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Cr (VI) induces cell growth arrest through hydrogen peroxide-mediated reactions

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Abstract

Cr (VI) compounds are widely used in industries and are recognized human carcinogens. The mechanism of carcinogenesis associated with these compounds is not well understood. The present study focused on Cr (VI)-induced cell growth arrest in human lung epithelial A549 cells, using flow cytometric analysis of DNA content. Treatment of the cells with Cr (VI) at 1 μM caused a growth arrest at G_2/M phase. An increase in Cr (VI) concentration enhanced the growth arrest. At a concentration of 25 μM, Cr (VI)-induced apoptosis became apparent. Superoxide dismutase (SOD) or sodium formate did not alter the Cr (VI)-induced cell growth arrest. While catalase inhibited growth, indicating H_2O_2 is an important mediator in Cr (VI)-induced G_2/M phase arrest. Electron spin resonance (ESR) spin trapping measurements showed that incubation of cells with Cr (VI) generated hydroxyl radical (•OH). Catalase inhibited the •OH radical generation, indicating that H_2O_2 was generated from cells stimulated by Cr (VI), and that H_2O_2 functioned as a precursor for •OH radical generation. The formation of H_2O_2 from Cr (VI)-stimulated cells was also measured by the change in fluorescence of scopoletin in the presence of horseradish peroxidase. The mechanism of reactive oxygen species generation involved the reduction of molecular oxygen as shown by oxygen consumption assay. These results support the following conclusions: (a) Reactive oxygen species are generated in Cr (VI)-stimulated A549 cells through reduction of molecular oxygen, (b) Among the reactive oxygen species generated, H_2O_2 played a major role in causing G_2/M phase arrest in human lung epithelial cells. (Mol Cell Biochem **222**: 77–83, 2001)

Key words: chromium, cell cycle, apoptosis, reactive oxygen species

Introduction

Chromate (Cr (VI)) compounds, widely used in industry, have been shown to have serious toxic and carcinogenic effects on humans [1, 2]. Epidemiological studies in workers occupationally exposed to Cr (VI) compounds provided evidence on the high incidence of respiratory tract cancers [3–5]. Cr (VI) has been demonstrated to induce a variety of DNA lesions, such as single-strand breaks, alkali-labile sites, and DNA protein cross-links. In contrast, most Cr (III) compounds, the final product in the reduction of Cr (VI), are relatively nontoxic, noncarcinogenic, and nonmutagenic [6–8]. Since Cr (VI) does not react with isolated DNA, the reduction of Cr (VI) by cellular reductants to lower oxidation states has been considered to be an important step in the mechanism of Cr (VI)-induced carcinogenesis [7]. Earlier studies have shown that a relatively long-lived Cr (V) species is formed from the reduction of Cr (VI) by various cellular reductants [9]. Since Cr (V) complexes are generally characterized as being labile and reactive, whereas Cr (III) com-

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plexes are relatively inert, the Cr (V) intermediates have been suggested to be the likely candidates as the carcinogenic form of chromium compounds [10].

While several studies have shown that the Cr (VI)-induced DNA damage is strongly dependent on the formation of Cr (V) intermediates, free radicals generated by Cr (V) intermediates may also play an important role [11–18]. We have shown earlier that chromium is able to generate ·OH radical through a Cr (VI)-mediated Fenton and Haber-Weiss cycle [17, 18]. Treatment of Chinese hamster V-79 cells with FAD and Cr (VI) resulted in an increase in DNA strand breaks over that observed upon treatment of cells with Cr (VI) alone [12]. This increase in DNA strand breakage was attributed to enhanced Cr (VI)-related hydroxyl (·OH) radical formation in the presence of FAD. In contrast, incubation of Chinese hamster V-79 cells with an antioxidant, vitamin E, prior to treatment with Cr (VI) led to a decrease in Cr (VI)-induced DNA strand breaks [12, 14]. Participation of free radicals in the generation of Cr (VI)-mediated DNA damage in both noncellular systems and cultured human cells was suggested by the observation that this damage was inhibited by antioxidants [15, 16, 19].

