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Generation of reactive oxygen species in the enzymatic reduction of $PbCrO_4$ and related DNA damage

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Abstract

Free radical reactions are believed to play an important role in the mechanism of Cr(VI)-induced carcinogenesis. Most studies concerning the role of free radical reactions have been limited to soluble Cr(VI). Various studies have shown that solubility is an important factor contributing to the carcinogenic potential of Cr(VI) compounds. Here, we report that reduction of insoluble $PbCrO_4$ by glutathione reductase in the presence of NADPH as a cofactor generated hydroxyl radicals (•OH) and caused DNA damage. The •OH radicals were detected by electron spin resonance (ESR) using 5,5-dimethyl-N-oxide as a spin trap. Addition of catalase, a specific H_2O_2 scavenger, inhibited the •OH radical generation, indicating the involvement of H_2O_2 in the mechanism of Cr(VI)-induced •OH generation. Catalase reduced •OH radicals measured by electron spin resonance and reduced DNA strand breaks, indicating •OH radicals are involved in the damage measured. The H_2O_2 formation was measured by change in fluorescence of scopoletin in the presence of horseradish peroxidase. Molecular oxygen was used in the system as measured by oxygen consumption assay. Chelation of $PbCrO_4$ impaired the generation of •OH radical. The results obtained from this study show that reduction of insoluble $PbCrO_4$ by glutathione reductase/NADPH generates •OH radicals. The mechanism of •OH generation involves reduction of molecular oxygen to H_2O_2, which generates •OH radicals through a Fenton-like reaction. The •OH radicals generated by $PbCrO_4$ caused DNA strand breakage. (Mol Cell Biochem **234/235**: 309–315, 2002)

Key words: $PbCrO_4$, electron spin resonance (ESR), hydroxyl radicals, glutathione reductase, NADPH

Introduction

Cr(VI) compounds are used widely in industry and are also found in the environment [1, 2]. Epidemiological, animal, and cellular studies have established that Cr(VI) compounds are toxic and carcinogenic [3, 4]. Occupational exposure to Cr(VI)-containing compounds is known to induce lung toxicity and increase incidence of cancers of the respiratory system [3, 5–7]. In experimental animals, Cr(VI) compounds also induce tumors at the injection and implantation sites [5–9]. For example, intra-bronchial implantation of chromate compounds in rats resulted in bronchial carcinomas, whereas intramuscular implantations and intra-pleural and subcutaneous injections in rats and mice result in injection site sarcomas [7, 8]. Cr(VI) causes mutation in bacteria and transformation in mammalian cells [10, 11]. Although the mechanism is still not well understood, it is generally considered that the carcinogenic effect of Cr(VI) involves cellular uptake because Cr(VI) and not Cr(III) actively enters cells by the sulfite transport mechanism [12]. Once inside the cells, Cr(VI) is reduced to its lower oxidation states, such as Cr(V) and Cr(IV). These reactive intermediates can directly

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cause DNA damage [13]. They are also able to generate hydroxyl radical ($^{\bullet}OH$) from H_2O_2 via a Fenton-like reaction [13]. During the Cr(VI) reduction process, molecular oxygen is consumed to generate superoxide radical ($O_2^{\bullet-}$) and H_2O_2 [13–15]. Thus, in the cellular system, Cr(VI) can generate a whole spectrum of reactive oxygen species (ROS). Through ROS mediated reactions, Cr(VI) causes DNA damage [16], activation of nuclear transcription factors (NF-κB, AP-1, and p53) [17–19], apoptosis [20], cell growth arrest [21], oncogene expression [22], and activation of certain family members of mitogen-activated protein (MAP) kinases [18].

While it appears that ROS play an important role in the mechanism of Cr(VI)-induced carcinogenesis, very few studies are available concerning the free radical generation and its role in cellular injuries caused by insoluble Cr(VI)-containing compounds. The results from earlier studies suggest that the carcinogenic activity of certain Cr(VI)-containing compounds is due to deposition and slow dissolution over time resulting in a localized chronic exposure to chromate [7, 23, 24]. Various studies have also shown that solubility is an important factor contributing to the carcinogenic potential of Cr(VI) compounds [23–29]. Slightly soluble to highly insoluble particle chromates of Pb, Zn, and Ca cause tumor induction at injection sites [24], induce transformation in Syrian hamster and C3H/10T1/2 mouse embryo cells [25], and cause chromosomal clastogenesis [26–28]. Using rodent fibroblasts, several studies have shown that insoluble $PbCrO_4$ can adhere to the cell membrane and release a high local concentration of chromate [28, 29]. Since there is substantial evidence to indicate that solubility of Cr(VI)-compounds is an important factor for the potency of Cr(VI)-induced carcinogenicity, the present study was undertaken to investigate the free radical generation by insoluble chromate and ensuing DNA damage using $PbCrO_4$ as a model compound.

