Molecular and Cellular Biochemistry **255:** 171–179, 2004.

$PbCrO_4$ mediates cellular responses via reactive oxygen species

Stephen S. Leonard,[1,2] Jenny R. Roberts,[1] James M. Antonini,[1] Vince Castranova[1] and Xianglin Shi[1]

[1]*Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV;* [2]*Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, WV, USA*

Abstract

Exposure to certain particulate hexavalent chromium [Cr(VI)] compounds, such as lead chromate ($PbCrO_4$), has been associated with lung cancer and respiratory tract toxicity. Previous studies indicate that the solubility of Cr(VI)-compounds is an important factor in Cr(VI)-induced carcinogenesis. The present study investigates reactive oxygen species (ROS) generation by $PbCrO_4$ particles and cellular responses using RAW 264.7 cells. A mixture containing $PbCrO_4$ and RAW 264.7 cells generated hydroxyl radical (·OH), using cellularly generated H_2O_2 as a precursor, as measured by electron spin resonance (ESR) spin trapping in combination with H_2O_2 and ·OH scavengers, catalase and sodium formate. The effect of ascorbic acid on ·OH radicals was also measured using ESR. Confocal microscopy showed that particles could become either bound to the cell surface or engulfed over a 120 min time period. H_2O_2 generation and O_2 consumption were also increased after treatment of the cells with $PbCrO_4$. Both NF-κB and AP-1 were activated after exposure to $PbCrO_4$ particles as measured by the NF-κB or AP-1 luciferase reporter plasmid assay. Our investigation thus demonstrated that the RAW 264.7 cells phagocytized the $PbCrO_4$ particles leading to accumulation of the particles within vacuoles in the cytoplasm. These particles could induce chronic production of ROS and activation of NF-κB and AP-1. Such induction of transcription pathways may be involved in the inflammatory and carcinogenic responses induced by Cr(VI)-containing particles. (Mol Cell Biochem **255**: 171–179, 2004)

Key words: $PbCrO_4$, RAW 264.7 cells, electron spin resonance (ESR), hydroxyl radicals, NF-kB, AP-1

Introduction

Cr(VI) compounds are widely used in industries, such as plating, paint, steel, tanning, and chrome ore processing [1, 2]. Previous studies have demonstrated a relationship between exposure to chromium [Cr(VI)] compounds, such as lead chromate, and respiratory tract toxicity as well as lung cancer [3]. Cr(VI) causes mutations in bacteria and transformations in mammalian cells [4, 5]. Cellular, animal and epidemiological studies have established that Cr(VI) compounds are toxic and carcinogenic [6, 7]. It has been shown that $PbCrO_4$ can be phagocytized by cells and can accumulate within vacuoles in the cytoplasm [8]. Light and scanning electron microscopy have been used to observe the cytoplasmic engulfment of $PbCrO_4$ particles [8, 9]. This internalization has been associated with the cellular stress response to $PbCrO_4$ exposure [10, 11]. These particles result in the chronic production of reactive oxygen species (ROS). Previous studies have shown that these species are genotoxic and clastogenic [12, 13]. Cr(VI) compounds use a complex metabolic reducing pathway, which generates a variety of reactive forms of chromium and ROS, which are believed to be responsible for their genotoxic and mutagenic characteristics [14, 15]. They are also able to generate hydroxyl radical (·OH) from H_2O_2 via a Fenton-like reaction [16]. During the cellular Cr(VI) reduction process, molecular oxygen is consumed to generate superoxide radical ($O_2^{\cdot -}$) and H_2O_2 [15–18]. Cr(VI)-generated ROS have been shown to cause DNA damage [19, 20], activation of nuclear

Address for offprints: X. Shi, Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505, USA (E-mail: xshi@cdc.gov)

transcription factors (NF-κB, AP-1) [21–23], apoptosis [24] and activation of mitogen-activated protein (MAP) kinases [22].

In contrast to the numerous studies with soluble Cr(VI) compounds there are few studies available on the generation of ROS and related toxicity by particulate Cr(VI) compounds. $PbCrO_4$ is designated as insoluble in H_2O and slightly soluble in alkaline or dilute acid solutions [25]. However, phagocytosis of $PbCrO_4$ particles can lead to localized chronic exposure to chromate [17, 26–29]. Highly insoluble or only slightly soluble chromate particles, such as Pb, Zn, and Ca, have been shown to cause tumor induction at sites of injection [28]. Previous studies indicate that the solubility of Cr(VI)-compounds is an important factor for the development of Cr(VI)-induced carcinogenesis, the present study used a cellular model to investigate the generation of ROS and cellular responses after exposure of the cells to $PbCrO_4$ particles.

Materials and methods

Reagents

Lead (II) chromate ($PbCrO_4$) and 5,5-dimethyl-l-pyrroline N-oxide (DMPO) were purchased from Aldrich (Milwaukee, WI, USA). Deferoxamine, sodium formate, H_2O_2, ascorbic acid, and nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) were purchased from Sigma (St. Louis, MO, USA). Catalase was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Phosphate-buffered saline (PBS) was purchased from Gibco BRL (Gaithersburg, MD, USA). Nile red was purchased from Molecular Probes (Eugene, OR, USA). The RAW 264.7 cell line was purchased from American Type Culture Collection (Rockville, MD, USA). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation. DMPO solution, thus purified, did not contain any ESR detectable impurities. Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA, USA). The PBS (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants.

Cell culture

RAW 264.7 cells were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50 mg/ml penicillin/streptomycin at 37°C in a 5% CO_2 incubator. Cells used for the luciferase assay were stably transfected with either the NF-κB or the AP-1 luciferase reporter plasmid and cultured under the same conditions as above [30].

Confocal microscopy

In order to prepare cells for confocal microscopy, RAW 264.7 cells were incubated on glass coverslips in 24-well plates with 1 ml phosphate buffered saline (PBS; pH 7.4) at a concentration of 2×10^5 cells/well for 1 h to allow for attachment. The cells were washed and treated with 50 μg/ml $PbCrO_4$ particles suspended in PBS. The cells were then incubated for 30, 60, and 120 min. After the incubation period, the cells were fixed with 2% paraformaldehyde for 30 min, and stained with the fluorochrome nile red (0.1 μg/ml) for 5 min.