Under normal circumstances the cell cycle proceeds without interruptions. However, when damage particular to DNA occurs, most normal cells have the capacity to arrest proliferation in G_1/S, or G_2/M and then resume proliferation after the damage is repaired [20]. The cell cycle controls the onset of DNA replication and mitosis in order to ensure the integrity of the genome [21, 22]. Lack of fidelity in DNA replication and maintenance can result in deleterious mutations, leading to cell death or, in multicellular organisms, cancer [20]. It has been reported that Cr (VI) not only decreased DNA synthesis in LL 24 cells, but also induced S phase arrest [23]. One to two μM $K_2Cr_2O_7$ produced a prolongation of the G_2 phase of the cell cycle in NHIK 3025 cells [24].

The goal of the present study was to answer the following questions. (a) Can Cr (VI) cause cell growth arrest in A549 cells? (b) If it can, does ROS play a role in Cr (VI)-induced cell growth arrest? (c) Among ROS, which species play a major role?

Materials and methods

Reagents

Potassium dichromate (Cr (VI)) and 5,5-diethylenetriaminepentaacetic acid (DMPO) were purchased from Aldrich (Milwaukee, WI, USA). RNase A, sodium formate, catalase, and superoxide dismutase (SOD) were purchased from Sigma (St. Louis, MO, USA). Propidium iodide (PI) was from Molecular probes (Eugene, OR, USA). Both F12K nutrient mixture medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Life Technologies, Gaithersburg, MD, USA). The spin trap, DMPO, was purified by charcoal decoloration and vacuum distillation and was free of ESR detectable impurities.

Cell culture

The human lung epithelial cell line, A549, was cultured in F12k nutrient mixture medium containing 10% FBS, 2 mM L-glutamine and 25 mg/ml gentamicin in an incubator at 5% CO_2 and 37°C.

Measurement of cell cycle/DNA content

DNA content in G_1/S and G_2/M phase were analyzed using flow cytometry [25, 26]. Cells were first fixed with 70% ice-cold ethanol for more than 2 h, followed by incubation with freshly prepared staining buffer (0.1% Triton X-100 in PBS, 200 mg/ml RNase A, and 20 mg/ml PI) for 15 min at 37°C. Cell cycle analysis was performed by flow cytometry with at least 10,000 cells for each sample. The percentage of cells in G_1/S and G_2/M phase were then analyzed using ModFit LT software.

Electron spin resonance (ESR) measurements

ESR spin trapping was used to examine hydroxyl radical generation. The spin adduct, a relatively long-lived free radical product formed by the reaction of a short-lived radical with a diamagnetic compound, can be measured by conventional ESR [27]. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped. All samples were measured using a Varian E9 ESR spectrometer and a flat cell assembly as described previously [9]. A549 cells (1×10^6) were mixed with DMPO (200 mM) to a total final volume of 0.5 ml of PBS. The reaction mixture was transferred to a flat cell for measurement for 5 min.

Cellular hydrogen peroxide (H_2O_2)

H_2O_2 was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase [16]. Fluorescence was measured at 37°C using 1×10^6 A549 cells suspended in 1 ml of PBS at an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a PerSeptive Biosystems Cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems Inc., Framingham, MA, USA).

Oxygen consumption assay

The reaction mixtures contained A549 cells (1×10^6 cells/ml) suspended in 1.5 ml of PBS [28]. Oxygen consumption was determined at 37°C using a Gilson oxygraph equipped with a Clark eletrode. The oxygraph was calibrated with medium equilibrated with oxygen of known concentrations.

Statistical analysis

All data were based on at least 3 independent experiments. H_2O_2 formation and oxygen consumption data were presented as means ± S.D. and analyzed using one-way ANOVA with the Scheffe's test. A p value less than 0.05 was considered statistically significant.

