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Role of Molecular Oxygen in the Generation of Hydroxyl and Superoxide Anion Radicals During Enzymatic Cr(VI) Reduction and its Implication to Cr(VI)-Induced Carcinogenesis

Stephen Leonard, Suwei Wang,* Lunyi Zang, Vince Castranova, Val Vallyathan, and Xianglin Shi*

Electron spin resonance (ESR) spin trapping measurements provide evidence for the generation of hydroxyl radicals (•OH) in the reduction of Cr(VI) by glutathione reductase (GSSG-R) in the presence of NADPH as a cofactor. Catalase inhibited the •OH generation, while the addition of H_2O_2 enhanced it, indicating that the •OH radical generation involves a Fenton-like reaction. The metal chelator, deferoxamine, inhibited the •OH generation with a concomitant generation of a deferoxamine nitroxide radical. EDTA and 1,10-phenanthroline also inhibited the •OH generation. Experiments performed under argon atmosphere decreased the yield of the •OH formation, showing that molecular oxygen plays a critical role. ESR spin trapping and measurements of fluorescence change of scopoletin in the presence of horseradish peroxidase show that reduction of Cr(VI) by GSSG-R/NADPH generates superoxide anion radicals ($O_2^{\bullet -}$) as well as H_2O_2. It can be concluded that •OH radical is generated by the reaction of H_2O_2 with Cr(V), which is produced by enzymatic one-electron reduction of Cr(VI). H_2O_2 is produced by the reduction of molecular oxygen via $O_2^{\bullet -}$ as an intermediate. The •OH radicals generated by these reactions are capable of causing DNA strand breaks, which can be inhibited by catalase, formate, and experiments performed under argon.

KEY WORDS: molecular oxygen, hydroxyl radicals, superoxide anion radicals, electron spin resonance, chromium carcinogenesis, metal chelators.

Introduction

Cr(VI)-containing compounds, used widely in industries, are established carcinogens.[1] These compounds have been found to exert toxic and carcinogenic effects on humans and animals[1] and to cause mutation in bacteria and transformation of mammalian cells.[2,3] Epidemiological studies have shown that workers exposed to Cr(VI) had a higher incidence of respiratory cancers than the general population.[4] Although the mechanism is still not well understood, it is generally considered that the carcinogenic effect of Cr(VI) is attributed to cellular uptake because Cr(VI) and not Cr(III) actively enters cells by the sulfite transport mechanism.[5] 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 cause DNA damage. They are also able to generate hydroxyl radicals (•OH) from H_2O_2 via Fenton-

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like reactions.[6–9] The •OH radicals generated by these reactions cause DNA strand breaks and dG hydroxylation[9] and induce activation of nuclear transcription factor, NF-κB.[10] It is generally believed that reactive chromium intermediates and reactive oxygen species (ROS) generated by these intermediates play a key role in the mechanism of Cr(VI)-induced carcinogenesis.[10]

Recent studies have shown that Cr(VI) can be reduced by various reductants, such as ascorbate,[9] glutathione,[11] and glutathione reductase (GSSG-R) with NAD(P)H as a cofactor.[6] Using electron spin resonance (ESR) with loop-gap resonator, we have shown that reduction of Cr(VI) by whole live animal generates Cr(V)-NAD(P)H complexes.[12] The flavoenzymes such as GSSG-R are likely candidates responsible for Cr(V) generation. We have shown that reduction of Cr(VI) by GSSG-R in the presence of NAD(P)H generates Cr(V)-NAD(P)H complexes as well as •OH radicals in vitro.[6] Although these studies provided important information regarding Cr(VI) reduction and its related •OH generation, many questions remain to be answered. For example, what is the mechanism of •OH radical generation? Does molecular oxygen play a key role in the •OH generation? Does reduction of Cr(VI) by GSSG-R/NADPH generate superoxide radicals ($O_2^{\bullet-}$) and/or H_2O_2? Do •OH radicals generated by this system cause DNA strand breaks? The major goals of the present study are focused on answering these questions.

Materials and Methods

Reagents

Potassium dichromate ($K_2Cr_2O_7$), deferoxamine, 1,10-phenanthroline, 5,5-dimethyl-l-pyrroline N-oxide (DMPO), glutathione reductase (GSSG-R), sodium formate, H_2O_2, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), nicotinamide adenine dinucleotide 3′-phosphate ($NADP^+$), and *N*-ethylmaleimide were purchased from Sigma (St. Louis, MO). Superoxide dismutase (SOD) and catalase were purchased from Boeh-ringer Mannheim (Indianapolis, IN). EDTA was purchased from Fisherbiotech (Pittsburgh, PA). Compressed argon gas was purchased from Butler Gas Products (McKees Rocks, PA). DNA/Hind III fragments and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD). 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). 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 intensity of the spin adduct signal represents the concentration of the radicals to be detected. 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 Varian E9 ESR spectrometer 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. An EPRDAP 2.0 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 except those under Argon. Samples were mixed and sealed in an Atmosbag® (Aldrich Chemical Co. Milwaukee, WI) filled with argon and all reagents were flushed with argon before use.

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 PerSeptive Biosystems Cytofluor multiwell plate reader se-

ries 4000 (PerSeptive Biosystems Inc., Framingham, MA).

DNA Strand Breakage Assay

The DNA strand breakage assay was carried out according to methods described earlier.[13] Briefly, reactions were performed in 10 m*M* phosphate-buffered saline (pH 7.4) in 1.5 ml polypropylene tubes at 37 °C. Each reaction mixture contained 10 mg DNA (Hind III fragments) in a total volume of 100 mL of buffer. To this solution, 2 mL of gel-loading buffer (50 m*M* EDTA, 2.5% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue) was added, and then electrophoresed in 0.7% agarose at 1 to 2 V/cm in 40 m*M* tris acetate buffer containing 2 m*M* EDTA (pH 8.0). Gels were stained with ethidium bromide (5 μg/ml) for 30 minutes and photographed under UV light using a Stratagene Eagle Eye II.

