p38 Signaling-mediated Hypoxia-inducible Factor 1α and Vascular Endothelial Growth Factor Induction by Cr(VI) in DU145 Human Prostate Carcinoma Cells*

Ning Gao†, Bing-Hua Jiang‡§, Stephen S. Leonard†, Linda Corum‡, Zhuo Zhang‡¶, Jenny R. Roberts¶, Jim Antonini†, Jenny Z. Zheng‡, Daniel C. Flynn‡, Vince Castranova¶, and Xianglin Shi¶

From the †Mary Babb Randolph Cancer Center, Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, West Virginia 26506-9300 and §Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

Chromium(VI) (Cr(VI)) is widely used in industry and is a potent inducer of tumors in animals. The present study demonstrates that Cr(VI) induces hypoxia-inducible factor 1 (HIF-1) activity through the specific expression of HIF-1α but not HIF-1β subunit and increases the level of vascular endothelial growth factor (VEGF) expression in DU145 human prostate carcinoma cells. To dissect the signaling pathways involved in Cr(VI)-induced HIF-1 expression, we found that p38 mitogen-activated protein kinase signaling was required for HIF-1α expression induced by Cr(VI). Neither phosphatidylinositol 3-kinase nor extracellular signal-regulated kinase activity was required for Cr(VI)-induced HIF-1 expression. Cr(VI) induced expression of HIF-1 and VEGF through the production of reactive oxygen species in DU145 cells. The major species of reactive oxygen species responsible for the induction of HIF-1 and VEGF expression is H2O2. These results suggest that the expression of HIF-1 and VEGF induced by Cr(VI) may be an important signaling pathway in the Cr(VI)-induced carcinogenesis.

Most mutations of human cancer are because of the activation of oncogenes and the loss of function of tumor suppressor genes, which commonly induce new blood vessels (angiogenesis) for tumors to grow beyond a few millimeters in diameter (1, 2). Vascular endothelial growth factor (VEGF) is an essential protein for angiogenesis (2, 3). Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic helix-loop-helix transcription factor, composed of HIF-1α and HIF-1β/aryl hydrocarbon nuclear translocator subunits (4). HIF-1α is unique to HIF-1 and is induced exponentially in response to a decrease in cellular O2 concentration (5). HIF-1β is identical to the aryl hydrocarbon nuclear translocator that forms a heterodimer with an aryl hydrocarbon receptor (4, 6) and is not regulated by cellular oxygen tension (5). HIF-1 regulates the expression of many genes including VEGF, erythropoietin, heme oxygenase 1, aldolase, enolase, and lactate dehydrogenase A (7). The levels of HIF-1 activity in cells correlate with tumorigenicity and angiogenesis in nude mice (8, 9). HIF-1 is induced by the expression of oncogenes such as v-Src and Ras (8, 10) and is overexpressed in many human cancers (11). Loss of function of HIF-1 results in defects in embryonic vasculization (12, 13). HIF-1α expression, but not HIF-1β, is induced by growth factors through the activation of PI3K signaling (14, 15). HIF-1 activates the expression of VEGF gene at the transcriptional level (16, 17). VEGF plays a key role in tumor progression and angiogenesis (2, 3). Both inhibition of VEGF expression and the function of its receptor decrease dramatically the tumor growth, invasion, and metastasis in animal models (18–26). Tissue hypoxia is a major inducer for the expression of VEGF in tumors (27, 28). Somatic mutations such as oncogene Ras activation and tumor suppressor gene p53 inactivation also increase VEGF expression (19, 29–31).

Although HIF-1α and VEGF are implicated in cancer development, the mechanisms of HIF-1α activation and stabilization of HIF-1α protein remain to be studied. The present study investigates Cr(VI)-induced expression of HIF-1α and VEGF in DU145 human prostate carcinoma cells and the role of individual reactive oxygen species (ROS). Cr(VI) is chosen, because it is a well-established carcinogen (32). Cr(VI)-containing compounds are widely used in industry under many occupational settings and are found in the environment in the soil and water. Epidemiological studies have shown that workers in the chrome production industry have a consistently high risk for cancer than the general population. Cr(VI) compounds are also potential inducers of tumors in experimental animals (32). Although the mechanisms of Cr(VI)-induced carcinogenesis are not understood fully, ROS are considered to play an important role (33–39). Previous studies have shown that upon reduction by cellular oxidants, Cr(VI) is able to generate a whole spectrum of ROS, i.e. O2, H2O2, and OH. It is well known that ROS play important roles in carcinogenesis induced by a variety of carcinogens. Through ROS-mediated reaction, Cr(VI) is able to activate p53 and induce apoptosis (37, 39, 40). It has been demonstrated that HIF-1 interacts with p53 and is involved in tumor progression (41–45). It is possible that Cr(VI) may be able to induce the expression of HIF-1α and VEGF. In the present study, we tested whether Cr(VI) is able to induce...
expression of HIF-1α and VEGF. The specific questions were as follows. (a) Is Cr(VI) able to induce HIF-1 and VEGF expression? (b) What are the up-stream signal pathways in Cr(VI)-induced expression of HIF-1α protein? (c) Are ROS species involved in Cr(VI)-induced HIF-1α and VEGF expression? (d) Which species among ROS play the critical roles in Cr(VI)-induced HIF-1α and VEGF expression?

