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Heme-oxygenase 1 Gene Expression is a Marker for Hexavalent Chromium-Induced Stress and Toxicity in Human Dermal Fibroblasts

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Several adverse health effects, including irritant and allergic contact dermatitis, have been reported among workers who are occupationally exposed to chromium-containing compounds. Human dermal fibroblasts were used as an *in vitro* experimental model to study the potential mechanisms underlying hexavalent chromium [Cr(VI)]–induced dermal toxicity. Exposure of the fibroblasts to 5μM Cr(VI) (LC50 for a 24-h exposure period) followed by microarray analysis of the gene expression profile revealed overexpression of several genes including those involved in cell stress response. The cellular level of glutathione, the major antioxidant molecule present in the cells, was significantly lower in the Cr(VI)-treated cells compared to the corresponding control cells. The Cr(VI)-induced overexpression of heme-oxygenase 1 messenger RNA (HO-1) in the fibroblasts was significantly blocked by actinomycin D and by inhibitors of MAP kinase pathways. The Cr(VI)-induced cytotoxicity and the overexpression of the HO-1 gene were dependent on the glutathione level of the fibroblasts. Buthionine sulfoximine-mediated GSH depletion resulted in enhanced Cr(VI) cytotoxicity and further overexpression of the HO-1 gene. On the other hand, elevated cellular levels of glutathione resulting from pretreating the cells with GSH significantly protected the cells against the Cr(VI)-induced cytotoxicity and blocked the HO-1 gene's overexpression. Pretreating the fibroblasts with N-acetyl cysteine also significantly reduced the Cr(VI)-induced cytotoxicity and overexpression of the HO-1 gene. In conclusion, depletion of GSH leading to cellular stress is a major mechanism responsible for Cr(VI)-induced cytotoxicity. Furthermore, the expression level of HO-1 gene is a marker for Cr(VI)-induced cell stress leading to cytotoxicity.

Key Words: **hexavalent chromium; cell stress; glutathione; cytotoxicity; heme-oxygenase 1 gene.**

Chromium, considered as an essential trace element, is required for several biological processes in the body (Cohen *et al*., 1993). However, elevated levels of hexavalent chromium in the body, which can occur in conditions of excessive environmental and occupational exposure to certain species (valencies) of chromium, can lead to toxicity and adverse health effects. Several regulatory and nonregulatory agencies, including the International Agency for Research on Cancer (IARC), the U.S. Environmental Protection Agency (USEPA), and the Agency for Toxic Substances and Diseases Registry (ATSDR), consider hexavalent chromium as toxic and carcinogenic to humans (ATSDR, 1993; IARC, 1990; USEPA, 1992).

Various industries, including, welding, chrome plating, chrome pigmenting, leather tanning, and the ferrochrome industry, use large quantities of compounds containing chromium (IARC, 1990). It is estimated that more than 300,000 workers are annually exposed to chromium and chromium-containing compounds in the workplace (ATSDR, 1993). Even though the principal route of human exposure to chromium is through inhalation, and the lung is the primary target organ, significant human exposure to chromium has also been reported to take place through the skin (Costa, 1997; Shelnutt *et al*., 2007). For example, the widespread incidence of dermatitis noticed among construction workers is attributed to their exposure to chromium present in cement (Shelnutt *et al*., 2007).

Considering the potential of Cr(VI) to cause health effects among workers following their occupational dermal exposure to it and the consequent socioeconomic impact, it is important to develop strategies to prevent or to intervene in the dermal toxicity of chromium. Any such attempt would essentially require a proper understanding of the mechanisms underlying the toxicity of chromium. It has previously been shown that lead chromate is cytotoxic to cultured mouse embryo fibroblasts and induces morphological, anchorage-independent, and neoplastic transformation in them (Patierno *et al*., 1988). It has also been shown that Cr(VI) compounds induce strong cytotoxicity to cultured human foreskin fibroblasts (Biedermann and Landolph, 1987, 1990), mutate them to 6-thioguanine

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resistance (Biedermann and Landolph, 1990), and also induce anchorage independence in them (Biedermann and Landolph, 1987, 1990). Cr(III) compounds also kill human fibroblasts and mutate them, but at 100-fold higher concentrations than Cr(VI) compounds (Biedermann and Landolph, 1990). Recent developments in toxicogenomics have demonstrated the potential of microarray studies investigating differential gene expression profiles to elucidate the cellular/molecular mechanisms of chemical toxicity (Hamadeh *et al*., 2002; Thukral *et al*., 2005). Presently, by employing human dermal fibroblasts as an *in vitro* experimental model, we have investigated the molecular/cellular mechanisms potentially underlying hexavalent chromium-induced dermal toxicity. The data presented in this communication demonstrate that induction of stress, as evidenced from a decreased cellular level of glutathione, is a major cellular mechanism responsible for hexavalent chromium-induced toxicity in human dermal fibroblasts. Furthermore, we have demonstrated that the cellular expression level of heme-oxygenase 1 is an indicator of the chromium-induced cell stress leading to toxicity.

MATERIALS AND METHODS

Cell Culture and Cytotoxicity Studies

Mycoplasma-free human dermal fibroblasts (Catalog number CRL 2076, ATCC, Manassas, VA) were cultured in Iscove's modified Dulbecco's medium under standard cell culturing conditions. Exponentially growing fibroblasts were used to determine the lethal concentration 50 (LC50) of hexavalent potassium dichromate ($K_2Cr_2O_7$, abbreviated as Cr(VI), Sigma Chemical Company, St Louis, MO). One hundred thousand cells each were plated in the individual wells of a 96-well cell culture plate for 24 h to allow for formation of a monolayer. A stock solution of Cr(VI) prepared in sterile, double distilled water was further diluted with serum-free medium to get the final concentrations of Cr(VI) ranging from 0 to 50μM. The cells were cultured in control or Cr(VI) containing medium for 24 h. The number of surviving cells at the end of the Cr(VI) exposure period was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay (ATCC) and the LC50 value for Cr(VI) was calculated based on the response of the cells to increasing concentrations of Cr(VI) that resulted in 0–100% cell death.

Gene Expression Profile of Fibroblasts Exposed to Cr(VI)

Microarray analysis of gene expression. Exponentially growing dermal fibroblasts were cultured in T25 cell culture flasks. When the cells were approximately 70% confluent, Cr(VI) was added to the medium at a final concentration of 5μM (LC50, see "Results" section for details) and the cells were further cultured for time intervals of 2, 6, and 24 h. Total RNA, free of contaminating DNA and proteins, was isolated from the cells, using the RNeasy Kit (Qiagen, Inc., Valencia, CA).

