

The role of hydroxyl radical as a messenger in Cr(VI)-induced p53 activation

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Wang, Suwei, Stephen S. Leonard, Jianping Ye, Min Ding, and Xianglin Shi. The role of hydroxyl radical as a messenger in p53 activation. *Am J Physiol Cell Physiol* 279: C868–C875, 2000.—The present study investigates whether reactive oxygen species (ROS) are involved in p53 activation, and if they are, which species is responsible for the activation. Our hypothesis is that hydroxyl radical ($\cdot\text{OH}$) functions as a messenger for the activation of this tumor suppressor protein. Human lung epithelial cells (A549) were used to test this hypothesis. Cr(VI) was employed as the source of ROS due to its ability to generate a whole spectrum of ROS inside the cell. Cr(VI) is able to activate p53 by increasing the protein levels and enhancing both the DNA binding activity and transactivation ability of the protein. Increased cellular levels of superoxide radicals ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and $\cdot\text{OH}$ radicals were detected on the addition of Cr(VI) to the cells. Superoxide dismutase, by enhancing the production of H_2O_2 from $\text{O}_2^{\cdot-}$ radicals, increased p53 activity. Catalase, an H_2O_2 scavenger, eliminated $\cdot\text{OH}$ radical generation and inhibited p53 activation. Sodium formate and aspirin, $\cdot\text{OH}$ radical scavengers, also suppressed p53 activation. Deferoxamine, a metal chelator, inhibited p53 activation by chelating Cr(V) to make it incapable of generating radicals from H_2O_2 . NADPH, which accelerated the one-electron reduction of Cr(VI) to Cr(V) and increased $\cdot\text{OH}$ radical generation, dramatically enhanced p53 activation. Thus $\cdot\text{OH}$ radical generated from Cr(VI) reduction in A549 cells is responsible for Cr(VI)-induced p53 activation.

Cr(VI) carcinogenesis; reactive oxygen species

THE TUMOR SUPPRESSOR PROTEIN p53 plays an important role in protecting cells from tumorigenic alteration (19, 22). Mutational inactivation of p53 has been found to be involved in various human cancers, which indicates the importance of p53 in human carcinogenesis (42). It has been reported that >50% of human cancers contain mutations in the p53 gene (15, 22). The p53 is activated in response to a variety of stimuli, such as UV, γ radiation, hypoxia, nucleotide deprivation, etc. (7, 10, 18, 23). The activation of p53 may cause either cell division cycle arrest or apoptosis (1, 9, 19, 40).

The p53 can be considered as one of the oxidative stress response transcription factors (42). There are several cysteine residues in the central domain of the p53 protein. These residues are crucial for the p53 protein binding to the specific DNA sequence. Redox modulation at a posttranslational level often occurs by reduction or oxidation of the disulfide bond. A reduced state is required for these cysteine residues to ensure that p53 protein would bind to specific consensus DNA and transactivate target genes (12, 27, 29). Among these genes, more than one-half of them are reported to be involved in the metabolism of reactive oxygen species (ROS) (28). ROS are also believed to be involved in the activation of p53 protein induced by UV and ion irradiation (11, 44). Questions remain to be answered concerning which species among ROS plays a key role in the process of p53 activation.

Cr(VI)-containing compounds are considered to be well-established carcinogens (4). They are potential inducers of tumors in experimental animals and active agents in the induction of DNA damage, such as DNA strand breakage (4, 13, 20, 21, 41, 43, 46). Industrial exposure to these compounds is reported to be associated with a higher incidence of human lung cancer (13, 21). Environmental exposure to Cr(VI) could induce lung toxicity in the short term and carcinogenicity over the long term (6). Cr(VI) and Cr(III) are the two stable chromium oxidation states found in nature. Unlike Cr(III), Cr(VI) can enter the cell through the anion transport system (3, 5). After it enters the cell, Cr(VI) is reduced by cellular reductants to its lower oxidation states, Cr(V) and Cr(IV) (31). These reactive chromium intermediates are capable of generating a whole spectrum of ROS (33, 35, 36, 39). ROS generated by these reactions can cause DNA strand breaks, base modification, lipid peroxidation, and nuclear transcription factor NF- κ B activation (30, 33, 35, 36, 39, 48). In the present studies, Cr(VI) was used as the source of ROS to investigate the role of ROS in p53 activation, and the following questions will be answered: 1) whether Cr(VI) is able to activate p53 protein, and 2) if so,

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whether ROS play a role in this process and which species is responsible for p53 activation.

MATERIALS AND METHODS

Materials. Sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7$) and chromium fluoride [Cr(III)] were purchased from Aldrich (Milwaukee, WI). Deferoxamine, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), superoxide dismutase (SOD), catalase, sodium formate, aspirin, NADPH, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO). Dihydroethidium (DE) was purchased from Molecular Probes (Eugene, OR). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation and was free of electron spin resonance (ESR) detectable impurities.

Cell culture. A human lung epithelial cell line (A549 cells) was obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in F-12K (Kaighn's modification) nutrient mixture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1,000 U/ml penicillin-streptomycin.