Materials and methods

Reagents

Lead (II) chromate ($PbCrO_4$) was purchased from Aldrich (Milwaukee, WI, USA). Deferoxamine, 5,5-dimethyl-l-pyrroline N-oxide (DMPO), glutathione reductase (GSSG-R), sodium formate, H_2O_2, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), and nicotinamide adenine dinucleotide 3′-phosphate ($NADP^+$) were purchased from Sigma (St. Louis, MO, USA). Catalase was purchased from Boehringer Mannheim (Indianapolis, IN, USA). DNA λ hind III fragments and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation. DMPO solution, thus purified, did not contain any ESR detectable impurities. Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA, USA). The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants.

Free radical measurements

ESR spin trapping was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which can then be studied using conventional ESR. The intensity of the signal is used to measure the amount of short-lived radicals trapped and the hyperfine couplings of the spin adduct are generally characteristic of the original trapped radicals. The spin trapping is a method of choice for detection and identification of free radical generation due to its specificity and sensitivity. All ESR measurements were conducted using a Bruker ESP 300E ESR spectrometer (Bruker Instruments Inc. Billerica, MA, USA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to peak width. A SPEX 300 program was used for data acquisitions and analyses.

Reactants were mixed in test tubes in a final volume of 500 µl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. Experiments were performed at room temperature and under ambient air.

H_2O_2 measurements

H_2O_2 was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase. Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a Cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems, Foster City, CA, USA).

DNA strand breakage assay

The DNA strand break assay was carried out according to methods described earlier [30]. Briefly, reactions were performed in 10 mM phosphate-buffered saline (pH 7.4) in

1.5 ml polypropylene tubes at 37°C. Each reaction mixture contained 10 μg DNA (λ Hind III fragments) in a total volume of 100 μl of buffer. To this solution, 2 μl of gel loading buffer (50 mM EDTA, 2.5 % sodium dodecyl sulfate (SDS), 0.1% bromophenol blue) was added, and then electrophoresis was performed in 0.7% agarose at 1–2 V/cm in 40 mM tris acetate buffer containing 2 mM EDTA (pH 8.0). Gels were stained in ethidium bromide (5 μg/ml) for 30 min and photographed under UV light using a Stratagene Eagle Eye II (Stratagene Inc. La Jolla, CA, USA).

Oxygen consumption measurements

Oxygen consumption measurements were carried out using a Gilson oxygraph (Gilson Medical Electronics, Middleton, WI, USA). Measurements were made on a system containing 50 μg/ml $PbCrO_4$, 1 mM NADPH and 0.5 mg/ml GSSG-R in pH 7.4 phosphate buffer. The oxygraph was calibrated with media equilibrated with gases of known concentrations.

Statistics

Data expressed as mean ± S.E.M. (n = 3) for each group. One way ANOVA test was employed to compare the responses between treatments. Statistical significance was set at $p < 0.05$.

Results

$^{\bullet}OH$ radical generation

Figure 1a shows a typical ESR spectrum generated from a mixture containing $PbCrO_4$, NADPH and GSSG-R in the presence of DMPO as a spin trap. This spectrum consists of a 1:2:2:1 quartet with hyperfine splittings of $a_H = a_N = 14.9$ G. Based on these splitting constants, the 1:2:2:1 quartet was assigned to a DMPO/$^{\bullet}OH$ adduct. $PbCrO_4$ alone with DMPO produced a weak DMPO/$^{\bullet}OH$ signal (Fig. 1b). Omission of either NADPH or GSSG-R resulted in a decrease in the overall spectral intensity (Figs 1c and 1d). Replacement of NADPH by $NADP^+$ reduced the generation of $^{\bullet}OH$ radicals (Fig. 1e). Replacement with heat denatured GSSG-R generated a very weak ESR signal (Fig. 1f). Addition of catalase, whose function is to remove H_2O_2, inhibited $^{\bullet}OH$ radical generation (Fig. 1g) with a concomitant generation of a peak at g = 1.9792 (marked with an * in Fig. 1g). This peak was assigned to a Cr(V)-NADPH complex as reported earlier [31–34]. The inhibition of $^{\bullet}OH$ generation with a concomitant appearance of Cr(V) signal upon addition of catalase indicates that $^{\bullet}OH$ radicals were generated via a Cr(V)-mediated Fenton-like reaction ($Cr(V) + H_2O_2 \rightarrow Cr(VI) + {}^{\bullet}OH + OH^-$) and H_2O_2 was