Results

Effects of Cr (VI) on the cell cycle

To investigate Cr (VI)-induced cell growth arrest, DNA content was measured by flow cytometry. Figure 1a shows the histogram of the A549 cells without Cr (VI) treatment as a control. It can be noted from this figure that 6.8% of the cell population was in G_2/M phase. Treatment of the cells with Cr (VI) at a concentration of 1 μM for 24 h increased the cell population at G_2/M phase to 10.02% (Fig. 1b). An increase in the Cr (VI) concentrations further increased the percentage of cells in G_2/M phase to 18.10% at 5 μM, and 26.16% at 10 μM (Figs 1c and 1d). At the concentration of 25 μM, cells underwent apparent apoptosis as a sub-G_1 peak appeared (Fig. 1e).

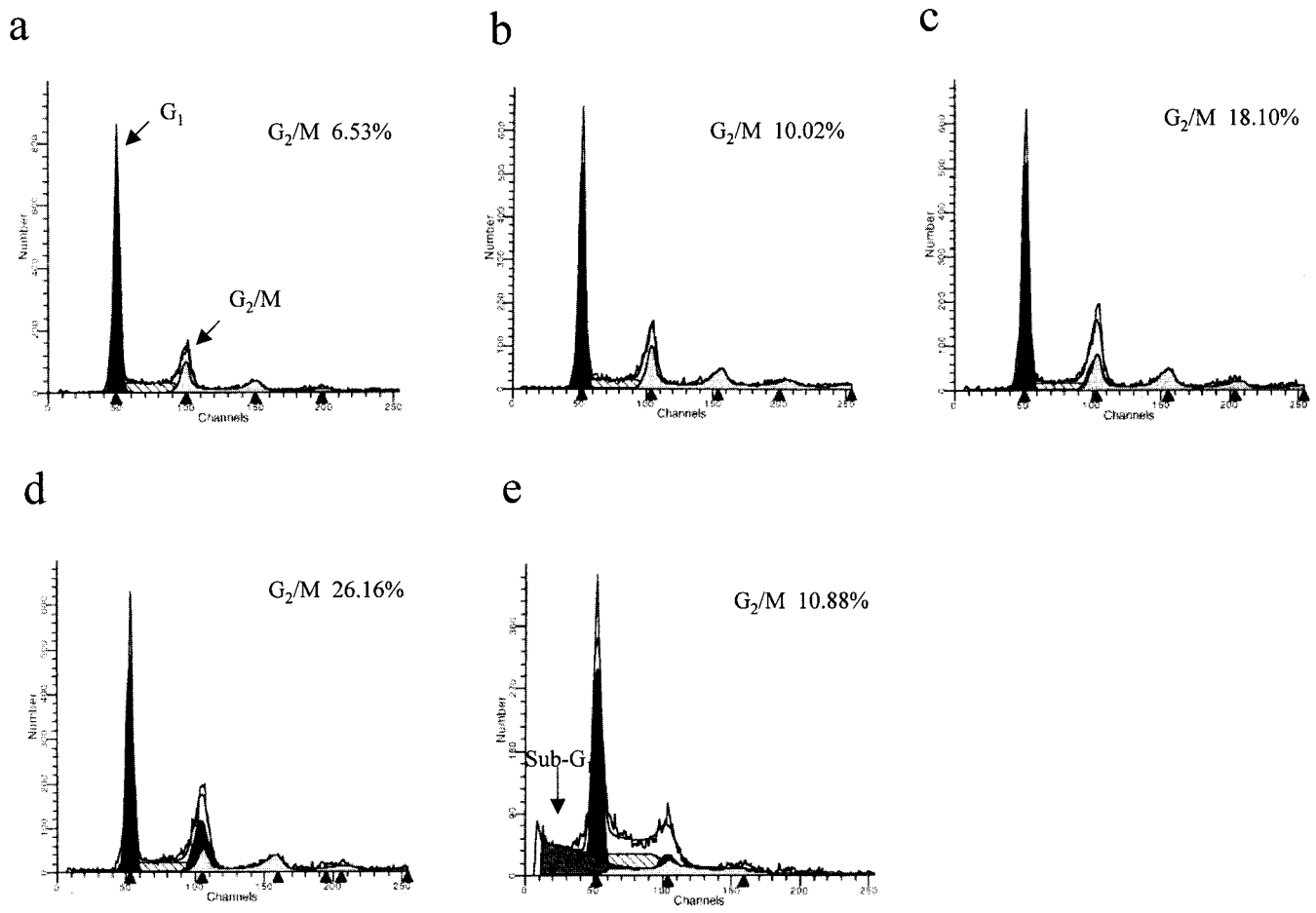

Fig. 1. Cr (VI)-induced cell growth arrest in human lung epithelial A549 cells. DNA content was measured by flow cytometry. A549 cells were suspended in 10% fetal bovine serum (FBS) F12 K nutrient mixture medium in a 100 mm dish. After 80–90% confluence, cells were washed with PBS for three times, and treated with various concentration of Cr (VI) at 37°C for 24 h: (a) cells only; (b) treatment with 1.0 μM Cr (VI); (c) treatment with 5.0 μM Cr (VI); (d) treatment with 10 μM Cr (VI); and (e) treatment with 25 μM Cr (VI). It was noted that at 25 μM Cr (VI), apoptotic cells became apparent as indicated by the sub-G_1 signal.

