In Vivo Effects of Chromium

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The production of reactive oxygen species on addition of hexavalent chromium (potassium dichromate, $K_2Cr_2O_7$) to lung cells in culture was studied using flow cytometer analysis. A Coulter Epics Profile II flow cytometer was used to detect the formation of reactive oxygen species after $K_2Cr_2O_7$ was added to A549 cells grown to confluence. The cells were loaded with the dye, 2',7'- dichlorofluorescein diacetate, after which cellular esterases removed the acetate groups and the dye was trapped intracellularly. Reactive oxygen species oxidized the dye, with resultant fluorescence. Increased doses of Cr(VI) caused increasing fluorescence (10-fold higher than background at 200 µM). Addition of Cr(III) compounds, as the picolinate or chloride, caused no increased fluorescence. Electron paramagnetic resonance (EPR) spectroscopic studies indicated that three (as yet unidentified) spectral "signals" of the free radical type were formed on addition of 20, 50, 100, and 200 µM Cr(VI) to the A549 cells in suspension. Two other EPR "signals" with the characteristics of Cr(V) entities were seen at field values lower than the standard free radical value. Liver microsomes from male Sprague-Dawley rats treated intraperitoneally with $K_2Cr_2O_7$ (130 µmole/kg every 48 hr for six treatments) had decreased activity of cytochromes P4503A1 and/or 3A2, and 2C11. Hepatic microsomes from treated female Sprague-Dawley rats, in contrast, had increased activities of these isozymes. Lung microsomes from male Sprague-Dawley rats had increased activity of P4502C11. — Environ Health Perspect 102(Suppl 3):169–176 (1994).

Key words: chromium, cytochrome P450 isozymes, electron paramagnetic resonance (EPR), reactive oxygen species (ROS), lung cells

Introduction

Previous studies in our laboratories (1–3) and those of others (4–6) have shown that there are several potentially toxic in vitro effects of hexavalent chromium [Cr(VI)] which are readily detected. However, some of these effects are not evident in vivo and others have not been tested in relevant systems. For example, it has been shown that when Cr(VI) compounds are reduced by H_2O_2 and/or glutathione (GSH), the pathway of reduction is dependent upon the concentrations of the reductants. The resultant cellular damage (to DNA) may depend upon the molar ratios of the reductants and Cr(VI) compound (4). Production of Cr(V) species in unidentified

forms, as well as the formation of a Cr(V)-tetraperoxo complex, can be detected with addition of Cr(VI) to several reductants in vitro (4,5,7,8). There is evidence that under slightly alkaline conditions, in vitro, hydroxyl radicals (•OH) are formed, which then react with deoxyguanine to initiate DNA strand breakage (4). The concentrations of reactants used to demonstrate these intermediates in vitro have generally been higher than those found in vivo. The carcinogenicity of chromium compounds also appears to result from attack on DNA by Cr(III), formed in situ by the reduction of Cr(VI). Paradoxically, there are beneficial effects of Cr(III) on glucose metabolism (9) and chromium is generally accepted (although controversially) as being a required trace element (10). Extracellular reduction of Cr(VI) to Cr(III), which cannot readily cross the cell membrane, is currently perceived as a protection reaction. The acute toxicity of Cr(VI), which is also little understood, can result in fatality to rats after intraperitoneal administration of several daily doses in the 200 µmole/kg range (2). This general toxicity must be separated from the carcinogenicity in studies of mechanisms of action, and is also not extensively studied. Current knowledge of chromate carcinogenicity and toxicity thus indicates that effects of in vivo exposure to

Cr(VI) compounds depend upon the uptake and metabolic fate of the chromate in target organs.

The lung has been well established as the principal target organ for chromium carcinogenicity (11–13), and as the site of some Cr(VI)-induced toxicity (3). However, little is known of the metabolic fate of hexavalent chromium in this organ, except that many reducing agents, such as ascorbate and glutathione, are present in this tissue and can ultimately produce Cr(III) (14–16). The liver, on the other hand, is the major site of xenobiotic metabolism in mammals. Effects of Cr(VI) on the liver enzymes of rats have been shown in our laboratory to differ in in vitro and in vivo studies (1,2). Addition of potassium dichromate ($K_2Cr_2O_7$) to microsomal fractions from livers of both male and female rats of two strains caused significant decreases in both cytochrome P450 (P450) content and cytochrome c reductase activity (a measure of cytochrome P450 reductase activity). These decreases were found in vitro in microsomes from untreated rats as well as in microsomes from rats that had been treated with inducing agents or with dichromate prior to removal of the livers (2). In in vivo studies, intraperitoneal administration of $K_2Cr_2O_7$ (130 µmole/kg body weight) to rats on alternate days for

six treatments showed that only livers from male Sprague-Dawley rats exhibited a decrease in P450 content, while the hepatic reductase activities remained unchanged in all rats (2). These results emphasize the fact that in vitro effects are often not mimicked in vivo, and indicate the need for further exploration of in vivo effects of Cr(VI) on the hepatic enzymes.

We have previously demonstrated that in the rat less than 2% of orally administered hexavalent chromium is absorbed (17,18). However, lung chromium concentration was measurable (12 µg/g tissue) 24 hr after 14 days of daily treatment with 240 µmole Cr(VI)/kg (as calcium chromate) (17). Intratracheal administration (45 µmole/kg, which mimics the exposure of workers in smelting plants, results in relatively high concentrations of chromium in lung 21 days after the single treatment (8.5 µg/g tissue) (E. Faria and C. Witmer, unpublished data). We also compared the effects of Cr(VI) on A549 cells and L1210 cells (19), using the alkaline elution technique (20). The A549 cells are a cell line from human lung tissue which has the characteristics of Type II cells, and L1210 cells are a murine cell line of leukemic origin. In this study, 5 µM chromate caused single-strand breaks as well as DNA-protein cross-links in A549 cells, but formed only DNA-protein cross-links in the L1210 cells. These findings support the premise that lung tissue and lung cells differ from other tissues in their response to Cr(VI)-initiated damage.