The gene expression profile of the cells was determined using the Toxicology and Drug Resistance Oligo GE Array (Catalog number OHS-401, Superarray Biosciences Corporation, Frederick, MD). The GE array contained 263 genes whose expression levels in general are indicative of stress and toxicity. In addition, the array contained several housekeeping genes as well as positive and negative controls for hybridization. A complete list of the genes represented on the array is available at http://www.superarray.com.

Total RNA isolated from the cells was used to synthesize double-stranded complimentary DNA (cDNA) using the One Cycle cDNA Synthesis Kit (Affymetrix, Inc., Santa Clara, CA) following the procedures provided by the manufacturer. The double-stranded cDNA synthesized was purified using the Qiagen MiniElute Reaction Cleanup Kit (Qiagen, Inc.). Biotin labeled cRNA was synthesized from the double-stranded cDNA using EnzoBioarray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Inc., Farmingdale, NY) and was purified using the RNeasy Mini Kit (Qiagen, Inc.). The arrays were prehybridized at 60°C for 4 h, hybridized with the biotin labeled cRNA probes at 60°C overnight, and washed following the guidelines of Superarray Biosciences Corporation. The arrays were incubated with alkaline phosphatase-conjugated streptavidin and the hybridization signals were developed with CDP star chemiluminiscent substrate (Superarray Biosciences Corporation). The images were captured using the Flourchem SP Image System (Alpha Innotech Corporation, San Leandro, CA) and the gene expression profile was analyzed with the GE Array Gene Expression Suite (Superarray Biosciences Corporation).

Real-time PCR analysis of gene expression. Forty-eight genes that exhibited more than 1.5-fold change in their expressions in response to Cr(VI) exposure as evidenced from the microarray data were further analyzed for their differential expressions by quantitative real-time PCR analysis. The PCR amplification, detection of the PCR amplified gene products, and their quantitations were performed with the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). The expression levels of the genes were normalized to that of the housekeeping gene, GAPDH, and were calculated using the formula $2^{(-(\Delta Ct\_target\ -\ \Delta Ct\_GAPDH))}$.

Cr(VI)-Induced Cell Stress and Cytotoxicity Studies

Cellular GSH level. Fibroblasts cultured in six-well cell culture dishes were treated with 5μM Cr(VI) for 6 h and their GSH contents were determined using the GSH/GSSG-412 Kit (Oxis International, Inc., Foster City, CA).

Role of cellular GSH and oxidative stress in Cr(VI)-induced cytotoxicity. Fibroblasts were grown in six-well cell culture dishes. The medium was changed and the cells were allowed to grow in fresh medium containing buthionine sulfoximine (BSO) (25 and 50μM) or GSH (1 and 5mM) for 24 h. The cells were harvested and GSH content was determined using the GSH/GSSG-412 Kit (Oxis International, Inc). In order to determine the role of cellular GSH content and reactive oxygen species (ROS) on the Cr(VI)-induced cytotoxicity, cells were plated at a density of 100,000 cells per well in a 96-well microplate. The cells were subsequently grown in medium containing 50μM BSO, 10mM GSH, or 20mM N-acetyl cysteine (NAC) for 24 h. The medium was removed and the cells were rinsed twice with serum-free growth medium. The cells were grown in medium containing 0, 1, 2.5, 5, or 10μM Cr(VI) for 6 h and cytotoxicity was determined.

Cr(VI)-Induced Cell Stress and HO-1 Gene Expression Studies

HO-1 gene expression in Cr(VI)-treated cells. Fibroblasts grown to 70% confluence in six-well cell culture dishes were treated with 5μM Cr(VI) for 6 h. At the end of the Cr(VI) exposure, total RNA was isolated from the cells using the RNeasy Mini Kit and HO-1 gene expression was determined by quantitative real-time PCR analysis.

Role of cellular GSH level on Cr(VI)-induced HO-1 gene expression. In order to determine the role of cellular GSH level on the Cr(VI)-induced cytotoxicity and overexpression of the HO-1 gene, fibroblasts were grown to 70% confluence in six-well cell culture dishes. The medium was changed and the cells were allowed to grow in fresh medium containing BSO (25 and 50μM) or GSH (1 and 5mM) for 24 h. The cells were rinsed twice with serum-free medium and further incubated with fresh medium containing 5μM Cr(VI) for 6 h. Total RNA was isolated from the cells and HO-1 gene expression was determined by real-time PCR analysis.

Role of GSH in the Cr(VI)-mediated regulation of HO-1 gene expression. The Luciferase Reporter Gene Expression System (Promega Corporation, Madison, WI) was employed to study the role of cellular GSH level on the Cr(VI)-mediated regulation of HO-1 gene expression. Genomic DNA isolated from the fibroblasts (Laird *et al*., 1991) was used as the template to PCR amplify approximately 1.9 kb of the HO-1 gene promoter immediately upstream to the start site of HO-1 gene transcription. The PCR amplification

was carried out using the Advantage GC2 PCR Kit (Clontech Laboratories, Mountainview, CA) and the primers: 5′-AGATCTCCAGGAAAGATCAAC-CAC-3′ (sense) and 5′-AGATCTAGGGTTGGGAGAGAAAGG-3′ (anti-sense). Each PCR cycle consisted of a 30-s melting step at 94°C, a 30-s annealing step at 58°C, and a 180-s extension step at 68°C, for a total of 25 cycles. The PCR product was subcloned into the PCR4-TOPO cloning vector following the standard protocol for the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). The nucleotide sequence of the TA-cloned HO-1 promoter fragment was confirmed by DNA sequencing and by matching the sequence to that available in the NCBI Database. The TA-cloned HO-1 plasmid DNA was digested with *Bgl*II restriction enzyme and the HO-1 gene promoter fragment was subcloned in the pGL3 basic vector. The resulting plasmid DNA, designated as pGL3 basic-HO-1 was used in the transfection experiment to determine the expression of Luciferase reporter gene which was regulated by the HO-1 gene promoter.