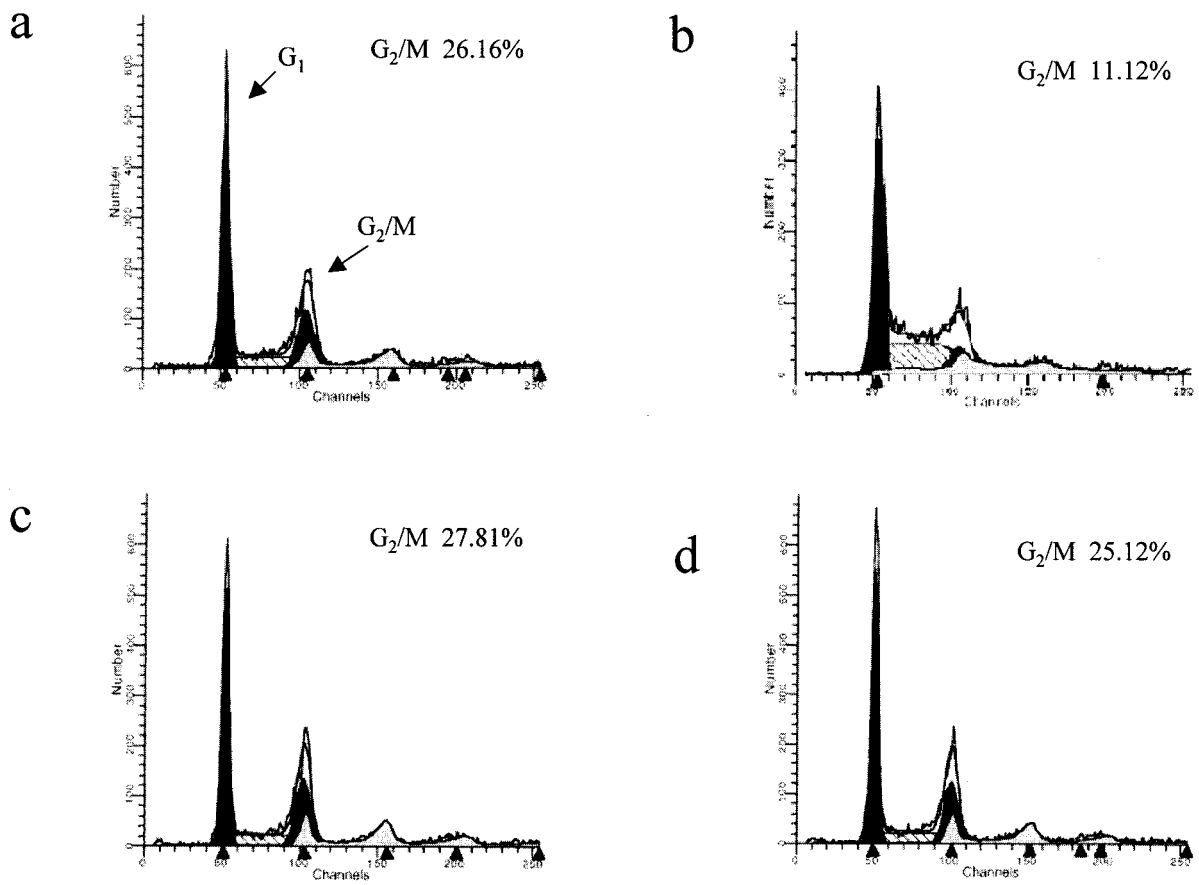

Fig. 2. Effect of antioxidants on Cr (VI)-induced cell growth arrest in human lung epithelial A549 cells. Cells were incubated in a 100 mm dish and pretreated with 50,000 units/ml catalase, 300 μM sodium formate, or 500 units/ml SOD for 0.5 h before Cr (VI) treatment (10 μM) for 24 h. (a) Cr (VI); (b) Cr (VI) + catalase; (c) Cr (VI) + sodium formate; (d) Cr (VI) + SOD.

Effects of antioxidants on Cr (VI)-induced cell growth arrest

Figure 2 shows the effects of antioxidants on Cr (VI)-induced G_2/M phase arrest. SOD, a specific $O_2^{\cdot-}$ radical scavenger, or formate, a specific $^{\cdot}OH$ radical scavenger, did not exhibit significant effect, i.e. the percentage of G_2/M phase was 26.16, 27.81 and 25.12% for Cr (VI), Cr (VI) plus formate and Cr (VI) plus SOD, respectively (Figs 2a, 2c and 2d), indicating that neither $O_2^{\cdot-}$ nor $^{\cdot}OH$ was directly involved in the mechanism of Cr (VI)-induced cell growth arrest. Catalase, a scavenger of H_2O_2, decreased Cr (VI)-induced G_2/M phase arrest from 26.16 to 11.12%, indicating that H_2O_2 was involved in Cr (VI)-induced cell growth arrest (Fig. 2b).

Hydroxyl radical formation from Cr (VI)-stimulated cells

