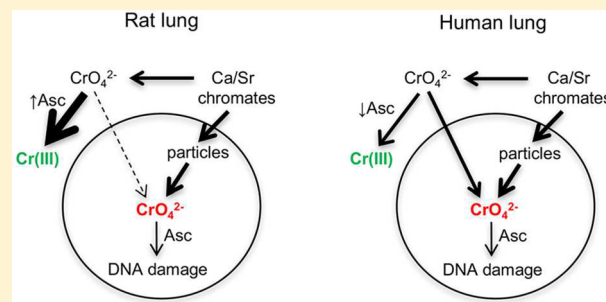


# Variation in Extracellular Detoxification Is a Link to Different Carcinogenicity among Chromates in Rodent and Human Lungs

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**ABSTRACT:** Inhalation of soluble chromium(VI) is firmly linked with higher risks of lung cancer in humans. However, comparative studies in rats have found a high lung tumorigenicity for moderately soluble chromates but no tumors for highly soluble chromates. These major species differences remain unexplained. We investigated the impact of extracellular reducers on responses of human and rat lung epithelial cells to different Cr(VI) forms. Extracellular reduction of Cr(VI) is a detoxification process, and rat and human lung lining fluids contain different concentrations of ascorbate and glutathione. We found that reduction of chromate anions in simulated lung fluids was principally driven by ascorbate with only minimal contribution from glutathione. The addition of 500  $\mu\text{M}$  ascorbate ( $\sim$ rat lung fluid concentration) to culture media strongly inhibited cellular uptake of chromate anions and completely prevented their cytotoxicity even at otherwise lethal doses. While proportionally less effective, 50  $\mu\text{M}$  extracellular ascorbate ( $\sim$ human lung fluid concentration) also decreased uptake of chromate anions and their cytotoxicity. In comparison to chromate anions, uptake and cytotoxicity of respirable particles of moderately soluble  $\text{CaCrO}_4$  and  $\text{SrCrO}_4$  were much less sensitive to suppression by extracellular ascorbate, especially during early exposure times and in primary bronchial cells. In the absence of extracellular ascorbate, chromate anions and  $\text{CaCrO}_4/\text{SrCrO}_4$  particles produced overall similar levels of DNA double-stranded breaks, with less soluble particles exhibiting a slower rate of breakage. Our results indicate that a gradual extracellular dissolution and a rapid internalization of calcium chromate and strontium chromate particles makes them resistant to detoxification outside the cells, which is extremely effective for chromate anions in the rat lung fluid. The detoxification potential of the human lung fluid is significant but much lower and insufficient to provide a threshold-type dose dependence for soluble chromates.



## INTRODUCTION

Hexavalent chromium has been firmly recognized as a human respiratory carcinogen.<sup>1–3</sup> Upon dissolution at neutral pH, Cr(VI) exists principally in the form of chromate anion ( $\text{CrO}_4^{2-}$ ) that structurally is very similar to physiological anions sulfate and phosphate. Because of this structural resemblance, chromate enters a broad variety of cells utilizing their sulfate and phosphate channels. Cr(VI) is unstable inside the cells where it undergoes reduction generating Cr(III) as the thermodynamically stable form.<sup>4</sup> Intracellular reduction of Cr(VI) is required for the formation of Cr–DNA damage, which includes DNA–protein cross-links<sup>5,6</sup> and several types of smaller Cr–DNA adducts.<sup>7–9</sup> Metabolism of Cr(VI) can also result in the production of oxidative DNA damage.<sup>10–12</sup> Cellular reduction of Cr(VI) *in vivo* is principally driven by direct electron transfer reactions with ascorbate (Asc),<sup>13,14</sup> which is present in low millimolar concentrations in cells,<sup>15,16</sup> glutathione (GSH) and, to a smaller extent, cysteine also contribute to the conversion of Cr(VI) to Cr(III) in tissues. Cultured cells are severely deficient in Asc and their metabolism of Cr(VI) is largely dependent on the non-enzymatic one-electron transfer reactions with GSH.<sup>4</sup> The initial reduction of Cr(VI) by Asc involves transfer of two electrons, which skips the formation of reactive Cr(V) intermediate<sup>17–19</sup> and suppresses oxidative DNA damage.<sup>20,21</sup>

In contrast to its activation role inside the cells, reduction of Cr(VI) outside the cells is the detoxification process producing membrane-impermeable Cr(III).<sup>4</sup>

Although all chemical forms of Cr(VI) are classified as human carcinogens,<sup>2</sup> the strength of experimental and epidemiological evidence for individual compounds varies significantly. Two large comparative studies using different strains of rats and delivery methods have found that Cr(VI) compounds of moderate solubility were highly tumorigenic in the lung whereas highly soluble and very poorly soluble chromates were not tumorigenic.<sup>22,23</sup> Epidemiological evidence for carcinogenicity of the most insoluble chromates is also weaker than for the moderately soluble group.<sup>1,3</sup> A frequently referenced first review of chromium carcinogenicity by the International Agency for Research on Cancer, which was released in 1990, did not identify reports with strong evidence for carcinogenicity of soluble chromates in humans.<sup>24</sup> However, subsequent epidemiological studies<sup>25–27</sup> among cohorts of workers exposed to soluble Cr(VI) all found significantly elevated lung cancer risks. The solubility-related dependence of Cr(VI) tumorigenicity in rodents remains unexplained, which limits the use of these standard laboratory animals for

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investigation of mechanistic aspects of Cr(VI) carcinogenicity and complicates application of animal results for human risk assessment. A comparison of cytotoxicity and chromosomal damage in standard cultures of telomerase-immortalized human fibroblasts has not uncovered large differences among highly soluble sodium chromate, moderately soluble zinc chromate and very poorly soluble barium and lead chromates,<sup>28</sup> suggesting similar causes of genotoxicity for these compounds under GSH-driven reduction conditions. Mechanistic considerations of DNA damage formation by various products of intracellular reduction of chromate anions<sup>10,29</sup> also cannot explain why solubility is such an important factor in rat lungs but not in human lungs.

In this work, we examined the significance of species differences in the concentrations of chromate reducers in the lung lining fluid for uptake and toxicity of Cr(VI) compounds of different solubility. Using human and rat lung epithelial cells, we obtained evidence that link variable carcinogenicity and toxicity of chromates to their different extracellular detoxification.

## EXPERIMENTAL PROCEDURES

**Materials.** L-Ascorbic acid (99.9% pure), dehydro-L-(+)-ascorbic acid dimer, potassium chromate ( $K_2CrO_4$ , 99% pure), L-glutathione (>98% pure), and nitric acid (>99.999% pure) were obtained from Sigma-Aldrich.  $CaCrO_4$  (43333) and  $SrCrO_4$  (89026) were purchased from Alfa Aesar. RPMI-1640 medium (11875–093) was from ThermoFisher Scientific.

**Cell Culture.** H460 human lung epithelial and RLE-6TN rat lung epithelial cells were obtained from the American Type Tissue Collection. H460 cells were grown in RPMI-1640 media (11875119, ThermoFisher) containing 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin. RLE-6TN rat cells were propagated in DMEM/F12 media (11320–033, ThermoFisher), 10% FBS, and 1% penicillin/streptomycin. Primary human bronchial epithelial (HBE) cells were purchased from Lonza and grown in the supplier's recommended medium (CC-3170) supplemented with growth factors and antibiotics. All three cell lines were kept in the atmosphere of 95% air/5%  $CO_2$ . Cells were treated with the indicated concentrations of Cr(VI) next day after seeding. Stock solutions of  $K_2CrO_4$  were freshly prepared in water for each experiment.  $CaCrO_4$  or  $SrCrO_4$  were used as suspensions of particles in ethanol.

