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Hypoxia impedes the formation of chromium DNA-adducts in a cell-free system

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Abstract

The metabolic reduction of hexavalent chromium [Cr(VI)] in the presence of DNA generates several lesions which impede DNA replication and gene transcription. However, the relative contribution of molecular oxygen to Cr-induced genetic damage is unclear. To elucidate the role of dioxygen in Cr genotoxicity, we studied the formation of Cr-induced lesions in DNA treated with either Cr(VI) and the physiological reductant, ascorbic acid (Asc), or Cr(III), under ambient and hypoxic (<1% oxygen) conditions. We found that hypoxia did not impede the reduction of Cr(VI) by Asc throughout a 2 h treatment. In contrast, Cr-DNA binding under these conditions was reduced up to 70% by hypoxia, and a 50–90% decrease in the frequency of Cr-induced *Taq* polymerase-arresting DNA adducts was also observed. In the presence of Cr(VI)/Asc, formation of Cr-DNA interstrand crosslinks (ICLs) under hypoxia was 50% or less of that under ambient conditions. Kinetic studies found that hypoxia reduced the *rate* at which Cr interacted with DNA, but not the ultimate steady state level of Cr-DNA binding. The inhibitory effect of hypoxia on Cr(VI)/Asc genotoxicity could not be explained solely by alterations in the reactivity of intermediate Cr(V) species because Cr(III)-DNA binding and Cr(III)-induced ICL formation were also impaired by hypoxia. Moreover, Cr(V) was generated to similar levels in ambient and hypoxic reactions. Hypoxia did not affect ICL formation by the inorganic chemotherapeutic agent cisplatin, suggesting that these effects were specific for Cr(III). Taken together, these results support a role for dioxygen in facilitating the formation of Cr-DNA coordination complexes.

Keywords: Chromium; Oxygen; Hypoxia; Cisplatin; DNA adducts

1. Introduction

Certain hexavalent chromium [Cr(VI)]-containing compounds have been implicated as occupational lung carcinogens [1,2]. Cr(VI) enters the cell through non-specific anionic transporters as a chromate oxyanion [3,4] where it is metabolically reduced to Cr(III) by ascorbic acid (Asc), reduced glutathione (GSH) or cysteine (Cys). The plasma membrane is relatively impermeable to Cr(III) and, consequently, Cr(III) accumulates within the cell [5]. The reductive metabolism of Cr(VI) also generates transient intermediate Cr species (Cr(V), Cr(IV)) [6,7]. Although Cr(VI) does not directly interact with nucleic acids, Cr(III) and Cr(V) display an affinity towards DNA [6,8]. In addition to the formation of reactive Cr species, carbon-based and oxygen radicals can be generated during Cr reduction [7,9–13].

Cr, generally in its trivalent form, interacts with DNA bases as well as the phosphodiester backbone. Specifically, Cr(VI) reduction has been implicated in the formation of Cr-DNA base monoadducts, DNA strand breaks, abasic sites, oxidatively damaged bases, Asc–Cr-DNA crosslinks, protein/amino acid–Cr-DNA crosslinks (Cr-DPCs) and

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DNA interstrand crosslinks (ICLs) (reviewed in [14]). There have been several reports on the guanine-specific inhibition of DNA polymerase by Cr-DNA damage, which has been associated with the formation of Cr-ICLs [15–19] involving either phosphate and/or bases (i.e. guanine) in complementary strands of DNA as well as oxidized bases arising from the further, Cr(V)-mediated, oxidation of 7,8-dihydro-8-oxoguanine (8-oxo-G) [20]. In addition, Cr(VI)-exposed cells exhibit a prolonged delay in the resumption of RNA synthesis after treatment [21,22], which may be associated with the physical inhibition of RNA polymerase elongation of nascent transcripts [21]. The formation of Cr-induced DNA/RNA polymerase arresting lesions (Cr-PALs) is believed to be closely linked to Cr toxicity and may represent pro-apoptotic lesions [23].

It is becoming increasingly clear that reductive activation of Cr(VI) to Cr(III) is necessary for the formation of toxicologically relevant DNA adducts, although the true nature of this diverse group of lesions is still poorly understood. The reduction of Cr(VI) to Cr(III) is a three electron process; hence, the inability of intracellular reductants to completely reduce Cr(VI) in a single step leads to the formation of highly oxidizing chromium intermediates with +5 or +4 oxidation states. These transient oxidation states of Cr are significantly more oxidizing than the parent Cr(VI) and have been shown to directly oxidize deoxyribose sugars and bases in DNA [20,24]. Unlike Cr(III), the ligands surrounding these intermediate oxidation states are kinetically labile [25].

The role of molecular oxygen or reactive oxygen species (ROS) in the generation of DNA lesions by Cr is unclear. The oxidation of DNA by Cr(VI) reduction may involve either a ROS- or metal-dependent [(Cr(V)] pathway (reviewed in [26]). Co-treatment of isolated DNA with H_2O_2 and either Cr(III) or Cr(VI) produces abasic sites, DNA strand breaks and 8-oxo-G [27–34]. However, other studies have indicated that the reduction of Cr(VI), in the presence of DNA, does not generate extensive strand breakage or abasic sites [35–37]. Furthermore, the oxidation of DNA by hypervalent Cr(V) complexes occurs independently of oxygen but requires phosphate coordination [38].