Images were recorded from a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA, USA) fitted with an argon-ion laser using 514 nm excitation light. Macrophages were imaged with an emission spectra >535 nm. When using reflected light <535 nm simultaneously passed to a separate optical path, the $PbCrO_4$ particles could also be imaged and recorded [31].

Oxygen consumption measurements

Oxygen consumption measurements were carried out using a Gilson oxygraph (Gilson Medical Electronics, Middleton, WI, USA). Measurements were made on a system containing 50 μg/ml $PbCrO_4$, 1×10^6 RAW 264.7 cells/ml in pH 7.4 phosphate buffer. The effects of the addition of catalase, deferoxamine and sodium formate were also measured. The oxygraph was calibrated with media equilibrated with gases of known concentrations.

H_2O_2 measurements

H_2O_2 was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase. Measurements were made on a system containing 1×10^6 RAW 264.7 cells/ml in pH 7.4 PBS and a cellular system with 50 μg/ml $PbCrO_4$ added. The effects of the addition of catalase, deferoxamine and sodium formate were also measured. Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a Cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems, Foster City, CA, USA).

Free radical measurements

ESR spin trapping was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical prod-

uct (spin adduct), which can then be studied using conventional ESR. The intensity of the signal is used to measure the amount of short-lived radicals trapped and the hyperfine couplings of the spin adduct are generally characteristic of the original trapped radicals. The spin trapping is the method of choice for detection and identification of free radical generation due to its specificity and sensitivity. All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA, USA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to peak-width. An Acquisit program (Bruker Instruments, Billerica, MA, USA) was used for data acquisitions and analyses.

RAW 264.7 cells (1×10^6), $PbCrO_4$ (50 µg/ml), DMPO (200 mM) and additional scavengers, listed in the figure legends, were mixed in test tubes to a final volume of 1.0 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. Experiments were performed at room temperature, under ambient air.

Luciferase assay of NF-κB or AP-1 activity in vitro

A confluent layer of RAW 264.7 cells transfected with either NF-κB or AP-1 luciferase reporter was detached using cell scrapers. Then 5×10^4 viable cells, suspended in 1 ml of Dulbecco's MEM supplemented with 10% FBS, were added to each well of a 24-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. 12 h later, cells were cultured in Dulbecco's MEM supplemented with 1.0% FBS for 12 h to minimize basal NF-κB /AP-1 activity and then exposed to $PbCrO_4$ particles (10 µg/ml) in the same media to monitor the effects on NF-κB /AP-1 induction. The cells were extracted with 200 µl of 1× lysis buffer provided in the luciferase assay kit (Promega, Madison, WI, USA). Luciferase activity was measured using a Monolight luminometer, model 3010 (Analytical Luminescence Laboratory, San Diego, CA, USA) [30]. The results were expressed as relative NF-κB or AP-1 activity compared with untreated controls.

Statistics

Data were expressed as mean ± S.E.M. (n = 3) for each group. A one way ANOVA test was performed using SigmaStat statistical software (Jandel Scientific, San Rafael, CA, USA) to compare the responses between treatments. Statistical significance was set at $p < 0.05$.

Results

Engulfment of particles by macrophages

Confocal microscopy

Plated RAW 264.7 cells (2×10^5/well) were exposed to $PbCrO_4$ particles for 30, 60, and 120 min and examined using confocal microscopy. $PbCrO_4$ particles were first bound to the surface and then internalized by the RAW 264.7 cells (Fig. 1). After 30 min, cells were observed in contact with some $PbCrO_4$ particles (Fig. 1, top panel arrow). At the 60 min time point, more particles were in contact with the RAW 264.7 cells, and pseudopodia were observed to be reaching out to engulf the particles (Fig. 1, middle panel arrow). At the 120 min time point, an increased number of particles were bound to the surface or engulfed by the cells (Fig. 1, lower panel arrow).

Oxygen consumption

Since macrophages are able to engulf $PbCrO_4$ particles, it is very likely that these phagocytic cells will consume molecular oxygen through induction of the respiratory burst. Oxygen consumption from a mixture containing RAW 264.7 cells (1×10^6/ml) in suspension and $PbCrO_4$ (50 µg/ml) was measured to verify this possibility. Figure 2 shows that $PbCrO_4$ stimulated cells consumed oxygen at a significantly higher rate than cells alone. Measurements were taken for 30 min after initial exposure of cells to $PbCrO_4$. Addition of catalase or deferoxamine returned the oxygen consumption rate to control levels. $PbCrO_4$ particles alone did not consume oxygen at a significant rate (data not shown).

H_2O_2 generation in RAW 264.7 cells

The increased consumption of molecular oxygen by macrophages after exposure to $PbCrO_4$ can generate H_2O_2 through one-electron reduction. H_2O_2 generation was measured from a suspension containing RAW 264.7 cells (1×10^6/ml) in suspension and $PbCrO_4$ (50 µg/ml) after a 20 min incubation period. As shown in Fig. 3, this mixture indeed caused a significant increase in H_2O_2 generation compared to the control of RAW 264.7 cells alone. Addition of catalase, deferoxamine or sodium formate decreased the H_2O_2 generation to control levels. $PbCrO_4$ particles alone did not generate significant amounts of H_2O_2 (data not shown).