Monkey kidney COS7 cells (Catalog number CRL 1651, ATCC), one of the commonly used and well characterized cell lines for studying reporter gene expression following transient transfection (Suzuki *et al*., 2005), were used to determine the role of cellular GSH level on Cr(VI)-mediated regulation of HO-1 gene expression. Fifty thousand cells each were cultured in medium containing 50μM BSO in the individual wells of a 24-well plate and the cells were transiently transfected with the pGL3 basic-HO-1 plasmid DNA using TransFast Transfection Reagent (Promega Corporation). phRL null plasmid DNA (Promega Corporation) capable of expressing renilla (*Renilla reinformis*) Luciferase was cotransfected to normalize the results obtained with the HO-1 promoter regulated firefly Luciferase reporter gene expression. Twenty-four hours after the transfection, the medium was changed and the cells were cultured in medium containing 5μM Cr(VI) for an additional 6 h. The cells were rinsed with PBS and lysed by the addition of 100 μl of passive lysis buffer (Promega Corporation). Firefly and *Renilla* Luciferase activities were measured in the same cell lysate sample using the Dual-Luciferase Reporter Assay System (Promega Corporation) and the luminescence was read in a microplate luminometer (Berthold Detection Systems USA, Oak Ridge, TN). The results were expressed as a ratio of firefly Luciferase activity to *Renilla* Luciferase activity.

Role of oxidative stress, transcription and MAPK pathways in Cr(VI)-induced overexpression of HO-1 gene. The roles of oxidative stress and transcription in the Cr(VI)-induced HO-1 gene overexpression in the fibroblasts was studied using NAC and actinomycin D—inhibitors of oxidative stress and transcription, respectively. PD98059 (mitogen-activated protein kinase [MAPK] kinase inhibitor), SB203580 (p38-MAPK inhibitor) and SP600125 (jun-N-terminal kinase inhibitor) were used to determine the role of MAPK pathways in Cr(VI)-induced HO-1 gene overexpression. Exponentially growing fibroblasts were treated with NAC (20mM final concentrations), actinomycin D (5 μg/ml final concentration), or with a cocktail containing the three MAPK inhibitors at final concentrations of 10μM each for 3 h. At the end of the treatment period, the cells were washed with PBS and further incubated with fresh growth medium containing 5μM Cr(VI) for 6 h. Total RNA isolated from the cells was used to determine the expression of the HO-1 gene by real-time PCR analysis.

Statistical Analysis of Data. Experiments were repeated at least four times. Statistical significance of the data presented as mean ± SE was analyzed by Student's *t* test or by one-way analysis of variance (ANOVA). *p* values less than 0.05 were considered statistically significant.

RESULTS

Cr(VI) Cytotoxicity in Human Dermal Fibroblasts

Exposure of human dermal fibroblasts to increasing concentrations of Cr(VI) resulted in a concentration-dependent cytotoxicity and cell death as evidenced from results of the MTT assay (Fig. 1). The LC50 value determined for Cr(VI) from the concentration–response curve was approximately 5μM (4.8 ± 0.32μM).

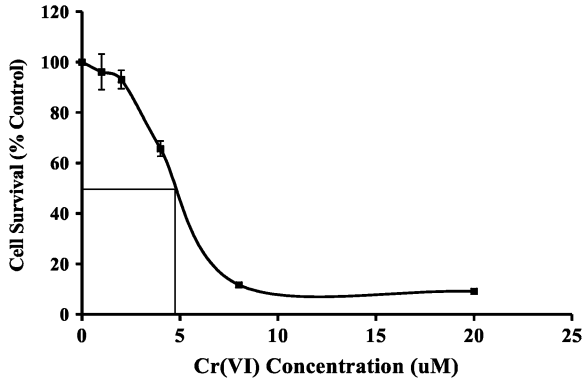

FIG. 1. Cr(VI) cytotoxicity in human dermal fibroblasts. Human dermal fibroblasts were exposed to increasing concentrations of Cr(VI) for 24 h. The number of surviving cells at the end of the treatment period was determined by MTT assay. The results are mean ± SE of four independent experiments.

Gene Expression Profile in Human Dermal Fibroblasts Exposed to Cr(VI)

Exposure of human dermal fibroblasts to Cr(VI) for time intervals of 2, 6, and 24 h resulted in differential expression of several genes that were represented on the microarray used in this study. A representative image of the hybridized microarrays, with some of the differentially expressed genes labeled, is presented in Figure 2. Microarray data revealed that 48 genes exhibited $\geq$ 1.5-fold change in their expressions in the Cr(VI) treated cells compared with the controls. Further analysis by real-time PCR confirmed that 32 out of the 48 genes selected exhibited a statistically significant differential expression ($p < 0.05$) in at least one of the three time intervals analyzed (Table 1). Genes belonging to every functional group that was represented on the microarray were found differentially expressed in response to exposure of the cells to Cr(VI). Of the 32 differentially expressed genes, 28 were found overexpressed while the expression of four genes were downregulated in response to the Cr(VI) exposure. A vast majority of the genes (88%) were found differentially expressed at the 24-h time interval, while fewer genes exhibited a statistically significant difference in their expression at earlier time intervals (17 and 7 genes at time intervals of 2 and 6 h, respectively). Six out of the 32 genes exhibited a statistically significant difference in their expression in the Cr(VI)-treated cells compared to the control cells at all three time intervals studied. Growth differentiation factor 15 gene exhibited the highest alteration in expression (288-fold) in the treated cells compared with the corresponding control cells.

Cr(VI)-Induced HO-1 Gene Expression and GSH Depletion

Exposure of the fibroblasts to 5μM Cr(VI) resulted in a significant overexpression of the HO-1 gene. The HO-1 gene

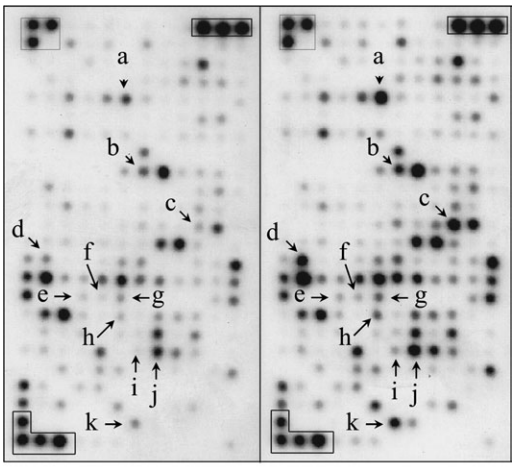

FIG. 2. Differential gene expression profile in human dermal fibroblasts treated with Cr(VI). Human dermal fibroblasts were treated with 5μM Cr(VI) for 2, 6, and 24 h. Total RNA isolated from the control and Cr(VI)-treated cells was analyzed for differential gene expression profile using the toxicity and drug metabolism microarray as described in the text. Some of the genes whose expression was significantly different between the control and the chemical treated cells are labeled (a–k) and they are: a—cyclin-dependent kinase inhibitor; b—dihydrofolate reductase; c—growth differentiation factor 15; d—heme-oxygenase 1; e—interleukin-1β; f—interleukin-6; g—lymphotoxin α; h—metallothionein 1; i—proteasome proliferative activated receptor γ; j—peroxiredoxin 1; k—tumor rejection antigen. The housekeeping genes and those used as hybridization controls are presented within the boxes.