On the basis of these previous findings, we have used the A549 lung cells and in vivo studies in rats to explore the effects of Cr(VI) exposure in in vivo systems. The purposes of the present work are to continue our studies of the effects of Cr(VI) treatment on the hepatic and lung mixed-function oxidase enzymes and to determine whether lung cells in culture respond to exposure to Cr(VI) compounds by producing reactive oxygen species and free radicals (including paramagnetic forms of chromium), as has been indicated by in vitro work (21,22). We have also concentrated on effects on P450 isozymes in hepatic and lung microsomes following treatment of the rats with $K_2Cr_2O_7$. The dose chosen was previously shown to decrease the total P450 in male Sprague-Dawley rats (2). To further study the mode of action of Cr(VI) in these in vivo systems, we used flow cytometry and electron paramagnetic resonance (EPR) spectroscopy. Flow cytometry is a powerful tool for detecting reactive oxygen species (ROS),

which have been implicated in metal-induced DNA strand breakage in other cell types (21,22). EPR spectroscopy can be used to detect, under favorable conditions, paramagnetic transition metal ion species, including Cr(V) species, as well as a variety of free radical entities that may be formed in these A549 lung cells by reactions with chromium compounds.

Materials and Methods

Animals and Cells

All rats used in these studies were obtained from Taconic Farms (Germantown, NY). They were kept in the animal facility for acclimatization at least 4 days prior to use and conditions in the animal facility were those recommended by the National Institutes of Health. The animals weighed between 120 and 200 g when used. Both food (Purina rodent chow) and water were supplied ad libitum. All water bottles were fitted with plastic tips and the food was added directly to the cages to avoid chromium contamination. The rats were kept in plastic cages in groups of three per cage.

A549 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in a 95% air, 5% CO_2 atmosphere, in RPMI 1640 medium (Gibco, Grand Island, NY) at 37°C, pH 7.2, with 10% fetal calf serum.

Reagents

Hexavalent chromium compounds (Na_2CrO_4, $K_2Cr_2O_7$) and all other chemicals not specifically noted were obtained from Fisher Chemical Company (Somerville, NJ). The 2',7'-dichlorofluorescein (DCFH) and the diacetate of DCFH (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). All chemicals were of the highest purity available. Chromium picolinate (98% purity) was generously donated by the Nutrition 21 Company (San Diego, CA). Testosterone and testosterone metabolites were obtained as referenced in Jayyosi et al. (23) and were were donated to us by Dr. Paul Thomas (Rutgers University).

Treatment of Animals

Potassium dichromate was administered intraperitoneally to the rats in groups of at least three per group at a dose of 130 µmole Cr/kg on alternate days for total of six treatments. Animals were sacrificed with ether anesthetic 24 hr after the last dose. Organs were removed and weighed, and microsomes were immediately prepared.

Preparation of Microsomes

Microsomes were prepared by methods used routinely in our laboratory (24), and the microsomal fractions were suspended in phosphate buffer, pH 7.4 (0.1 M phosphate, 0.15 M KCl) so that the resulting suspensions contained the equivalent of approximately 15 g of original liver/ml. The microsomes were either used immediately or frozen at –80°C for future use.

Testosterone Metabolism (Assay for Activities of Cytochrome P450 Isozymes)

The isozymes of cytochrome P450 hydroxylate testosterone in a regio- and stereo-specific manner, and thus the metabolites are indicative of the specific isozyme activities. We used the method described by Jayyosi et al. (23) for this assay. Specifically, testosterone (0.25 µmole, dissolved in methanol) was incubated in a final volume of 1.0 ml of 0.05 M phosphate buffer (pH 7.4, containing 0.15 M KCl and 1 mM EDTA), with liver microsomes (0.250 mg protein) or lung microsomes (0.250 mg protein), and an NADPH-generating system, in a shaking water bath for 10 and 30 min, respectively, at 37°C. Each assay was carried out in duplicate, and the appropriate blanks were run. The reaction was stopped by the addition of methylene chloride and an aliquot of the organic layer (containing the hydroxylated metabolites) was dried under nitrogen and then dissolved in the mobile phase and analyzed by using high-pressure liquid chromatography (HPLC). The HPLC column was a reverse-phase C_{18} column, used as described by Jayyosi et al. (23) to separate testosterone, androstenedione and the eight monohydroxylated testosterone isomers at room temperature. The isomers were identified by comigration with authentic standards. It should be noted that, in this assay, the microsomes used were from the same animals as those used to study the in vitro effects of dichromate (2).

Generation of Reactive Oxygen Species

The Coulter Epics Profile II flow cytometer was used to study the Cr(VI)-induced formation of reactive oxygen species (ROS), such as H_2O_2 and hydroperoxides, by measuring the fluorescence of the oxidized form of 2',7'-dichlorofluorescein (DCF). This dye is fluorescent only in the oxidized state. The reduced dye (DCFH) is introduced into the cells and is oxidized to its fluorescent form by any ROS produced after Cr(VI) addition. The fluorescence