**Particle Preparation.** Commercial  $CaCrO_4$  or  $SrCrO_4$  was placed in glass containers (7-mL scintillation vial for Ca-chromate and Erlenmeyer flask for Sr-chromate), combined with 100% ethanol, sealed with parafilm, and stirred overnight. The next day, chromate solutions were passed through 30 mm nylon syringe filters with 5  $\mu m$  pores (F2500–50, ThermoFisher) and stored at 4 °C. When necessary, solutions of chromate particles were concentrated at 30 °C using a vacuum centrifuge (Eppendorf Vacufuge 5301). The volumes were monitored over time, and the samples were occasionally taken out and vortexed to remove the accumulated chromium from the side of the vials. When the ethanol was at the desired volume, the solution was vigorously vortexed to break up any aggregates. For the determination of Cr(VI) concentrations, Ca-chromate and Sr-chromate particles were dissolved in 250 mM NaOH (final concentration) followed by absorbance measurements at 372 nm. Sodium chromate solubilized in a mixture of 50% ethanol/50% 500 mM NaOH was used for standard curves. To correct for light scattering and other nonspecific effects, A<sub>372</sub> values were adjusted by subtracting absorbance at 550 nm in the same sample. Ethanol suspensions of chromate particles were stored at 4 °C and used within a few days after preparation.

**Ascorbate Determination.** Asc was extracted with 50 mM methanesulfonic acid/5 mM diethylenetriaminepentaacetic acid and detected as a fluorescent product after conjugation with 1,2-diamino-4,5-dimethoxybenzene dihydrochloride.<sup>30</sup>

**Restoration of Cellular Ascorbate.** Cells were incubated with dehydroascorbic acid in Krebs-HEPES buffer [30 mM HEPES (pH 7.5), 130 mM NaCl, 4 mM  $KH_2PO_4$ , 1 mM  $MgSO_4$ , 1 mM  $CaCl_2$ ] supplemented with 10% FBS and 0.5 mM D-glucose. Stock solutions of dehydroascorbic acid were freshly prepared in the same buffer and kept on ice before use.

**Chromate Reduction.** Dissolved  $K_2CrO_4$  was used a source of chromate anions in all reactions. Reduction was measured at 37 °C by recording chromate absorbance at 372 nm. Equal volumes of 2× concentrated chromate and reducer solutions were rapidly mixed in 96-well plates followed by the immediate determination of the initial A<sub>372</sub>. Absorbance values were recorded every 10 s in kinetics experiments. Plates were maintained at 37 °C inside the SpectraMax M5 microplate reader.

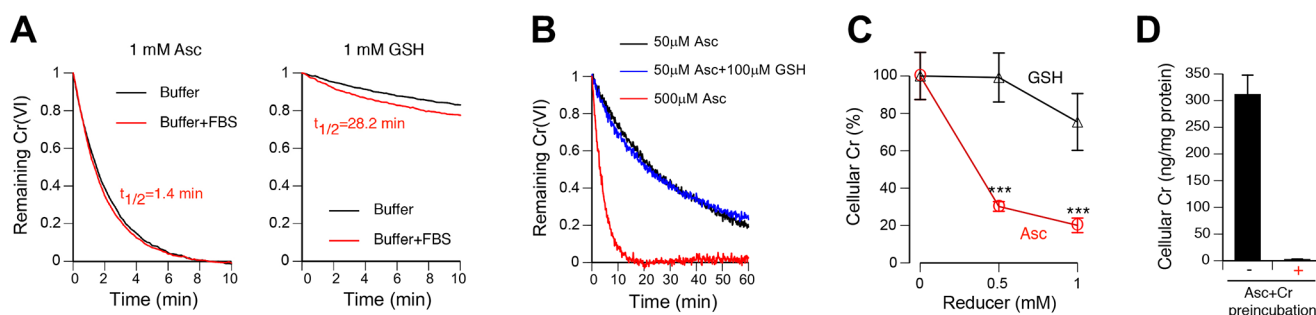
**Cr(VI) Uptake.** The determination of cellular Cr was based on a previously described procedure.<sup>12</sup> Cells were seeded into six-well plates and allowed to grow overnight before the addition of Cr(VI). After removal of Cr-containing media, cells were rinsed twice with warm PBS and collected by trypsinization in the presence of EDTA (Trypsin-EDTA solution from ThermoFisher Scientific, 15400–054). Cells were pelleted and washed twice with cold PBS (5 min at 800 × g, 4 °C) prior to extraction with 5% (v/v) nitric acid at 50 °C for 30 min. Insoluble debris was removed by centrifugation at 10 000 × g for 10 min at 4 °C. Supernatants were diluted to 2% (v/v) nitric acid prior to Cr measurements by graphite furnace atomic absorption spectroscopy (AAAnalyst600 Atomic Absorption Spectrometer, PerkinElmer). Cellular precipitates formed after nitric acid extraction were washed twice with cold 5% (v/v) nitric acid (10 000 × g for 5 min, 4 °C), dissolved in 0.5 M NaOH, and used for the determination of protein content of each sample. Final Cr uptake values were protein-normalized. Control experiments showed that Ca-chromate and Sr-chromate were rapidly solubilized in 2% or 5% nitric acid even in the absence of heating, which indicated that the employed nitric extraction procedure did not underestimate the amounts of cellular Cr due to a potential inability to recover internalized but not yet solubilized chromate particles.

**Western Blotting.** At the time of collection, cells were washed twice with cold PBS and detached from the dishes by scraping in PBS. Attached and floating cells were combined, pelleted, and then boiled for 10 min in a lysis buffer containing 2% SDS, 50 mM Tris, pH 6.8, 10% glycerol, and protease/phosphate inhibitors (78425, ThermoFisher Scientific). Samples were cleared from occasional debris by centrifugation at 10 000 × g for 10 min at room temperature. Proteins were separated on 10 or 12% SDS-PAGE gels and electrotransferred to PVDF membranes (162–0177, Bio-Rad). Primary antibodies were from Santa Cruz for detection of p53 (sc-125, 1:1000 dilution), from Cell Signaling Technology for Ser15-phosphorylated p53 (9284, 1:1000), cleaved caspase 7 (8438, 1:500), PARP (9542, 1:1000), and Ser139-phosphorylated histone H2AX (2577, 1:1000), from Abcam for fibrillarin (ab5821, 1:5000) and from Sigma for  $\gamma$ -tubulin (T6557, 1:2000).

**Cell Viability.** Cytotoxic effects of Cr(VI) treatments were measured using the CellTiter-Glo luminescent assay (Promega). Cells were seeded into 96-well plates (3000 per well for all cell lines) and treated with Cr(VI) on the next day. Cytotoxicity was determined following 48 h post-Cr recovery for H460 and RLE-6TN cells and 72 h recovery for HBE cells.

**Colony Formation.** H460 cells were grown on six-well plates overnight followed by preloading with Asc by incubation for 90 min with 2 mM (as monomer) dehydroascorbic acid as described above. Cells were switched to the standard growth media and treated with Cr(VI) compounds for 3 h in the presence of 0, 50, or 500  $\mu M$  Asc. After 7–8 days of growth with one change of media, colonies were visualized by Giemsa staining.

