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REDUCTION OF CHROMIUM(VI) AND ITS RELATIONSHIP TO CARCINOGENESIS

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Although Cr(VI)-containing compounds are well-documented carcinogens, their mechanism of action is still not well understood. Recent studies have suggested that reduction of Cr(VI) to its lower oxidation states and related free-radical reactions play an important role in carcinogenesis. This article summarizes recent studies on (1) the reduction of Cr(VI) by ascorbate, diol- and thiol-containing molecules, certain flavoenzymes, cell organelles, intact cells, and whole animals; (2) free-radical production with emphasis on hydroxy radical generation via Fenton or Haber–Weiss type reactions; and (3) free-radical-induced cellular damage, such as DNA strand breaks, hydroxylation of 2′-deoxyguanosine, and activation of nuclear transcription factor κB.

Chromate [Cr(VI)] compounds, widely used in industry, have been shown to have serious toxic and carcinogenic effects on humans (Cohen et al., 1993; Costa, 1977; IARC, 1987, 1990). Epidemiological studies among industrially exposed chromium workers have identified chrome plating, chrome pigment, leather tanning, and stainless steel production as sources of potential exposure to this metal (Cohen et al., 1993). Because of its wide industrial use, environmental contamination is an additional source of human exposure to this metal. While chromium(VI) is now a well-estab-

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lished carcinogen, the mechanism for chromium-induced carcinogenesis remains largely unknown. Cr(VI) has been shown to induce chromosomal aberration, mutations, and transformation in cultured mammalian cells (De Flora et al., 1990; De Flora & Wetterhahn, 1989; Sugiyama, 1992), and a variety of DNA lesions such as strand breaks, DNA protein cross-links, and DNA base modification (Luo et al., 1996b; Shi et al., 1992, 1994b; Standeven & Wetterhahn, 1991). In contrast, most Cr(III) compounds are relatively nontoxic, noncarcinogenic, and nonmutagenic (De Flora & Wetterhahn, 1989). In a number of studies (Connett & Wetterhahn, 1983; Kortenkamp et al., 1987), it has been shown that Cr(VI) readily enters cells while Cr(III) does not. An "uptake-reduction" model of Cr(VI) metabolism has been proposed to explain the carcinogenicity of chromate (Connett & Wetterhahn, 1983; Jennett, 1979). According to this model, Cr(VI) in the form of tetrahedral chromate anion crosses the cell membrane using an anion transport system. A nonspecific anion channel is the common mechanism for Cr(VI) uptake in exposed hosts. Being predominantly octahedral, Cr(III) ions can cross the membranes only very slowly via simple diffusion or phagocytosis. Since Cr(VI) itself does not react readily with isolated DNA (Tsapakos & Wetterhahn, 1983), the reduction of Cr(VI) by cellular reductants to its lower oxidation states, Cr(V), Cr(IV), and Cr(III), has been considered an important step (Jennett, 1982). The levels of reactive chromium intermediates inside cells may be associated with the induction of Cr(VI)-induced damage. Thus, intensive studies have been carried out to examine the formation of reactive chromium intermediates during the reduction of Cr(VI) by cells. Cr(V) intermediates have been reported to induce DNA strand breaks in vitro and mutation in bacterial systems (Barr-David et al., 1992; Kortenkamp et al., 1989; Rodney et al., 1989; Standeven & Wetterhahn, 1991). Data show that these reactive chromium intermediates are capable of generating reactive oxygen species (ROS) via Fenton or Haber–Weiss type reactions (Liu et al., 1997; Shi & Dalal, 1989, 1990a, 1990b, 1990c, 1990d, 1992; Shi et al., 1994b). Free radicals generated by these reactions can cause DNA strand breaks (Shi et al., 1992, 1994b), base modification (Luo et al., 1996b; Shi et al., 1992, 1994b), lipid peroxidation, and nuclear transcription factor (NF)-κB activation (Chen et al., 1997; Ye et al., 1995). It was hypothesized that chromium-mediated ROS generation may cause a persistent oxidative stress that may play a key role in the mechanism of Cr(VI)-induced carcinogenesis. This article summarizes evidence for Cr(VI) reduction and for free radical generation in biological systems and discusses the role of free radicals in various potential mechanisms for the initiation of carcinogenesis induced by this metal.

REDUCTION OF Cr(VI)

Reduction of Cr(VI) by Low-Molecular-Weight Molecules

Various low-molecular-weight cellular constituents have been shown to reduce Cr(VI) in vitro at physiological pH. These substances include

glutathione (GSH) (Dalal & Shi, 1989; Shi & Dalal, 1988), cysteine (Shi et al., 1994a), lipoic acid (Chen et al., 1997), and diol-containing molecules, such as NAD(P)H, ribose, fructose, and arabinose (Shi & Dalal, 1990a, 1990b). Only those constituents that react with Cr(VI) at a significant rate are likely to contribute substantially to Cr(VI) reduction in cellular systems. Among these substances, ascorbate and GSH were considered the most likely candidates as nonenzymatic Cr(VI) reductants (Standeven & Wetterhahn, 1991), especially because of their ubiquitous occurrence in mammalian cells.

