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CHROMIUM (V) AND HYDROXYL RADICAL FORMATION DURING THE GLUTATHIONE REDUCTASE-CATALYZED REDUCTION OF CHROMIUM (VI)[1]

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ABSTRACT: Electron spin resonance measurements provide evidence for the formation of long-lived Cr(V) intermediates in the reduction of Cr(VI) by glutathione reductase in the presence of NADPH and for the hydroxyl radical formation during the glutathione reductase catalyzed reduction of Cr(VI). Hydrogen peroxide suppresses Cr(V) and enhances the formation of hydroxyl radicals. Thus Cr(V) intermediates catalyze generation of hydroxyl radicals from hydrogen peroxide through a Fenton-like reaction. Thus the mechanism of Cr(VI) toxicity might involve the interaction between macromolecules and the hydroxyl radicals.

C(VI) compounds have been found to exert serious toxic and carcinogenic effects on humans and animals (1-3). In contrast, most Cr(III) compounds are relatively nontoxic, noncarcinogenic, and nonmutagenic (1,4). Cr(VI) and Cr(III) oxidation states are different in their metabolic pathways: Cr(VI) ions are rapidly transported across cellular membrane (1,4,5), while Cr(III) moieties do not easily penetrate cells and are not oxidized by cellular constituents (1). Since it is known that the reduction of Cr(VI) ion is required for its reaction with DNA (1), the molecular mechanisms for the intracellular Cr(VI) reduction has been the focus of current studies but the details are still not understood.

Earlier studies on the mechanism of Cr(VI) reduction include those on the reduction of Cr(VI) by glutathione (GSH) (1,6-8), ascorbic acid (9), glucose (10), lactose (10), galacturonic acid (11), microsome (12), and mitochondrial electron transport chain complexes (13). These studies have shown that the Cr(VI) reduction involves the formation of Cr(V)-containing species which are often considered to be the toxic form of chromium. Thus far, however, no information is available on the subsequent fate of those biologically formed

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Cr(V) species, in particular employing well defined biochemical systems. In this context, we report here our investigations of the reduction of Cr(VI) by glutathione reductase (GSSG-R). This enzyme was selected because it is ubiquitously present in cellular systems and it has been postulated to be involved in the Cr(VI) reduction (14-17) but the underlying mechanism has not been clarified. We find that the reduction process involves not only Cr(V) but also ·OH radicals. We further investigate the reactivity of Cr(V) and find that it catalyzes generation of ·OH radical from H_2O_2 through a Fenton-like reaction. The role of ·OH in Cr(VI) carcinogenicity is suggested from this study.

MATERIALS AND METHODS: Dimethyl sulfoxide (DMSO), 1,3-dimethyl-2-thiourea (DMTU), ethanol, hydrogen peroxide, and Potassium dichromate were purchased from Fisher. Glutathione reductase (GSSG-R) from bovine intestinal mucosa, superoxide dismutase (SOD) from bovine blood, catalase from bovine liver, sodium formate, NADPH, and N-ethylmaleimide were purchased from Sigma and used as received. Spin traps, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), were purchased from Aldrich, and were used without further purification, since very week or no spin adduct signal was obtained from the purchased sample when used alone. The buffer of pH = 7.2 was purchased from Fisher while those of pH 4.0-6.5 were obtained from Aldrich.

ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER 200D ESR spectrometer. For accurate measurements of the g-values and the hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ER035A) and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, Model 5340A). An ASPECT 2000 computer was used for data acquisition and analysis. The concentrations given in the Figure legends are final concentrations. All experiments were carried out at room temperature.

