ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 292, No. 1, January, pp. 323–327, 1992

COMMUNICATION

The Role of Superoxide Radical in Chromium(VI)-Generated Hydroxyl Radical: The Cr(VI) Haber–Weiss Cycle[1]

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Received September 19, 1991, and in revised form October 10, 1991

To understand the role of the superoxide (O_2^-) radical in chromate-related genotoxicity, we investigated whether Cr(VI) can catalyze the Haber–Weiss cycle *in vitro:*

$$O_2^- + Cr(VI) \rightarrow Cr(V) + O_2$$

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^-.$$

ESR and spin trapping techniques were utilized to monitor the O_2^- (produced using xanthine/xanthine oxidase), $\cdot$OH, and Cr(V) species. Superoxide dismutase as well as catalase inhibited the $\cdot$OH radical radical formation, attesting to the direct involvement of O_2^- and H_2O_2 in the process. ESR measurements also provided direct evidence for the formation of Cr(V). Kinetic measurements were consistent with the role of Cr(V) and H_2O_2 as intermediates in $\cdot$OH formation. These results indicate that in cellular media, especially during chromate phagocytosis, the O_2^- radical can become a significant source of $\cdot$OH radicals and hence a significant factor in the biochemical mechanism of cellular damage due to Cr(VI) exposure.

The biochemical mechanism of the carcinogenic effect of chromate exposure on bacteria (1), mammalian cells (2), animals (3), and humans (4, 5) is still not fully understood (6). Recent studies have suggested, nevertheless, that some Cr(V) species, as well as hydroxyl ($\cdot$OH) radicals, play a significant role (7–20). As to the mechanism of $\cdot$OH generation in cellular media by chromate, henceforth referred to as Cr(VI), it has been suggested that one of the important steps is the reaction of a Cr(V) species with hydrogen peroxide (H_2O_2) through a Fenton-like mechanism (17–20):

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^-. \quad [1]$$

Earlier studies have also shown that in biochemical systems the Cr(V) species are generated via the reduction of Cr(VI) by a variety of cellular components, such as microsomes (9), mitochondria (21), mitochondrial electron transfer complexes (22), and flavoenzymes (glutathione reductase (GSSG-R), lipoyl dehydrogenase, and ferredoxin–$NADP^+$ oxidoreductase) (17, 23, 24). The Cr(V) species thus produced react with cellular H_2O_2 to generate the $\cdot$OH radical (Eq. [1]). Molecular oxygen (O_2) has also been found to play a critical role in Cr(VI) toxicity (25); therefore we thought that O_2^- might also be involved in these reactions. While no previous study has reported on the role of O_2^- in Cr(VI) toxicity, its investigation is considered important because O_2^- is produced in significant amounts by cellular organisms, especially during the phenomenon of phagocytosis, which results in so-called "respiratory burst" (26–28). The respiratory burst involves a large oxygen uptake and a concomitant generation of O_2^- and other toxic oxygenated species. Generally, however, O_2^- is relatively inert unless some metal ion catalyst is also present near the site of the O_2^- formation.

The purpose of this Communication is to point out that the O_2^- radical must be considered in the biochemical mechanism of cellular injury caused by exposure to Cr(VI). The current study indicated that Cr(VI) can catalyze the formation of $\cdot$OH radical from O_2^- via a Haber–Weiss type of cycle (29), i.e.,

$$O_2^- + Cr(VI) \rightarrow Cr(V) + O_2 \quad [2]$$

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^-. \quad [3]$$

Overall

$$O_2^- + H_2O_2 \xrightarrow{Cr(VI)} O_2 + \cdot OH + OH^-. \quad [4]$$

We employed ESR and spin trapping techniques to monitor the formation of Cr(V) from the reduction of Cr(VI) by O_2^- and the subsequent $\cdot$OH generation via the reaction of the Cr(V)

[1] This research has been supported by the Department of the Interior's Mineral Institutes program administered by the Bureau of Mines through the Generic Mineral Technology Center for Respirable Dust under Grant G1135142 and by NIOSH Grant U60-CCU306149-01-1.

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0003-9861/92 $3.00

species with H_2O_2. The xanthine/xanthine oxidase was utilized as a standard *in vitro* source of O_2^-, with superoxide dismutase (SOD)[3] as a control (30). Following an earlier report (24) NADH was utilized to chelate the Cr(V) intermediate to form an ESR-detectable Cr(V)–NADH complex. Catalase was utilized for confirming the role of H_2O_2. It is felt that the results described below clearly support the validity of Eqs. [2] and [3], thus indicating the role of O_2^- in the generation of the toxic Cr(V) and ·OH radical species in Cr(VI)-exposed cellular media.

