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The pro-oxidant chromium(VI) inhibits mitochondrial complex I, complex II, and aconitase in the bronchial epithelium: EPR markers for Fe-S proteins

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Abstract

Hexavalent chromium [Cr(VI)] compounds (e.g. chromates) are strong oxidants that readily enter cells where they are reduced to reactive Cr species that also facilitate reactive oxygen species (ROS) generation. Recent studies demonstrated inhibition and oxidation of the thioredoxin system, with greater effects on mitochondrial thioredoxin (Trx2). This implies that Cr(VI)-induced oxidant stress may be especially directed at the mitochondria. Examination of other redox-sensitive mitochondrial functions showed that Cr(VI) treatments that cause Trx2 oxidation in human bronchial epithelial cells also result in pronounced and irreversible inhibition of aconitase, a TCA cycle enzyme that has an iron-sulfur (Fe-S) center that is labile with respect to certain oxidants. The activities of electron transport complexes I and II were also inhibited, whereas complex III was not. Electron paramagnetic resonance (EPR) studies of samples at liquid helium temperature (10 K) showed a strong signal at $g = 1.94$ that is consistent with the inhibition of electron flow through complexes I and/or II. A signal at $g = 2.02$ was also observed which is consistent with oxidation of the Fe-S center of aconitase. The $g = 1.94$ signal was particularly intense and remained after extracellular Cr(VI) was removed, whereas the $g = 2.02$ signal declined in intensity after Cr(VI) was removed. A similar inhibition of these activities and analogous EPR findings were noted in bovine airways treated *ex vivo* with Cr(VI). Overall, the data support the hypothesis that Cr(VI) exposure has deleterious effects on a number of redox-sensitive core mitochondrial proteins. The $g = 1.94$ signal could prove to be an important biomarker for oxidative damage resulting from Cr(VI) exposure. The EPR spectra simultaneously showed signals for Cr(V) and Cr(III) which verify Cr(VI) exposure and its intracellular reductive activation.

Keywords

chromate; chromium(VI); mitochondria; iron-sulfur proteins; aconitase; complex I; complex II

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1. Introduction

Human exposure to hexavalent chromium, Cr(VI), largely results from industrial use and release. Common uses include stainless steel machining and welding, chrome plating, chromate pigments, corrosion inhibitors, zinc chromate primer paints, and others. Contact with various sources of Cr(VI) has demonstrated its ability to cause an array of cytotoxic effects and pathologies [1-9]. Inhalation of Cr(VI) compounds (e.g. chromates) as dusts, particles, and fumes is a common form of exposure that can cause multiple respiratory effects (e.g. pulmonary fibrosis, chronic bronchitis, occupational asthma, and lung cancer) [2,6,10-13]. Environmental exposure is of increasing concern because more than 10^5 tons of Cr are released annually, and Cr is a significant contaminant at many sites in the U.S.A. [14,15].

Among the stable oxidation states of Cr, Cr(III) species are often insoluble and do not easily enter cells [16]. In contrast, many Cr(VI) compounds are much more soluble and readily enter cells via an anion transporter [17]. However, even some less soluble chromates that are used industrially are implicated in toxicity. Once inside cells, there are several chemical and enzymatic reductants that can reduce Cr(VI), including ascorbate, cysteine, glutathione, glutathione reductase, and multiple microsomal enzymes including cytochrome *b*₅ [18-26]. These 1- and 2-electron reductants generate reactive Cr intermediates, Cr(V) and Cr(IV), which are important for the cytotoxic effects [8,27-33]. Oxidative damage is one likely outcome given that Cr(VI) reduction results in several oxidants: (a) Cr(V) can directly oxidize cell components [34,35]; (b) Cr(V) and Cr(IV) catalyze robust hydroxyl radical (HO•) generation in Fenton-like reactions with H₂O₂ [26,29,36-39]; and (c) some enzymes simultaneously reduce Cr(VI) to Cr(V) and generate superoxide (O₂^{•-}) [26,40]. Such pro-oxidant effects could disrupt intracellular redox status and control. Consistent with this, exposure of human bronchial epithelial cells to Cr(VI) results in the inhibition and oxidation of the thioredoxin (Trx) system [41,42] which has a key role in maintaining normal intracellular thiol redox balance and in controlling redox-sensitive cell signaling [43,44]. Mitochondrial Trx2 was more susceptible than cytosolic Trx1 [41,42] which implies that the Cr-mediated pro-oxidant effects may be greater in the mitochondria, or that the mitochondria may be more susceptible. Other evidence for the potential mitochondrial effects of Cr(VI) in cells or tissues is limited, although it has been reported to decrease the mitochondrial transmembrane potential [45,46] and decrease the mitochondrial reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [47].

The studies reported here explore the hypothesis that other potentially redox-sensitive activities that are central to normal mitochondrial function may also be adversely affected by Cr(VI) treatment of the bronchial epithelium. Mitochondrial activities that are potentially susceptible to various reactive oxygen and nitrogen species include aconitase and the respiratory electron transport complexes I, II, and III [48-54]. Depending on the specific oxidants and other conditions, both reversible and irreversible inhibition of these activities has been noted. These proteins/complexes all have iron-sulfur (Fe-S) clusters that are critical to their activity. Most Fe-S proteins are in the mitochondria which is where Fe-S clusters are generated. However, some Fe-S clusters are exported to the cytosol, e.g. for the cytosolic isoform of aconitase [55]. There is also a mitochondrial aconitase that catalyzes the conversion of citrate to isocitrate (via aconitate), an early step in the TCA cycle. Aconitase is susceptible to oxidative inactivation, particularly by O₂^{•-}, which results in the release of a labile iron (Fe) from its catalytic 4Fe-4S center resulting in an inactive [3Fe-4S]¹⁺ species that can be detected by EPR [56-59]. While the activities of complexes I, II, and III can be inhibited by a number of oxidants (see above), their Fe-S centers are generally resistant to oxidants [52,60]. Complex I activity, in particular, is quite sensitive to the thiol redox status of mitochondria [61,62], which could explain its inhibition under some conditions of oxidant

stress. The disruption of electron flow through complexes I, II, and III can result in changes to the redox state of some of their Fe-S centers, some of which yield characteristic EPR signals that provide information about their functional state (see more below). Complex IV is, however, quite resistant to several oxidants [48].

The studies reported here discovered that Cr(VI) treatment of bronchial epithelium results in inhibition of aconitase and complexes I and II, whereas complex III was not inhibited. EPR studies showed a strong signal that is consistent with the inhibition of electron flow through complexes I and/or II, and another signal that is consistent with oxidation of the Fe-S center of aconitase. The former signal was especially pronounced and remained after Cr(VI) was removed; it could prove to be a useful marker for Cr(VI)-mediated oxidative damage. Together with the previous reports on its disruption of mitochondrial thioredoxin, Cr(VI)-induced oxidant stress results in the inhibition of multiple redox-sensitive mitochondrial proteins that are essential for energy generation and the maintenance of mitochondrial redox status.

2. Experimental

2.1 Chemicals and reagents

LHC-9 medium and Hanks' Balanced Salt Solution (HBSS) were from Invitrogen (Carlsbad, CA). BEAS-2B cells were obtained from the American Type Culture Collection. Sodium chromate (99+%) was the highest purity available from Aldrich Chemical (Milwaukee, WI). Zinc chromate was from Pfaltz & Bauer (Waterbury, CT). Chromates are known carcinogens and should be handled accordingly. All other chemicals and reagents were purchased from Sigma-Aldrich or from the sources indicated below.