**Statistics.** Differences between the groups were evaluated by two-tailed, unpaired *t* test.



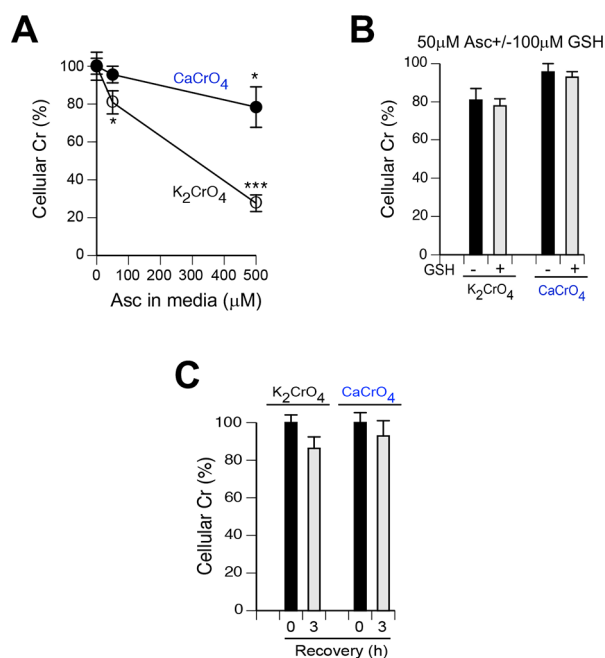
**Figure 1.** Impact of ascorbate (Asc) and glutathione (GSH) on reduction and cellular uptake of chromate anion. All reactions used solubilized  $K_2CrO_4$  as a source of chromate anions. (A) Time-course of  $50 \mu M$  chromate reduction at  $37^\circ C$  (buffer,  $50 \text{ mM}$  HEPES,  $100 \text{ mM}$  NaCl,  $\text{pH}$  7.0; buffer+FBS, HEPES buffer containing 10% fetal bovine serum). Data are means of triplicate measurements. Error bars were smaller than 5% of the mean and are not shown for clarity. (B) Chromate reduction in HEPES buffer by reducer concentrations that are typically found in lung lining fluids. Reactions contained  $5 \mu M$  Cr(VI). Data are means of triplicate measurements. (C) Uptake of chromate by H460 cells in the presence of extracellular Asc or GSH. Cells were incubated with  $10 \mu M$  Cr(VI) for 1 h. Data are means  $\pm$  SD,  $n = 3$ ,  $***p < 0.001$  relative to 0 mM reducer. (D) Elimination of Cr accumulation by a preincubation of  $10 \mu M$  chromate in culture media with 1 mM Asc (1 h,  $37^\circ C$ ) prior to the addition to H460 cells. Cells were incubated with Cr-containing media for 1 h. Data are means  $\pm$  SD,  $n = 3$ .

## RESULTS

**Chromate Reduction by Asc and GSH.** Asc and GSH are two most important reducers of chromate *in vivo* in tissues,<sup>13,14</sup> and they both are present in human and rat bronchoalveolar lining fluids. Cysteine, another biological thiol that is capable of Cr(VI) reduction,<sup>31–33</sup> is generally absent in extracellular milieu. Human lung lining fluid contained on average  $109 \mu M$  GSH and  $40 \mu M$  Asc.<sup>34</sup> Relative to humans, respiratory tract lining fluid from rats had approximately two-times lower GSH but almost 10-times higher Asc concentrations.<sup>35</sup> Other investigators have estimated that rat lung lining fluid contained at least 1 mM Asc.<sup>36</sup> The protein content of human respiratory lining fluid is approximately 10% of that in plasma.<sup>34</sup> The rate of chromate reduction in HEPES buffer with physiological pH and ionic strength was dramatically faster by Asc relative to GSH (Figure 1A). The inclusion of 10% serum to mimic the protein content of human respiratory fluid slightly increased reduction rates for both Asc (1.1-fold) and GSH (1.3-fold). The initial rate of chromate reduction in 10% serum-containing samples by 1 mM Asc was 20.1-times higher than that by 1 mM GSH, which points to a minor role of GSH in Cr(VI) reduction in the bronchoalveolar fluid. The use of both reducers at concentrations that approximate those found in human ( $50 \mu M$  Asc,  $100 \mu M$  GSH) and rat lung lining fluids ( $500 \mu M$  Asc) confirmed the dominant role of Asc in chromate reduction, as evidenced by practically identical rates of Cr(VI) metabolism in  $50 \mu M$  Asc versus  $50 \mu M$  Asc+ $100 \mu M$  GSH reactions (Figure 1B). The rate of chromate reduction by  $500 \mu M$  Asc was 7.4-times faster in comparison to  $50 \mu M$  Asc, which modestly deviated from the linear relationship expected for the first-order reaction kinetics in the Asc-driven reduction.<sup>37</sup> A relatively low absorbance of  $5 \mu M$  Cr(VI) used to ensure the pseudo-first order conditions for all reactions ( $\geq 10$ -times excess of reducers over Cr) probably diminished the accuracy of our rate measurements, particularly for the fast reduction with  $500 \mu M$  Asc. To test the relative abilities of extracellular Asc and GSH to convert chromate to nontoxic cell-impermeable Cr(III), we measured accumulation of Cr in H460 human lung epithelial cells in their standard medium supplemented with 10% serum (Figure 1C). In agreement with chromate reduction results, Asc was also dramatically more effective than GSH in suppressing cellular uptake of Cr(VI). A preincubation of solubilized  $K_2CrO_4$  (chromate anion-containing solution)

with 1 mM Asc in culture media for 1 h prior to the addition to cells completely abolished Cr accumulation, confirming Cr(VI)-dependence of our metal uptake measurements (Figure 1D).

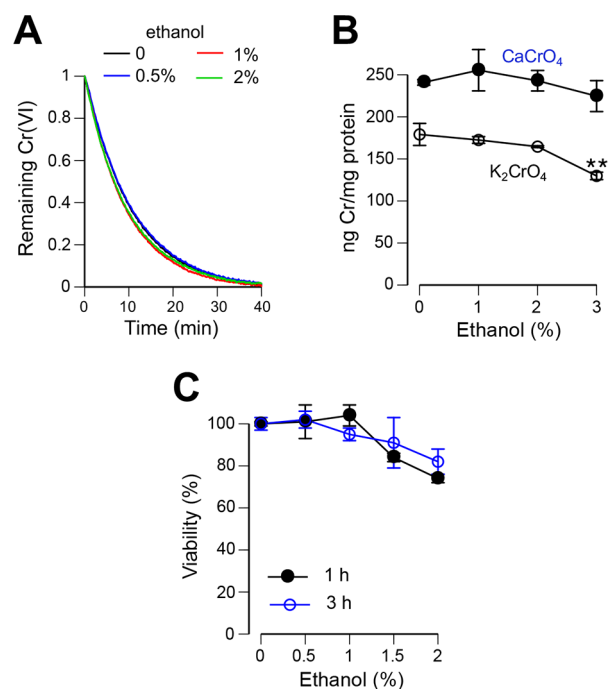
**Uptake of Solubilized Chromate and Chromate Particles in H460 Cells.** A poorly soluble calcium chromate has been found as one of the most potent tumorigenic Cr(VI) compounds in rat lungs using intrapleural injections<sup>22</sup> and intrabronchial implants.<sup>23</sup> Using filtration through nylon filters with  $5 \mu m$  pores, we prepared ethanol suspensions of insoluble  $CaCrO_4$  particles of the respirable size. In part due to clogging of filters, the yield of  $CaCrO_4$  particles was low, which necessitated preparation of fresh batches for each experiment. The use of multiple batches of particles helped avoid sample-specific and “aging”-related effects. As expected, we found that the presence of Asc in media at the rat lung lining fluid concentration ( $500 \mu M$ ) strongly inhibited cellular accumulation of Cr using chromate anions (solubilized  $K_2CrO_4$ ) as a source of Cr(VI) (Figure 2A). A small decrease in chromate uptake was also observed with  $50 \mu M$  Asc, which corresponds to the concentration of this reducer in the human lung fluid. In contrast to chromate anion solutions, cellular accumulation of Cr after incubations with  $CaCrO_4$  particles was only minimally decreased by  $500 \mu M$  Asc, indicating that internalization of particles was the primary uptake mode as particle dissolution and release of chromate anions would have made them susceptible to reduction by Asc to cell-impermeable Cr(III). Consistent with its very slow reduction kinetics, the addition of the human lung fluid-relevant concentration of GSH ( $100 \mu M$ ) to  $50 \mu M$  Asc-supplemented media had no appreciable effects on cellular Cr levels after incubations with solubilized chromate or  $CaCrO_4$  particles (Figure 2B). Uptake and cellular reduction of chromate lead to a long-term retention of Cr due to binding of Cr(III) to various macromolecules.<sup>4</sup> To assess a potential “stickiness” of  $CaCrO_4$  particles to cells without the actual uptake, we measured the levels of cellular Cr immediately after Cr(VI) exposures and following 3 h recovery in complete media (Figure 2C). The amounts of cellular Cr were practically unchanged after 3 h of recovery for both soluble chromate and  $CaCrO_4$  particles, which argues against a significant nonspecific absorption of particles in our uptake measurements. To prevent their solubilization, chromate particles were prepared and stored in ethanol, which raises a question whether the addition



**Figure 2.** Uptake of chromate anions and CaCrO<sub>4</sub> particles by human H460 cells. Cells were incubated for 1 h with 5 μM Cr(VI) in the form of solubilized K<sub>2</sub>CrO<sub>4</sub> or CaCrO<sub>4</sub> particles (0.8% final ethanol concentration). (A) Cellular Cr levels after incubations with soluble and particulate Cr(VI) in the presence of 0, 50, and 500 μM Asc in media. Data are means ± SD, *n* = 3, \**p* < 0.05, \*\*\**p* < 0.001 relative to 0 μM Asc. (B) Cellular Cr accumulation from soluble and particulate Cr(VI) in the presence of extracellular 50 μM Asc with and without 100 μM GSH. Data are means ± SD, *n* = 3. (C) Cellular amounts of Cr at 0 and 3 h postexposure. Data are means ± SD, *n* = 3.