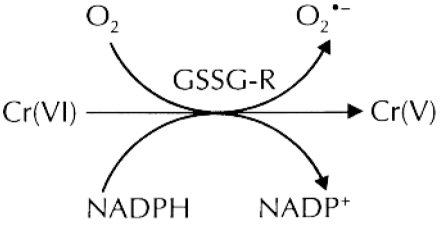
Reduction of Cr(VI) by GSH generates glutathionyl radical ($GS^{\bullet}$) (Dalal & Shi, 1989; Shi & Dalal, 1988) and Cr(V) as well as Cr(IV) complexes (Bose et al., 1992; Liu et al., 1997; Shi & Dalal, 1990b). Both of these complexes can be isolated in solid forms (Shi & Dalal, 1988; Liu et al., 1997). These stable Cr(V) and Cr(IV) solids can be used as model compounds to study the role of intracellular Cr(IV) and Cr(V) in the mechanism of Cr(VI)-induced carcinogenesis. One of the observations indicating that GSH may be involved in Cr(VI) metabolism was the finding of a correlation between Cr(VI)-induced DNA damage and GSH levels in cultured chick embryo hepatocytes (Cupo & Wetterhahn, 1985a, 1985b). Specifically, depletion of GSH with buthionine sulfoximine decreased Cr(VI)-induced DNA damage, while increasing GSH levels with *N*-acetylcysteine increased Cr(VI)-induced DNA damage.

Another important Cr(VI) reductant is believed to be ascorbate (Standeven & Wetterhahn, 1991). Reduction of Cr(VI) by ascorbate is kinetically favored over GSH, and intratracheal injection of Cr(VI) has been reported to deplete ascorbate but not GSH in rat lung (Suzuki & Fukuda, 1990). It has been found that ascorbate is more reactive than GSH for reduction of Cr(VI) in rat lung (Standeven & Wetterhahn, 1991, 1992). In addition, ascorbate has been shown to be the major reductant of Cr(VI) in rat lung, kidney, and liver ultrafiltrates (Standeven & Wetterhahn, 1992). Binding of Cr to DNA resulted from the reaction of Cr(VI) with DNA in the presence of rat lung ultrafiltrates and was correlated with ascorbate-dependent metabolism of Cr(VI). On the basis of kinetic studies, it has been suggested that GSH and ascorbate may act synergistically to reduce Cr(VI) (Suzuki, 1994). In noncellular systems, reduction of Cr(VI) by ascorbate has been reported to generate ascorbate-derived free radicals, Cr(V) and Cr(IV) (Shi et al., 1994b). The relative yield of these species was dependent on the relative concentrations of Cr(VI) and ascorbate. The Cr(V) and Cr(IV) generated by ascorbate reduction have been shown to react with H_2O_2 to produce hydroxyl radical ($^{\bullet}OH$), which caused DNA strand breaks (Shi et al., 1994b).

Reduction of Cr(VI) by Cellular Reductants

A variety of enzymatic and nonenzymatic factors function as Cr(VI) reductants. These factors include microsomes (Jennett, 1982; Shi et al., 1991), mitochondria (Shi et al., 1991), cytochromes P-450, cytochrome b_5, the electron transport complex of the inner mitochondrial membrane

(Rossi & Wetterhahn, 1989), and several flavoenzymes, such as glutathione reductase (GSSG-R), lipoyl dehydrogenase, and ferredoxin-$NADP^+$ oxidoreductase (Shi & Dalal, 1989, 1990c, 1991). Among these reductants, glutathione reductase is discussed here as an example. In the presence of NADPH, glutathione reductase reduces Cr(VI) to generate Cr(V), which was identified as a Cr(V)–NADPH complex. During the reduction process, molecular oxygen is reduced to $O_2^{\bullet -}$, which generates H_2O_2 via dismutation. The Cr(V)–NADPH complex in turn reacts with H_2O_2 to generate $^{\bullet}OH$ radical. The reaction steps are described in Eqs. (1)–(3) (Shi & Dalal, 1989, 1990c, 1991).



(1)

$$2O_2^{\bullet -} + 2H^+ \longrightarrow O_2 + H_2O_2 \quad (2)$$

$$Cr(V) + H_2O_2 \longrightarrow Cr(VI) + {}^{\bullet}OH + OH^- \quad (3)$$

Reduction of Cr(VI) in Intact Cells

Our earlier study showed that incubation of *Escherichia coli* with $K_2Cr_2O_7$ generated a Cr(V)–NADPH complex (Shi et al., 1991). Addition of NADPH enhanced the Cr(V)–NADPH formation, indicating that NADPH-dependent reductases are likely to be involved in this Cr(V) formation. It has also been reported that incubation of cultured Chinese hamster V-79 cells with $K_2Cr_2O_7$ resulted in the formation of both Cr(V) and Cr(III) (Sugiyama, 1991, 1994; Sugiyama et al., 1991c). These paramagnetic chromium species were measured by electron spin resonance (ESR) spectroscopy at a temperature of 153 K. The linewidth for Cr(III) generated by these cells was between 700 and 800 G. Since the linewidth of the Cr(III) hexaaqueous complex signal was about 150 G, it was believed that the increased line width of Cr(III) in the cells may be related to the formation of Cr(III) complexes with cellular components, resulting in decreased mobility of this metal (Sugiyama et al., 1991c).