Oxygen Consumption Measurements

Oxygen consumption measurements were carried out using Gilson oxygraph (Gilson Medical Electronics, Middleton, WI). Measurements were made in a system containing 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R and various controls in pH 7.4 phosphate buffer. The oxygraph was standardized in media equilibrated with gases of known concentrations.

Results

•OH Radical Generation

Figure 1*a* shows a typical ESR spectrum generated from a mixture containing Cr(VI), NADPH and GSSG-R in the presence of DMPO as a spin trap. This spectrum consists of a 1:2:2:1 quartet with splittings of $a_H = a_N = 14.9$ G. Based on these splittings constants, the 1:2:2:1 quartet was assigned to a DMPO/•OH adduct. The peak at g = 1.9792 was assigned to a Cr(V)-NADPH complex as reported earlier.[6–9] Replacement of NADPH by $NADP^+$ abolished the generation of both Cr(VI) and hydroxyl radical (Figure 1*b*). Inactivated GSSG-R generated a very weak ESR signal (Figure 1*c*). Omission of either GSSG-R or NADPH results in a decrease in the overall spectral intensity (Figures 1*e* and 1*f*). Figure 1*g* shows the inhibitory effect of *N*-ethylmaleimide, a GSSG-R inhibitor, on the generation of both •OH and Cr(V). A confirmational piece of evidence for the •OH radical trapping was obtained through the •OH scavenging competition experiment involving formate. In this competition experiment, •OH radical abstracts a hydrogen atom from formate to form a new radical, which will be 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 (Figure 1*h*). These splittings are typical of those of DMPO/$•COO^-$ adduct,[14] demonstrating the generation of the •OH radicals. Addition of catalase, whose function is to remove H_2O_2, inhibited •OH radical generation (Figure 1*i*), whereas the addition of H_2O_2 enhanced it (Figure 1*j*), showing that •OH radicals were generated via Cr(V)-mediated Fenton-like reaction ($Cr(V) + H_2O_2 \rightarrow Cr(VI) + •OH + OH^-$).

Effect of Deferoxamine on •OH Radical Generation

In the earlier section, it has been demonstrated that Cr(V) is involved in the •OH radical generation via Fenton-like reaction. In this section, chemical properties of Cr(V) were modified using chelator to examine the effect on •OH radical generation. Figure 2*a* shows an ESR spectrum generated from a mixture containing Cr(VI), NADPH, GSSG-R, and DMPO. The addition of 0.5 m*M* deferoxamine decreased the DMPO/•OH signal with the appearance of deferoxamine nitroxide radical (marked by plus signs) and enhanced Cr(V) signal. An increase in deferoxamine concentration (1 m*M*) further decreased the DMPO/ •OH signal. At 2 m*M*, deferoxamine abolished both DMPO/•OH and Cr(V) signals with an enhancement of DMPO adduct of deferoxamine nitroxide radical signal. The hyperfine splittings of this adduct are $a_H = 7.7$ G and $a_N = 6.2$ G and are in agreement with those reported in the literature.[15] Other metal chelators, 1,10 phenanthroline and EDTA, at concentrations of 2 m*M* blocked both the DMPO/•OH and Cr(V) signals (Figures 2*e* and 2*f*).

H_2O_2 Generation

The previous section shows that H_2O_2 was involved in the •OH radical generation via Fenton-like reaction. In this section, the H_2O_2 generation was measured from a mixture containing Cr(VI), NADPH, and GSSG-R. As shown in Figure 3, this mixture generated H_2O_2, which caused a change in fluorescence of scopoletin in the presence of horseradish peroxidase. Replacement of NADPH