·OH radical generation

Our previous studies using soluble Cr(VI) have shown that H_2O_2 can function as a precursor for ·OH generation [15–17]. The possible generation of free radicals from the interaction of $PbCrO_4$ (50 µg/ml) with RAW 264.7 cells (1×10^6/ml) was examined by ESR spin trapping. Measurements were taken 5 min after exposure of cells to $PbCrO_4$. Figures 4a and 4b show ESR spectra observed from the spin trap DMPO alone and DMPO with cells, respectively. Figure 4c shows a typi-

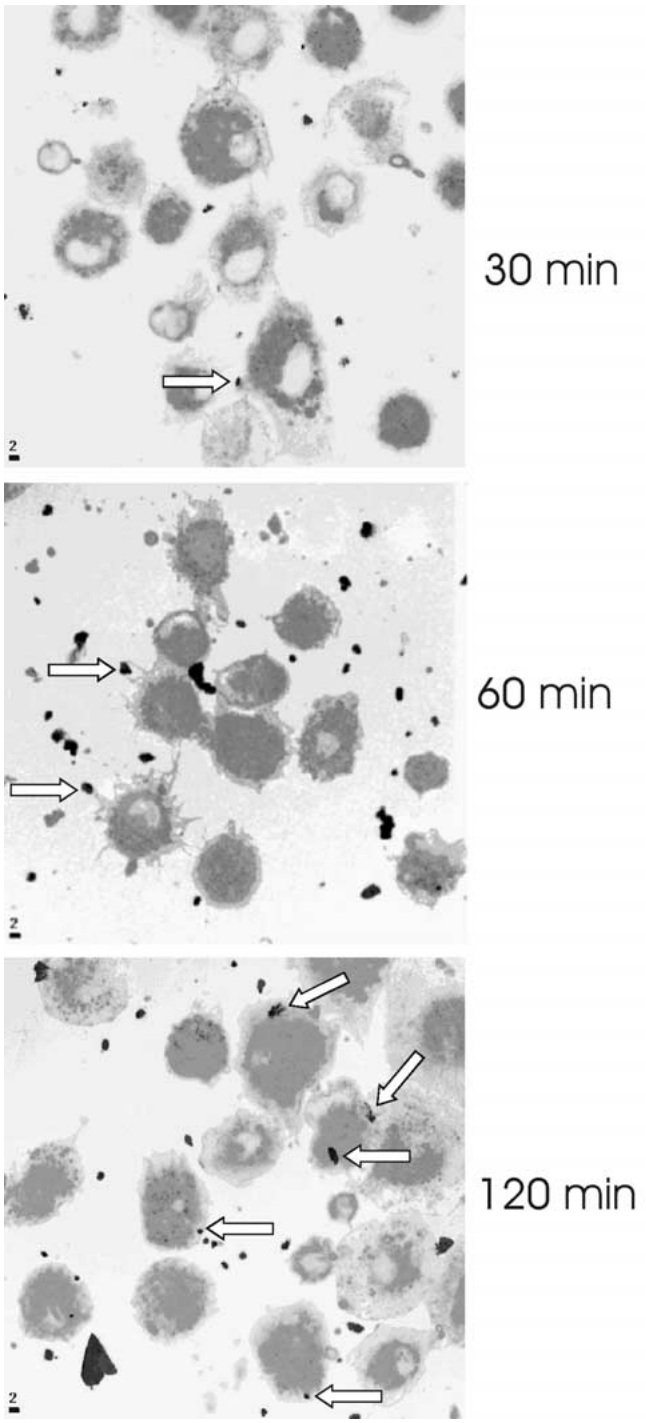

Fig. 1. Confocal micrograph of RAW 264.7 cells (2×10^5/ml) exposed to $PbCrO_4$ particles (arrows) for 30, 60, and 120 min. The final concentration of particle samples was 50 μg/ml. Bar = 2 μm.

cal ESR spectrum generated from a mixture containing $PbCrO_4$ and RAW 264.7 cells in the presence of DMPO. This spectrum consists of a 1:2:2:1 quartet with hyperfine coupling of $a_H = a_N = 14.9$. Based on these splitting constants and the

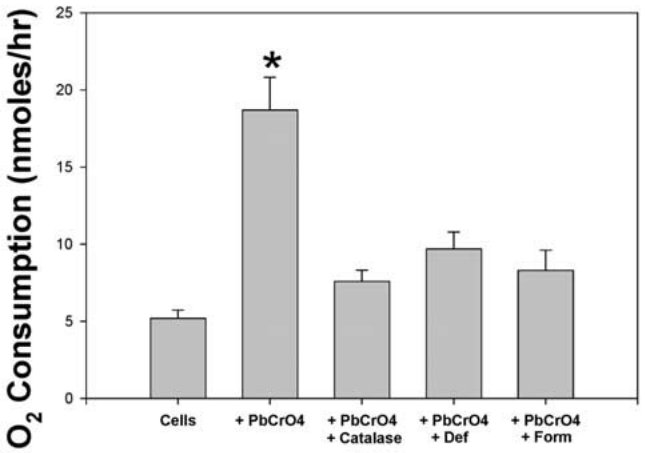

Fig. 2. O_2 consumption in incubation mixtures containing 1×10^6 RAW 264.7 cells alone, cells and 50 μg/ml $PbCrO_4$, and effects of 2000 U/ml catalase, 2 mM deferoxamine or 500 mM sodium formate. The data represent mean ± S.E.M. values of 3 independent experiments. *Significant difference from the control ($p < 0.05$).

lineshape, the 1:2:2:1 quartet was assigned to a DMPO/·OH adduct. Addition of NADPH increased the DMPO/·OH signal (Fig. 4d). Addition of catalase, whose function is to remove H_2O_2, inhibited ·OH radical generation (Fig. 4e). Since DMPO/·OH could, in principle, arise from many sources other than ·OH trapping we performed the competition experiment using sodium formate as a ·OH radical scavenger and a source of a secondary radical to verify the presence of ·OH radicals. In this competition experiment, ·OH radical abstracts a hydrogen atom from formate to form a new radical, which can be trapped by DMPO to generate a new spin adduct signal. As expected, addition of formate decreased the intensity of

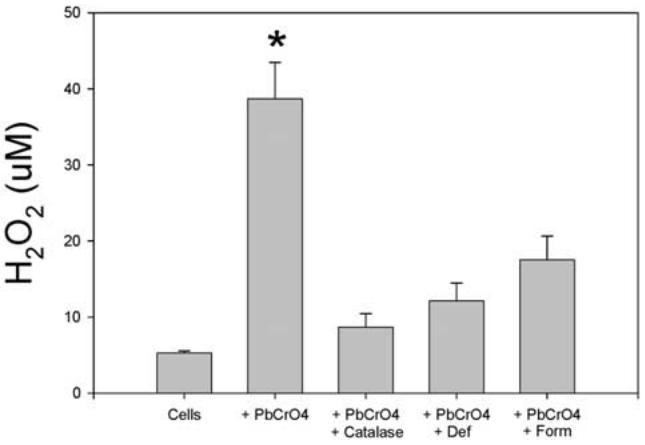

Fig. 3. H_2O_2 production in incubation mixtures containing 1×10^6 RAW 264.7 cells alone, cells and 50 μg/ml $PbCrO_4$, and effect of 2000 U/ml catalase, 2 mM deferoxamine or 500 mM sodium formate. The data represent mean ± S.E.M. values of 3 independent experiments. *Significant difference from the control ($p < 0.05$).