intensity is thus directly proportional to the ROS produced. To carry out these experiments, A549 cells were grown to confluence, harvested by centrifugation at $1000g$ for 10 min, and suspended in phosphate buffer, pH 7.4 (0.1 M, containing 0.15 M KCl) at a concentration of 2×10^6 cells/ml. The reduced nonfluorescent dye (DCFH), as the diacetate (DCFH-DA), which is nonpolar and enters the cells rapidly (25) was added to the cells (5 μM final concentration) and the mixture incubated for 30 min at 37°C. Intracellular esterases immediately hydrolyze the DCFH-DA leaving a resultant polar compound which is trapped in the cells in the reduced, nonfluorescent state. $K_2Cr_2O_7$ (in phosphate-buffered saline) at several concentrations (20, 100, 200 μM) was then added in 200-μl volumes. (Total volume in the tube was 2.2 ml). The resulting ROS were determined at 10-min intervals for 60 min. The excitation wavelength of the dye is 488 nm; the emission wavelength, 525 nm. The data (number of cells containing the oxidized fluorescent dye and the log fluorescence) were automatically recorded on a disc in the cytometer, and histograms of the data were generated using Epics Cytologic. Control samples contained either buffer (no chromium compound) or the trivalent compound, $CrCl_3$, which does not enter cells readily, or chromium picolinate (dissolved in methanol), which enters the cells (both at 200 μM). Positive controls were H_2O_2 or phorbol myristate. To detect any direct oxidation of the reduced dye by the hexavalent chromium, $K_2Cr_2O_7$ was added to the reduced dye directly in the Perkin-Elmer fluorimeter (Perkin-Elmer, South Plainfield, NJ) with a 525-nm bandpass filter, and any fluorescence of the mixture at 525 nm was recorded for 60 min. Experiments were repeated three times with the same results.

Electron Paramagnetic Resonance

The electron paramagnetic resonance (EPR) data were generated using a Varian E-109 spectrometer (Vairan Instruments, Palo Alto, CA) to detect spectra from free radicals and/or paramagnetic Cr(V) species, following addition of hexavalent chromium (as $K_2Cr_2O_7$) to the A549 lung cells. The cells were grown to confluence and harvested immediately prior to the EPR experiments. The cells were resuspended in physiologic saline (by dilution of a stock solution in phosphate-buffered saline, unless otherwise designated) at a concentration of 1 to 2×10^7 cells in a total volume of 0.18 ml, and chromate was added
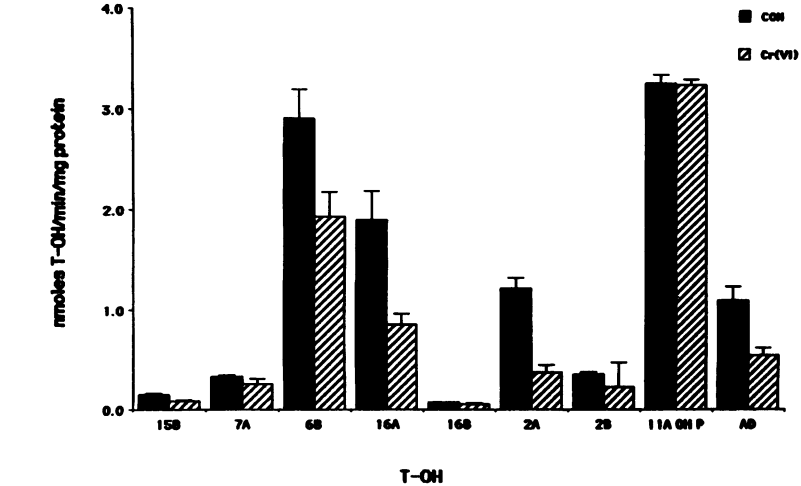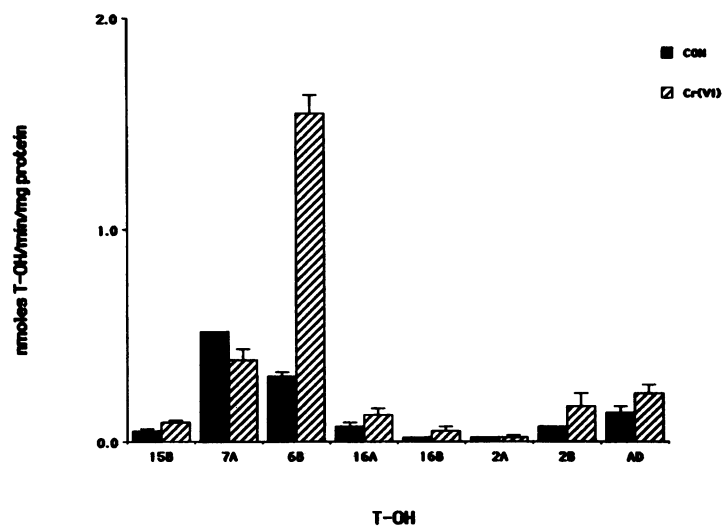

Figure 1. Hydroxylation of testosterone by cytochrome P450 isozymes in hepatic microsomes from dichromate-treated male Sprague-Dawley rats. Microsomes were prepared from dichromate-treated male rats as described in the text. The testosterone assay and identification of metabolites were carried out as described by Jayyosi et al. (21). The abscissa indicates the position of hydroxylation of the testosterone.

in a volume of 0.250 ml. The concentrations of chromate added are given in the legends for Figures 7 to 9. Immediately after addition of the chromate and mixing, the suspension was transferred to an EPR tube of the "flat cell" type (with minimal dimension in the E-field plane). The cell was inserted in the pretuned microwave cavity, and the instrument was retuned as necessary. In most cases, this entire procedure required less than 1.5 min.