ESR study was used to detect the $^{\cdot}OH$ formation from Cr (VI)-stimulated cells. A549 cells alone did not produce any detectable amount of free radicals (Fig. 3a), whereas addition of 2 mM Cr (VI) generated a 1:2:2:1 quartet ESR spin adduct signal (Fig. 3b). The splittings of this spectrum were $a_H = a_N = 14.9$ G, where a_H and a_N denote hyperfine splittings of the α-hydrogen and the nitroxyl nitrogen, respectively, indicating the DMPO/$^{\cdot}OH$ adduct (Shi and Dalal, 1992a). The detection of this DMPO/$^{\cdot}OH$ spin adduct is evidence for $^{\cdot}OH$ generation. The peak at the right side was assigned to a Cr (V) intermediate based on the lineshape and the g value. Addition of formate (50 mM), an $^{\cdot}OH$ scavenger, reduced the signal intensity by 50% (Fig. 3d). Catalase (2000 units/ml), a specific scavenger of H_2O_2, inhibited the generation of $^{\cdot}OH$ by 90% (Fig. 3c). The inhibition of $^{\cdot}OH$ generation upon addition of catalase indicates that H_2O_2 was generated, and that it was a precursor for $^{\cdot}OH$ generation. SOD (5 μg/ml), a scavenger of $O_2^{\cdot-}$ radical, did not show any significant inhibition, showing that the 1:2:2:1 quartet signal is not due to the $O_2^{\cdot-}$ radical trapping.