Cellular reductants can modify the levels of paramagnetic chromium and alter chromium-induced DNA damage. It has been reported that an increase of the cellular content of α-tocopherol, riboflavin, and ascorbic acid by pretreatment of V-79 cells with these vitamins resulted in alterations of levels of paramagnetic chromium species (Sugiyama, 1989, 1991, 1994; Sugiyama et al., 1991a). An increase of either α-tocopherol or ascorbic acid in cells decreased the level of Cr(V) complex, whereas

an increase of riboflavin increased the level of Cr(V) complex. With regard to Cr(III), an increase of the content of ascorbic acid increased the Cr(III) level. The levels of Cr(V) formation correlated with Cr(VI)-induced DNA strand breaks and/or alkali-labile sites. Both α-tocopherol and ascorbic acid decreased Cr(V) formation and decreased the DNA damage, whereas an opposite effect was observed for riboflavin. The effects of increased levels of α-tocopherol or riboflavin on the induction of chromosomal aberrations and mutations by Cr(VI) in V-79 cells have been reported (Sugiyama et al., 1991b, 1992). An increase of α-tocopherol suppressed the clastogenic and mutagenic action of chromate compounds, while the increased riboflavin resulted in an enhancement of both actions of this metal, indicating that the Cr(V) may be associated with these actions.

Certain metal chelators, such as *o*-phenanthroline (a cell-permeable metal chelator), can also modify Cr(V) formation (Shi et al., 1992; Sugiyama et al., 1993). Sugiyama (1994) has reported that *o*-phenanthroline suppressed the formation of Cr(V). Parallel studies have found that the decrease of Cr(V) levels resulted in a decrease of chromate-induced DNA strand breaks and/or alkali-lable sites.

Reduction of Cr(VI) In Vivo

One-electron reduction of Cr(VI) has been observed in chick embryo liver and red blood cells (Liebross & Wetterhahn, 1990, 1992). The most direct evidence for Cr(VI) reduction was demonstrated by the generation of Cr(V) in whole animals treated with Cr(VI). Using ESR with a low-frequency microwave bridge and a cylinder-shaped loop gap resonator, we were able to show that a Cr(V) intermediate can be generated by one-electron reduction of Cr(VI) in whole living animals (Liu et al., 1994). The Cr(V) was found predominantly in the liver with a small amount in the blood. Liver homogenates from Cr(VI)-treated animals generated essentially the same Cr(V) species as that obtained from the whole living mice. This Cr(V) species was identified to be a Cr(V)–NADPH complex with an oxygen bond to Cr(V) (Liu et al., 1995, 1996). Pretreatment of the animals with ascorbate or GSH decreased the Cr(V) formation, while pretreatment with NADPH enhanced it. Metal chelators, such as 1,10-phenanthroline and diethylenetriamine pentaacetic acid (DTPA), inhibited the formation of Cr(V)–NADPH complex. These results suggest that NADPH/flavoenzymes and not GSH or ascorbate are the major one-electron Cr(VI) reductants in vivo.

Summary of Cr(VI) Reduction

The reduction of Cr(VI) to Cr(III) can occur by a multiplicity of mechanisms, dependent on the nature of reducing agents. Cr(V) and Cr(IV) are reactive intermediates generated in these reduction processes. The Cr(VI) reducing agents include small molecules (i.e., ascorbate and GSH), cer-

tain flavoenzymes (i.e., glutathione reductase and ferredoxin $NADP^+$ oxidoreductase), cell organelles (i.e., microsomes and mitochondria), and unified systems (i.e., cells and whole animals). The reaction equations can be described as follows:

$$Cr(VI) \xrightarrow{e} Cr(V) \quad (4)$$

$$Cr(V) \xrightarrow{e} Cr(IV) \quad (5)$$

$$Cr(IV) \xrightarrow{e} Cr(III) \quad (6)$$

$$Cr(IV) + Cr(VI) \longrightarrow 2Cr(V) \quad (7)$$

$$2Cr(IV) \longrightarrow Cr(III) + Cr(V) \quad (8)$$

FREE-RADICAL GENERATION

Thiyl Radical Generation

Using ESR spin trapping, Shi and Dalal (1988) detected the formation of Cr(V) and glutathione-derived thiyl radicals ($GS^\bullet$) in the reaction of Cr(VI) with GSH. An increase in GSH concentration enhanced the $GS^\bullet$ radical generation. Reaction of Cr(VI) with cysteine or penicillamine also generates corresponding thiyl radicals (Shi et al., 1994a). The reaction equation is:

$$Cr(VI) + GSH \longrightarrow Cr(V) + GS^\bullet \quad (9)$$

The thiyl radicals generated by this reaction may cause direct cellular damage. These radicals may also react with other thiol molecules to generate $O_2^{\bullet-}$ radicals [Eqs. (10) and (11)] (Barton & Packer, 1970; Quintiliani et al., 1977).

$$GS^\bullet + RSH \longrightarrow RSSR^{\bullet-} + H^+ \quad (10)$$

$$RSSR^{\bullet-} + O_2 \longrightarrow RSSR + O_2^{\bullet-} \quad (11)$$

The generation of $O_2^{\bullet-}$ radicals leads to the formation of H_2O_2. $O_2^{\bullet-}$ is able to cause additional oxygen radical generation, for example, by reducing Cr(VI) to Cr(V) and a subsequent reaction with H_2O_2, as discussed in the following sections.