MATERIALS AND METHODS

Reagents and Cell Culture—Potassium dichromate (Cr(VI)) was purchased from Aldrich (Milwaukee, WI). NAPDH, superoxide dismutase, sodium formate, deferoxamine, diphenylene iodonium (DPI), and rotenone were purchased from Sigma. Catalase was purchased from Roche Molecular Biochemicals. Hydroethidine (HE) was from Molecular Probes (Eugene, OR). Antibodies against HIF-1α and HIF-1β were from Transduction Laboratories (Lexington, KY). The human VEGF immunosay kit was from R & D Systems (Minneapolis, MN). The human prostate cancer cell line DU145 was maintained in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum, 3% chicken serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and cultured at 37 °C in a 5% CO2 incubator. The cells form a monolayer at confluence. Trypsin (0.25%) EDTA solution was used to detach the cells from the culture flask for plating and passing the cells.

Immunoblot Analysis—The cells were plated in a 60-mm culture dish and treated with Cr(VI). Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, and 2 mM peptatin A on ice for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70 °C. The protein concentration was determined using Bio-Rad protein assay reagent (Richmond, CA). The nuclear protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with antibodies against HIF-1α and HIF-1β. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (PerkinElmer Life Sciences) and visualized through enhanced chemiluminescence reagent (PerkinElmer Life Sciences).

VEGF Immunoinnassy—DU145 cells were plated in a 6-well plate at a density of 1 × 10⁵ cells/well in MEM medium and incubated overnight before the cells were subjected to treatment. After treatment, the cell culture media were removed and stored at −80 °C. VEGF protein concentration in the media was determined by ELISA using a commercial kit (R & D Systems). Briefly, 200 µl of standards or cell culture supernatant were pipetted into the wells of a microplate that had been coated with a monoclonal antibody specific for VEGF and incubated for 2 h at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody against VEGF conjugated to horseradish peroxidase was added to the wells and incubated for 2 h at room temperature. Following a wash, 200 µl of substrate solution was added to the wells and incubated for 30 min and then 50 µl of stop solution was added to stop color development. The optical density of each well was determined using a microplate reader at 450 nm. A mean value of duplicate samples for each experiment and two separate experiments were used for analysis.

Electron Spin Resonance (ESR) Measurements—ESR spin trapping technique was used to detect free radical generation. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relative long-lived free radical product (spin adduct), which can be studied by conventional ESR (46). The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct are generally characteristic of the original trapped radicals. All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly as described (47). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K₃CrO₄) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Reactants were mixed in test tubes in a total final volume of 500 µl and transferred to a flat cell for ESR measurement.

Cellular Hydrogen Peroxide (H₂O₂) Measurement—Cellular H₂O₂ was determined with a quantitative H₂O₂ assay kit (BIOXYTECH® H₂O₂, 560™; Portland, OR). The assay was performed according to the protocol provided by the manufacturer. This assay is based on the oxidation of ferrous ions (Fe²⁺) to ferric ions (Fe³⁺) by H₂O₂ under acidic conditions. The ferric ion binds with the indicator dye xylenol orange to form a stable colored complex that can be measured at 560 nm. Briefly, cells were pretreated with Cr(VI) and with or without antioxidants. The aliquots of media were incubated at 25 °C for 30 min with 100 µl of a 10× xylenol orange stock solution (xylenol orange (10 µM), Fe(NH₄)₂(SO₄)₂·6H₂O (2.5 µM), H₂SO₄ (25 mM)) after which the absorbance was read at 560 nm. The concentration of H₂O₂ was determined from a standard curve (0–100 µM).

Superoxide Anion (O₂⁻) Assay—HE is a specific dye for O₂⁻. HE is oxidized by O₂⁻ to a blue dye that stains nucleus to form a bright fluorescent red (37, 39). DU145 cells were cultured in a 6-well plate at 5 × 10⁵ cells/well for 24 h before treatment. After the cells were treated with Cr(VI), HE was added into the cell culture 30 min before the treatment was completed. Then the cells were washed twice in 1× PBS buffer and harvested for flow cytometric analysis. For cellular image assay, the cells were plated onto a glass slip in the 12-well plate at 1 × 10⁵ cells/well for 24 h before treatment. HE was added into the cell culture 30 min before treatment was completed. After being stained, the cells were washed in PBS and fixed with 10% buffered formalin. The slip was mounted on a glass slide and observed using a Saratoga 2000 (Molecular Dynamics, Sunnyvale, CA) laser scanning confocal microscope (Optiphot-2; Nikon, Melville, PA) fitted with an argon-ion laser.

