Chromium(VI) Enhances (±)-*anti*-7*β*,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-Induced Cytotoxicity and Mutagenicity in Mammalian Cells through Its Inhibitory Effect on Nucleotide Excision Repair†

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Received July 7, 2004; Revised Manuscript Received August 30, 2004

ABSTRACT: Chromium(VI) [Cr(VI)], a ubiquitous environmental contaminant, is a well-known carcinogen to both humans and experimental animals, although it is a weak mutagen by itself. Occupational exposure to Cr(VI) is strongly associated with a high incidence of lung cancer, but the underlying mechanisms remain unclear. Tobacco smoking is the major cause of lung cancer, and polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke are the major etiological agents. Since humans are frequently exposed to both Cr(VI) and PAHs, it is possible that Cr(VI) and PAHs have a synergistic effect on mutagenecity and cytotoxicity that contributes to the high incidence of lung cancer associated with exposure to both agents. In this study, we tested this possibility by determining the effect of Cr(VI) exposure on (±)-*anti*-7*β*,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE, an active metabolite of PAHs) induced cytotoxicity, mutagenicity, and DNA adduct formation in Chinese hamster ovary (CHO) cells. Using the adenine phosphoribosyltransferase (*APRT*$^+$) → *APRT*$^-$ forward mutation assay, we found that while Cr(VI) alone induced low mutation frequency, it greatly enhanced BPDE-induced mutations in nucleotide excision repair (NER)-proficient CHO cells. Cr(VI) exposure also greatly enhanced BPDE-induced killing in NER-proficient cells. It is known that the cytotoxicity and mutagenicity of BPDE are mainly caused by the formation of DNA adduct, which are removed by NER. To test the possibility that the enhancement of cytotoxicity and mutagenicity by Cr(VI) is caused by the inhibition of NER, NER-deficient cells were used, and the enhancement effects of Cr(VI) were not observed in those cells. We further found that while Cr(VI) exposure does not change the total BPDE−DNA adduct formation, it significantly inhibited the repair of BPDE−DNA adducts from genomic DNA in NER-proficient cells. Using a host cell reactivation assay, we found that the repair of BPDE−DNA adduct in a luciferase reporter gene is greatly inhibited after Cr(VI) exposure in NER-proficient cells while not in NER-deficient cells. Together these results clearly demonstrate that Cr(VI) exposure can greatly enhance the mutagenicity and cytotoxicity of PAHs by inhibiting the cellular NER pathway, and this may constitute an important mechanism for Cr(VI)-induced human carcinogenesis.

Chromium(VI) [Cr(VI)]-containing compounds are well-known carcinogens in both humans and animal models (*1*−*3*). These compounds are widespread in cigarette smoke, automobile emissions, and in the environment. The compounds are also commonly used in the chemical industry, artistic paints, anticorrosion paints, electroplating, and stainless steel welding (*1*, *4*, *5*). It is estimated that several million workers are potentially exposed to high levels of Cr(VI)[1] (*1*, *6*). There are also concerns about Cr(VI) exposure by the general population through consumption of Cr(VI)-contaminated drinking water (*7*) and by other routes (*8*, *9*). It has been shown that the lower respiratory tract is the target organ of Cr(VI)-containing compound exposure, and chromium accumulation in lung tissue is found in workers with occupational exposure to Cr(VI) and in cigarette smokers. Epidemiological studies have consistently shown that occupational exposure to Cr(VI) is strongly associated with a higher incidence of lung cancer (*1*, *4*, *10*−*12*). Cr(VI) has been shown to cause various forms of genetic damage, such as cell transformation, chromosomal aberration, sister-chromatid exchange, DNA strand breaks, DNA−DNA cross-links, and DNA−protein cross-links (*1*−*4*, *12*−*17*). However, Cr(VI), by itself, has been shown to be a weak mutagen in various bacterial and mammalian cell systems (*1*−*3*), and

† This work was supported by Public Health Service grants ES03124, ES10344, ES00260, and CA99007.

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[1] Abbreviations: BPDE, (±)-*anti*-7*β*,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; Cr(VI), chromium(VI); PAH, polycyclic aromatic hydrocarbon; NER, nucleotide excision repair; 8-AA, 8-azaadenine; APRT, adenine phosphoribosyltransferase; CHO, Chinese hamster ovary.

10.1021/bi048560o CCC: $27.50
Published on Web 10/16/2004

the underlying molecular mechanisms of Cr(VI)-induced carcinogenesis remain unclear.