Nuclear extraction. Nuclear extracts were prepared by a modified method of Ye et al. (47). A549 cells suspended in F-12K plus 10% FBS were cultured in 35-mm cell culture plates at 5×10^6 cells/plate for 24 h. The cells were treated with chromium and other agents for 3 h. After washing the cells with 5 ml PBS twice, we added fresh F-12K medium with 10% FBS. The cells were then incubated at 37°C for various times. At the end of the culture period, cells were harvested and treated with 500 μl of lysis buffer [50 mM KCl, 0.5% Nonidet P-40 (NP-40), 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 100 μM dithiothreitol (DTT)] on ice for 4 min. After centrifugation at 14,000 rpm for 1 min, we saved the supernatant as a cytoplasmic extract. The nuclei were washed once with the same buffer without NP-40. The washed nuclei were suspended in a 100- μl volume of extraction buffer (500 mM KCl, 10% glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin, and DTT as the lysis buffer) and pipetted three times for proper mixing. After centrifugation at 14,000 rpm for 5 min, we harvested the supernatant and stored this nuclear protein extract at -70°C . The concentration was determined using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Western blot. The nuclear extraction proteins were used for Western blot analysis to determine the p53 protein level. This analysis was carried out as described before (8). Briefly, samples (20 μg protein) denatured with SDS were electrophoretically separated on 10% Tris-glycine gels (Novex, San Diego, CA) and were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The membrane was preblocked in milk buffer [Tris-buffered saline Tween (TBST) containing 5% nonfat milk] for 20 min and exposed for 1 h to 0.8 $\mu\text{g}/\text{ml}$ affinity-purified mouse antibody to p53 protein in fresh milk buffer. The membrane was rinsed and incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h. The membrane was then washed with TBST, and antibody binding sites were visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). All the antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA synthesizer

(model 392; Applied Biosystems, Foster City, CA). A p53 binding sequence (5'-AGACATGCCTAGACATTGT-3') was used to synthesize a p53 binding oligonucleotide. The synthesized single-stranded oligonucleotides were deprotected at 50°C overnight, dried in a speed vacuum, and then dissolved in the Tris-EDTA buffer. Complementary strands were denatured at 80°C for 5 min and annealed at room temperature. The double-stranded probe was labeled with [α - ^{32}P]ATP (Amersham, Arlington Heights, IL) using a T4 kinase (Bethesda Research Laboratories, Gaithersburg, MD).

Electrophoretic mobility shift assay. The DNA-protein binding reaction was conducted in a 24- μl reaction mixture including 1 μg poly[dI-dC] (Sigma), 3 μg nuclear protein extract, 3 μg BSA, 4×10^4 cpm of ^{32}P -labeled oligonucleotide probe (1 μg), 0.1 μg p53 antibody Ab-1 (Oncogene, Cambridge, MA), 3 μl distilled water, and 12 μl of 2 \times reaction buffer (24% glycerol, 24 mM HEPES, pH 7.9, 8 mM Tris \cdot HCl, pH 7.9, 2 mM EDTA, and 2 mM DTT). This mixture was incubated on ice for 10 min in the absence of the radiolabeled probe, then incubated for 20 min at room temperature in the presence of the radiolabeled probe. After the incubation, the DNA-protein complexes were resolved on a 3.5% acrylamide gel that had been prerun at 210 V for 30 min with 0.5 \times Tris-boric acid-EDTA buffer. The loaded gel was run at 210 V for 60 min, then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) for autoradiography. The film was developed after overnight exposure at -70°C .

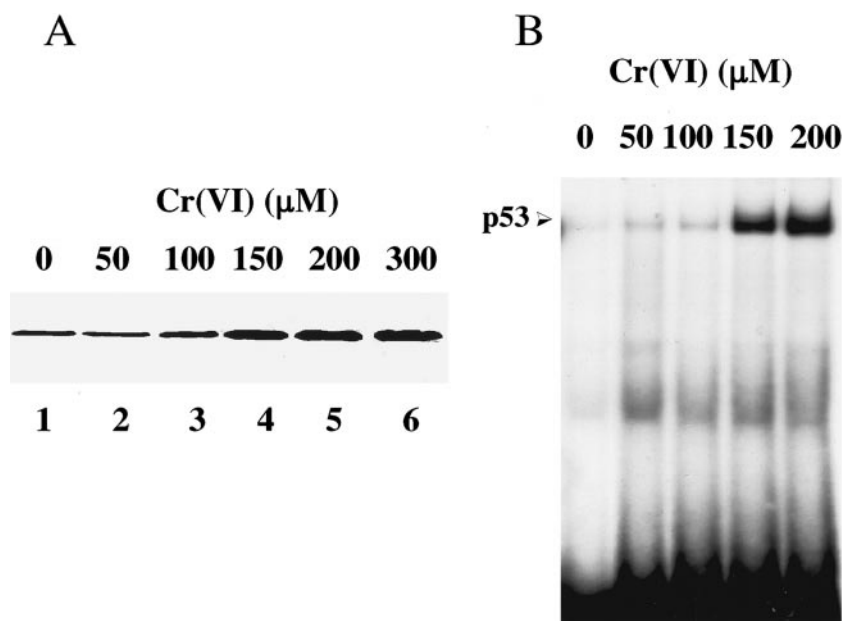
The concentrations given in the figure legends are final concentrations. All experiments were performed at room temperature and under ambient air except those specifically indicated otherwise.