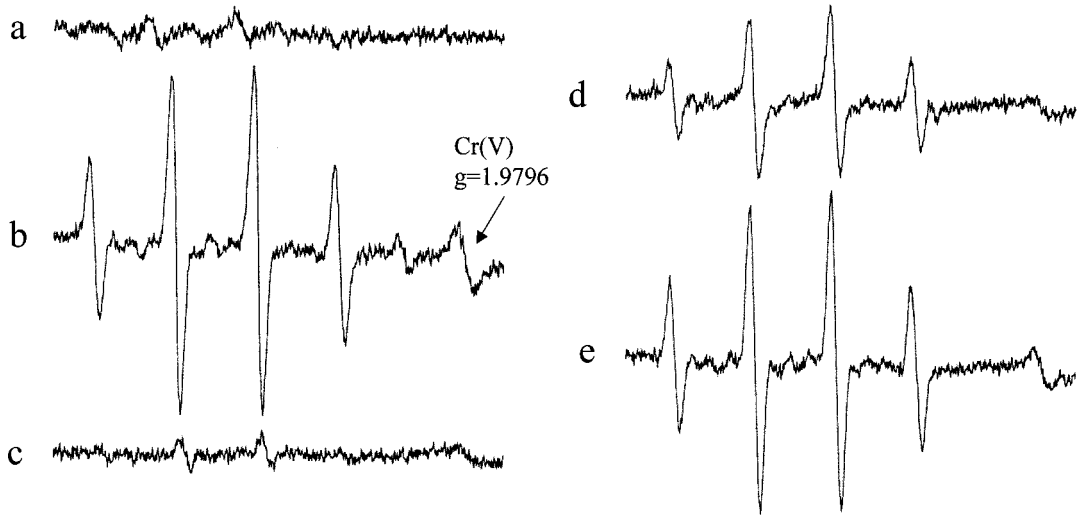

Fig. 3. Cr (VI)-induced free radical formation and the effects of antioxidants. 1×10^6 A549 cells were suspended in 0.5 ml of PBS and mixed with 200 mM DMPO and Cr (VI) with or without antioxidants. ESR spectra were recorded for 5 min at operational conditions. (a) A549 cells only; (b) cells + Cr (VI); (c) cells + Cr (VI) + catalase; (d) cells + Cr (VI) + sodium formate; (e) cells + Cr (VI) + SOD. The final concentration were: Cr (VI), 2 mM; catalase, 2,000 units/ml; sodium formate, 50 mM; SOD, 5 μg/ml.

Formation of hydrogen peroxide

As discussed in the previous section, catalase inhibition of ·OH generation from A549 cells stimulated by Cr (VI) implied that H_2O_2 was a precursor of ·OH production. In this section, the H_2O_2 generation was measured directly as the change in fluorescence of scopoletin in the present of horseradish peroxidase. Figure 4 shows that stimulation of A549 cells with 2 mM Cr (VI) increased H_2O_2 production by 3-fold above the sum of the fluorescence for cells alone and Cr (VI) alone.

Oxygen consumption by the Cr (VI)-stimulated cells

Since both H_2O_2 and ·OH were generated in the Cr (VI)-stimulated cells, it would be expected that H_2O_2 was generated from the reduction of molecular oxygen via $O_2^{\cdot-}$ radical as an intermediate. The O_2 consumption from cells was measured using an oxygraph. O_2 consumption was 176 nmol/5 × 10^5 cells in control cells, whereas it was 235 nmol/5 × 10^5 cells after Cr (VI) treatment, i.e. an increase in O_2 consumption by 33% (Fig. 5).