The EPR spectra (X-band, 9.1 GHz) were measured with 100 KHz field modulation. The H_0 magnetic field was centered at 3230 Oersted. Maximal field scan range
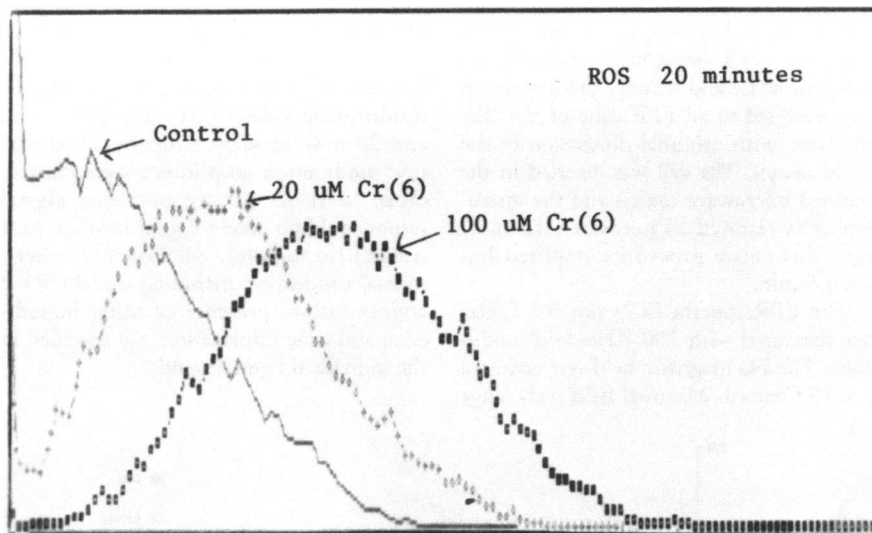
was 200 Oersted; typical scan rates were 50 Oe/min. Nominal filter time constants (RC) of 0.1 or 0.3 sec were used. Microwave power levels were set at low, nonlimiting values (typically between 5 and 20 mW incident radiation), and narrow modulation amplitudes were chosen, close to those giving maximal signal response. High receiver gain settings were needed throughout. All essential experimental conditions, including initial Cr(VI) concentration, presence of other ingredients, and time information, are specified in the individual figure legends.

Figure 2. Hydroxylation of testosterone by cytochrome P450 isozymes in hepatic microsomes from dichromate-treated female Sprageu-Dawley rats. Microsomes were prepared from dichromatre-treated female rats as described in the text. The testosterone assay and identification of metabolites were carried out as referenced in Figure 1. See text for identification of isozymes catalyzing the hydroxylation of testosterone at the positions indicated.
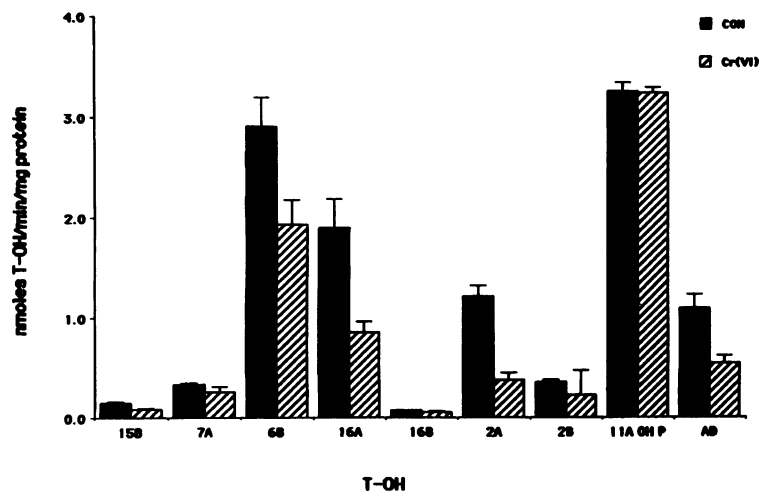
Figure 3. Hydroxylation of testosterone by cytochrome P450 isozymes in lung microsomes from dichromate-treated male Sprague-Dawley rats. Lung microsomes were prepared from the group of rats referred to in Figure 1. The testosterone assay and identification of metabolites were carried out as referenced in Figure 1.

Figure 5. Production of reactive oxygen species (ROS) after the addition of Cr(III) compounds (as $CrCl_3$ and Cr picolinate) and Cr(VI) as potassium dichromate. The histograms for the Cr(VI) compounds are the same as those shown in Figure 4. The Cr(III) compounds (200 µM) were added to the A549 cells as described in the text. The histograms represent the appearance of ROS; measurements were made 20 min after the addition of each compound. Conditions were as for Figure 4. Control was buffer vehicle.
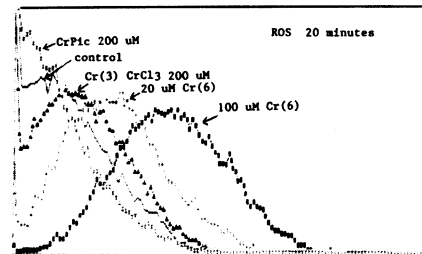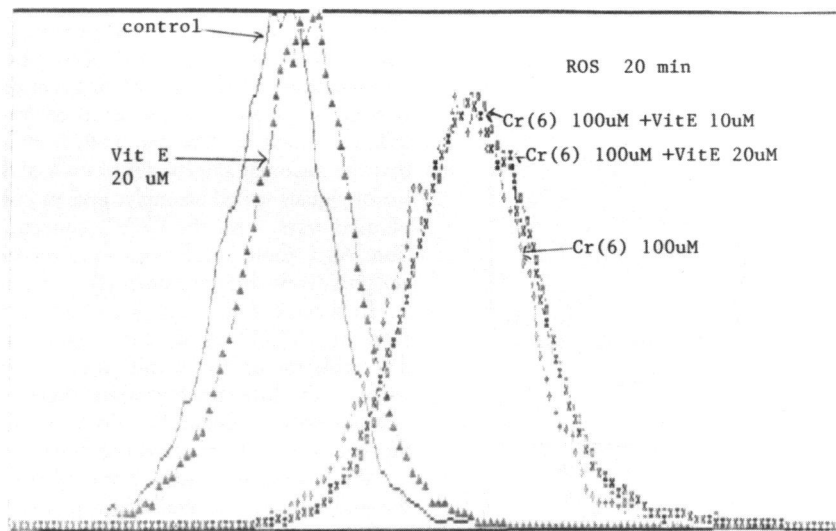

Figure 4. Production of reactive oxygen species (ROS) after addition of Cr(VI) as potassium dichromate to A549 lung cells. The histograms, made using the program, EPICS Cytologic, represent the appearance of ROS after the addition of buffer, and 20 and 100 µM Cr(VI) to A549 cells. Measurements were made using the Coulter Epics Profile flow cytometer. Measurements were made 20 min after the addition of the compounds. Control was buffer vehicle.