Hydroxyl Radical Generation by Cr(V) Reaction

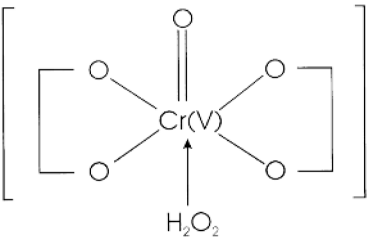
Shi and Dalal (1990b) have reported that addition of H_2O_2 to a mixture of GSH and Cr(VI) decreased the ESR signal of Cr(V) and generated

$^{\bullet}OH$ radicals. The $^{\bullet}OH$ generation was believed to be the result of reaction of Cr(V) with H_2O_2 via a Fenton-like reaction:

$$Cr(V) + H_2O_2 \longrightarrow Cr(VI) + {}^{\bullet}OH + OH^- \quad (12)$$

Cr(V)-NADPH generated by reaction of Cr(VI) with NADPH was used to verify this reaction [Eq. (7)] (Shi & Dalal, 1990a, 1990b, 1990c, 1990d). A mixture of Cr(VI), NADPH, and H_2O_2 generated both Cr(V) and $^{\bullet}OH$ radical. An increase in H_2O_2 concentration enhanced $^{\bullet}OH$ generation with a concomitant decrease in the Cr(V) formation. Addition of superoxide dismutase (SOD) did not significantly affect the $^{\bullet}OH$ generation, indicating that $O_2^{\bullet -}$ was not significantly involved in the mechanism of $^{\bullet}OH$ generation. The reactivity of Cr(V) species depends on their structure (Shi & Dalal, 1994). For example, tetraperoxochromate $\{[Cr(O_2^{2-})_4]^{3-}\}$ has a tetrahederal structure with all covalent bonds fully occupied by O_2^{2-} moieties as shown:

The $[Cr(O_2^{2-})_4]^{3-}/H_2O_2$ complex did not easily split H_2O_2 to generate $^{\bullet}OH$ radical. On the other hand, a Cr(V) complex, such as Cr(V)–NADPH, is expected to be octahedral, with one vacant site as shown:



H_2O_2 can attach to the vacant coordination site and form a long-lived complex to generate $^{\bullet}OH$ radicals. This mechanism is similar to the oxidation of Fe(II) with H_2O_2, as the production of $^{\bullet}OH$ radical from H_2O_2 via the Fenton reaction is facilitated by the formation of Fe(II) complexes that have vacant sites for H_2O_2 coordination (Craf et al., 1984).

Hydroxyl Radical Generation by Cr(IV) Reaction

Cr(IV) is the other reactive chromium intermediate generated in the reduction of Cr(VI) by cellular reductants such as ascorbate and GSH. Shi

et al. (1994b) have demonstrated that Cr(IV) is able to generate $^{\bullet}OH$ radical from H_2O_2 via a Fenton-like reaction:

$$Cr(IV) + H_2O_2 \longrightarrow Cr(V) + {}^{\bullet}OH + OH^- \quad (13)$$

Reaction of Cr(VI) with ascorbate was used as a source of Cr(IV). The following experimental observations (Shi et al., 1994b) support the validity of Eq. (13). (1) A mixture of Cr(VI) and ascorbate generated both Cr(V) and Cr(IV) intermediates. (2) Addition of H_2O_2 produced $^{\bullet}OH$ radicals with a significant enhancement of Cr(V) generation. In a system with both Cr(V) and Cr(IV) present, H_2O_2 reacted with both Cr(V) and Cr(IV) competitively. If the reaction rate of Cr(IV) with H_2O_2 were larger, the Cr(V) signal could increase upon addition of H_2O_2. Otherwise it would decrease. The observed enhancement of Cr(V) upon addition of H_2O_2 indicates that the reaction between Cr(IV) and H_2O_2 occurs and that the rate constant is larger than that of Cr(V) with H_2O_2. (3) Synthesized Cr(IV) compounds, such as Cr(IV)–GSH and Cr(IV) ester of 2,4-dimethyl-2,4-pentanediol, were used to show that Cr(IV) was indeed able to generate $^{\bullet}OH$ radical via the Fenton-like reaction (Liu et al., 1997; Luo et al., 1996a, 1996b).