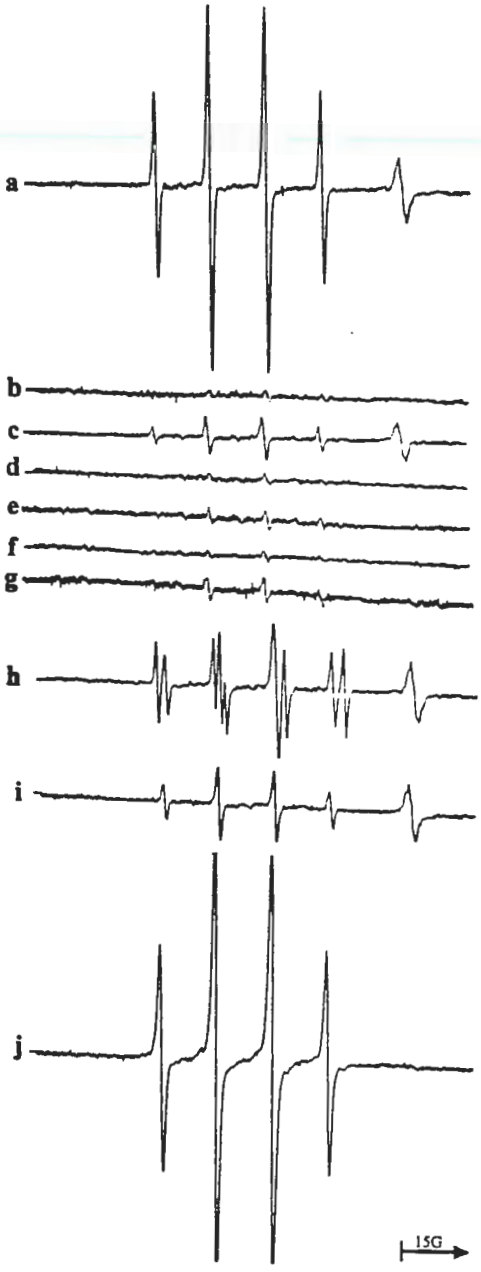

FIGURE 1. ESR spectra recorded 3 min after reaction initiation from a pH 7.4 phosphate buffer solution of 50 m*M* DMPO and the following reactants: (a) 2 m*M* Cr(VI), 1 m*M* NADPH and 0.5 mg/mL GSSG-R; (b) 2 m*M* Cr(VI), 1 m*M* $NADP^+$ and 0.5 mg/mL GSSG-R; (c) 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL heat-denatured GSSG-R; (d) 2 m*M* Cr(VI); (e) 2 m*M* Cr(VI) and 1 m*M* NADPH; (f) 2 m*M* Cr(VI) and 0.5 mg/mL GSSG-R; (g) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2 m*M* *N*-ethylmaleimide; (h) 2 m*M* Cr(VI), 1m*M* NADPH , 0.5 mg/mL GSSG-R, and 50 m*M* formate; (i) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2000 U/mL catalase; (j) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2 m*M* H_2O_2. The ESR spectrometer settings were receiver gain, 2.5×10^5; time constant 0.25 s; modulation amplitude, 0.5 G; scan time, 8 min; magnetic field, 3375 ± 80 G.

by $NADP^+$ decreased the H_2O_2 generation and so did heat-denatured (HD) GSSG-R. Omission of any one component resulted in the decrease in H_2O_2 production. *N*-ethylmeimide, a GSSG-R inhibitor, also inhibited H_2O_2 generation, indicating that an enzymatic reaction is involved in the mechanism of H_2O_2 production.

Role of Molecular Oxygen in •OH Radical Generation

As shown in the previous sections, •OH radicals are generated via Cr(V)-mediated Fenton-like reaction. It can be assumed that H_2O_2 was generated by one-electron reduction of molecular oxygen during the enzymatic reduction of Cr(VI). Figure 4*a* shows the ESR spectrum recorded from a mixture containing Cr(VI), NADPH, and GSSG-R. Both DMPO/•OH and Cr(V) signals decreased when the same experiment was performed in argon atmosphere, demonstrating the role of molecular oxygen in the mechanism of •OH radical generation.

Oxygen Consumption

Oxygen consumption from the mixture containing Cr(VI), NADPH, and GSSG-R was measured to support the role of molecular oxygen in the mechanism of •OH generation from the reduction of Cr(VI) by GSSG-R in the presence of NADPH. As shown in Figure 5, a mixture of Cr(VI), NADPH, and GSSG-R consumed molecular oxygen. Cr(VI) alone or omission of any one component resulted in a sharp decrease in molecular oxygen consumption.

Superoxide Radical Generation

From the studies described above, it is evident that •OH radicals are generated by reaction of Cr(V) with H_2O_2, which was produced by reduction of molecular oxygen. $O_2^{\bullet-}$ was implied in the generation of H_2O_2. In this section, we attempted to detect $O_2^{\bullet-}$ radical generation. From Figure 1*a*,

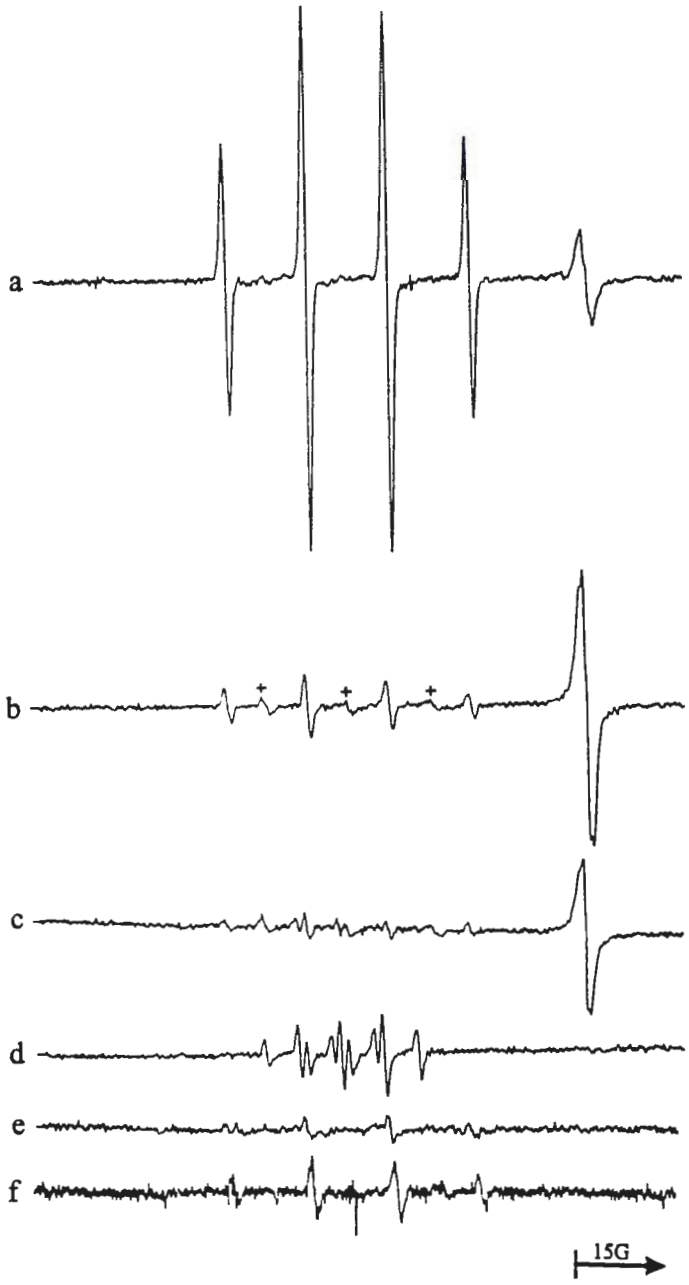

FIGURE 2. ESR spectra recorded 3 min after reaction initiation from a pH 7.4 phosphate buffer solution of 50 m*M* DMPO and the following reactants: (a) 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R; (b) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 0.5 m*M* desferoxamine; (c) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 1.0 m*M* desferoxamine; (d) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2.0 m*M* desferoxamine; (e) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2.0 m*M* EDTA; (f) 2 m*M* Cr(VI), 1 m*M* NADPH, 0.5 mg/mL GSSG-R, and 2.0 m*M* 1,10-phenanthroline. The ESR spectrometer settings were receiver gain, 2.5×10^5; time constant 0.25 s; modulation amplitude, 0.5 G; scan time, 8 min; magnetic field, 3375 ± 80 G.