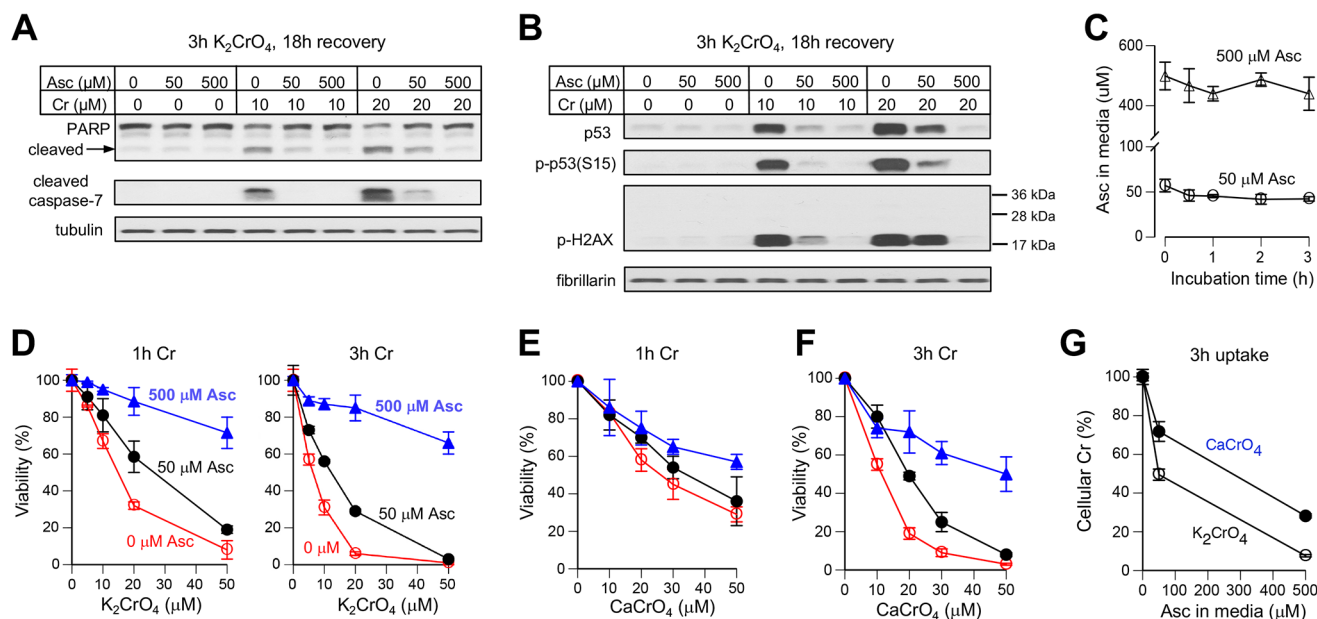
of this solvent (always <1%) altered chromate metabolism or related cellular functions. We found that the rate of chromate reduction by Asc was not significantly different in the range of 0–2% ethanol concentration (Figure 3A), excluding a possibility that ethanol interfered with chromate detoxification in the experiments with Cr(VI) particles. Cellular uptake of chromate anions and CaCrO<sub>4</sub> particles was also unaffected in the presence of 0–2% ethanol (Figure 3B). However, the addition of 3% ethanol significantly diminished cellular accumulation of chromate anions (to 73.7% of control) but not that of CaCrO<sub>4</sub> particles, further pointing to the different mechanisms of cellular entry for these two forms of Cr(VI). H460 cells incubated with ethanol for 1 h (as in Cr uptake experiments) or 3 h displayed no detectable cytotoxic effects for 0.5 and 1% concentrations but showed small and gradually increasing losses of viability at 1.5% and higher concentrations (Figure 3C). As all of our experiments with Cr(VI) particles contained <1% ethanol in culture media, its presence at these levels does not affect Cr(VI) metabolism or cell viability.

**Cytotoxicity of Chromate Anions and Chromate Particles in H460 Cells.** To assess the effects of extracellular Asc on toxicity of chromate anions, we measured apoptotic and DNA damage-related responses in H460 cells treated with Cr(VI) for 3 h followed by 18 h recovery. We found that the presence of 500 μM Asc in media completely abolished apoptotic cleavage of PARP and the formation of the active form of executioner caspase-7 (Figure 4A). Even the addition of 50 μM Asc to media was very protective against chromate-induced apoptosis. DNA damage-related markers assessed by levels of the transcription factor p53, its Ser15 phosphorylation,

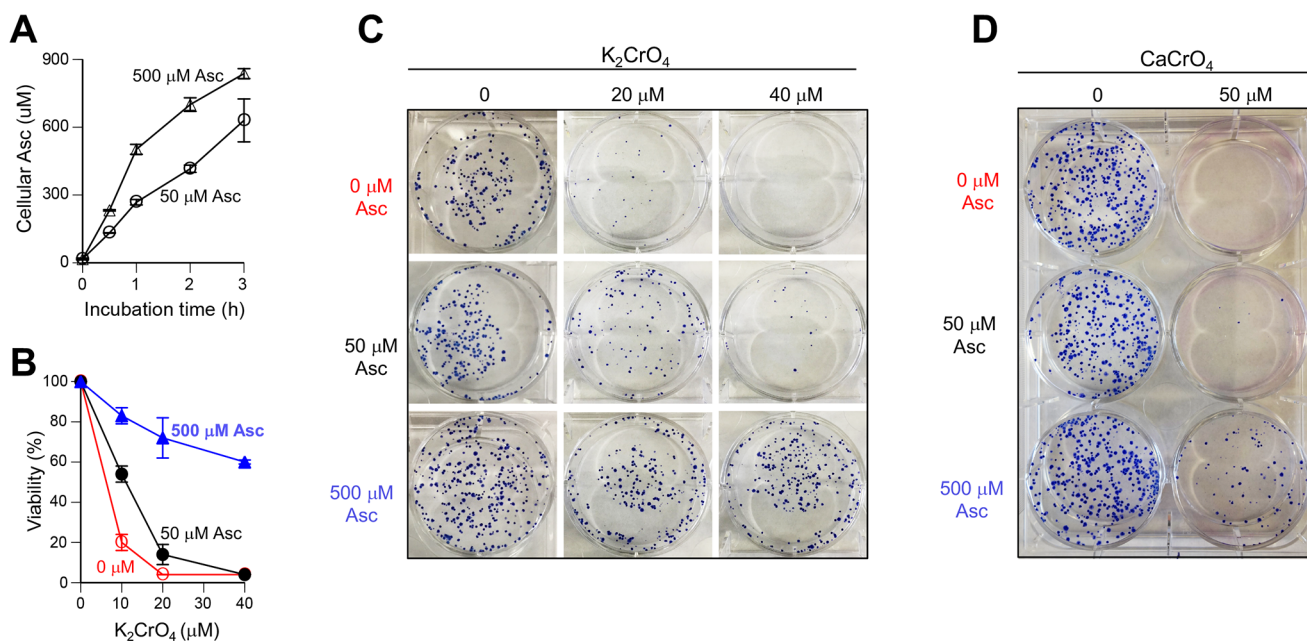


**Figure 3.** Effects of ethanol on Cr(VI) metabolism and cell viability. (A) Kinetics of chromate reduction by Asc in the presence of ethanol. Reactions contained 0–2% ethanol, 200 μM Asc, 20 μM chromate in HEPES-NaCl buffer, pH 7.0. Data are means of triplicate measurements. (B) Uptake of chromate anions (solubilized K<sub>2</sub>CrO<sub>4</sub>) and CaCrO<sub>4</sub> particles by H460 cells. Cells were incubated with 5 μM of each Cr(VI) form for 1 h. Means ± SD, *n* = 3, \*\*\**p* < 0.01 relative to controls without ethanol. The addition of CaCrO<sub>4</sub> gave 0.07% ethanol in media. (C) Viability of H460 cells treated with ethanol for 1 or 3 h. Cell viability measurements were taken at 48 h postexposure. Data are means ± SD, *n* = 3.