The true contribution of oxygen to Cr genotoxicity, be it as molecular oxygen or ROS, is variable and depends upon the reaction conditions and Cr concentrations employed. It is difficult to investigate this mechanism in cell systems as the measurement of ROS production is often complicated by the accompanying cytotoxicity associated with Cr(VI) exposure. To further elucidate the potential role of oxygen in the development of Cr-DNA damage, we have examined the impact of hypoxia on the formation of Cr-induced DNA damage in a cell-free system. Our results reveal that the formation of Cr-DNA adducts displays a unique dependence on molecular oxygen.

2. Materials and methods

2.1. Cell culture and reagents

Human lung fibroblasts (HLF) (ATCC LL-24 cells, number 151-CCL) were grown in F12K medium (Life Technologies, Gaithersburg, MD) containing 15% fetal bovine serum (FBS) and supplemented with 0.5% penicillin–streptomycin in a humidified incubator under 5% CO_2 at 37 °C. Solutions of sodium chromate [Na_2CrO_4, Cr(VI)] (J.T. Baker Chemical, Phillipsburg, NJ) and ascorbic acid (Asc) (Fisher Scientific, Fair Lawn, NJ) and chromium chloride [$CrCl_3$, Cr(III)] (Sigma Chemical, St. Louis, MO) were freshly prepared in sterile water prior to each experiment. Cisplatin (*cis*-diammineplatinum(II) dichloride; CDDP), Sigma Chemicals) was freshly rehydrated with 0.9% aqueous NaCl.

2.2. Preparation and treatment of DNA

HLF cells were harvested and DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Life Science, Madison, WI) per the manufacturer's protocol, and digested with *Eco*R1. Plasmid [pSV2neoTS [39]] or HLF DNA (0.04 μg/μl) was incubated in the presence of Cr(III) alone, or Cr(VI) plus Asc, at 37 °C for 2 h at a constant Asc:Cr(VI) molar ratio of 0.5. Hypoxic experiments (<1% oxygen in solution, Dissolved Oxygen Meter, Hanna Instruments, Woonsocket, RI) were carried out in an anaerobic chamber (Forma Scientific, Marietta, OH). All reagents and supplies were equilibrated overnight in the hypoxic environment (50% [5% CO_2/10% hydrogen/nitrogen balanced]):(50% [100% nitrogen]) and were freshly prepared/dissolved immediately prior to each experiment. Reactions performed under ambient conditions were otherwise identical. Additional experiments, in which the hypoxic environment was created, using 100% nitrogen, yielded similar results. This indicates that the presence of small amounts of carbon dioxide and/or hydrogen in the hypoxic environment did not affect the results.

2.3. Measurement of Cr(VI) reduction

Cr(VI) reduction by Asc under ambient or hypoxic environments was monitored by absorption spectroscopy (OD_{372}) as previously described [37].

2.4. Measurement of total Cr-DNA binding

Total Cr-DNA binding ($Na_2\ ^{51}CrO_4$ and $^{51}CrCl_3$; ICN, Irvine, CA) was analyzed as previously described [37]. Briefly, [^{51}Cr]-labeled Cr(VI) or Cr(III) solutions were incubated with pSV2neoTS DNA (0.04 μg/μl) in 100 mM HEPES (pH 7.2) for 2 h at 37 °C in a final volume of 25–100 μl. Following treatment, DNA was precipitated with ice-cold 5% trichloroacetic acid (TCA) for 30 min

and filtered (Whatman GF/A glass micro fiber filters) using a vacuum apparatus. After correction for non-specific filter binding, Cr-DNA adducts were calculated and expressed as total Cr adducts/1.5 kbp (Cr molecules/molecules DNA bases, 660 Da/bp) based on the [^{51}Cr]-labeled Cr stock solution. It should be noted that we excluded samples from our analysis where the non-specific ^{51}Cr binding was greater than 50% even though the hypoxia-dependent effects were still evident. In more recent experiments, we have reduced the non-specific binding by the inclusion of a filter-dialysis step prior to precipitation/filtration. However, the hypoxic effects on Cr-DNA binding were quantitatively identical to our previous methodologies (i.e. no dialysis).

2.5. Quantitative PCR (QPCR) analysis of Cr-treated human DNA

Following treatment, genomic DNA was filter-dialyzed against sterile, deionized water using microcon filter columns (10,000 MWCO) (Millipore, Bedford, MA) to remove unreacted Cr and other compounds from the reaction mixture. DNA concentration was determined by absorption spectroscopy (A_{260}). Quantitative PCR analysis (QPCR) of the *p53* gene and PAL frequency calculation were performed as previously described [37].

2.6. DNA interstrand crosslink detection in plasmid DNA

The existence of DNA interstrand crosslinks in *EcoR*1-linearized pSV2neoTS DNA was detected using renaturing agarose gel electrophoresis (RAGE) [37,39]. Reactions containing cisplatin were performed under the same conditions (100 mM HEPES, pH 7.2, 2 h at 37 °C) as those using Cr(III).

2.6.1. Electron paramagnetic spectroscopy (EPR)

Reactions were performed with 2 mM Cr(VI)/1 mM Asc in the presence of mannitol (50 mM) or with 10-fold higher Cr(VI)/Asc concentrations in the absence of mannitol for the indicated times. After incubation, samples were immediately frozen in liquid nitrogen until analysis. The samples were examined at room temperature using an X-band EPR spectrometer (Bruker ER-200, Washington, DC). EPR conditions were as follows: microwave frequency: 9.71 GHz, microwave power 10 mW, modulation frequency 100 KHz, modulation amplitude 1 G, center field 3472 G, scan rate 30 G/min, time constant 0.2 s.