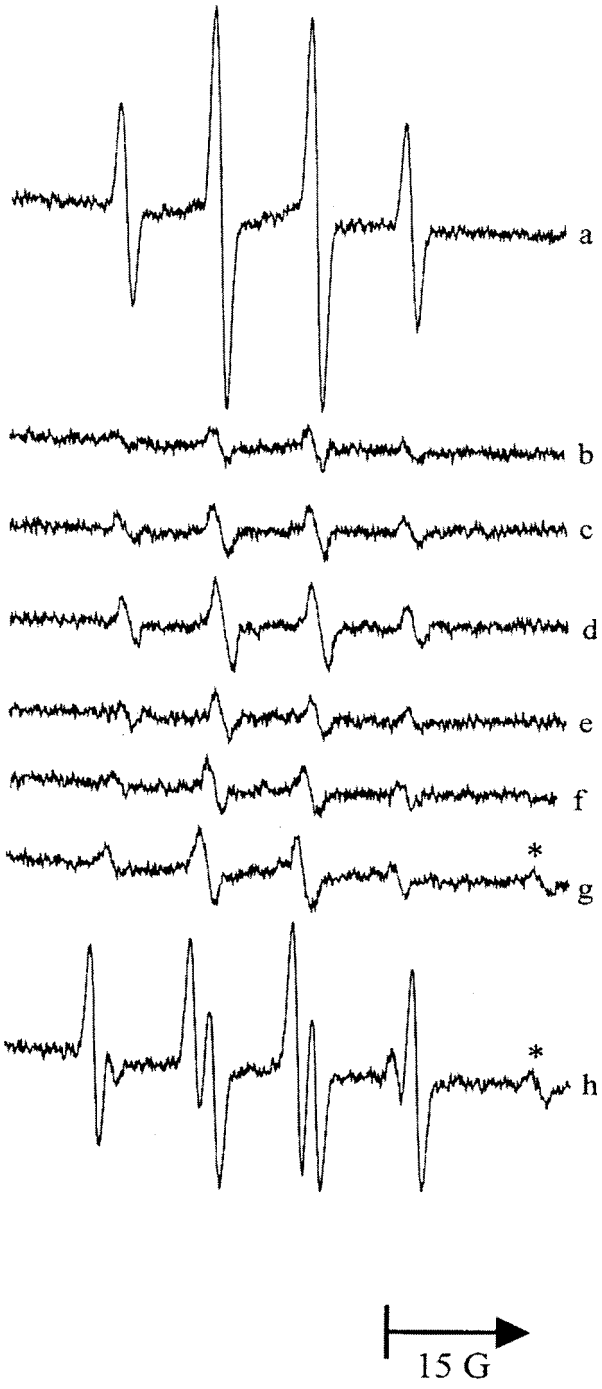

Fig. 1. ESR spectra recorded 3 min after reaction initiation from a pH 7.4 phosphate buffer solution containing 200 mM DMPO and the following reactants: (a) 50 μg/ml $PbCrO_4$, 5 mM NADPH, and 0.5 mg/ml GSSG-R; (b) 50 μg/ml $PbCrO_4$; (c) 50 μg/ml $PbCrO_4$ and 0.5 mg/ml GSSG-R; (d) 50 μg/ml $PbCrO_4$ and 5 mM NADPH; (e) 50 μg/ml $PbCrO_4$, 5 mM $NADP^+$, and 0.5 mg/ml GSSG-R; (f) 50 μg/ml $PbCrO_4$, 5 mM NADPH, and 0.5 mg/ml heat-denatured GSSG-R; (g) 50 μg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 2000 U/ml catalase; (h) 50 μg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 500 mM sodium formate. The ESR spectrometer settings were: receiver gain, 2.5×10^4; time constant, 0.04 sec; modulation amplitude, 1.0 G; scan time, 1 min; magnetic field, 3440 ± 100 G.