and the appearance of the biochemical marker of DNA double-strand breaks (DSBs), Ser139-phosphorylated histone H2AX (also known as γ-H2AX), showed a complete loss of genotoxicity when cells were treated with chromate in the presence of 500 μM extracellular Asc (Figure 4B). The suppression of DNA damage responses was also clearly evident for 50 μM extracellular Asc. The protective effects of extracellular Asc were not underestimated due to its instability, as Asc losses during 3-h long incubations were minimal for both 50 and 500 μM concentrations (Figure 4C). Apoptotic responses reflect only one form of cell death. Therefore, we next examined a longer-term viability of cells, which integrates apoptotic and other forms of cell death. We found that 500 μM extracellular Asc was highly protective against chromate cytotoxicity, which provided a very high viability even at doses of Cr(VI) that were 100% lethal to cells without media supplementation with Asc (Figure 4D). The lower 50 μM extracellular Asc concentration also produced clear protective effects at a magnitude that was more similar to DNA damage markers (p53 and phospho-H2AX, Figure 4B) than apoptosis (Figure 4A). Consistent with the uptake measurements (Figure 2A), examination of cell viability showed that extracellular Asc offered a much more limited shield against toxicity of CaCrO<sub>4</sub> particles using 1 h treatments of cells (Figure 4E). On the basis of the comparison of the dose–response curves, 500 μM extracellular Asc diminished cytotoxicity of CaCrO<sub>4</sub> particles by 1.89-fold whereas its protective effect against chromate anions was 7.5-fold. The presence of Asc in media exhibited a much



**Figure 4.** Effects of extracellular Asc on responses to Cr(VI) in H460 cells. Cells were treated for 1 or 3 h in media containing 0, 50, or 500  $\mu\text{M}$  Asc. (A) Westerns for apoptotic markers and (B) DNA damage-related responses at 18 h after 3 h-long treatments with solubilized chromate. (C) Stability of Asc in media during incubations with cells. Data are means  $\pm$  SD,  $n = 3$ . (D) Cell viability at 48 h after treatments with solubilized chromate or (E)  $\text{CaCrO}_4$  particles for 1 h or (F) 3 h. Data are means  $\pm$  SD,  $n = 3$ . Controls for  $\text{CaCrO}_4$ -treated samples were treated with the corresponding concentrations of ethanol, which was present at  $<0.7\%$  at the highest Cr(VI) dose. (G) Cr uptake after 3 h incubations with chromate anions (solubilized  $\text{K}_2\text{CrO}_4$ ) and particulate  $\text{CaCrO}_4$  in the presence of 0, 50, and 500  $\mu\text{M}$  Asc in media. Data are means  $\pm$  SD,  $n = 3$ . When not visible, error bars were smaller than symbols.



**Figure 5.** Cytotoxicity of Cr(VI) in H460 cells preloaded with Asc. (A) Accumulation of Asc in cells during incubations in culture media supplemented with Asc. Data are means  $\pm$  SD,  $n = 3$ . (B) Viability of Asc-preincubated cells treated with solubilized  $\text{K}_2\text{CrO}_4$  (chromate anions) for 3 h in the presence of different concentrations of Asc. Cells were incubated with 500  $\mu\text{M}$  Asc for 3 h before the addition of chromate. Cell viability was measured at 48 h after chromate removal. Data are means  $\pm$  SD,  $n = 3$ . (C) Colony formation by cells preloaded with 6.1 mM Asc and then treated for 3 h with chromate anions or (D)  $\text{CaCrO}_4$  particles in the presence of different concentrations of extracellular Asc. Cells were preloaded with Asc by incubations with 2 mM dehydroascorbic acid as described in [Materials and Methods](#). In the experiment with  $\text{CaCrO}_4$ , control dishes were treated with 0.7% ethanol to match its concentration in Cr(VI) samples.

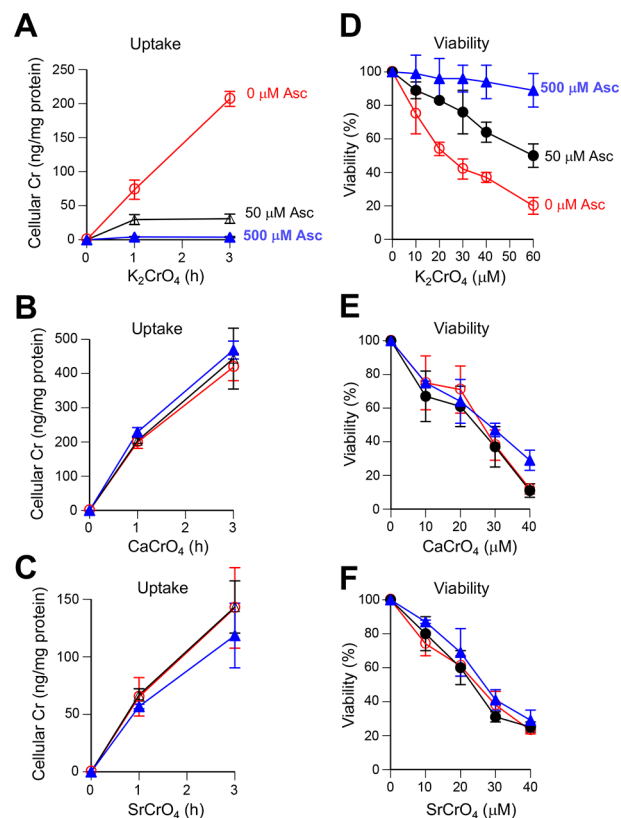
stronger cytoprotective power when cells were treated with  $\text{CaCrO}_4$  particles for 3 h (Figure 4F). In this case, 500  $\mu\text{M}$  Asc decreased cytotoxicity of  $\text{CaCrO}_4$  by 5.5-fold, which was still less dramatic than protection against chromate anions in 3-h

long treatments (18.9-fold, from slopes in Figure 4D). Longer 3 h treatments with  $\text{CaCrO}_4$  were clearly much more toxic in the absence of Asc in media (2.8-fold increase in the slope for 3 h versus 1 h), whereas 500  $\mu\text{M}$  Asc-supplemented samples

showed no further declines in cell viability (slopes ratio = 1.02) (Figure 4E,F). These results suggest that Cr(VI) uptake in longer than 1 h incubations is dominated by chromate anions released during dissolution of  $\text{CaCrO}_4$  particles. In agreement with this interpretation, 500  $\mu\text{M}$  extracellular Asc strongly suppressed cellular accumulation of Cr during 3 h incubations with  $\text{CaCrO}_4$  (Figure 4G), which was only minimally affected in shorter 1 h treatments (Figure 2A). The uptake-suppressive effects of Asc in 3 h incubations were still higher for chromate anions than for  $\text{CaCrO}_4$  (2.0-fold differences in slopes), which can be attributed to the detoxification-resistant internalization of particles during the early exposure time.