Cigarette smoking is known to be the major cause of human lung cancer, with up to 90% of lung cancer deaths attributable to cigarette smoking (*18*, *19*). It has been suggested that polycyclic aromatic hydrocarbons (PAHs), carcinogens present in cigarette smoke and in all products of combustion of organic matter, contribute greatly to the initiation and development of lung cancer (*18*−*20*). Previously, we have shown that PAHs can form DNA adducts preferentially at mutational hotspots in the *p53* and *K-ras* genes in smoking-related lung cancer, and DNA adducts formed at these sites are poorly repaired (*21*−*25*). These results provide strong molecular evidence linking PAHs with lung carcinogenesis.

Humans are frequently exposed to both Cr(VI) and PAHs under both occupational and environmental conditions (*26*). Epidemiological studies have also shown that exposure to Cr(VI) increases the incidence of lung cancer in cigarette smokers (*27*−*31*). It has been reported that the lung cancer morbidity rate for ex-chromate workers with 9 or more years of exposure is 21.6 times higher than that of nonsmokers (*29*). Together these results raise the possibility that Cr(VI) and cigarette smoke may have a synergistic effect on lung carcinogenesis. However, the effect of Cr(VI) exposure on the mutagenicity and cytotoxicity of PAHs in mammalian cells remains unclear. To address this question, in this study we used the adenine phosphoribosyltransferase (*APRT*$^+$) → *APRT*$^-$ forward mutation system to detect the effect of Cr(VI) exposure on the mutagenesis induced by (±)-*anti*-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene (BPDE), a major activated metabolite of PAHs in cigarette smoke, in Chinese hamster ovary (CHO) cells. We found that Cr(VI) preexposure greatly increased the frequency of BPDE-induced *APRT*$^+$ → *APRT*$^-$ mutations in nucleotide excision repair (NER)-proficient but not in NER-deficient CHO cells. We also found that Cr(VI) preexposure greatly enhanced BPDE-induced cytotoxicity in NER-proficient but not in NER-deficient CHO cells. We further confirmed that Cr(VI) preexposure caused a significant decrease in the removal of BPDE-adduct from genomic DNA and in the repair of a transfected BPDE-damaged reporter gene in NER-proficient cells. NER is the most important DNA repair pathway to repair various kinds of bulky DNA damage and plays important roles in human carcinogenesis and other disease processes (*32*, *33*). Our results clearly demonstrate that Cr(VI) exposure causes the inhibition of NER, which consequently enhances carcinogen-induced mutagenicity and cytotoxicity greatly in mammalian cells. We propose that the inhibition of NER is an important mechanism for Cr(VI)-induced human carcinogenesis.

MATERIALS AND METHODS

Cell Culture and Cr(VI) Exposure. The NER-deficient CHO cell line UVL-1 (ERCC2 mutant) was derived from NER-proficient CHO AT3-2 cells (*34*). Both cell lines contain a single functional APRT allele. Cells were grown in minimal essential medium α-modification supplemented with 10% fetal bovine serum. Cells at 50% confluence in 150-mm dishes were treated with different concentrations of potassium chromate (K_2CrO_4, Sigma, Saint Louis, MO) at 37 °C for 48 h. After treatment, K_2CrO_4 was removed, and the cells were incubated in fresh culture medium.

BPDE Treatment. Cells with or without K_2CrO_4 preexposure were treated with different concentrations of BPDE or [^{3}H]BPDE (2210 mCi/mmol) (Chemsyn Science Laboratories, Lenexa, KS) in medium without serum, at 37 °C for 30 min in the dark, as described previously (*35*). After treatment, BPDE was removed, and cells were incubated in fresh culture medium for different time periods.

Colony Formation Ability Assay and APRT$^+$→*APRT* $^-$ *Forward Mutation Assay.* Logarithmically growing NER-proficient AT3-2 cells and NER-deficient UVL-1 cells were rinsed with phosphate-buffered saline (PBS) (68 mM NaCl, 1.94 mM KCl, 1.07 mM KH_2PO_4, pH 7.4) and then subjected to the following treatments: (1) various concentrations of K_2CrO_4 for 48 h, (2) various concentrations of BPDE in serum-free medium at 37 °C for 30 min in the dark, or (3) K_2CrO_4 at 37 °C for 48 h, rinsing with PBS to remove K_2CrO_4, and then BPDE treatment as described above. For colony formation ability assay, cells after treatment were rinsed with PBS, trypsinized, seeded (300 cells/dish) in fresh culture medium, and incubated in a CO_2 incubator at 37 °C. After 9 days of incubation, the colonies were fixed with methanol, stained with crystal violet, and counted. Colony formation ability was calculated on the basis of the plating efficiency of treated cells versus the plating efficiency of untreated control cells.