The A549 cells were carefully examined before and after each of the experimental runs. In no case was there less than 80% survival as tested by Trypan Blue exclusion and cell count determinations.

Results

Our studies of the effects of Cr(VI) treatment on testosterone metabolism in hepatic microsomal preparations from male and female rats are summarized in Figures 1 and 2. In microsomes from dichromate-treated male rats there was a significant decrease in the 6β-hydroxylation of testosterone; the

2β-hydroxylation was also decreased. These specific hydroxylations are carried out almost exclusively by both cytochrome P4503A1 (CYP3A1) and cytochrome P450 3A2 (CYP3A2), isozymes previously designated as cytochromes P450p and P450l respectively. As there is no constitutive CYP3A1 enzyme in livers of mature male rats (26), the initial (and perhaps the reduced) activities at these positions appear to be due to the CYP3A2 isozyme. The hydroxylation of testosterone at the 16α position, catalyzed by cytochrome P4502C11 (CYP2C11), previously designated P450h, a

male-specific isozyme, was also significantly diminished in the male rats following dichromate treatment.

The results of the dichromate treatment of the female rats with Cr(VI) prior to isolation of hepatic microsomes were directly in contrast to the results in hepatic microsomes from the male rats similarly treated. The hydroxylations catalyzed by isozymes CYP3A1 and CYP3A2 (at both the 2β and 6β positions) were significantly increased (Figure 2), as was the testosterone hydroxylation by CYP2B1 (at the 16α position, and in the formation of androstenedione). This latter enzyme is the type that is also induced by phenobarbital. The two isoforms of the 3A family (CYP3A1 and CYP3A2) are both present in the female rat. These two isoforms are very similar in amino acid content (89% homology) and in substrate specificity, and their activities cannot be satisfactorily separated by this method. For further separation, we are currently carrying out SDS gel electrophoresis of the microsomal fractions and making Western blots of the P450 proteins, followed by the use of monoclonal antibodies to the two isozymes as probes. The monoclonal antibodies were recently developed by Dr. Paul Thomas and colleagues (personal communication). These results have been discussed elsewhere (3).

In lung microsomes from the dicromate-treated male rats, the specific change was a significant increase in hydroxylations carried out by cytochrome P4502B1 (CYP2B1), previously called P450b. Specifically there were increases in the hydroxylation activities at the 16α, and 16β positions and in the production of adrostenedione (Figure 3).This isozyme

Figure 6. Effect of vitamin E succinate on production of reactive oxygen species (ROS) in A549 cells after addition of Cr(VI) as potassium dichromate. Vitamin E succinate (10 and 20 µM) was incubated with the A549 cells for 24 hr prior to measurement of the ROS produced by the addition of the Cr(VI) as potassium dichromate to the vitamin-treated cells. Vitamin E succinate in buffer and the control buffer were also added to the A549 cells at zero time. Cr(VI) was added at 100 µM concentration, as indicated. Measurements were made as described in legend for Figure 1.

was also induced in the hepatic microsomes from the dichromate-treated female rats.
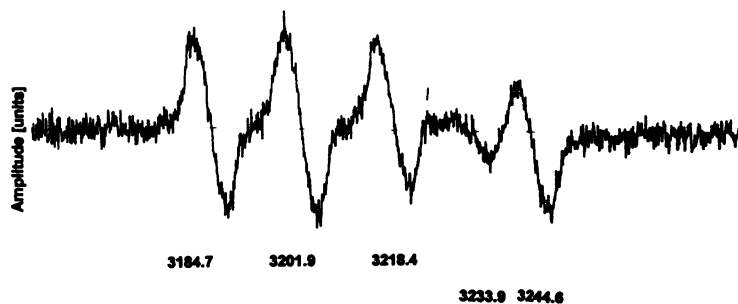
Generation of ROS Following Addition of Cr(VI) to A549 Cells

Figures 4 to 6 are representative histograms generated by the Epics Cytologic program, indicating a significant formation of ROS after $K_2Cr_2O_7$ addition to A549 cells, at 20, 100, and 200 µM concentrations, with an increase in ROS with increased dichromate. These figures all show the response at 20 min. The ROS produced at 30 min were almost identical to those at 20 min, indicating that the reaction was maximal by 20 min. No drop in the ROS levels appears until the 40-min period, indicating some leakage of the dye from the cells by this period, since the oxidized dye is stable. No ROS were seen with addition of the chromium compounds (the chloride and picolinate) to the cells. The fluorescence of cells containing the picolinate actually was less than that of untreated control cells. We detected no effect of preincubation of the cells with vitamin E succinate (20 µM, 24 hr) on the ROS produced with addition of Cr(VI) (20 or 200 µM) to the A549 cells (Figure 6). The cells maintained their homogeneity for the 60-min period of exposure to dichromate (figure not shown), indicating no damage detectable by this measure. All results shown in these figures are from a typical experiment.

EPR Spectroscopy

We have used electron paramagnetic resonance spectroscopy (EPR) as a probe to study the chemical and biochemical events that occur in A549 lung cells with exposure to Cr(VI). A representative example of the application of EPR techniques to these cells is illustrated in Figure 7. This spectrum is a good representative of results obtained with the A549 cells under the conditions outlined in "Materials and Methods."