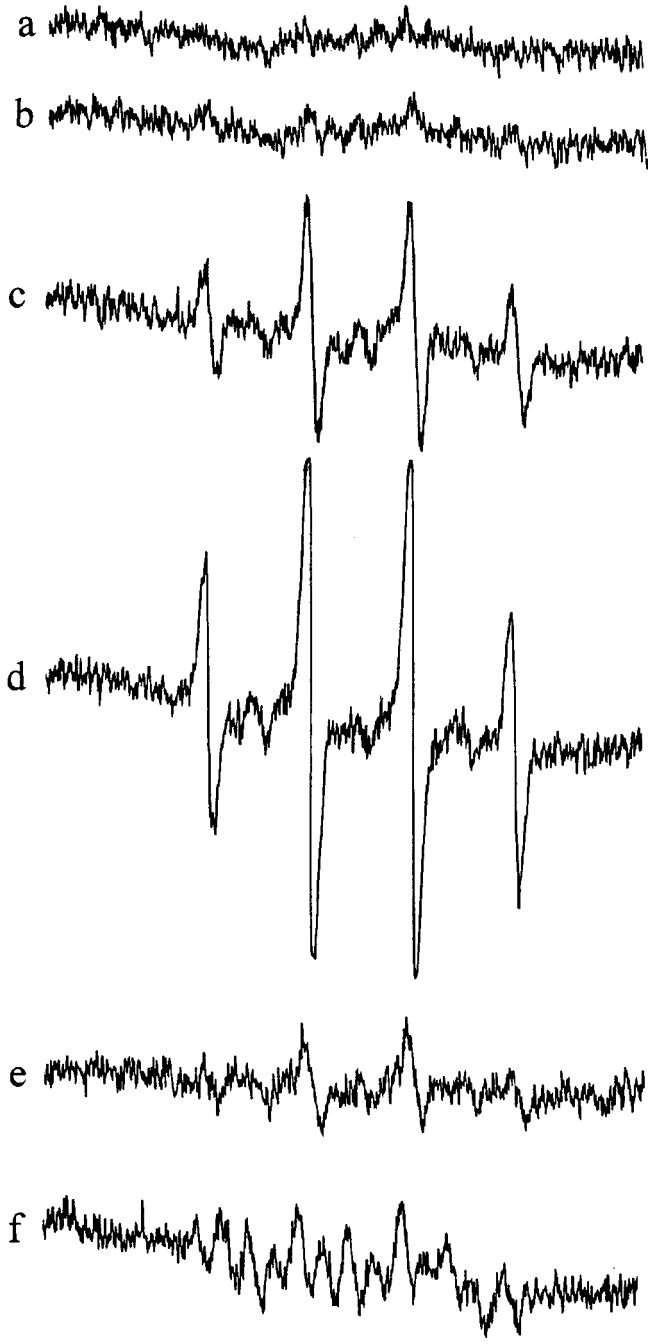

Fig. 4. ESR spectra recorded 5 min after addition of $PbCrO_4$ to cell suspension in a phosphate buffer solution (pH 7.4) containing the following reactants: (a) 200 mM DMPO; (b) 200 mM DMPO and 1×10^6 RAW 264.7 cells; (c) 200 mM DMPO, 1×10^6 RAW 264.7 cells and 50 µg/ml $PbCrO_4$; (d) 200 mM DMPO, 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 5 mM NADPH; (e) 200 mM DMPO, 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 2000 U/ml catalase; and (f) 200 mM DMPO, 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 500 mM sodium formate. The ESR spectrometer settings were: receiver gain, 2.5×10^4; time constant, 0.04 sec; modulation amplitude, 1.0 G; scan time, 1 min; magnetic field, 3440 ± 100 G.

DMPO/·OH adduct signal and resulted in the appearance of a new spin adduct signal with a hyperfine splitting of $a_H = 15.8$ G and $a_N = 18.8$ G (Fig. 4f). These splittings are typical of those of DMPO/·COO^- adduct, demonstrating that the ·OH radicals are indeed generated. $PbCrO_4$ alone did not produce an ESR detectable signal (data not shown).

Ascorbic acid effects on ·OH

The effect of ascorbic acid on ·OH generation was investigated by addition of different concentrations of this antioxidant to the $PbCrO_4$ and RAW 264.7 cells suspension. Figure 5a shows a spectrum obtained from a mixture containing $PbCrO_4$ and RAW 264.7 cells in the presence of DMPO. With