RESULTS: An aqueous Cr(VI) solution at pH = 7.2 containing low (0.9 mM) concentration of NADPH did not give a detectable ESR signal (Figure 1(a)), that of Cr(VI) containing GSSG-R gave only a weak ESR signal (Figure 1(b)), but that containing both NADPH and GSSG-R gave a much stronger ESR spectrum (Figure 1(c)). In order to better analyze Figure 1(c), a spectrum was recorded at a higher modulation amplitude and a wider scan width. The spectrum obtained (Figure 1d) was similar to those reported earlier for Cr(V) complexes with ethylene glycol (18,19), ascorbic acid (9), glucose or lactose (10), and galacturonic acid (11): the center of the spectrum centered at g = 1.9785 with four satellite signals due to ^{53}Cr (9.55% abundance, I = 3/2) hyperfine structure. The observed ^{53}Cr hyperfine coupling of 17.8 G (indicated in Figure 1(d) is very similar to those observed for Cr(V) complexes with oxygen ligands (9-11,19). The spectra in Figure 1(c) and (d) were thus assigned to Cr(V) species. When the spectrum was recorded 10 minutes after the reaction initiation, the overall signal intensity was found to decrease significantly (Figure 1(e)), showing that the Cr(V) species are reactive but fairly long-lived reactive intermediates. Figure 1(f) shows the effect of

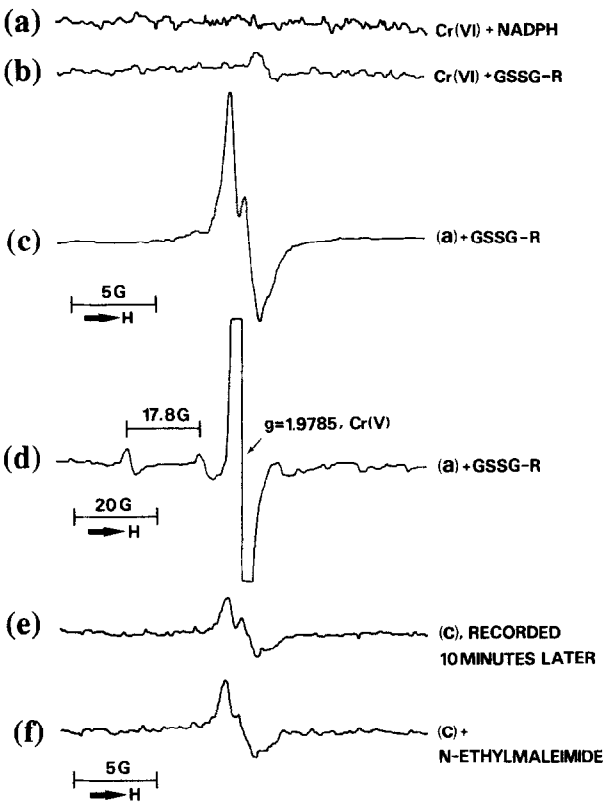

Figure 1. ESR spectra recorded 2 minutes after mixing 1.9 mM $K_2Cr_2O_7$ buffer solution (pH = 7.2) with (a) 0.9 mM NADPH; (b) 12 units/ml GSSG-R; (c) 0.9 mM NADPH and 12 units/ml GSSG-R; (d) same as (c) but the spectrum was recorded at a higher gain and a wider scan width; (e) same as (c) but the spectrum was recorded 10 minute after mixing; (f) same as (c) but with 200 mM N-ethylmaleimide added.

N-ethylmaleimide, the GSSG-R inhibitor (14,15). By comparing the spectra in Figure 1(c) and 1(f), it can be seen that N-ethylmaleimide significantly inhibits the Cr(V) formation, showing that the reaction proceeds enzymatically.

Figure 2 shows some typical spectra of the ESR spin trapping measurements. A aqueous solution of the spin trap DMPO with Cr(VI) and GSSG-R did not give a detectable ESR spectrum (Figure 2(a)). When DMPO, Cr(VI) and NADPH were mixed, a relatively small spin adduct signal was observed (Figure 2(b). The addition of GSSG-R to the solution containing DMPO, Cr(VI) and NADPH generates an ESR spectrum, consisting of a 1:2:2:1 quartet with splittings of $a_N = a_H = 14.9$ G (Figure 2(c), and a Cr(V) peak as discussed above. Based on the splitting constants, the 1:2:2:1 quartet was assigned to the DMPO-OH adduct (20,21). Since the DMPO-OH adduct could, in principle, arise from many sources, we have carried out the standard competition experiments in which the ·OH radical abstracts a hydrogen atom from ethanol or formate, with the trapping of new radical (20,21). Thus, if the production of the DMPO-OH adduct were due to the