Once Cr(V) is generated, it reacts with H_2O_2 to generate more $^{\bullet}OH$ radicals [Eq. (12)]. By adding Eqs. (12) and (13), the net reaction becomes:

$$Cr(IV) + 2H_2O_2 \longrightarrow Cr(VI) + 2{}^{\bullet}OH + 2OH^- \quad (14)$$

Hydroxyl Radical Generation by Cr(III) Reaction

Although Cr(III) compounds are not significantly toxic to intact cells, they have been found to be very effective in genotoxicity assays using cell-free systems (Snow, 1992). The lack of toxicity and carcinogenicity of Cr(III) in intact cells has been explained by the poor uptake of this cation into cells. However, cells are permeable to Cr(VI). Once inside cells, Cr(VI) is eventually reduced to Cr(III). Our earlier studies have shown that Cr(III) is also able to generate $^{\bullet}OH$ radical from H_2O_2 (Shi et al., 1993). The $^{\bullet}OH$ radical yield depends on pH, reaching its highest value at pH 10. This pH dependence indicates that high pH may promote oxidation of Cr(III) with H_2O_2. The latter is consistent with the observed decrease of Cr(III) concentration upon addition of H_2O_2 with a concomitant increase in $^{\bullet}OH$ radical generation.

Hydroxyl Radical Generation by Cr(VI)- and Cr(III)-Mediated Haber–Weiss Reaction

Shi and Dalal (1992) have studied the role of $O_2^{\bullet-}$ radical in Cr(VI)-generated $^{\bullet}OH$ radicals via a Cr(VI)-dependent Haber–Weiss cycle. Xanthine and xanthine oxidase were used as a source of $O_2^{\bullet-}$ radicals. NADH

was used to chelate the Cr(V) intermediate to form an ESR-detectable Cr(V)-NADH complex. Catalase was used to confirm the role of H_2O_2. The results show that $O_2^{\bullet-}$ was able to reduce Cr(VI) to Cr(V), which in turn reacted with H_2O_2 to generate $^{\bullet}OH$ and Cr(VI) [Eqs. (12), (15), and (16)].

$$O_2^{\bullet-} + \text{Cr(VI)} \longrightarrow \text{Cr(V)} + O_2 \tag{15}$$

$$\text{Cr(V)} + H_2O_2 \longrightarrow \text{Cr(VI)} + {}^{\bullet}OH + OH^- \tag{12}$$

Overall,

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Cr(VI)/Cr(V)}} O_2 + {}^{\bullet}OH + OH^- \tag{16}$$

Similar to Cr(VI), we have shown that Cr(III) is also able to generate $^{\bullet}OH$ radical via the Haber–Weiss cycle (Shi et al., 1998). The reaction steps are:

$$O_2^{\bullet-} + \text{Cr(III)} \longrightarrow \text{Cr(II)} + O_2 \tag{17}$$

$$\text{Cr(II)} + H_2O_2 \longrightarrow \text{Cr(III)} + {}^{\bullet}OH + OH^- \tag{18}$$

Overall

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Cr(III)/Cr(II)}} O_2 + {}^{\bullet}OH + OH^- \tag{19}$$

The metal chelators deferoxamine, 1,10-phenanthroline, and ethylenediamine tetraacetic acid (EDTA) decreased the $^{\bullet}OH$ generation, showing that proper coordination of Cr(V) is required for Cr(III)-mediated $^{\bullet}OH$ generation. The relative yield of the $^{\bullet}OH$ radical generation by Cr(III) was lower than that generated by Cr(VI) through similar reactions. However, it has been reported that Cr(III) is able to bind to DNA (Tsapakos & Wetterhahn, 1983). Thus, this bound Cr(III) may significantly damage DNA via site-specific $^{\bullet}OH$ radical generation.

The Haber–Weiss mechanism of $^{\bullet}OH$ generation could become particularly significant during phagocytosis, when macrophages and other cellular constituents generate large quantities of $O_2^{\bullet-}$ radicals in the so-called respiratory burst (Freeman & Crapo, 1982). It has been reported that a significant portion of oxygen consumed by phagocytes is first converted to $O_2^{\bullet-}$ (Freeman & Crapo, 1982). However, further conversion of $O_2^{\bullet-}$ to $^{\bullet}OH$ is too slow to be physiologically significant, unless a suitable metal ion [i.e., Fe(II)] is present as a Haber–Weiss catalyst (Weinstein & Bielski, 1979). The finding that Cr(VI) or Cr(III) can act as a Haber–Weiss

catalyst may provide a basis for the known critical role of molecular oxygen in the genotoxic and carcinogenic reactions of Cr(VI)-containing particles.

Hydroxyl Radical Generation in Intact Cells

Using an ESR spin trapping technique, we have studied $^{\bullet}OH$ radical generation from intact (Jurkat) cells stimulated by Cr(VI) (Ye et al., 1995). Both $^{\bullet}OH$ and Cr(V) were produced from this system. The Cr(V) generated was identified as a Cr(V)–NADPH complex. Addition of NADPH or glutathione reductase enhanced the Cr(V) generation, indicating that NADPH-dependent flavoenzymes were likely to be involved in the formation of the Cr(V)–NADPH complex. Addition of H_2O_2 enhanced $^{\bullet}OH$ radical generation and also increased the ESR signal intensity of Cr(V) peak. The observed enhancement of Cr(V) formation upon addition of H_2O_2 indicates that in this cellular system the reaction rate of Cr(IV) with H_2O_2 [Eq. (8)] is larger than that of Cr(V) with H_2O_2 [Eq. (7)].