Oxygen Consumption Measurements—Oxygen consumption measurements were carried out with a Gilson oxygraph equipped with a Clark microelectrode (Gilson Medical Electronic, Middleton, WI). These measurements were made from mixture containing 1 × 10⁵ cells/ml and various treatments in a total volume of 1.5 ml. The oxygraph was calibrated with media equilibrated with oxygen of known concentrations.

Plasmid Constructs and VEGF Reporter Activity—The dominant-negative form of HIF-1, HIF-1DN, was subcloned into pCEP4 as described previously (14). A VEGF reporter containing a 2.65-kb KpnI-BssIII fragment of the human VEGF gene promoter was cloned into the pGL2 basic vector as described previously (16). DU145 cells were cultured as described above. VEGF reporter plasmid (1.5 µg) was transfected into the cells using LipofectAMINE (Invitrogen), and the relative luciferase activity was assayed as described previously (14). Briefly, after transfection, cells were cultured in fresh medium for 12 h and then the cells were switched to medium in the absence or presence of 1.5 µM Cr(VI) for 24 h or were switched to the medium containing 1.5 µM Cr(VI) and superoxide dismutase (1000 units/ml), catalase (10,000 units/ml), or sodium formate (2 mM) as indicated for 24 h. The cells were co-transfected with the dominant-negative form of HIF-1, HIF-1DN, were cultured in fresh medium for 12 h, followed by the incubation with the medium in the presence of 1.5 µM Cr(VI) for 24 h. Light production was measured for 15 s, and the values were corrected by subtracting the readings obtained with nontransfected cells and were normalized to the control in each experiment.
RESULTS

Induction of HIF-1 Expression—To determine whether Cr(VI) could induce the expression of HIF-1 in DU145 cells, cells were exposed to Cr(VI) at different concentrations for 3 h, and total cellular protein extracts were prepared for immunoblot assays of HIF-1α and HIF-1β protein levels. As shown in Fig. 1A, the levels of HIF-1α protein in Cr(VI) exposed cells were increased markedly in a dose-dependent manner, whereas the levels of HIF-1β protein were not altered. The maximum induction of HIF-1α expression was caused by Cr(VI) at a concentration of 5 μM, whereas further increase in Cr(VI) concentration resulted in a slight decrease, possibly because of Cr(VI)-induced apoptosis. A time course study of the expression of HIF-1α revealed that Cr(VI) (5 μM) induced HIF-1α expression in a time-dependent manner, whereas it did not affect HIF-1β expression (Fig. 1B). The maximum induction of HIF-1α expression in response to Cr(VI) occurred at 3 h after exposure and began to decrease after 6 h.

PI3K and MAPK/Extracellular Signal-regulated Kinase Pathways Are Not Involved in Induction of HIF-1α by Cr(VI), and p38 MAPK Pathway Is Required for HIF-1α Expression—To determine whether PI3K signaling pathway was required for HIF-1α expression, DU145 cells were treated with LY294002 or wortmannin, inhibitors of PI3K. The cells were then exposed to different concentrations of Cr(VI) for 3 h, and nuclear extracts were prepared for immunoblot assays for HIF-1α and HIF-1β protein levels. As shown in Fig. 2, A and B, neither LY294002 nor wortmannin inhibited HIF-1α expression induced by Cr(VI), suggesting that PI3K activity is not required for induction of HIF-1α by Cr(VI). We also investigated whether MAPK activity was required for HIF-1α expression. Cells were pretreated with PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, or SB202190, an inhibitor of p38 MAP kinase. Cells were then treated with 5 μM Cr(VI). Protein levels were determined using Western blot. The results (Fig. 2C) indicated that PD98059 did not have any inhibitory effect on HIF-1α expression, whereas SB202190 caused a dose-dependent inhibition of HIF-1α expression induced by Cr(VI) (Fig. 2D). These results suggest that p38 MAP kinase activity is required for HIF-1α expression, whereas mitogen-activated protein kinase/extracellular signal-regulated kinase kinase activity is not involved in HIF-1α activation.

Induction of VEGF Expression—VEGF is regulated by HIF-1 in response to hypoxia. To investigate whether the induction of HIF-1 by Cr(VI) increased VEGF expression, the level of VEGF protein produced by DU145 cells was analyzed by ELISA. As shown in Fig. 3, VEGF protein levels in untreated cells were increased over a period of 48 h. Treatment with 2.5 μM Cr(VI) markedly increased the induction of VEGF expression in a time-dependent manner.