MATERIALS AND METHODS

Xanthine and xanthine oxidase (bovine milk), purchased from Sigma, were used as a source of O_2^-. $K_2Cr_2O_7$ (as a source of Cr(VI)), phosphate buffer (pH 7.2), and ethanol were purchased from Fisher. Catalase (bovine liver) was purchased from Boehringer-Mannheim. NADH, H_2O_2, sodium formate, bovine superoxide dismutase, and diethylenetriamine pentaacetic acid (DETAPAC) were purchased from Sigma. The spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was purchased from Aldrich and was purified by charcoal decolorization (31).

ESR measurements were made utilizing a Bruker ER 200D X-band (9.7 GHz) ESR spectrometer, essentially as reported earlier (17–19). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separations. For accurate measurements of the *g*-values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ER 035A) and the microwave frequency was measured with a digital frequency counter (Hewlett–Packard, Model 5340A). For the measurement of relative concentrations of the free radicals and the Cr(V) species, care was taken to use the same quartz flat cell and to maintain the same orientation of the flat cell in the ESR cavity.

RESULTS

A. ·OH Formation in the Reaction of Cr(VI) with O_2^-

The xanthine/xanthine oxidase system was utilized as an *in vitro* method of generating O_2^-, and DMPO as the spin trap for measuring its concentration. The ESR spectrum obtained from a pH 7.2 phosphate buffer solution of 0.6 mM xanthine, 0.1 unit/ml xanthine oxidase, 100 mM DMPO, and 0.2 mM DETAPAC, in order to minimize the dismutation of O_2^- by any extraneous transition metal ion, was essentially identical with that reported earlier for the DMPO–OO^- adduct (32, 33). Further, addition of SOD to the reaction mixture eliminated the DMPO–OO^- signal, thus confirming O_2^- formation, and supporting our O_2^--detecting methodology.

Figure 1a shows a typical ESR spectrum obtained from an aqueous solution of $K_2Cr_2O_7$ (1 mM), xanthine (0.3 mM), xanthine oxidase (0.05 unit/ml), and DMPO (60 mM) in a pH 7.2 phosphate buffer solution. The spectrum consists of a 1:2:2:1 quartet with hyperfine splitting of $a_H = a_N = 14.9$ G (Fig. 1a). Based on these splitting constants (34), the 1:2:2:1 quartet was assigned to a DMPO–OH adduct. A confirmational piece of evidence for the ·OH radical trapping was obtained through the ·OH scavenging competition experiments, in which the ·OH radical abstracts a hydrogen atom from ethanol or formate, and the thus-formed radical is trapped by DMPO (35–37). As expected, addition of formate to the reaction mixture caused a dose-dependent decrease in the intensity of the DMPO–OH adduct signal and led to the appearance of a spin adduct signal with measured hyperfine splitting of $a_H = 18.7$ G and $a_N = 15.8$ G (data not shown). These splittings are typical of those of the DMPO–COO^- adduct (34), attesting to the trapping of ·OH radicals. Furthermore the relative amounts of the DMPO–COO^- and DMPO–OH spin adducts depend on the amount of formate added: an increase in formate concentration increases DMPO–COO^- while decreasing the DMPO–OH adduct as expected (35–37). Similar results were obtained using ethanol (data not shown), confirming that the DMPO–OH spin adduct formation (Fig. 1a) is indeed the result of trapping of the ·OH radical formed during the reaction. Omission of either xanthine or xanthine oxidase results in a sharp decrease in spectral intensity (Figs. 1a and 1b). The ·OH radical generation is SOD inhibitable (Fig. 1d), showing that O_2^- participates in the ·OH generation. Addition of catalase, whose function is to remove H_2O_2, causes a substantial reduction in the ·OH generation (Fig. 1e), while addition of H_2O_2 (2 mM) causes about a 10-fold increase (Fig. 1f).