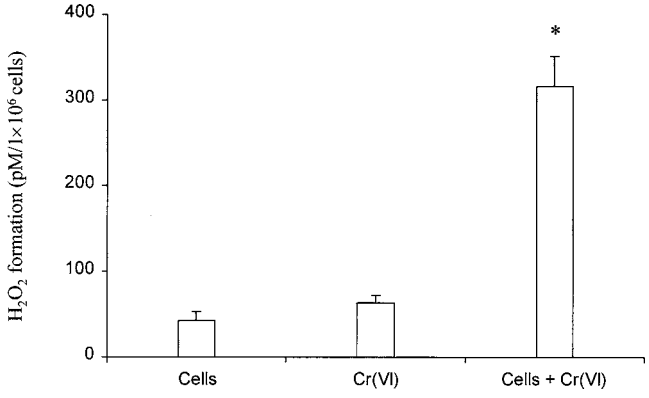

Fig. 4. Cr (VI)-induced H_2O_2 formation from human lung epithelial A549 cells. H_2O_2 was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase. The samples contained 1.0×10^6 cells suspended in 1 ml of PBS with and without 2 mM Cr (VI). Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. *$p < 0.05$ compared to control (one-way ANOVA with Scheffe's test).

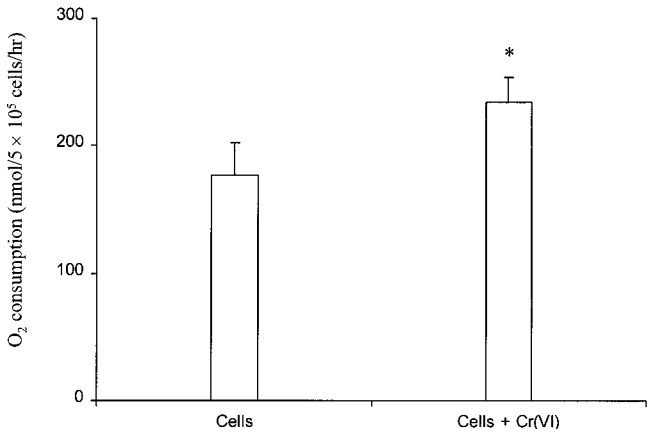

Fig. 5. Cr (VI)-stimulated oxygen consumption from human lung epithelial A549 cells. 1×10^6 cells were suspended in 1.5 ml of PBS and of oxygen consumption measured at 37°C using an oxygraph. The final concentration of Cr (VI) was 2 mM. *$p < 0.05$ compared to control (one-way ANOVA with Scheffe's test).

Discussion

ROS-mediated reactions are believed to be involved in various pathological processes. Initiation of carcinogenesis can be associated with an imbalance caused by the excessive generation of ROS. ROS can cause DNA strand breaks, base modification, lipid peroxidation and protein modification, resulting in oxidative stress. Various signal pathways are involved in the mechanism of ROS-induced oxidative stress, including activation of nuclear transcription factors, NF-κB [29], AP-1 [30], and p53 [31]. Under oxidative stress, dividing cells will exhibit a cell cycle checkpoint response [32, 33]. The division of cells must be carefully regulated and coordinated with both cell growth and DNA replication in order to ensure the formation of progeny cells containing intact genomes [20]. In higher eukaryotes, the cell machinery is itself regulated by the growth factors that control cell proliferation, allowing the division of individual cells to be coordinated with the needs of the organism as a whole. Defects in cell cycle regulation are a common cause of the abnormal proliferation of cancer cells. The cell cycle regulation and carcinogenesis are believed to be closely interconnected.

