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NADPH Oxidase Activation Is Required in Reactive Oxygen Species Generation and Cell Transformation Induced by Hexavalent Chromium

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Hexavalent chromium [Cr(VI)] is a well-known human carcinogen associated with the incidence of lung cancer. Although overproduction of reactive oxygen species (ROS) has been suggested to play a major role in its carcinogenicity, the mechanisms of Cr(VI)-induced ROS production remain unclear. In this study, we investigated the role of NADPH oxidase (NOX), one of the major sources of cellular ROS, in Cr(VI)-induced oxidative stress and carcinogenesis. We found that short-term exposure to Cr(VI) (2μM) resulted in a rapid increase in ROS generation in Beas-2B cells, and concomitantly increased NOX activity and expression of NOX members (NOX1–3 and NOX5) and subunits ($p22^{phox}$, $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$). Cr(VI) also induced phosphorylation of $p47^{phox}$ and membrane translocation of $p47^{phox}$ and $p67^{phox}$, further confirming NOX activation. Knockdown of $p47^{phox}$ with a short hairpin RNA attenuated the ROS production induced by Cr(VI). Chronic exposure (up to 3 months) to low doses of Cr(VI) (0.125, 0.25, and 0.5μM) also promoted ROS generation and the expression of NOX subunits, such as $p47^{phox}$ and $p67^{phox}$, but inhibited the expression of main antioxidant enzymes, such as superoxidase dismutase (SOD) and glutathione peroxidase (GPx). Chronic Cr(VI) exposure resulted in transformation of Beas-2B cells, increasing cell proliferation, anchorage independent growth in soft agar, and forming aggressive tumors in nude mice. Stable knockdown of $p47^{phox}$ or overexpression of SOD1, SOD2, or catalase (CAT) eliminated Cr(VI)-induced malignant transformation. Our results suggest that NOX plays an important role in Cr(VI)-induced ROS generation and carcinogenesis.

Key Words: **hexavalent chromium; NADPH oxidase; ROS generation; carcinogenesis.**

Chromium (Cr) compounds are widely used in industries, such as plating, paint, steel, tanning, and chrome ore processing (Leonard *et al*., 2004). Environmental chromium (Cr) contamination is ubiquitous. Both epidemiologic and clinical studies indicate that exposure to hexavalent chromium [Cr(VI)] compounds is linked to the high incidence of respiratory cancers (Gibb *et al*., 2000; International Agency for Research on Cancer [IARC], 1990; Langard 1990). IARC (1990) has classified Cr(VI) as a class I carcinogen. It has been postulated that overproduction of reactive oxygen species (ROS) play a major role in carcinogenicity of Cr(VI) (O′Brien *et al*., 2003). Intracellular reduction of Cr(VI) to the stable Cr(III) is associated with the production of ROS and a series of oxidative intermediates. The subsequent oxidative damage to different intracellular molecules like DNA, proteins, and lipids is believed to contribute to Cr(VI) carcinogenesis (Quievryn *et al*., 2003; Shi *et al*., 1999).

Although Cr(VI)-induced oxidative stress is well established, the molecular mechanism by which Cr(VI) induces ROS generation and its contribution to carcinogenesis remains to be investigated. NADPH oxidase (NOX) is one of the major sources of cellular ROS. The NOX family consists of the homologous enzymes NOX1–4 and the more distantly related NOX5, Duox1, and Duox2 (Bedard and Krause, 2007). NOX1 and NOX3 are the closest homologs of NOX2 in terms of structure and function (Cheng *et al*., 2004; Geiszt *et al*., 2004). NOX2-based system is a multisubunit enzyme that catalyzes the reduction of molecular oxygen and oxidation of NADPH to generate superoxide radicals ($O_2^{\cdot-}$), which undergoes dismutation to form H_2O_2 (Babior, 2004; Lambeth 2004). The subunits are distributed in plasma membrane (cytochrome b558, comprised of $p22^{phox}$ and $gp91^{phox}$) as well as cytoplasm ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) (Babior, 2004). Upon activation, a low–molecular-weight G protein (Rac1 or Rac2) and phosphorylated cytoplasmic $p47^{phox}$ subunit initiate migration of the cytosolic elements to the plasma membrane, whereby a functional complex forms that generates $O_2^{\cdot-}$ (Lambeth *et al*., 2004). The $p47^{phox}$ acts as a regulatory subunit of NOX to initiate assembly of active oxidase, which is important for NOX activation. Inhibition of $p47^{phox}$ abrogated NOX function and diminished ROS production in ovarian and prostate cancer cells

(Xia *et al*., 2007). All NOX members share common structural characteristics, including six hydrophobic transmembrane domains, conserved motifs in the cytoplasmic domains involved in NADPH and flavin-adenine dinucleotide binding, and two heme moieties, which are located to the intramembranous domain (Brown and Griending, 2009). In addition to these common features, Nox5 contains an N-terminal extension with four Ca^{2+}-binding EF-hand domains (Bánfi *et al*., 2004).