The spectrum in Figure 7 shows at least five major spectral features (signals) which we have labeled Peaks I through V, with
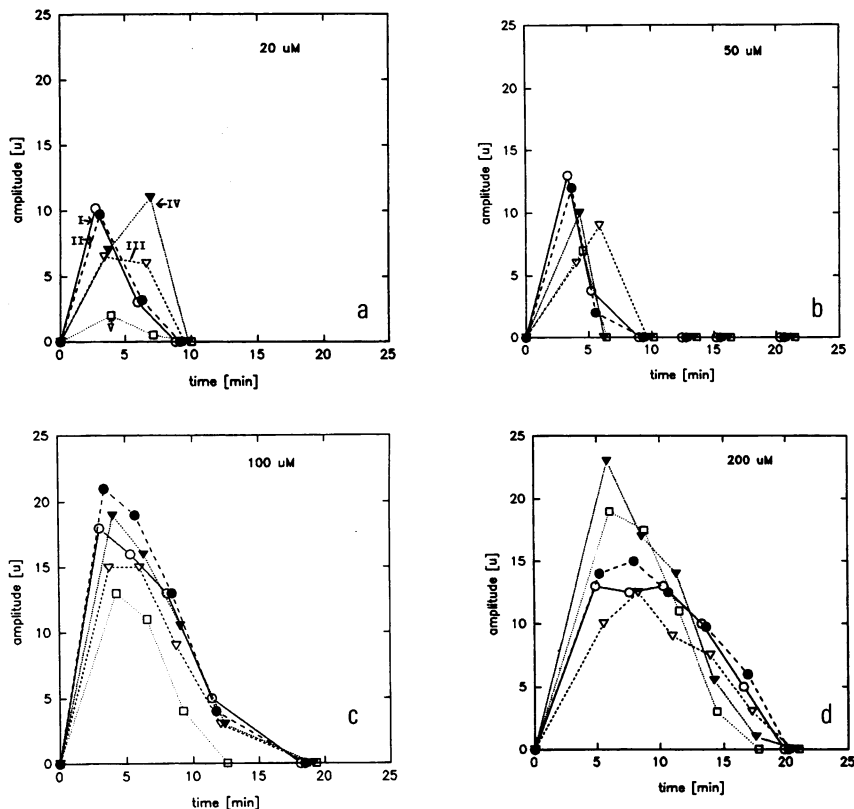
field strength increasing from left to right. Actual H_0 values and the exact times (after mixing) when these signals were recorded are given in the legend. The spectrum represents, in approximation, the first derivative of the resonance absorption, since magnetic field modulation and phase-sensitive detection were employed. The intensities of all observed spectral features (peak amplitude, or integrated areas, when used) exhibit an absolute dependence on the concentration of the A549 cells. With buffer medium alone, only a flat "baseline" spectrum is seen. Furthermore, all signals also depend on the initial Cr(VI) concentration (see below). A free radical standard (DPPH) has been included in the measurement. Under the instrumental conditions used, this resonance was centered at 3204.9 Oe (Figure 7).

Three of the major EPR signals (Peaks I–III) were observed at magnetic fields lower than that of the marker, and thus with g-values greater than that of the free electron value. Together with other information, this suggests that the species responsible for this part of the EPR spectrum have the characteristics of free radical species. On the other hand, the rest of EPR signals, which are found at higher fields (Peaks IV and V), and thus to the right of the marker position, are consistent with complexes of paramagnetic transition ions and are no doubt Cr(V) species.

Some of the chemical and biochemical complexities caused by the exposure of the A549 cells to Cr(VI) are seen in Figure 8*a-d*. Peak amplitudes of the five major EPR peaks previously defined are plotted as a function of time. These data were obtained

Figure 7. EPR spectrum of A549 cells after exposure to Cr(VI). Signal amplitudes, in relative units (au), are plotted as a function of the H0 field in [Oersted]. The reaction was started by adding Cr(VI) (as $K_2Cr_2O_7$ in buffered solution) to harvested and resuspended A549 cells at "zero time." A total of 2.2×10^7 cells was used, in a volume of 0.43 ml. Final concentrations at zero time were: Cr(VI) 116 µM, Na+ (as NaCl) 57 mM, Na_2-EDTA 4.2 mM, phosphate 4.0 mM. Field values of the prominent spectral features (at constant microwave frequency) are indicated in the graph (Peaks I–V). They were recorded —actual times after mixing—as follows: Peak I (3184.7 Oe), at 2.98 min; Peak II (3201.9 Oe), at 3.3 min; Peak III (3218.4 Oe), at 3.7 min; Peak IV (3233.9 Oe), at 3.9 min; and Peak V (3244.6 Oe), at 4.17 min. All these measurements were performed at 21°C. The inserted marker indicates the field position of an organic free radical standard (crystalline 1,1-diphenyl-2-picryl-hydrazyl, DPPH, with a g-value, g = 2.0036 ± 0.0003) under the conditions of this experiment. This narrow-line resonance signal of this marker was observed at 3204.9 Oe.