expression was 4- and 15-fold higher in the fibroblasts following their exposure to Cr(VI) for 2- and 6 h, respectively (Fig. 3A). Exposure for longer time intervals of 12 and 24 h also resulted in the induction of HO-1 gene overexpression (data not presented). Exposure of the fibroblasts to 5μM Cr(VI) for time intervals of 2 and 6 h resulted in 24 and 38% depletion in their GSH content compared to untreated control cells (Fig. 3B).

Involvement of Transcription, Oxidative Stress and MAPK Pathways in Cr(VI)-Induced HO-1 Overexpression

Exposure of the fibroblasts to actinomycin D, an inhibitor of transcription, prior to their exposure to Cr(VI) resulted in approximately a 95% reduction in the Cr(VI)-induced overexpression of the HO-1 gene (Fig. 4A). Pre-exposure of the cells to MAPK inhibitors also significantly blocked the Cr(VI)-induced HO-1 overexpression (Fig. 4B). Similarly, pre-exposure of fibroblasts to NAC, a scavenger of ROS, resulted in approximately a 75% decrease in the Cr(VI)-induced overexpression of the HO-1 gene (Fig. 4C).

Cr(VI)-Induced HO-1 Overexpression—Modulation by Cellular GSH Level

The cellular level of GSH in the fibroblasts was modulated by culturing the cells in medium containing BSO or GSH. Compared to the control cells, exposure to BSO at concentrations of 25 and 50μM resulted in the depletion of cellular GSH levels by 78 and 89%, respectively, in the fibroblasts (data not presented). On the other hand, culturing the cells in medium containing 1 and 5mM GSH resulted in 2.2 and 3.3-fold increases, respectively, in the cellular GSH content (data not presented). The Cr(VI)-induced HO-1 gene overexpression was significantly influenced by the cellular GSH level. Thus, depletion of cellular GSH by pretreating the cells with 25 (data not shown) and 50μM BSO resulted in a further enhancement in the Cr(VI)-induced overexpression of the HO-1 gene compared to the control cells not pretreated with BSO (Fig. 5A). On the other hand, the Cr(VI)-induced overexpression of the HO-1 gene was significantly reduced in the cells pretreated with GSH compared to the corresponding control cells not pretreated with GSH (Fig. 5B).

Cr(VI)-Mediated Regulation of HO-1 Gene Expression—Role of Cellular GSH

Results obtained from the Luciferase reporter gene expression experiment further confirmed the role of cellular GSH level on the Cr(VI)-induced HO-1 gene overexpression. The Luciferase activity, which was regulated by the HO-1 gene promoter, was induced by Cr(VI) (Fig. 6). Depletion of cellular GSH content by pretreating the cells with BSO resulted in a further enhancement in the HO-1 regulated Luciferase activity (Fig. 6). On the other hand, pretreating the cells with 10mM GSH resulted in a moderate decline in the Cr(VI)-induced overexpression of the Luciferase reporter gene (data not presented).

Cr(VI)-Induced Cytotoxicity—Modulation by NAC and Cellular GSH Level

Culturing the dermal fibroblasts in medium containing NAC, BSO, or GSH significantly influenced the response of the cells to Cr(VI)-induced cytotoxicity. Pre-exposure of fibroblasts to 20mM NAC or 10mM GSH resulted in a significant protection of the cells against the cytotoxicity induced by Cr(VI) at concentrations of 1, 2.5, 5 and 10μM compared to the control cells (Figs. 7A and 7B). On the other hand, pre-exposure of the cells to 50μM BSO resulted in a significant enhancement in the Cr(VI)-induced cytotoxicity (Fig. 7C).

DISCUSSION

Exposure to chromium and its compounds is known to result in toxicity (Mathur and Gupta, 1994; Nielsen *et al*., 2000; Rudolf *et al*., 2005). The potential of chromium to induce dermal toxicity has been reported previously (reviewed by Shelnutt *et al*., 2007). Fibroblasts and other cell types of dermal origin have been used in the past as *in vitro* experimental models to study the potential of chromium and other toxic

TABLE 1
Differentially Expressed Genes in Human Dermal Fibroblasts Exposed to 5μM Cr(VI) for Time Intervals of 2, 6, and 24 h