2.7. Statistical analysis

A Student's *t*-test ($P < 0.05$) was used to compare differences of means between ambient and hypoxic samples.

3. Results

3.1. Cr(VI) reduction by Asc under ambient and anaerobic conditions is similar

To determine whether reduced levels of oxygen inhibited Cr(VI) reduction by Asc, we measured the disappearance of Cr(VI) in the reaction mixture spectrophotometrically under both ambient and hypoxic conditions following a 2 h incubation. As seen in Fig. 1, the percentage of unreacted Cr(VI) remaining in the reactions was unaffected by reduced oxygen levels. During the 2 h incubation, the kinetics of Cr(VI) reduction was also unaltered by oxygen deprivation (data not shown). Our previous work determined that the lowest molar ratio of Asc:Cr(VI) capable of producing maximal levels of Cr-induced replication-blocking lesions was 0.5 [37]. Consequently, this molar ratio (0.5) was used in all subsequent experiments.

3.1.1. Cr-DNA binding is reduced under hypoxia

We next measured total Cr-DNA binding in reactions performed under ambient and hypoxic environments. Fig. 2 shows that the formation of Cr-DNA adducts in plasmid DNA treated with ^{51}Cr(VI) and Asc (2 h) was significantly ($P < 0.05$) reduced by up to 70% under hypoxia compared to ambient samples. For example, at 30 μM Cr(VI), under hypoxic conditions there were 5.8 Cr-DNA adducts/1.5 kbp, compared to 15.3 Cr-DNA adducts/1.5 kbp under ambient conditions.

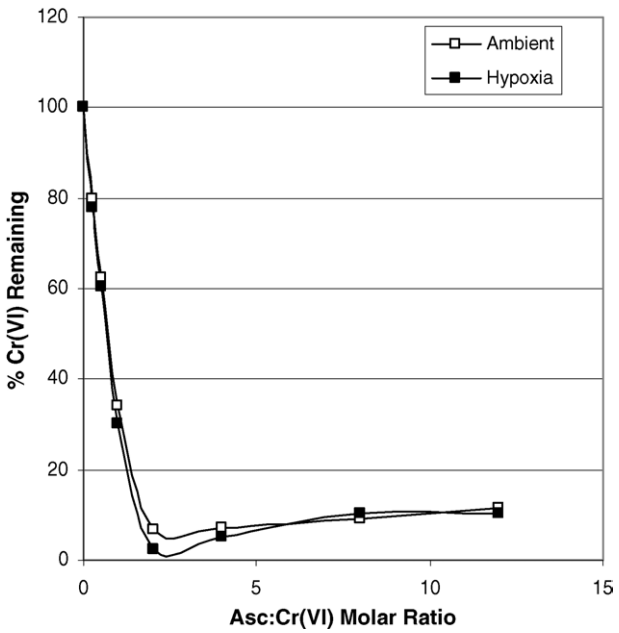

Fig. 1. Lack of effect of hypoxia on Cr(VI) reduction. Cr(VI) (60 μM) was incubated with Asc at the indicated molar ratios (2 h at 37 °C) under ambient (open squares) or hypoxic conditions (closed squares). Percent Cr(VI) remaining following incubation was measured spectrophotometrically. Data points are the mean of two independent experiments.

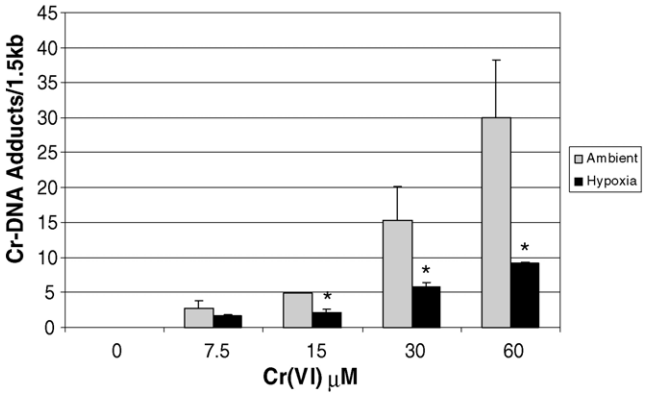

Fig. 2. Diminution of Cr-DNA binding by hypoxia. pSV2neoTS DNA was treated with the indicated concentrations of Cr(VI) containing ^{51}Cr(VI) (Asc:Cr(VI) molar ratio of 0.5) under ambient (gray columns) or hypoxic (black columns) reaction conditions. The data are the mean ± standard error from three independent experiments. Asterisks (*) indicate significant differences from respective ambient samples at $P < 0.05$.

3.2. Hypoxia reduces the development of Cr-induced PALs

The hypoxia-sensitive formation of Cr-DNA adducts indicated that the formation of lesions that inhibit DNA polymerase elongation might similarly be affected by reduced oxygen levels. To test this, we next measured the frequency of replication-blocking lesions in DNA treated with Cr(VI)/Asc under ambient or hypoxic conditions. Similar to the DNA binding data (Fig. 2), the development of Cr-PALs within a target sequence of the *p53* gene (exons 5–8) after a 2 h treatment of HLF DNA with 7.5–30 μM Cr(VI) was significantly ($P < 0.05$) reduced by 50–90% in comparison to ambient conditions (Fig. 3A and B). In contrast, at 60 μM Cr(VI), the formation of Cr-PALs was indistinguishable under ambient and hypoxic conditions (~4.0 PALs/1.5 kbp). This concentration-dependent discrepancy suggests that the Cr-DNA adduct levels (~5–10 Cr adducts/1.5 kbp) achieved at doses above 30 μM Cr(VI) under hypoxia, produce sufficient levels of PALs (i.e. ICLs) to still inhibit DNA replication. Indeed, these levels of Cr-DNA adducts are similar to those (5–15 Cr adducts/1.5 kbp) attained at 15–30 μM Cr(VI) in an ambient environment, the concentration range in which amplification is completely inhibited.

