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The Effects of Hexavalent Chromium on Thioredoxin Reductase and Peroxiredoxins in Human Bronchial Epithelial Cells

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Abstract

Inhalational exposure to hexavalent chromium [Cr(VI)] compounds (e.g. chromates) is of concern in many Cr-related industries and their surrounding environments. The bronchial epithelium is directly exposed to inhaled Cr(VI). Cr(VI) species gain easy access inside cells where they are reduced to reactive Cr species which may also contribute to the generation of reactive oxygen species (ROS). The thioredoxin (Trx) system promotes cell survival and has a major role in maintaining intracellular thiol redox balance. Previous studies with normal human bronchial epithelial cells (BEAS-2B) demonstrated that chromates cause dose- and time-dependent oxidation of Trx1 and Trx2. The Trxs keep many intracellular proteins reduced including the peroxiredoxins (Prx). Prx1 (cytosolic) and Prx3 (mitochondrial) were oxidized by Cr(VI) treatments that oxidized all, or nearly all, of the respective Trxs. Prx oxidation is therefore likely the result of a lack of reducing equivalents from Trx. Trx reductases (TrxR) maintain the Trxs largely in the reduced state. Cr(VI) caused pronounced inhibition of TrxR, but the levels of TrxR protein remained unchanged. The inhibition of TrxR was not reversed by removal of residual Cr(VI) or by NADPH, the endogenous electron donor for TrxR. In contrast, the oxidation of Trx1, Trx2, and Prx3 were reversible by disulfide reductants. Prolonged inhibition of TrxR in Cr(VI)-treated cells might contribute to the sustained oxidation of Trxs and Prxs. Reduced Trx binds to an N-terminal domain of apoptosis signaling kinase (ASK1), keeping ASK1 inactive. Cr(VI) treatments that significantly oxidized Trx1 resulted in pronounced dissociation of Trx1 from ASK1. Overall, the effects of Cr(VI) on the redox state and function of the Trxs, Prxs, and TrxR in the bronchial epithelium could have important implications for redox-sensitive cell signaling and tolerance to oxidant insults.

Keywords

Chromate; Chromium; Thioredoxin Reductase; Peroxiredoxin; BEAS-2B cells

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Introduction

Hexavalent chromium [Cr(VI)] compounds cause several toxic effects [1-13]. Given that inhalation is a prominent form of Cr(VI) exposure, respiratory effects (e.g. pulmonary fibrosis, chronic bronchitis, lung cancer) are of concern [3,7,14-17]. Bronchial epithelial cells, which line the airways, are directly exposed to inhaled chromium. The highest exposures are typically associated with industrial uses, e.g. chromate (CrO_4^{2-}) pigments, chromate-based corrosion inhibitors, stainless steel machining and welding, chrome plating, leather tanning, and others. Industrial uses result in the annual release of more than 10^5 tons of Cr to the environment, and Cr contamination is a significant concern at many sites [18-20].

Cr(VI) compounds are generally cell-permeable via an anion carrier [21]. A variety of chemical and enzymatic intracellular reductants [22-29] reduce Cr(VI) to Cr(III), the next stable oxidation state. However, during this reduction, reactive Cr species [Cr(V) and/or Cr(IV)] are formed that can directly cause oxidative-like damage [30,31] or they can generate ROS via redox cycling [12,28,32-36].

The redox balance of cellular thiols is critical for normal function and viability. The thioredoxins (Trx) and glutathione contribute to thiol redox balance, but they are not in redox equilibrium with each other [37,38]. Trx1 (cytosolic) and Trx2 (mitochondrial) are distinct proteins [39], but they share a conserved active site (WCGPCK) that is largely maintained in the reduced (dithiol) form in normal cells [39-43]. A major role of Trxs is to maintain several intracellular proteins in their reduced state [44]. In doing so, the Trx active site is converted to the oxidized (disulfide) form. Trx reductases (TrxRs) regenerate reduced (active) Trxs [39]. TrxR1 (cytosolic) and TrxR2 (mitochondrial) are NADPH-dependent homodimers that contain selenium (Se) as SeCys within the active site -Cys-SeCys- motif [39,43].

The Trxs are essential for cell survival. Knockout mice lacking either Trx1 or Trx2 do not survive [39,45]. Trx redox status may be more critical to cell survival than glutathione [46]. Inhibition or suppression of Trx promotes oxidant stress, increased sensitivity to oxidants, and apoptosis [38,47]. Conversely, cells that overexpress Trx are more resistant to oxidant-induced apoptosis [38,47]. Trxs are directly dependent on TrxRs, and TrxR inhibition enhances oxidant susceptibility and favors apoptosis [43]. Proteins directly dependent on Trxs are also important for cell survival. For example, the peroxiredoxins (Prx) are ubiquitous peroxidases that are dependent on the Trxs for reducing equivalents to support their active site cysteine [39,43, 48], and depletion of Prx3 enhances susceptibility to apoptotic insults [49]. Another protein that is dependent upon Trx redox state is apoptosis signaling kinase (ASK1), a ubiquitously expressed MAP kinase kinase kinase [50]. In unstressed cells, reduced Trx negatively regulates ASK1 by binding to an N-terminal domain, whereas Trx oxidation results in its dissociation from ASK1 facilitating ASK1 activation and thereby promoting apoptosis [43,51,52]. Overall, significant oxidation or inhibition of TrxR, Trxs, and Prxs would be expected to decrease cell survival.