Cell cycle checkpoints monitor movement through the cell cycle, survey for cell damage, and induce a pause in cell cycle progression when necessary. Damage to growing cells causes a temporary pause in G_1/S, or G_2/M phase until the damage is repaired. When damage is severe, cells may either undergo apoptosis or enter a dormant G_0 state. Regulation of cell cycle progression is achieved by events including cyclin accumulation and degradation; phosphorylation of Cdks, cyclins, and other proteins; regulation of cyclin/Cdk dimerization; and the binding of a number of Cdk inhibitory proteins [34–36]. Movement of cells from G_2 to M is regulated by cyclin A and cyclin B/Cdc2. Cylin B/Cdc2 kinase activity peaks in late G_2 and remains high until its degradation [37]. Previous study has shown that Cr (VI) treatment of normal human lung cells results in guanine-specific DNA polymerase arrest, DNA-DNA cross-links and S-phase blockade of the cell cycle [23]. The present study shows that Cr (VI) is able to cause cell growth arrest at G_2/M phase in the human lung epithelial cell line, A549 cells. The percentage of cells in G_2/M phase increased in a dose-dependent manner following treatment with Cr (VI). ROS generated in the reduction of Cr (VI) by the cells are involved in the Cr (VI)-induced cell growth arrest. Among ROS generated by Cr (VI) stimulation of A549 cells, H_2O_2 appears to be the species responsible for Cr (VI)-induced cell growth arrest at the G_2/M phase. The following experimental observations support this conclusion. (a) Catalase, a specific H_2O_2 scavenger, decreased Cr (VI)-induced growth arrest, (b) Neither SOD, a $O_2^{\cdot-}$ scavenger, nor sodium formate, an $^{\cdot}OH$ radical scavenger, exhibited any effect.

The generation of ROS by Cr (VI) stimulated A549 cells is demonstrated in the present study by ESR spin trapping. Molecular oxygen is the source of ROS generation in Cr (VI)-stimulated A549 cells as demonstrated by the oxygen consumption assay. Molecular oxygen was consumed to generate $O_2^{\cdot-}$, which produced H_2O_2 upon dismutation. H_2O_2 produced $^{\cdot}OH$ via a Fenton-like reaction (Cr (V) + $H_2O_2 \rightarrow$ Cr (VI) + $^{\cdot}OH + OH^-$). Catalase scavenged H_2O_2, a precursor of $^{\cdot}OH$, and inhibited $^{\cdot}OH$ generation.

It may be noted that, while at 1–10 μM, Cr (VI) caused cell growth arrest at G_2/M phase, Cr (VI) induced-apoptosis became apparent at a concentration of 25 μM. It appears that at this concentration cell growth arrest was not sufficient for cells to repair the damage. Thus the apoptosis mechanism was initiated. Apoptosis is a process in which cell death occurs in an orderly manner through activation and/or synthesis of gene products necessary for cell destruction. It is a response to physiologic and pathologic stress that disrupts the balance between the rate of cell division and elimination. In diseases such as cancer, there is imbalance between the rate of cell proliferation and cell death. Agents that promote or suppress apoptosis can alter the rates of cell division and death, influencing the anomalous accumulation of neoplastic cells. Using morphological and DNA fragment analyses, our laboratory has shown that Cr (VI) is able to cause apoptosis through both p53-dependent and p53-independent mechanisms [38]. The results obtained from the present study using flow cytometry show that at a relative low concentration, Cr (VI) caused cell growth arrest while at relatively high concentration apoptosis was initiated.

It may also be noted that many other metal ions, mineral particles and chemical carcinogens, such as cobalt, nickel, vanadium, asbestos and silica are known to be cable of generating ROS. It is possible that these agents may have the same function as Cr (VI); i.e. they may cause cell growth arrest and apoptosis. Because both cell growth arrest and apoptosis are important mechanisms in repair of damaged cells and elimination of the cells which are severely damaged cells, cell growth arrest and apoptosis induced by Cr (VI) and other carcinogens could have an important influence on the induction of carcinogenesis.

The results obtained from the present study support the following conclusions: (a) Cr (VI) is able to induce cell growth arrest at the G_2/M phase in human lung epithelial A549 cells, (b) While at relatively low concentrations Cr (VI) causes cell growth arrest and relatively high concentrations induce apoptosis, (c) ROS generated by Cr (VI)-stimulated cells are involved in Cr (VI)-induced cell growth arrest and among ROS, H_2O_2 plays a key role, (d) H_2O_2 is generated by the reduction of molecular oxygen by cells. From the results it can be speculated that other metal carcinogens, such as vanadium, cobalt and nickel, which are ROS-promoting agents, may cause cell growth arrest and apoptosis by a mechanism similar to that of Cr (VI).

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