All NOX enzymes have been implicated in ROS production and tumorigenicity in various cancer cells (Ushio-Fukai and Nakamura, 2008). NOX1 is highly expressed in human colon cancers and prostate cancers (Bedard and Krause, 2007). NOX1-generated H_2O_2 can trigger an "angiogenic switch" that includes the induction of angiogenic factors promoting tumor cell vascularization and proliferation (Arbiser *et al*., 2002). NOX4- and NOX5-medicated generation of ROS has been shown to prevent apoptosis and promote tumor cell growth in melanoma cells and in pancreatic cancer cells, respectively (Govindarajan *et al*., 2007; Vaquero *et al*., 2004).

Oxidative stress may be caused by either excess ROS production and/or deficient antioxidant capacity. We hypothesized that the activation of NOX and/or the reductions of enzymes that scavenge of ROS, such as superoxidase dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) might play an important role in Cr(VI)-induced oxidative stress and carcinogenesis. We demonstrated that NOX activation played an important role in ROS generation induced by both short-term and chronic exposure of Cr(VI) in immortalized human lung epithelial cell, Beas-2B cell. Cr(VI) exposure induced phosphorylation and translocation of $p47^{phox}$ which are important for activation of NOX activation. Chronic exposure to low doses of Cr(VI) resulted in cell transformation with the characteristic of increasing cell proliferation, cell anchorage–independent growth in soft agar, and tumor growth in nude mice. Upregulation of NOX and downregulation of the main antioxidant enzymes, such as SOD and GPx, contributed to ROS generation induced by chronic Cr(VI) exposure. Inhibition of ROS overproduction by stable overexpression of antioxidant enzymes, such as SOD and CAT, or stable downregulation of NOX activation by $p47^{phox}$ short hairpin RNA (shRNA), abolished Cr(VI)-induced the transformation and tumor formation.

MATERIALS AND METHODS

Animals and reagents. Athymic nude mice were obtained from The Jackson Laboratory. All animals were housed in a specific pathogen-free room within the animal facilities at the University of Kentucky. Animals were allowed to acclimatize to their new environments for 1 week prior to use. All animals were handled according to the Institutional Animal Care and Use Committee, University of Kentucky. Dichlorodihydrofluoresceine acetate (DCFDA) and dihydroethidium (DHE) were obtained from Molecular Probes (Eugene, OR). Protein G-Sepharose beads were purchased from Amersham Biosciences (Pittsburgh, PA). Other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise mentioned. Catalase and NOX4 antibody were from Novus Biologicals, Inc. (Littleton, CO). Other antibodies were purchased from Santa Cruz (Santa Cruz, CA). Lipofectamine 2000 was purchased from Invitrogen Corporation (Carlsbad, CA). pSUPER-neo vector was from Oligoengine (Seattle, WA). NOX1 small interfering RNA (h) and NOX3-siRNA (h) were purchased from Santa Cruz. Plasmids DNA encoding human SOD1, SOD2, and catalase were purchased from Origene (Rockville, MD).

Cell lines and cell culture. Human bronchial epithelial cells (Beas-2B; ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 5% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2 in air.

Sodium dichromate dihydrate ($Na_2Cr_2O_7{\cdot}2H_2O$) was used for Cr(VI) treatment. For short-term exposure of Cr(VI), cells were grown to 80–90% confluent, then the medium was replaced with DMEM medium containing 0.1% FBS for overnight before indicated Cr(VI) treatment. In some experiments, cells were first pretreated with various inhibitors for 2 h and then exposed to Cr(VI). For chronic exposure of Cr(VI), the cells were continuously cultured in growth medium with indicated Cr(VI).

SOD1_Beas-2B, SOD2_Beas-2B, CAT_Beas-2B were generated by stable transfection of SOD1, SOD2, and CAT in Beas-2B, respectively; shRNA-$p47^{phox}$_Beas-2B were created by stable transfection of $p47^{phox}$ shRNA. The transfected cells were maintained in growth medium with 800 μg/ml G418.

Cell viability assay. Cell viability was determined using 3-(4,5-dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide (MTT) method as described previously (Wang *et al*., 2007).

Clonogenicity assay. Beas-2B cells were seeded at 10^5 cells per 60-mm^2 dish and incubated for 24 h prior to treatment. After the indicated exposure, cells were collected by trypsinization, counted, and reseeded at 1×10^3 cells per 60-mm^2 dish in triplicate. The plates were incubated for 9–10 days, then rinsed with PBS and incubated with crystal violet stain for 15–30 min at room temperature. The plates were thoroughly rinsed with dH_2O and allowed to dry. Colonies with 20 or greater cells were counted, and the triplicates were averaged.

ROS determination. Detection of ROS was performed using the fluorescent dye DCFDA and DHE as described previously (Wang *et al*., 2010). DHE is selectively oxidized by superoxide anion (O_2^{-}) into fluorescent ethidium, and DCFDA indicates oxidation by hydrogen peroxide (H_2O_2), peroxynitrite, or hydroxyl radical into fluorescent DCF. ROS generation induced by Cr(VI) was confirmed by cotreatment of Beas-2B cells with antioxidant enzymes, SOD (500 U/ml) or CAT (1000 U/ml). Potential superoxide sources were assessed by experiments performed in the presence of apocynin (APO, 50μM; NOX inhibitor), *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100μM; inhibits superoxide from dysfunctional NOS), oxypurinol (100μM; xanthine oxidase inhibitor), or rotenone (20μM; mitochondrial inhibitor). The fluorescence intensity of DCF was measured at an excitation wavelength of 492 nm and an emission wavelength of 517 nm. The fluorescence intensity of DHE was measured at an excitation wavelength of 535 nm and an emission wavelength of 610 nm.