Various types of oxidants or agents that promote oxidant stress and cell death result in Trx oxidation, including *t*-butyl hydroperoxide, arsenic, mercury, diamide, and some anti-cancer agents diamide [38,46,47,53,54]. The redox-active nature of Cr(VI), and its ability to generate pro-oxidant species (above) suggests that it could influence the redox status and intracellular thiol redox balance. A previous report that used redox western blotting noted the oxidation of Trx1 and Trx2 in Cr(VI)-exposed cells [42]. The oxidation of Trx may be direct, whereas indirect effects are also possible, e.g. inhibition of TrxR could diminish the ability to keep the Trxs reduced. No studies to date have examined the effects of Cr(VI) on TrxR. In addition, the oxidation of Trx would imply effects on proteins directly dependent on Trx, but studies to date

have only examined the effects of Cr(VI) on Trx redox state, but have not examined the effects on Trx-dependent proteins.

The purpose of the studies reported here was to determine the effects of Cr(VI) treatment on TrxR, Trxs, and Prxs in human bronchial epithelial cells. The studies show that Cr(VI) treatment inhibits TrxR and causes the oxidation of both cytosolic and mitochondrial Trxs and Prxs. The inhibition of TrxR was not readily reversed. The data demonstrate a correlation between TrxR inhibition and the oxidation of Trx1 and Trx2. Both cytosolic and mitochondrial peroxiredoxins were oxidized for treatments that oxidized the vast majority of the respective Trxs. Prx oxidation may therefore largely result from the loss of reducing equivalents from the respective Trxs. Prx oxidation served as a cellular indicator to substantiate the loss of Trx function in cells. This loss of Trx function was also substantiated by the dissociation of Trx1 from ASK1 under conditions which caused significant oxidation of Trx1.

Materials and methods

Reagents

The following were purchased from Invitrogen Corp. (Carlsbad, CA): Hank's balanced salt solution (HBSS), 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS), and pre-cast gels (12% Bis-Tris, 16% Tris-Glycine) and matching electrophoresis and loading buffers. Phenylmethylsulfonyl fluoride (PMSF) and Tris were from Research Organics (Cleveland, OH). EDTA, guanidine-HCl, and trichloroacetic acid (TCA) were obtained from Fisher Scientific (Hampton, NH). Sodium chromate (Na_2CrO_4 ; 99+%) was the highest purity available from Aldrich Chemical (Milwaukee, WI). Chromates are known carcinogens and should be handled accordingly. Primary antibodies specific for Trx1, Trx2, TrxR1, Prx1, and Prx3 were obtained from Abcam (Cambridge, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to ASK1 (apoptosis signal-regulating kinase 1) was obtained from Santa Cruz. HRP-conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA) or Promega (Madison, WI). The Supersignal West Pico Chemiluminescent substrate was obtained from Pierce (Rockford, IL). All other chemicals and reagents were purchased from Sigma Chemical or from the sources indicated.

Cell culture

BEAS-2B cells (American Type Culture Collection no. CRL-9609) were grown at 37°C in humidified air containing 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with 25 mM HEPES and 4.5 g/L glucose (BioWhittaker 12-709F, Lonza, Inc.), 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/ cm^2 .

Redox blots for thioredoxins and peroxiredoxins

Cells were grown to 70–90% of confluence (typically in T-75 or T-25 flasks), washed once in pre-warmed HBSS, and treated with Cr(VI) in HBSS at 37°C for the indicated times. Untreated cells were exposed to HBSS without Cr(VI). Following treatment, the cells were washed once in HBSS and immediately processed for redox blots.

The redox status of Trx1 was determined according to Halvey et al. [53]. Following treatment, cells were washed once in HBSS and scraped into chilled 6 M guanidine-HCl, 100 mM Tris-HCl pH 8.3, 3 mM EDTA, 0.5 % Triton X-100, 50 mM iodoacetic acid. The samples were briefly sonicated on ice, and guanidine and unreacted iodoacetate were removed using Microspin G25 columns (GE Healthcare, Piscataway NJ). The column eluates were mixed with

an equal volume of loading buffer, separated by native PAGE (16% Tris-Glycine), transferred to nitrocellulose, blocked with 5% bovine serum albumin (BSA) and probed with anti-Trx1 and HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA). Iodoacetate covalently labels the –SH groups of reduced Trx1; the resulting negative charge increases migration in native PAGE relative to oxidized Trx1. Reduced Trx1 refers to the form in which both dithiols are reduced and represents the active form. Reduced Trx1 migrates the fastest because it binds iodoacetate at both dithiols. Oxidation of one or both dithiols will result in slower migration in native PAGE.

Trx2 redox status was determined according to [47]. Following treatment, cells were washed and scraped into 10% TCA. After >30 min on ice, they were briefly sonicated and centrifuged (10 min, 12000 × g, 4°C). The pellets were resuspended in ice-cold 100% acetone, iced for 30 min, and centrifuged (15 min, 12000 × g). The pellets were dried and resuspended in 80 mM Tris-HCl pH 7, 2% SDS, 1 mM PMSF, 25 mM AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate). After incubation (15 min on ice, 10 min at 37°C), the samples were centrifuged (5 min, 12000 × g), mixed with loading buffer and separated by SDS-PAGE (12% Bis-Tris) under non-reducing conditions, and transferred to nitrocellulose. The blots were blocked with 5% milk (Carnation nonfat) and probed with anti-Trx2 and HRP-conjugated goat anti-rabbit IgG. The isolation of mitochondria and their use as standards for oxidized and reduced Trx2 has been previously described [41,42]. Trx2 has a single dithiol (the active site). Reduced Trx2 migrates slower in these gels because AMS forms covalent bonds with the –SH groups which increases its mass [38].

