Reductive Activation with Cysteine Represents a Chromium(III)-Dependent Pathway in the Induction of Genotoxicity by Carcinogenic Chromium(VI)

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Induction of DNA damage by carcinogenic hexavalent chromium compounds [Cr(VI)] results from its reduction to lower oxidation states. Reductive metabolism of Cr(VI) generates intermediate Cr(V/IV)species, organic radicals, and finally Cr(III), which forms stable complexes with many biological ligands, including DNA. To determine the biological significance of different reaction products, we examined genotoxic responses and the formation of DNA damage during reduction of Cr(VI) by its biological reducer, cysteine. We have found that cysteine-dependent activation of Cr(VI) led to the formation of Cr–DNA and cysteine–Cr–DNA adducts as well as interstrand DNA cross-links. The yield of binary and ternary DNA adducts was relatively constant at different concentrations of Cr(VI) and averaged approximately 54 and 45%, respectively. Interstrand DNA cross-links accounted on average for 1% of adducts, and their yield was even less significant at low Cr(VI) concentrations. Reduction of Cr(VI) in several commonly used buffers did not induce detectable damage to the sugar–phosphate backbone of DNA. Replication of Cr(VI)-modified plasmids in intact human fibroblasts has shown that cysteine-dependent metabolism of Cr(VI) resulted in the formation of mutagenic and replication-blocking DNA lesions. Selective elimination of Cr–DNA adducts from Cr(VI)-treated plasmids abolished all genotoxic responses, indicating that nonoxidative, Cr(III)-dependent reactions were responsible for the induction of both mutagenicity and replication blockage by Cr(VI). The demonstration of the mutagenic potential of Cr–DNA adducts suggests that these lesions can be explored in the development of specific and mechanistically important biomarkers of exposure to toxic forms of Cr. *Key words:* biomarker, chromium, cysteine, DNA adduct, mutagenesis. *Environ Health Perspect* 110(suppl 5):729–731 (2002).

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Hexavalent chromium compounds [Cr(VI)] are recognized human and animal carcinogens (*1,2*). The cell transformation ability of Cr(VI) is believed to result from its mutagenic, clastogenic, and other genotoxic activities (*3,4*). Cr(VI) itself is unreactive toward DNA at physiological pH and requires reductive activation to produce DNA-damaging species. The most important intracellular reducers of Cr(VI) are ascorbate and nonprotein thiols such as glutathione and cysteine (Cys) (*5–7*). The reductive metabolism of Cr(VI) generates intermediate Cr(V) and Cr(IV) forms and finally yields thermodynamically stable Cr(III) (*8,9*). The relative amount of these intermediate Cr forms varies dramatically depending on the experimental conditions, such as the nature of the reducing agent and its ratio to Cr(VI). Other products of Cr(VI) reduction include sulfur- and carbon-based radicals (*9–11*). Hydrogen peroxide, either added intentionally or generated incidentally from iron-contaminated reagents, is responsible for the formation of hydroxyl radicals and Cr(V)-peroxo complexes during reduction of Cr(VI) (*10,12*). These unstable Cr intermediates and radical species can potentially induce oxidation of DNA (*13*), which could subsequently lead to mutations and chromosomal damage. DNA-damaging activity of hydroxyl radicals is well known, whereas the reactivity of thiyl and carbon-based radicals is expected to be much lower (*11,14*). Treatment of cells or reduction of Cr(VI) *in vitro* also generates numerous Cr–DNA adducts. These include binary Cr(III)–DNA adducts, Cr(III)-mediated DNA cross-links with Cys, histidine, or glutathione (*15*), as well as interstrand DNA cross-links (*16*). Ternary Cr(III)–DNA adducts have been found to be mutagenic during replication in human fibroblasts (*17*).

The presence of several transient species and the potential formation of both oxidative and Cr–DNA lesions raise two important questions: *a*) what is the nature of DNA-attacking species, and *b*) what is the relative significance of oxidative and Cr(III)-dependent pathways in the induction of genotoxicity by Cr(VI)? To address these questions, we have conducted detailed studies of Cys-based reactions in which direct determinations of DNA damage and the assessment of genotoxic consequences were performed (*18–20*). Here, we summarize our findings that nonoxidative mechanisms involving Cr(III)–DNA binding are responsible for the induction of the genotoxicity during reductive activation of Cr(VI) by Cys.