Assay for p53 transcriptional activation. Human lung epithelial A549 cells (3×10^5) suspended in 1 ml of 10% FBS F-12K medium were seeded into each well of a six-well plate. After being incubated at 37°C for 24 h, the cells were transit transfected by 1 μg p53-luciferase reporter plasmid and 1 μg β -gal plasmid in reduced-serum medium. The cells were incubated overnight and then washed. Fresh 10% FBS F-12K medium was added. Twenty-four hours later, the cells were exposed to various concentrations of Cr(VI) for 3 h. After being washed, the transfected cells were incubated overnight in fresh F-12K medium. The cells were then extracted with 400 μl reporter lysis buffer in 4°C for 2 h. The luciferase activity was measured with 80 μl of cell lysate using a Monolight luminometer (model 3010; Analytical Luminescence Laboratory, Sparks, MD). β -gal activity was determined by using a method described before (47). The results are expressed as relative p53 activity compared with controls after normalizing by β -gal activity.

ESR measurements. ESR spin trapping was used to examine free radical generation. The use of this method is necessary because of the reactive nature of the free radicals to be studied. This technique involves an addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived trapped radical.

All measurements were conducted with a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 Gauss) directly from magnetic field separation with the use of potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. An EPRDAP 2.0 program (US EPR, Clarksville, MD) was employed for data acquisition and analysis. Reac-

Fig. 1. Dose-dependent p53 activation induced by Cr(VI). The A549 cells were adjusted to a density of 1×10^6 /ml and treated for 3 h with different concentrations of Cr(VI). The cells were washed, added to fresh medium, and incubated overnight. The nuclear extraction proteins were used for Western blot and DNA binding activity assay. **A:** Western blot for p53 protein level. *Lane 1*, untreated cells; *lane 2*, cells + 50 μ M Cr(VI); *lane 3*, cells + 100 μ M Cr(VI); *lane 4*, cells + 150 μ M Cr(VI); *lane 5*, cells + 200 μ M Cr(VI); *lane 6*, cells + 300 μ M Cr(VI). **B:** DNA binding assay for p53 protein. *Lane 1*, untreated cells; *lane 2*, cells + 50 μ M Cr(VI); *lane 3*, cells + 100 μ M Cr(VI); *lane 4*, cells + 150 μ M Cr(VI); *lane 5*, cells + 200 μ M Cr(VI).



tants were mixed in test tubes to a total final volume of 0.5 ml. The reaction mixture was then transferred to a flat cell for ESR measurement.

Cellular O_2^- and H_2O_2 assay. DE is a superoxide radical (O_2^-)-specific dye (2, 26). DE was dissolved in DMSO at 2 mM and kept at -20°C . The cells were plated onto a glass slip in the 24-well plate at 1×10^4 /well 16 h before treatment. DE was added to the cell culture together with the various treatments, and the cells were incubated at 37°C for 30 min. The final DMSO concentration in the cell culture was 0.1%. After the cells were stained, they were washed with PBS and fixed with 10% buffered formalin. The slip was mounted on a glass slide and observed with the use of a Saratrol 2000 (Molecular Dynamics, Sunnyvale, CA) laser scanning confocal microscope (Optiphot-2; Nikon, Melville, PA) fitted with an argon-ion laser. DCFH-DA at 2 μ M was used to monitor the H_2O_2 level inside the cells according to the methods reported earlier (14, 17, 49).

Oxygen consumption measurements. Oxygen consumption measurements were carried out with a Gilson oxygraph equipped with a Clark electrode (Gilson Medical Electronics, Middleton, WI). These measurements were made from mixtures containing 1.0×10^6 /ml cells and various treatments in a total volume of 1.5 ml. The oxygraph was calibrated with media equilibrated with oxygen of known concentrations.

RESULTS

The p53 activation by Cr(VI) is dose and time dependent. Human lung epithelial A549 cells were used to study induction of p53 activation by Cr(VI). The cells were incubated with different concentrations of Cr(VI) for 3 h. After washing the cells, fresh F-12K medium supplemented with 10% FBS was added. The cells were then incubated at 37°C overnight. The p53 protein level and DNA binding activity were analyzed in the nuclear extracts. As shown in Fig. 1A, the p53 protein level increased after exposure to increasing concentrations of Cr(VI). The DNA binding activity of the same protein was also investigated by electrophoretic mobility shift assay. The results are shown in Fig. 1B. From

this figure, a similar concentration-dependent pattern was found, i.e., the higher the concentration of Cr(VI), the higher the binding activity of p53 protein.