3.3. The formation of Cr-ICLs in plasmid DNA is reduced by hypoxia

Because Cr-ICLs are the primary lesions responsible for Cr-induced polymerase arrest under these conditions [37], we next measured the formation of these lesions on plasmid DNA treated with Cr(VI)/Asc under ambient and hypoxic conditions. As can be seen in Fig. 4, the percentage of dsDNA (Cr-ICLs) increased in a concentration-dependent manner in both normal and hypoxic reactions. However, the overall percentage of DNA containing ICLs under hypoxia was ~50% of that observed in ambient reactions. For example, at 30 μM Cr(VI)/15 μM Asc, the amount of crosslinked plasmid was ~70% in the presence of oxygen and ~40% under hypoxia.

3.4. The rate of Cr-DNA binding is slower under hypoxia

To test whether the inhibition of Cr-DNA lesion formation by hypoxia was related to an alteration in the rate of Cr-DNA binding, we quantified Cr-DNA adduct levels in plasmid DNA immediately after a 2 h incubation or following a 20 h treatment with 30 μM Cr(VI)/Asc to permit steady state levels of Cr-DNA binding to be achieved. Under ambient conditions (Fig. 5) the longer incubation produced only small increases (<1.3-fold) in Cr-DNA binding, relative to the 2 h treatment. This is consistent with recent work, which showed that the majority of in vitro Cr-DNA ternary adducts form within the first 2 h of treatment [36]. In contrast, total Cr-DNA binding increased at least 2-fold in samples reacted overnight under hypoxia, which was significantly ($P = 0.007$) greater than the increase observed for ambient samples at 60 μM Cr(VI). ICL formation also increased to a greater degree in hypoxic samples after 20 h in relation to the 2 h incubation. However, the levels of Cr-ICLs under hypoxia were still slightly lower than under ambient conditions (data not shown).

3.5. Cr(V) generation is unaffected by hypoxia

Although Cr(VI) reduction occurs normally under hypoxic conditions (Fig. 1), it is possible that the generation of DNA reactive Cr(V) species might be attenuated by hypoxia. To test this possibility, we followed the formation of Cr(V) by EPR spectroscopy. Because mannitol is known to act as a spin-trap for Cr(V) [40,41], we initially examined the temporal formation, and accumulation, of this complex under ambient and hypoxic conditions. As can be seen in Fig. 6A, the reaction of 2 mM Cr(VI), 1 mM Asc and 50 mM mannitol generated the characteristic Cr(V)–mannitol signal (g-value = 1.98, A_H = 1.1 G) irrespective of molecular oxygen. The signal increased slightly from 2 to 10 min, but remained constant up to 120 min. We note that the hyperfine splitting pattern of the Cr(V)–mannitol signal (~1:4:6:4:1) seemed to be slightly distorted due to aggregation of the paramagnetic particles resulting from the high Cr(V) concentrations present in the reaction mixture. Reactions were also conducted using 10-fold higher levels of reactants to permit detection of the Cr(V) EPR signal in the absence of mannitol. Similar to reactions containing mannitol, we found no difference in the generation of Cr(V) between the ambient and hypoxic conditions (Fig. 6B). In general, the concentration of Cr(V) produced in the presence and absence of mannitol was ~1.27 mM (63.5% of total Cr) and ~4.30 μM (0.02% of total Cr), respectively, under either set of conditions after 120 min.