Cultured cells contain very low amounts of intracellular Asc due to its absence in the commonly used media and its irreversible oxidation during preparation and storage of serum. We found that the overnight cultures of H460 cells contained only  $17 \pm 4 \mu\text{M}$  Asc ( $n = 3$ ) in comparison to its millimolar concentrations in cells *in vivo*.<sup>15,16</sup> Asc-driven metabolism of Cr(VI) produces a different spectrum of intermediate Cr forms relative to thiols<sup>17–19</sup> and Asc restoration in cells altered cytotoxic and DNA damage responses to Cr(VI).<sup>20,21</sup> Supplementation of culture media with Asc is expected to raise intracellular concentrations of this reducer, which could have differentially affected cytotoxic responses to chromate anions and chromate particles. We found that the addition of both 50 and 500  $\mu\text{M}$  Asc strongly increased intracellular concentrations of Asc during 1–3 h incubations (Figure 5A), which was probably responsible for the modest decreases in the extracellular Asc over the same time (Figure 4C). To assess potential effects of changes in intracellular Asc, we preincubated H460 cells with 500  $\mu\text{M}$  extracellular Asc for 3 h, which raised cellular Asc to 0.84 mM, and then treated them with chromate anions in the presence of different concentrations of Asc in media. This experimental setup also showed dramatically protective effects of extracellular Asc on cytotoxicity of solubilized chromate (Figure 5B). Next, we preloaded cells with even higher Asc concentrations ( $6.1 \pm 0.3 \text{ mM}$ ,  $n = 3$ ), which minimized contributions from the inflow of extracellular Asc, and then assessed their ability to form colonies after treatments with chromate anions and  $\text{CaCrO}_4$  particles. Again, extracellular Asc was highly protective against chromate anions, with 500  $\mu\text{M}$  Asc rescuing colony formation even at a dose of Cr(VI) that was completely lethal in the Asc-free medium (Figure 5C). In contrast, 500  $\mu\text{M}$  extracellular Asc offered only a partial protection against lethality of  $\text{CaCrO}_4$  particles in Asc-loaded cells (Figure 5D). Thus, protection by extracellular Asc against cytotoxicity of chromate anions and chromate particles is unrelated to changes in intracellular Asc.

**Uptake and Cytotoxicity of Cr(VI) in Primary Human Bronchial Epithelial (HBE) Cells.** Bronchial cells are the main transformation target for Cr(VI) in occupationally exposed individuals.<sup>3</sup> Therefore, we next examined interactions of Cr(VI) compounds with primary HBE cells. To expand investigation of poorly soluble chromates, we also prepared respirable-size particles of  $\text{SrCrO}_4$ , which is another potent carcinogenic Cr(VI) compound in rat lungs.<sup>22,23</sup> Uptake measurements in normal HBE cells found striking effects of extracellular Asc on suppression of the cellular entry of chromate anions, as essentially no uptake was detectable in the presence of 500  $\mu\text{M}$  Asc in culture media (Figure 6A). Although less dramatic, supplementation of media with 50  $\mu\text{M}$  Asc was also very effective in inhibition of cellular accumulation of chromate anions, as evidenced by a significantly ( $p < 0.01$ )

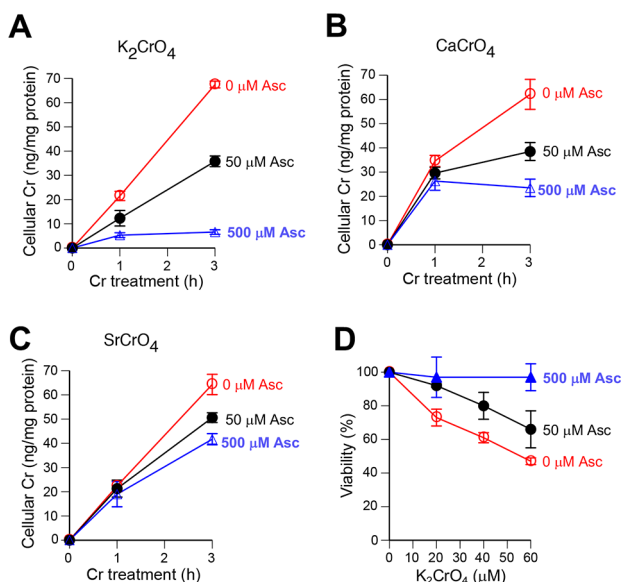


**Figure 6.** Uptake and cytotoxicity of different forms of Cr(VI) in primary HBE cells. All panels show means  $\pm$  SD,  $n = 3$ . (A) Time-dependent accumulation of Cr in cells incubated with chromate anions (solubilized  $\text{K}_2\text{CrO}_4$ ), (B)  $\text{CaCrO}_4$  particles, or (C)  $\text{SrCrO}_4$  particles in the presence of 0, 50, or 500  $\mu\text{M}$  Asc in culture media. Panels A–C use the same symbols and colors for line labeling. (D) Viability of cells treated with chromate anions (solubilized  $\text{K}_2\text{CrO}_4$ ), (E)  $\text{CaCrO}_4$  particles, or (F)  $\text{SrCrO}_4$  particles in the presence of 0, 50, or 500  $\mu\text{M}$  Asc in culture media. Cells were treated with Cr(VI) compounds for 3 h and their viability was measured 72 h later. Panels D–F use the same symbols and colors for line labeling.

decreased uptake during 1 h incubation and no further changes during additional 2 h treatments. In contrast, uptake of  $\text{CaCrO}_4$  particles by HBE cells was practically unaffected by either 50 or 500  $\mu\text{M}$  extracellular Asc (Figure 6B). Cellular accumulation of Cr during incubations with  $\text{SrCrO}_4$  particles was also unchanged by the addition of low or high Asc concentrations to the media (Figure 6C). To validate our findings on Cr accumulation, we examined a long-term viability of HBE cells after Cr(VI) treatments. In full agreement with uptake measurements, we found that 500  $\mu\text{M}$  extracellular Asc completely eliminated cytotoxicity of chromate anions, whereas 50  $\mu\text{M}$  Asc was strongly cytoprotective (Figure 6D). Although uptake of chromate anions by H460 (Figure 3B) and HBE cells (Figure 6A) was similar, cytotoxic responses in HBE cultures without Asc addition were much lower than those in H460 cells treated under the same conditions. For example, 3 h-long treatment of H460 cells with 20  $\mu\text{M}$  chromate resulted in almost complete loss of viability (Figure 4D), whereas the same dose produced less than 50% loss of viability in HBE cells (Figure 6D). We attribute a lower toxicity of chromate in HBE cells to their slow proliferation (typical for cultures of primary cells). The formation of DNA-double stranded breaks (DSBs) in human lung cells by chromate anions has been found to be

replication-dependent,<sup>38,39</sup> indicating that poorly cycling cells would produce fewer toxic lesions.

**Effects of Extracellular Asc in Rat Alveolar Cells.** Since rats were used as the model species for testing lung carcinogenicity of chromates of different solubility,<sup>22,23</sup> we next assessed the effects of extracellular Asc in spontaneously immortalized rat lung epithelial cells, the RLE-6TN (rat lung epithelial-T-antigen negative) cell line. Similar to human cells, rat cells accumulated progressively less Cr when incubated with chromate anions in the presence of increasing concentrations of Asc in media (Figure 7A). The presence of 500  $\mu\text{M}$  Asc



**Figure 7.** Treatments of rat alveolar cells with different Cr(VI) forms. Data in all panels are means  $\pm$  SD ( $n = 3$ ). (A) Accumulation of Cr by RLE-6TN cells after incubations with 5  $\mu\text{M}$  solubilized  $\text{K}_2\text{CrO}_4$ , (B)  $\text{CaCrO}_4$  particles, or (C)  $\text{SrCrO}_4$  particles in the presence of 0, 50, or 500  $\mu\text{M}$  extracellular Asc. (D) Viability of RLE-6TN cells treated with solubilized  $\text{K}_2\text{CrO}_4$  for 6 h. Cell viability was measured at 48 h post-Cr treatments.