Figure 2 shows the effect of time on the p53 protein level and its DNA binding activity. A549 cells were

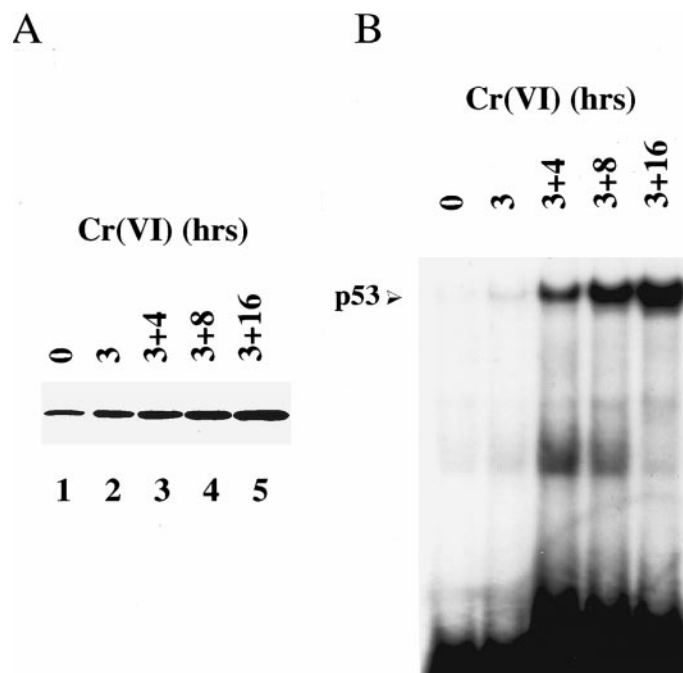


Fig. 2. Time-dependent p53 activation induced by Cr(VI). The A549 cells were adjusted to a density of 1×10^6 /ml and treated for 3 h with 150 μ M Cr(VI). The cells were washed, added to fresh medium, and collected at different times. The nuclear extraction proteins were used for Western blot and DNA binding activity assay. **A:** Western blot for p53 protein level. *Lane 1*, 0 h; *lane 2*, 3 h; *lane 3*, 4 h after the 3 h of treatment; *lane 4*, 8 h after the 3 h of treatment; *lane 5*, overnight after the 3 h of treatment. **B:** DNA binding assay for p53 protein. The experimental conditions are the same as A.

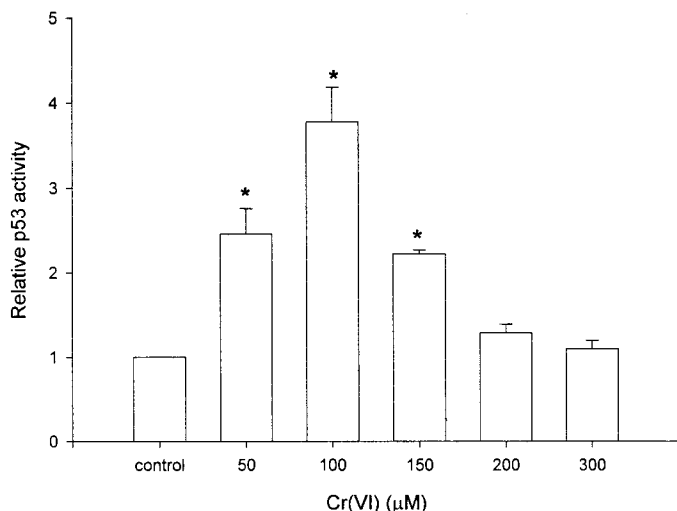


Fig. 3. Luciferase assay for Cr(VI)-induced p53 activation. Human lung epithelial A549 cells (3×10^5) suspended in 1 ml of 10% FBS F-12K (Kaighn's modification) medium were seeded into a 6-well plate. After being incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 h, the cells were transiently transfected by p53-luciferase reporter plasmid and β -gal plasmid in reduced-serum medium. The cells were incubated overnight, then washed, added to fresh 10% FBS F-12K medium, and incubated for 24 h. The cells were exposed to various concentrations of Cr(VI) for 3 h, washed, added to fresh F-12K medium, and incubated overnight. The p53 activity was measured by luciferase activity assay as described in MATERIALS AND METHODS. Results are presented as relative p53 induction compared with the untreated control cells (means and SD of 3 repeated assays). *Indicates a significant increase from control ($P < 0.05$).

treated with 150 μ M Cr(VI) for 3 h. Fresh F-12K medium with 10% FBS was added. The cells were then collected at different times. The same nuclear extractions were used for measuring protein level (Fig. 2A) and DNA binding activity (Fig. 2B). The results indicate that both p53 protein level and DNA binding activity were enhanced in a time-dependent fashion from 0 to 16 h postexposure.

Figure 3 shows the results of the luciferase assay for p53 activity. Increasing the Cr(VI) concentration up to 100 μ M enhanced p53 activation, whereas further increase in Cr(VI) concentration resulted in lower p53 activation, possibly due to Cr(VI)-induced apoptosis.