produced in the reaction system. Since DMPO/•OH could, in principle, arise from many sources other than •OH trapping we performed the competition experiment using sodium formate as an •OH radical scavenger and a source of a secondary radical to verify the presence of •OH radicals. In this competition experiment, •OH radical abstracts a hydrogen atom from formate to form a new radical, which was trapped by DMPO to generate a new spin adduct signal. As expected, addition of formate decreased the intensity of DMPO/•OH adduct signal and resulted in the appearance of a new spin adduct signal with a hyperfine splittings of a_H = 15.8 G and a_N = 18.8 G (Fig. 1h). These splittings are typical of those of DMPO/•COO^- adduct [35], demonstrating that the •OH radicals are indeed generated.

Effect of deferoxamine on •OH radical generation

In these experiments, chemical properties of $PbCrO_4$ were modified using chelator to examine the effect on •OH radical generation. Figure 2a shows an ESR spectrum generated from a mixture containing $PbCrO_4$, NADPH, GSSG-R, and DMPO. Addition of 0.5 mM deferoxamine decreased the DMPO/•OH signal (Fig. 2b). An increase in deferoxamine concentration (1 and 2 mM) further decreased the DMPO/•OH signal (Figs 2c and 2d).

H_2O_2 generation

H_2O_2 generation was measured from a mixture containing $PbCrO_4$, NADPH, and GSSG-R. As shown in Fig. 3, this mixture indeed generated H_2O_2. Replacement of NADPH by $NADP^+$ decreased the H_2O_2 generation as did heat-denatured (HD) GSSG-R. Omission of any one component resulted in a decrease in H_2O_2 production. N-ethylmeimide, a GSSG-R inhibitor, also inhibited H_2O_2 generation, indicating that H_2O_2 production was enzymatic.

Oxygen consumption

Since the reaction mixture of $PbCrO_4$, NADPH and GSSG-R generated •OH radical and no exogenous H_2O_2 was added, it is expected that the precursor of •OH is molecular oxygen. Oxygen consumption from a mixture containing $PbCrO_4$, NADPH, and GSSG-R was measured to verify the role of molecular oxygen in the mechanism of •OH generation from the reduction of $PbCrO_4$ by GSSG-R in the presence of NADPH. As shown in Fig. 4, a mixture of $PbCrO_4$, NADPH, and GSSG-R consumed molecular oxygen. $PbCrO_4$ alone or omission of any one component resulted in a significant decrease in molecular oxygen consumption.