Figure 8 EPR spectra of A549 cells after exposure to Cr(VI): time course. Peak amplitudes (arbitrary units, au), corresponding to the Peak I to V signals defined in Figure 7, are plotted as a function of time after mixing, in min. They were determined directly from the recorded spectra; no attempts were made here to obtain and use "refined values" using nonlinear curve-fitting procedures. The spectra were recorded as described. Approximately 2×10^7 freshly harvested cells were resuspended in 0.18 ml phosphate-buffered saline. Cr(VI), as $K_2Cr_2O_7$, was added in phosphate buffer (0.1 M, pH 7.2) to obtain the final Cr(VI) concentrations specified below. Final phosphate concentration was about 58 mM. All four panels have identical X- and Y- axes. In each panel, the following codes are used: Peak I, open circles, solid line; Peak II, filled circles, long dashes; Peak III, open triangles, medium-length dashes; Peak IV, filled triangles, short dashes; Peak V, open squares, dotted line. Final Cr(VI) concentrations for exposure at "zero time" were (a) 23 μM; (b) 58 μM; (c) 116 uM; (d) 233 μM.
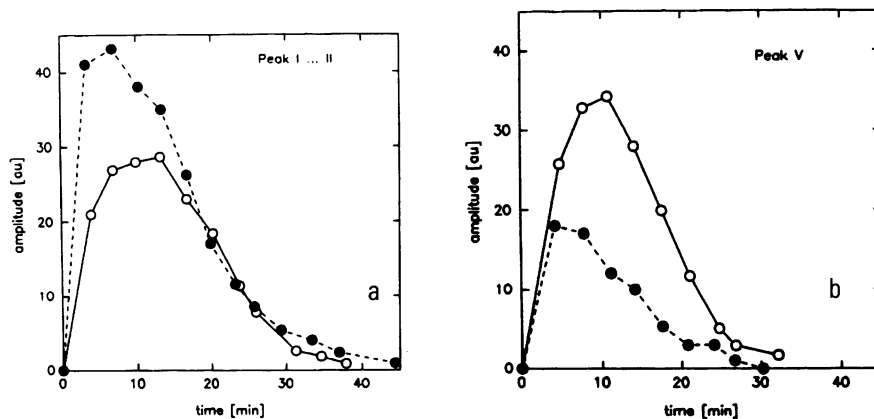
Figure 9. EPR spectra of A549 cells after exposure to Cr(VI): effects of dimethylsulfoxide (DMSO). (a) Mean of the observed amplitudes of Peak I and Peak II is plotted as a function of time [min] after mixing. DMSO sample: open circles, solid line; control sample (no DMSO): filled circles, broken line. (b) The amplitude of Peak V is plotted, in analogous fashion as a function of time after mixing. DMSO sample: open circles, solid line; control sample (no DMSO): filled circles, broken line. Experimental conditions used: 2.0×10^7 cells in 0.43 ml final volume. Cr(VI) 116 μM; DMSO 320 mM.

from a set of experiments in which the initial Cr(VI) concentration was varied about 10-fold, from 23 to 233 μM final concentration, while the concentration of A549 cells was constant. Detailed analysis shows that the maximal amplitudes of each of the major signals varied strongly, and in complicated ways, with the Cr(VI) concentration. Also, these amplitudes were reached at significantly different times after mixing.

In spectra from the highest concentration of Cr(VI) used, the signals are detectable for up to 20 min after mixing, while at the low concentration, these signals last only for about 10 min, indicating that the rates of decay are also different.

We have also examined the effects of the addition of a several additional compounds, including dimethylsulfoxide and inorganic anions (phosphate and sulfate) to the A549 cells, by adding them to the extracellular medium. In Figure 9, we show the effects on the Cr(VI)-induced spectra (233 μM Cr), of the addition of dimethylsulfoxide (DMSO) to the cellular medium. Panel A shows the effects seen on the mean of the amplitudes of Peaks I and II; panel B shows that the opposite effect is seen on the amplitude of Peak V. Details are outlined in the figure legend.

Discussion

The results seen in the studies of *in vivo* effects of Cr(VI) were of two kinds: a change in critical isozyme activities, which may be detrimental for the animal under some circumstances; and the formation of ROS and paramagnetic species which are known to be toxic. That these effects on the isozymes are seen *in vivo* in both liver and lung after treatment of rats with relatively low doses of Cr(VI) indicates that the liver at least responds somewhat differently than predicted by the *in vitro* studies. Relatively high Cr(VI) concentrations were required in the *in vitro* system to reduce the total cytochrome P450 content (2).

The findings of significant changes in the rat hepatic and lung microsomal isozymes of the P450 family of enzymes following *in vivo* exposure to dichromate indicate that the effects of hexavalent chromium compounds can include important indirect effects, such as changes in the first step of metabolism (the hydroxylation) of other xenobiotics. The liver enzymes, and specifically the P450 family are the major site of xenobiotics metabolism. The substrates metabolized by the 3A P450 gene family include many carcinogens, i.e., CYP3A1 metabolizes aflatoxin B_1 and acetylaminofluorene, as well as cortisol,

progesterone, and testosterone. This gene family is induced by steroids and has the most widespread substrate activities of all the P450 families. Aflatoxin B_1 is a xenobiotic to which we are frequently exposed (in peanut butter), so changes in its rate of metabolism could cause concern.

The overall effects of these changes in specific hepatic P450s on metabolism of other xenobiotics cannot be effectively assessed, however, without information on the fate of the phase I products (epoxides and/or hydroxylated metabolites) resulting from P450 oxidations. Any such changes could alter the equilibria of the initial reactions. For example, chromate has been shown to inhibit glutathione reductase activity in erythrocytes (27) and perhaps this is also true in other tissues. Our results emphasize the differing effects of chromate treatment on lung. and on one other tissue, liver in male rats. The interesting opposing effects of Cr(VI) on liver enzymes in male and female rats, however, were not unexpected, as steroids are metabolized differently in the two sexes.

CYP2B1, which was increased in activity in lungs of Cr(VI)-treated male rats, is active in lung Clara cells. The lung is the first site of inactivation of inhaled xenobiotics, and the increased activity of this form of P450 appears to be a defense action. Increases in CYP2B1 can cause increased metabolism of xenobiotics such as aniline, barbiturates, and benzo[a]-pyrene and other compounds in the lung. However, if the companion enzyme, epoxide hydrolase, is not also increased, there could be increased production of toxic or carcinogenic intermediates, such as epoxides. This enzyme is also induced by phenobarbital treatment and is relatively nonspecific in its substrate activities. The mechanism of this increased activity was not addressed in the current study.