Detection of free radicals. ESR spin trapping was used to detect free radical generation from Cr(VI)-stimulated A549 cells. Fig. 4A shows the spectrum recorded from a mixture containing Cr(VI), A549 cells, and DMPO (a spin-trapping reagent). This spectrum consists of a 1:2:2:1 quartet with splittings of $a_H = a_N = 14.9$ G, where a_H and a_N denote hyperfine splittings of the α -hydrogen and the nitroxyl nitrogen, respectively. On the basis of these splitting constants, the 1:2:2:1 quartet was assigned to DMPO \cdot /OH adduct. The arrow at the right side shows a small peak that was assigned to a Cr(V)-NADPH complex as reported earlier (38). Addition of SOD, a O₂ \cdot^- scavenger whose function is to convert O₂ \cdot^- to H₂O₂, dramatically increased the DMPO \cdot /OH adduct signal (Fig. 4B). Catalase, an H₂O₂ scavenger, and sodium formate, a scavenger of \cdot OH radical, decreased the generation of \cdot OH

radical (Fig. 4, C and D). A metal chelator, deferoxamine, suppressed the DMPO \cdot /OH signal (Fig. 4E). NADPH, a cofactor of certain flavoenzymes, such as GSSG-R (glutathione reductase), which catalyzes the conversion of Cr(VI) to Cr(V), enhanced the generation of both Cr(V) and \cdot OH radical (Fig. 4F). H₂O₂ dramatically increased the DMPO \cdot /OH signal with reduction of the Cr(V) peak (Fig. 4G).

Specific fluorescent dyes were used to directly visualize free radical generation inside the A549 cells. DE, a specific fluorescent dye for O₂ \cdot^- , and DCFH-DA, a fluorescent dye for H₂O₂, were used to detect the generation of O₂ \cdot^- and H₂O₂, respectively. Both O₂ \cdot^- and

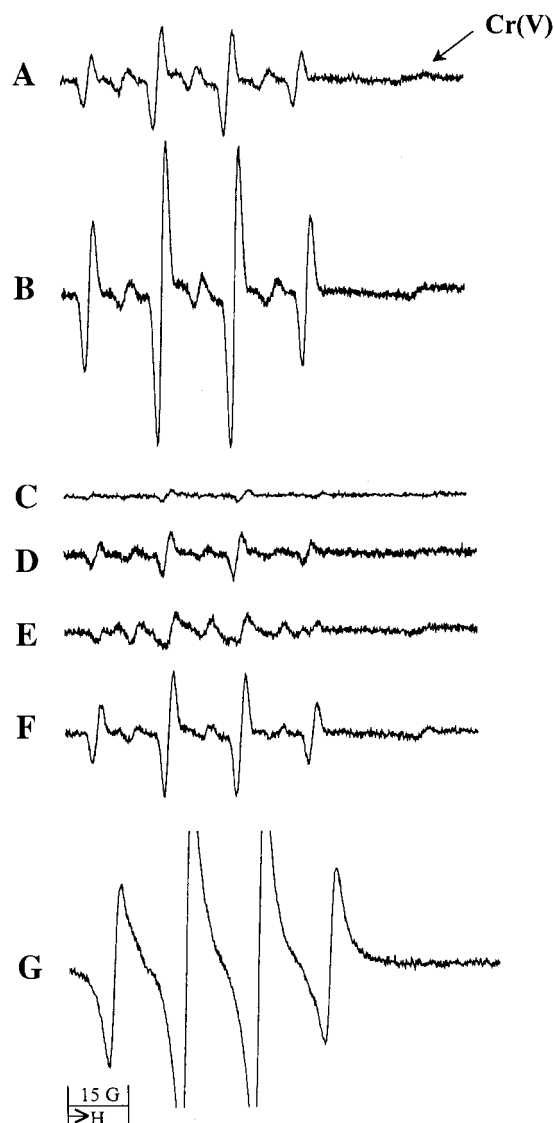


Fig. 4. Generation of free radicals from Cr(VI)-stimulated A549 cells. A: electron spin resonance (ESR) spectrum recorded 10 min after the addition of 1 mM Cr(VI) to 1×10^6 cells/ml and 100 mM 5,5-dimethyl-1-pyrroline 1-oxide in a phosphate-buffered solution (pH 7.4). B: same as A but with 1,000 U/ml superoxide dismutase (SOD) added. C: same as A but with 10,000 U/ml catalase added. D: same as A but with 50 mM sodium formate added. E: same as A but with 2 mM deferoxamine added. F: same as A but with 2 mM NADPH added. G: same as A but with 2 mM H₂O₂ added.