Immunoblotting and immunoprecipitation. The procedure for immunoblotting and immunoprecipitation has been previously described (Wang *et al*., 2010). Aliquots of proteins (50 μg) were loaded into the lanes of a sodium dodecyl sulfate-polyacrylamide gel. The proteins were separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in 0.01M Tris-buffered saline (TBS) (pH 7.4) and 0.05% Tween-20 (TBST) at 23°C for 1 h. Subsequently, the membrane was incubated with primary antibodies directed against target proteins overnight at 4°C. The final dilutions for primary antibodies were: NOX1–5, 1:1000; phosphoserine, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, 1:1000. After two quick washes in TBST, the membranes were incubated with secondary antibodies

conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) diluted at 1:5000 in TBST for 1 h. The immunocomplexes were detected by the enhanced chemiluminescence method (Amersham). The density of immunoblotting was quantified with the software of ImageJ (version 1.42; National Institutes of Health, Bethesda, MD). The activation of $p47^{phox}$ by serine phosphorylation was detected using an anti-phosphoserine antibody.

Isolation of membrane and cytosolic fractions. Subcellular fraction was isolated by differential centrifugation according to the previously described method (Wei *et al*., 2006). In brief, cells from three 75% confluent 10-cm plates were harvested with cell dissociation solution (PBS-EDTA), washed once with ice-cold PBS, and centrifuged for 5 min at 700 × g. Supernatant was discarded, and the pellet was resuspended in 2.5 ml of ice-cold Tris-sucrose buffer, containing 10mM Trizma base, 340mM sucrose, 1mM ethylene glycol tetraacetic acid (EGTA), 10 μg/ml protease inhibitor mixture (Sigma), pH 7.1 and sonicated by four 15-s bursts. The cellular homogenates were clarified by centrifugation at 1475 × g at 4°C to remove nuclei and unbroken cells. The supernatant was then ultracentrifuged at 100,000 × g for 1 h at 4°C. The pellet, which was referred to as the membrane fraction, was resuspended in Tris-sucrose buffer and stored at −80°C. The supernatant was referred to as cytosolic fraction.

NOX activity assay. NOX activity was measured by the lucigenin-enhanced chemiluminescence method as described (Block *et al*., 2007). Briefly, cultured cells were homogenized in lysis buffer (20mM KH_2PO_4, pH 7.0, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) by using a Dounce homogenizer (100 strokes on ice). Homogenates were centrifuged at 800 × g at 4°C for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100-μl aliquots of homogenates were added to 900 μl of 50mM phosphate buffer, pH 7.0, containing 1mM EGTA, 150mM sucrose, 5μM lucigenin, and 100μM NADPH. Photon emission in terms of relative light units was measured in a luminometer every 30 s for 5 min. There was no measurable activity in the absence of NADPH. Superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent. SE was calculated for each set of samples.

RNAi-mediated silencing of $p47^{phox}$. Silencing of $p47^{phox}$ was performed using $p47^{phox}$-specific shRNAs in pSUPER, a Neo-resistant vector. Three sequences of 19 nucleotides in length specific for human $p47^{phox}$ cDNA (GenBank accession number NM_000265.4) were selected for synthesis of shRNA. Their sequences (5′-GATCTACGAGTTCCATAAA-3′, 5′-GGTGGTTCTGTCAGATGAA-3′, and 5′-GCAGACGCAGCGCTCTAAA-3′), corresponding to nucleotides relative to the ATG translational initiation site (138–156, 579–597, and 1062–1080), were cloned into *Bgl*II and *Hind*III sites of the vector; these vectors were named pSUPER-$p47^{phox}$-138, pSUPER-$p47^{phox}$-579, and pSUPER-$p47^{phox}$-1080, respectively. The constructs were sequenced to confirm their identities. The plasmid DNA was prepared by using a kit for plasmid DNA extraction (Ultrapure; Qiagen) according to the manufacturer's protocol.

Cell transfection. DNA and siRNA transfections were performed using Lipofectamine 2000 according to the manufacturer's recommendations. For general silencing experiments, nonsilencing siRNA (50nM) or gene-specific siRNA (50nM) was transfected into Beas-2B. The nonsilencing siRNA was used as a negative control. After 48 h transfection, the cells were starved for 16 h followed by treatment with or without Cr(VI). For generation of stable cell lines, after transfection of SOD1, SOD2, CAT, shRNA-$p47^{phox}$, or their responding vectors using Lipofectamine 2000, cells were cultured in complete medium in the presence of 1000 μg/ml G418. After 3 weeks, cell clones resistant to G418 were isolated. Transfected cells were routinely cultured in the presence of 800 μg/ml of G418. Overexpression of SOD1, SOD2, CAT, and suppressed $p47^{phox}$ protein production were confirmed by immunoblotting as described previously (Wang *et al*., 2010).