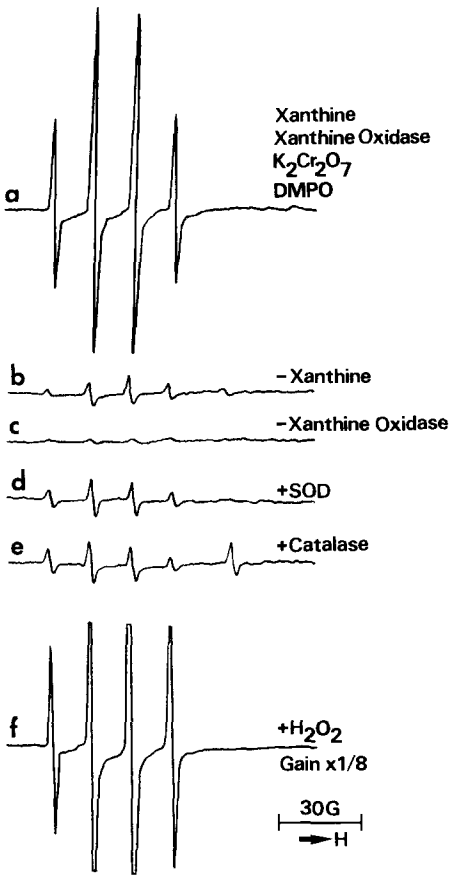

FIG. 1. (a) ESR spectrum recorded, 1 min after reaction initiation, from a pH 7.2 phosphate solution of 0.3 mM xanthine, 0.05 unit/ml xanthine oxidase, 1 mM $K_2Cr_2O_7$, and 60 mM DMPO. (b) Same as (a) but without xanthine. (c) Same as (a) but without xanthine oxidase. (d) Same as (a) but with 500 units/ml SOD added. (e) Same as (a) but with 2000 units/ml catalase added. (f) Same as (a) but with 2 mM H_2O_2 added. The spectrometer settings were receiver gain, 3.2×10^5; modulation [except 4.0×10^4 for [part of]]; modulation amplitude, 1.0 G; scan time, 8 min; time constant, 0.5 s.

[3] Abbreviations used: SOD, superoxide dismutase; DETAPAC, diethylenetriamine pentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

The time course of ·OH generation in the above discussed reactions is shown in Fig. 2. The concentration of DMPO–OH adduct generated in the reaction mixture of xanthine/xanthine oxidase, DMPO, and Cr(VI) increased during the first 2 min and then gradually decreased (plot b). Addition of H_2O_2 to this mixture markedly enhanced the DMPO–OH formation (plot a), with the reaction kinetics following a similar pattern. Omission of either xanthine or xanthine oxidase, or addition of either SOD or catalase caused a substantial reduction in DMPO–OH generation at all time intervals, as may be noted from plots c and d. The above kinetics study together with the results in Fig. 1 suggest that O_2^- and H_2O_2 play a seminal role in the ·OH formation and that the reaction of Cr(V) (generated in the reduction of Cr(VI) by O_2^-) with H_2O_2 is the likely mechanism.

B. Evidence for Cr(V) Formation by Cr(VI) and O_2^-

While the above-discussed measurements utilizing xanthine/xanthine oxidase suggested that O_2^- reacts with Cr(VI) to form Cr(V), we were unable to detect any Cr(V) species because of their short lifetime. It is known that NADH is a substrate for xanthine oxidase, albeit less effective than xanthine (38–40). However, since NADH can also chelate Cr(V) to form a relatively long-lived, ESR-detectable, Cr(V)–NADH complex (24), we used NADH/xanthine oxidase as the O_2^- source. We verified that NADH itself does not react significantly with O_2^- (data not shown). As shown in Fig. 3a, a reaction mixture containing NADH (1 mM), xanthine oxidase (0.05 unit/ml), $K_2Cr_2O_7$ (1 mM), and DMPO (60 mM) in a pH 7.2 phosphate buffer solution generates the DMPO–OH spin adduct as well as the long-lived Cr(V)–NADH complex (24) (g = 1.9792, as indicated). Omission of any one component results in a strong decrease in the overall spectral intensity (Figs. 3b–3d), showing that the whole reaction