2.2 Cell culture and Cr(VI) treatment

BEAS-2B cells were grown at 37°C in humidified air containing 5% CO₂ in Dulbecco's Modified Eagle's Medium with 25 mM HEPES and 4.5 g/L glucose (BioWhittaker 12-709F, Cambrex BioScience) supplemented with 10% LHC-9 medium, 10% fetal bovine serum (Valley Biomedical, Winchester, VA), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were fed every 48 h, and were subcultured prior to reaching confluence using the Reagent Pak system (Clonetics, CC-5034). Normal plating density was 3000 to 5000 cells/cm².

Cells were grown to ca. 60–80% confluence in T-75 flasks. The medium was removed and the cells were washed with pre-warmed HBSS. Each flask was then treated with 7.5 ml HBSS (control) or HBSS containing sodium chromate as indicated in the results. Just prior to harvest, the treatment solution was removed, the cells were washed with HBSS, and harvested as described below.

2.3 Electron Paramagnetic Resonance

The treated and washed cells were scraped into HBSS and pelleted by centrifugation (5 min, 100 × g). The cell pellet was resuspended in 0.3 ml HBSS, transferred to a 4-mm quartz EPR tube, immersed in liquid nitrogen (77 K) and stored, typically for less than one week. EPR spectra were obtained at liquid helium temperature (10 K) using a Bruker E500 ELEXSYS spectrometer (Silberstreifen, Germany) with an Oxford Instruments ESR-9 helium flow cryostat (Oxfordshire, UK) and a Bruker DM0101 cavity. Instrument settings are indicated in the results. EPR spectra were confirmed in replicate experiments. The *g* values were determined by comparison to the 2,2-diphenyl-1-picrylhydrazyl radical which has a *g* value of 2.0036.

2.4 Aconitase activity

Aconitase activity was measured as the conversion of isocitrate to *cis*-aconitate as described [63]. Briefly, the washed cells were scraped into 1 ml HBSS and pelleted by centrifugation (5 min, $100 \times g$). The cell pellets were washed three times in HBSS and lysed in phosphate-buffered saline (PBS) with 5 mM citrate, 0.1 mM DTPA, and 0.2% Triton X-100. Aconitase activity in these lysates was measured in 0.1 M Tris-HCl pH 8.3 with 20 mM trisodium DL-isocitrate (extinction coefficient for *cis*-aconitate at 240 nm is $3.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The reference cuvet was identical except that isocitrate was omitted.

2.5 Complex I activity

Complex I activity was measured in isolated mitochondria as the NADH-dependent reduction of ubiquinone-1 (coenzyme Q_1) as described [63]. Briefly, the washed cells from six T-75 flasks were scraped into HBSS and pelleted by centrifugation (5 min, $100 \times g$). The cell pellets were resuspended in 2.5 ml TES buffer (10 mM triethanolamine pH 7.0, 1 mM EGTA, 0.25 M sucrose). Cells were lysed on ice using a Teflon-on-glass Potter-Elvehjem homogenizer (30 passes). The resulting lysate was centrifuged for 10 min at 4°C ($1500 \times g$), and the post-nuclear supernatant was centrifuged (10 min, 4°C , $10,000 \times g$) to obtain the mitochondria-enriched pellet. The pellet was washed in TES buffer, frozen at -80°C , and subjected to three freeze-thaw cycles to facilitate lysis. Complex I activity was measured at 37°C using 15 μl aliquots of these mitochondrial lysates as described [63]. The rate of NADH oxidation that was stimulated by ubiquinone-1 was attributed to complex I activity. Rotenone inhibited essentially all of this activity as expected.

2.6 Complex II activity assays

Succinate dehydrogenase (SDH) activity was determined using iodonitrotetrazolium chloride (INT) as the final electron acceptor as described [64]. Briefly, the washed cells were scraped into 1 ml HBSS and pelleted by centrifugation (5 min, $100 \times g$). The cell pellets were washed three times in HBSS and frozen at -80°C . The pellets were thawed on ice, resuspended in 50 μl 0.1 M sodium phosphate pH 7.4, and sonicated two times (10 sec each) on ice. SDH activity was measured at 37°C using 10 μl aliquots of these lysates in a reaction mixture of 0.1 M triethanolamine HCl pH 8.3, 0.5 mM EDTA, 1.2% (w/v) Cremophor EL, 2 mM KCN, 2 mM INT, and 20 mM succinate [64]. The reference cuvet lacked succinate and absorbance was followed at 500 nm over time (extinction coefficient for reduced INT is $19,300 \text{ M}^{-1} \text{ cm}^{-1}$).

Complex II activity (succinate-dependent reduction of ubiquinone) was measured in mitochondria isolated from treated and washed cells (see above). The mitochondria-enriched pellet was stored in TES buffer at -80°C , and was subjected to three freeze-thaw cycles before analysis. Succinate-dependent ubiquinone reduction was measured at 37°C using 15 μl aliquots of these lysates in a reaction mixture of 0.1 M triethanolamine HCl pH 8.3, 0.5 mM EDTA, 1.2% (w/v) Cremophor EL, 2 mM KCN, and 50 μM ubiquinone-1 [65]. The formation of ubiquinol-1 was followed at 278 nm for 3 min. Succinate was then added to a final concentration of 20 mM and the absorbance at 278 nm was followed for another 3 min. The rate of ubiquinone-1 reduction that was stimulated by succinate was attributed to complex II (extinction coefficient for ubiquinol-1 is $14.7 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.7 Complex III activity

Complex III activity was measured as decylubiquinol cytochrome *c* reductase as described [66]. Mitochondria were isolated and prepared as described above for the complex I assay. Decylubiquinol was prepared from decylubiquinone as described [66] and aliquots of decylubiquinol were stored at -80°C in 90% ethanol with 10 mM HCl. Complex III activity

was measured at 37°C using 15 μ l aliquots of mitochondrial lysates in a reaction mixture of 50 mM Tris-HCl pH 7.4, 4 mM NaN₃, 40 μ M cytochrome *c* (horse heart), and 50 μ M decylubiquinol. The reduction of cytochrome *c* was followed at 550 nm for 2 min. The portion of cytochrome *c* reduction that was inhibited by 10 μ M antimycin A was attributed to complex III (extinction coefficient for reduced cytochrome *c* is 29.5 mM⁻¹ cm⁻¹).

2.8 Ex vivo treatment of bovine airways with Cr(VI)

Bovine lungs were obtained from freshly sacrificed animals at the local slaughterhouse. Bronchi (~1.5 cm diameter) were dissected from the lungs as quickly as possible, and rinsed in HEPES buffer (10 mM HEPES pH 7.4, 148.9 mM NaCl, 5 mM KCl, 5.5 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂). The bronchi were bisected longitudinally to expose the airway epithelial surface; one half was used for the control and the other for Cr(VI) treatment. Sections of bronchi with ca. 12 cm² of airway epithelium were treated with HEPES buffer (control) or with solutions of sodium chromate as indicated in the results. In some experiments, zinc chromate was used as an alternative chromium treatment and was distributed as ZnCrO₄ powder on the airway epithelial surface (0.62 mg per cm²). After treatment for 3 hr at 37°C, the bronchi were washed with HEPES buffer, and the bronchial epithelial cells were harvested by scraping the cells from the airway surface using a small metal spatula. The cells were assayed by EPR or for enzyme activities using the assays described above for cultured cells.

2.9 Miscellaneous

Protein was determined by a modified Lowry method, with bovine serum albumin as the standard [67].

For quantitative data, differences between three or more groups of data were assessed using one-way ANOVA and the Tukey-Kramer post test (Prism software, Graphpad). Differences between two groups were assessed using the unpaired *t* test (Prism software). Significance was assumed at *P* < 0.05.