Redox western blots for Prx1 and Prx3 were done according to Cox et al. [55]. After Cr(VI) treatment, the cells were washed in HBSS and scraped into 0.5 ml of 40 mM HEPES pH 7.4, 50 mM NaCl, 100 mM *N*-ethylmaleimide (NEM), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 10 µg/ml catalase. After 15 min at room temperature, the cells were lysed by adding CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) to 1%. The supernatants were run on non-reducing SDS-PAGE, and the blots were probed with anti-Prx1 or anti-Prx3, followed by the appropriate and HRP-conjugated secondary antibody.

TrxR activity

Cells were treated with HBSS (untreated) or Cr(VI) as indicated, washed and scraped into HBSS, pelleted by centrifugation (800 × g, 5 min) and frozen (–80°C). The pellets were thawed, suspended in 0.1 M sodium phosphate pH 7.4/5 mM EDTA and sonicated twice (15 sec each) on ice. Following centrifugation (30 min at 12000 × g), the supernatants were dialyzed overnight against 0.1 M sodium phosphate pH 7.4. TrxR activity in these dialysates was measured as the NADPH-dependent reduction of DTNB (5,5'-dithiobis(2-nitrobenzoic) acid) [56,57], except that the assay was done at 37°C. The sample to be tested was pre-incubated (10 min 37°C) in buffer (0.1 M sodium phosphate pH 7.4, 5 mM EDTA) for 10 min. Ethanol, the vehicle for the TrxR inhibitor auranofin, was included at a volume equal to that for the auranofin used below. DTNB was added to 3 mM and incubation was continued for 5 min to allow non-specific thiols to react with DTNB. NADPH was added to 0.2 mM to the sample cuvet and the rate of increase in absorbance at 412 nm was measured. The assay was repeated in the presence of 4 µM auranofin, a strong inhibitor of TrxR [58]. The amount of NADPH-dependent activity that was inhibited by auranofin is attributed to TrxR. Auranofin inhibited essentially all the activity in the samples we examined.

Immunoprecipitation

Cells were untreated or treated with Cr(VI) (as Na₂CrO₄) as indicated. Cells were washed twice in room temperature HBSS. They were scraped into 0.5 ml HBSS, pelleted (800 × g, 5 min), and the pellets were resuspended in cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl,

1% Triton X-100, 0.5% deoxycholate, 1 mM Na_3VO_4 , 10 mM NaF, 12 mM β -glycerophosphate, 5 mM EGTA, 1 mM PMSF, Roche complete protease inhibitor) and rocked at 4°C for 30 min. After passage several times through a 22-ga needle, the lysates were clarified by centrifugation ($15000 \times g$, 10 min, 4°C). The supernates were incubated with anti-Trx1 for 1 hr and then with protein A/G agarose overnight (4°C). The agarose was pelleted ($1000 \times g$, 5 min, 4°C), washed three times in lysis buffer, mixed with loading buffer and reducing agent, boiled for 2–3 min, and the supernate was run on SDS-PAGE. The blots were probed with anti-Trx1 and anti-ASK1. Because Trx1 can oxidize under room air in cell lysates, all steps from the second HBSS wash prior to cell harvest through the final preparation for loading on gels were conducted in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) under an atmosphere of 4 to 5% H_2 (balance N_2). The chamber contains circulating fans with palladium catalyst units to maintain O_2 at 0 ppm (continuously monitored using a model 10 gas analyzer). All solutions were pre-equilibrated in the anaerobic chamber for >1 hr prior to use. Samples were sealed in capped tubes for steps which were conducted outside the chamber (e.g. centrifugation, incubation at 4°C).

Clonogenic assay

Clonogenic survival was determined using the method of Wise et al. [59] adapted for the growth and treatment conditions described for the other experiments in this paper. Briefly, 200 BEAS-2B cells were seeded per 60-mm dish, and allowed to grow for 24 hr. They were then treated with Cr(VI) as Na_2CrO_4 for the indicated times, washed with HBSS, and incubated for 2 weeks in complete medium. The dishes were then stained with 2% crystal violet, and the number of colonies per dish was determined.

Miscellaneous

Protein was determined by a modified Lowry method, with bovine serum albumin as the standard [60].

Differences between multiple groups 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 $P < 0.05$.

Results

Reversibility of Trx oxidation

It was previously shown that treatment of BEAS-2B cells with chromates results in the oxidation of Trx1 (cytosolic) and Trx2 (mitochondrial) [42] that is dependent on both the Cr (VI) concentration and duration of exposure. Trx1 has two dithiols, only one of which is oxidized in Cr(VI)-treated cells [42]. Since the active site dithiol (C32/C35) is the more easily oxidized of the two [40], it is probable that the “partially oxidized” form of Trx1 in Cr(VI)-exposed cells represents oxidation of the active site.

The potential reversibility of the Trx oxidation in Cr(VI)-exposed cells has not been determined, however. If it represents conversion of active site sulfhydryls to disulfides, then a disulfide reductant should reverse Trx oxidation. Alternatively, if Cr or some other reactive species form an adduct with the sulfhydryls, reversal by disulfide reductants would not be expected. In untreated cells, >90% of Trx1 was reduced, whereas 52% was in the partially oxidized form in cells treated with 50 μM Cr(VI) (Fig. 1A). The disulfide reductant Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) [61] fully reversed Trx1 oxidation (Fig. 1A). Thus, the oxidation of Trx1 following Cr(VI) exposure is consistent with the disulfide form, and not an adduct or some other irreversible modification to the dithiol.