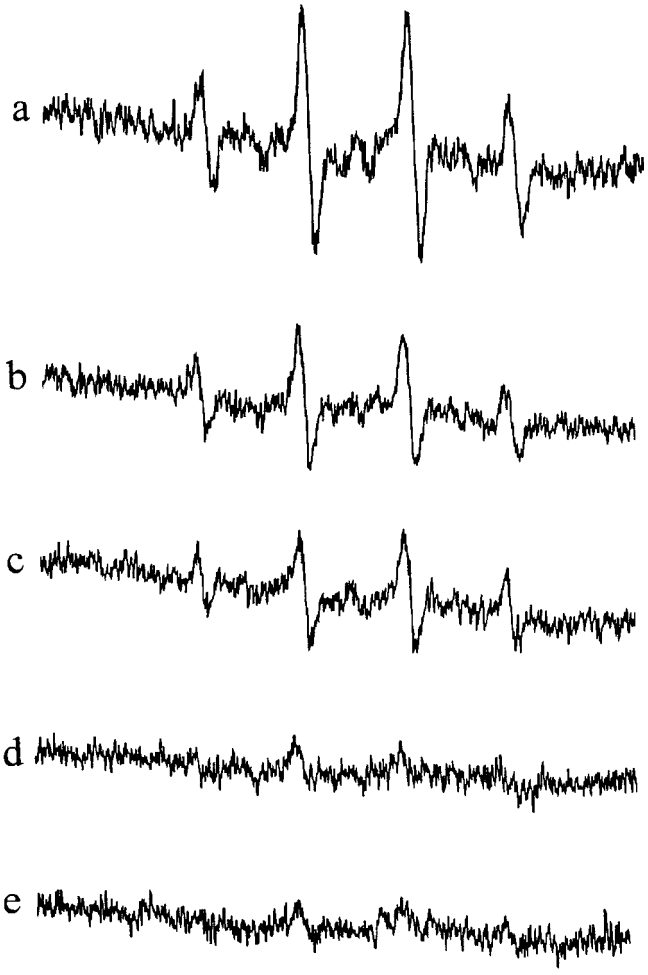

Fig. 5. ESR spectra recorded 5 min after addition of $PbCrO_4$ to cell suspension in a phosphate buffer solution containing 200 mM DMPO and the following reactants: (a) 1×10^6 RAW 264.7 cells and 50 µg/ml $PbCrO_4$; (b) 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 0.1 mM ascorbic acid; (c) 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 0.5 mM ascorbic acid; (d) 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 2.0 mM ascorbic acid; and (e) 1×10^6 RAW 264.7 cells, 50 µg/ml $PbCrO_4$ and 10 mM ascorbic acid. The ESR spectrometer settings were as described in Fig. 4.

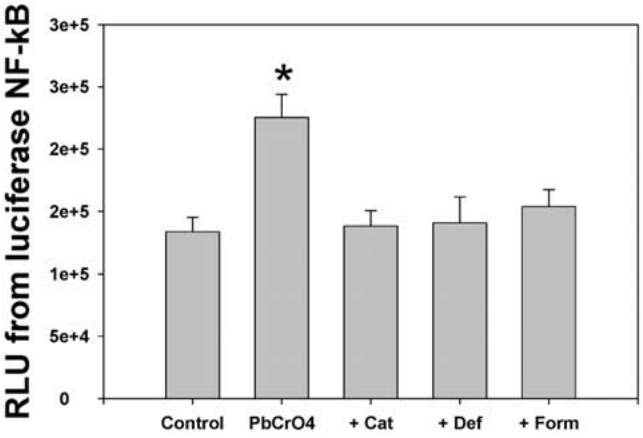

Fig. 6. Activation of NF-κB by $PbCrO_4$. RAW 264.7 cells (5×10^4/ml), transfected with a NF-κB dependent luciferase reporter plasmid, were treated with $PbCrO_4$ (10 μg/ml), for an additional 12 h. The luciferase activity was determined as described in Materials and methods. The data presented are mean ± S.E.M. values of 4 independent experiments. *Significant difference from the control ($p < 0.05$). RLU = relative light units.

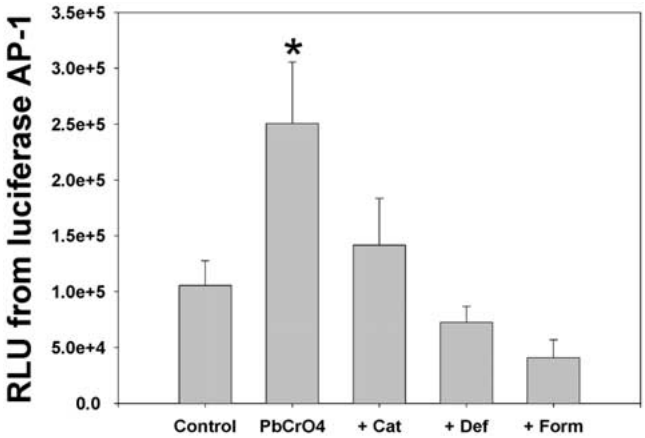

Fig. 7. Activation of AP-1 by $PbCrO_4$. RAW 264.7 cells (5×10^4/ml), transfected with a AP-1 dependent luciferase reporter plasmid, were treated with $PbCrO_4$ (10 μg/ml), for an additional 12 h. The luciferase activity was determined as described in Materials and methods. The data presented are mean ± S.E.M. values of 4 independent experiments. *Significant difference from the control ($p < 0.05$). RLU = relative light units.

the addition of ascorbic acid (0.1 mM), we observed a reduction in the DMPO/·OH signal (Fig. 5b). Increasing the concentration of ascorbic acid (0.5, 2.0, and 10 mM) led to a decrease in the DMPO/·OH signal (Figs 5c, 5d and 5e).

Assay for NF-kB activity using a luciferase reporter

The NF-κB activity in RAW 264.7 cells stimulated by $PbCrO_4$ was measured by a luciferase reporter assay. $PbCrO_4$ treatment of RAW 264.7 cells induced an increase in luciferase reporter gene activity as shown in Fig. 6. Catalase, which catalyzes the reaction of H_2O_2 into H_2O and O_2, deferoxamine (a metal chelator) and sodium formate (a hydroxyl radical competitor) all exhibited an inhibitory effect (Fig. 6).

Assay for AP-1 activity using a luciferase reporter

Analysis of AP-1-dependent luciferase activity indicates that $PbCrO_4$ treatment of RAW 264.7 cells induced an increase in luciferase reporter gene activity (Fig. 7). This $PbCrO_4$ stimulation the AP-1 luciferase reporter was inhibited by catalase, deferoxamine, and sodium formate (Fig. 7).

Discussion

Our results demonstrate that RAW 264.7 cells engulf $PbCrO_4$ particles by phagocytosis. In addition to engulfment, cells show increased O_2 consumption followed by a significant increase in production of H_2O_2. ESR measurements showed ·OH generation after cellular exposure to $PbCrO_4$ particles. Our results further indicate that through ROS these particles are able to activate NF-κB and AP-1, which are involved in inflammatory and carcinogenic signaling pathways.