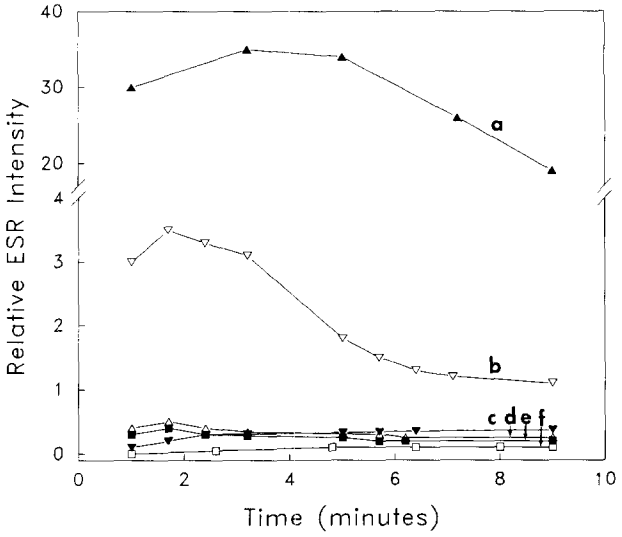

FIG. 2. Time course of DMPO–OH formation in a pH 7.2 phosphate buffer solution containing the following reaction mixtures. (▽) 0.3 mM xanthine, 0.05 unit/ml xanthine oxidase, 1 mM $K_2Cr_2O_7$, and 60 mM DMPO. (▼) Same as (▽) but without xanthine. (□) Same as (▽) but without xanthine oxidase. (■) Same as (▽) but with 500 units/ml SOD added. (△) Same as (▽) but with 2000 units/ml catalase added. (▲) Same as (▽) but with 2 mM H_2O_2 added.

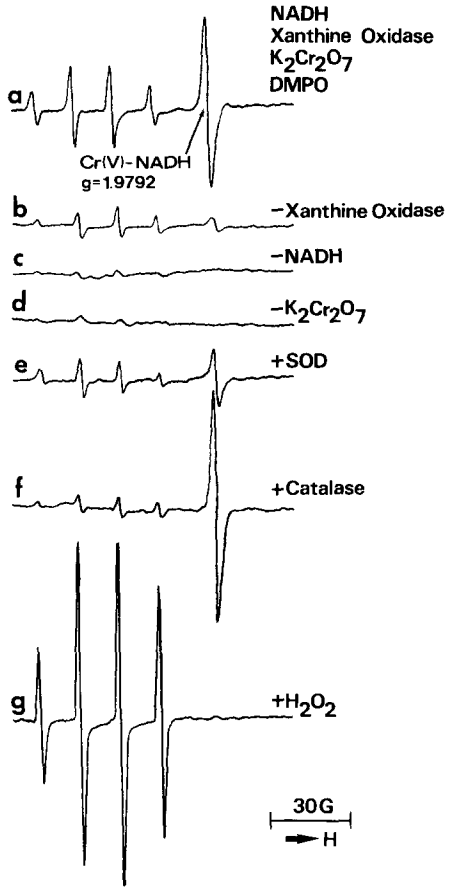

FIG. 3. (a) ESR spectrum recorded, 1 min after reaction initiation, from a pH 7.2 phosphate solution of 1 mM NADH, 0.05 unit/ml xanthine oxidase, 1 mM $K_2Cr_2O_7$, and 60 mM DMPO. (b) Same as (a) but without xanthine oxidase. (c) Same as (a) but without NADH. (d) Same as (a) but without $K_2Cr_2O_7$. (e) Same as (a) but with 500 units/ml SOD added. (f) Same as (a) but with 2000 units/ml catalase added. (g) Same as (a) but with 2 mM H_2O_2 added. The spectrometer settings were the same as those in Fig. 1.

system is required for the generation of both the ·OH radical and the Cr(V)–NADH complex. Addition of SOD also causes a significant decrease in the overall spectral intensity (Fig. 3e), showing that the generation of both the Cr(V)–NADH complex and the ·OH radical depends on O_2^-. Addition of catalase essentially eliminates the ·OH formation and enhances the Cr(V)–NADH generation, Fig. 3f indicating that the Cr(V)–NADH generation is due to the reduction of Cr(VI) to Cr(V) by O_2^- and subsequent chelation of Cr(V) by NADH. The H_2O_2 generated by the dismutation of O_2^- as well as via a two-electron reduction of O_2 by the xanthine oxidase (27–30) reacts with the Cr(V)–NADH complex to generate ·OH radical. This conjecture is further supported by the observation (Fig. 3g) that addition of H_2O_2 enhances the ·OH generation while essentially eliminating the Cr(V)–NADH complex.