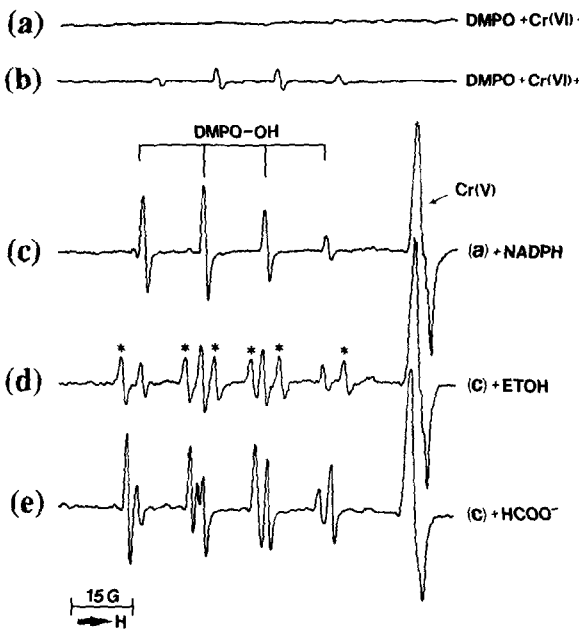

Figure 2. ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO and 2 mM $K_2Cr_2O_7$ with (a) 12 units/ml GSSG-R; (b) 2 mM NADPH; (c) 12 units/ml GSSG-R and 2 mM NADPH; (d) same as (c) but with 5% ethanol added; (e) same as (c) but with 500 mM sodium formate added. Spectrometer settings were: receiver gain, 1.25 x 10^5; modulation amplitude, 1.25 G; scan time, 200 seconds; field, 3480 ± 50 G; time constant, 0.5 second. The asterisks indicate the DMPO-$CHOHCH_3$ spin adduct signal.

trapping of ·OH radicals, the addition of either ethanol or sodium formate to the solution should inhibit the production of the DMPO-OH adduct and result in the appearance of a new ESR signal from DMPO adduct of the secondary radical. As expected, addition of ethanol decreases the intensity of the DMPO-OH spin adduct signal and results in the appearance of a new spin adduct signal (as indicated by the asterisks in Figure 2(d)) with measured splitting constants, a_N = 15.8 G and a_H = 22.8 G. These splitting constants are typical of those of the DMPO-$CHOHCH_3$ adduct (21-23). Similar results were obtained using sodium formate (Figure 2(e)). These results indicate that the DMPO-OH adduct formation (Figure 2c) is a result of the trapping of the ·OH radicals formed during the reaction and not from an artifact, such as the possible hydrolysis of the nitrone spin trap.

Additional support for the ·OH radical identification was obtained by using ·OH radical scavenger, DMTU. The spectrum in Figure 3(a) shows the result without DMTU and that in Figure 3(b) in the presence of DMTU. As can be seen from Figure 3(c), the addition of catalase enhances Cr(V) and substantially suppresses the ·OH radical formation, implying the possible involvement of H_2O_2 in the mechanism of the ·OH radical generation. An addition of SOD, however, effects neither Cr(V) nor ·OH radical generation,