Soft agar growth assay. Anchorage-independent growth was determined by assaying colony formation in soft agar as previously described (Biedermann and Landolph, 1987). Beas-2B cells were cultured in the presence or absence of 0.125, 0.25, or 0.5μM Cr(VI) for 3 months. After 3-month treatment, cells were harvested, and 5×10^3 cells were suspended in 2 ml of medium containing 0.35% agar and seeded into six-well plates with 2 ml of a 0.5% agar base layer and maintained in a 37°C, 5% CO_2 incubator. Four weeks later, cells were stained with 1 mg/ml iodonitrotetrazolium violet in PBS overnight, and colonies greater than 0.1 mm in diameter were scored by microscopic examination.

When cultures were established from anchorage-independent colonies, soft agar colonies were plucked under sterile conditions and then dispersed in trypsin and replated into culture dishes. Transformed Beas-2B cells were cultured in DMEM growth medium containing 10% FBS.

Tumorigenesis studies. Nude mouse xenograft assays were performed as described (Block *et al*., 2007). In brief, cells were released by trypsinization and resuspended in PBS. Cell suspension (2×10^6) was injected sc into the flanks of 6-week-old male BALB/cathymic nude mice. Tumors were allowed to grow for 6 weeks. Tumor volume (mm^3) was monitored daily by externally measuring tumors in two dimensions using a caliper. Tumor volume (V) was determined by the equation $V = L \times W^2 \times 0.5$, where L is the length and W is the width of the xenograft. At the end of the experiment, mice were sacrificed and the tumors excised and snap frozen.

Statistical analysis. Differences among treatment groups were tested using ANOVA. Differences in which p value was < 0.05 were considered statistically significant. In cases where significant differences were detected, specific *post hoc* comparisons between treatment groups were examined with Student-Newman-Keuls tests. The analyses were performed using SPSS software (SPSS, Chicago, IL).

RESULTS

ROS Generation *Induced by Short-Term Cr(VI) Exposure*

First, we evaluated the effect of Cr(VI) on cell viability (Fig. 1A). Both MTT and clonogenic assay revealed that Cr(VI) exposure for 48 h decreased cell viability/proliferation in a dose-dependent manner; 2.5μM of Cr(VI) induced 53% of cell death or 40% colony formation inhibition in Beas-2B cells. Based on these results, we selected 2μM of Cr(VI) for our following short-term experiment. Cell death induced by 2μM Cr(VI) was inhibited by cotreatment with antioxidant, vitamin E, or NOX inhibitor, APO, suggesting that ROS play a role in the Cr(VI)-induced toxicity (Fig. 1B). We quantified the Cr(VI)-induced ROS production by flow cytometry using the fluorescent probes DCFDA and DHE. The fluorescence intensity produced by DCFDA and DHE was significantly higher in Cr(VI)-exposed Beas-2B cells than that in untreated control cells (Fig. 1C). ROS modulators used in combination with Cr(VI) verified these results (Fig. 1C). DHE signal was increased by Cr(VI) and LY83853 ($O_2^{\cdot-}$ donor) and inhibited by the addition of the SOD ($O_2^{\cdot-}$ scavenger). Similarly, DCF signal was increased by Cr(VI) and H_2O_2 and inhibited by CAT (H_2O_2 scavenger). The fluorescence intensity stimulated by Cr(VI) was also abolished by APO. Taken together, the results suggested that Cr(VI) exposure induced ROS production in Beas-2B cells, and NOX might play an important role in this process.