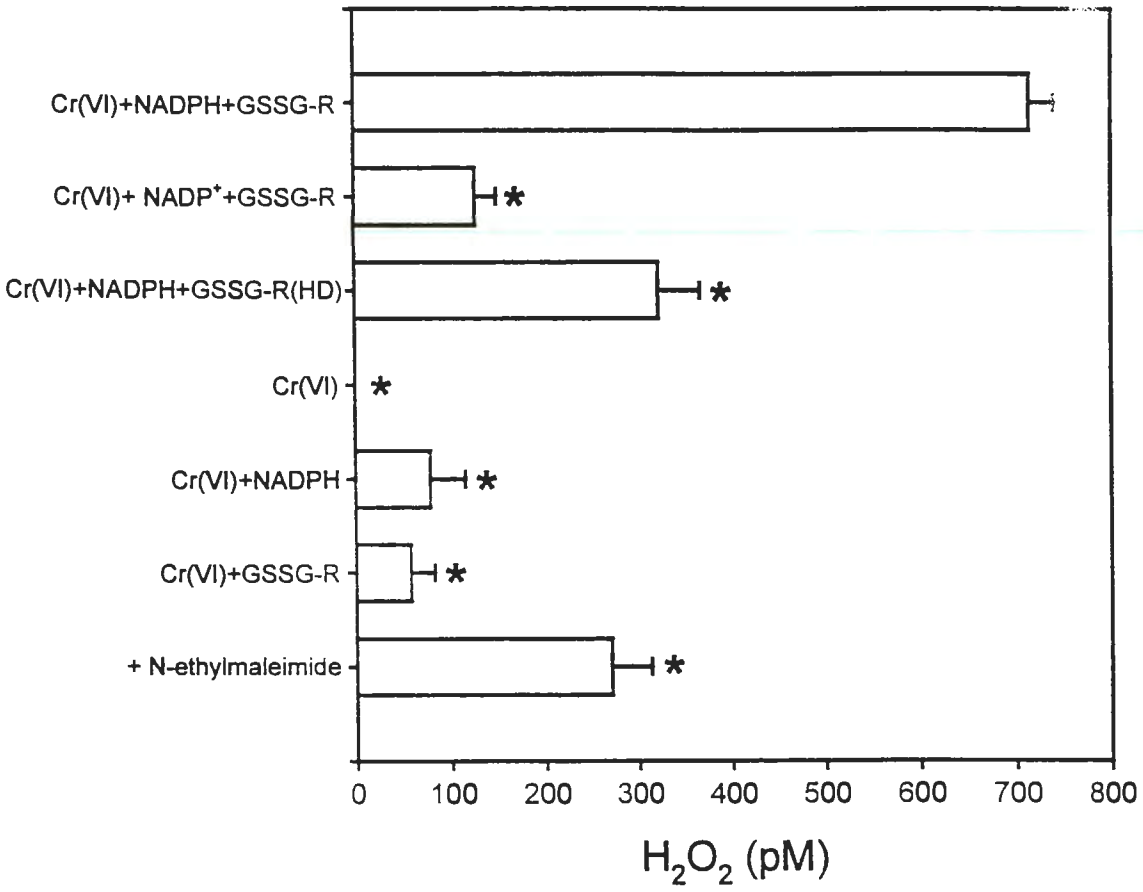

FIGURE 3. H_2O_2 production in incubation mixtures containing 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R, various controls, and effect of 2 m*M* *N*-thylmaleimide.

it may be noted that only DMPO/$^{\bullet}$OH and Cr(V) signals were detected from a mixture containing Cr(VI), NADPH, and GSSG-R. No DMPO/$O_2^{\bullet-}$ signal was observed. In earlier studies,[16,17] we have shown that Cr(VI) and especially Cr(V) can be reduced by $O_2^{\bullet-}$. It is possible that $O_2^{\bullet-}$ radicals were generated in the mixture and reacted with Cr(V). In the present attempt to detect $O_2^{\bullet-}$ radicals, deferoxamine at different concentrations was added to the mixture containing Cr(VI), NADPH, and GSSG-R to modulate the reaction of Cr(V) toward $O_2^{\bullet-}$. Figure 6*a* shows the spectrum recorded from a mixture of Cr(VI), NADPH, GSSG-R, and 2 m*M* deferoxamine. This spectrum consisted of three spin adduct signals, DMPO/$^{\bullet}$OH, DMPO/$O_2^{\bullet-}$ (marked by circles), and DMPO adduct of deferoxamine nitroxide radical (marked by plus signs). The addition of SOD did not effect DMPO/$^{\bullet}$OH, DMPO adduct of deferoxamine nitroxide radical, or Cr(V) signals but abolished the DMPO/$O_2^{\bullet-}$ signal, further showing the $O_2^{\bullet-}$ radical generation.

DNA Strand Breakage

In the previous sections we have shown that $^{\bullet}$OH radical was generated in the reduction of Cr(VI) by GSSG-R in the presence of NADPH as a cofactor. The mechanism involves reaction of Cr(VI) with H_2O_2, which is generated in the reduction of molecular oxygen with $O_2^{\bullet-}$ as an intermediate. In this section, we examined whether $^{\bullet}$OH radicals generated by these reactions are able to cause DNA strand breaks. Figure 7, lane 1 shows that incubation of Cr(VI) alone with DNA did not cause any observable DNA strand breaks. NADPH plus GSSG-R without Cr(VI) did not produce any observable DNA strand breaks either (Figure 7, lane 2). A mixture containing Cr(VI), GSSG-R, and NADPH caused DNA strand breaks (Figure 7, lane 3). Sodium formate inhibited the DNA

strand breaks (Figure 7, lane 4), whereas H_2O_2 enhanced them (Figure 7, lane 5). The DNA strand breaks caused by a mixture of Cr(VI), GSSG-R, and NADPH were inhibited if the experiment was carried out under argon (Figure 7, lane 6).