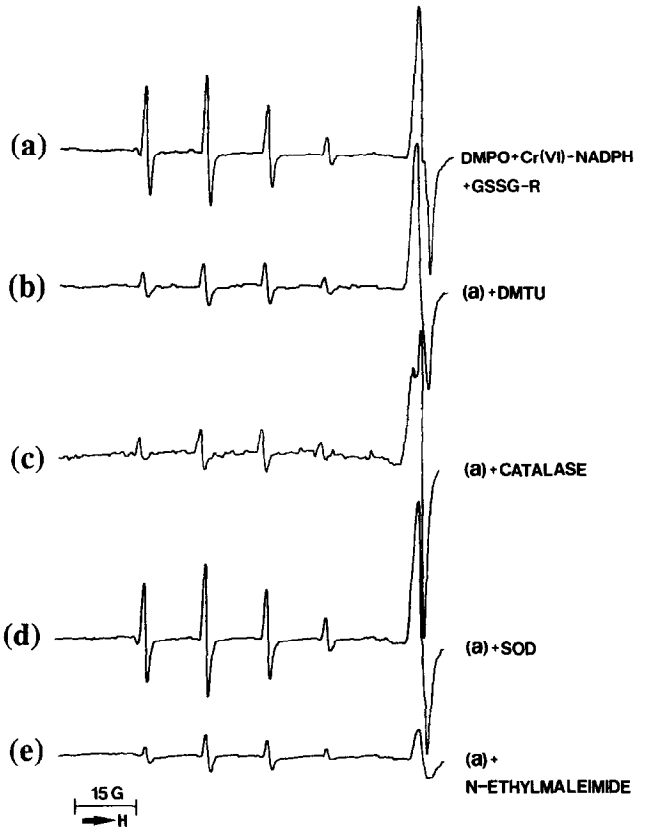

Figure 3. (a) ESR spectrum recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO, 2 mM $K_2Cr_2O_7$, 12 units/ml GSSG-R, and 2 mM NADPH; (b) same as (a) but with 200 mM DMTU added; (c) same as (a) but with 5000 units/ml catalase added; (d) same as (a) but with 200 units/ml SOD added; (e) same as (a) but with 200 mM N-ethylmaleimide added. Spectrometer settings were the same as those in Figure 3.

showing that O_2^- was not involved in the reaction mechanism (Figure 3(d)). Figure 3(e) shows the result of adding N-ethylmaleimide, a GSSG-R inhibitor. This spectrum clearly shows the inhibitive effect of N-ethylmaleide on the formation of both Cr(V) and ·OH radicals, providing additional evidence that the generation of both Cr(V) and ·OH radicals proceeds enzymatically.

Figure 4(a) shows the ESR spectrum from a mixture containing H_2O_2, GSSG-R and DMPO. Figure 4(b) corresponds to a reaction mixture containing H_2O_2, DMPO, and Cr(VI) and Figure 4(c) to a reaction mixture containing H_2O_2, DMPO, GSSG-R, and Cr(VI). Figure 4(d) shows the ESR spectrum from a reaction mixture containing H_2O_2, DMPO, Cr(VI), and NADPH. The 1:2:2:1 quartet in Figure 4(d) was assigned to the DMPO-OH adduct, as discussed earlier. On adding GSSG-R, the intensity of the DMPO-OH adduct (Figure 4(e)) increased substantially. For the convenience of direct comparison of the effect of H_2O_2 on the ·OH radical generation, the ESR spectrum observed from the reaction containing DMPO, GSSG-R, Cr(VI), and NADPH without H_2O_2 is provided in Figure 4(f). From the