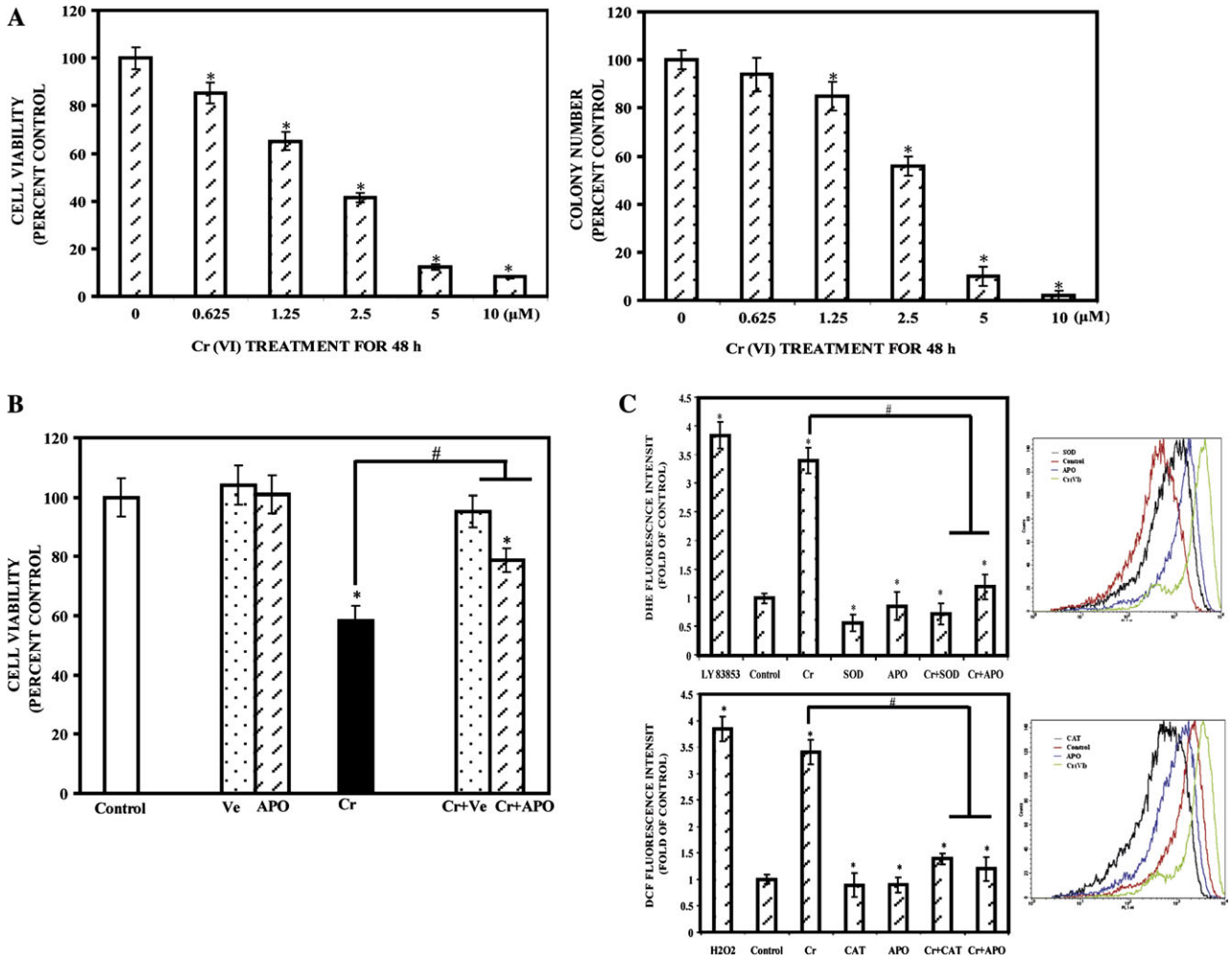

FIG. 1. Cr(VI)-induced ROS generation in Beas-2B cells. (A) Effect of Cr(VI) on the viability of Beas-2B cells by MTT assay and clonogenic assay. Beas-2B cells were treated with Cr(VI) (0, 0.625, 1.25, 2.5, 5, or 10μM) for 48 h. (B) Effect of vitamin E or APO on the viability of Beas-2B cells. Beas-2B cells were pretreated with vitamin E (Ve: 0 or 2μM) or APO (0 or 50μM) for 2 h and exposed to Cr(VI) (0 or 2μM) for 48 h. (C) Cr (VI)-induced ROS production in Beas-2B cells. Beas-2B cells were exposed to Cr(VI) (0 or 2μM) or were pretreated with SOD (500 U/ml), CAT (1000 U/ml), or APO (50μM) for 2 h followed by Cr(VI) (2μM) treatment for 6 h and then were labeled with DCFDA (10μM) or DHE (5μM) as described under the ''Materials and Methods'' section. LY83853 (10μM) and H_2O_2 (0.1mM) were used as positive controls for DHE and DCF measurements, respectively. For graph (A–C), the data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control cells. #$p < 0.05$, statistically significant difference from Cr(VI)-treated cells.

NOX Activation *Induced by Cr(VI)*

To study NOX activation induced by Cr(VI), we first measured the effect of Cr(VI) on NOX activity. Exposed Beas-2B cells to 2μM Cr(VI) resulted in a time-dependent increase in NOX activity. As shown in Figure 2A, Cr(VI) exposure induced a robust increase in NOX activity within 6 h and lasted for up to 48 h. It is noted that NOX activity in control cells were also increased. This was probably because of the absence of serum in cell culture conditions. To further determine which NOX is activated by Cr(VI), we analyzed the expression of NOX family and subunits in Beas-2B cells in response to Cr(VI) exposure. As shown in Figure 2B, Cr(VI) dramatically increased the expression level of NOX1, NOX2, NOX3, and NOX5 but not NOX4. Similarly, the expression levels of NOX subunits, such as $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, were also increased by Cr(VI). A cytoplasmic p40-p47-$p67^{phox}$ complex to the membrane is important for NOX activation, including NOX2, NOX1, and NOX3 (Groemping, 2003). Serine phosphorylation of $p47^{phox}$ is a critical step for this complex formation and a strong indication of NOX activation (Babior, 1999; Chinen *et al.*, 2007). As shown in Figure 2C, Cr(VI) exposure induced an increase in serine phosphorylation of $p47^{phox}$ within 1 h and remained at a high level up to 24 h. Concomitantly, Cr(VI) exposure for 6 h caused an increase in the membrane-associated $p47^{phox}$ and $p67^{phox}$, compared with untreated cells in which a small amount of $p47^{phox}$ and $p67^{phox}$