Experimental Procedures

All buffers and reagents for electrophoresis were from Sigma (St. Louis, MO, USA). Chelex-100 resin and Bio-Gel P-30 columns were obtained from Bio-Rad (Hercules, CA, USA). K_2CrO_4 (A.C.S. reagent) was from Aldrich (Milwaukee, WI, USA), 2′,7′-dichlorofluoroscin diacetate was supplied by Molecular Probes (Eugene, OR, USA). $Na_2{}^{51}CrO_4$ and [^{35}S]-labeled L-cysteine were from Amersham (Arlington Heights, IL, USA). ΦX174 DNA was obtained from New England Biolabs (Beverly, MA, USA). L-Cysteine was from Gibco (Rockville, MD, USA). All reagents were purified by Chelex-100 chromatography as described previously (*18*). Solutions of Cys were used within 30 min of preparation. A standard Cr(VI) reduction mixture contained 2 μg of supercoiled pSP189 DNA, 25 mM 3-[*N*-morpholino]-propanesulfonic acid (MOPS; pH 7.0), 2 mM Cys, and various concentrations of K_2CrO_4 in a final volume of 50 μL. In some reactions 5 mM ethylenediamine tetraacetic acid (EDTA) was also added or MOPS buffer was replaced by 25 mM phosphate, 2-[*N*-morpholino]ethanesulfonic acid (MES) or HEPES (pH 7.0). Samples used for the detection of DNA breakage contained 0.3 μg of supercoiled ΦX174 DNA in a final volume of 25 μL. All samples were incubated for 60 min at 37°C, and DNA-unbound reactants were removed by Bio-Gel P30 columns and precipitation with ethanol. The amounts of DNA-bound Cr and Cys were determined using trace quantities of radioactive ^{51}Cr-chromate and ^{35}S-Cys, respectively. The number of interstrand DNA cross-links was calculated from the relative amounts of double-stranded DNA after denaturation in 0.2 M NaOH and separation of DNA by agarose electrophoresis (*18*). Dissociation of DNA-bound Cr was achieved by incubating adducted DNA in the presence of 50 mM sodium phosphate (pH 7.0) for 24 hr at 37°C (*19*). Released Cr was removed by Bio-Gel P30 columns. The presence of nicked DNA was analyzed by agarose electrophoresis.

The genotoxic significance of DNA damage arising from Cr(VI) reduction by Cys was examined using the pSP189 shuttle-vector

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(*19*). This vector is capable of replicating in intact human cells, which permits its use for the biological assessment of the presence of mutagenic and replication-blocking DNA lesions after *in vitro* treatments with Cr(VI). In brief, control and Cr(VI)-treated plasmids were transfected into human fibroblasts and were allowed to replicate for 42–48 hr. Replicated plasmids was isolated and then used to transfect an indicator *Escherichia coli* strain, MBL50. The efficiency of plasmid replication in human cells was determined by the number of *E. coli* transformants (ampicillin-resistant colonies). The mutation frequency at the *supF* gene was calculated by dividing the number of arabinose/ampicillin-resistant colonies by the number of ampicillin-resistant colonies.

Results and Discussion

Kinetic studies of Cys-dependent reduction of Cr(VI) at neutral pH have indicated that the initial step in the reduction could proceed through either one- or two-electron transfer (*20*). The relative contribution of each reduction pathway depends on the concentration of Cys, with one-electron transfer the dominant mechanism in the physiological range of Cys concentrations (>90% reduction). Transfer of one electron to Cr(VI) would generate a sulfur-based thiyl radical and Cr(V) as the first products. Increased oxidation of 2′,7′-dichlorofluoroscin in the reactions containing low Cys concentrations was consistent with the enhanced production of Cr(V) under these conditions (*20*). The formation of Cr(V) species and thiyl radicals in Cr(VI)/Cys mixtures has been detected using electron spin resonance (*8,10*). A second reduction step most likely involves the production of Cr(IV), but this has not yet been established experimentally. Cr(III)–$(Cys)_2$ complex was the major stable product of Cr(VI) reduction by Cys (*21*). Addition of DNA to the reduction mixtures resulted in the formation of Cr(III)–DNA adducts (*18*). Therefore, the induction of genotoxicity in Cys-based reactions can potentially result from oxidative damage by Cr(V) and thiyl radicals or/and is caused by Cr(III)–DNA binding.

The sugar–phosphate backbone of DNA is one of the most common sites of damage by oxidizing species. To detect the presence of DNA-oxidizing activity in Cr(VI)–Cys reactions, we analyzed the production of DNA single-strand breaks (Figure 1A). No significant DNA breakage was detected using sodium phosphate or three organic buffers. Reduction of Cr(VI) in phosphate or MOPS buffer using a 10-fold range of Cys concentrations also failed to produce abasic sites (*20*). Thus, thiyl radicals and Cr(V) species generated in Cr(VI)–Cys reactions appeared unable to cause oxidative damage to the sugar–phosphate backbone of DNA.