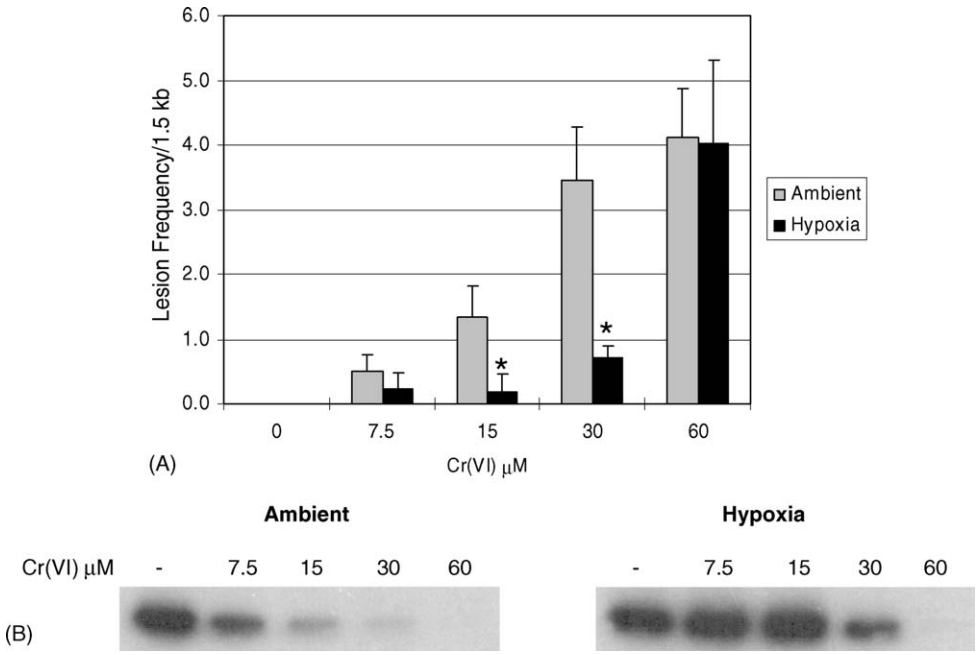

Fig. 3. Reduction of Cr-PAL formation by hypoxia. HLF DNA was incubated with Cr(VI) and Asc at the indicated concentrations as described in Fig. 1. (A) Quantification of QPCR target sequence (1.5 kb) amplification in Cr(VI)/Asc-treated HLF DNA under ambient (gray columns) and hypoxic (black columns) conditions. All data points are the mean ± standard error of at least three independent experiments. Asterisks indicate significant differences compared to respective ambient samples at $P < 0.05$. (B) Representative autoradiograms showing Cr-induced replication arrest in Cr(VI)/Asc-treated HLF DNA under ambient (left gel) and hypoxic (right gel) conditions.

3.6. The direct interaction of Cr(III) with DNA is inhibited by hypoxia

Because the rate of Cr-DNA binding in Cr(VI)/Asc reactions was decreased by hypoxia (Fig. 5A), we next determined whether hypoxia affected Cr(III)-DNA binding. To accomplish this, we incubated plasmid DNA directly with Cr(III), thereby eliminating any contribution of Cr(IV) or Cr(V) to the observed Cr-DNA binding data. As seen in Fig. 7, similar to reactions containing Cr(VI) and Asc (Fig. 2), hypoxia inhibited the formation of Cr(III)-DNA adducts as well. For example, the total bind-

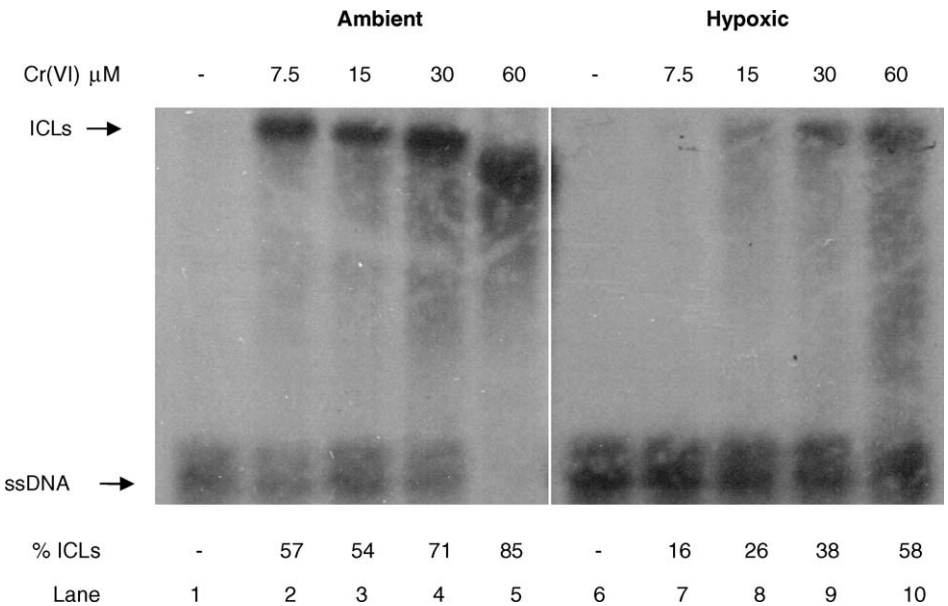

Fig. 4. Decreased formation of Cr-induced DNA interstrand crosslinks (Cr-ICLs) in plasmid DNA by hypoxia. Representative autoradiograms illustrating ICL formation at the indicated Cr(VI) concentrations. *Eco*R1-linearized plasmid DNA was reacted with Cr(VI)/Asc under ambient (lanes 1–5) and hypoxic conditions (lanes 6–10). Twenty-five nanograms of plasmid DNA was loaded per lane. ICLs were detected by RAGE as described in Section 2. dsDNA: double-stranded DNA; ssDNA: single-stranded DNA; ICLs: Cr-DNA interstrand crosslinks. ICLs were quantified by densitometry and expressed as the percentage of dsDNA remaining following heat denaturing.

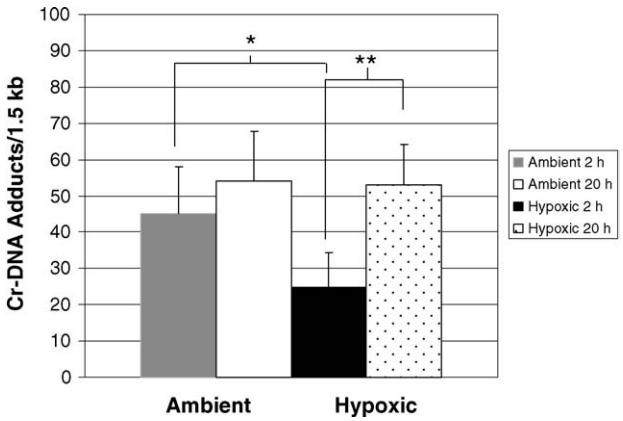

Fig. 5. Hypoxia inhibits the initial interaction of Cr with DNA. Comparison of initial (2 h incubation) and steady state (20 h incubation) ^{51}Cr-DNA binding under ambient and hypoxic conditions. DNA was reacted with 60 μM Cr(VI) and 30 μM Asc as described in Section 2. '*': Significantly different ($P = 0.019$) from corresponding 60 μM Cr(VI) treatment under ambient conditions. '**': Incubation (20 h) produced significant increase ($P = 0.007$) in Cr-DNA binding relative to 2 h reaction under hypoxic conditions.