The formation of an abundance of reactive oxygen species caused by Cr(VI) addition to the A549 lung cells indicates that the reduction of hexavalent chromium is not a simple process in which some Cr(V) may be formed which is then reduced to Cr(III) complex(es) that react with DNA. Rather, there is a possibility of many interactions of reactive species with intracellular moieties. Several methods of identifying these species are now being tried in our laboratory. The lack of effect of vitamin E on the ROS formation was unexpected and suggests that the species produced do not result from lipid peroxidation. Vitamin E added to V79 cells in culture at the same concentration by

Sugiyama (28) reduced the number of single strand breaks in DNA caused by hexavalent chromate. This suggests that the strand breaks are not caused directly by the reactive oxygen species.

We have also carried out preliminary studies in which we exposed A549 cells to several concentrations of Cr(VI) as dichromate for 3 hr. The dichromate exposure was followed by washout of the chromate with subsequent growth in a dichromate-free medium for 24 hr, followed by RNAse treatment and staining of the cells with propidium iodide. We found an abnormal accumulation of cells in S-phase in cells exposed to 5 µM dichromate. This is in agreement with Costa (29) who also reported a build-up of S-phase cells in Chinese hamster ovary cells after treatment with several metals. Our work with the cell cycle analysis is still in progress.

EPR Spectroscopy

EPR spectroscopy detects the presence of paramagnetic entities, allows their quantitation, and affords the characterization of kinetic features of their formation as well as their decay processes. Compounds that can be investigated exhibit paramagnetism that is derived either from the electron configuration of transition metal ions in certain valence states and specific coordination geometries, or from molecular unpaired electrons in other molecules, i.e., free radical species (S=1/2). Different species can be observed side by side, and their individual characteristics can be evaluated. However, a definitive chemical characterization of the molecules causing the observed resonance absorption solely on a spectroscopic basis may be difficult.

EPR spectroscopy has been successfully applied to studies of the transition element, chromium, in its many states and levels of complexation for many years. Most chromium compounds were found to have g-values close to the "free electron" value, often at slightly higher fields (i.e., lower g-values); observed line widths are often narrow, especially for Cr(V) species. As is characteristic for transition metal ions, the actual spectral features are quite sensitive to the nature of the ligands, the geometry of coordination, and even the matrix in which these complexes are studied. These factors influence the shape of the resonance absorption; relaxation times and temperature play additional important roles. Finally, for different, purely geometric reasons, the actual EPR spectra for any given material obtained in liquid solution (as we have used here) differ substantially

from spectra obtained in the frozen state (with "immobilized" quasi-random orientation of the spins). Thus the g-values in immobilized and randomly oriented samples are inherently very different from the g-values of solution spectra.

Of particular interest in these studies were paramagnetic entities containing chromium in a formal (V) valence state with a $3d^1$ electron configuration. These coordination complexes play recognized, but not understood, roles as intermediates in the reduction of Cr(VI) compounds to complexes of the thermodynamically stable Cr(III). Recent reports include the observation of tetraperoxo-chromate(V) as a product of the reduction of Cr(VI) (4,5) and a number of studies of Cr(V) entities in a variety of chemical and biochemical systems, with reported g-values around 1.98 (4–6). Frozen samples were usually employed to facilitate detection and to circumvent problems of chemical stability of the Cr(V) complexes.

The aim of our application of EPR spectroscopy was to investigate effects of Cr(VI) in A549 cells, under conditions that are as close as possible to physiological conditions. These techniques provide the most direct experimental access to the measurements of the paramagnetic species. The approach requires the operation of the EPR spectrometer close to the fundamental limitations of the method, especially regarding detection sensitivity. It necessitates compromises in virtually all instrumental settings (choice of microwave power levels, modulation amplitudes, scan rates and amplifier gain). Use of cell suspensions in solution allowed us to look closely at the reactions in which the observed paramagnetic species [including Cr(V) species] respond kinetically in real time, under nearly physiological conditions, and relatively unperturbed by the measurements. We have not used spin-trapping agents, so as not to shift equilibria or alter kinetic information.

The EPR spectrum seen in Figure 7 is a good example of our results obtained with the A549 cells under our defined conditions (see Materials and Methods). Five major spectral features (signals) can be identified (Peaks I to V). Each of these exhibits its own complex kinetics of formation and decay. A representative summary of these kinetic aspects [amplitude vs time, as a function of initial Cr(VI) concentration] is shown in Figure 8a–d.

Peaks I to III can be classified, spectroscopically, as generated by free radical species. Like all species we have observed

in the A549 cells, they are transient species, varying greatly in rates of formation and decay. It must be stressed, however, that no definite assignments of chemical structures from the signals is possible. More information is required than the spectroscopic data provide. Likewise, the kinetic results which can provide crucial information for understanding of some of the dynamic intracellular processes involving the paramagnetic species, do not allow definition of the chemical structures. Clearly, much further work is needed to positively identify the species responsible for Peaks I to III in the A549 cells. Work toward this aim is in progress.

The three signals (Peaks I to III) which we have found in these lung cells following Cr(VI) addition have not been reported previously in any known system . It is not known whether these signals are unique to lung cells, because other organs have not been examined by EPR techniques. Peaks IV and V, however, indicate by their resonance absorption at higher magnetic fields than the free electron value that they are caused by a Cr(V) entity. Thus, our findings are in agreement with the reports of others (4,5,7,8) that some form of Cr(V) is found in chemical and biochemical systems on the reduction of Cr(VI).

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