For the *APRT*$^+$ → *APRT*$^-$ mutation assay, cells after treatment were incubated in regular medium for a 3-day period to allow phenotypic expression. The cells were then rinsed with PBS, trypsinized and either seeded at a density of 200,000 cells/dish in medium containing 0.6 mM 8-aza-adenine (8-AA), which is toxic only to cells containing a functional *aprt* gene, to select 8-AA-resistant *APRT*$^-$ mutant colonies or seeded at a density of 300 cells/dish in regular medium for determining colony formation ability as described above. After 3 weeks, the 8-AA-resistant mutant colonies were fixed with methanol, stained with crystal violet, and counted. The mutation frequency (MF) was calculated based on MF = number of *APRT*$^-$ mutants/number of clonable cells.

Measurement of Total BPDE−*DNA Adduct Formation and Repair in Genomic DNA.* To determine the effect of Cr(VI) preexposure on BPDE−DNA adduct formation and repair, NER-proficient AT3-2 cells with and without preexposure to K_2CrO_4 (0.5 μM) for 48 h were treated with 1.5 μM [^{3}H]-BPDE (2210 mCi/mmol) at 37 °C for 30 min in the dark. Cells were either harvested immediately or further incubated in fresh medium for various time periods to allow DNA repair to take place. Genomic DNA was then isolated as described previously (*36*). Briefly, cells were lysed with lysing buffer (0.5% SDS, 10 mM Tris, pH 7.5, 10 mM NaCl, 100 μg/mL proteinase K) at room temperature for 2 h. RNA was removed by treatment with RNase A (50 μg/mL) at 37 °C for 1 h followed by repeated phenol and diethyl ether extractions. The genomic DNA was ethanol precipitated and dissolved in TE (10 mM Tris, pH 7.5, 1 mM EDTA). A known quantity of purified genomic DNA was mixed thoroughly with LSC cocktail (Fisher Scientific Co., Pittsburgh, PA), and the amount of ^{3}H radioactivity in the DNA was measured with a 1219 RACKBETA scintillation counter (LKB Wallac, Turku, Finland). The number of BPDE−DNA

adducts in a 10-kb genomic DNA fragment was calculated on the basis of the ^3H specific activity of the DNA.

Host Cell Reactivation Assay. The pGL3-luciferase reporter plasmids containing a coding region for firefly luciferase (Catalog number E1741) and the pSV-β-galactosidase control vector containing the bacterial *lacZ* gene which codes for β-galactosidase were purchased from Promega (Madison, WI). Purified plasmids were modified with BPDE as previously described (*37*). Briefly, for BPDE treatment, purified plasmid DNA was modified with 15 μM BPDE at 25 °C for 2 h, and the reactions were stopped by repeated phenol and diethyl ether extractions to remove the unreacted BPDE. DNA was then ethanol precipitated and dissolved in TE buffer. NER-proficient AT3-2 cells and NER-deficient UVL-1 cells were plated in triplicate in 60 mm dishes at a density of 3×10^5 cells/dish and exposed to various concentrations of K_2CrO_4 at 37 °C for 48 h. Cells were then rinsed with PBS, and transfected with 2 μg of pGL3-luciferase reporter plasmid modified with BPDE using the FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN). The untreated pSV-β-galactosidase control vector (0.5 μg) was cotransfected into human cells as an internal control to normalize transfection efficiency. After transfection for 3 h, medium containing the transfection mixture was removed and cells were incubated in fresh complete medium for another 24 h. The cells were then lysed with 600 μL of Reporter Lysis Buffer (Promega). Transient expression of luciferase was determined by mixing 50 μL of cell extracts with 100 μL of luciferase assay reagent (Promega) and measuring the light emission with a luminometer (Wallac 1420 Victor 2 multilable counter system, Gaithersbery, MD). Transient expression of β-galactosidase was determined using a β-galactosidase enzyme assay system (Promega). Values of luciferase expression were normalized to the β-galactosidase control and averaged over the triplicates. Since the reporter gene will not express unless BPDE−DNA adducts are repaired by cells, this assay can be used to detect the repair capacity of the cells. The relative luciferase activity (i.e., reactivation of the damaged plasmids by the host cells) from BPDE-treated pGL3-luciferase reporter plasmids is expressed as a percentage of luciferase activity from untreated pGL3-luciferase reporter plasmids and is used to represent the repair capacity of cells. The relative repair capacity of cells was calculated as the percentage of the relative luciferase activity of the plasmids transfected into K_2CrO_4-exposed cells compared to the activity found in untreated cells.