3. Results

3.1 Low Temperature EPR of Cr(VI)-treated cells

It was previously shown that 25 μ M Cr(VI) for 3 hr resulted in essentially complete oxidation of Trx2 (mitochondrial) in cultured BEAS-2B cells, with about 55% of Trx1 (cytosolic) oxidized [41]. BEAS-2B cells subjected to these same treatments showed several EPR signals when analyzed at liquid helium temperature (Fig. 1). The signal at *g* = 1.989 is consistent with a mixture of Cr(V)-thiol, Cr(V)-GSH like species, or Cr(V)-diol-thiol species. Examples of the *g*-values for Cr(V) complexes are *g* = 1.996 for [Cr(V)O(SR)₄]⁻, *g* = 1.985 for [Cr(V)OL(SR)₂]⁻, *g* = 1.983 for [Cr(V)(O)₂(SR)₂]⁻, and *g* = 1.979 for [Cr(V)OL₂]⁻, where LH₂ is a biological 1,2-diol and RSH is a thiol [68]. Signals for Cr(III) were observed at *g*_{eff} = 5.4, 5.2 and 4.3 (Fig. 1). The lines in the *g* = 5 region are characteristic of Cr(III) complexes with a large ZFS (zero field splitting), signifying a distorted geometry. The line with *g*_{eff(z)} = 5.4 should have a *g*_{eff(y)} = 2.0 and a broad line at about *g* = 1.4 for *g*_{eff(x)}, and the line with *g*_{eff(z)} = 5.2 should have *g*_{eff(y)} = 2.3 and a broad line at about 1.6 for *g*_{eff(x)} as determined from a rhombogram [69]. E/D (the ratio of E to D, which are the axial and rhombic ZFS parameters, respectively) for the cells spectrum is 0.33 and 0.25 for *g*_{eff} = 5.4 and 5.2, respectively, signifying a large rhombic component. However, the broad lines for *g*_{eff(y)} and *g*_{eff(x)} are superimposed on broad lines in the *g* = 2 region assigned to unresolved Cr(III) complexes with a weak ZFS (Fig. 1A). In Fig. 1A, a signal at ca. *g* = 2.3 could be the line for *g*_{eff(y)} associated with the *g* = 5.2 signal, or could be attributed to a compound with weak ZFS. The three Cr(III) signals in cells, the *g*_{eff} = 5.4

and 5.2 just described and the $g_{\text{eff}} = 4.3$ which has an E/D of about 0.04, indicate Cr(III) species with a large ZFS. While the exact composition of these Cr(III) species is unknown, the spectral features suggest complex(es) with ligands from cellular components. Overall, these Cr signals demonstrate that at least some of the Cr(VI) was reduced to Cr(V) and Cr(III) in these cells.

These cellular spectra also showed an intense signal at $g = 1.94$ and another whose peak was at $g = 2.02$ (Fig. 1). The $g = 1.94$ signal is most consistent with reduced forms of the 2Fe-2S centers in mitochondrial electron transport complexes I and II, and the signal at $g = 2.02$ is most consistent with oxidized inactivated $[3\text{Fe-4S}]^{1+}$ aconitase. However, certain Fe-S centers in the mitochondrial electron transport chain could contribute to $g = 2.02$ as well (see more below).

Focusing on this high field region, there is good resolution of the signals at $g = 2.02$, 1.989 and 1.94 (Fig. 1B). The intensity of the signal at $g = 2.02$ (actually the maximum of the S-shaped line is at $g = 2.02$) does not vary much between 30 K and 20 K (Fig. 1B), which is consistent with saturation of the line for $[3\text{Fe-4S}]^{1+}$ [52]. This signal is consistent with mitochondrial aconitase because it does not have a shoulder to the left of the $g = 2.02$ signal that would be expected for cytosolic aconitase [70]. The intensity of the $g = 2.02$ signal (e.g. $g_{z,y,x} = 2.022, 1.938, 1.923$ [71,72]) does approximately double, however, when going from 20 K to 10 K (Fig. 1B), which demonstrates a contribution from the low field line of mitochondrial 2Fe-2S centers at 10 K ([73-75] and references therein). Also, the line shape of $g = 2.02$ is narrower at 10 K than at 20 or 30 K, supporting an additional component to the signal at 10 K. By contrast, the intensity of the signal at $g = 1.94$ [76] progressively increases as the temperature goes from 30 K to 20 K to 10 K, which is consistent with mitochondrial 2Fe-2S centers [73,77]. The difference in the temperature dependence for the line at $g = 2.02$ versus the line at $g = 1.94$ therefore indicates that both $[3\text{Fe-4S}]^{1+}$ (probably from aconitase and/or aconitase-like signals), and the signals for reduced $[2\text{Fe-2S}]^{1+}$ centers contribute to the $g = 2.02$ signals in Cr(VI)-treated cells (Fig. 1). The line at $g = 1.94$ has a $P_{1/2}$ (the power at which the signal obtains half of its unsaturated value) of about 80 mW at 10 K, and the line at $g = 2.02$ has a $P_{1/2}$ of about 16 mW confirming that the signals from both $[2\text{Fe-2S}]^{1+}$ and $[3\text{Fe-4S}]^{1+}$ centers are present. A $P_{1/2}$ of 80 mW for the $g = 1.94$ signal is consistent with conditions where there is a nearby paramagnetic state, e.g. the 4Fe-4S cluster of complex II [73]. In complex I, the 4Fe-4S centers (N3, N4) on either side of its 2Fe-2S center (N1b) can influence the EPR behavior of the latter [74]. The $g = 1.94$ and $g = 2.02$ signals were not seen in control cells treated with just HBSS (no chromate) for 3 hr (Fig. 2).

Since it was previously shown that prolonged (16 hr) incubation with only 5 μM Cr(VI) also causes significant oxidation of mitochondrial Trx2 [42], cells treated in this manner were also examined by EPR (Fig. 3). The signals for Cr(III) at $g = 5.4, 5.2$, and 4.3 were again seen, as was the $g = 1.989$ signal indicative of Cr(V)-thiol-diol complexes. The $g = 1.989$ signal was very small in cells treated with just 2.5 μM Cr(VI), however (Fig. 3). It is possible that most of the Cr had been reduced beyond Cr(V) given the low amounts of Cr(VI) and the prolonged incubation. For the cells treated with 5 μM Cr(VI), there was also a very broad shallow signal centered around $g = 2$ at high field indicative of non-specific Cr(III) complexes (Fig. 3A). This signal was not detectable in cells treated with 2.5 μM for 16 hr (Fig. 3A), but was seen in cells treated for 3 hr with 25 μM Cr (Fig. 1A). Together, these signals for Cr species indicate that Cr(VI) reduction occurred in these cells. A prominent $g = 1.94$ signal was seen in cells treated with 2.5 or 5 μM Cr(VI) for 16 hr (Fig. 3), which again is consistent with the $[2\text{Fe-2S}]^{1+}$ centers in mitochondrial electron transport complexes I and II.

Together, these EPR data for the various Cr(VI) exposures suggest that mitochondrial electron transport complexes I and/or II, and the 4Fe-4S center in aconitase are the most likely species to be compromised in Cr(VI)-treated cells. However, we cannot exclude the possibility that a portion of the $g = 2.02$ signal could result from removal of an Fe from other mitochondrial 4Fe-4S centers [52].

3.2 Activities of aconitase and mitochondrial electron transport complexes in Cr(VI)-treated cells

Activity assays were done to determine if aconitase and mitochondrial electron transport complexes are indeed inhibited in Cr(VI)-treated cells, as would be predicted from the EPR signals. Aconitase activity was strongly inhibited in cells treated with 25 or 50 μM Cr(VI) for 3 or 6 hr (Fig. 4A). This decline in activity would be consistent with oxidation of its $[\text{4Fe-4S}]^{2+}$ center (active) to $[\text{3Fe-4S}]^{1+}$ (inactive); this inactive state yields the $g = 2.018$ EPR signal (peak at $g = 2.02$) [78] that was observed in Figs. 1 and 2. Western blots for mitochondrial aconitase showed that the aconitase protein does not decrease as a result of Cr(VI) treatment (not shown), which is consistent with enzyme inactivation.