Gene	GenBank no.	Fold change		
		2 h	6 h	24 h
Group 1—apoptosis				
1. Cyclin-dependent kinase inhibitor 1A	NM_000389	1.51 ± 0.13*	2.41 ± 0.36**	21.14 ± 2.19**
2. Lymphotoxin alpha (tumor necrosis factor [TNF] superfamily member 1)	NM_000595	1.58 ± 0.26	4.85 ± 2.05*	84.47 ± 26.13*
3. Clusterin	NM_001831	1.09 ± 0.13	0.66 ± 0.13*	0.70 ± 0.10
4. Growth arrest and DNA-damage–inducible, alpha	NM_001924	1.58 ± 0.36	1.12 ± 0.17	97.44 ± 19.32**
5. Interleukin 1, beta	NM_000576	0.93 ± 0.34	2.98 ± 0.83*	15.04 ± 4.02*
Group 2—cell cycle				
1. Cyclin-dependent kinase inhibitor 1A	NM_000389	1.51 ± 0.13*	2.41 ± 0.36**	21.14 ± 2.19**
2. Growth arrest and DNA-damage–inducible, alpha	NM_001924	1.58 ± 0.36	1.12 ± 0.17	97.44 ± 19.32**
3. V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	1.00 ± 0.12	1.20 ± 0.14	10.04 ± 3.14*
4. Cyclin G1	NM_004060	0.87 ± 0.13	0.82 ± 0.29	148.89 ± 28.42**
5. Chaperonin containing TCP1, subunit 2 (beta)	NM_006431	1.35 ± 0.19	1.25 ± 0.18	3.16 ± 0.41**
6. DnaJ (HSP40) homolog, subfamily A, member 2	NM_005880	1.17 ± 0.11	1.41 ± 0.19	14.70 ± 2.37**
7. Interleukin 1, beta	NM_000576	0.93 ± 0.34	2.98 ± 0.83*	15.04 ± 4.02*
Group 3—cell growth, proliferation and differentiation				
1. Growth differentiation factor 15	NM_004864	2.55 ± 0.49*	15.58 ± 3.49**	733.25 ± 139.98**
2. Interleukin 1, beta	NM_000576	0.93 ± 0.34	2.98 ± 0.83*	15.04 ± 4.02*
3. Lymphotoxin alpha (TNF superfamily, member 1)	NM_000595	1.58 ± 0.26	4.85 ± 2.05*	84.47 ± 26.13*
4. Interleukin 6	NM_000600	1.33 ± 0.38	7.94 ± 1.58**	13.96 ± 2.49**
5. Cyclin-dependent kinase inhibitor 1A	NM_000389	1.51 ± 0.13*	2.41 ± 0.36**	21.14 ± 2.19**
6. DnaJ (HSP40) homolog, subfamily A, member 1	NM_001539	1.08 ± 0.27	1.55 ± 0.57	24.20 ± 6.77*
7. V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	1.00 ± 0.12	1.20 ± 0.14	10.04 ± 3.14*
8. Peroxiredoxin	NM_002574	6.40 ± 1.96*	192.15 ± 55.55*	24.84 ± 9.31*
Group 4—response to stress				
1. Superoxide dismutase 1	NM_000454	1.02 ± 0.04	1.10 ± 0.34	4.64 ± 0.62*
2. Glutathione *S*-transferase theta 1	NM_000853	1.27 ± 0.17	1.88 ± 0.11**	1.09 ± 0.37
3. Metallothionein 1×	NM_005952	1.61 ± 0.28*	1.66 ± 0.05**	4.66 ± 0.69**
4. Peroxisome proliferative activated receptor, gamma coactivator 1 Alpha	NM_013261	1.22 ± 0.36	4.56 ± 1.41*	20.16 ± 10.37 *
5. Serpin peptidase inhibitor, clade H (heat shock protein 47)	NM_001235	1.29 ± 0.12	0.84 ± 0.09	0.30 ± 0.02**
6. Tumor rejection antigen (gp96) 1	NM_003299	1.25 ± 0.51	4.91 ± 1.45*	47.6 ± 15.45*
Group 5—chaperons and heat shock proteins				
1. Calnexin	NM_001746	2.11 ± 1.39	0.97 ± 0.09	24.33 ± 6.17**
2. Chaperon containing TCP1, subunit 2 (beta)	NM_006431	1.35 ± 0.19	1.25 ± 0.18	3.16 ± 0.41**
3. DnaJ (HSP40) homolog, subfamily A, member 1	NM_001539	1.08 ± 0.27	1.55 ± 0.57	24.20 ± 6.77*
4. DnaJ (HSP40) homolog, subfamily A, member 2	NM_005880	1.17 ± 0.11	1.41 ± 0.19	14.70 ± 2.37**
5. Heat shock 70-kDa protein 1A	NM_005345	1.39 ± 0.07*	1.11 ± 0.05	0.32 ± 0.04*
6. Heat shock 60-kDa protein 1 (chaperonin)	NM_002156	1.12 ± 0.16	1.28 ± 0.15	23.79 ± 3.30**
7. Serpin peptidase inhibitor, clade H (heat shock protein 47) Member 1	NM_001235	1.29 ± 0.12	0.84 ± 0.09	0.30 ± 0.02**
8. Tumor rejection antigen (gp96) 1	NM_003299	1.25 ± 0.51	4.91 ± 1.45*	47.6 ± 15.45*
Group 6—transcription factors and regulators				
1. Peroxisome proliferative activated receptor, gamma coactivator 1, alpha	NM_013261	1.22 ± 0.36	4.56 ± 1.41*	20.16 ± 10.37 *
2. Early growth response 1	NM_001964	1.87 ± 0.22**	0.70 ± 0.10*	0.88 ± 0.10
3. V-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467	1.00 ± 0.12	1.20 ± 0.14	10.04 ± 3.14*
Group 7				
1. Acetyl-coenzyme A acetyltransferase 1	NM_000019	1.09 ± 0.06	0.72 ± 0.08*	16.42 ± 2.41**
2. Catechol-*O*-methyltransferase	NM_000754	1.14 ± 0.11	1.43 ± 0.14*	5.09 ± 0.51**
3. *O*-6-methylguanine-DNA methyltransferase	NM_002412	1.22 ± 0.21	1.41 ± 0.28	17.72 ± 0.97**

TABLE 1—*Continued*

Gene		GenBank no.	Fold change 2 h	Fold change 6 h	Fold change 24 h
4.	Nicotinamide N-methyltransferase	NM_006169	0.78 ± 0.14	2.15 ± 0.52*	11.33 ± 2.02**
5.	Cytochrome P450, family 2, subfamily B, polypeptide 6	NM_000106	1.22 ± 0.19	1.14 ± 0.15	43.40 ± 13.33*
6.	Dihydrofolate reductase	NM_000791	1.33 ± 0.52	5.38 ± 1.91*	128.35 ± 18.81**
7.	Heme-oxygenase 1 (decycling) 1	NM_002133	4.22 ± 0.19**	15.10 ± 3.15**	1.15 ± 0.13
8.	Peroxiredoxin 1	NM_002574	6.40 ± 1.96*	192.15 ± 55.55**	24.84 ± 9.31*
9.	Prostaglandin-endoperoxide synthase 1	NM_000962	0.79 ± 0.14	1.10 ± 0.20	40.94 ± 5.45**
10.	Superoxide dismutase 1, soluble	NM_000454	1.02 ± 0.04	1.10 ± 0.34	4.64 ± 0.62*
11.	Glutathione *S*-transferase theta 1	NM_000853	1.27 ± 0.17	1.88 ± 0.11**	1.09 ± 0.37

Note. The genes are categorized into various functional groups. Data are presented as mean ± SE ($n = 4$ independent experiments). Only those genes which exhibited a statistically significant difference in their expressions compared to the corresponding time-matched controls in at least one of the time points analyzed are presented in the table. Some of the genes are represented in more than one functional group. *$p < 0.05$; **$p < 0.01$.

chemicals to induce toxicity including dermal toxicity (Biedermann and Landolph, 1987, 1990; Carlisle *et al*., 2000; Patierno *et al*., 1988; Rudolf *et al*., 2005). Therefore, human dermal fibroblasts were used in the present study as an *in vitro* experimental model to investigate the mechanisms of Cr(VI)-induced dermal toxicity.