Reduction of Cr(VI) results in the formation of three types of Cr–DNA adducts: binary Cr–DNA, ternary Cys–Cr(III)–DNA adducts, and interstrand DNA cross-links (*18*). Binary and ternary adducts represented the majority of Cr–DNA complexes, and their yield was essentially constant over a 25- to 100-μM range of Cr(VI) (Figure 1B). The average yields of binary and ternary DNA adducts were 53.8 ± 1.8% and 45.3 ± 2.1%, respectively. In contrast to monoadducts, the formation of DNA–DNA cross-links was progressively higher with increasing Cr(VI) concentrations [0.2, 1.0, and 2.2% yield at 25, 50, and 100 μM Cr(VI), respectively]. Samples containing 10 μM Cr(VI) had undetectable amounts of DNA cross-links (*18*). Although DNA cross-links are considered to be potent polymerase-blocking lesions (*22*), their sparse formation at low concentrations

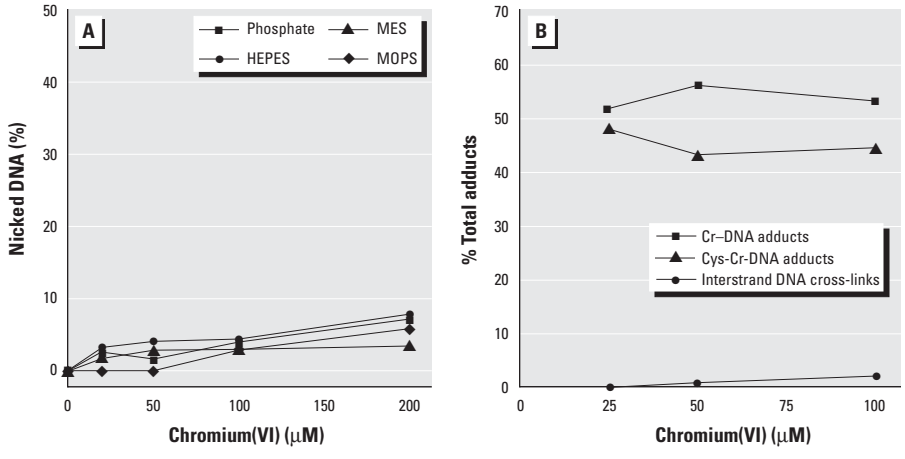

Figure 1. DNA damage resulting from Cr(VI) reduction by Cys. Each reaction mixture contained supercoiled plasmid DNA, 25 mM buffer (pH 7.0), 2 mM Cys, and various concentrations of Cr(VI). Samples were incubated for 60 min at 37°C and then analyzed for DNA breakage and Cr–DNA adducts. All determinations of DNA adducts were performed using supercoiled pSP189 DNA. Overall yield of Cr adducts was determined using ^{51}Cr-chromate. The number of Cys–DNA cross-links was determined using ^{35}S-Cys. Interstrand DNA cross-links were quantified by renaturing agarose gel electrophoresis (*18*). DNA breakage was analyzed by agarose electrophoresis of ΦX174 DNA. (*A*) DNA strand breakage: means from four independent experiments. Error bars are not shown for clarity. (*B*) Yield of different Cr–DNA adducts. Percentage of each type of adducts was calculated using mean values from four to six independent determinations.