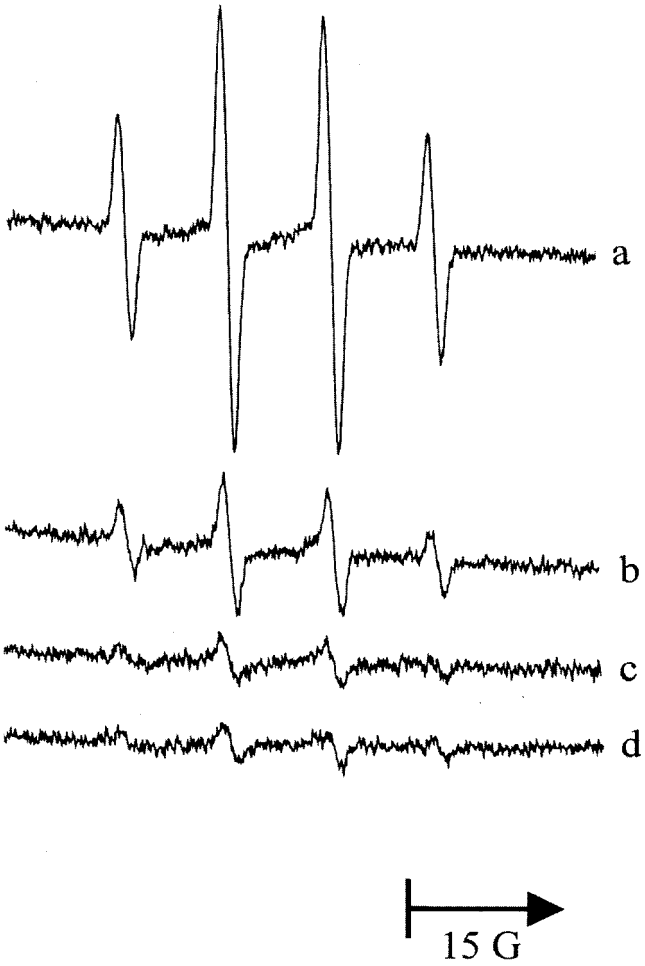

Fig. 2. ESR spectra recorded 3 min after reaction initiation from a pH 7.4 phosphate buffer solution containing 200 mM DMPO and the following reactants: (a) 50 μg/ml $PbCrO_4$, 5 mM NADPH, and 0.5 mg/ml GSSG-R; (b) 50 μg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 0.5 mM deferoxamine; (c) 50 μg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 1.0 mM deferoxamine; (d) 50 μg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 2.0 mM deferoxamine. The ESR spectrometer settings were: receiver gain, 2.5×10^4; time constant, 0.04 sec; modulation amplitude, 1.0 G; scan time, 1 min; magnetic field, 3440 ± 100 G.

DNA strand breaks

We examined whether •OH radicals generated by $PbCrO_4$ are able to cause DNA strand breaks. Figure 5, lane 1 shows that incubation of $PbCrO_4$ alone with DNA did not cause any observable DNA strand breaks. A mixture containing $PbCrO_4$, GSSG-R, and NADPH caused DNA strand breaks as seen by the smearing of the bands (Fig. 5, lane 2). Lane 3 shows $PbCrO_4$ and added H_2O_2 alone without GSSG-R or NADPH did not produce substantial strand breaks. $PbCrO_4$ plus H_2O_2, with GSSG-R and NADPH, enhanced the DNA strand breaks (Fig. 5, lane 4). Catalase inhibited the DNA strand breaks (Fig. 5, lane 5). Sodium formate inhibited the DNA strand

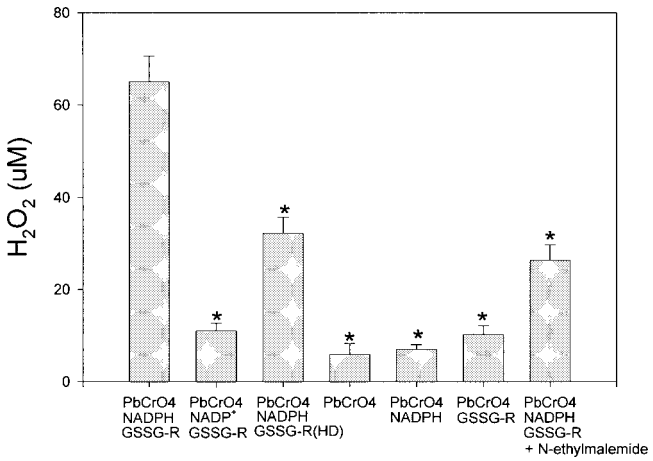

Fig. 3. H_2O_2 production in incubation mixtures containing 50 µg/ml $PbCrO_4$, 5 mM NADPH and 0.5 mg/ml GSSG-R, various controls, and effect of 2 mM N-ethylmaleimide. The data represent the mean values of 3 independent experiments. *indicates a significant difference from the control ($p < 0.05$).

breaks both without H_2O_2 present or with H_2O_2 present (Fig. 5, lanes 6 and 7).

Discussion

Results demonstrate that reduction of insoluble $PbCrO_4$ by GSSG-R in the presence of NADPH as a cofactor generates ·OH radicals. The mechanism involves a Fenton-like reaction. H_2O_2 was generated via reduction of molecular oxygen with $O_2^{\cdot-}$ as an intermediate. The ·OH radicals generated by $PbCrO_4$ are able to cause DNA strand breaks.

