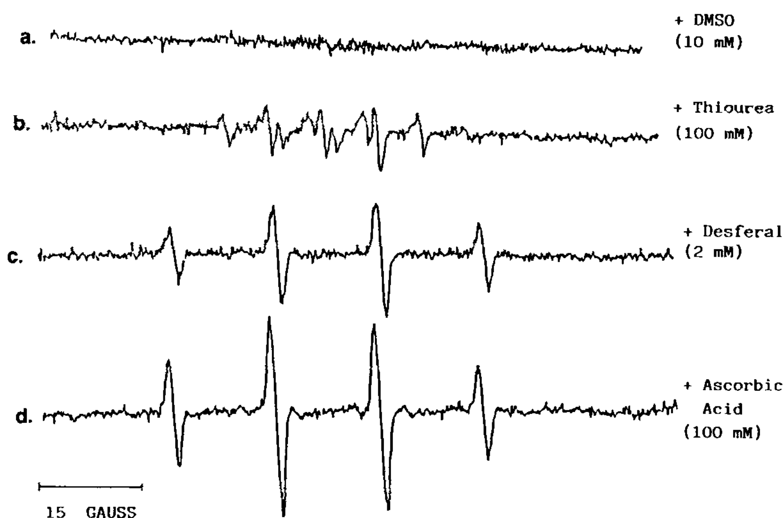

Fig. 3. ESR spectra recorded 1 min after mixing in a Tris buffer solution (pH 7.4) of 2 m*M* Cr^{6+}, 2 m*M* NADPH, 2 m*M* H_2O_2, 12 U/mL GRx, and 500 m*M* DMPO with (a) 10 m*M* DMSO (b) 100 m*M* Thiourea, (c) 2 m*M* Desferal, and (d) 100 m*M* ascorbic acid. Spectrometer settings were same as Fig. 1.

EFFECT OF DIFFERENT CONCENTRATIONS OF CR ON LIPID PEROXIDATION

The effects of Cr^{6+} or Cr^{3+} over the range of 1–10,000 μ*M* were studied on peroxidation in rat heart homogenate. At these concentrations, lipid peroxidation was induced only by Cr^{6+}, whereas Cr^{3+} has no

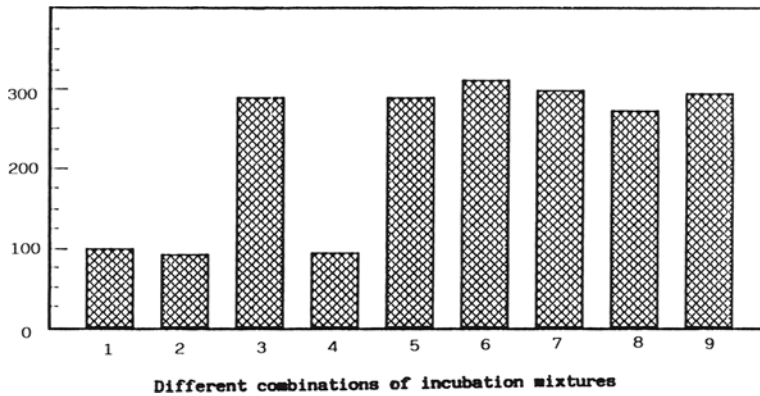

Fig. 4. Importance of each reagent in chromium-induced lipid peroxidation system. Different combinations of incubation mixtures are: (1) heart homogenate, (2) 1 + 1 m*M* Cr^{3+}, 1 m*M* NADPH, 1 m*M* H_2O_2, 2 U/mL GRx. (3) Same as (2) but Cr^{3+} is replaced by Cr^{6+}. (4) Same as (3) without Cr. (5) Same as (3) without NADPH. (6) Same as (3) without GRx. (7) Same as (3) without H_2O_2. (8) Same as (3) without NADPH and GRx. (9) Same as (3) without NADPH, GRx, and H_2O_2 (only Cr^{6+} and homogenate).

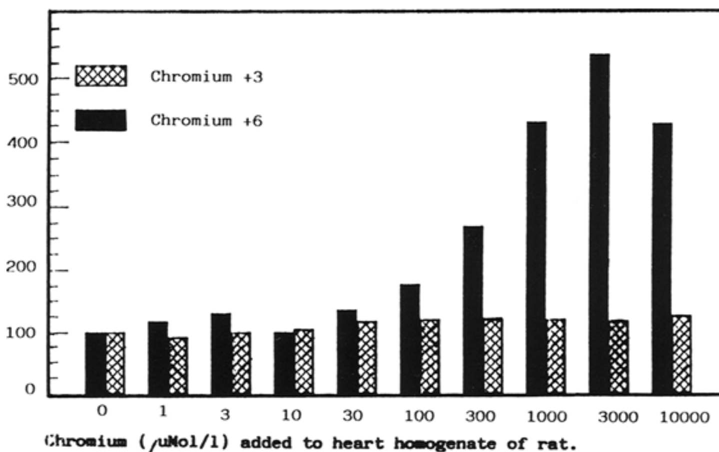

Fig. 5. Effect of different concentrations of Cr (Cr^{3+}, Cr^{6+}) on lipid peroxidation. Rat heart homogenate was incubated with increasing concentrations of Cr^{3+} or Cr^{6+}. Lipid peroxides (MDA) were measured after 1 h of incubation.

effect. The lipid peroxidation levels increase with an increase in the concentrations of Cr^{6+} added (Fig. 5).