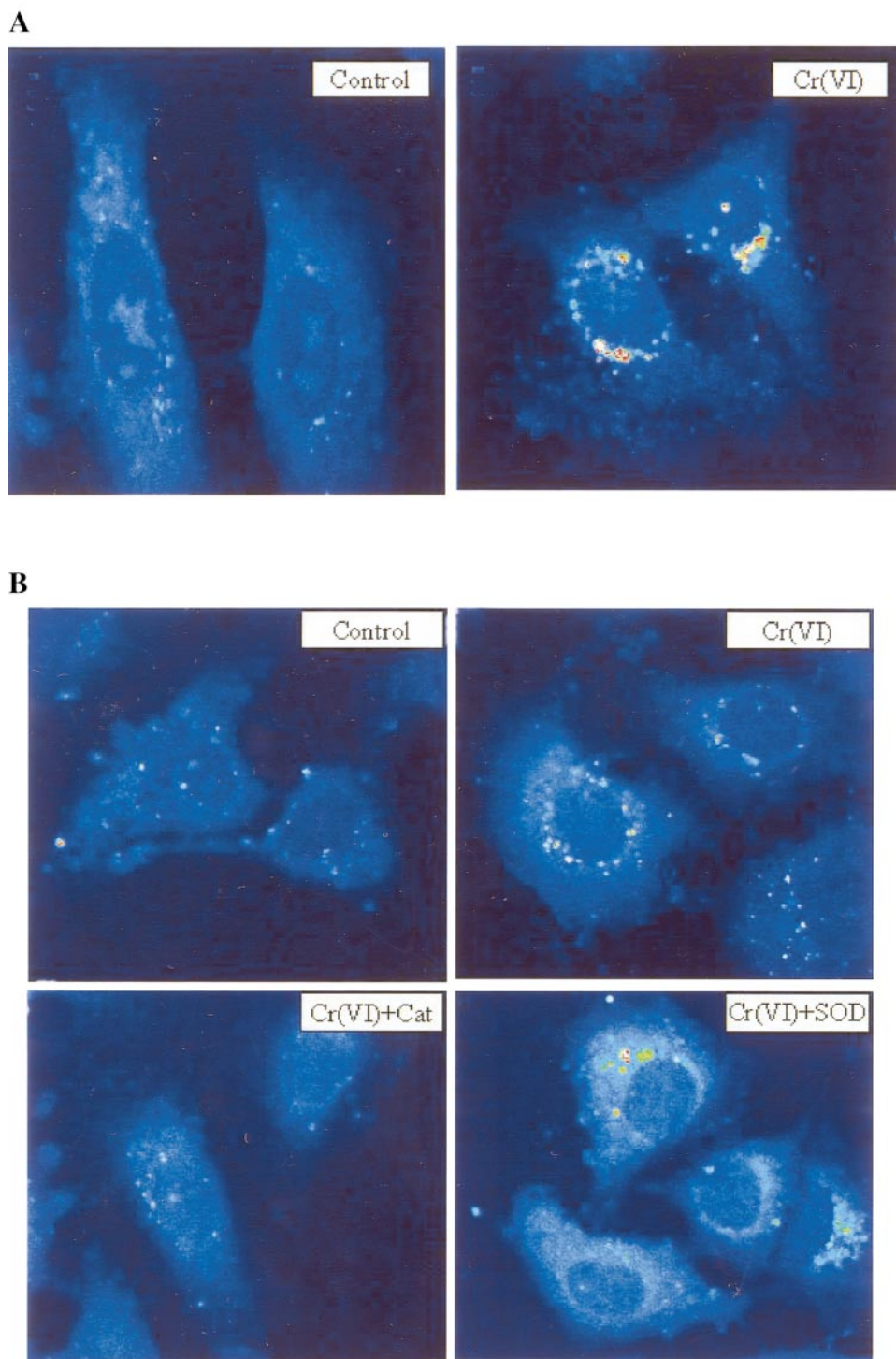


Fig. 5. Generation of $O_2^{\cdot-}$ radicals and H_2O_2 inside the A549 cells. *A*: fluorescent staining for $O_2^{\cdot-}$ radicals. The cells were incubated in the absence (*left*) or presence (*right*) of 150 μ M Cr(VI) in the presence of 2 μ M dihydroethidium for 30 min. *B*: fluorescent staining of H_2O_2 . The cells were incubated with 5 μ M 2',7'-dichlorofluorescein diacetate in the absence (control) or presence of 150 μ M Cr(VI), Cr(VI) + 10,000 U/ml catalase, and Cr(VI) + 1,000 U/ml SOD for 30 min. The cells were washed once in PBS and fixed with 10% formalin. The images were captured with a confocal microscope.

H_2O_2 can be visualized in untreated cells (Fig. 5). On stimulation with Cr(VI), $O_2^{\cdot-}$ radicals were dramatically enhanced (Fig. 5A). The H_2O_2 level also increased, although not as significantly as $O_2^{\cdot-}$ radicals (Fig. 5B). Addition of catalase decreased the amount of H_2O_2 inside the cell [Fig. 5B, Cr(VI) + Cat], whereas addition of SOD increased its level [Fig. 5B, Cr(VI) + SOD]. The changes occurred within 30 min. The fluorescent dyes exhibited a red or orange color after being oxidized by ROS.

Oxygen consumption. As shown before, \cdot OH radicals were produced via a Cr(V)-mediated Fenton-like reaction [$H_2O_2 + Cr(V) \rightarrow Cr(VI) + \cdot$ OH + OH^-]. The H_2O_2 was generated by the dismutation of $O_2^{\cdot-}$ radicals. $O_2^{\cdot-}$ radicals were generated by one-electron reduction of molecular oxygen during the reduction of Cr(VI) by flavoenzymes in the presence of NADPH. A Gilson oxygraph equipped with a Clark electrode was used to measure oxygen consumption in Cr(VI)-stimulated A549 cells. As shown in Fig. 6, addition of Cr(VI)