Similar but more intense signals were observed from a reaction mixture containing xanthine/xanthine oxidase as a (stronger) source of O_2^-, but to which NADH was added to ligate Cr(V). In fact the Cr(V) peak was strong enough that it exhibited five partially resolved principal components with 0.84 G spacing as

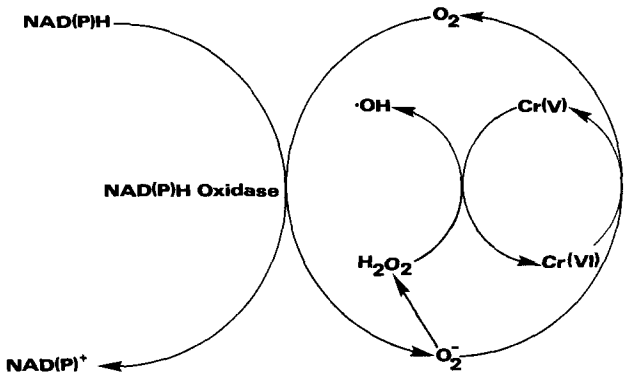

FIG. 4. A schematic representation of the proposed Cr(VI)-mediated Haber–Weiss type of reaction.

well as ^{53}Cr hyperfine satellites (data not shown), essentially identical with that of a Cr(V)–NADH complex reported earlier (18, 24). All the tests based on SOD and catalase yielded results similar to those in Fig. 3. Moreover, the time course of DMPO–OH formation under various conditions (data not shown) was essentially similar to that in Fig. 2. Together, the above results support the conjecture that ·OH radicals are generated from the reaction of an O_2^--generated Cr(V)–NADH complex with H_2O_2.

DISCUSSION

Based on the above-described ESR spectroscopic and enzymatic control measurements, we conclude that freely available O_2^- can reduce Cr(VI) to generate Cr(V), which can react with H_2O_2 to produce ·OH radical and regenerate Cr(VI). Thus, here Cr(VI) simply catalyzes the formation of ·OH from O_2^- through a Haber–Weiss cycle (Eq. [4]).

The above result is considered to be significant because earlier studies (10–20) have indicated that the ·OH radical plays a significant role in the mechanism of the genotoxic reactions of Cr(VI) compounds. As to the mechanism of ·OH generation, previous studies (17–20) have indicated that one significant pathway is the Fenton-type reaction: Cr(V) + H_2O_2 → Cr(VI) + ·OH + OH^- (Eq. [1]). While the exact pathway of the *in vivo* Cr(VI) reduction has not yet been clarified, the present work clearly indicates that a Haber–Weiss type of mechanism involving the generation of Cr(V) via a one-electron transfer from O_2^- should be considered. The present study clearly indicates that O_2^- could make a significant contribution to the total *in vivo* generation of Cr(V) as well as ·OH, both of which have been implicated in the mechanism of Cr(VI) genotoxicity (7–20). To our knowledge, this important role for O_2^- in Cr(VI) metabolism has not been addressed in any prior work.

The Haber–Weiss mechanism of ·OH generation could become particularly significant during phagocytosis when macrophages and other cellular constituents generate large quantities of the O_2^- radical in the so-called respiratory burst (27–29). It has been demonstrated that virtually all of the oxygen consumption by phagocytes is first converted to O_2^- (27). However, further conversion of O_2^- to ·OH is too slow to be biologically significant, unless a suitable ion (e.g., Fe^{2+}) is present as a Haber–Weiss catalyst (41). Thus the present finding that Cr(VI) can act as a Haber–Weiss catalyst may provide a basis for the known critical role of molecular oxygen in the toxic reactions of Cr(VI) vapor/particulates (25).

A scheme for the proposed Cr(VI)-mediated ·OH generation from O_2^- and H_2O_2 is presented in Fig. 4. As a corollary this diagram suggests that Cr(VI)-related toxicity might be controlled through the utilization of antioxidants (to combat O_2^- and ·OH) and also by using catalase (to remove H_2O_2 from the vicinity of Cr(V)). Additionally, Cr(V) chelators, such as deferoxamine (42), may be utilized for inhibiting the ·OH generation via Eq. [3].

In conclusion, this work provides the first experimental evidence that Cr(VI) is capable of catalyzing a Haber–Weiss cycle to generate ·OH radicals. This finding provides a clue as to why molecular oxygen plays a fundamentally important role in the mechanism of genotoxic reactions resulting from exposure to Cr(VI)-containing compounds. The results also imply that antioxidants could prove to be effective antagonists toward Cr(VI)-related genotoxicity.

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