RESULTS

Cr(VI) Exposure Enhances BPDE-Induced Cell Killing in NER-Proficient but not NER-Deficient CHO Cells. It has been shown that after entering cells, Cr(VI) is reduced progressively to Cr(V), then Cr(IV), and finally to Cr(III), which can interact with amino acids such as cyteine, histidine, and glutathione to form binary products (*15*−*17*). Cr(III) alone or the binary products can interact with DNA to form stable Cr(III)−DNA binary or Cr(III)−amino acid−DNA ternary complexes; these DNA adducts have been shown to be mutagenic (*15*−*17*). To mimic the environmental exposure, we purposely chose low levels of Cr(VI) to treat cells that would cause minimal cell killing and cytotoxicity but still allow for a detectable level of effect on BPDE-induced mutagenesis and DNA repair. NER-proficient AT3-2 cells and NER-deficient UVL-1 cells were treated with various doses of K_2CrO_4 for 48 h, and the colony formation ability was measured. Results in Table 1 show that the colony formation ability of cells treated with Cr(VI) was reduced slightly but in a dose-dependent fashion. There is no statistically significant differences in Cr(VI)-induced cell killing between NER-deficient and NER-proficient cells ($P > 0.05$). On the basis of the results presented in Table 1, a subtoxic dosage up to 2 μM K_2CrO_4 was used in the following mutagenesis and repair studies.

To determine the effect of Cr(VI) exposure on BPDE-induced cytotoxicity, both NER-proficient AT3-2 cells and NER-deficient UVL-1 cells with and without exposure to different concentrations of Cr(VI) for 48 h were treated with two concentrations of BPDE for 30 min, and the colony formation ability of these cells was then measured. This sequence of treatment was chosen because the uptake of Cr(VI) by cultured cells is a slow process; a 48 h exposure time is needed to ensure that the Cr(VI) uptake by cells has plateaued. Results in Figure 1A show that NER-deficient

Table 1: Cytotoxicity of K_2CrO_4 in NER-Proficient AT3-2 and NER-Deficient UVL-1 Cells[a]

	colony formation ability (% of control (ISD)	
$[K_2CrO_4]$ (μM)	NER-proficient (AT3-2)	NER-deficient (UVL-1)
0	100	100
0.1	97 ± 6	95 ± 6
0.5	93 ± 5	90 ± 4
1	91 ± 4	85 ± 6
1.5	87 ± 5	82 ± 4
2	85 ± 4	79 ± 5

[a] Logarithmically growing cells were treated with K_2CrO_4 for 48 h, trypsinized, and reseeded for the determination of the colony formation ability. The data represent three independent experiments. The statistical difference between NER-proficient AT3-2 and NER-deficient UVL-1 cells is $P > 0.05$.

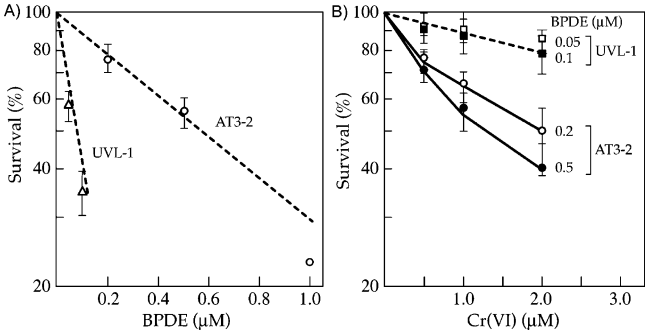

FIGURE 1: Effect of K_2CrO_4 preexposure on the BPDE-induced cell killing. NER-proficient AT3-2 cells and NER-deficient UVL-1 cells without (A) and with (B) preexposure to different concentrations of K_2CrO_4 for 48 h were treated with BPDE (0.2, 0.5, and 1 μM for AT3-2 cells and 0.05 and 0.1 μM for UVL-1 cells) for 30 min at 37 °C, and their colony-forming abilities were measured as described in Materials and Methods. The survival (%) in panel A is expressed as a percentage of colony formation ability of BPDE-treated cells from untreated cells, while the survival (%) in panel B is expressed as a percentage of colony formation ability of cells with K_2CrO_4 and BPDE combined treatment from cells with BPDE treatment alone. The data represent three independent experiments. The statistical difference between AT3-2 cells with and without K_2CrO_4 exposure is $P < 0.01$.