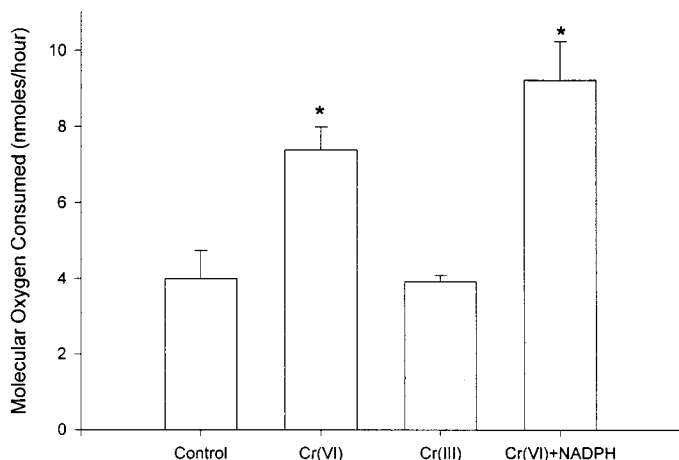


Fig. 6. Oxygen consumption by Cr(VI)-stimulated A549 cells. An incubation mixture contained 1 mM Cr(VI), 1×10^6 cells/ml cells, and various reagents [1 mM NADPH; 1 mM Cr(III)] as indicated. Values are means and SD of 3 repeated experiments. *Indicates a significant increase from the control ($P < 0.05$).

enhanced the oxygen consumption, and NADPH slightly accelerated it. Cr(III) did not have any significant effect.

·OH radical is responsible for Cr(VI)-induced p53 activation. A549 cells were incubated with Cr(VI) in the presence of various reagents for 3 h (Fig. 7). After the cells were washed, the fresh F-12K medium with 10% FBS was added. The cells were then incubated overnight and were harvested for extraction of nuclear proteins. The protein level was analyzed by Western blot. The results are shown in Fig. 7. As shown in this figure, the p53 protein level in the untreated A549 cells (lane 1) was below the detection limits. Cells treated with Cr(VI) showed an elevated p53 protein level (lane 2). Cr(III), because of its inability to enter the cell, had

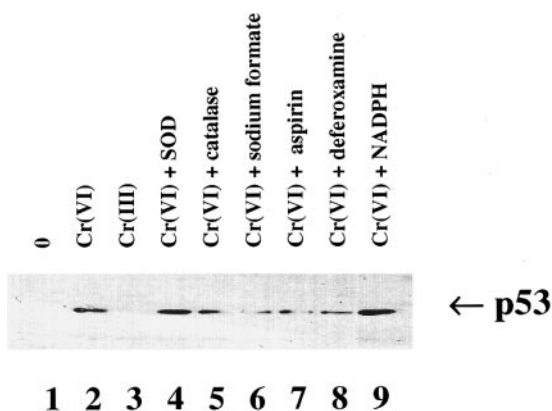


Fig. 7. Effects of reactive oxygen species on Cr(VI)-induced p53 activation. The A549 cells were adjusted to a density of 1×10^6 /ml and treated for 3 h with different stimuli. The cells were washed, added to fresh medium, and incubated overnight. The nuclear extraction proteins were used for Western blot. Lane 1, untreated cells; lane 2, cells + 150 μ M Cr(VI); lane 3, cells + 150 μ M Cr(III); lane 4, cells + 150 μ M Cr(VI) + 1,000 U/ml SOD; lane 5, cells + 150 μ M Cr(VI) + 10,000 U/ml catalase; lane 6, cells + 150 μ M Cr(VI) + 50 mM sodium formate; lane 7, cells + 150 μ M Cr(VI) + 2 mM aspirin; lane 8, cells + 150 μ M Cr(VI) + 2 mM deferoxamine; lane 9, cells + 150 μ M Cr(VI) + 2 mM NADPH.

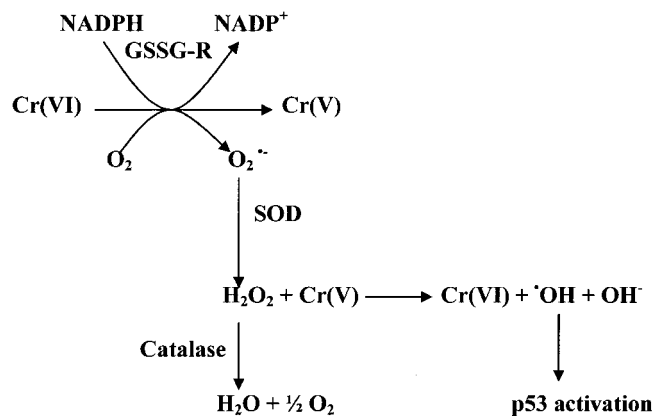


Fig. 8. Schematic representation of possible mechanism of \cdot OH radical generation in Cr(VI)-stimulated A549 cells. GSSG-R, glutathione reductase.

no effect (lane 3). SOD increased the protein level through the generation of H_2O_2 and \cdot OH radical (lane 4), whereas catalase, sodium formate, and aspirin scavenged \cdot OH radical and diminished the protein level (lanes 5–7). Deferoxamine chelated Cr(V) and decreased p53 protein induction (lane 8). NADPH, on the other hand, enhanced the generation of \cdot OH radical and increased p53 protein induction in A549 cells (lane 9). These results suggest that \cdot OH radical is responsible for Cr(VI)-induced p53 protein activation.

Results shown above support the generation of ROS in this cellular system. The reaction scheme presented in Fig. 8 may best accommodate the pathways for \cdot OH radical generation in Cr(VI)-stimulated A549 cells and the resultant p53 activation.

DISCUSSION

The present studies show that Cr(VI) is able to induce the activation of p53 protein in the epithelial cell line A549. \cdot OH radicals play a key role in this Cr(VI)-induced p53 activation.