We next examined whether lipid peroxide formation at 1 m*M* of Cr^{6+} could be inhibited by metal chelators (EDTA, Desferal) or antioxidant compounds (ascorbic acid). As we could note in Fig. 6, lipid peroxidation

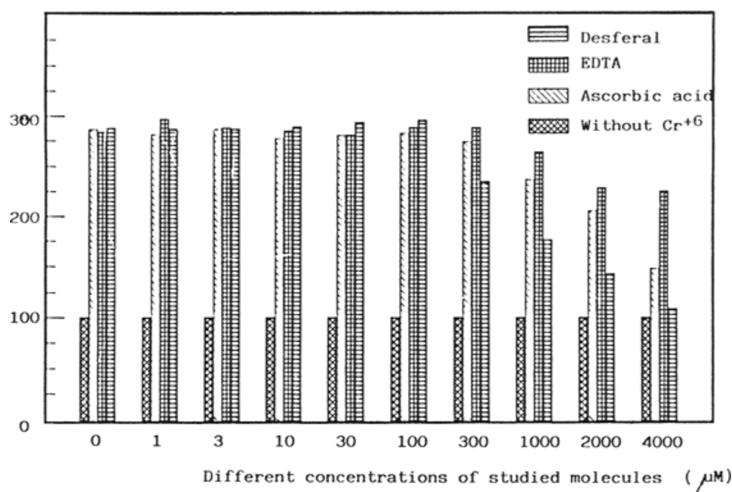

Fig. 6. Effect of metal chelators or antioxidant molecules on Cr^{6+}-induced lipid peroxidation. Rat heart homogenate was incubated with increasing concentrations of metal chelators or antioxidant molecules during 30 min. Lipid peroxidation was then induced by 1 m*M* of Cr^{6+} as described under materials and methods.

was slightly inhibited by EDTA, but highly inhibited by Desferal and moderately by ascorbic acid. However, a concentration of at lest 1 m*M* of studied molecules is necessary to inhibit partly or largely (according to the case) the Cr^{6+}-induced lipid peroxidation in the presence of 1 m*M* added Cr^{6+}.

DISCUSSION

In the present study, we have investigated the ability of Cr^{6+} to be reduced to Cr^{5+} by biological systems (GRx and NADPH) and its ability to trigger off the lipid peroxidation process in rat heart homogenates. The results reported here provide direct evidence for the NADPH- and GRx-dependent reduction of Cr^{6+} with Cr^{5+} as an intermediate, indicating that at least one of the initial steps in the Cr^{6+} reduction involves a one-electron transfer process.

Since Cr^{5+} complexes are generally characterized as being labile and reactive, the detection of Cr^{5+} formation led Jennette (1981) (*11*) to suggest that the Cr^{5+} intermediates are the likely candidates for the ultimate carcinogenic forms of carcinogenic chromium species. Our results show that Cr^{6+} can be enzymatically metabolized to Cr^{5+} with simultaneous formation of °OH radical. This indicates that the Cr^{5+} generated has an ability to react with H_2O_2 to generate °OH radicals. This Cr^{5+} intermediate catalyzes generation of °OH radicals from H_2O_2

through a Fenton-like reaction. So, under in vivo conditions, °OH radicals might be the ultimate carcinogenic species in the mechanism of Cr^{6+} carcinogenesis.

In order to complete our study, we linked this Cr^{5+} intermediate formation with in vivo lipid peroxidation enhancement by Cr^{6+}. The effects of Cr^{6+} and Cr^{3+} in the presence of enzymatic system (GRx/NADPH) and H_2O_2 on lipid peroxidation were studied in rat heart homogenate. Our data show that only Cr^{6+} is able to enhance lipid peroxidation, not Cr^{3+}. These data agree with those obtained by Ueno et al. (1988) (*12*). Moreover, the enzymatic system plus H_2O_2 are not essential for this peroxidative reaction. On the other hand, this lipid peroxidation enhancement seems dose-dependent. It could mean that Cr^{6+} reduced to Cr^{5+} could catalyze the lipid peroxidation process. This lipid peroxidation was highly inhibited by Desferal, a powerful metal chelator.

Susa et al. (1989) (*13*) reported that lipid peroxidation in the mice liver showed a significant increase at 24 and 48 h after Cr^{6+} injection (20 mg/kg ip), whereas administration of Cr^{3+} resulted in a decrease in TBA-RM formation in the mice liver. Ueno et al. (1987) (*12*) showed that cellular injury, which was estimated as LDH-leakage from isolated hepatocytes into incubation medium, was induced by the addition of Cr^{6+} at concentrations $> 125\ \mu M$. They also demonstrated that lipid peroxidation induced by Cr^{6+} was enhanced remarkably in isolated hepatocytes pretreated with GSH-depleting agent. In contrast, Yonaha et al. in 1980 (*7*) indicated that at lower concentrations at the range of 1–100 μM, both Cr^{6+} and Cr^{3+} inhibited lipid peroxidation induced by ascorbate or NADPH system in rat liver microsomes. However, since lipid peroxidation has the characteristics of a chain reaction, it may be possible to determine the mechanism of chromium-induced lipid peroxidation by means of a search for the effect of chromium VI on different systems related to lipid peroxidation, such as superoxide dismutase, glutathione peroxidase, catalase, or on intracellular GSH levels.

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