Recent studies using chromium salts have indicated that reduction of Cr(VI) to its lower oxidation states, such as Cr(V) and Cr(IV), is a key step for Cr(VI)-induced ROS generation and carcinogenesis [13, 32, 33]. Previous studies have indicated that soluble Cr(VI) is able to enter cells in a dose dependent manner at low concentrations [34]. Once inside the cells, Cr(VI) is reduced to Cr(V) and Cr(IV). During redox cycling, molecular oxygen is reduced to $O_2^{\cdot -}$, H_2O_2, and ·OH radical. The reactive Cr intermediates and ROS have been reported to cause DNA damage, activation of p53 and NF-κB, inhibition of cell proliferation, and apoptosis [34].

Particulate $PbCrO_4$ is a highly water-insoluble cytotoxic and carcinogenic agent, and its mechanism of action remains to be investigated [35]. It has been suggested that the unique physiochemical properties of $PbCrO_4$ particles lead to their internalization and the resultant associated cellular stress response, which may be related to the transformation induced by these particles [36]. Light and scanning electron microscopy showed a progressive engulfment of $PbCrO_4$ particles by macrophages and extensive vacuolization of cells in contact with these particles [10]. When particles were incubated in culture media the solubility of $PbCrO_4$ increased 2-fold compared to $PbCrO_4$ in H_2O at 37°C, and extracellular concentrations of chromium increased 7-fold when $PbCrO_4$ was incubated in the presence of cells compared to culture media alone [37, 38]. This increase in the solubility of $PbCrO_4$ in the presence of cells could contribute significantly towards toxic and carcinogenic effects of these particles, as well as effecting the rate of the oxidative burst response.

Previous work by our and other laboratories [13, 39] show that Cr(VI) can be reduced by various reductants, such as glutathione, ascorbate, and glutathione reductase (GSSG-R), as well as in microsomes and mitochondria [16]. Our recent studies have demonstrated, using soluble Cr(VI), that the reduction by GSSG-R generated ROS and caused cellular injury [40–42]. The results of the present study show that particulate $PbCrO_4$ is phagocytized by RAW 264.7 cells. Confocal microscopy showed that over a period of 2 h $PbCrO_4$ particles were engulfed by RAW cells. This result indicates that these particles are not only able to cross the cell's membrane barrier but are also able to cause a concentrated exposure at the point of particle engulfment.

Oxygen consumption was significantly increased in cells exposed to $PbCrO_4$ particles through induction of a respiratory burst. This burst results in an increase in the generation of ROS and is an important mechanism by which phagocytes attempt to deal with invading organisms and other foreign substances [43]. We also demonstrated that this stimulation and engulfment of the $PbCrO_4$ particles results in the increased production of ROS. The reduction of Cr(VI) and the generation of ROS may contribute to the overall mechanisms of Cr(VI)-induced cell damage and carcinogenesis [34, 35, 44]. The present study shows that production of H_2O_2 is significantly increased in RAW cells upon exposure to $PbCrO_4$ particles. H_2O_2 production is part of the inflammatory response in cells elicited by their exposure to a stimulant. H_2O_2 is an important member of ROS. It can react with Cr(V) to form ·OH and Cr(VI). Cr(VI) can be reduced by cellular reductants to Cr(V) and thus a feedback cycle can result [8].

The results from the present study showed the increased generation of the ·OH radical in RAW cells exposed to $PbCrO_4$ particles. The ·OH radical is one of the products of the Cr(VI) + H_2O_2 reaction and has been shown to cause damage in biological systems [40, 45]. The ·OH radical is able to cause DNA strand breaks. Although the ·OH radical is highly reactive the location of generation and the proximity of DNA makes the DNA damage possible [46]. The ·OH radical has also been shown to cause lipid peroxidation and activation of the NF-κB and AP-1 pathways [22].

The results obtained from our present study show that ascorbate is able to decrease ·OH radicals in a dose-dependent manner. Ascorbate can not only directly react with ROS but also reduce Cr(VI) to its lower oxidation states, Cr(V), Cr(IV) and Cr(III). The ratio of these Cr species depends on the concentration of ascorbate as well as that of Cr(VI) [19]. If a soluble part of Cr(VI) or the Cr(VI) associated with the surface of $PbCrO_4$ is reduced to Cr(III), its ability to produce ROS and to penetrate into certain particular cell compartments will be decreased. Thus, ascorbate can protect tissues from $PbCrO_4$-induced ROS generation and related cellular injury.

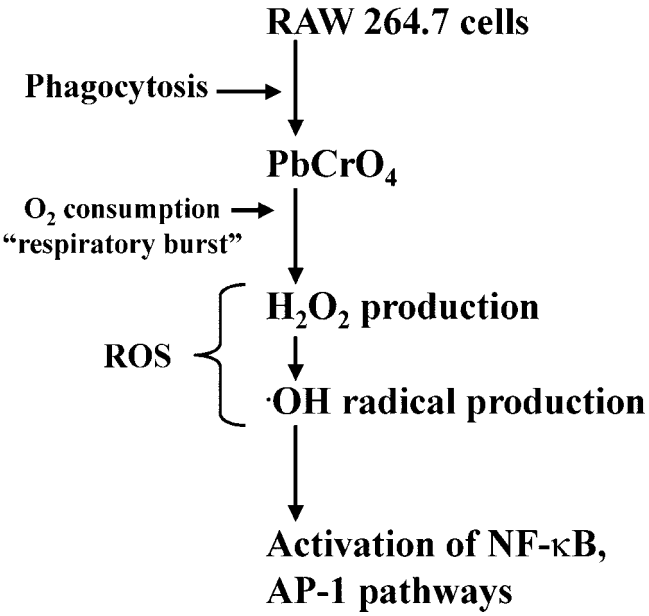

Fig. 8. Schematic representation of $PbCrO_4$ induced generation of reactive oxygen species and cellular responses.