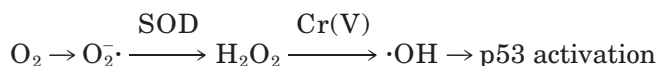
With the use of ESR with a loop-gap resonator, previous studies have demonstrated that reduction of Cr(VI) in live animals generates Cr(V) (24). Flavoenzymes, such as GSSG-R, are likely reductants for Cr(V) generation. With the use of a noncellular chemical system, our earlier studies have shown that reduction of Cr(VI) by GSSG-R in the presence of NADPH generates Cr(V) (31). The results obtained from the present studies show that Cr(VI) can be reduced by the whole cells to Cr(V). During the reduction process, molecular oxygen is reduced to $O_2\cdot^-$ radical, which generates H_2O_2 via dismutation. Cr(V) reacts with H_2O_2 to generate \cdot OH radicals via a Fenton-like reaction [$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^-$] (Fig. 8).

The \cdot OH radical is the key species among ROS responsible for p53 activation. The following experimental observations support the above conclusion. 1) Sodium formate, an \cdot OH radical scavenger, inhibited p53 activation. 2) Deferoxamine, which chelated Cr(V) to make it incapable of generating \cdot OH radical from H_2O_2 , diminished p53 activation. 3) SOD enhanced the p53

activation instead of attenuating it. This is due to the fact that SOD increased the H_2O_2 generation by catalyzing O_2^- dismutation, as demonstrated by fluorescent staining, and increased the $\cdot OH$ radical generation through a Fenton-like reaction, as demonstrated by ESR spin trapping. 4) A mixture of Cr(VI) and A549 cells increased molecular oxygen consumption. 5) Catalase, which depleted H_2O_2 and blocked $\cdot OH$ radical generation, inhibited the p53 activation.

H_2O_2 is an endogenous oxidant that is commonly used to investigate the role of ROS in p53 activation. However, inconsistent observations were reported from different laboratories (16, 27). For example, it has been observed that H_2O_2 can activate p53 protein and its target gene Bax (16). It has also been reported that H_2O_2 decreased p53 transactivation by weakening its binding to the specific DNA sequence (27). The results obtained from present study show that whereas H_2O_2 itself is unable to cause p53 activation, it can indirectly activate p53 through its ability to generate $\cdot OH$ radical on reaction with metal ions. The following experimental observations support this conclusion. 1) Sodium formate, an $\cdot OH$ radical scavenger, inhibited p53 activation. This $\cdot OH$ radical scavenger did not react with H_2O_2 . Similar results were obtained with the use of a different $\cdot OH$ radical scavenger, aspirin. 2) Deferoxamine, which inhibited p53 activation due to chelation of Cr(V) to make this metal ion unable to generate $\cdot OH$ radical from H_2O_2 , did not react with H_2O_2 . Although H_2O_2 is not a direct p53 stimulating agent, it functions as a precursor for $\cdot OH$ radical generation. Thus elimination of H_2O_2 inhibited p53 activation. This may explain the reported controversy concerning the role of H_2O_2 in p53 activation.

As for O_2^- radical, it is not a direct p53 stimulating agent either. As shown in the present studies, a specific O_2^- radical scavenger, SOD, enhanced p53 activation instead of causing inhibition. The enhancement of p53 activation is due to the generation of H_2O_2 from O_2^- radical catalyzed by SOD. O_2^- radical is generated via reduction of molecular oxygen as demonstrated by the oxygen consumption assay. Thus the following scheme can best accommodate p53 activation induced by ROS reaction



Cr(VI) is a well-established carcinogen. Although the mechanism of its action is still unclear, free radical reactions mediated by this metal ion are believed to play a key role. For example, $\cdot OH$ radicals generated by reactive chromium intermediates, such as Cr(V) and Cr(IV), can cause NF- κ B activation, DNA strand breakage, and cell injury. These radicals also function as messengers to induce p53 activation in response to Cr(VI)-induced cellular injury. The process of p53-dependent cell cycle arrest or apoptosis functions to repair or remove the damaged cells. Because of the fundamental importance of cell cycle arrest and apoptosis, which are regulated by p53, alternations of these path-

ways can enhance cancer development. Thus understanding of p53 activation, especially the role of ROS, is essential for understanding the overall carcinogenic process induced not only by Cr(VI), but also by other chemical carcinogens as well.

It may be noted that many other metal ions, mineral particles, and chemical carcinogens, such as cobalt, nickel, vanadium, asbestos, and silica are reported to be capable of generating $\cdot OH$ radical (25, 32, 34, 37, 45). It is possible that those agents may have the same function as Cr(VI); i.e., they may cause p53 activation through $\cdot OH$ radical as a common messenger. Because p53 is involved in various biological processes such as regulation of genes involved in the cell cycle, cell growth arrest after DNA damage, and apoptosis, $\cdot OH$ radical could be an important messenger in signal transduction pathways involved in carcinogenesis processes.

In conclusion, 1) Cr(VI) is able to induce the p53 activation, 2) ROS is involved in its mechanism of activation, and 3) $\cdot OH$ radical functions as a messenger in the p53 activation processes.

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