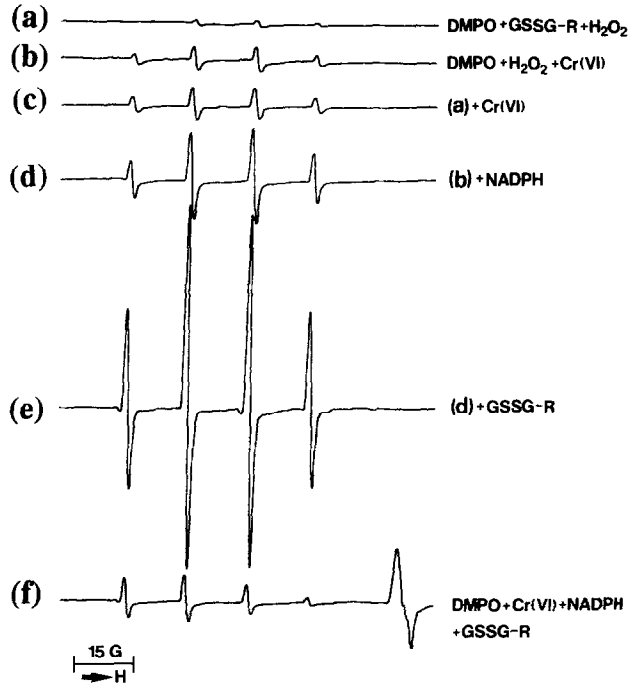

Figure 4. ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO with (a) 12 units/ml GSSG-R and 3 mM H_2O_2; (b) 3 mM H_2O_2 and 2 mM $K_2Cr_2O_7$; (c) 12 units/ml GSSG-R, 3 mM H_2O_2 and 2 mM $K_2Cr_2O_7$; (d) 3 mM H_2O_2, $K_2Cr_2O_7$ and 2 mM NADPH; (e) same as (d) but with 12 units/ml GSSG-R added; (f) same as (e) but without H_2O_2.

Spectrometer settings were: receiver gain, 4.0×10^4; modulation amplitude, 1.25 G; scan time, 200 seconds; field, 3480 ± 50 G; time constant, 0.5 second.

spectra obtained with and without H_2O_2 (Figure 4(e) and (f)), it can be noted that H_2O_2 greatly enhances the ·OH radical formation (7 times), and reduces the Cr(V) formation, indicating that the formation of ·OH radicals involves a reaction between the Cr(V) species and H_2O_2.

DISCUSSION: GSSG-R, a ubiquitous FAD-containing enzyme, uses NADPH to reduce oxidized glutathione (GSSG) and generate GSH. The enzyme's general function is to maintain a high level of GSH in the cytosol, but it might have additional roles. Earlier investigators reported the NADPH dependent inhibition of GSSG-R by Cr(VI) (14,15). The mechanism of inhibition of GSSG-R by Cr(VI) was hypothesized to be linked to the reduction of chromium from Cr(VI) to Cr(III) (14,15), but the mechanistic details (such as the formation of Cr(V)) were not established. The results reported here provide direct evidence for the NADPH dependent reduction of Cr(VI) by GSSG-R with Cr(V) as an intermediate, thus indicating that at least one of the the initial steps in the Cr(VI) reduction involves a one-electron transfer process.

It has been reported that certain enzymes of the cytoplasm, endoplasmic reticulum, and mitochondria of mammalian cells catalyze Cr(VI) reduction

(1,12,13,24). Jennette reported Cr(V) formation in the incubation of Cr(VI) with liver microsomes in the presence of NADPH (12). Even though the details were not clear, Jennette suggested that a direct one-electron transfer from the microsomal electron-transport cytochrome P-450 system to Cr(VI) is a likely mechanism (12). Since Cr(V) complexes are generally characterized as being labile and reactive, whereas Cr(III) complexes are substitutionally inert, the detection of Cr(V) formation led Jennette (1,12) to suggest that the Cr(V) intermediates are the likely candidates for the "ultimate" carcinogenic forms of carcinogenic chromium species. This suggestion has led to many further investigations of the formation of Cr(V) in the reduction of Cr(VI) under biologically relevant conditions (6-13,25,29). Howerer, to the best of our knowledge, no information is available on the subsequent fate of those biologically formed Cr(V) species. The results presented here show that Cr(VI) can be enzymatically metabolized to Cr(V) with simultaneous formation of ·OH radical. It is also found that addition of H_2O_2 to the above mentioned enzymatic Cr(VI) reaction system yields much higher concentration of ·OH radicals, with concommitant suppression of the Cr(V) formation. This indicates that the Cr(V) complexes generated in the enzymatic Cr(VI) reduction have an ability to react with H_2O_2 to generate ·OH radicals. Thus Cr(V) intermediates catalyze generation of ·OH radicals from hydrogen peroxide through a Fenton-like reaction. Since Cr(V) species are known to be generated in the reduction of Cr(VI) by various biological reductants (6-13,25,26) and H_2O_2 is a normal biological metabolite, the reaction of Cr(V) complexes with H_2O_2 could become an important source of ·OH radicals. The present work implies that under *in vivo* conditions, ·OH radicals might be the "ultimate" carcinogenic species in the mechanism of Cr(VI) carcinogenesis. Thus strategies for combating Cr(VI) toxicity should take into account the role of the ·OH radicals, and hence steps for blocking its chain propagation.

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