ing of Cr(III) with DNA was reduced by ~60% at 15 and 30 μM Cr(III). Consistent with these results, and those with Cr(VI)/Asc (Fig. 4), we also found that the formation of Cr(III)-ICLs was impeded by hypoxia (Fig. 8). We also examined whether hypoxia can affect the stability of preformed Cr(III)-DNA adducts. We found that incubation of Cr(III)-reacted DNA, following dialysis, in a hypoxic environment had no effect on Cr(III) DNA binding levels (data not shown).

3.7. Hypoxia-sensitive ICL formation is unique to Cr, but not another inorganic DNA interstrand crosslinking agent, cisplatin

The formation or reactivity of a Cr(III)-DNA binding complex was found to be dependent upon the presence of oxygen (Fig. 7). In order to separately confirm these findings using a different assay, and to determine whether hypoxia was modifying the general reactivity of DNA, we compared the formation of ICLs with equimolar concentrations of Cr(III) and another inorganic crosslinking agent, cisplatin (CDDP), under ambient and hypoxic conditions. As can be seen in Fig. 8, the levels of CDDP–ICLs were unaltered by hypoxia following 120 min incubation (Fig. 8). In contrast, the percentage of DNA containing Cr(III)-ICLs under hypoxia was approximately half that compared to ambient conditions.

4. Discussion

Much attention has centered on the potential involvement of an ROS-mediated mechanism in Cr genotoxicity. Despite this interest, the contribution of oxygen, including ROS, to the mutagenicity and carcinogenicity of Cr(VI) is

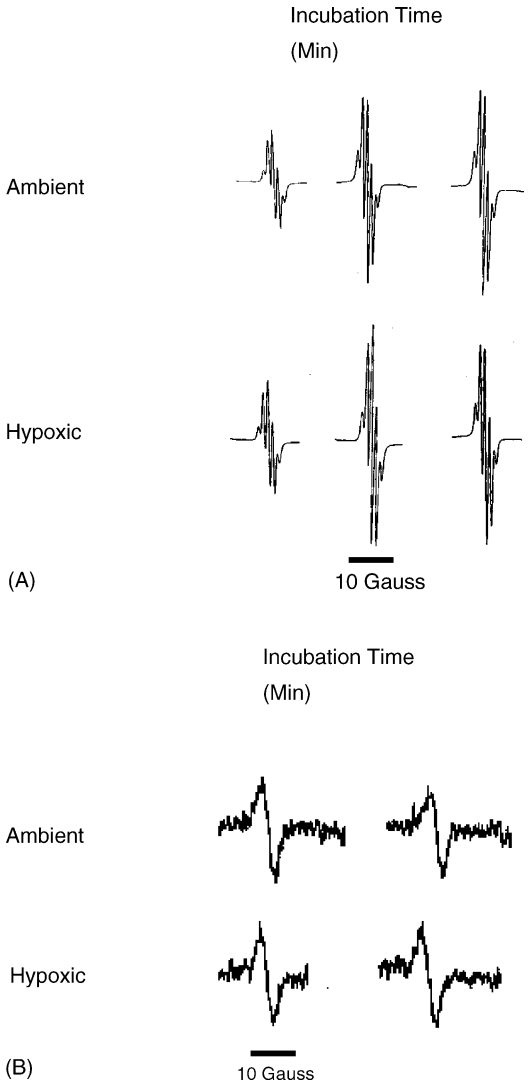

Fig. 6. Hypoxia has no effect on the generation of hypervalent Cr(V). Reactions were carried out with Cr(VI) (2 mM) and Asc (1 mM) in the presence of mannitol (50 mM) (A) or with Cr(VI) (20 mM) and Asc (10 mM) in the absence of mannitol (B) for the indicated time points in HEPES buffer at 37 °C. Shown are representative spectra of at least two independent experiments. Note that the gain in (B) is 100-fold greater than in part (A); bar ≈ 10 G.

still not clear. Because certain ROS-induced lesions (DNA strand breaks, oxidized bases, abasic sites) may potentially interfere with DNA replication in vitro, the aim of this investigation was initially to determine whether DNA damage produced by the Asc-mediated reduction of Cr(VI) displays oxygen-dependence. Interestingly, hypoxia inhibited the formation of replication-arresting lesions by reducing the rate of Cr-DNA adduct formation independent of alterations in Cr(VI) reduction or Cr(V) generation. Taken together, these results illustrate the importance of oxygen, not in damaging DNA directly via a Fenton-like reaction with Cr, but in generating DNA-reactive Cr species.