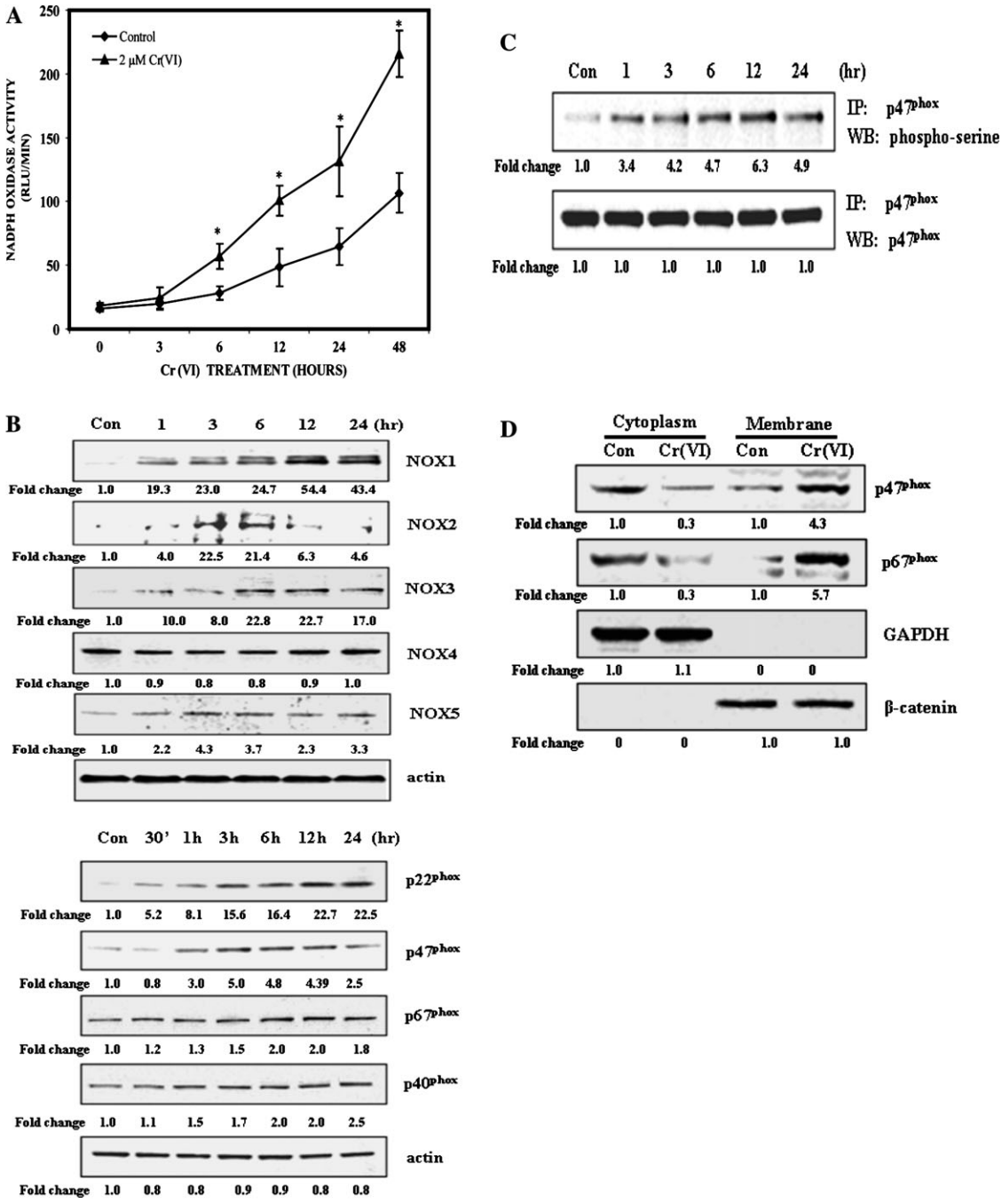

FIG. 2. Nox activation induced by Cr(VI) in Beas-2B cells. (A) NOX activity was measured by lucigenin chemiluminescence assay with Cr(VI) (0 or 2μM) exposure for specified times. The data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control cells. (B) Effect of Cr(VI) on expression of NOX members and subunits. Beas-2B cells were treated with Cr(VI) (0 or 2μM) for specified times. The expression of NOX members (NOX1, NOX2, NOX3, NOX4, and NOX5) and NOX subunits ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) were determined with immunoblotting. The expression of actin served as an internal loading control. Con: control. (C) Effect of Cr(VI) on serine phosphorylation of $p47^{phox}$ in Beas-2B cells. Beas-2B cells were stimulated with Cr(VI) (0 or 2μM) for specified times. After stimulation, the cells were lysed and the lysates were immunoprecipitated (IP) with an anti-$p47^{phox}$ antibody. The immunoprecipitation reactions were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, followed by Western blotting analysis using an anti-phosphoserine antibody. (D) Effect of Cr(VI) on $p47^{phox}$ and $p67^{phox}$ distribution. Cytoplasm and membrane proteins were extracted as described under the ''Materials and Methods'' section. The expression of $p47^{phox}$ and $p67^{phox}$ in the cytoplasm/membrane fractions was determined with immunoblotting. The expression of GAPDH and β-catemin served as internal loading control for cytoplasmic and membrane proteins, respectively. Con, control.

was found in the membrane fraction (Fig. 2D). Taken together, these results indicated that Cr(VI) exposure activated NOX in Beas-2B cells. The phosphorylation and translocation of $p47^{phox}$ may play an important role in NOX activation.