Generation of ROS—ROS are implicated to play a major role in Cr(VI)-induced carcinogenesis. To determine whether Cr(VI) could induce HIF-1α and VEGF expression though ROS, we examined the ROS generation in the Cr(VI)-treated cells. The ability of Cr(VI) to generate ‘OH radicals was examined using an ESR spin trapping method. DU145 cells alone did not produce any detectable amount of free radicals (Fig. 4A). DU145 cells incubated with Cr(VI) generated a 1:2:2:1 quartet ESR spectrum (Fig. 4B). The splittings of this spectrum were aH =
Cr(VI) Induces HIF-1 and VEGF Expression

Fig. 4. Effects of antioxidants on Cr(VI)-induced ROS generation. Aliquots of 1 ml DU145 cells (1 x 10⁶ cells/ml) were incubated in 1 × PBS containing 100 mM DMPO and 2 mM Cr(VI) in the absence or presence of antioxidants, DPI, and rotenone. ESR spectra were recorded during the initial 5 min. a, DU145 cells alone; b, the cells + Cr(VI); c, cells + Cr(VI) + superoxide dismutase (1000 units/ml); d, cells + Cr(VI) + catalase (10,000 units/ml); e, cells + Cr(VI) + sodium formate (2 mM); f, cells + Cr(VI) + deferoxamine (2 mM); g, cells + Cr(VI) + NADPH (1 mM); h, cells + Cr(VI) + DPI (20 μM); i, cells + Cr(VI) + rotenone (50 μM).

α₂ = 14.9 G, where α₂ and hλ denote hyperfine splittings of the nitroxyl nitrogen and α-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, this spectrum was assigned to the DMPO/OH adduct (34, 49, 50), which is evidence for ‘OH radical generation. Addition of superoxide dismutase, an O₂⁻ scavenger, slightly decreased the DMPO/OH adduct signal (Fig. 4C). Catalase, an H₂O₂ scavenger, decreased the generation of ‘OH radical induced by Cr(VI) (Fig. 4D). Sodium formate, a scavenger of ‘OH radical, also reduced the intensity of DMPO/OH signal (Fig. 4E). Deferoxamine, a metal chelator, suppressed the DMPO/OH signal with a concomitant generation of deferoxamine-derived free radical (Fig. 4F). NADPH, a cofactor of certain flavoenzymes, which catalyzes the conversion from Cr(VI) to Cr(V) and ‘OH radical (Fig. 4G). To determine whether NADPH oxidase or the mitochondria electron transport chain could play an important role in Cr(VI)-induced ROS generation, DPI, NADPH oxidase inhibitor, and rotenone, a mitochondrial electron transport inhibitor, were used. As shown in Fig. 4, H and I, both DPI and rotenone reduced ‘OH radical generation induced by Cr(VI). These results indicated that both NADPH oxidase and the mitochondrial electron transport chain were involved in Cr(VI)-induced ROS generation.

The ability of Cr(VI) to generate both H₂O₂ and O₂⁻ in DU145 cells was analyzed by quantitative H₂O₂ assay kit, which determines H₂O₂ generation. In the presence of Cr(VI), H₂O₂ generation was dramatically enhanced, whereas catalase markedly suppressed the formation of H₂O₂ induced by Cr(VI) (Fig. 5). Specific fluorescent dye was used directly to visualize free radical generation inside the DU145 cells. HE, a specific fluorescent dye for O₂⁻ was used to detect the generation of O₂⁻. The cells were visualized with a laser scanning confocal microscope. When comparing to control, Cr(VI) markedly enhanced O₂⁻ radical production inside the cell, which exhibited a red or orange color after being oxidized by O₂⁻. Addition of superoxide dismutase decreased it (Fig. 6A). The generation of O₂⁻ was also examined by flow cytometry analysis of the stained cells. For O₂⁻ production, the relative fluorescence intensity increased from 200 to 550 after Cr(VI) treatment (Fig. 6B). Because dye staining is not specific for the detection of O₂⁻ specific antioxidants and metal chelator were used to verify that O₂⁻ radicals were indeed detected. Superoxide dismutase, a specific scavenger of O₂⁻, decreased the relative fluorescence intensity from 550 to 350, whereas catalase, sodium formate, and defereroxamine had only minor effects (Fig. 6B). Because O₂⁻ is the one-electron reduction product of molecular oxygen, the O₂ consumption in cells treated with Cr(VI) was measured using an oxygraph. As shown in Fig. 7, Cr(VI) increased the O₂ consumption, and addition of NADPH enhanced it.

Reactive Oxygen Species Generated by Cr(VI) Are Involved in HIF-1α and VEGF Expression—To understand whether ROS generated by Cr(VI) in DU145 play a role in Cr(VI)-induced HIF-1α expression, the effects of various antioxidants on Cr(VI)-induced HIF-1α expression were determined using immunoblot assay. As shown in Fig. 8A, treatment of cells with Cr(VI) at a concentration of 5 μM for 3 h induced HIF-1α expression. Catalase, a H₂O₂ scavenger, inhibited Cr(VI)-induced HIF-1α expression. Sodium formate, an ‘OH radical scavenger, slightly inhibited it. In contrast, treatment of cells...
with superoxide dismutase did not inhibit Cr(VI)-induced HIF-1α expression. The results indicated that H₂O₂ played a key role in Cr(VI)-induced HIF-1α expression. To further confirm the role of ROS in biological activity of HIF-1α induced by Cr(VI), the effects of various specific antioxidants on Cr(VI)-induced VEGF expression were determined using ELISA. Treatment of cells with Cr(VI) induced a significant increase in VEGF protein level secreted by the culture cells (Fig. 8B). Pretreatment of cells with catalase decreased the VEGF protein level induced by Cr(VI), and sodium formate slightly inhibited it, whereas superoxide dismutase did not exhibit any effect on VEGF expression (Fig. 8B). In contrast, deferoxamine, a metal chelator, enhanced the protein level of VEGF induced by Cr(VI). These results suggest that H₂O₂ generated by Cr(VI) plays an important role in Cr(VI)-induced HIF-1α and VEGF expression.