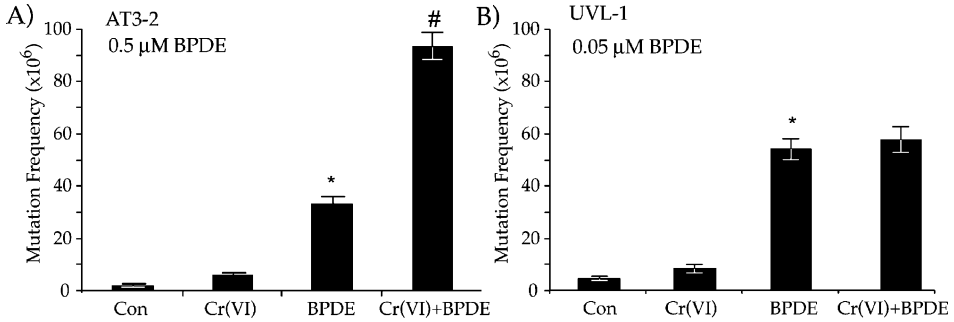

FIGURE 2: K_2CrO_4 preexposure synergistically enhances BPDE-induced *APRT*$^-$ mutations in NER-proficient cells but not in NER-deficient cells. NER-proficient AT3-2 (A) and NER-deficient UVL-1 (B) cells were preexposed with or without 0.5 μM K_2CrO_4 for 48 h followed by treatment with or without BPDE (0.5 μM for AT3-2 cells and 0.05 μM for UVL-1 cells) for 30 min. The mutation frequency of the *APRT*$^-$ gene was then measured as described in Materials and Methods. The data represent three independent experiments. $*$: the statistical difference between cells with and without BPDE treatment is $P < 0.01$. #: the statistical difference between AT3-2 cells with K_2CrO_4 and BPDE combined treatment and with BPDE treatment alone is $P < 0.01$.

UVL-1 cells are much more sensitive to BPDE-induced cell killing than NER-proficient AT3-2 cells. These results are consistent with the findings that NER is the major pathway for repair of BPDE−DNA adducts in mammalian cells (*38*, *39*). Interestingly, in NER-proficient AT3-2 cells preexposure to Cr(VI) causes much more severe BPDE-induced cell killing compared with cells without Cr(VI) exposure (Figure 1B). However, this enhanced killing effect induced by Cr(VI) preexposure is not observed in NER-deficient cells (Figure 1B). Since NER is the major pathway for repair of BPDE−DNA adducts, these results suggest that Cr(VI) exposure reduces NER efficiency.

Cr(VI) Exposure Enhances the Frequency of BPDE-Induced APRT$^+ \rightarrow$ *APRT*$^-$ *Forward Mutations in NER-Proficient but Not NER-Deficient CHO Cells.* To determine the effect of K_2CrO_4 preexposure on the BPDE-induced mutagenicity, *APRT*$^+ \rightarrow$ *APRT*$^-$ forward mutants in both NER proficient AT3-2 cells and NER-deficient UVL-1 cells were measured after preexposure to K_2CrO_4 for 48 h followed by BPDE treatment for 30 min. The results were then compared to cells treated with K_2CrO_4 for 48 h only or treated with BPDE for 30 min only. Thus, 0.5 μM Cr(VI) was chosen to treat both types of cells, because it induced similar cytotoxicity (≈90% survival) in both NER-proficient and -deficient cells. Then, 0.5 and 0.05 μM BPDE were chosen to treat NER-proficient and -deficient cells, respectively (≈56% survival) for the same reason. As shown in Figure 2, the spontaneous mutation frequency of *APRT*$^+ \rightarrow$ *APRT*$^-$ in NER-proficient AT3-2 cells is $(1.5 \pm 0.3) \times 10^{-6}$, and $(4.2 \pm 0.9) \times 10^{-6}$ in NER-deficient UVL-1 cells; Cr(VI) treatment induces *APRT*$^-$ mutations at the frequency of $(6.2 \pm 0.8) \times 10^{-6}$ and $(8.7 \pm 1.6) \times 10^{-6}$ in NER-proficient AT3-2 and NER-deficient UVL-1 cells, respectively. These results show that Cr(VI) induces a low frequency of mutation in both NER-proficient and NER-deficient cells, which is consistent with previous reports that Cr(VI) itself is a weak mutagen (*1*−*3*). In contrast, 0.5 μM BPDE treatment induces *APRT*$^-$ mutation frequency at $(33.4 \pm 2.7) \times 10^{-6}$ in NER-proficient AT3-2 cells, and 0.05 μM BPDE induces *APRT*$^-$ mutation frequency at $(54.8 \pm 4.2) \times 10^{-6}$ in NER-deficient UVL-1 cells, indicating that BPDE is much more mutagenic in NER-deficient cells than in NER-proficient cells. Interestingly, while Cr(VI) preexposure greatly enhances BPDE-induced mutation frequency [from $(33.4 \pm 2.7) \times 10^{-6}$ to $(93.7 \pm 5.2) \times 10^{-6}$] in NER-proficient AT3-2 cells, it does not change BPDE-induced mutation frequency significantly in NER-deficient UVL-1 cells [from $(54.8 \pm 4.2) \times 10^{-6}$ to $(57.9 \pm 5.1) \times 10^{-6}$]. These results demonstrate that Cr(VI) exposure can significantly enhance the mutagenicity of BPDE in NER-proficient cells but has no effect on NER-deficient cells and strongly suggest that the enhancement of BPDE-induced mutagenesis by Cr(VI) is the result of inhibition of the NER of BPDE−DNA adducts by Cr(VI).