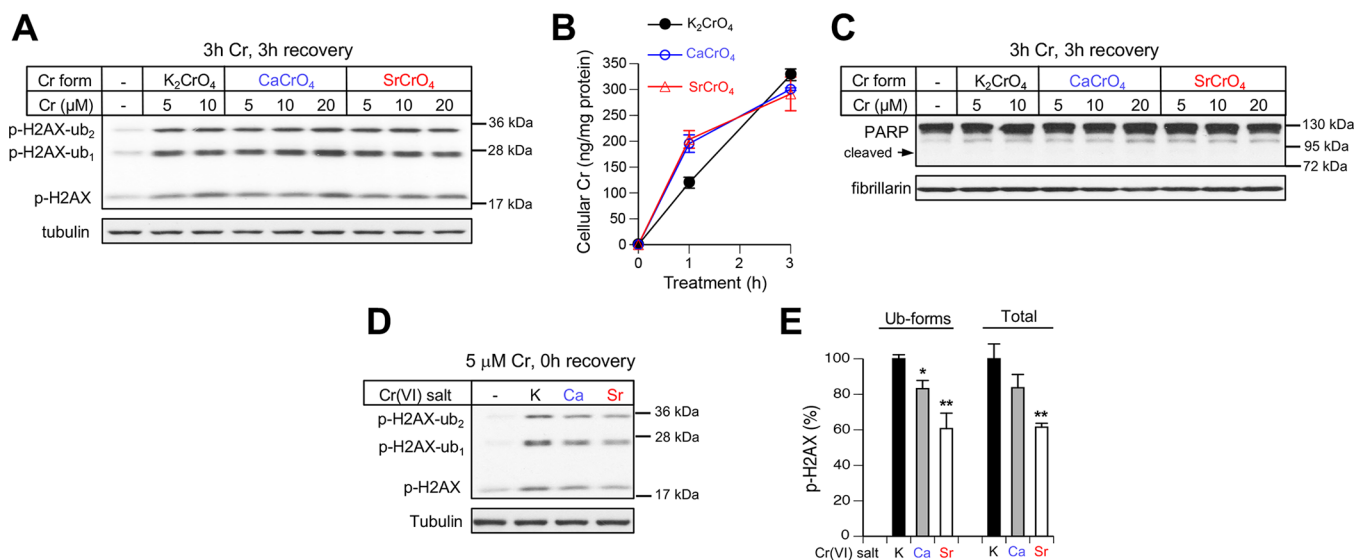
severely suppressed uptake of Cr during the initial 1 h incubation and completely prevented entry of chromate anions into cells during the additional 2 h-long incubation. Using  $\text{CaCrO}_4$  particles, we found that extracellular Asc only minimally diminished Cr accumulation by cells during the first 1 h, but it was much more effective in inhibition of Cr uptake during the next 2 h (Figure 7B). As found for chromate anions, 500  $\mu\text{M}$  Asc completely suppressed further Cr accumulation during the post-1 h treatment time. These results indicate that uptake of Cr(VI) from  $\text{CaCrO}_4$  initially occurs via internalization of particles whereas the subsequent Cr accumulation in cells is caused by entry of chromate anions arising from dissolution of particles. Consistent with its lower water solubility in comparison to  $\text{CaCrO}_4$ , accumulation of cellular Cr during incubations with  $\text{SrCrO}_4$  was more modestly diminished by extracellular Asc (Figure 7C). In agreement with uptake results, cytotoxic effects of chromate anions in rat cells were significantly decreased by the addition of 50  $\mu\text{M}$  Asc to the media and completely prevented by 500  $\mu\text{M}$  extracellular Asc (Figure 7D). We used 6 h-long incubations with chromate anions for examination of their cytotoxicity in rat cells because shorter exposure times, such as 1 h or even 3 h used for human cells, did not produce sufficient losses of viability that are

necessary for a reliable assessment of cytoprotective effects of extracellular Asc. Since prolonged incubations with  $\text{CaCrO}_4$  or  $\text{SrCrO}_4$  particles in their dissolution, cytotoxicity studies with these Cr(VI) forms would not be informative and have not been performed.

**Formation of DNA Double-Stranded Breaks (DSBs).** DSBs are critical genetic lesions produced by chromate anions in Asc-restored human lung cells.<sup>38–40</sup> Mechanistically, DSBs arise from recognition and processing of Cr-DNA adducts by mismatch repair in replicated DNA.<sup>38,39,41</sup> Thus, formation of DSBs can serve as an important test for the assessment of genotoxic activity of Cr(VI) compounds and intracellular delivery of chromate anions either directly or as a result of particle dissolution inside the cell. We have previously validated Ser139-phosphorylated H2AX (also known as  $\gamma\text{-H2AX}$ ) as a marker of DSBs in chromate-treated cells by parallel physical measurements of these lesions.<sup>39,40</sup> Using westerns for phospho-Ser139-H2AX, we found that chromate anions (solubilized  $\text{K}_2\text{CrO}_4$ ) and  $\text{CaCrO}_4$  and  $\text{SrCrO}_4$  particles produced similar levels of DSBs in Asc-restored H460 cells at 3 h recovery after 3 h-long treatments (Figure 8A). For example, in 5  $\mu\text{M}$  Cr-treated cells, the combined amounts of mono- and diubiquitinated forms of phospho-H2AX were 100, 94.1, and 106.3% for  $\text{K}_2\text{CrO}_4$ ,  $\text{CaCrO}_4$ , and  $\text{SrCrO}_4$ , respectively. At this concentration, all three Cr(VI) compounds also showed similar cellular levels of Cr accumulation at the end of 3 h-long incubations (Figure 8B). The observed abundance of mono- and diubiquitinated forms of phospho-H2AX at short postexposure times is typical for chromate anions-induced DSBs in cells with physiological levels of Asc.<sup>42</sup> This nonproteolytic ubiquitination acts as signal for the recruitment of DNA proteins involved in repair of DSBs.<sup>43</sup> Apoptotic DNA cleavage products can also stimulate H2AX phosphorylation but not its ubiquitination (as shown earlier in Figure 4B). A lack of apoptosis at the time of collection of cells for DSB detection is further confirmed by the absence of a caspase-mediated cleavage of PARP (Figure 8C). Thus, comparable levels of phospho-H2AX forms for all three Cr(VI) compounds indicate similarity in their genotoxic potencies when cellular uptake is not modulated by the presence of extracellular Asc. Since solubilized and particulate chromates deliver Cr(VI) into cells via different routes and in different forms, we also examined the formation of DSBs immediately after 3 h-long treatments. We found that at this time the amounts of ubiquitinated forms or total phospho-H2AX were the highest for chromate anions and the lowest for the less soluble  $\text{SrCrO}_4$  (Figure 8D,E). Although at the end of 3 h-long treatments the overall Cr accumulation was comparable for all three compounds, uptake of  $\text{CaCrO}_4$  and  $\text{SrCrO}_4$  particles occurred faster in comparison to chromate anions (Figure 8B). A gradual dissolution of internalized particles releasing Cr(VI) is the likely cause for the observed slower kinetics of DSB formation by  $\text{SrCrO}_4$  despite its faster uptake in comparison to chromate anions-treated cells.