The specific activity for Complex I, measured as the NADH-dependent reduction of ubiquinone-1 [63], was inhibited by an average of 66% in mitochondria isolated from cells treated with 25 μM Cr(VI) for 3 hr (Fig. 4B). The inhibition of complex I is consistent with the $g = 1.94$ EPR signal in Figs. 1–3 which represent the reduced form of 2Fe-2S centers in complexes I and/or II [73,75,79].

Two assays for complex II activity were done. The first measured SDH activity as the succinate-driven reduction of INT. This activity was not affected by treating cells with 25 or 50 μM Cr(VI) for 3 hr (Fig. 4C). However, the reduction of artificial electron acceptors such as INT only requires the proximal redox centers in SDH, i.e. the FAD (which is reduced by succinate) and S1, the 2Fe/2S center that is reduced by the FADH_2 and that then reduces INT [80]. The second assay for complex II measured the succinate-dependent reduction of ubiquinone which requires electron flow through all of its redox centers, including the FAD, and the S1 (2Fe-2S) and S3 (3Fe4S) centers [77]. This measure of complex II activity was significantly inhibited by an average of 35% ($P = 0.0157$) in mitochondria isolated from cells treated with 25 μM Cr(VI) for 3 hr (Fig. 4D). Disruption of electron flow at sites between S1 and ubiquinone reduction would result in reduction of the 2Fe-2S center (S1) and thus a $g = 1.94$ signal.

The specific activity for complex III, measured as decylubiquinol cytochrome *c* reductase, was not affected by treating cells with 25 μM Cr(VI) for 3 hr (Fig. 4E). If complex III had been inhibited, we would have expected to see the EPR signals for the Rieske 2Fe-2S center in complex III ($g = 2.03$, 1.90, and a broad line at $g = 1.75$) [81]. These signals were not observed, consistent with no significant inhibition of complex III activity.

3.3 Potential recovery of cells once Cr(VI) is removed

Since occupational and environmental Cr(VI) exposure is predicted to be intermittent, studies were conducted to determine if the mitochondrial indicators would return toward normal once Cr(VI) was removed. Aconitase was of particular interest because it recovers with a half-life of ca. 12 min in cells once the superoxide ($\text{O}_2^{\bullet-}$)-generating agent phenazine methosulfate is withdrawn [56]. Cells were treated with buffer for 6 hr, 25 μM Na_2CrO_4 for 3 or 6 hr, or Na_2CrO_4 for 3 hr followed by 3 hr in buffer. In the high-field region, the two most prominent signals in cells treated with Cr for 3 or 6 hr were $g = 1.989$ representing Cr(V) species, and $g = 1.938$ (2Fe-2S centers of complex I and/or II) (Fig. 5A). The line shape for Cr(V) is skewed on the high field side indicative of multiple Cr(V) species. The

signal for Cr(V) decreased dramatically following the 3-hr recovery in Cr-free buffer and shifted to $g = 1.985$ (Fig. 5A). This shift suggests a change in Cr(V) species or ligands. The marked decline in the Cr(V) signal intensity implies that the Cr(V) species in cells are not long-lived and that the strong signals observed in the samples treated with Cr for 3 or 6 hr largely represent continual generation of Cr(V) from chromate. Previous *in vitro* work estimated that Cr(V) species generated by the enzymatic reduction of Cr(VI) turn over in 1–2 min [24] and some hydroxamic acid complexes of Cr(V) decompose within minutes in aqueous environments [82]. Cr(V) species stabilized by some ligands have longer half-lives (e.g. 45–90 min [83]), and up to several hours in non-aqueous compartments [68]. The decay of Cr(V)-thiol species in aqueous biological systems is much more rapid, although Cr(V)-diols ($g = 1.979$) can exhibit enhanced stability [68].

While the Cr(V) signal significantly declined during the 3 hr recovery, the $g = 1.938$ signal was essentially the same in cells treated with Cr for 6 hr and cells treated for 3 hr followed by the 3 hr recovery (Fig. 5A). This implies that the blocks in electron flow through complex I and/or II are not readily reversed once Cr(VI) is removed. The signals for Cr(III) at $g = 5.4$ and 4.3 were similar for all cells that were Cr(VI)-treated (Fig. 5B); this verifies similar exposure of the treated cells as well as the intracellular reduction of Cr(VI) to Cr(III). Since Cr(III) is a stable oxidation state, its persistence is expected, unlike Cr(V) which is an unstable intermediate in the stepwise reduction of Cr(VI) to Cr(III). The $g = 2.02$ signal, which is consistent with the $[3\text{Fe-4S}]^{1+}$ center of inactive aconitase (or the $[3\text{Fe-4S}]^{1+}$ of S3 in complex II) was again observed in cells treated for 3 or 6 hr with Na_2CrO_4 ; however, it was not seen in cells following the 3 hr recovery (Fig. 5A). These samples were also analyzed at 0.2 mW microwave power (not shown), and the relative $g = 2.02$ signal intensities matched those in Fig. 5A. Since the $g = 2.02$ signal did not persist during the Cr(VI)-free recovery period, whereas the $g = 1.938$ signal indicative of 2Fe-2S from complex I and/or II did persist, the bulk of the $g = 2.02$ signal likely represents the inactive aconitase $[3\text{Fe-4S}]^{1+}$ center. The loss of the $g = 2.02$ signal during Cr(VI)-free recovery could either represent reactivation of aconitase to the $[4\text{Fe-4S}]^{2+}$ state, or oxidative disassembly of the cluster to EPR silent species. Aconitase activity was determined to distinguish between these possibilities. Since there was no significant recovery of activity after Cr(VI) was removed from the cells (Fig. 6), the loss of the $g = 2.02$ signal during the 3-hr Cr(VI)-free period therefore likely represents oxidative disassembly of the Fe-S cluster.

3.4 Cr(VI) treatment of bovine airways

To determine if the Cr-mediated effects we observed with cultured human bronchial epithelial cells would similarly occur with an analogous animal tissue, *ex vivo* experiments were done using bronchi from bovine lungs. These bronchi were treated with either soluble Na_2CrO_4 as used for the human cells above, or with a less soluble ZnCrO_4 powder to model the effects of particulate chromates that are used in some industries. Exposure times were 3 hr to match those most commonly used for the cells above. Both forms of chromate generated EPR signals in the bovine airways that were similar to those seen in the cells above. Focusing on the high field region, a marked signal at $g = 1.938$ (1.94) was noted at 10 K in samples that were treated with either Na_2CrO_4 or ZnCrO_4 (Fig. 7A). As noted above, this signal is consistent with the 2Fe-2S centers in mitochondrial electron transport complexes I and II. The ZnCrO_4 treatment of the airways in particular also caused a marked increase in the signal whose peak is at $g = 2.027$ (Fig. 7A). This signal could represent oxidized inactivated $[3\text{Fe-4S}]^{1+}$ aconitase, and/or other Fe-S centers in the mitochondrial electron transport chain (see elsewhere). Signals at $g = 1.986$ and 1.982 were noted in the airways treated with Na_2CrO_4 and ZnCrO_4 , respectively (Fig. 7A); these signals represent a mix of Cr(V) species, and the Cr(V) signal was particularly intense with ZnCrO_4 treatment (Fig. 7A). When analyzed at 40 K, the $g = 1.94$ and $g = 2.02$ signals were broadened and/or

less intense such that they were difficult to discern from background in ZnCrO_4 -treated bovine airway. At 40 K, the signals for Cr(V) and Cr(III) were still present, although at lower intensity as expected for the warmer temperature (not shown).