Does Cr(VI) Generate $^{\bullet}OH$ Radical from H_2O_2?

It has been frequently suggested that Cr(VI) could react with cellular H_2O_2 to generate $[Cr(O_2^{2-})_4]^{3-}$ ions, which would decompose to produce $^{\bullet}OH$ radicals (Aiyar et al., 1990; Lefebvre & Pezerat, 1992; Kawanishi et al., 1986):

$$Cr(VI) + H_2O_2 \longrightarrow [Cr(O_2^{2-})_4]^{3-} \longrightarrow Cr(VI) + {}^{\bullet}OH \qquad (20)$$

The first evidence for the role of $[Cr(O_2^{2-})_4]^{3-}$ in the Cr(VI)-mediated $^{\bullet}OH$ radical generation was suggested by Kawanishi et al. (1986). They observed $[Cr(O_2^{2-})_4]^{3-}$ formation by ESR from a mixture containing 40 m*M* Na_2CrO_4 and 400 m*M* H_2O_2 at pH 8.0. However, the concentrations used for both Cr(VI) and H_2O_2 were several orders of magnitude higher than any in vivo estimates. Later Aiyar et al. (1990) reaffirmed the Kawanishi model of $^{\bullet}OH$ radical generation. Lefebvre and Pezerat (1992) considered the $^{\bullet}OH$ radical generation from $[Cr(O_2^{2-})_4]^{3-}$ as a support for the "tetraperoxochromate(V) theory of carcinogenesis from chromate."

Using ESR and spin trapping approaches, Shi and Dalal (1994) examined the possibility of $[Cr(O_2^{2-})_4]^{3-}$ and $^{\bullet}OH$ radical generation under physiological conditions. The results obtained showed: (1) $[Cr(O_2^{2-})_4]^{3-}$ is not formed in any significant quantity in the reaction of chromate with biologically relevant reductants such as GSH, glutathione reductase, NADPH, ascorbate, or vitamin B_2. (2) Decomposition of $[Cr(O_2^{2-})_4]^{3-}$ or its reaction with H_2O_2 did not generate a significant amount of $^{\bullet}OH$ radicals. (3) The major Cr(V) species formed were complexes of Cr(V) with reductant moieties as ligands. (4) These Cr(V) complexes would generate $^{\bullet}OH$ radical from H_2O_2 via a Fenton-like reaction.

Generation of Lipid Hydroperoxide-Derived Free Radicals

At physiological pH, Cr(V), Cr(IV), and Cr(III) were capable of generating lipid hydroperoxide-derived free radicals from the model lipid hydroperoxide cumene hydroperoxide (Mao et al., 1995; Shi et al., 1993). These radicals were identified as carbon-centered cumene alkyl radical ($R^{\bullet}$) and the oxygen-centered cumene alkoxyl radical ($RO^{\bullet}$). Similar results were obtained using *t*-butyl hydroperoxide as another model lipid peroxide.

Summary of Chromium-Mediated $^{\bullet}OH$ Radical Generation

All chromium oxidation states are able to generate $^{\bullet}OH$ radical from H_2O_2, with Cr(V) and Cr(IV) exhibiting a high potency. A summary scheme for chromium-mediated $^{\bullet}OH$ radical generation is provided in Figure 1.

ROLE OF FREE RADICAL REACTIONS IN Cr(VI)-INDUCED CARCINOGENESIS

DNA Strand Breaks

Using λ Hind III DNA digest, our laboratory assessed DNA damage induced by a mixture of Cr(VI) and ascorbate with and without H_2O_2 (Shi et al., 1994b). DNA strand breaks were detected by agarose gel electrophoresis. A significant amount of DNA strand breaks occurred when the DNA was incubated with Cr(VI) and ascorbate. The amount of DNA strand