Discussion

The results obtained from the present study show that reduction of Cr(VI) by GSSG-R in the presence of NADPH as a cofactor generates $^{\bullet}OH$ radicals. The mechanism involves reaction of Cr(V) with H_2O_2, which was generated via reduction of molecular oxygen with $O_2^{\bullet-}$ as an intermediate. The $^{\bullet}OH$ radicals generated by these reactions are able to cause DNA strand breaks.

GSSG-R is a ubiquitous flavoenzyme, using NADPH as a cofactor to reduce oxidized glutathione (GSSG) and generate reduced glutathione (GSH). The general function of this enzyme is to maintain a high level of GSH in the cytosol. The mechanism of the enzymatic function of GSSG-R was proposed to involve the activation of the thiolate at Cys-46, which is stabilized by a nearby protonated His in the stable reduced form of the enzyme.[18] The reduced Cys-41 was proposed to attack GSSG, release GSH, and form a mixed disulfite and GSH. The inhibitory effect of *N*-ethylmaleimide suggests that the adjacent Cys residues are involved in the reduction of Cr(VI) by GSSG-R.[12] Recent studies have shown that reduction of Cr(VI) by whole live animals generates Cr(V)-NADPH complexes. GSSG-R is a likely candidate for the generation of these complexes. Because reduction of Cr(VI) by GSSG-R generates not only Cr(V) but also a whole spectrum of ROS ($^{\bullet}OH$, $O_2^{\bullet-}$, and H_2O_2), the reduction of Cr(VI) and related free radicals generated by this enzyme may represent an important pathway in the mechanism of Cr(VI)-induced carcinogenesis.

This study shows that all metal chelators tested (deferoxamine, 1,10-phenanthroline, and EDTA)

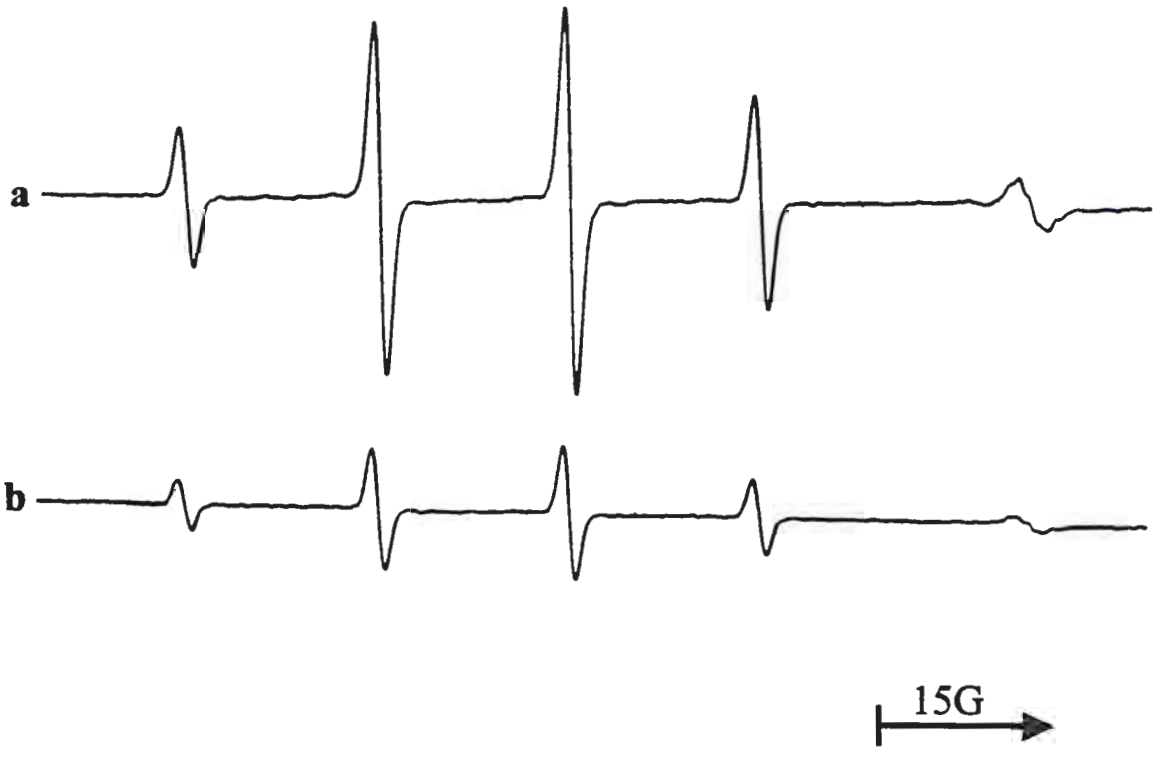

FIGURE 4. ESR spectra recorded 3 min after reaction initiation from a pH 7.4 phosphate buffer solution of 50 m*M* DMPO and the following reactants: (a) 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R; (b) same as (a) but experiment performed under Argon atmosphere. The ESR spectrometer settings were receiver gain, 5×10^4; time constant 0.5 s; modulation amplitude, 0.5 G; scan time, 8 min; magnetic field, 3375 ± 50 G.