The signal at $g = 2.006$ was larger in the chromate-treated samples (Fig. 7A) and is assigned to a free radical, probably ubisemiquinone [52,71,84]. The signal at $g = 5.9$ (Fig. 7B) is consistent with a high spin heme and was much larger in the Cr(VI)-treated samples relative to the buffer-treated controls. In the high field region, samples without chromate were noted to have signals at $g = 2.04$ and $g = 2.013$ (Fig. 7A). These signals are nicely assigned to specific Fe-S clusters [85]. The $g = 2.04$ signal was of similar size in untreated airway, and it was also noted with ZnCrO_4 treatment although the $g = 2.04$ was not fully resolved from the large signal at $g = 2.027$ (Fig. 7A). The $g = 2.04$ signal is consistent with center N3 of complex I, but its line at $g = 1.86$ were not observed. The $g = 2.013$ signal is S-shaped and could indicate a 3Fe-4S site (possibly S3 from complex II [52,60,86]) but this assignment is tentative. A signal at $g = 2.85$ and weak lines between $g = 2.85$ and $g = 4.3$ were noted in the airways treated with Na_2CrO_4 but were not seen with ZnCrO_4 treatment (Fig. 7B); the identity of these signals is yet to be determined. The signal at $g = 4.3$ is most likely non-heme iron in the absence of Cr, and in the presence of Cr this non-heme iron signal is most likely superimposed on the signal for Cr(III) in this region.

The full-scale EPR spectra show that some of the Cr(VI) was reduced to Cr(III) in the bovine airways treated with either Na_2CrO_4 or ZnCrO_4 (Fig. 7B). The signals at $g_{\text{eff}} = 5.4$, 5.2 and 4.3 were particularly noted following ZnCrO_4 treatment and are characteristic of Cr(III) complexes with a large ZFS. The spectral features of these signals suggest complex(es) with cellular ligands. These signals were also noted at a lesser intensity in bovine airways treated with Na_2CrO_4 (Fig. 7B). As with cells, there was a broad signal in the $g = 2$ region attributed to Cr(III) complexes with weak ZFS.

EPR spectra were also obtained for bovine airways treated ex vivo with lower concentrations of Na_2CrO_4 (25 or 100 μM) (Fig. 8). Both concentrations of Na_2CrO_4 caused an increase in the signal intensity at $g = 1.937$ (Fig. 8) suggesting some disruption of electron flow through complexes I and/or II. A very small signal indicative of Cr(V) was seen at $g = 1.986$ in the 100 μM Na_2CrO_4 samples but this signal was not seen with 25 μM Na_2CrO_4 (Fig. 8). Since Cr(V) is transient, it is possible that the bulk of Cr(VI) reduction occurred earlier. In full-scale scans at 20 mW microwave power, it was difficult to see the Cr(III) signals ($g = 5.2$) above background in these samples (not shown). While a signal at $g = 2.02$ was noted in these samples (Fig. 8), the background prevented a quantitative assessment for possible changes in its intensity.

In airways treated with 2-fold and 4-fold lower density of ZnCrO_4 , the signals for Cr(V) and Cr(III) were observed (Supplemental Figs. 1 and 2). Relative to the control without Cr, the signal at $g = 2.026$ was 40–55% larger in samples treated with ZnCrO_4 , and the signal at $g = 1.938$ (1.94) was very prominent in ZnCrO_4 -treated samples (Supplemental Figs. 1 and 2). Overall, the data show that lower concentrations of Na_2CrO_4 and ZnCrO_4 are sufficient to generate a readily detectable $g = 1.94$ signal in bovine airways. With Na_2CrO_4 treatments, this signal proved even more sensitive than those for Cr(V) and Cr(III), so it could represent an important biomarker of Cr-mediated effects on mitochondrial function.

Lastly, activity assays were done to determine if aconitase and mitochondrial electron transport complexes I and II were inhibited in Cr(VI)-treated bovine airways, as would be predicted from the EPR signals. Indeed, the activities of complex I, aconitase, and complex II were all significantly inhibited in bovine airways treated with ZnCrO_4 for 3 hr (Fig. 9). Complex I was inhibited an average of 71% (Fig. 9A) which is similar to the 66% inhibition

noted for human cells (Fig. 4B). Aconitase activity was inhibited an average of 68% in ZnCrO₄-treated bovine airways (Fig. 9B); this inhibition is consistent with the increased $g = 2.02$ signal seen in Fig. 7A. Complex II activity (succinate-dependent reduction of ubiquinone) was inhibited by an average of 49% (Fig. 9C). The inhibition of complexes I and II is consistent with the $g = 1.94$ signal observed in Fig. 7. Overall, the major EPR and activity findings are consistent between the cultured human bronchial cells and the bovine airways suggesting that the cultured cells are representative of the relevant biological tissue.

4. Discussion

4.1 Utility of the 10 K EPR signals as markers relevant to Cr(VI) exposure

While the distribution of intracellular Cr between the mitochondria and other cell compartments is not well understood, the inhibition of aconitase and complexes I and II, and the resulting EPR signals at $g = 1.94$ and $g = 2.02$ imply that there are major effects on core mitochondrial proteins in cultured human airway cells and in bovine airways. Some earlier studies that looked for specific mitochondrial effects of Cr(VI) used isolated mitochondria, and determined that they rapidly accumulate ⁵¹Cr(VI), with about 70% of the maximal uptake within 10 min [87]. This is consistent with our findings that imply significant entry of Cr into mitochondria in cultured cells and in bovine airway epithelium. Isolated mitochondria can reduce Cr(VI) to Cr(V) and this is stimulated by succinate (directly reduces complex II), and by malate and glutamate (which support NADH generation and therefore complex I) [87]. Therefore, reactive Cr intermediates can be generated within mitochondria. Studies with electron transport inhibitors suggested that Cr(VI) may be reduced at complexes I, II, and IV, although ca. 75% of total mitochondrial Cr(VI) reduction is not affected by these inhibitors [87]. Therefore, mitochondria have multiple ways to generate reactive Cr intermediates. We previously reported that human microsomal cytochrome *b*₅ has significant Cr(VI)-reducing activity [25,26]. There is a mitochondrial isoform of *b*₅ that has a very similar three-dimensional structure and heme environment to the microsomal form, although the redox potential of mitochondrial *b*₅ is approximately 100 mV more negative [88]. While the function(s) of mitochondrial *b*₅ are less well known, it is possible that it contributes to mitochondrial NADH-dependent Cr(VI) reduction.

In ruptured rat liver mitochondria, $\geq 20 \mu\text{M}$ Cr(VI) was noted to cause a partial inhibition of complex I activity, and a partial inhibition of complex II was noted in isolated mitochondria [89]. It is important to emphasize that these observations were not extended to cells but were limited to isolated/ruptured mitochondria. They are, however, consistent with the significant inhibition we noted in Cr(VI)-treated cells and airways. Other evidence for the potential mitochondrial effects of Cr(VI) in cells or tissues has been limited and indirect. For example, with human keratinocytes, 33–182 μM K₂Cr₂O₇ caused a 50% decrease in the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is one indicator of mitochondrial activity [47]. Cr(VI) has been noted to cause a decrease in the mitochondrial membrane potential in immortalized human fibroblasts [45] and in A549 lung cancer cells [46], although it is unknown if this resulted from intra- or extra-mitochondrial events.