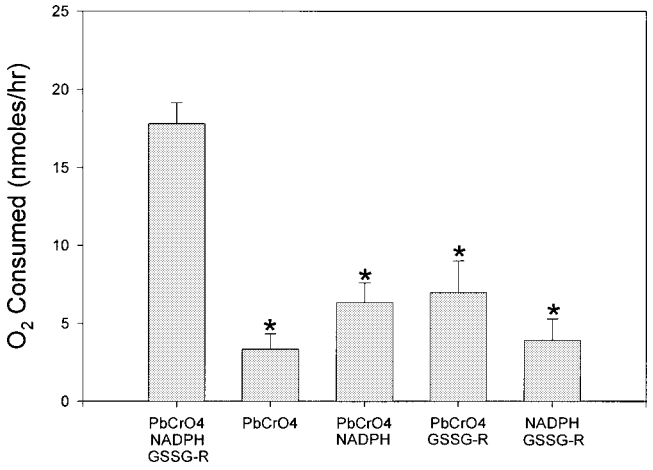

Fig. 4. O_2 consumption in incubation mixtures containing 50 µg/ml $PbCrO_4$, 5 mM NADPH, and 0.5 mg/ml GSSG-R and various controls. The data represent the mean values of 3 independent experiments. *indicates a significant difference from the control ($p < 0.05$).

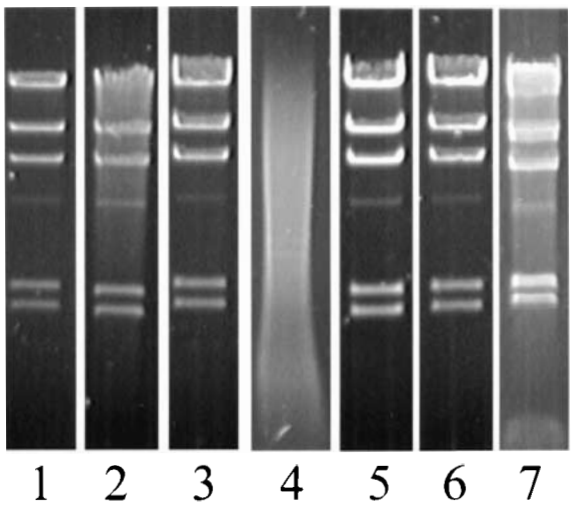

Fig. 5. DNA damage by $PbCrO_4$-mediated reactions. Lane 1 DNA and 50 µg/ml $PbCrO_4$; lane 2, DNA, 50 µg/ml $PbCrO_4$, 5 mM NADPH, and 0.5 mg/ml GSSG-R; lane 3, DNA, 50 µg/ml $PbCrO_4$, and 2 mM H_2O_2; lane 4, DNA, 50 µg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and H_2O_2; lane 5, DNA, 50 µg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 2000 U/ml catalase; lane 6, DNA, 50 µg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, and 500 mM sodium formate; lane 7, DNA, 50 µg/ml $PbCrO_4$, 5 mM NADPH, 0.5 mg/ml GSSG-R, 500 mM sodium formate, and 2 mM H_2O_2. Other experimental conditions are described in 'Materials and methods'.

It is generally believed that reduction of Cr(VI) to low oxidation states, such as Cr(V) and Cr(IV), is a key step for Cr(VI)-induced ROS generation and carcinogenesis [13]. Cr(VI) can be reduced by various reductants, such as glutathione, ascorbate, microsomes, mitochondria, and GSSG-R [13]. Using low frequency ESR, we have shown that reduction of Cr(VI) in live animals generates Cr(V)-NADPH complexes. GSSG-R is a likely candidate for the generation of these complexes. Using soluble Cr(VI), we have shown that reduction of Cr(VI) by GSSG-R generates a whole spectrum of ROS. The ROS generation by this enzyme may represent an important pathway in the mechanism of Cr(VI)-induced carcinogenesis. Thus in the present study, GSSG-R was used as a model Cr(VI) reductant. This enzyme is a ubiquitous flavoenzyme using NADPH as a cofactor to reduce oxidized glutathione (GSSG) and generate reduced glutathione (GSH). The cysteine residue is a key amino acid in the catalytic center of the enzyme. The present study shows that the $PbCrO_4$-mediated H_2O_2 and ·OH generation by GSSG-R/NADPH is through enzymatic reactions based on the following observations. (1) NADPH is required for this reaction. (2) Heat-denatured enzyme failed to generate ·OH radicals. (3) N-ethymalemide, a thiol blocker, inhibited the ROS generation. The inhibitory effect of N-ethymalemide also suggests that the adjacent cys residues were involved in the function of GSSG-R to reduce $PbCrO_4$ [15].