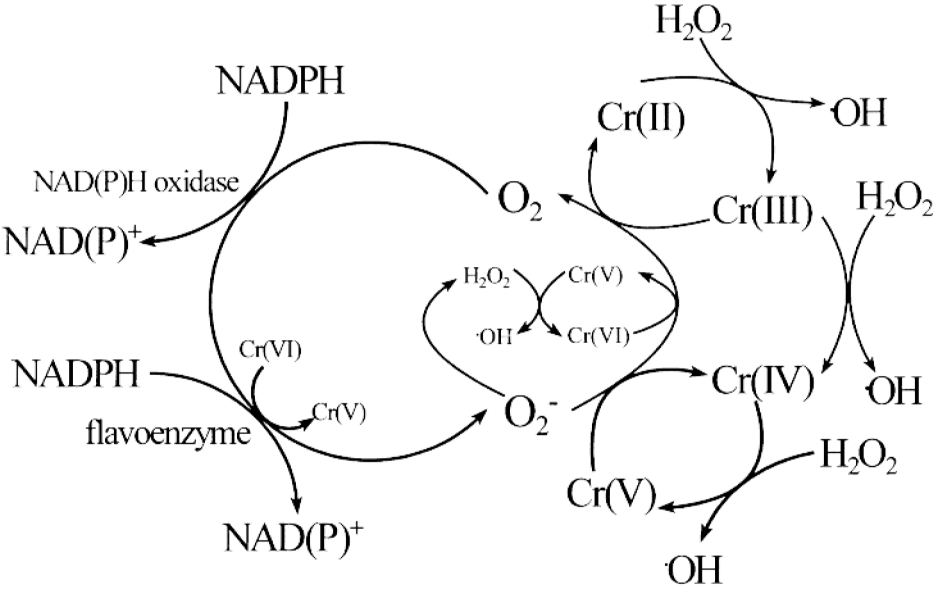


FIGURE 1. A scheme of chromium-mediated $^{\bullet}OH$ generation.

breaks depended on the relative concentrations of Cr(VI) and ascorbate. Addition of H_2O_2 drastically enhanced the DNA damage. Addition of Mn(II), which can remove Cr(IV) and inhibit Cr(IV)-mediated Fenton-like reactions, inhibited DNA damage. The amount of DNA strand breaks correlated with the amount of free radicals generated.

In vivo treatment with Cr(VI) has been shown to produce persistent DNA strand breaks in chick embryo red blood cells (Hamilton & Wetterhahn, 1986). The major Cr(VI)-induced DNA lesions in vivo appeared to be DNA interstrand cross-links, DNA protein cross-links, and formation of Cr–DNA adducts (Cupo & Wetterhahn, 1985a; Hamilton & Wetterhahn, 1986; Tsapakos et al., 1983). In a cultured cell system, Cr(VI) has been shown to produce DNA strand breaks that could be efficiently repaired (Whiting et al., 1979). The level of Cr(VI)-induced DNA strand breaks in human diploid fibroblasts was decreased by superoxide dismutase and catalase but was enhanced by GSH (Snyder, 1988). The level of Cr(VI)-induced DNA strand breaks in cultured chick embryo hepatocytes was enhanced by increased intracellular concentration of GSH and was decreased by depletion of GSH (Cupo & Wetterhahn, 1985b). Vitamin E and ascorbate decreased the level of Cr(VI)-induced DNA strand breaks and the levels of Cr(V) formation, whereas vitamin B_2 (a diol-containing molecule) increased the level of Cr(VI)-induced DNA strand breaks and the level of Cr(V) in Chinese hamster V79 cells (Sugiyama et al., 1989, 1991c). These results suggest that the levels of Cr(VI)-induced DNA strand breaks are correlated with the levels of Cr(V) produced.

8-OHdG Formation

The $^{\bullet}$OH radical can interact with guanine residues at several positions to generate a range of products, of which the most studied one is 8-hydroxy-deoxyguanosine (8-OHdG) (Dizadaroglu, 1991). The formation of this adduct is considered a marker to implicate ROS in the mechanism of toxicity and carcinogenicity of a variety of agents. Using high-performance liquid chromatography (HPLC) with electrochemical detection, it was found that $^{\bullet}$OH radicals generated by Cr(V)- and Cr(IV)-mediated reactions caused 2′-deoxyguanosine (dG) hydroxylation to form 8-OHdG (Shi et al., 1992, 1994b).

Lipid Peroxidation

Lipid peroxidation, which can be defined broadly as the oxidative deterioration of polyunsaturated components of membrane lipids, has been implicated in the etiology of many disease processes, including cancer (Sunderman, 1986). Since lipid peroxidation is initiated by hydrogen atom abstraction from unsaturated lipids, lipid peroxidation could potentially be mediated by ROS. Ueno et al. (1988) reported that intraperitoneal injection of rats with Cr(VI) resulted in lipid peroxidation, which was demonstrated by an increase in thiobarbituric acid-reactive sub-

stances (TBARS) in liver and kidney, although the reliability of simple TBARS tests in tissues has been questioned (Halliwell & Chirico, 1993).

The generation of lipid hydroperoxide-derived free radicals by the reaction of chromium with lipid hydroperoxides is significant because there is substantial evidence to implicate free radicals, generated during lipid peroxidation, in the process of carcinogenesis (Brambilla et al., 1989; Halliwell & Gutteridge, 1998; Marnett, 1987). Radical reactions can cause cell membrane damage, leading to increased intracellular levels of catalytically active iron and, in turn, increased generation of ROS. These ROS may interact with chromatin and thereby act as tumor initiators and/or promoters. The lipid hydroperoxide-derived free radicals can also cause site-specific cleavage of double-stranded DNA. It is well documented that chromium as chromate anion is efficiently taken up by living cells via anion transport channels and is gradually reduced to Cr(III) as a final product (Connett & Wetterhahn, 1983). Cr(III) accumulates in cells due to its limited ability to diffuse through the cell membrane. Cr(III), generated by Cr(VI) reduction, may react with lipid hydroperoxides to produce lipid hydroperoxide-derived free radicals as demonstrated by in vitro studies (Mao et al., 1995; Shi et al., 1993). It has been shown that Cr(III) can bind to isolated nuclei and interact with nucleotides and nucleic acids (Tsapakos & Wetterhahn, 1983). Thus, $^{\bullet}OH$ and lipid hydroperoxide-derived free radicals generated by chromium can cause DNA damage.