**H₂O₂ Generation Is Required for the Cr(VI)-induced Transcriptional Activation of VEGF**—We transfected a VEGF reporter containing a 2.5-kb fragment of the 5′ flanking region from the human VEGF gene to determine whether Cr(VI)-induced transcriptional activation of VEGF in the cells. Cr(VI) increased the reporter activity by 50% in the presence of 1.5 μM Cr(VI) (Fig. 9A), and this induction and basal activity of the reporter were completely inhibited by catalase (Fig. 9B). Superoxide dismutase and sodium formate exhibited some inhibition. Cotransfection of the dominant-negative form of HIF-1, HIF-1DN, also inhibited both induction and basal activity of the reporter. These results suggest that both H₂O₂ generation...
and HIF-1 are important for the transcriptional activation of VEGF.

**DISCUSSION**

The results obtained from the present study show that Cr(VI) was able to induce HIF-1α and VEGF protein expression in dose- and time-dependent manner in DU145 cells, whereas HIF-1β protein levels were not affected by Cr(VI) treatment. It has been reported that HIF-1β is not affected significantly by cellular oxygen tension (5), whereas HIF-1α protein is degraded rapidly under normoxic conditions by the ubiquitin-proteasome system (51–53). Cr(VI) may increase HIF-1α expression by a similar signal pathway as hypoxia. The functional HIF-1α/HIF-1β complex is regulated primarily by the abundance of the HIF-1α subunit. It is known that HIF-1 activates VEGF expression by binding directly to VEGF promoter in response to hypoxia. Similarly, the induction of HIF-1

**FIG. 7.** Oxygen consumption in the cells. DU145 cells were incubated without or with 1 mM Cr(VI) and 1 mM NADPH. Oxygen consumption was measured with a Gilson oxygraph equipped with a Clark microelectrode as described under “Materials and Methods.” * indicates that the oxygen consumption in the cells was increased significantly compared with the control (p < 0.01). Values are means + S.E. (n = 3). # indicates that the oxygen consumption in the cells was increased significantly compared with the Cr(VI)-treated group (p < 0.05).

**FIG. 8.** Effects of antioxidants on Cr(VI)-induced HIF-1α and VEGF expression. A, the cells were plated in a 60-mm culture dish and pretreated with or without antioxidants for 30 min and then treated with 5 μM Cr(VI) for 3 h. The total protein extracts were separated by SDS-PAGE, transferred to a membrane, and detected using the antibodies against HIF-1α and HIF-1β. B, the cells were seeded in a 6-well plate for 24 h and pretreated with or without antioxidants for 30 min as indicated. Cells were treated with 2.5 μM Cr(VI) for 24 h, and VEGF protein concentrations in the cell culture media were determined by ELISA. The results represent the mean value of VEGF concentrations from three independent experiments with three replicates per experiment. * indicates that the VEGF level in the cells was increased significantly compared with the control (p < 0.05). # indicates that the VEGF level in the cells was decreased significantly compared with the Cr(VI)-treated group (p < 0.01).

**FIG. 9.** VEGF transcriptional activity is mediated by the H$_2$O$_2$ generation. A, the cells were transfected with VEGF reporter (1.5 μg/well) and incubated with the medium for 12 h. The cells were switched to medium in the presence or absence of 1.5 μM Cr(VI) for 24 h. Relative luciferase activity was determined as described previously (48). B, after the transfection of the cells with VEGF reporter, cells were cultured in fresh medium for 12 h and then to medium in the presence of 1.5 μM Cr(VI) for 24 h or to medium containing 1.5 μM Cr(VI) and superoxide dismutase (1000 units/ml), catalase (10,000 units/ml), or sodium formate (2 mM) as indicated for 24 h. In the same experiment, VEGF reporter plasmid was cotransfected with 1.5 or 3.0 μg of HIF-1αDN plasmid, respectively. After transfection, the cells were cultured as above in the presence of 1.5 μM Cr(VI) for 24 h. Values are means + S.E. (n = 3). * indicates a significant increase compared with control (p < 0.05). # indicates a significant decrease compared with the Cr(VI)-treated group (p < 0.01).
Cr(VI) induces HIF-1 and VEGF expression

By Cr(VI) resulted in an increased level of VEGF expression. HIF-1 and VEGF expression are associated with cancer progression and metastasis. There is a possibility that the induction of HIF-1 and VEGF may play a major role in Cr(VI)-induced carcinogenesis.