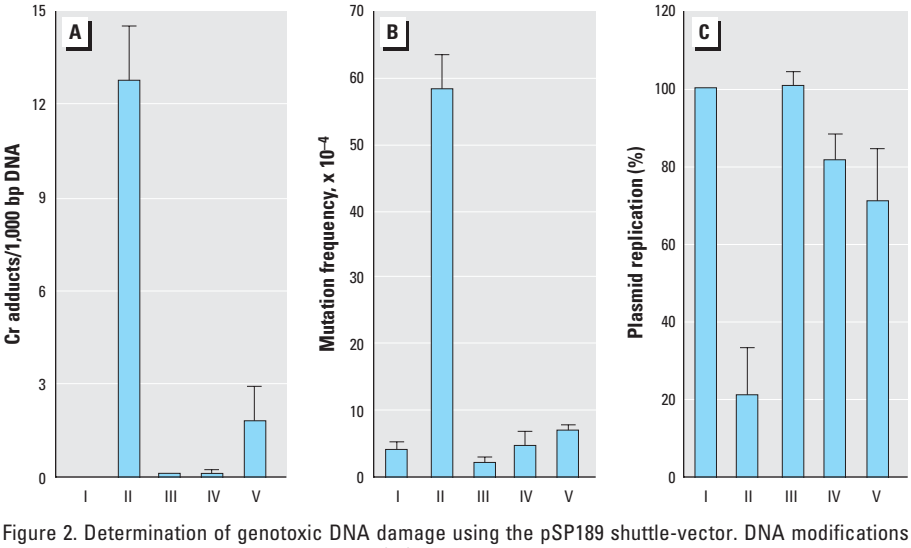

Figure 2. Determination of genotoxic DNA damage using the pSP189 shuttle-vector. DNA modifications were performed in the presence of 50 μM Cr(VI) and 2 mM Cys as described in Figure 1. DNA was purified by Bio-Gel P-30 chromatography and ethanol precipitation and then transfected into human fibroblasts. Mutation frequency and recovery of replicated plasmids were determined as described by Zhitkovich et al. (*19*). Columns: I, Cr-untreated DNA; II, Cr(VI) reduction in 25 mM MOPS buffer; III, Cr(VI) reduction in 25 mM MOPS + 5 mM EDTA; IV, Cr(VI) reduction in 25 mM phosphate; V, Cr(VI) reduction in 25 mM MOPS followed by dissociation of Cr from DNA in the presence of 50 mM phosphate (*19*). Shown are means ± SD from six to eight independent experiments. (*A*) Cr–DNA binding. (*B*) Mutagenic responses. (*C*) Yield of replicated plasmids.

of Cr(VI) suggests that these lesions are not very likely to play a major role in the genotoxicity induced by Cys-dependent activation of Cr(VI).

To determine whether reduction of Cr(VI) in the presence of Cys produced genotoxic DNA modifications, we employed a pSP189 shuttle-vector approach (*19*). In this assay, the pSP189 DNA is incubated in Cr(VI)/Cys reduction mixtures, purified, and then transfected into human cells to assess the presence of premutagenic and replication-blocking DNA lesions. Using this methodology, it is possible to examine the importance of different types of DNA damage by controlling their formation during *in vitro* reduction of Cr(VI). Figure 2A shows that the formation of Cr–DNA adducts can be completely blocked when reaction mixtures contained the Cr(III)-chelating agents EDTA or inorganic phosphate. We have also found (*19*) that the majority of Cr(III)–DNA adducts formed in the MOPS-based reactions can be dissociated by the subsequent incubation in the presence of 50 mM phosphate (Figure 2A, bar V). When pSP189 plasmids modified in Cr(VI)–Cys reactions were replicated in human fibroblasts, we found a strong mutagenic response in samples containing Cr–DNA adducts but not in those lacking them (Figure 2B). These results demonstrate that Cys-dependent metabolism of Cr(VI) did produce mutagenic DNA damage and that Cr–DNA adducts were responsible for the induction of mutagenicity. In addition to mutagenic effects, Cr(VI)-induced damage also caused a strong inhibition of plasmid replication in intact human cells (Figure 2C, bar II). Blocking of Cr(III)–DNA binding in the reactions containing EDTA or phosphate essentially restored template properties of pSP189 plasmids. Release of approximately 80% of DNA-bound Cr by the treatment with phosphate resulted in almost normal yield of replicated plasmids, which further confirmed a critical role of Cr–DNA adducts in the replication-blocking activity of Cr(VI).

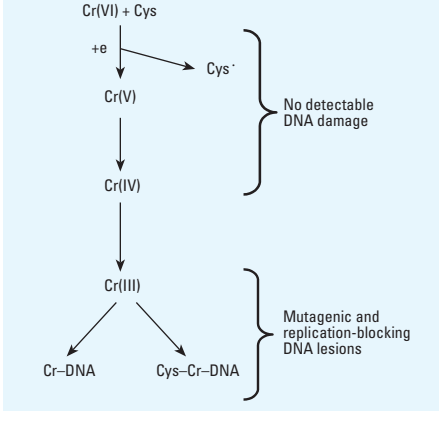

Figure 3. Metabolism of Cr(VI) by Cys and the formation of genotoxic DNA lesions.

The shuttle-vector experiments showed that two very important forms of Cr(VI)/Cys-induced genotoxicity, mutagenesis and replication blockage are caused by nonoxidative mechanisms through the generation of Cr(III)–DNA adducts (Figure 3). These findings provide an example of the biologically relevant metabolic system in which Cr(III)–DNA adducts are responsible for the major genotoxic activities of Cr(VI). The absence of detectable oxidative DNA damage in Cr(VI)/Cys reactions indicates that thiyl radicals and intermediate Cr(V/IV) forms are relatively unreactive toward DNA. We expect that the glutathione thiyl radical produced in Cr(VI)/glutathione reactions (*23*) will have a similarly low reactivity. The demonstration of the mutagenic activity of Cr(III)–DNA adducts identifies a biologically important class of Cr(VI)-specific DNA lesions that can be explored in the development of useful biomarkers of exposure to toxic forms of Cr.

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