In cells treated with 25 or 50 μM Cr(VI) for 3 hr, all of Trx2 was in the oxidized form. However, in several attempts of treating these cells with 1 mM or 10 mM TCEP, or with 10 mM DTT (dithiothreitol) for 15 min after Cr(VI) was removed did not reverse the oxidation of Trx2 in these cells (not shown). TCEP was also unable to reduce Trx2 in cells that had been simultaneously treated with TCEP and 1% Triton X-100 (not shown). However, in Cr(VI)-treated cells that were lysed in 1% CHAPS, both 20 mM TCEP and 10 mM DTT were able to fully reverse Trx2 oxidation (Fig. 1B). Thus, the oxidation of Trx2 following Cr(VI) exposure is also consistent with the disulfide form, and not an adduct or some other irreversible modification to the dithiol. However, the different conditions required to reverse the oxidation of Trx1 vs. Trx2 imply that while TCEP is readily cell-permeable, it does not gain ready access into intact mitochondria.

TrxR is inhibited in Cr(VI)-treated cells

TrxR1 and TrxR2 maintain their respective Trxs in the reduced/active state. Trx oxidation could result from effects on Trxs or on TrxR. The activity of TrxR in Cr(VI)-treated cells was therefore examined. In cells treated with 25 or 50 μM Cr(VI) for 90 min, TrxR activity was inhibited by 71 and 77% respectively (Fig. 2A). After 180 min of the same treatments, TrxR was inhibited by 97 and 85%, respectively (Fig. 2A). Additional time course analyses showed that TrxR inhibition is dependent on both Cr(VI) concentration and duration of exposure (Fig. 2C). Simple oxidation of TrxR, such as occurs when it reduces Trx, is reversed by electrons donated by NADPH, the endogenous electron donor for TrxR. Since the TrxR activity assay includes 0.2 mM NADPH, the loss of activity in Cr(VI)-treated cells is not restored by NADPH. Furthermore, these cell lysates had been dialyzed overnight prior to the activity assay, so free Cr had been removed. This suggests a prolonged inhibition that is not dependent on the continued presence of Cr(VI). TrxR1 protein levels as determined by western blot did not change as a result of Cr(VI) treatment (Fig. 2B) so the activity declines represent inhibition and not protein degradation.

Cr(VI) treatment results in Prx1 and Prx3 oxidation

Trxs are responsible for maintaining Prxs in their reduced/active state. The oxidation of Trxs in Cr(VI)-treated cells might therefore prevent the cells from keeping their Prxs reduced. The redox status of Prx1 (cytosolic) and Prx3 (mitochondrial) in Cr(VI)-treated cells was therefore examined in conjunction with their respective Trxs (Trx1 and Trx2). In untreated cells, Prx1 and Trx1 were in the reduced state (Fig. 3A). After 6 hr with 25 or 50 μM Cr(VI), Trx1 was mostly in the partially oxidized state, and Prx1 was also mostly oxidized (Fig. 3A). Thus, under conditions in which Trx1 is mostly oxidized, Prx1 also shifts to the oxidized state.

Examination of the mitochondrial counterparts indicated that essentially all Trx2 was reduced in untreated cells, whereas Prx3 was a mix of reduced and oxidized forms (Fig. 3B). After 6 hr with 25 or 50 μM Cr(VI), Trx2 was 100% oxidized, and the vast majority of Prx3 similarly shifted to the oxidized form (Fig. 3B).

The effects of 6-hr Cr(VI) treatments on TrxR activity, Trx and Prx redox status, and clonogenic survival are shown together in Fig. 4. Both 25 and 50 μM Cr(VI) had a significant effect on all of these parameters. As with the shorter treatments (e.g. Fig. 2), TrxR activity was inhibited by $\geq 80\%$, and there was a pronounced shift in the redox status of both Trxs and both Prxs to the oxidized state (Fig. 4). These treatments were also cytotoxic as indicated by lack of clonogenic survival (Fig. 4). After these 6 hr treatments, the cells remained attached, but in separate experiments in which they were returned to complete medium after the 25 or 50 μM Cr(VI) treatment, they will subsequently detach and die by the following day, which is in agreement with the lack of clonogenic survival.

Prolonged exposure to low micromolar Cr(VI)

The Cr(VI) exposures described above and previously [42] were of shorter duration. Since prior studies indicated that Trx oxidation was dependent on both time and Cr concentration, overnight (16 hr) exposures to lower Cr(VI) concentrations (2.5 and 5 μ M) were also examined. An example of the relationship between Trx2 and Prx3 redox status is shown in Fig. 5. 2.5 μ M Cr(VI) caused only a partial oxidation of Trx2 and essentially no change in Prx3 redox state, whereas 5 μ M Cr(VI) resulted in essentially complete oxidation of both Trx2 and Prx3 (Fig. 5). Longer exposure to low μ M Cr(VI) can therefore also result in Trx2 and Prx3 oxidation.

The quantitative effects of 16-hr Cr(VI) treatments on TrxR activity, Trx and Prx redox status, and clonogenic survival are shown together in Fig. 6. Both 2.5 and 5 μ M Cr(VI) resulted in significant inhibition of TrxR activity (87 and 76% inhibition, respectively) (Fig. 6). Clonogenic survival was also markedly decreased by both treatments (Fig. 6). These treatments showed some interesting differential effects on Trx and Prx redox status. While there was a significant shift in Trx1 redox state with both treatments, the cells maintained 60% of Trx1 in the reduced state after 2.5 μ M Cr(VI) vs. just 21% reduced after 5 μ M Cr(VI) (Fig. 6). Interestingly, Prx1 redox state was not changed in cells treated with 2.5 μ M, whereas there was significant Prx1 oxidation with 5 μ M Cr(VI) (Fig. 6). There was a similar relationship between the mitochondrial counterparts Trx2 and Prx3. After 2.5 and 5 μ M Cr(VI), 49% and 0% of Trx2 was in the reduced state, as compared to 100% for untreated cells. However, there was significant oxidation of Prx3 only with the 5 μ M treatment, but no significant change for the 2.5 μ M treatment (Fig. 6).