The present study also shows that ROS were involved in Cr(VI)-induced HIF-1α and VEGF expression. Among them, we suggest that H2O2 plays a key role based on several lines of evidence as follows. (a) Treatment of cells with Cr(VI) caused an increase in the generation of H2O2 using quantitative H2O2 assay. (b) ESR spin trapping measurements show that cells pretreated with Cr(VI) generated ‘OH radical using H2O2 as a precursor. (c) Cr(VI) increased the rate of cellular oxygen consumption. (d) Catalase, a specific scavenger of H2O2, decreased the generation of ROS. (e) Catalase inhibited HIF-1α or VEGF expression induced by Cr(VI), whereas sodium formate, a scavenger of ‘OH, showed little effect. (f) Superoxide dismutase did not inhibit Cr(VI)-induced HIF-1α and VEGF expression. Among these ROS, H2O2 appears to be the major species responsible for Cr(VI)-induced HIF-1α and VEGF expression. Our results indicate the possibility that Cr(VI)-mediated HIF-1α and VEGF expression may be involved in carcinogenesis induced by this metal. The mechanism of ROS generation induced by Cr(VI) could be that in the presence of NADPH and several cellular flavoenzymes (such as glutathione reductase, lipoyl dehydrogenase, ferredoxin-NADP+, and NADPH oxidase), the mitochondria electron transport chain is able to reduce Cr(VI) to Cr(V). During the reduction process, molecular oxygen is consumed to generate O2 which subsequently is converted to H2O2 through superoxide dismutase dismutation. Indeed, the oxygen consumption assay shows that molecular oxygen is the original source of ROS generation in DU145 under Cr(VI) stimulation. The major pathways involved in ROS generation are both the flavoprotein-containing NADPH oxidase complex and the mitochondrial electron transport chain. This conclusion was supported by the inhibition of ROS generation by DPI, a flavoprotein inhibitor, as well as rotenone, an inhibitor of the mitochondrial electron transport chain as determined by ESR measurements.

Previous studies have shown that both hypoxia and CoCl2 elicited an increase in dichlorofluorescin fluorescence and that oxidation of the fluorescence probe increases prior to the stabilization of HIF-1α suggesting that ROS are required for stabilization and activation of HIF-1α (54). The antioxidant pyridine-ridicolinate abolished the stabilization of HIF-1α in response to hypoxia and CoCl2, which suggests the involvement of ROS in stabilization and activation of HIF-1α. It has also been reported that treatment with exogenous H2O2 induced expression of HIF-1α, and catalase, a H2O2 scavenger, abolished H2O2-induced HIF-1α activation (55). Other studies have found that mitochondria-derived ROS are both required and sufficient to initiate HIF-1α stabilization during hypoxia. Hypoxia increases mitochondrial ROS generation at ComplexIII, which causes accumulation of HIF-1α protein (55). These results are consistent with our observations that catalase, a specific scavenger, abolished the expression of HIF-1α. Although oxygen sensor for HIF-1α expression is not yet known, the possible involvement of an NADPH oxidoreductase, such as flavoprotein, was proposed (56). O2-heme binding proteins and cytochrome P-450 were also proposed as possible oxygen sensors. Interestingly, in Hep3B hepatoma cells incapable of mitochondrial respiration, HIF-1 expression in the cells is inhibited in response to hypoxia (57), indicating that ROS produced by the mitochondria may play an important role in inducing HIF-1 expression. The present study demonstrates that Cr(VI) is able to induce HIF-1α and VEGF expression through ROS-mediated reactions. Among these species, H2O2 plays a major role.

The results obtained from the present study have demonstrated that neither P38 nor MAPK/extracellular signal-regulated kinase pathway is involved in induction of HIF-α by Cr(VI). Instead, p38 pathway is required. Although the relationship between p38 and HIF-α has not been established, from the present study it appears that Cr(VI) induces generation of ROS, which cause p38 phosphorylation, leading to HIF-α activation (Cr(VI) → ROS → p38 → HIF-1α). p38 is an oxidative stress-sensitive MAP kinase. Previous study from our laboratories has shown that Cr(VI) is able to cause p38 phosphorylation in mouse macrophage cell line RAW 264.7 cells (58). We have also shown that Cr(VI) is able to activate p38 in DU145 cells, and catalase inhibits Cr(VI)-induced p38 phosphorylation (data not shown).

It may noted that many other mineral particles and chemical carcinogens, such as asbestos and silica, are reported to be capable of generating H2O2 upon stimulation of the cells (59–61). It is possible that those agents may have the same function as Cr(VI); i.e. they may induce HIF-1α and VEGF expression through H2O2-mediated reaction. Because HIF-1α and VEGF are involved in cancer development, H2O2-induced HIF-1α and VEGF expression could be an important signal transduction pathway involved in carcinogenesis induced by these carcinogens. However, it should be noted that different agents may induce HIF-α and VEGF through different pathways. In our recent study (49), we have shown that vanadate is able to induce HIF-α and VEGF through P38/MAPK pathway. In Cr(VI)-induced HIF-α and VEGF induction, neither P38 nor Akt is involved. Instead, p38 signaling plays a key role.