The current studies show that Cr(VI) exposure significantly inhibits the activity of core mitochondrial functions (aconitase, complex I and II) in both cultured cells and bronchial epithelium. The 10 K EPR spectra simultaneously show several important signals in these cells including those for Cr(V) and Cr(III), and the Fe-S signals at $g = 1.94$ (complexes I and II) and at $g = 2.02$ (aconitase, complexes I and II). Since Cr(III) is a stable oxidation state, its signals persist and therefore reflect the cumulative net reduction of Cr(VI) over time. They also serve to confirm Cr exposure and cellular uptake. Since there are no 3-electron donors in biological systems, the reduction of Cr(VI) to Cr(III) implies the formation of

Cr(V) and/or Cr(IV), which are short-lived reactive intermediates. The Cr(V) EPR signal intensity therefore represents the relative level of Cr(V) formation near the time the sample was collected [90]. Along these lines, we noted that the Cr(V) signal largely disappeared after the source of Cr(VI) was removed.

The formation of reactive Cr intermediates in cells could also promote reactive oxygen species generation by the redox cycling of Cr [26]. Both Cr(V) and Cr(IV) rapidly generate HO• via Fenton-like reactions with H₂O₂ [26,29,36-39]. While Cr(III) might participate in such reactions [91], it is relatively slow when compared with Cr(IV) and Cr(V). Prior studies with BEAS-2B cells showed significant mitochondrial redox stress (as reflected in the oxidation of mitochondrial thioredoxin-2 and peroxiredoxin-3) following Cr(VI) treatments that were identical to those noted here to cause inhibition of aconitase and complex I and II [41,42]. These effects on multiple proteins could represent independent indicators of pronounced mitochondrial redox stress, or they could reflect a relationship between these proteins. The thioredoxins are important mediators of intracellular thiol redox balance [92] so it is possible that thioredoxin-2 has a role, either direct or indirect, in maintaining aconitase and/or complexes I and II in their active state, either through maintenance of their thiols in a reduced state or protection of their Fe-S centers or thiols from oxidative inactivation. In yeast that were depleted of glutathione reductase, the overexpression of thioredoxin-2 restored Fe-S enzymes (e.g. aconitase, sulfite reductase) to their normal activity/state [55], implying that mitochondrial thioredoxin-2 may protect certain Fe-S proteins from oxidative inactivation. Because thioredoxin-2 and its dependent peroxiredoxin-3 are oxidized following Cr(VI) treatment [41,42], total mitochondrial oxidant stress could also be increased though the loss of a major mitochondrial peroxidase, specifically peroxiredoxin-3 [93]. The resulting increase in peroxides might facilitate the inactivation of some mitochondrial proteins, and could enhance HO• generation by Fenton-like reactions involving reduced forms of Fe or Cr.

The $g = 1.94$ and $g = 2.02$ EPR signals that result from Cr(VI) exposure could serve as important biomarkers for Cr(VI)-mediated mitochondrial oxidative damage. Of these two, the $g = 2.02$ signal was less pronounced and it diminished after the Cr(VI) source was withdrawn. In contrast, the $g = 1.94$ signal was very strong and persisted after Cr(VI) removal. It is therefore a potentially more sensitive and useful marker. Inhalational Cr(VI) exposure results in a non-homogeneous distribution of Cr in the airways [2]. This would be expected to yield great diversity in the exposure of airway epithelial cells, ranging from zero to potentially very high levels in cells that are in direct contact with inhaled Cr(VI) particulates. While activities of individual enzymes or electron transport complexes were an important part of the studies reported here, such studies would be more difficult to extrapolate to in vivo exposures because the normal activity in areas receiving little or no Cr(VI) might mask any inhibition in those areas that received higher Cr(VI). The EPR studies could be more useful in this regard. The $g = 1.94$ signal is very small to absent in untreated cells and airways, but it increases dramatically following Cr(VI) exposure. Thus the low or absent signal in areas receiving little or no Cr(VI) should not mask a $g = 1.94$ signal from areas receiving higher Cr(VI). With our ex vivo use of particulate ZnCrO₄, “sprinkling” the ZnCrO₄ powder on the airways results in a non-homogeneous distribution that is expected to yield highly variable Cr exposure to different cells. Thus, it is likely that only some of the cells were in direct contact with ZnCrO₄ particles and yet we observed very strong $g = 1.94$ signals. In experiments in which only 50 or 25% of the airway surface was treated with ZnCrO₄, and the rest of the surface was deliberately untreated, we still observed the signals at $g = 1.94$ and $g = 2.02$, as well as those for Cr(V) and Cr(III) (Supplemental Fig. 3).

4.2 Implications for the inhibition of aconitase

To our knowledge, this is the first report noting the inhibition of aconitase in Cr(VI)-treated cells. The EPR signal at $g = 2.02$ could largely result from inhibited aconitase, although other $[3\text{Fe}4\text{S}]^{1+}$ signals (e.g. the oxidized state of the constitutive 3Fe-4S center S3 in complex II) may also contribute [52,60,86]. While both the mitochondrial and cytosolic forms of aconitase are sensitive to inactivation by various oxidants [51,57], the signal we observed in cells was consistent with mitochondrial aconitase (above). However, given the near complete inactivation of total aconitase activity in these cells (Fig. 4), it is possible that both forms of aconitase were inhibited. Since aconitase activity is typically much higher in the mitochondrial compartment [56], the EPR signal for inactivated cytosolic aconitase may have been present, but below the limit of detection.

Given the role of m-aconitase in the TCA cycle, its inhibition would be predicted to slow the production of NADH and thus reduce the source of electrons for complex I [94]. Such a decrease in NADH generation could slow mitochondrial electron transport under conditions of significant oxidant stress. Consistent with this, the inhibition of aconitase by hyperoxia or fluoroacetate results in a net decrease in cellular O_2 consumption [49]. Complex I is known to generate significant amounts of $\text{O}_2^{\bullet-}$ from O_2 [95,96], so the decreased ability to generate NADH could help to limit $\text{O}_2^{\bullet-}$ generation that would be expected to result from the inhibition of electron flow through complex I [58,97].

In other ways, however, aconitase inactivation could promote oxidant generation. Since one Fe atom of the aconitase $[4\text{Fe}-4\text{S}]^{2+}$ center is labile with respect to oxidants such as $\text{O}_2^{\bullet-}$, the $\text{O}_2^{\bullet-}$ -mediated oxidation of this center results in the release of Fe(II) and H_2O_2 [57,59] which can directly promote $\text{HO}^\bullet$ generation [59]. The formation of the $[3\text{Fe}-4\text{S}]^{1+}$ signal ($g = 2.02$) in Cr(VI)-treated cells implies that at least some labile Fe was released from active aconitase or other 4Fe-4S centers. Since even low levels of Fe can also markedly stimulate the reductive activation of Cr(VI) [98], this release of Fe might further enhance Cr-mediated oxidant generation. The inhibition of cytosolic aconitase could promote its role as an iron regulatory protein and further increase intracellular Fe [51,94,99].

Since aconitase activity is not restored following Cr(VI) removal, its inactivation is irreversible or difficult to reverse in cells. The corresponding loss of the $g = 2.02$ signal during this Cr(VI)-free recovery suggests that its Fe-S cluster may be oxidatively disassembled. As a result, TCA cycle activity may be diminished long-term. The oxidative disassembly of its Fe-S cluster could release additional Fe and further promote Fe-mediated reactions.