Cr(VI) Preexposure Inhibits the Repair of BPDE−DNA Adducts from Genomic DNA in NER-Proficient CHO Cells. It is known that the cytotoxicity and mutagenicity of BPDE is mainly caused by the formation of BPDE−DNA adduct, which is removed by NER in mammalian cells (*32*, *33*, *38*−*40*). The findings that Cr(VI) preexposure can enhance BPDE-induced cytotoxicity and mutagenicity in NER-proficient cells but not in NER-deficient cells strongly suggest that the enhancement effect by Cr(VI) is the result of inhibition of the NER of BPDE−DNA adducts by Cr(VI). To further test the possibility that Cr(VI) inhibits the repair of BPDE−DNA adduct, NER-proficient AT3-2 cells with and without Cr(VI) preexposure (0.5 μM for 48 h) were treated with 1.5 μM [^{3}H]BPDE for 30 min, and the removal of [^{3}H]BPDE−DNA adducts from genomic DNA in cells at different incubation times was determined. Although the initial total BPDE−DNA adducts formed at 0 h after BPDE treatment in cells with and without Cr(VI) preexposure was very similar (≈0.55 adduct/10 kb), Cr(VI) preexposure significantly reduced the repair efficiency of BPDE−DNA adducts; after 24 h of incubation only 55% of the BPDE−DNA adducts were removed in cells with Cr(VI) preexposure, while over 80% of the BPDE−DNA adducts were removed in cells without Cr(VI) preexposure (Figure 3). These results clearly demonstrate that while Cr(VI) preexposure does not affect the efficiency of BPDE−DNA adduct formation, but it greatly inhibits the repair of BPDE−DNA adducts in mammalian cells.

Cr(VI) Preexposure Inhibits the Repair of BPDE−DNA Adducts from Reporter Gene in NER-Proficient but Not NER-Deficient Cells. To further confirm the inhibitory effect Cr(VI) on NER repair, a host cell reactivation assay was performed to determine the repair capacity for BPDE−DNA adducts in both NER-proficient AT3-2 cells and NER-deficient UVL-1 cells. Cells treated with various concentrations of Cr(VI) for 48 h at 37 °C were transfected with the

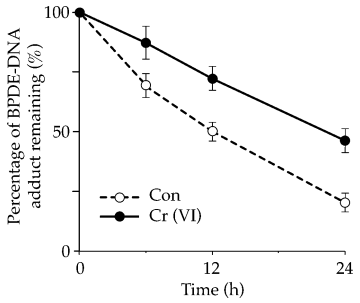

FIGURE 3: Effect of K_2CrO_4 preexposure on BPDE−DNA adduct repair in NER-proficient AT3-2 cells. AT3-2 cells with (solid line) or without (broken line) preexposure to 0.5 μM K_2CrO_4 for 48 h were treated with 1.5 μM [^{3}H]BPDE for 30 min at 37 °C, followed by incubation in fresh medium for various times. Cells were harvested, genomic DNA was isolated, and the amount of BPDE−DNA adducts in the genomic DNA was determined as described in Materials and Methods. The data represent three independent experiments. The statistical difference between cells with and without K_2CrO_4 exposure at each time point is $P < 0.01$.

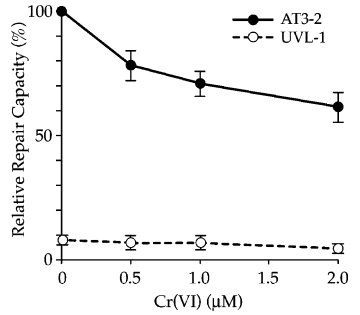

FIGURE 4: Effect of K_2CrO_4 preexposure on host cell reactivation of BPDE-damaged luciferase reporter gene in NER-proficient AT3-2 (solid line) and NER-deficient UVL-1 (broken line) cells. BPDE (15 μM)-modified luciferase reporter plasmids (pGL3-luciferase) and unmodified pSV-β-galactosidase gene-containing plasmids (pSV-β-galactosidase) were cotransfected into AT3-2 and UVL-1 cells with or without preexposure to various concentrations of K_2CrO_4 for 48 h. After transfection for 3 h, the cells were incubated in fresh medium for 24 h to allow DNA repair. Cells were then harvested and lysed, and the luciferase activity in the cells was measured. The luciferase activity was first normalized to the β-galactosidase activity. The relative luciferase activity, which represents the relative extent of BPDE−DNA adduct repair under each condition, was determined as the percentage of luciferase activity expressed from BPDE-treated plasmids to that from untreated plasmids. The relative repair capacity was calculated as the percentage of the relative luciferase activity of the plasmids transfected in cells with K_2CrO_4 exposure to that transfected into cells without K_2CrO_4 exposure. The data represent three independent experiments. The statistical difference between AT3-2 cells with and without K_2CrO_4 exposure is $P < 0.01$.