The oxidation of Prx3 in Cr(VI)-treated cells could be reversed by a disulfide reductant (Fig. 6). This implies that Prx oxidation represents the formation of disulfide links between the two subunits to form the Prx dimers observed in the gels.

Dissociation of Trx1 from ASK1

The oxidation of Prxs following Cr(VI) treatments that caused substantial Trx oxidation implies that Trx function is compromised in these cells. Another function of reduced Trx is to bind and inhibit ASK1, whereas oxidized Trx dissociates from ASK1 [43,51,52]. We therefore examined Trx1/ASK1 association as another cellular indicator of the functional redox state of Trx1. In untreated cells in which >95% of Trx1 is reduced (Fig. 4), ASK1 is bound to Trx1 (Fig. 8). Following a 3-hr treatment with either 25 or 50 μ M Cr(VI), the relative percent of ASK1 bound to Trx1 decreased markedly (Fig. 8). Total ASK1 did not change in these cells (not shown). These ASK1 data provide another cellular indicator consistent with the disruption of Trx function in Cr(VI)-treated cells.

Discussion

To our knowledge, this is the first report demonstrating that Prxs are oxidized and that TrxR is inhibited in Cr(VI)-treated cells. Since the TrxR protein level remains constant in these cells (Fig. 2B), the decline in TrxR activity represents enzyme inhibition. This inhibition was not reversed by NADPH, its endogenous electron donor, or by removal of free Cr by overnight dialysis. The effects on TrxR may therefore be prolonged in cells, extending beyond the time of Cr(VI) exposure. Even intermittent exposure to Cr(VI), which would be consistent with occupational exposures, could therefore have prolonged effects on TrxR in the bronchial epithelium.

Since this is the first report on TrxR inhibition following Cr(VI) exposure, the mechanism by which TrxR is inhibited is not known. Cr(VI) is redox-active and there are multiple redox-

active sites within TrxR, including the flavin (FAD), the C-terminal active site Cys-SeCys, and the N-terminal domain dithiol (-CVNVGC-) [43]. All of these sites are necessary for electron flow within TrxR and therefore its activity, and disruption of any one of these could theoretically inhibit its activity. Of these, the active site is likely the most reactive. It is exposed on the surface of the enzyme [62] and the selenol of the SeCys residue (pK_a ca. 5.2) [63] should be ionized to selenolate at physiological pH making it more reactive. As an example, one inhibitor of TrxR, 2,4-dinitrochlorobenzene, irreversibly binds the SeCys [56], but it does not modify the N-terminal dithiol [64].

Previous work demonstrated the potential for purified rat TrxR1 to reduce Cr(VI) to Cr(V) [42]. Diversion of electrons to Cr(VI) therefore represents one potential mechanism of inhibition, but it cannot explain the results here because free Cr(VI) had been removed by dialysis and NADPH did not restore enzyme activity. The inhibition of TrxR observed here is therefore not likely due to competitive inhibition, or to simple oxidation of the redox-active thiols or selenol as would occur when TrxR reduces endogenous substrates such as Trx. Instead, the results are consistent with a more prolonged inactivation of the enzyme. The intracellular reduction of Cr(VI) is known to lead to a variety of reactive species which can act as potent oxidants, including Cr(V), Cr(IV), and reactive oxygen species including $HO^\bullet$ [28,30,31,65]. Reaction of TrxR with one or more of these reactive species represents one possible mechanism for TrxR inhibition. Other mechanisms must also be considered, however, including indirect effects resulting from reactive species generation. We are not aware of studies which have examined interactions between Cr species and SeCys. However, Cr(III) potassium sulfate in high concentration in vitro with 30% H_2O_2 has been reported to convert thiols to disulfides in a reaction mechanism that may involve $HO^\bullet$ generation [66]. However, such disulfides within TrxR, or the thioselenide of its active site, should be reversible by NADPH, the endogenous electron donor for TrxR. Since NADPH did not restore TrxR activity, a disulfide type of TrxR oxidation is not predicted in Cr(VI)-treated cells.

A prolonged inhibition of TrxR could, however, contribute to Trx oxidation or to a decreased capacity to keep Trx reduced. Since Prxs are dependent on Trxs, they are therefore indirectly dependent on TrxR. In these studies, the examination of TrxR, Trxs, and Prxs within the same cells allowed for direct examination of the relationship of the effects of Cr(VI) exposure on these proteins. Given the dependence of Prxs on Trxs, the oxidation of Trxs could influence the redox status and function of the Prxs. Similarly, TrxR inhibition could influence the redox status and function of the Trxs. The effects of Cr(VI) on the Trx redox status may therefore be due to its ability to both enhance oxidant generation and to inhibit TrxR. Even though the TrxR assay does not distinguish between TrxR1 and TrxR2, there was a significant correlation between the % TrxR inhibited and the % Trx1 oxidized (Fig. 9A) (Spearman nonparametric correlation $r = 0.7245$, $P < 0.0001$). This might imply that an inability of TrxR to maintain Trx1 in a reduced state contributes to Trx1 oxidation. This type of Trx1 oxidation should be reversible by disulfide reductants, which proved to be the case (Fig. 1). However, it was previously noted that Cr(VI) can oxidize purified Trx1 in vitro [42], as do some other oxidants. Trx1 oxidation may therefore be initiated by Cr(VI) and/or other oxidizing species that are generated following Cr(VI) exposure, and this oxidation may be sustained or enhanced by the decreased ability of TrxR to regenerate reduced Trx1. In the absence of oxidant stress, however, TrxR inhibition on its own may not be sufficient to cause Trx oxidation. This proved to be the case in HeLa cells in which inhibition of TrxR by aurothioglucose, or TrxR knockdown by siRNA, were not sufficient to cause Trx1 oxidation [67]. However, monomethylarsonous acid, which both inhibits TrxR and increases ROS generation, did cause Trx1 oxidation [67].