The potential of Cr(VI) to induce cytotoxicity depends on the cell's ability to facilitate the reductive metabolism of Cr(VI) as well as its ability to detoxify and thus to defend against the toxicity of various reductive metabolites and by-products such as ROS that are generated during the metabolism of Cr(VI). While cytochrome P450 (Mikalsen *et al*., 1991), nicotinamide adeneine diphosphate (reduced) cytochrome *c* reductase (Gunaratnam *et al*., 2002), cytochrome b5 reductase (Borthiry *et al*., 2007), DT-diaphorase (De Flora *et al*., 1985), and glutathione reductase (Gunaratnam and Grant, 2001) are mostly responsible for the enzymatic reduction of Cr(VI), glutathione—a major antioxidant and reducing agent present in millimolar concentrations in cells, is mainly responsible for its nonenzymatic reduction (Meister and Anderson, 1983; O'Brien *et al*., 2003; Wiegand *et al*., 1984). Whereas the role of glutathione in the metabolism and toxicity of Cr(VI) has been intensely investigated, its actual role in Cr(VI) toxicity still remains controversial. Glutathione-mediated reduction of Cr(VI) can result in the generation of toxic chromium species of lower valences (O'Brien *et al*., 2003; Sugiyama, 1992) and may thus represent a major mechanism responsible for its toxicity. This view is further supported by the observations that (1) Cr(VI) toxicity is enhanced by elevated GSH levels in cells (Cupo and Wetterhahn, 1985) and in animal tissues (Cheng *et al*., 2000), and (2) BSO-mediated GSH depletion in cells and in experimental animals is protective against its toxicity (Cheng *et al*., 2000; Hojo and Satomi, 1991; Ning and Grant, 2000; Ueno *et al*., 1995). Contrary to these findings, several other investigators have reported a protective role for glutathione in Cr(VI) toxicity. Accordingly, supplementation of glutathione precursors is protective against Cr(VI)-induced toxicity (Hojo and Satomi, 1991; Ning and Grant, 2000). The results of the current study further support a protective role for glutathione against Cr(VI)-induced cytotoxicity.

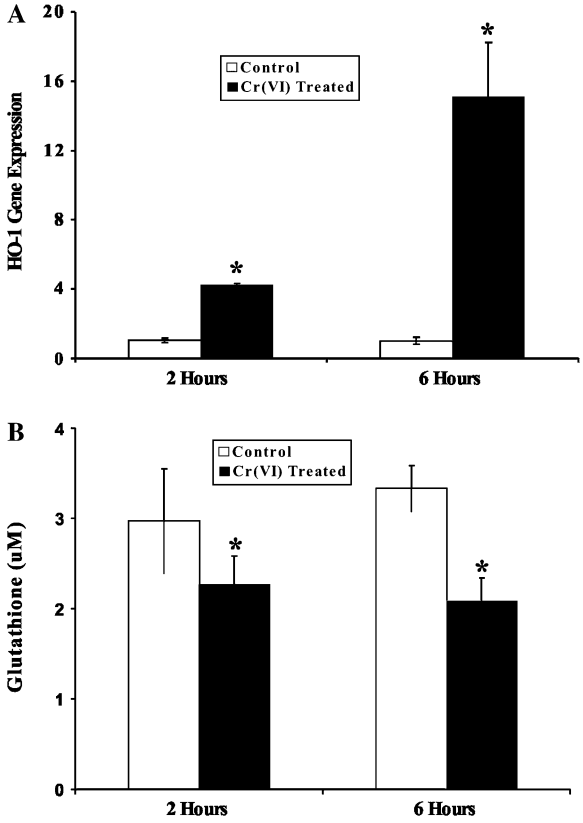

FIG. 3. Heme-oxygenase 1 expression and glutathione level in cells treated with Cr(VI). Fibroblasts were treated with 5μM Cr(VI) for 2 or 6 h. Total RNA was isolated and used to determine heme-oxygenase 1 expression by quantitative real-time PCR (A). Glutathione levels of the cells were determined using the cell lysates as described in the text (B). Results are presented as fold overexpression compared with the corresponding controls. Data is presented as mean ± SE ($n = 4$). *Statistically significant difference ($p < 0.05$).

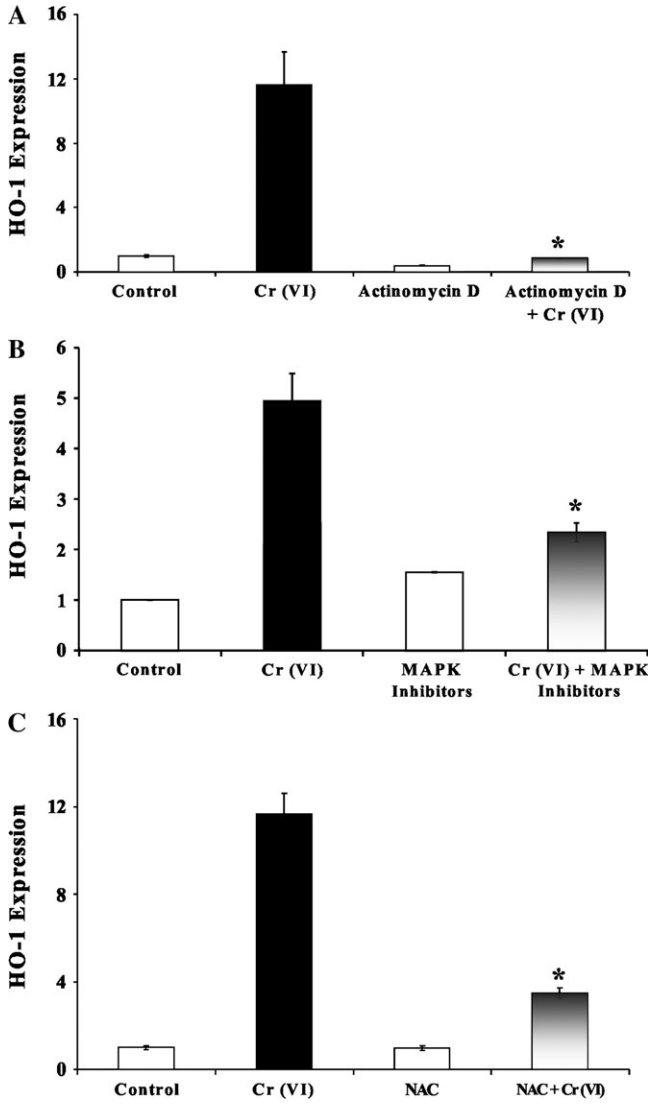

FIG. 4. Effect of actinomycin D, inhibitors of MAPK pathway and N-acetyl cysteine on Cr(VI)-induced heme-oxygenase 1 expression. Dermal fibroblasts were treated with the transcriptional inhibitor—actinimycin D (A), the MAPK inhibitors—PD98059, SB203580, and SP600125 (B), or with a scavenger for ROS—NAC (C), followed by 5μM Cr(VI) for 6 h. Heme-oxygenase 1 expression was determined by quantitative real-time PCR analysis using the total RNA isolated from the control and Cr(VI)-treated cells. Results are presented as fold overexpression compared with the corresponding control cells. Data represents mean ±SE (n= 4). *Statistically significant difference (p<0.05) compared to the respective Cr(VI) treated cells.