NF-κB Activation

Nuclear transcription factor (NF)-κB is considered a primary oxidative stress response transcription factor that functions to enhance the transcription of a variety of genes (Baldwin, 1996; Baeuerle & Henkel, 1994). In several cell types, reactive ROS have been shown to activate this transcription factor (Schreck et al., 1992; Sun & Oberley, 1996). It has been shown that Cr(VI) was able to induce NF-κB activation in Jurkat cells (Ye et al., 1995). The reduction of Cr(VI) to low oxidation states is required for Cr(VI)-induced NF-κB activation. Hydroxyl radicals generated by Cr(V)- and Cr(IV)-mediated Fenton-like reactions play a prominent role in the mechanism of Cr(VI)-induced NF-κB activation. It may be noted that NF-κB binding sites serve as an enhancer element in the c-*myc* oncogene, and this gene is associated with the formation of Burkitt's lymphoma (Ji et al., 1994). Cr(VI) could induce expression of c-*myc* proto-oncogene via NF-κB activation. It is possible that NF-κB activation and a subsequent expression of proto-oncogenes, such as c-*myc*, may play a role in the induction of neoplastic transformation by Cr(VI).

Summary of the Role of Free-Radical Reactions in Cr(VI)-Induced Carcinogenesis

Free radicals generated by chromium-mediated reactions can cause a variety of cell injuries. DNA strand breaks and hydroxylation of dG

residues are typical examples. These types of DNA damage can lead to mutation. Chromium induces lipid peroxidation, and the products can cause DNA damage and also a rise in intracellular level of Ca^{2+}, leading to cell injury. Chromium also causes activation of nuclear transcription factor, NF-κB. This transcription factor is involved in regulation of expression of growth factors and oncogenes. Figure 2 summarizes the role of free radical reactions in Cr(VI)-induced carcinogenesis.

CONCLUSIONS

Cr(VI)-containing compounds are major occupational and environmental contaminants and are well-established carcinogens. Although the mechanism of Cr(VI)-induced carcinogenesis is still not well understood, it is generally believed that reduction of Cr(VI) to its lower oxidation states, particularly Cr(V) and Cr(IV), is an important step. The reduction of Cr(VI) occurs by a multiplicity of mechanisms, depending on the nature of reducing agents. These reducing agents include cellular small molecules (such as ascorbate and GSH), certain flavoenzymes (such as glutathione reductase), cell organelles (such as microsomes and mitochondria), intact cells, and whole animals. All the chromium oxidation states tested are able to generate $^{\bullet}OH$ radicals from H_2O_2, with Cr(V) and Cr(IV) exhibiting strong potency. The reaction mechanisms involve Haber–Weiss or Fenton-like reactions. The free radicals generated by chromium-mediated reactions cause DNA damage, lipid peroxidation, and activation of NF-κB. Biological systems are normally protected against oxidative injury caused by

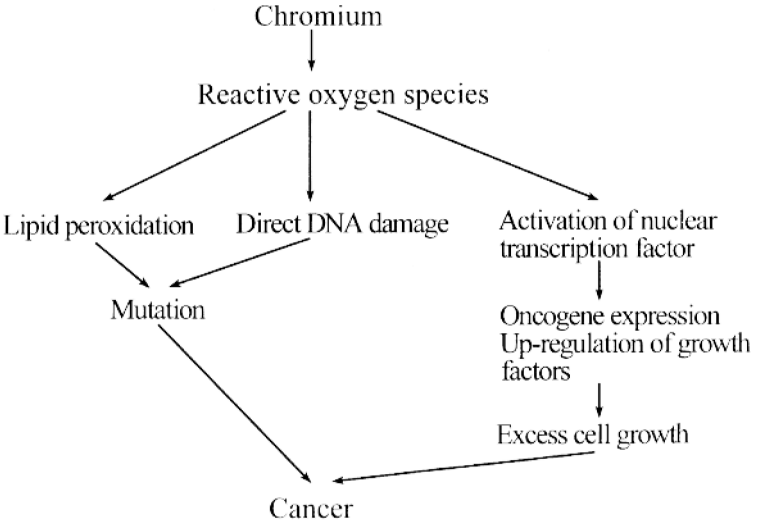


FIGURE 2. A schematic representation of the role of free-radical reactions in Cr(VI)-induced carcinogenesis.

free-radical reactions by enzymatic and nonenzymatic antioxidants. When the balance between pro-oxidants and antioxidants shifts in favor of pro-oxidants, chromium-induced oxidative injury occurs. Development of therapeutic agents to enhance intra- and extracellular antioxidant levels and to block chromium-mediated generation of ROS may prevent or attenuate Cr(VI)-induced carcinogenesis.

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