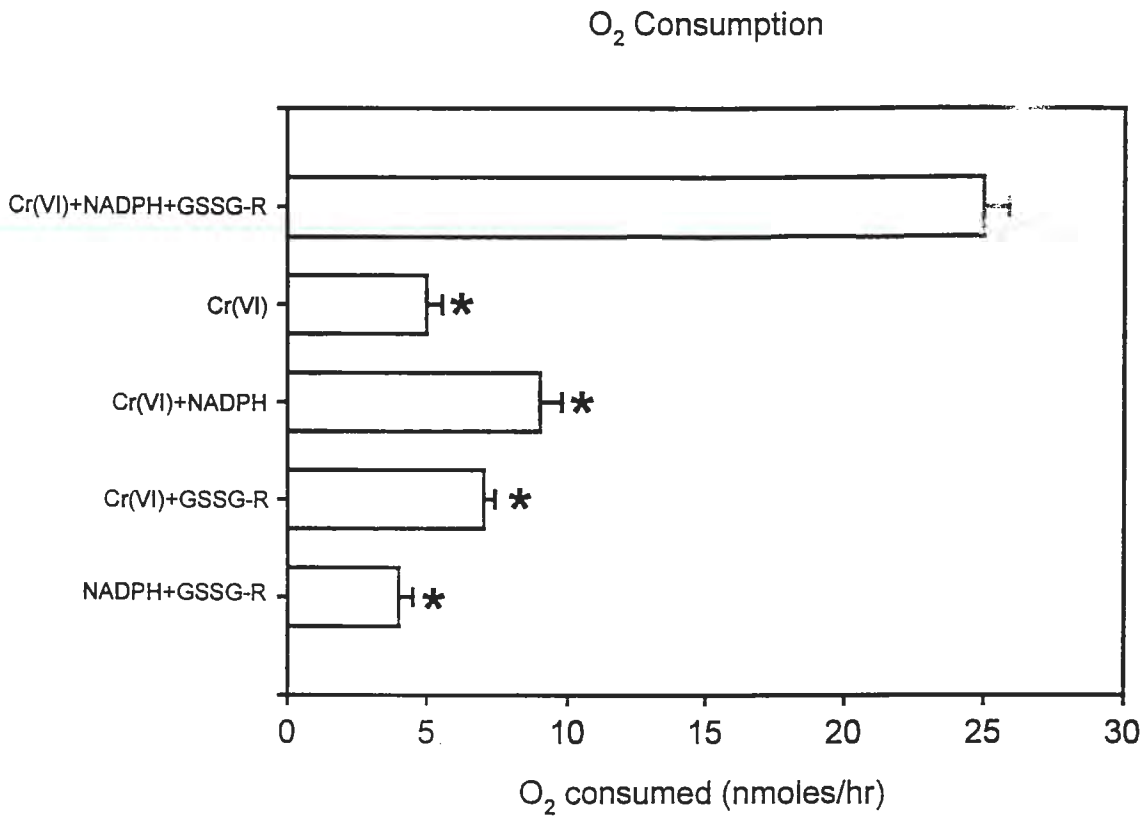

FIGURE 5. O_2 consumption in incubation mixtures containing 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R and various controls.

inhibited •OH radical generation. Among these chelators, deferoxamine is used for the prevention and treatment of iron overload[19] as well as for combating toxic effect of vanadium.[20] The formation of deferoxamine nitroxide radical with a concomitant disappearance of DMPO/•OH indicates that deferoxamine site-specifically reacts with •OH radical. Deferoxamine nitroxide radical is much less reactive than •OH radical. Because deferoxamine is bound to Cr(V), it scavenges •OH radical at its site of generation via a site-specific reaction, leading to the protection of •OH radical-initiated reactions. With regard to 1,10-phenanthroline, it is a membrane-permeable chelating agent, which has been reported to inhibit H_2O_2-induced DNA damage,[21] mutation, and transformation.[22] It is able to form complex with transition metal ions, such as iron to block •OH radical generation. This chelator has been reported to protect cells from either Cr(VI)-induced alkali-labile sites or the combination of alkali-labile sites plus DNA damage.[23] Thus, it appears that proper chelation may be used to inhibit Cr(VI)-induced •OH radical generation and to prevent or attenuate Cr(VI)-induced carcinogenicity.

The results obtained from the present study demonstrate that molecular oxygen is a primary source of •OH radical generation. The following experimental results support this conclusion. (1) A mixture of Cr(VI), GSSG-R, and NADPH consumed molecular oxygen. (2) The same mixture containing Cr(VI), GSSG-R, and NADPH under argon atmosphere generated a low level of •OH radicals compared with those under ambient atmosphere. (3) Under argon atmosphere, this mixture did not cause any significant DNA strand breaks. It may be noted that previous study has shown that Cr(VI) mutagenesis is strongly oxygen dependent.[24] The oxygen-dependent •OH radical generation, as demonstrated by the present study, further supports the free radical hypothesis in the mechanism of Cr(VI)-induced carcinogenicity.

Another important result obtained from the present study is the observation of $O_2^{\bullet-}$ radical

generation. A previous study has demonstrated that $O_2^{\bullet-}$ radical is capable of reducing Cr(VI) to generate Cr(V), which reacts with H_2O_2 to generate •OH radical via a Fenton-like reaction.[16,17] Similar reactions can occur for Cr(V) and Cr(III).[16,17] In those studies, xanthine and xanthine oxidase have been used as a source of $O_2^{\bullet-}$ radicals. In reactions involving Cr(VI), Cr(V), Cr(III), or possibly Cr(IV), $O_2^{\bullet-}$ radicals react with these ions. These reactions decrease the concentration of $O_2^{\bullet-}$ radicals and make these radicals hard to be detected. In the present study, deferoxamine was used to decrease the reactivity of Cr(V). By choosing the right concentration of deferoxamine, $O_2^{\bullet-}$ radical generation was detected. Although this radical may not directly cause cellular damage, it can enhance •OH radical generation via Haber-Weiss cycles, as illustrated in Figure 8. From this figure it can be noted that chromium in different oxidation states may function as a Haber-Weiss catalyst. Thus, a trace amount of Cr(VI) could catalytically provide a continued supply of the highly reactive •OH radicals and act as a radiomimetic •OH radical generating system.