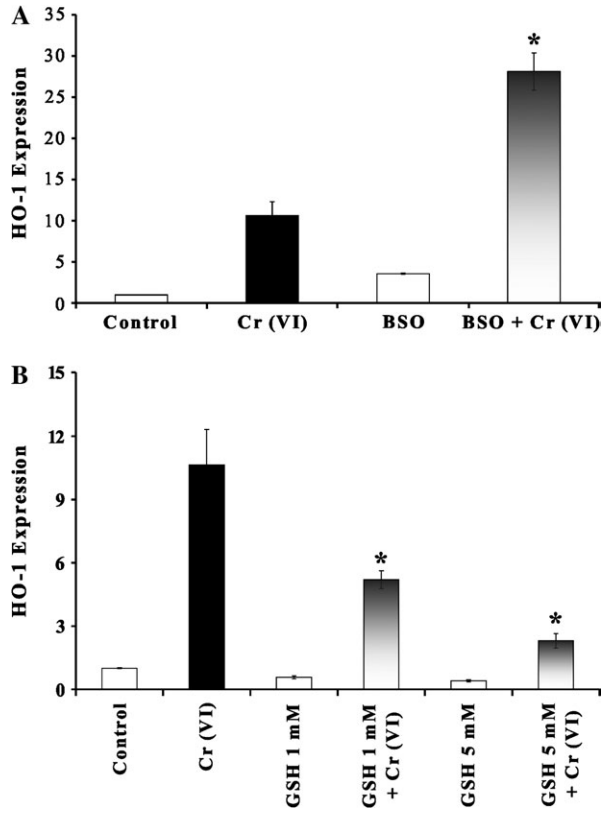

FIG. 5. Effect of BSO (A) and GSH (B) on Cr(VI)-induced heme-oxygenase 1 expression. The fibroblasts were treated with BSO (50μM) or GSH (1 and 5mM) to result in the depletion or elevation, respectively, of cellular GSH level. Subsequently, the cells were treated with 5μM Cr(VI) for 6 h. Heme-oxygenase 1 expression was determined by quantitative real-time PCR analysis of the total RNA isolated from the cells. Results are presented as fold overexpression compared with the corresponding control cells. *Statistically significant difference ($p < 0.05$) compared to the respective control cells treated with Cr(VI) alone.

Induction of cell stress is a major mechanism responsible for the toxicity of Cr(VI). The reductive metabolism of Cr(VI) results in the generation of toxic metabolites and ROS that are capable of inducing cell stress leading to toxicity (Standeven and Wetterhahn, 1991; Wetterhahn *et al.*, 1989). In general, cells possess protective mechanisms capable of defending against the cell stress induced by toxic intermediates and ROS. Glutathione functions as a major antioxidant capable of neutralizing the toxicity of reactive metabolites and ROS in cells (Hayes and McLellan, 1999). In addition, glutathione S-transferases catalyze the conjugation of reactive toxic metabolites with glutathione facilitating, in general, their detoxification and elimination from the body (Hayes and Pulford, 1995). Several lines of evidence obtained in this study have demonstrated that cell stress resulting from the exposure of human dermal fibroblasts to Cr(VI) was in fact responsible for the ultimate cytotoxicity of the chemical. These include:

1. Exposure of fibroblasts to Cr(VI) resulted in a significant reduction in the cellular level of glutathione (Fig. 3B)—one of the major stress-responsive antioxidants, present in the cells.

2. Expression of several stress-response genes, including that of HO-1, was significantly induced by Cr(VI) in the fibroblasts (Figs. 2 and 3A and Table 1).

3. Pre-exposure of the fibroblasts to NAC—a scavenger of ROS, was protective against the Cr(VI)-induced cytotoxicity (Fig. 7A) while BSO-mediated GSH depletion resulted in a significant increase in Cr(VI)-induced cytotoxicity (Fig. 7C), and

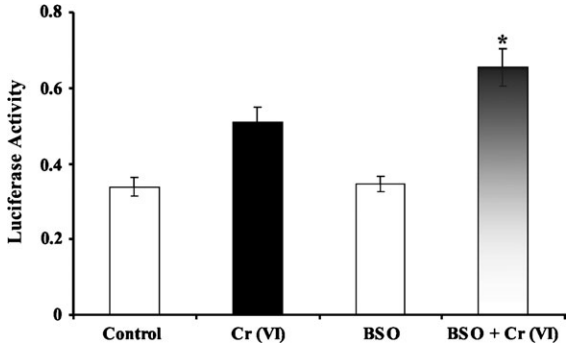

FIG. 6. Effect of BSO on Cr(VI)-mediated regulation of heme-oxygenase 1 gene expression. The luciferase expression vector containing part of the heme-oxygenase 1 gene promoter was constructed as described in the text. Monkey kidney COS7 cells were transfected with the luciferase plasmid DNA and treated with 5μM Cr(VI) and 50μM BSO as described in the text. Luciferase activity was determined in the cell lysates. The data is presented as mean ± SE ($n = 4$). *Statistically significant difference ($p < 0.05$) compared with the corresponding cells treated with Cr(VI) alone.

4. Elevated cellular levels of glutathione resulted in significant protection of the cells against Cr(VI)-induced cytotoxicity (Fig. 7B).