Previous studies have established that ROS generated by Cr(VI) are able to cause hydroxylation of dG residues, protein modification, DNA strand breaks and lipid peroxidation [16]. Soluble forms of Cr(VI) have been shown to cause activation of nuclear transcription factors (NF-κB, AP-1 and p53) [21–23], apoptosis [24], cell growth arrest [44], oncogene expression [18], and activation of mitogen-activated protein (MAP) kinases [22]. We have also demonstrated in non-cellular systems that reduction of soluble Cr(VI) by GSSG-R in the presence of NADPH as cofactor generate ROS [15]. In the present study, we have also shown that reduction of particulate $PbCrO_4$ by the cells produces ROS and addition of NADPH enhanced the generation, indicating that NADPH-dependent GSSG-R may also play an important role in cellular reduction of particulate $PbCrO_4$. We have also shown that through ROS, $PbCrO_4$ particles are able to cause activation of NF-κB and AP-1, which are important mediators in the inflammatory signaling pathways. Our results are outlined in Fig. 8.

In conclusion, the present study demonstrates the following: (1) Macrophages are able to contact and engulf particulate $PbCrO_4$. (2) Cellular reduction of particulate $PbCrO_4$ is able to generate ROS, and the GSSG-R and NADPH systems play a key role in $PbCrO_4$-induced ROS generation. (3) Through ROS, $PbCrO_4$ can cause activation of NF-κB and AP-1. (4) Since ROS can cause various cellular injuries, it can be postulated that $PbCrO_4$-induced ROS generation may be an important pathway in toxicity and carcinogenicity induced by particulate $PbCrO_4$.

References

1. Molina D, Abell MT: An ion chromatographic method for insoluble chromates in paint aerosol. Am In Hygiene Assoc J 48: 830–835, 1987
2. McAughey JJ, Samuel AM, Baxter PJ, Smith NJ: Biological monitoring of occupational exposure in the chromate pigment production industry. Sci Total Environ 71: 317–322, 1988
3. Singh J, Pritchard DE, Carlisle DL, Mclean JA, Monstaser A, Orenstein JM, Patierno SR: Internalization of carcinogenic lead chromate particles by cultured normal human lung epithelial cells: Formation of intracellular lead-inclusion bodies and induction of apoptosis. Toxicol Appl Pharmacol 161: 240–248, 1999
4. Petrilli FL, De Flora S: Toxicity and mutagenicity of hexavalent chromium on *Salmonella typhimurium*. Appl Environ Microbiol 33: 805–809, 1977
5. Majone F, Levis AG: Chromosomal abberrations and sister-chromatid exchanges in Chinese hamster cells treated *in vitro* with hexavalent chromium compounds. Mutat Res 67: 231–238, 1979
6. De Flora S, Bagnasco M, Serra D, Zanacchi P: Genotoxicity of chromium compounds: A review. Mutat Res 238: 99–172, 1990
7. Aiyar J, De Flora S, Wetterhahn KE: Reduction of chromium(VI) to chromium(V) by rat liver cytosolic and microsomal fractions: Is DT-diaphorase involved? Carcinogenesis 13: 1159–1166, 1992
8. Sidhu MK, Fernandez C, Khan MY, Kumar S: Induction of morphological transformation, anchorage-independent growth and plasminogen activators in non-tumorigenic human osteosarcoma cells by lead chromate. Anticancer Res 11: 1045–1053, 1991
9. Wise JP, Leonard JC, Patierno SR: Clastogenicity of lead chromate particles in hamster and human cells. Mutat Res 278: 69–79, 1992
10. Patierno SR, Landolph JR: Soluble vs. insoluble hexavalent chromate. Relationship of mutation to *in vitro* transformation and particle uptake. Biol Trace Elem Res 21: 469–474, 1989
11. Shi X, Dalal NS: On the hydroxyl radical formation in the reaction between hydrogen peroxide and biologically generated chromium(V) species. Arch Biochem Biophys 277: 342–350, 1990
12. Blankenship LJ, Carlisle DL, Wise JP, Orenstein JM, Dye LE, Patierno SR: Induction of apoptotic cell death by particulate lead chromate: Differential effects of vitamins C and E on genotoxicity and survival. Toxicol Appl Pharmacol 146: 270–280, 1997
13. Leonard SS, Wang S, Zang L, Castranova V, Vallyathan V, Shi X: Role of molecular oxygen in the generation of hydroxl and superoxide anion radicals during enzymatic Cr(VI) reduction and its implication to Cr(VI)-induced carcinogenesis. J Environ Pathol Toxicol Oncol 19: 49–60, 2000
14. Yang JL, Hsieh YC, Wu CW, Lee TC: Mutational specificity of chromium(VI) compounds in the hprt locus of Chinese hamster ovary-K1 cells. Carcinogenesis 13: 2053–2057, 1992
15. Leonard SS, Vallyathan V, Castranova V, Shi X: Generation of reactive oxygen species in the enzymatic reduction of $PbCrO_4$ and related DNA damage. Mol Cell Biochem 234/235: 309–315, 2002
16. Shi X, Chiu A, Chen CT, Halliwell B, Castranova V, Vallyathan V: Reduction of chromium(VI) and its relationship to carcinogenesis. J Toxicol Environ Health 2: 101–118, 1998
17. Shi X, Dalal NS: Chromium (V) and hydroxyl radical formation during the glutathione reductase-catalyzed reduction of chromium (VI). Biochem Biophys Res Commun 163: 627–634, 1989
18. Ye X, Shi X: Gene expression profile in response to chromium-induced cell stress in A549 cells. Mol Cell Biochem 222: 189–197, 2001
19. Shi X, Mao Y, Knapton AD, Ding M, Rojanasakul Y, Gannett PM, Dalal NS, Liu K: Reaction of Cr(VI) with ascorbate and hydrogen peroxide generates hydroxyl radicals and causes DNA damage: role of Cr(IV)-mediated Fenton-like reaction. Carcinogenesis 15: 2475–2478, 1994
20. Watanabe M, Takayama Y, Koike M, Yamamoto M: *In vivo* clastogenicity of lead chromate in mice. Tohoku J Exp Med 146: 373–374, 1985
21. Ye J, Zhang X, Young HA, Mao Y, Shi X: Chromium(VI)-induced nuclear factor-κB activation in intact cells via free radical reactions. Carcinogenesis 16: 2401–2405, 1995
22. Chen F, Ding M, Lu Y, Leonard SS, Vallyathan V, Castranova V, Shi X: Participation of MAP kinase p38 and IκB kinase in chromium(VI)-induced NF-κB and AP-1 activation. J Environ Pathol Toxicol Oncol 19: 231–238, 2000
23. Wang S, Leonard SS, Ye J, Ding M, Shi X: The role of hydroxyl radical as a messenger in Cr(VI)-induced p53 activation. Am J Physiol 279: C868–C875, 2000
24. Ye J, Wang S, Leonard SS, Sun Y, Butterworth L, Antonini J, Ding M, Rojanasakul Y, Vallyathan V, Castranova V, Shi X: Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis. J Biol Chem 274: 34974–34980, 1999
25. Lide DR: Handbook of Chemistry and Physics. CRC Press, New York, 1999, pp 4–65
26. Langard S: One hundred years of chromium and cancer: A review of epidemiological evidence and selected case reports. Am J Ind Med 17: 189–215, 1990
27. Hayes RB: Review of occupational epidemiology of chromium chemicals and respiratory cancer. Sci Total Environ 71: 331–339, 1988
28. IARC. Chromium, nickel and welding. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. IARC, Lyon, France, 1990, 49, pp 49–256
29. Perrault G, Dufresne A, Strati G, McNeil M, Michaud D, Baril M, Begin R, Labbe J, Lariviere P, Eeckhaoudt S: Physico-chemical fate of chromium compounds in the sheep lung model. J Toxicol Environ Health 44: 247–262, 1995
30. Ding M, Shi X, Dong Z, Chen F, Lu Y, Castranova V, Vallyathan V: Freshly fractured crystalline silica induces activation protein-1 activation through Erks and p38 mitogen-activated protein kinase. J Biol Chem 274: 30611–30616, 1999
31. Antonini JM, Yang H, Ma JYC, Roberts J, Barger M, Butterworth L, Charron T, Castranova V: Subchronic silica exposure enhances respiratory defense mechanisms and the pulmonary clearance of *Listeria monocytogenes* in rats. Inhal Toxicol 12: 1017–1036, 2000
32. Wang S, Leonard SS, Ye J, Ding M, Shi X: The role of hydroxl radical as a messenger in p53 activation. Am J Physiol 279, C868–C875, 2000
33. Chen F, Bower J, Leonard SS, Ding M, Lu Y, Rojanasakul Y, Kung H, Vallyathan V, Castranova V, Shi X: Protective roles of NF-κB for chromium (VI)-induced cytotoxicity is revealed by expression of IκB kinase-b mutant. J Biol Chem 277: 3342–3349, 2002
34. Liu K, Husler J, Ye J, Leonard SS, Cutler D, Chen F, Wang S, Zhang Z, Ding M, Wang L, Shi X: On the mechanism of Cr(VI)-induced carcinogenesis: Dose dependence of uptake and cellular responses. Mol Cell Biochem 222: 221–229, 2001
35. Xu J, Wise JP, Patierno SR: DNA damage induced by carcinogenic lead chromate particles in cultured mammalian cells. Mutat Res 280: 129–136, 1992
36. Patierno SR, Banh D, Landolph JR: Tranformation of C3H/10T1/2 mouse embryo cells to focus formation and anchorage independently by insoluble lead chromate but not soluble calcium chromate: Relationship to mutagenesis and internalization of lead chromate particles. Cancer Res 48: 5280–5288, 1988
37. Wise JP, Stearns DM, Wetterhahn KE, Patierno SR: Cell-enhanced dissolution of carcinogenic lead chromate particles: The role of individual dissolution products in clastogenesis. Carcinogenesis 15: 2249–2254, 1994
38. Wise JP, Orenstein JM, Patierno SR: Inhibition of lead chromate clasto-