In conclusion, the present study demonstrates that Cr(VI) is able to increase the levels of HIF-1α and VEGF expression through p38 MAP kinase pathway. H2O2 generated during the Cr(VI) reaction is the major species responsible for Cr(VI)-induced expression of HIF-1α and VEGF. Further studies concerning the mechanism of HIF-1α and VEGF expression induced by Cr(VI) may yield important clues regarding the role of HIF-1α and VEGF in carcinogenesis induced by Cr(VI).

REFERENCES

1. Folkman, J. (1995) N. Engl. J. Med. 333, 1757–1763
2. Folkman, J. (1995) Nat. Med. 1, 27–31
3. Carmeliet, P., and Jain, R. K. (2000) Nature 407, 249–257
4. Mashiach, G., Li, J., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5930–5934
5. Jiang, B. H., Semenza, G. L., Bauer, C., and Marti, H. H. (1998) Am. J. Physiol. 271, C1172–C1180
6. Hoffman, R. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991) Science 252, 954–958
7. Semenza, G. L. (2000) Crit. Rev. Biochem. Mol. Biol. 35, 71–103
8. Jiang, B. H., Agani, F., Pascualini, A., and Semenza, G. L. (1997) Cancer Res. 57, 5238–5235
9. Maxwell, P. H., Dachs, G. U., Giedel, J. M., Nicholls, L. G., Harris, A. L., Strafford, J. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8104–8109
10. Maruyama, K., Chen, E. Y., Laderoute, K. R., and Giaiella, A. J. (1997) Blood 90, 3222–3331
11. Zhong, H., DeMarco, A. M., Laughner, E., Lim, M., Hilton, D. A., Zaggia, D., Buechler, P., Uecke, W. B., Semenza, G. L., and Simonas, J. W. (1999) Cancer Res. 59, 5830–5835
12. Iyer, N. V., Kitch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gammass, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1996) Genes Dev. 12, 149–162
13. Ryan, H. E., Lo, J., and Johnson, R. S. (1998) EMBO J. 17, 3005–3015
14. Jiang, B. H., Jiang, G., Zheng, J. Z., Lu, Z., Hunter, T., and Vogt, P. K. (2001) Cell Growth & Differ. 12, 363–369
15. Zhong, H., Chiles, K., Kudler, D., Laughner, E., Hanahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. (2000) Cancer Res. 60, 1541–1545
16. Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1998) Mol. Cell. Biol. 18, 4604–4613
17. Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995) Circ. Res. 77, 638–643
18. Angelov, L., Salihia, B., Roncalli, L., McMahon, G., and Guha, A. (1999) Cancer Res. 59, 5336–5342
19. Borghard, P., Hillan, K. J., Sirlaroman, P., and Ferrara, N. (1996) Cancer Res. 56, 4032–4039
Cr(VI) Induces HIF-1 and VEGF Expression