HO-1 gene overexpression in response to Cr(VI) exposure appears to be the result of the gene's transcriptional activation as evidenced by a significant loss of its overexpression when the cells were pretreated with actinomycin D, a general inhibitor of mRNA transcription (Fig. 4A). The MAPKs are components of signaling cascades that respond to extracellular stimuli, such as that which can result from the exposure of cells to xenobiotics (Morse and Choi, 2002; Ryter *et al.*, 2002). Pretreating the cells with PD98059, SB203580, and SP600125, chemical inhibitors of MAPK pathways (Bennett *et al.*, 2001; Dudley *et al.*, 1995; Schultz *et al.*, 1997), resulted in a significant loss of Cr(VI)-induced HO-1 gene overexpression (Fig. 4B) suggesting the potential involvement of MAPK pathways in the Cr(VI)-induced HO-1 gene overexpression. Similarly, the significant loss of Cr(VI)-induced HO-1 gene overexpression in cells pretreated with NAC, a scavenger of ROS, was indicative of the involvement of ROS and the resulting cell stress. Data obtained from the luciferase reporter gene experiments further supported the likely role of ROS and GSH in the Cr(VI)-induced overexpression of the HO-1 gene. Overexpression of the Luciferase reporter gene observed in response to exposure of the cells to Cr(VI) was further enhanced by pretreating the cells with BSO suggesting that in the absence of an adequate cellular level of GSH, the ROS generated from Cr(VI) were not readily detoxified and, thus, remained freely available in the cells and resulted in enhanced Luciferase expression. However, additional studies are required to understand the precise cellular mechanisms that are responsible for the ROS-mediated induction of HO-1 gene expression particularly with respect to the cellular GSH level in dermal fibroblasts treated with Cr(VI).

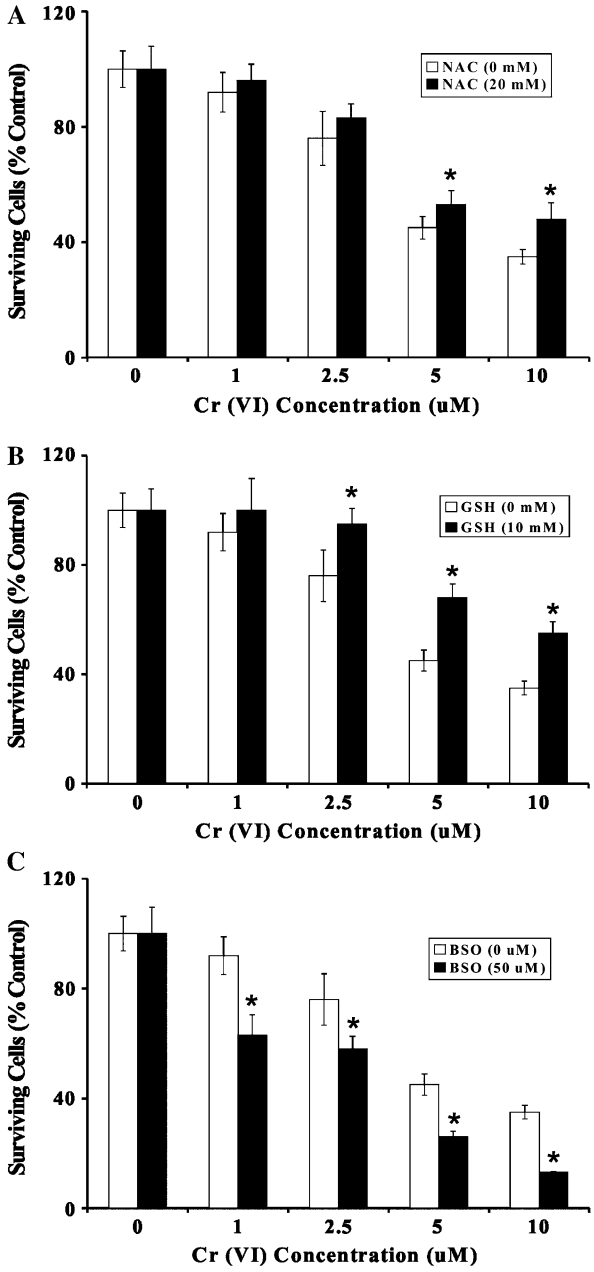

FIG. 7. Role of NAC (A), GSH (B), and BSO (C) on Cr(VI)-induced cytotoxicity in human dermal fibroblasts. Human dermal fibroblasts were treated with 20mM NAC (A), 10mM GSH (B), or 50μM BSO (C) for 6 h. Subsequently, the cells were treated with 5μM Cr(VI) for 6 h and cytotoxicity was determined by MTT assay. The data are presented as mean ± SE ($n = 4$). *Statistically significant difference ($p < 0.05$) compared with the corresponding samples not treated with NAC, GSH, or BSO.

Induction of HO-1 gene expression has been characterized as a general response to oxidative stress and an important cellular defense mechanism against the ensuing toxicity. HO-1 gene expression is upregulated in response to oxidative stress (Ewing and Maines, 1993; Keyse and Tyrrell, 1989). Bilirubin, the final product of heme catabolism by heme-oxygenase is an

antioxidant (Stocker *et al*., 1987). Oxidative stress and the associated toxicity in ultraviolet A irradiated human skin fibroblasts is enhanced by antisense HO-1 oligonucleotide mediated blockade of HO-1 gene expression (Vile *et al*., 1994). Furthermore, studies involving HO-1 gene knockout mice have confirmed that upregulation of the HO-1 gene serves as an adaptive protective mechanism against oxidative damage (Poss and Tonegawa, 1997). The induction of HO-1 gene expression, as noticed in the present study in response to exposure of fibroblasts to Cr(VI), is in agreement with its established role as an oxidant response gene and as a marker for cellular stress. Thus, rather than being a marker for Cr(VI) toxicity, HO-1 overexpression appears to be a marker for its mechanism (induction of cell stress) of toxicity. The Cr(VI)-induced cytotoxicity and overexpression of the HO-1 gene in the fibroblasts correlated well with the cellular GSH level. Both the Cr(VI)-induced cytotoxicity and HO-1 overexpression were enhanced when the cellular GSH level was depleted by pre-exposure of the cells to BSO. On the other hand, elevated cellular levels of GSH resulted in a significant protection against Cr(VI)-induced cytotoxicity and significantly blocked the Cr(VI)-induced HO-1 gene overexpression. Taken together our results indicate that (1) oxidative stress is a major mechanism responsible for Cr(VI) toxicity in human dermal fibroblasts, (2) cellular glutathione level is an important determinant of Cr(VI)-induced cell stress and cytotoxicity, and (3) HO-1 gene expression is a marker for Cr(VI)-induced cell stress leading to toxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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