There was also a correlation between TrxR inhibition and Trx2 oxidation (Fig. 9B, Spearman nonparametric correlation $r = 0.7003$, $P < 0.0001$). It should be noted that in Fig. 9B there are seven superimposed points at 0% TrxR inhibited and 0% Trx2 oxidized. Therefore most of the

points cluster at the lower left and upper right of this graph, which could contribute to the apparent correlation between TrxR and Trx2. Also note that there were two points for which TrxR inhibition was almost complete but where Trx2 oxidation was only partial, and three for which Trx2 oxidation was complete but TrxR was only partially inhibited. Since the TrxR assay measures total TrxR, it does not reflect just the mitochondrial isoform (TrxR2) on which Trx2 depends. However, the relationship in Fig. 9B and the ability to reverse Trx2 oxidation by disulfide reductants (Fig. 1) suggest that Trx2 oxidation may be enhanced or sustained by a loss of reducing equivalents from TrxR2.

Cytosolic Prxs such as Prx1 should be dependent on Trx1 to maintain their redox state. For Cr (VI) treatments that oxidized up to 75% of Trx1, the redox state of Prx1 largely resembled that of untreated cells (Fig. 9C). Therefore, even a minority of active Trx1 is sufficient to keep Prx1 largely reduced. However, when >75% of Trx1 was oxidized, Prx1 abruptly shifted to being mostly oxidized (Fig. 9C). Thus, these cells can maintain Prx1 redox status until nearly all of the Trx1 is oxidized. This suggests that Prx1 oxidation largely results from lack of reducing equivalents from Trx1. Prx1 oxidation in these cells therefore serves as a functional indicator that reflects the loss of the vast majority of Trx1 activity.

Mitochondrial Prxs such as Prx3 should be dependent on Trx2 to maintain their redox state. In untreated cells and for Cr(VI) treatments that did not cause Trx2 oxidation, Prx3 redox state was maintained at $56.7 \pm 10.3\%$ oxidized (mean $\pm$ SD) (Fig. 9D). The Prx3 redox state shifted to $94.4 \pm 7.2\%$ oxidized (mean $\pm$ SD) for treatments that fully oxidized Trx2 (Fig. 9D). The Prx3 oxidation for these two groups was significantly different ($P < 0.0001$). There were only a few points for which Trx2 oxidation was intermediate (between 0 and 100% oxidized), which were the result of $2.5 \mu\text{M}$ Cr(VI) for 16 hr; for these, Prx3 oxidation ($65.5 \pm 6.8\%$) was not different from treatments in which Trx2 was fully reduced ($P = 0.153$), but it was different from those with 100% Trx2 oxidation ($P < 0.0001$). Overall, the data imply that Prx3 redox state can be maintained when there is partial Trx2 oxidation, but is not maintained once Trx2 becomes fully oxidized. There was a correlation between Trx2 oxidation and Prx3 oxidation (Fig. 9D, Spearman nonparametric correlation $r = 0.8860$, $P < 0.0001$). However, given that 20 of the 24 points clustered at either 0% or 100% Trx2 oxidized, the relationship between Trx2 and Prx3 redox states may not necessarily be linear. What is clear is that Prx3 oxidation was significantly less in cells without Trx2 oxidation ($56.7\% \pm 10.2\%$) than in cells in which Trx2 oxidation was complete ($94.4\% \pm 7.2\%$) ($P < 0.0001$). The reversibility of Prx3 oxidation (Fig. 7) implies that Prx3 oxidation represents conversion to the disulfide form. This form would be expected to result from lack of reducing equivalents from Trx2, but other reasons for Prx3 oxidation remain possible. While peroxide substrates gain ready access to the active site thiols of Prxs, these thiols may be somewhat protected from other species. For example, the Prx2 active site thiols are not readily alkylated with iodoacetamide or various amino acid chloroamines, despite the high reactivity of these agents with other cellular -SH groups [68].

The cytosolic and mitochondrial thioredoxin systems are not in redox equilibrium with each other, so it is possible to discern differential effects of oxidants on these subcellular compartments [38,53]. Previous work with shorter Cr(VI) exposure demonstrated that Trx2 was more susceptible in that it could all be converted to the oxidized form, whereas a small amount of reduced Trx1 remained even after higher Cr concentrations [42]. The additional exposures tested here support the greater susceptibility of Trx2, e.g. 100% of Trx2 was oxidized after $25 \mu\text{M}$ (6 hr)(Fig. 4) or $5 \mu\text{M}$ (16 hr)(Fig. 6), whereas 83% and 79% of Trx1 was oxidized by these same treatments, respectively. The differential oxidation of Prx3 vs. Prx1 further supports the enhanced susceptibility of the mitochondrial system, e.g. 98% of Prx3 was oxidized after $5 \mu\text{M}$ Cr(VI) (16 hr), vs. 56% oxidation of Prx1 (Figs. 4,6). Similarly, mitochondrial Trx2 is more susceptible than cytosolic Trx1 to oxidation by *t*-butyl hydroperoxide or diamide [38,47]. The data are in contrast to acrolein, however, which

preferentially oxidizes Trx1 [41]. Overall, these redox blots provide a means to assess the relative extent of redox stress in the cytosolic vs. mitochondrial compartments of Cr(VI)-exposed cells. The greater effects on the mitochondrial compartment could indicate enhanced oxidant generation inside the mitochondria and/or a decreased ability of the mitochondrial system to protect itself.