pGL3-luciferase plasmids containing a luciferase reporter gene that had been damaged by BPDE. The luciferase activity, which is proportional to the extent of expression of the reporter gene, was determined 24 h after transfection. Since BPDE−DNA adducts block transcription, the full-length transcript is not produced unless BPDE−DNA adducts in this gene are repaired (*41*−*43*). The luciferase activity is therefore proportional to the extent of BPDE−DNA adduct repair, which in turn reflects the cellular repair capacity of BPDE−DNA adducts (*44*−*46*). The relative repair capacity detected in NER-proficient AT3-2 cells and NER-deficient UVL-1 cells with or without Cr(VI) exposure is presented in Figure 4. These results show that in NER-proficient AT3-2 cells, compared with cells without Cr(VI) exposure, the relative luciferase activity is much lower in cells with Cr(VI) exposure, indicating that the repair capacity for BPDE−DNA adducts in cells exposed to Cr(VI) was significantly reduced. Results in Figure 4 also show that the repair inhibition by Cr(VI) is dose-dependent. In NER-proficient AT3-2 cells, compared with cells without Cr(VI) exposure, 0.5 μM Cr(VI) decreases the relative repair capacity to 78 ± 6%, 1 μM Cr(VI) decreases the relative repair capacity to 71 ± 5%, and 2 μM Cr(VI) decreases the relative repair capacity to 62 ± 6%. In Cr(VI)-untreated NER-deficient UVL-1 cells, the relative repair capacity is less than 10% of that in Cr(VI)-untreated NER-proficient AT3-2 cells, which indicates that the NER-proficient AT3-2 cells can efficiently remove the BPDE−DNA adducts from luciferase reporter plasmids while NER-deficient UVL-1 cells do not. Compared with Cr(VI)-untreated UVL-1 cells, a very similar relative repair capacity was detected in NER-deficient UVL-1 cells pre-exposed to various concentrations of Cr(VI). These results further confirm that Cr(VI) exposure inhibits NER capacity in mammalian cells.

DISCUSSION

Chromium and PAHs are environmental contaminants that are also abundant in cigarette smoke (*1*−*3*, *18*−*20*). Cigarette smokers and certain human populations are constantly exposed to both PAHs and Cr(VI). It is thus important to understand the biological effects, such as cytotoxicity, mutagenicity, cell transformation, and carcinogenicity, that result from exposure to both agents. PAHs are both mutagenic and tumorigenic, and ample evidence has demonstrated that the interactions of metabolically activated PAHs with DNA trigger mutagenesis and carcinogenesis (*20*, *21*). Although Cr(VI) by itself is a weak mutagen, it has been long recognized that Cr(VI) is carcinogenic for both humans and animals (*1*−*3*). Chromium accumulation in lung tissue is found in workers with occupational exposure to Cr(VI) and in cigarette smokers, and furthermore, workers with occupational exposure to Cr(VI) have a higher incidence of lung cancer (*10*−*12*, *27*−*29*). Together these results strongly suggest that Cr(VI) contamination is involved in human lung carcinogenesis. In this study, our results clearly demonstrate that Cr(VI) exposure can greatly enhance both BPDE-induced mutagenicity and cytotoxicity in mammalian cells, and we propose that these effects contribute greatly to Cr(VI)-induced lung carcinogenesis.

Several possible mechanisms could account for the enhancement effect of Cr(VI) on BPDE-induced mutagenicity and cytotoxicity. We have found that Cr(VI) preexposure did not affect the total BPDE-DNA adduct formation in either NER-proficient or NER-deficient cells, thus excluding the possibility that the enhancement of BPDE-induced mutageneicity and cytotoxicity by Cr(VI) preexposure is due to changes in the efficiency of BPDE−DNA adduct formation in cells. However, we have shown that Cr(VI) preexposure greatly enhanced BPDE-induced mutagenicity and cytotoxicity and significantly decreased the removal of BPDE−DNA adducts from genomic DNA and the host cell reactivation of BPDE-modified luciferase reporter gene in NER-proficient cells but not in NER-deficient cells. These results clearly demonstrate that Cr(VI) preexposure significantly decreases the cellular NER capacity. Since measuring BPDE−DNA adduct removal from