Aconitase can be inactivated by a number of oxidants including $\text{O}_2^{\bullet-}$, H_2O_2 , and peroxynitrite [51,56,57]. Of these, $\text{O}_2^{\bullet-}$ is considered to be the most robust [56,57,97,100,101] with a second order rate constant of ca. $10^7 \text{ M}^{-1} \text{ s}^{-1}$ [102,103]. Because of this, aconitase inactivation is often thought to reflect $\text{O}_2^{\bullet-}$ generation in cells [57,101]. The enzymatic reduction of Cr(VI) in vitro generates both $\text{O}_2^{\bullet-}$ and $\text{HO}^\bullet$ [26,40], and both $\text{HO}^\bullet$ and thiyl radicals have been detected in Cr(VI)-treated cells [41,46]. However, $\text{HO}^\bullet$ scavengers did not protect aconitase from inactivation in vitro [59]. Since aconitase inhibition by $\text{O}_2^{\bullet-}$ is often reversible [56,99,101], the irreversible inactivation of aconitase in Cr(VI)-treated cells suggests that other oxidants may contribute to the overall outcome. However, the prolonged exposure of mitochondria to steady state $\text{O}_2^{\bullet-}$ can result in Fe-S cluster disassembly [58], so it is still possible that $\text{O}_2^{\bullet-}$ may contribute to the prolonged inactivation associated with Cr(VI).

A number of other oxidants are also generated as a result of Cr(VI) reduction. These include H_2O_2 , which does have some ability to inhibit aconitase [51], and Cr(V) and Cr(IV) species

which are robust direct oxidants of some cellular components [34,35] and are generated during Cr(VI) reduction by human enzymes and in BEAS-2B cells [24,26,33]. However, the potential effects of reactive Cr species on aconitase are not known. Other direct or indirect oxidants in Cr(VI)-treated cells, including peroxynitrite, could contribute to aconitase inactivation [51,104]. Cr(VI) increases nitrotyrosine levels in HUVECs implying that peroxynitrite generation is increased [105].

Other cellular components may also contribute to aconitase inactivation. For example, a significant portion of aconitase inactivation in H₂O₂-treated cardiac mitochondria depends on a H₂O₂-responsive mitochondrial membrane component [58]. In addition, m-aconitase contains a highly reactive Cys residue that is inactivated by sulfhydryl reactive agents that are able to block access of the substrate to the active site [106,107]. Therefore, effects on this critical sulfhydryl have to be considered as a potential contributor to aconitase inactivation following Cr(VI) treatment. Since different mechanisms of inactivation can occur with different oxidants [58], there may be multiple ways in which aconitase is inactivated given the diverse array of oxidants generated by Cr(VI) exposure.

4.3 Effects on complex I and/or II

The inhibition of complex I and II activity in Cr(VI)-treated cells implies that there are changes to one or more components that diminish electron flow through these complexes, or their ability to transfer electrons to ubiquinone. There are multiple potential consequences for the cell. The most obvious is that diminished electron transport could decrease the ability to maintain the mitochondrial transmembrane potential ($\Delta\psi$) and thus to generate ATP. As noted above, Cr(VI) treatment has been associated with decreased $\Delta\psi$. Depending on where the specific blocks are, diminished electron transport could also result in enhanced O₂^{•-} generation. Consistent with this, Cr(VI) increases cellular O₂ consumption in A549 cells with the concomitant generation of reactive oxygen species [46]. The inhibition of complex I is known to increase its generation of O₂^{•-} [95,96]. This might be partially offset, however, if the generation of NADH is diminished as a result of the inhibition of aconitase (above).

The EPR data are consistent with diminished electron flow through complex I and/or II. The $g = 1.94$ signal was essentially absent in control cells and tissues indicating that the 2Fe-2S centers of complexes I and II are normally in the oxidized state, [2Fe2S]²⁺. However, their decreased ability to reduce ubiquinone following Cr(VI) exposure would be expected to result in the back-up of electrons within these complexes, thereby generating the reduced forms, [2Fe-2S]¹⁺ ($g = 1.94$). While assignment of EPR signals to distinct Fe-S centers is possible with individual complexes purified from mitochondria, their assignment in cells or tissues is complicated by the signal overlap for some of these centers. Since both complex I and II are inhibited following Cr(VI) treatment, the $g = 1.94$ signal could result from [2Fe-2S]¹⁺ centers in either complex I (N1b, $g_{\perp} = 1.939$ [108]) or complex II (S1, $g = 1.93$ [86]), or both. The other 2Fe-2S center in complex I (N1a) does not likely contribute to this signal because it is unlikely to be reduced in biological systems given its very low redox potential [71,74].

Complex I has several 4Fe-4S centers whose reduced forms, [4Fe4S]¹⁺, might also contribute to signals at $g = 1.92$ – 1.94 at <20 K [71,108,109]. Several of these (N4, N5, N6a, N6b, N2) are downstream of N1b [74,95,96,108,109]. Signals representing the extreme g -values (g_z and g_x) for these other Fe-S centers were not, however, observed in Cr-treated human or bovine samples, although the absence of such signals under our conditions is not sufficient to conclude if these centers were reduced or not. It is therefore difficult to define the specific site(s) at which electron flow is compromised in complex I, although the block is most likely downstream of center N1b.

Given that both complex I and II are inhibited after Cr(VI) treatment, we propose that the bulk of the $g = 1.94$ signal represents a superposition of $[2\text{Fe}2\text{S}]^{1+}$ signals from Complexes I and II. In previous studies with the BEAS-2B cells in which 10 K EPR was used to examine the Cr(III) signals, a small $g = 1.94$ signal was observed following 5 min treatment with 400 μM Na_2CrO_4 [33]. The significance of this signal was not pursued at that time. Although the 400 μM Cr(VI) in this prior study was 16-fold higher than the 25 μM used for the 3 hr exposures here, it is now clear that Cr(VI) treatment results in the $g = 1.94$ signal over a wide range of Cr concentrations depending on the time of exposure. While it was a small component of the spectrum after 5 min with 400 μM Cr(VI) [33], it is the dominant feature after 3 hr with 25 μM , or 16 hr with 2.5 or 5 μM . This implies that both brief exposure to high concentrations of Cr(VI), and prolonged exposure to lower concentrations, can result in significant disruption of electron flow through complexes I and/or II.

The inhibition of complex I and II activities are not likely due to direct effects on the Fe-S centers, because these centers in complexes I, II, and III are remarkably resistant to repeated exposure to a number of oxidants including nitric oxide, peroxynitrite, and H_2O_2 [52,60,110]. However, complex I and II activities are susceptible to $\text{HO}^\bullet$ and $\text{O}_2^{\bullet-}$ [48,52]. These and other oxidants may be involved following Cr(VI) treatment, and the potential effects of reactive Cr intermediates, in particular, are not well understood in this regard. While the $g = 1.94$ signal implies the back-up of electrons within complex I and/or II, the site of inhibition does not necessarily have to be at Fe-S centers themselves. Effects at other sites within these complexes could account for the signals we observed as long as they result in a decreased electron flow. Since complex I, in particular, is sensitive to thiol redox status within mitochondria [61,62], oxidation of critical thiols might contribute to its inhibition in Cr(VI)-treated cells.

While the temperature and other data suggest that the $[3\text{Fe-4S}]^{1+}$ from aconitase is likely a significant component of the $g = 2.02$ signal, some centers in complex I and II could also contribute to signals in this region. The reduced $[2\text{Fe-2S}]^{1+}$ cluster N1b from complex I has a $g_{\parallel} = 2.03$ (2.02 in some species) [108], and reduced S1 of complex II has a $g_z = 2.025$ [86]. However, the ratios of the intensities of $g = 1.94$ (major) to $g = 2.02$ (minor) for S1 should be constant under different conditions [80]. Since both our temperature and recovery data show that this ratio is not constant, we do not believe that the $g = 2.02$ signal can be accounted for by a single species. While the exact site(s) of the block in complex II cannot be discerned from the current data, we predict it is downstream of S1. This is consistent with the fact that the succinate-driven reduction of INT (which requires S1 but not the other Fe-S centers) was not affected, whereas the succinate-dependent reduction of ubiquinone (which requires all of the Fe-S centers in complex II) was inhibited.