ROS Production Induced by Cr(VI) Is Mainly Mediated by NOX

To determine the origin of Cr(VI)-induced ROS, we first compared the effects of different inhibitors of ROS-generating systems on Cr(VI)-induced ROS generation. As shown in Figure 3A, APO most effectively inhibited ROS generation induced by Cr(VI) in Beas-2B cells. Rotenone partially reduced Cr(VI)-medicated ROS generation, whereas L-NAME and oxypurinol had little effect. None of these inhibitors exhibited any significant toxicity in cells (data not shown). These results suggested that NOX might be a mediator of Cr(VI)-induced ROS generation in Beas-2B cells.

To further confirm the role of NOX in Cr(VI)-induced ROS generation, we used RNA interference to inhibit NOX activation. Cr(VI) exposure increased the expression of several NOX members. Compensatory mechanisms were known to exist in NOX family. NOX4 or NOX2 knockdown with siRNA upregulated the mRNA and protein expression of the other in human lung endothelial cells (Pendyala *et al*., 2009). Our results showed that knockdown of NOX1 or NOX3 with siRNA upregulated the protein expression of the other in Beas-2B cells exposed to Cr(VI) for 24 h (Fig. 3B). To avoid these potential compensatory effects, we generated an shRNA targeting $p47^{phox}$ mRNA. As shown in Figure 3C, transfection with pSUPER-$p47^{phox}$-138 or pSUPER-$p47^{phox}$-579 significantly reduced levels of endogenous $p47^{phox}$ protein, whereas pSUPER-$p47^{phox}$-1062 had no apparent effect. These observations correlated with significant inhibition of NOX activity by the effective shRNA-$p47^{phox}$ vectors, pSUPER-$p47^{phox}$-138, and pSUPER-$p47^{phox}$-579. In particular, the most potent shRNA-$p47^{phox}$ vector, pSUPER-$p47^{phox}$-579, abolished Cr(VI)-induced ROS generation in Beas-2B cells; it decreased the DCF level by 82% (Fig. 3D). Taken together, these results indicated that NOX is an important mediator of ROS generation stimulated by Cr(VI).

Besides upregulation of ROS-generating system, impaired antioxidant capacity also contributes to oxidative stress. We next determined whether Cr(VI) exposure caused down-regulation of the main antioxidant enzymes, such as SOD, GPx, and CAT. Our results showed that Cr(VI) exposure (up to 48 h) had little effect on the expression of SOD1, SOD2, CAT, or GPx in Beas-2B cells (data not shown). Therefore, the ROS generation induced by Cr(VI), at least in a short term, is mainly due to upregulation of NOX.

Chronic exposure to low dose of Cr(VI) leads to malignant transformation of Beas-2B cells and tumor growth in nude mice.

We next examined the ability of Cr(VI) to activate NOX when cells were exposed to low dose of Cr(VI) in a chronic manner, which is thought to mimic more closely human exposure in Cr(VI)-contaminated areas. Transformation ability was assessed by anchorage-independent growth in soft agar (Carney *et al*., 1980). To elucidate the role of NOX in Cr(VI)-induced carcinogenesis, we first evaluated the ability of Cr(VI) on transformation of Beas-2B cells. We continuously exposed Beas-2B cells to low doses of Cr(VI) (0.125, 0.25, or 0.5μM) for up to 3 months. There was no obvious cytotoxicity in these concentrations of Cr(VI) (data not shown). Chronic exposure of Beas-2B cells to Cr(VI) increased cell proliferation and exhibited anchorage-independent growth in a dose-dependent manner (Figs. 4A and 4B). There was a noticeable increase in both the number and the size of colony compared with the control. It is noticed that control cells also formed a small number of slow-growing colonies in soft agar. This could be due to the fact that Beas-2B cells are reported to possess a mutation or dysfunction in p53 tumor suppressor gene (Gerwin *et al*., 1992). In addition, the American Type Culture Collection indicates that the colonies formed by Beas-2B cell line in semisolid medium were nontumorigenic in immunosuppressed mice. To extend our above findings, we examined the ability of Beas-2B cells with or without chronic Cr(VI) exposure to generate solid tumor *in vivo*. In this study, nude mice were injected sc with control cells, transformed Beas-2B, or chronic Cr(VI)-exposed Beas-2B cells. Over a period of 6-week postinjection, we observed visible tumor formation that progressively increased in size in mice injected with Cr(VI)-treated Beas-2B cells and transformed Beas-2B cells but not in those injected with control cells (Fig. 4C). Consistent with our *in vitro* findings above, chronic Cr(VI) exposure induced tumors growth in a dose-dependent manner. Transformed Beas-2B cells, which were obtained from colonies in soft agar, induced tumor growth at a much faster rate, compared with chronic Cr(VI)-exposed cells. Taken together, our results demonstrated that chronic Cr(VI) exposure led to malignant transformation of Beas-2B cells.

Suppression of ROS generation by stable knockdown of $p47^{phox}$ or overexpression of antioxidant enzymes inhibits the Cr(VI)-induced malignant transformation of Beas-2B cells.