Under the conditions employed in this study, Cr(VI) metabolism would have likely followed an initial one-electron reduction pathway involving Cr(V). In support

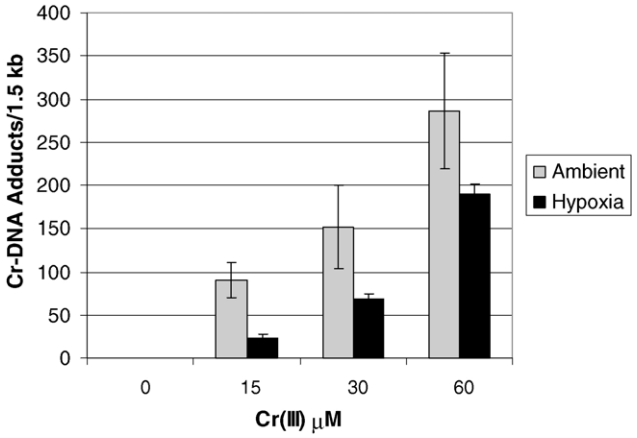

Fig. 7. Cr(III)-DNA interaction is impeded by hypoxia. Plasmid DNA was treated with the indicated concentrations of ^{51}Cr(III) for 2 h 37 °C under ambient (gray columns) or hypoxic (black columns) conditions. The data are the mean values ± standard deviation from two independent experiments.

of this, we have previously showed that mannitol, a hydroxyl radical scavenger and Cr(V) trapper [40,41], is an effective inhibitor of Cr-DNA adduct formation in reactions containing Cr(VI)/Asc, but not Cr(III) [37]. Hypoxia had no effect on the reduction of Cr(VI) or the generation of intermediate Cr(V) species (Figs. 1 and 6). These findings are in-line with previous work in which the generation of DNA single strand breaks by Cr(VI)/GSH [42] and the oxidation of formate by Cr(VI)/Asc [43] were found to depend upon molecular oxygen. In those studies, the generation of Cr(V) was unaffected by conditions of low-oxygen, which is consistent with our data. However, the role of oxygen in the formation of Cr-DNA adducts was not investigated in either study. It should be noted that the collective work by several other groups suggests that certain oxygenated Cr(V) complexes (Cr(V) superoxo/peroxo) exhibit a greater reactivity with DNA than non-oxygenated Cr(V) species [12,44–47]. Thus, while the net production of hypervalent Cr species is unaffected by hypoxia, we cannot exclude the possibility that the hypoxia-mediated inhibition of Cr-DNA binding could be at least partially due to the decreased formation of certain DNA reactive oxo-Cr(V) species.

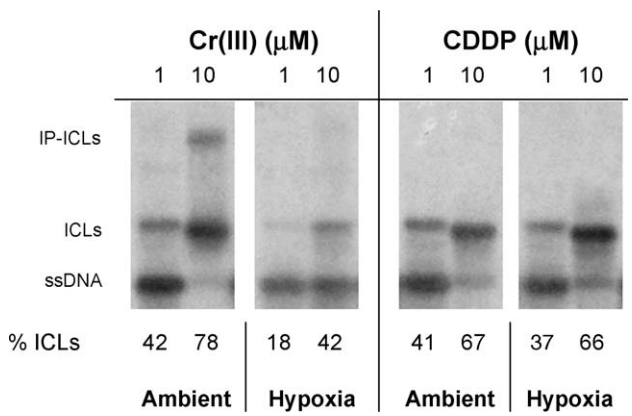

Fig. 8. Hypoxia impedes Cr(III), but not cisplatin (CDDP), ICL development. Plasmid DNA was reacted with either Cr(III) or CDDP (1 or 10 μM each) for 2 h at 37 °C under ambient or hypoxic conditions. ICLs were detected by RAGE as described Section 2. Autoradiograms representative of two experiments illustrating the presence of ICLs in Cr(III) (gels on left) or CDDP (gels on right)-reacted DNA. IP-ICLs: interplasmid ICLs.

A surprising finding of this work was the abrogation of Cr(III)-DNA binding by hypoxia (Figs. 5 and 8). This was not due to an alteration in the general reactivity of DNA because the generation of CDDP-induced ICLs was unaffected by hypoxia (Fig. 8). Cr-DNA binding is believed to consist of ionic electrostatic interactions of Cr(III) with the phosphodiester backbone and coordinate covalent complexation with DNA bases. It is unclear whether hypoxia differentially impacted the formation of either of these binding modes in this study. Earlier work by Hneihen et al. [48] found that the reactivity of Cr(III) complexes (aquo and amino acid) was highest for aquo-Cr(III) compounds and, specifically, for those containing ligands that were more labile (i.e. $CrCl_3{\cdot}6H_2O$, $Cr(NO_3)_3{\cdot}9H_2O$). With regard to the $CrCl_3{\cdot}6H_2O$ used in this study, the initial species present in solution is *trans*-$[Cr(H_2O)_4Cl_2]^+$ [49] which is capable of reacting, electrostatically, with the phosphodiester DNA backbone and can presumably participate in coordinately covalent Cr-DNA adducts [48,50]. The extreme reactivity of this aquo-Cr(III) complex towards DNA is thought to be related to the facile lability of the water and chloride ligands [48]. In addition, at pH ∼ 7.0, the aquo ligands of this complex may deprotonate to hydroxyl groups and result in olation of the Cr(III) complexes [49]. However, it is likely that the interaction between HEPES and Cr(III) might preclude the hydrolysis of Cr(III). It remains to be tested whether hypoxia impacts the lability or deprotonation of the ligands in the aquo-Cr(III) complex, either one of which could alter Cr(III) polymerization and binding to DNA. With regard to Cr(III), and possibly Cr(VI)/Asc, we note that the transient redox cycling of Cr(III) ⇆ Cr(II), in an oxygen-dependent manner, might also explain the decreased binding of Cr to DNA under hypoxia. Such a mechanism has been proposed for DNA strand breakage by Cr(III)–picolinate complexes [51].