Using gel electrophoresis, we further showed that •OH radicals generated in the reduction of Cr(VI) by GSSG-R in the presence of NADPH caused DNA strand breaks. It may be noted that •OH radicals generated in the reaction of H_2O_2

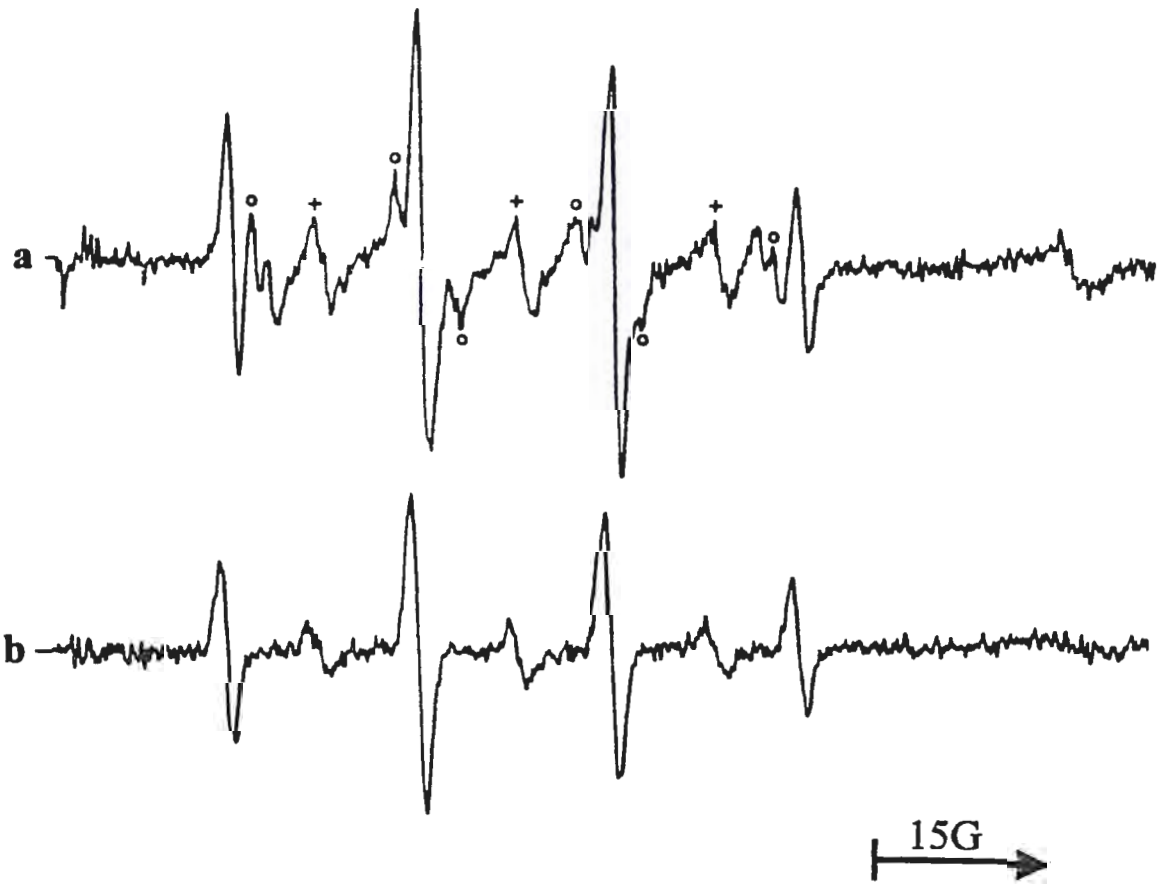

FIGURE 6. ESR spectra recorded 1 min after reaction initiation from a pH 7.4 phosphate buffer solution of 50 m*M* DMPO and the following reactants: (a) 2 m*M* Cr(VI), 1 m*M* NADPH 0.5 mg/mL GSSG-R, and 2 m*M* desferoxamine; (b) same as (a) but with 5 mg/mL superoxide dismutase added. The ESR spectrometer settings were receiver gain, 2.5×10^5; time constant 0.25 s; modulation amplitude, 0.5 G; scan time, 2 min; magnetic field, 3375 ± 50 G. "Circles" indicate DMPO/$O_2^{\bullet-}$ adduct peaks. "Plus signs" indicate DMPO adduct of deferoxamine nitroxide radical peaks.

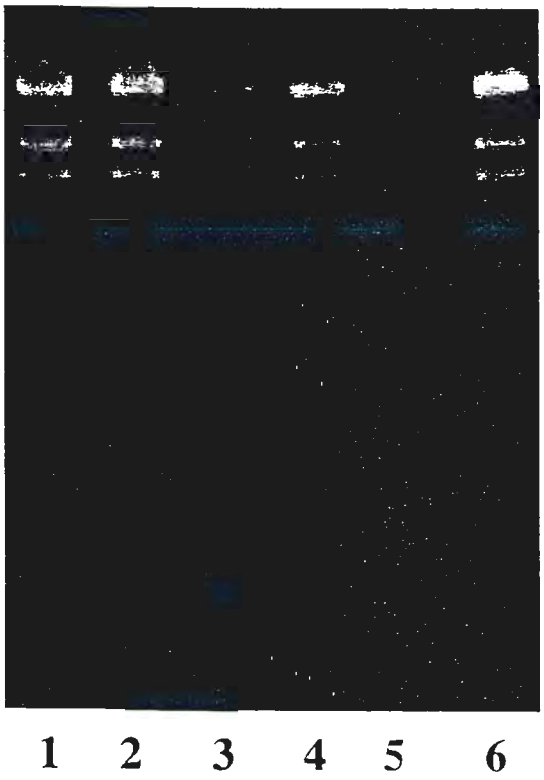

FIGURE 7. DNA damage by Cr(VI)-mediated reactions. Lane 1, untreated control DNA; lane 2, 1 m*M* NADPH only; lane 3, 0.5 mg/mL GSSG-R only; lane 4, 2 m*M* Cr(VI) only; lane 5, 1 m*M* NADPH and 0.5 mg/mL GSSG-R; lane 6, 2 m*M* Cr(VI), 1 m*M* NADPH, and 0.5 mg/mL GSSG-R; lane 7, same as lane 6, but with mg/mL superoxide dismutase added; lane 8, same as lane 6, but with 2000 units/mL catalase added; lane 9, same as lane 6, but with 50 m*M* sodium formate added; lane 10, same as lane 6, but with 2 m*M* H_2O_2 added; lane 11, same as lane 6, but experiment was performed under argon atmosphere. Other experimental conditions are described in Materials and Methods.