In summary, the increased $g = 2.02$ signal (crossover at $g = 2.01$) following Cr(VI) exposure is probably a superposition of the $[3\text{Fe-4S}]^{1+}$ signal from oxidized aconitase with contribution from the $g = 2.02$ component of the signals for reduced $[2\text{Fe-2S}]^{1+}$ centers from complex I and/or II. These assignments are consistent with the activity changes that we observed. We cannot discount the possibility that other 3Fe-4S centers contribute to the $g = 2.02$ signal, including the oxidized form of the constitutive 3Fe-4S (S3) center of complex II, and/or one or more 4Fe-4S centers whose transient oxidation may have resulted in 3Fe-4S species [52,54].

4.4 Summary

Exposure of bronchial epithelium to the pro-oxidant Cr(VI) causes pronounced and irreversible inhibition of aconitase. Electron transport complexes I and II are also inhibited, whereas complex III is not. EPR data were consistent with the inhibition of aconitase and complexes I/II. The EPR signal at $g = 1.94$ was particularly intense and remained after

Cr(VI) was removed, and could therefore prove to be an important stable biomarker for Cr(VI) exposure and the resulting oxidative damage to mitochondria. Overall, the data support the hypothesis that Cr(VI) exposure has deleterious effects on a number of redox-sensitive core mitochondrial functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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5. Abbreviations

Cr	chromium
EPR	electron paramagnetic resonance
Fe	iron
Fe-S	iron-sulfur
HBSS	Hank's balanced salts solution
HO[•]	hydroxyl radical

INT	iodonitrotetrazolium
O₂^{•-}	superoxide
SDH	succinate dehydrogenase
Trx	thioredoxin
Trx1	thioredoxin-1
Trx2	thioredoxin-2
ZFS	zero field splitting

Fig. 1.

EPR spectra of Cr(VI)-treated cells that were analyzed at 10, 20, and 30 K. Cells were treated with 25 μM Na_2CrO_4 for 3 hr at 37°C, washed and harvested as described in the Methods. The final cell suspension (ca. 2×10^7 cells in 0.3 ml HBSS in a 4-mm quartz EPR tube) was immediately frozen in liquid nitrogen. Each sample was analyzed at the three temperatures indicated. Each spectrum was corrected for background as in Fig. 2. The modulation amplitude was 10.0 G for A, and 5.0 G for B. Other instrument settings were: 2.00 mW microwave power, 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency = 100 kHz, scan time = 83.9 sec; number of scans, 9.

Fig. 2.

EPR spectra of cells that were treated with 25 μM Na_2CrO_4 or vehicle (No Cr) for 3 hr at 37°C. The treated cells were washed and harvested as described in the Methods. The final cell suspension (ca. 7×10^6 cells in 0.3 ml HBSS in a 4-mm quartz EPR tube) was immediately frozen in liquid nitrogen. The samples were analyzed at 10 K using the following instrument settings: 5 G modulation amplitude, 20 mW microwave power, 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency = 100 kHz, scan time = 83.9 sec; number of scans, 9. The “difference” spectrum at the bottom was generated by subtraction of the No Cr sample from the 25 μM Cr sample.

Fig. 3.

Representative EPR spectra of Cr(VI)-treated cells that were analyzed by EPR at 10 K. Cells were treated with 2.5 or 5 μM Na_2CrO_4 for 16 hr at 37°C, washed, harvested, and frozen in liquid nitrogen as described for Fig. 1. The final suspension contained ca. 2×10^7 cells in 0.3 ml HBSS. Panel (A) shows the full scan from 0 to 4500 G using the following instrument settings: 10 G modulation amplitude, 20 mW microwave power, 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency = 100 kHz, scan time = 83.9 sec, 1st order baseline correction; number of scans, 9. Panel (B) shows the high field region from 3100 to 3700 G using a 5 G modulation amplitude and 0.633 mW microwave power. The signal at $g = 2.06$ is a background signal that was observed in some samples when analyzed at higher powers. It was not unique to samples treated for 16 hr, and was also seen in some samples and controls in other experiments.

Fig. 4.

The effects of Cr(VI) treatment on the specific activities of (A) aconitase, (B) complex I, (C) SDH (succinate-driven reduction of INT), (D) complex II (succinate-dependent reduction of ubiquinone), and (E) complex III. BEAS-2B cells were treated with the indicated concentrations of Na_2CrO_4 for 3 and 6 hr (panel A) or 3 hr (panels B–E). Data are the mean $\pm$ S.D. ($n = 3$ for panels A, B, and E; $n = 4$ for panels C and D). In A, *** $P < 0.001$ relative to the 0 μM Cr controls. In B, * $P = 0.014$ relative to the 0 μM Cr control. In D, * $P = 0.0157$ relative to the 0 μM Cr control. In C and E, there was no significant difference between the control and treatments.

Fig. 5.

Representative EPR spectra collected at 10 K to determine if the signals returned to normal once Cr(VI) was removed from the cells. BEAS-2B cells were treated with buffer (HBSS) for 6 hr, 25 μ M Na₂CrO₄ for 3 or 6 hr, or Na₂CrO₄ for 3 hr followed by HBSS for 3 hr. For this last treatment, the cells were washed with HBSS after the 3 hr with Cr(VI) to ensure removal of extracellular Cr(VI) for the subsequent HBSS recovery period. Samples were analyzed at 5 mW (A), 20 mW (B), and 0.2 mW (not shown). The modulation amplitude was 5 G for A, and 10 G for B. The other instrument settings were: 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency = 100 kHz, scan time = 83.9 sec; number of scans, 9.

Fig. 6.

Aconitase activity does not recover once Cr(VI) is removed. BEAS-2B cells were treated with (left to right): HBSS for 6 hr (control), 25 μM Na_2CrO_4 for 6 hr, 25 μM Na_2CrO_4 for 3 hr followed by washing the cells with HBSS and incubating with HBSS for another 3 hr, and 25 μM Na_2CrO_4 for 3 hr as indicated. The specific activity of aconitase was determined. Data are the mean $\pm$ S.D. ($n = 3, 5, 6$, and 5 for treatments from left to right). *** $P < 0.001$ relative to the HBSS 6 hr control. There was no significant difference between the three Cr(VI) treatments ($P > 0.05$).

Fig 7.

Representative EPR spectra collected at 10 K of bovine airways treated ex vivo with 400 μM Na_2CrO_4 , particulate ZnCrO_4 , or buffer (no Cr) for 3 hr at 37°C. Following treatment, the airways were washed with buffer, the bronchial epithelium was harvested as described in the Methods, and the final cell suspension was immediately frozen in liquid nitrogen in a 4-mm quartz EPR tube. Each spectrum was corrected for background. The microwave power was 0.2 mW (A) or 20 mW (B). The modulation amplitude was 5 G (A), or 10 G (B). Other instrument settings were: 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency 100 kHz, scan time 83.9 sec; number of scans, 9.

Fig. 8.

EPR spectra collected at 10 K of bovine airways treated ex vivo with 100, 25, or 0 μM Na_2CrO_4 for 3 hr at 37°C. Following treatment, the airways were washed with buffer, the bronchial epithelium was harvested as described in the Methods, and the final cell suspension was immediately frozen in liquid nitrogen in a 4-mm quartz EPR tube. Each spectrum was corrected for background. The microwave power was 0.2 mW. Other instrument settings were: 5 G modulation amplitude, 60 dB receiver gain, 82 msec time constant, 9.633 GHz microwave frequency, modulation frequency = 100 kHz, scan time = 83.9 sec; number of scans, 9.

Fig. 9.

The ex vivo treatment of bovine bronchi with ZnCrO_4 for 3 hr inhibits the specific activities of complex I (A), aconitase (B), and complex II (C) (succinate-dependent reduction of ubiquinone). Data are the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ relative to the no Cr control.