As indicated above, the oxidation of Prxs following Cr(VI) exposure likely reflects the loss of reducing equivalents from the respective Trxs. This is in keeping with a recent report by Cox et al. [69] in which the pronounced inhibition of TrxR by auranofin or dinitrochlorobenzene, which are well-known inhibitors of TrxR, resulted in the preferential oxidation mitochondrial Prx3 vs. cytosolic Prx1 in human Jurkat cells. This differential effect did not correspond with a greater inherent sensitivity of mitochondrial TrxR, as the TrxR in isolated cytosol was marginally more sensitive to auranofin than was TrxR in isolated mitochondria [69]. It is plausible that Trx2 was oxidized in cells in which Prx3 oxidation occurred, but this was not examined [69]. Auranofin and dinitrochlorobenzene treatments that caused pronounced TrxR inhibition and Prx3 oxidation in Jurkat cells resulted in cell death [69]. In this study, Cr(VI) treatments that caused prominent changes in TrxR activity also caused loss of cell viability. However, the 16 hr data suggest that Prx oxidation is not required for cytotoxicity, e.g. clonogenic survival under these conditions was significantly less with 2.5 μ M Cr(VI) (16 hr), but Prx1 and Prx3 redox status were not significantly altered (Fig. 6). With the 6 hr treatments (25 or 50 μ M Cr), there was pronounced cytotoxicity and pronounced effects on TrxR, Trxs and Prxs (Fig. 4).

Given the complexity of Cr chemistry and its potential to affect multiple cellular components, Cr(VI) cytotoxicity is likely multifactorial. The Cr(VI)-induced changes to the activity of TrxR and to the redox state of Trxs and Prxs have not been previously explored, but they could contribute to the cytotoxic effects of Cr(VI). Inhibition of TrxR increases the susceptibility to oxidants and favors apoptosis [43], and inhibition or genetic suppression of Trx enhances oxidant sensitivity, oxidant stress and apoptosis [38,47]. The oxidation of Prxs can also enhance apoptosis [49,70]. Changes to each of these components could affect cell survival following Cr(VI) exposure. It remains to be determined if effects on some of these proteins are more critical than others in these cells. Loss of protection from oxidant stress is just one of the potential consequences of the effects of Cr(VI) on the TrxR/Trx/Prx system. Since mitochondria lack catalase, and they contain 30 times more Prx3 than glutathione peroxidase [49], Prx3 may be a critical regulator of mitochondrial H₂O₂ signaling [49]. Treatments which markedly oxidize Prx3 could therefore have important effects on this signaling. Besides compromising Prx function, the oxidation of Trxs could have other effects as well. Trx is an important hydrogen donor for ribonucleotide reductase [71], so Trx oxidation could lead to growth arrest. Also, reduced Trx binds and inhibits ASK1, and we noted the dissociation of ASK1 from Trx1 for treatments that oxidized Trx1 (Fig. 8). This dissociation could imply the activation of ASK1 which could promote apoptosis. The effects on Prxs and ASK1 imply that other Trx-dependent proteins may also be affected by these treatments.

While TrxR inhibition could facilitate the oxidation of Trxs and Prxs, TrxR inhibition could have other effects as well. While the mechanism of inhibition of TrxR in Cr(VI)-treated cells remains to be determined, treatments that target the SeCys of the TrxR active site can induce cell death [72]. Given these multiple possible effects, further studies on the consequences of the effects of Cr(VI) on the TrxR/Trx/Prx system are therefore warranted.

In summary, this report describes the effects of Cr(VI) on the thioredoxins, peroxiredoxins, and TrxR in human bronchial epithelial cells. Low micromolar concentrations of Cr(VI) caused oxidation of cytosolic and mitochondrial Trxs and Prxs and inhibited TrxR. The oxidation of Trx1, Trx2, and Prx3 were reversible in vitro, but TrxR inhibition was not easily reversed.

These effects of Cr(VI) could compromise the normal function and survival of the bronchial epithelium, and could alter redox-sensitive cell signaling and the ability of these cells to tolerate other oxidant insults.

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Abbreviations

AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate
ASK1	apoptosis signaling kinase-1
BSA	bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DMEM	Dulbecco's Modified Eagle's Medium
DTNB	5,5'-dithiobis(2-nitrobenzoic) acid
DTT	

	dithiothreitol
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NEM	<i>N</i> -ethylmaleimide
PMSF	phenylmethylsulfonyl fluoride
Prx	peroxiredoxin
Prx1	peroxiredoxin-1
Prx3	peroxiredoxin-3
ROS	reactive oxygen species
TCA	trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
Trx	thioredoxin
Trx1	thioredoxin-1
Trx2	thioredoxin-2
TrxR	thioredoxin reductase

Fig. 1.

The oxidation of Trx1 and Trx2 in Cr(VI)-treated cells is reversible by disulfide reductants.

A: In the two lanes at left, the redox state of Trx1 was assessed in cells immediately following treatment with 50 μM Cr(VI) as Na_2CrO_4 for 3 hr, or HBSS (untreated). For the two lanes at right, cells were given these same treatments, but were then washed in HBSS, and incubated with the disulfide reductant TCEP (1 mM) for 15 min. After a final wash, the Trx1 redox status was determined. **B:** Cells were treated for 3 hr with 0, 25, or 50 μM Na_2CrO_4 as indicated.