20. Brecken, R. A., Overholser, J. P., Staton, V. A., Waltenberger, J., Minna, J. D., and Thorpe, P. E. (2000) Cancer Res. 60, 5117–5124
21. Cheng, S. Y., Huang, H. J., Nagane, M., Ji, X. D., Wang, D., Shih, C. C., Arap, W., Huang, C. M., and Caveness, W. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8562–8567
22. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841–844
23. Paveo, P. A., Bouhana, K. S., Gallegos, A. M., Agrawal, A., Blanchard, K. S., Grimm, S. L., Jensen, K. L., Andrews, L. E., Wincott, F. E., Pirol, P. A., Tressler, R. J., Cushman, C., Reynolds, M. A., and Parry, T. J. (2000) Clin. Cancer Res. 6, 2094–2103
24. Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., Winkler, M., and Ferrara, N. (1997) Cancer Res. 57, 4595–4599
25. Saleh, M., Stacker, S. A., and Wilks, A. F. (1996) Cancer Res. 56, 393–401
26. Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A., and Ferrara, N. (1995) Cancer Res. 55, 1146–1151
27. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 834–835
28. Shweiki, D., Neeman, M., Itin, A., and Keshet, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 768–772
29. Arbiser, J. L., Moses, M. A., Fernandez, C. A., Ghiso, N., Cao, Y., Klauber, N., Frank, D., Brownlee, M., Flynn, E., Parangi, S., Byers, H. R., and Folkman, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 861–866
30. Konishi, T., Huang, C. L., Adachi, M., Taki, T., Inufusa, H., Kodama, K., Kohn, N., and Miyake, M. (2000) Int. J. Oncol. 16, 501–511
31. Volpert, O. V., Dameren, K. M., and Bouck, N. (1997) Oncogene 14, 1495–1502
32. Rojas, E., Herrera, L. A., Parier, L. A., and Ostronsky-Wegman, P. (1999) Mutat. Res. 443, 157–181
33. Shi, X., and Dalal, N. S. (1994) Environ. Health Perspect. 102, Suppl. 3, 231–236
34. Shi, X. L., and Dalal, N. S. (1989) Biochem. Biophys. Res. Commun. 163, 627–634
35. Shi, X. L., and Dalal, N. S. (1990) Arch. Biochem. Biophys. 281, 90–95
36. Shi, X. L., and Dalal, N. S. (1992) Arch. Biochem. Biophys. 292, 323–327
37. Wang, S., Leonard, S. S., Ye, J., Ding, M., and Shi, X. (2000) Am. J. Physiol. Cell Physiol. 279, C868–C875
38. Ye, J., Zhang, X., Young, H. A., Mao, Y., and Shi, X. (1995) Carcinogenesis 16, 2401–2405
39. Ye, J., Wang, S., Leonard, S. S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojansaksuk, Y., Vallyathan, V., Castranova, V., and Shi, X. (1999) J. Biol. Chem. 274, 34974–34980
40. Wang, S., and Shi, X. (2001) Carcinogenesis 22, 757–762
41. An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V., and Neckers, L. M. (1998) Nature 392, 405–408
42. Blagosklonny, M. V. (2001) Oncogene 20, 395–398
43. Carmeliet, P., Der, Y., Herbert, J. M., Fukumura, D., Brusselkans, K., Dewerchin, M., Neeman, M., Iono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Colen, D., Keshet, E., and Ferrara, N. (1998) Nature 394, 485–490
44. Halterman, M. W., Miller, C. C., and Fedderoff, H. J. (1999) J. Neurosci. 19, 6818–6824
45. Ravi, R., Mookerjee, B., Buhvujallia, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L., and Bedi, A. (2000) Genes Dev. 14, 34–44
46. Britigan, B. E., Rosen, G. M., Thompson, B. Y., Chai, Y., and Cohen, M. S. (1986) J. Biol. Chem. 261, 17026–17032
47. Shi, X., Deng, Z., Huang, C., Ma, W., Liu, K., Ye, J., Chen, F., Leonard, S. S., Ding, M., Castranova, V., and Vallyathan, V. (1999) Mol. Cell. Biochem. 194, 63–70
48. Jiang, B. H., Rue, E., Wang, G. L., Roe, R., and Semenza, G. L. (1996) J. Biol. Chem. 271, 17771–17778
49. Gao, N., Ding, M., Zheng, J. Z., Zhang, Z., Leonard, S. S., Liu, K. J., Shi, X., and Jiang, B. (2002) J. Biol. Chem. 277, 31963–31971
50. Shi, X., Ding, M., Ye, J., Wang, S., Leonard, S. S., Zang, L., Castranova, V., Vallyathan, V., Chiu, A., Dalal, N., and Liu, K. (1999) J. Inorg. Biochem. 75, 37–44
51. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7987–7992
52. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. (1999) J. Biol. Chem. 274, 6519–6525
53. Salceda, S., and Caro, J. (1997) J. Biol. Chem. 272, 23642–23647
54. Salnikow, K., Shi, W., Blagosklonny, M. V., and Costa, M. (2000) Cancer Res. 60, 3375–3378
55. Chand, S. N., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) J. Biol. Chem. 275, 25120–25138
56. Bunn, P. H., and Poyton, R. O. (1996) Physiol. Rev. 76, 839–885
57. Chand, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
58. Chen, F., Ding, M., Lu, Y., Leonard, S. S., Vallyathan, V., Castranova, V., and Shi, X. (2000) J. Environ. Pathol. Toxicol. Oncol. 19, 231–238
59. Ding, M., Shi, X., Lu, Y., Huang, C., Leonard, S., Roberts, J., Antonini, J., Castranova, V., and Vallyathan, V. (2001) J. Biol. Chem. 276, 9108–9114
60. Huang, C., Ding, M., Li, J., Leonard, S. S., Rojansaksuk, Y., Castranova, V., Vallyathan, V., Jiu, G., and Shi, X. (2001) J. Biol. Chem. 276, 22397–22403
61. Vallyathan, V., and Shi, X. (1997) Environ. Health Perspect. 105, Suppl. 1, 165–177
p38 Signaling-mediated Hypoxia-inducible Factor 1α and Vascular Endothelial Growth Factor Induction by Cr(VI) in DU145 Human Prostate Carcinoma Cells
Ning Gao, Bing-Hua Jiang, Stephen S. Leonard, Linda Corum, Zhuo Zhang, Jenny R. Roberts, Jim Antonini, Jenny Z. Zheng, Daniel C. Flynn, Vince Castranova and Xianglin Shi

J. Biol. Chem. 2002, 277:45041-45048.
doi: 10.1074/jbc.M202775200 originally published online September 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202775200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 61 references, 35 of which can be accessed free at http://www.jbc.org/content/277/47/45041.full.html#ref-list-1