Based on the results obtained from the present study, it can be speculated that in the cellular system, the insoluble Cr(VI)-mediated free radical generation can occur by two pathways. (1) Free radicals can be generated through the reduction of Cr(VI) by GSSG-R in the presence of NADPH as a cofactor. Other reductants, such as ascorbate, thiol-containing molecules, microsomes, and mitochondria, may also contribute to Cr(VI) reduction and ROS generation by a similar mechanism. (2) Free radicals can also be generated by cells stimulated by Cr(VI)-containing insoluble particles. The generation of ROS represents one of the important mechanisms by which phagocytes kill invading organisms and other foreign substances. ROS production increases in response to stimulation and/or phagocytosis of microorganisms, particles, and chemicals, resulting in an increase in oxygen consumption called the 'respiratory burst'. Insoluble Cr(VI) containing particles could be potent stimulators of the respiratory burst in alveolar macrophages. The respiratory burst is associated with an elevated production of ROS. The ROS generation by both direct Cr(VI) reduction and Cr(VI) stimulation of cells can contribute to the overall mechanisms of Cr(VI)-induced carcinogenesis. Thus, it may be expected that the insoluble Cr(VI) compound may be more potent than soluble Cr(VI) in carcinogenesis.

This study also shows that $^{\bullet}OH$ radicals generated by $PbCrO_4$ are able to cause DNA strand breaks. It is known that $^{\bullet}OH$ radical reaction is extremely rapid. The $^{\bullet}OH$ radicals generated may not escape beyond the solvent cage before reacting with DNA in solution. For example, the $^{\bullet}OH$ radicals generated in the reaction of H_2O_2 with certain metal ions, such as nickel- [36], copper-, or zinc-containing SOD [37], exhibit very little reactivity. For example, $^{\bullet}OH$ radicals produced by these systems cannot be scavenged by ethanol or formate [36, 37]. The possible reason for this non-reactivity is believed to be that $^{\bullet}OH$ radicals are generated within the domain of certain macromolecules, and hence are not 'free' to exhibit significant reactivity. The results obtained from the present study show that $^{\bullet}OH$ radicals generated the reduction of $PbCrO_4$ by GSSG-R in the presence of NADPH have the ability to cause DNA damage.

It is well established that ROS can cause various types of cellular damage, for example, DNA strand breaks, hydroxylation of dG residue, lipid peroxidation, and protein modification. Using soluble Cr(VI), we have shown that through free radical reactions Cr(VI) is able to cause activation of nuclear transcription factors (NF-κB, AP-1, and p53) [17–19], apoptosis [20], cell growth arrest [21], oncogene expression [22], and activation of certain family members of mitogen-activated protein (MAP) kinases [18]. Accumulative evidence suggests that ROS can also alter cell function by acting as, mimicking, or affecting the intermediates or second messengers in signal transduction. Since insoluble Cr(VI) compounds are also capable of generating ROS, it is expected these insoluble Cr(VI) compounds may cause cell injury and activate signal transduction events via a mechanism similar to that of soluble Cr(VI) compounds.

Our results show that deferoxamine inhibited the $^{\bullet}OH$ radical generation. This chelator is used for the prevention and treatment of iron overload [38] as well as for combating the toxic effect of vanadium [39]. It appears that proper chelation of the surface of the insoluble Cr(VI)-containing particle can be used to inhibit $^{\bullet}OH$ radical generation and decrease the potency of these insoluble Cr(VI) particles to induce cancer.

In conclusion, the results obtained from the present study show the following. (a) Reduction of $PbCrO_4$ by GSSG-R in the presence of NADPH as a cofactor generates $^{\bullet}OH$ radical. (b) The $^{\bullet}OH$ radical generation involves a Fenton-like reaction. H_2O_2 is generated by the reduction of molecular oxygen via $O_2^{\bullet-}$ as an intermediate. The $^{\bullet}OH$ radicals generated by the enzymatic $PbCrO_4$ reduction are capable of causing DNA damage. Metal chelator, deferoxamine, inhibited the $^{\bullet}OH$ radical generation caused by $PbCrO_4$. Since insoluble Cr(VI) compounds are able to generate ROS, it is expected that these compounds may cause oxidative damage and act on cell signal transduction systems by a mechanism similar to that of soluble Cr(VI) compounds.

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