genomic DNA adducts detects the global genomic repair capacity of cells and the host cell reactivation assay reflects transcription-coupled repair capacity of cells, the results in this study suggest that Cr(VI) preexposure significantly decreases both global genomic repair and transcription-coupled repair, two major NER pathway in mammalian cells (*32*, *33*, *38*). It is worth noting that BPDE-induced mutation frequency in AT3-2 cells is doubled with Cr(VI) exposure while the inhibition of repair capacity in AT3-2 cells with Cr(VI) exposure does not appear to be as dramatic. However, there is no a prior reason that the relationship between adduct-induced mutation frequency and repair capacity has to be linear. Ample evidence shows that DNA damage-induced mutation frequency is monotonically related to DNA damage dose, but this relationship is by no means linear. Since the enhancement of BPDE-induced mutations is only observed in NER-proficient cells but not in NER-deficient cells, the results suggest that Cr(VI) enhances BPDE-induced mutagenicity mainly through inhibition of NER. It is likely that Cr(VI) may affect mechanisms other than NER, but it seems that these effects do not influence BPDE-induced mutations. The results that no Cr(VI)-enhanced mutagenesis of BPDE was observed in NER-deficient cells also exclude the possibility that Cr(VI) preexposure affects the DNA replication fidelity in cells. These results strongly suggest that inhibition of the NER of BPDE adducts by Cr(VI) leads to the enhancement of BPDE-induced mutagenicity and cytotocity in mammalian cells.

It is well-established that the NER is the most important repair pathway in mammalian cells to repair various kinds of bulky DNA damage and plays important roles in human carcinogenesis and other disease processes (*32*, *33*, *47*). Individuals who have genetic defects in NER repair genes, such as xerodema pigmentosum (XP) and Cockayne syndrome (CS) patients, have greatly increased chances of developing cancers (1000−2000-fold increase in skin cancer and 20-fold increase in internal organ cancers incidence) as well as neural and immunological diseases in their lifetimes (*32*, *33*, *47*). The reduced NER capacity of BPDE has been reported in lung cancer patients (*40*), and moreover, individuals carrying certain polymorphisms in the *XPC* gene appear to have a higher incidence of cancer (*48*). These results are consistent with the hypothesis that compromised DNA repair capacity increases susceptibility to cancer. Hence, it is reasonable to propose that individuals constantly exposed to Cr(VI) suffer from reduction of their DNA repair capacity and may have increased susceptibilities to cancers and other disorders. Most recently, it was reported that NER also plays a principal role in the repair of Cr−DNA adducts in human cells, which has been shown to be mutagenic (*49*). If NER is compromised by Cr(VI) and Cr−DNA adducts cannot be repaired efficiently, this may further contribute to the mutagenecity and carcinogenecity of Cr(VI).

The mechanisms of how Cr(VI) inhibits BPDE−DNA adduct repair are yet to be understood, and several possible mechanisms may account for it. Cr(VI) may alter expression of genes involved in NER. For example Cr(VI) causes down-regulation the expression of hOGG1, a glycosylase for 8-oxo-deoxyguanine, in human cells and the lung tissue of rats exposed to Cr(VI) (*50*, *51*). Reduction of Cr(VI) to Cr(III) in cells could lead to the formation of Cr(III)−protein cross-links. It is possible that Cr(III) may form cross-links with NER proteins or proteins involved in NER regulation and thereby impair NER function. It has also been reported that nickel(II) and arsenate(II) can inhibit DNA repair by competing with and displacing magnesium and zinc ions, essential metal ions for DNA repair proteins (*52*, *53*). The magnesium ion mediates DNA−protein interactions in the damage recognition and incision steps and is also a cofactor for DNA polymerases and DNA ligases in the polymerization and/or ligation steps (*53*). Several repair proteins, such as XPA and NEH1, contain zinc fingers, and zinc is thus essential for maintaining the structural integrity of these proteins (*54*−*56*). It has been found that the inhibitory effect of nickel(II) on repair of cyclobutane pyrimidine dimers can be partially reversed by the addition of magnesium (*52*, *53*). Nickel(II) has also been found to be able to displace zinc ions from zinc finger structures (*57*). Whether Cr(VI) inhibits NER by competing with and displacing magnesium and zinc ions, similar to nickel(II), remains to be determined.

In summary, in this study we have clearly demonstrated that Cr(VI) can greatly enhance BPDE-induced mutagenicity and cytotoxicity through the inhibition of NER in mammalian cells. We propose that this inhibition of DNA repair by Cr(VI) may play an important role in Cr(VI)-induced human carcinogenesis, especially human lung carcinogenesis.

ACKNOWLEDGMENT

We wish to thank Dr Chuanshu Huang for technical assistance in measuring the expression of luciferase. We wish to thank Drs. C. Klein and Yen-Yee Tang for their critical review of the manuscript.

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BI048560O