It was interesting that the generation of Cr(III), but not CDDP, ICLs was sensitive to hypoxia. CDDP has unique coordination geometry, acid–base properties and ligand lability that allow this metal to bind to the purine bases of DNA. This is primarily a consequence of the Jahn–Teller distortion of square planar Pt(II) that significantly lowers the d_{z^2} orbital energy of this complex. Cisplatin binds primarily to the lone pair electrons at the N^7 of guanine. This electron pair on guanine is delocalized into the purine ring, making it a poor ligand for the binding of most metals. However, the lowered d_{z^2} orbital energy induced by the Jahn–Teller distortion in cisplatin allows coordinative bonding to this poor ligand as well as increasing the ligand lability. In contrast, Cr(III) has octahedral geometry and hard acid/base properties that make it considerably more

oxophilic than cisplatin and also result in slower ligand exchange kinetics. Octahedral Cr(III) does not have the Jahn–Teller distortion of Pt(II) and, not surprisingly, shows poorer binding to nucleic acid bases, which is consistent with its lower mutagenicity [52].

The role that molecular oxygen plays in the Cr genotoxicity is still controversial but has been traditionally been attributed to the formation of ROS. An alternative to activation of molecular oxygen to an oxygen radical species is the known propensity for oxygen to undergo an addition reaction at carbon-based radicals [53,54]. These pathways are well documented for oxidation reactions occurring at the deoxyribose sugar and the nucleic acid bases for a number of antibiotics, metal complexes, and metalloenzymes [20,24,55,56]. Depending upon whether these oxidation reactions occur under an aerobic environment or under conditions of hypoxia, a different subset of DNA cleavage products is observed [53–56]. Based on the current and previous studies on the binding and crosslinking of Cr to DNA, we propose a concerted oxidation/binding mechanism to explain the time-sensitivity (2 h versus 20 h) of our hypoxia-dependent results using Cr(VI) and Asc. Under ambient conditions, an initial DNA oxidation event, by either high valent chromium or an oxygen-based radical, generates a DNA lesion that can act as a Cr chelator. Oxidation of the nucleic acid base guanine, for instance can lead to the formation of a ring opened species such as imidazalone or 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G) that has less electron delocalization and thus is a better ligand for Cr(III) binding. Consistent with this we do observe the generation of *E. coli* formamidopyrimidine-DNA glycosylase (Fpg)-sensitive sites on plasmid DNA damaged by Cr(VI)/Asc (data not shown), but not Cr(III) alone, in HEPES buffer [57]. In addition, an oxygen-dependent pathway might also involve an initial oxidation event occurring at the deoxyribose sugar to form a terminal phosphate moiety. While Cr(III) binds weakly and transiently to oxygens in the phosphodiester backbone of DNA [58], the formation of a terminal phosphate at the site of a frank strand break in DNA may result in a significantly stronger Cr(III) coordination complex at this site. However, we have previously failed to observe any induction of DNA strand breaks by Cr(VI)/Asc or Cr(III) [37]. Nevertheless, either of these scenarios demonstrate how an initial oxidation event on DNA during the reduction of Cr(VI), followed by an oxygen addition to the DNA radical, may yield sites on DNA that are amenable towards chelation by Cr(III). The fact that the high valent oxidation states of Cr can induce this type of DNA damage and display significantly greater ligand lability than Cr(III) would support this type of mechanism applying more likely to reactions containing Cr(VI)/Asc rather than Cr(III).

The data from this investigation have identified at least two potential steps in the process of Cr-DNA adduct formation that require the presence of molecular oxygen. One mechanism suggests that the generation of transient, but highly DNA reactive, hypervalent oxo-Cr species (i.e. Cr(V), Cr(IV)), might display a dependence upon oxygen. However, the marked inhibition of Cr(III)-DNA adduct formation by hypoxia (Fig. 6) strongly indicates that an additional role of oxygen in facilitating the formation of Cr-DNA adducts occurs at the level of Cr(III). In support of this, there are several factors (i.e. Cr(III)-ligand lability, DNA radical formation, Cr(III) ⇆ Cr(II) redox-cycling) that influence the interaction of Cr(III) with DNA which might function at a slower rate under conditions of reduced oxygen. It should be noted that the amount of oxygen under our hypoxic conditions was less than 1%, but not absolute; thus, it is possible, but not likely, that, given enough time, small amounts of residual oxygen may be sufficient to facilitate Cr-DNA binding. All of these proposed scenarios would at least partially explain the temporal nature of the oxygen-dependent formation of Cr-DNA adducts (Fig. 5). Additional studies are required to elucidate the relative importance of these additional mechanisms in the oxygen-dependent formation of Cr-DNA adducts.

Although a complex array of DNA reactive species is produced as a consequence of Cr(VI) reduction, the direct interaction of Cr(III) with DNA is critical for the formation of lesions (i.e. ICLs) capable of obstructing DNA replication. The work presented here lends further support for a non-ROS related role of molecular oxygen in the metal-dependent genotoxicity of Cr by facilitating the interaction between Cr and DNA. These data imply that, in addition to possibly affecting the formation of DNA-reactive oxo-Cr complexes, the rate of direct coordinate complex formation of Cr(III) with DNA represents an additional oxygen-sensitive step in the formation of Cr-DNA adducts. This work adds important information to our understanding of the biochemical factors involved in the formation of Cr-DNA adducts and, accordingly, provides novel insight into the molecular mechanisms of Cr(VI)-induced carcinogenesis.

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