Some were processed immediately for Trx2 redox status, and others were treated as follows to determine potential reversal of Trx2 oxidation by TCEP or DTT: the cells were washed in cold HBSS, and scraped into 0.25 ml of CHAPS lysis buffer (1% CHAPS in 40 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, Roche complete mini protease inhibitor cocktail) containing either 20 mM TCEP or 10 mM DTT. After a 30-min incubation at room temp., and equal volume of 20% TCA was added to the samples, they were sonicated for 15 sec on ice, and processed for Trx2 redox status. For Trx2, reduced Trx2 runs slower in SDS-PAGE because AMS bonds to the two $-\text{SH}$ groups reduced Trx2, increasing its mass by approximately 1 kDa [38]. Standards for the migration of oxidized (oxid.) and reduced (red.)

Trx2 are included, and consist of isolated mitochondria that had either been stored frozen (“mito, oxid.”) (Trx2 oxidizes in storage), or had been pre-treated with DTT which reduces Trx2 (“mito, DTT”) [42]. Relative to the cell lysates, only small amounts of the purified mitochondria were loaded. Both figures are representative of replicate experiments.

Fig. 2.

A, TrxR is inhibited in Cr(VI)-treated cells (mean $\pm$ S.D. for 3 independent experiments). *** ($p < 0.001$) relative to the corresponding results for 0 μM Na₂CrO₄. **B**, Representative western blot for TrxR1 in the cell lysate dialysates used in **A**. Each lane was loaded with 5.9 μg total protein. **C**, Time course analysis of TrxR activity in Cr(VI)-treated cells (mean $\pm$ S.D., error bars shown in one direction only for each).

Fig. 3.

Redox western blots indicating the relationship between Trx and Prx oxidation in cells treated for 6 hr with Cr(VI) as indicated. **A**, redox status of the cytosolic proteins Prx1 and Trx1. **B**, redox status of the mitochondrial proteins Prx3 and Trx2. For the Prx redox blots, *N*-ethylmaleimide (NEM) reacts with the -SH groups of reduced Prx to prevent their oxidation during cell harvest/lysis. Reduced Prx therefore runs as monomers in SDS-PAGE. If one Cys-SH of Prx is oxidized to Cys-SOH, it will react with the nearby Cys-SH of the other subunit to create an intermolecular Prx disulfide (Prx-S-S-Prx). Oxidized Prx therefore runs as dimers. For Trx2, standards for the migration of oxidized (oxid.) and reduced (red.) Trx2 are included, and consist of isolated mitochondria that had either been stored frozen ("mito, oxid.") (Trx2 oxidizes in storage), or had been pre-treated with DTT which reduces Trx2 ("mito, DTT") [42]. Relative to the cell lysates, only small amounts of the purified mitochondria were loaded.

Fig. 4.

The effects of 6-hr treatments with Cr(VI) on TrxR activity, the redox states of Trxs and Prxs, and clonogenic survival. All data are the mean $\pm$ S.D. for independent experiments ($n = 4$ for Trx and Prx redox status, and $n = 3$ for TrxR and survival). ‡($p < 0.01$) and §($p < 0.001$) relative to the corresponding results for 0 μM Na_2CrO_4 .

Fig. 5. Redox western blots of Prx3 (left) and Trx2 (right) in cells treated for 16 hr with Cr(VI) as indicated.

Fig. 6.

The effects of 16-hr treatments with Cr(VI) on TrxR activity, the redox states of Trxs and Prxs, and clonogenic survival. Data are the mean $\pm$ S.D. for independent experiments ($n = 5$ for Trx and Prx redox status, and $n = 3$ for TrxR and survival). ‡ ($p < 0.01$) and § ($p < 0.001$) relative to the corresponding results for 0 μM Na_2CrO_4 .

Fig. 7.

The oxidation of Prx3 following 16 hr exposure to Cr(VI) is reversible by disulfide reduction. In cells treated with HBSS (untreated) or 5 μM Cr(VI) for 16 hr, the redox state of Prx3 was assessed using the standard protocol with non-reducing gel conditions (left). Alternatively, aliquots of the same samples were first incubated for 10 min at 70°C with NuPAGE reducing agent (Invitrogen) prior to loading on the gel (right).

Fig. 8.

Immunoprecipitation for Trx1 shows that Trx1 dissociates from ASK1 in cells treated with Cr (VI). The cell lysates were immunoprecipitated with anti-Trx1 and protein A/G agarose as described in the methods. The immunoprecipitates were run on SDS-PAGE, and the upper half of the blot was probed with anti-ASK1 (top), and the bottom half with anti-Trx1. The numbers in italics at the bottom show the relative ratio of ASK1 bound to Trx1. An average of 55% and 85% of Trx1 is oxidized by 3-hr treatment with 25 and 50 μ M Cr(VI), respectively. The blots are representative of replicate experiments.

Fig. 9.

Relationship between the inhibition/oxidation of various proteins in Cr(VI)-exposed cells: TrxR and Trx1 (A), TrxR and Trx2 (B), Trx1 and Prx1 (C), and Trx2 and Prx3 (D). All points represent individual datapoints, including different treatments and their replicates. In some cases, the replicate points are superimposed. Panels A and B show treatments with Na_2CrO_4 for 0.5, 1.5, 3, and 6 hr (0, 25, 50 μM), and 16 hr (0, 2.5, 5.0 μM). In panel B, there are seven superimposed points at 0% TrxR inhibited, 0% Trx2 oxidized. Panels C and D show treatments with Na_2CrO_4 for 6 hr (0, 25, and 50 μM) and 16 hr (0, 2.5, 5.0 μM).