## DISCUSSION

All Cr(VI)-containing compounds are considered as human respiratory carcinogens based on a large number of epidemiological studies demonstrating elevated lung cancer risks among occupational groups with inhalation exposures to more than one form of Cr(VI), including soluble compounds.<sup>1–3</sup> Because of their superior quality, data from two cohorts of US workers exposed to soluble Cr(VI)<sup>27,44</sup> were

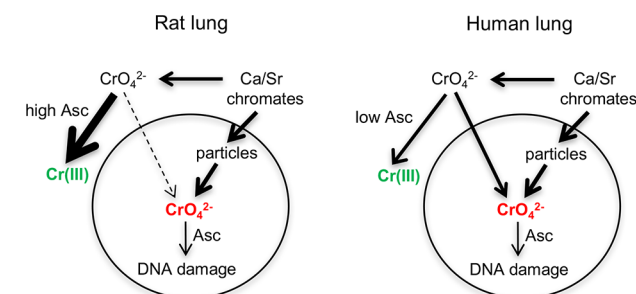


**Figure 8.** Histone H2AX phosphorylation by solubilized and particulate Cr(VI) in Asc-restored cells. H460 cells were preincubated with 0.2 mM dehydroascorbic acid and then treated with Cr(VI) for 3 h. (A) Ser139-phosphorylated histone H2AX (p-H2AX) in cells collected at 3 h recovery post-Cr. p-H2AX-ub<sub>1</sub> and p-H2AX-ub<sub>2</sub> indicate mono- and diubiquitinated forms of phospho-H2AX. Tubulin was used as a loading control. (B) Time-dependent accumulation of Cr in cells treated with different Cr(VI) forms (all at 5  $\mu$ M Cr). Data are means  $\pm$  SD ( $n = 3$ ). (C) Absence of apoptotic PARP cleavage in cells treated as in panel A. "Cleaved" indicates the expected position of the caspase-generated 89 kDa product. Fibrillarlin was used as a loading control. (D) Representative Western blot for Ser139-phosphorylated histone H2AX in cells treated with 5  $\mu$ M Cr(VI) for 3 h and collected immediately. K, solubilized K<sub>2</sub>CrO<sub>4</sub>; Ca, CaCrO<sub>4</sub> particles; Sr, SrCrO<sub>4</sub> particles. (E) Relative amounts of phospho-H2AX in cells treated as in panel D. The amounts of ubiquitinated (ub<sub>1</sub>+ub<sub>2</sub>) or all three p-H2AX forms (total) for K<sub>2</sub>CrO<sub>4</sub> were taken as 100%. Means  $\pm$  SD,  $n = 3$ , \* $p < 0.05$ , \*\* $p < 0.01$  relative to K<sub>2</sub>CrO<sub>4</sub>.

used by the Occupational Safety and Health Administration in the United States to estimate lung cancer risks for airborne Cr(VI) under a current permissible exposure limit.<sup>45</sup> However, comparative studies using intrapleural injections or intrabronchial implantations in rats have found a striking dependence of lung tumorigenicity on the solubility of Cr(VI) compounds.<sup>22,23</sup> Readily solubilized Cr(VI) forms releasing chromate anions upon dissolution produced no tumors, whereas moderately soluble Cr(VI) compounds such as CaCrO<sub>4</sub> and SrCrO<sub>4</sub> were highly tumorigenic. Our results suggest that the disparity between humans and rats in carcinogenicity of Cr(VI) compounds of different solubility was probably caused by the species differences in Asc concentrations in the lung extracellular fluid. We found that the Asc concentration corresponding to that in the rat lung fluid practically completely eliminated uptake of chromate anions and their toxicity in human and rat lung epithelial cells. In contrast, uptake and cytotoxicity of CaCrO<sub>4</sub> and SrCrO<sub>4</sub> particles were significantly less sensitive to the presence of high extracellular Asc, indicating their internalization in the form of particles. The protective effects of the 10-times lower Asc corresponding to its level in human lung fluids were proportionally smaller. Thus, the dramatically lower Asc content of the human lung extracellular fluid makes it a much less effective at detoxification of chromate anions through their reduction to cell-impermeable Cr(III). A relatively slow dissolution of CaCrO<sub>4</sub> and SrCrO<sub>4</sub> particles and their rapid internalization limit the detoxification potential of even high extracellular Asc, which explains high lung tumorigenicity of these compounds in rats.

Similar to highly soluble chromate salts, very poorly soluble Pb-chromate and Ba-chromate were also nontumorigenic in rats using intrapleural injections<sup>22</sup> or intrabronchial implantation models.<sup>23</sup> We suggest that high carcinogenicity of

moderately soluble chromate forms in the rat lung can be attributed to two factors (Figure 9):



**Figure 9.** Model for carcinogenicity of Cr(VI) compounds of different solubility in rat and human lungs. High concentrations of ascorbate (Asc) in the rat lung lining fluid rapidly detoxify extracellular chromate (CrO<sub>4</sub><sup>2-</sup>) produced by highly soluble Cr(VI) compounds or during a gradual dissolution of less soluble Cr(VI) particles. Dissolution of internalized Cr(VI) particles releases chromate that undergoes reduction to Cr(III) leading to Cr-DNA damage. A much lower (~1/10th) concentration of Asc in the human lung fluid provides a more slow and limited detoxification of chromate anions, permitting their uptake by lung epithelial cells and the subsequent formation of Cr-DNA damage.

- (1) Relative resistance to detoxification by high extracellular Asc due to their limited dissolution outside of cells, permitting their uptake during early exposure times in the form of particles.
- (2) Dissolution and release of chromate anions inside the cells leading to DNA damage.

Intracellular dissolution of CaCrO<sub>4</sub> or SrCrO<sub>4</sub> particles was evidenced by their cytotoxicity even in the presence of high extracellular Asc. The release of chromate anions from



internalized particles appeared to be relatively rapid, as the amounts of DNA breaks induced by exposures to particulate and solubilized chromates were similar at 3 h postexposure (Figure 8A). Studies with very poorly water-soluble  $\text{PbCrO}_4$  showed that its genotoxicity was caused by cellular uptake of chromate anions released during prolonged incubations in culture media, which promoted the solubilization process.<sup>46</sup> Although  $\text{PbCrO}_4$  particles were also internalized, they remained insoluble inside the cells and did not produce toxic effects.<sup>46,47</sup> High extracellular Asc in the rat lung fluid can effectively detoxify slowly released chromate anions preventing carcinogenicity of  $\text{PbCrO}_4$ . This slow release of chromate anions should promote their detoxification even by low Asc levels in the human lung fluid, which is consistent with epidemiological findings on weaker carcinogenicity of  $\text{PbCrO}_4$  relative to more soluble chromates.<sup>1,2</sup>

Our data showed that high extracellular Asc corresponding to its concentration in the rat lung lining fluid acted as a very effective shield against uptake and toxicity of chromate anions. Exposure to moderately insoluble forms of Cr(VI) avoided this defense mechanism by delivery of chromate anions into the cells via internalization and dissolution of particles. These observations lead us to suggest that the differences in rat lung tumorigenicity of Cr(VI) compounds are largely caused by different intracellular doses of chromate anions. One prediction of this model is that soluble chromates should be tumorigenic even in rats when they are delivered in sufficiently high doses to deplete extracellular Asc. The results of a study by Steinhoff et al.<sup>48</sup> on a high threshold dose dependence of tumorigenicity of solubilized dichromate delivered via intratracheal instillations are consistent with this prediction. Intratracheal instillations deposited concentrated Cr(VI) solutions onto a small area, which would create a localized exhaustion of Asc (due to its oxidation by massive amounts of Cr-6) permitting uptake of chromate anions into the cells. Despite their much lower levels, Asc concentrations found in the human lung fluid were clearly protective against chromate anions, particularly in primary HBE cells. A preferential deposition of inhaled Cr(VI) in a small region of human lungs (area of bronchial bifurcation)<sup>49</sup> increases a risk for depletion of extracellular Asc during periods of particularly heavy exposures or in situations with coexposures to other oxidants, further weakening the detoxification potential of the lining fluid. Bypass of extracellular detoxification by chromate particles suggests that the effectiveness of a frequently used chemoprotective agent *N*-acetylcysteine, which primarily acts via reduction of chromate anion outside the cells,<sup>50</sup> will be beneficial largely for exposures to Cr(VI) compounds with high solubility. Effective Cr(VI) detoxification by extracellular Asc raises a question whether its levels in the lung lining fluid could be manipulated via dietary intake of vitamin C. This possibility has never been tested directly but the concentrations of Asc in plasma and the lung fluid did not show any significant correlation,<sup>34</sup> suggesting that the amounts of extracellular Asc in the lung are unrelated to its systemic levels. Concentrations of GSH in the lung fluid was approximately 100-times higher in comparison to plasma,<sup>34</sup> further demonstrating that the levels of antioxidants in the human lung fluid are regulated independently and do not exist in equilibrium with plasma levels.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

Asc, ascorbate; DSBs, DNA double-stranded breaks; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

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