genesis by ascorbate: Relationship to particle dissolution and uptake. Carcinogenesis. 14: 429–434, 1993
39. Qian Y, Jiang B, Leonard SS, Wang S, Zhang Z, Ye J, Chen F, Wang L, Flynn DC, Shi X: Cr(VI) increases tyrosine phosphorylation through reactive oxygen species-mediated reactions. Mol Cell Biochem 222: 199–204, 2001
40. Shi X, Dong Z, Huang C, Ma W, Liu K, Ye J, Chen F, Leonard SS, Ding M, Castranova V Vallyathan V. The role of hydroxyl radical as messenger in the activation of nuclear transcription factor NF-kB. Mol Cell Biochem 194: 63–70, 1999
41. Shi X, Ding M, Ye J, Wang S, Leonard SS, Zang L, Castranova V, Vallyathan V, Chiu A, Dalal NS, Liu K: Cr(IV) causes activation of nuclear transcrition factor NF-κB, DNA strand breaks and dG hydroxylation via free radical reactions. J Inorg Biochem 20: 663–668, 1999
42. Wang S, Leonard SS, Castranova V, Vallyathan V, Shi X: The role of superoxide anion radical in TNF-α induced NF-κB activation. Ann Clin Lab Sci 29: 192–199, 1999
43. Halliwell B, Gutteridge JC: Free Radicals in Biology and Medicine. Oxford Science Publication, Oxford, 2000, pp 455
44. Zhang Z, Leonard SS, Wang W, Vallyathan V, Castranova V, Shi X: Cr(VI) induces cell growth arrest through hydrogen-peroxide-mediated reactions. Mol Cell Biochem 222: 77–83, 2001
45. Ye J, Wang S, Leonard SS, Sun Y, Butterworth L, Antonini J. Ding M, Rojanasakul Y, Vallyathan V, Castranova V, Shi X: Role of reactive oxygen species and p53 in chrominum (VI)-induced apoptosis. J Biol Chem 274: 34974–34980, 1999
46. Shi X, Ding M, Dong Z, Chen F, Leonard SS, Castranova V, Vallyathan V: Antioxidant properties of aspirin: Characterization of the ability of aspirin to inhibit silica-induced lipid peroxidation, DNA damage, NF-κB activation, and TNF-α production. Mol Cell Biochem 199: 93–102, 1999