with certain metal ions, such as nickel-,[25] copper-, or zinc-containing SOD,[26] exhibit very little reactivity. For example, the •OH radicals produced by these systems cannot be scavenged by ethanol or formate. The reason for this nonreactivity is believed to be that •OH radicals are generated within the domain of certain macromolecules, and hence are not "free" to exhibit significant reactivity.[25,26] The results obtained from the present study show that •OH radicals generated by the reduction of Cr(VI) by GSSG-R in the presence of NADPH have the potential to cause DNA damage. It should be noted that H_2O_2, •OH, and $O_2^{\bullet-}$ may cause other cellular damage via mechanisms typically associated with reactions initiated by reactive oxygen species, for example, lipid peroxidation, dG hydroxylation, and protein-DNA cross-links. They may also cause activation of nuclear transcription factors, such as NF-κB, overexpression of certain oncogenes and induction of p53 mutation.

In conclusion, (1) reduction of Cr(VI) by GSSG-R in the presence of NADPH as a cofactor generates •OH radical. (2) The mechanism of •OH radical generation involves reaction of H_2O_2 with Cr(V). (3) H_2O_2 is generated by the reduction of molecular oxygen via $O_2^{\bullet-}$ as an intermediate. (4) $O_2^{\bullet-}$ generated in the enzymatic Cr(VI) reduction is detectable by ESR spin trapping.[5] Metal chelators, deferoxamine, 1,10-phenanthroline and EDTA, inhibit •OH radical generation.[6] •OH radicals generated by the enzymatic Cr(VI) reduction are capable of causing DNA strand breaks.

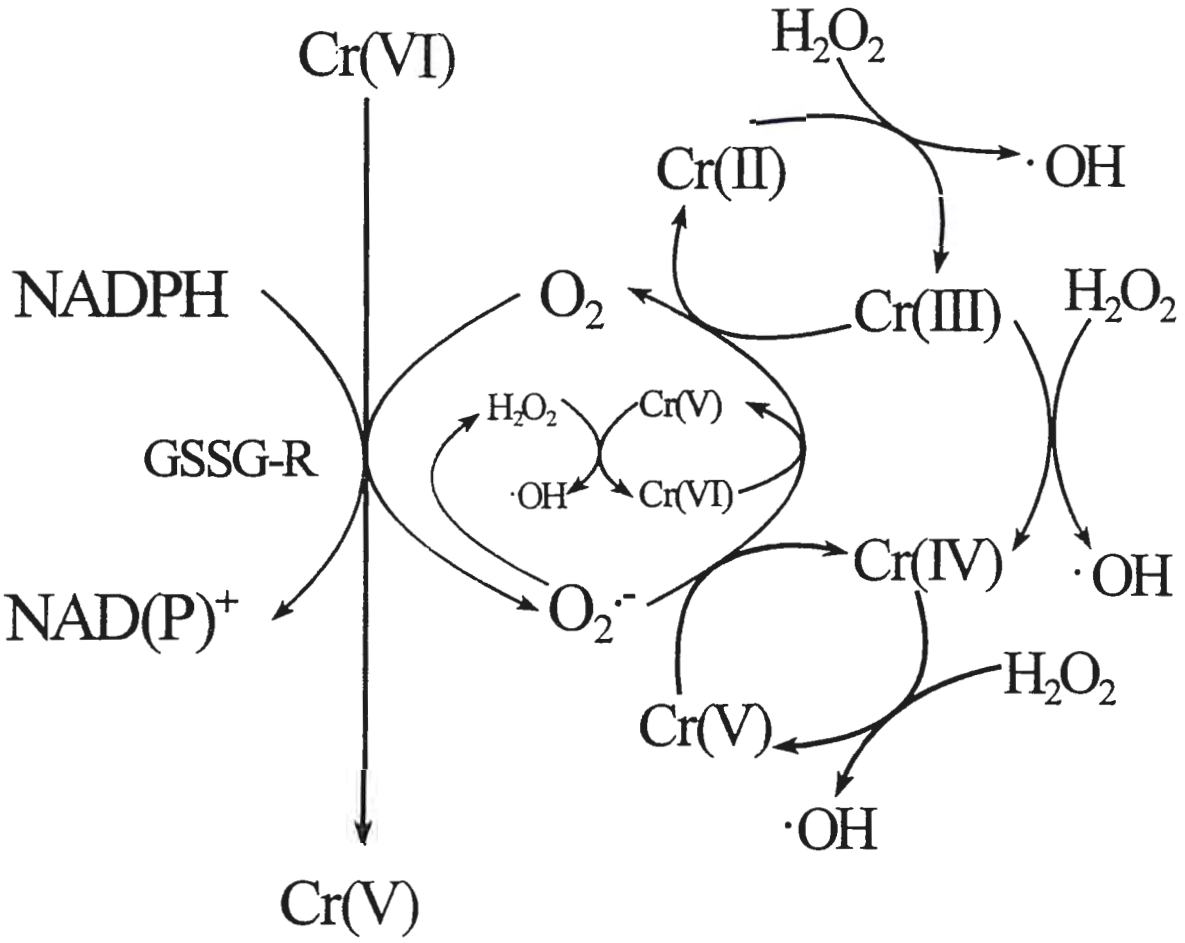

FIGURE 8. Schematic representation of possible mechanism of •OH radical generation in Cr-mediated reactions.

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