We first measured the ROS production induced by chronic Cr(VI) exposure. As shown in Figure 5A, chronic Cr(VI) exposure resulted in a progressive increase of ROS level in a time- and dose-dependent manner in Beas-2B cells. After 3-month exposure, the ROS level in Beas-2B exposed to 0.5μM Cr(VI) was almost sevenfold higher than that in the control cells. Similarly, the expression of NOX subunits, $p47^{phox}$ and $p67^{phox}$, were upregulated by chronic Cr(VI) exposure (Fig. 5B). Antioxidant enzymes, SOD1, SOD2, and GPx but not CAT, were rapidly decreased to low levels until the end of 3 months in response to chronic low doses of Cr(VI) exposure. Thus, it appeared that the increased ROS generation in Beas-2B cells during transformation was mediated by upregulation of NOX and impaired antioxidant defense capacity.

To determine the role of ROS and NOX in malignant transformation induced by Cr(VI), Beas-2B cells were stably

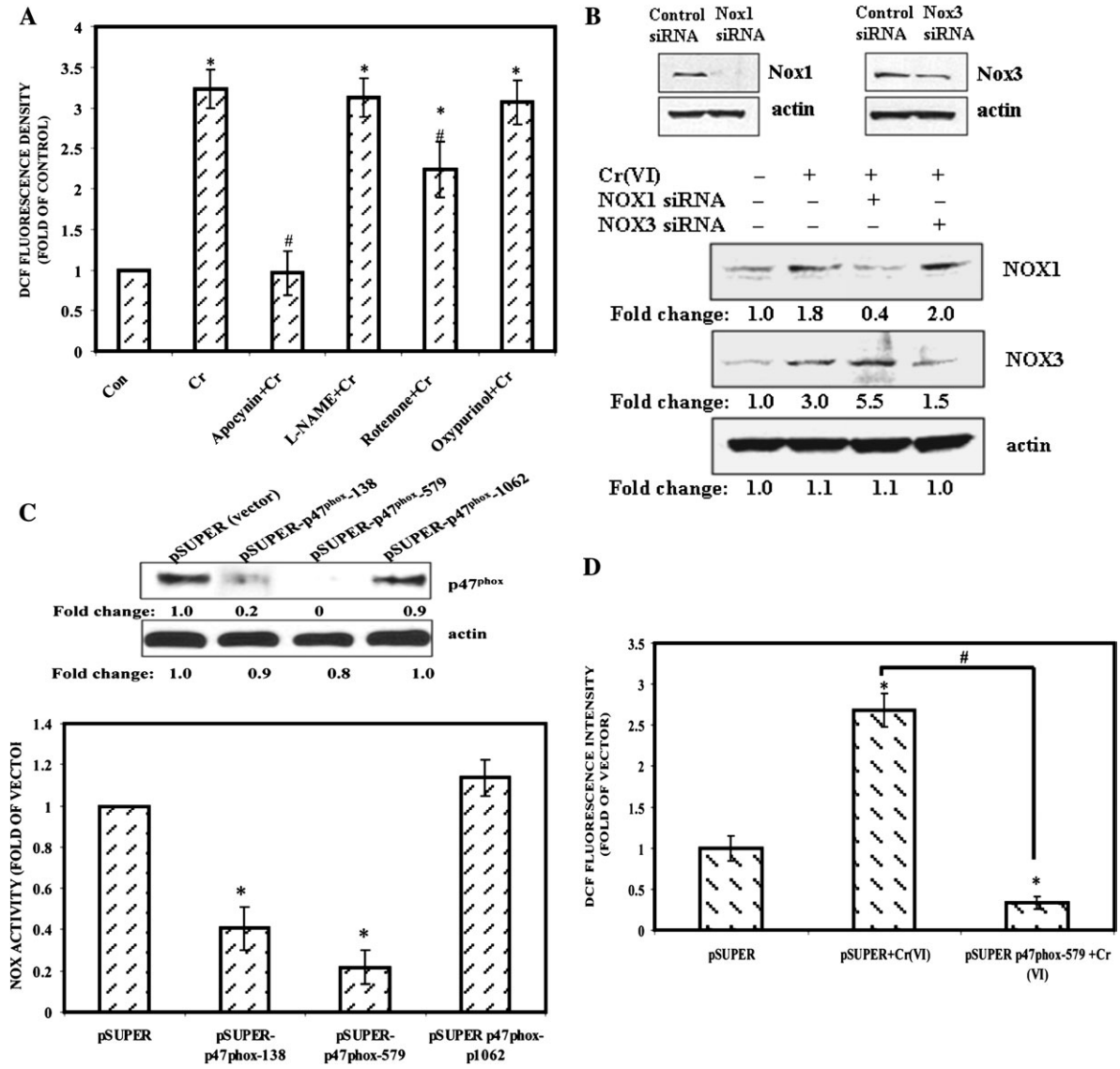

FIG. 3. Cr(VI)-induced ROS generation was mediated by NOX. (A) The effects of different inhibitors of ROS-generating systems in the Cr(VI)-induced ROS generation. Beas-2B cells were treated with Cr(VI) (0 or 2μM) or pretreated with 50μM APO, 50μM L-NAME, 1μM rotenone, or 50μM oxypurinol for 2 h followed by Cr(VI) exposure for 6 h. After exposure, cells were labeled with DCFDA (10μM) as described under the ''Materials and Methods'' section. (B) The compensatory effect of NOX1 and NOX3 under Cr(VI) exposure. Beas-2B cells were transfected with NOX1 or NOX3-siRNA as described under the ''Materials and Methods'' section. Twenty-four hours after transfection, cells were exposed to Cr(VI) (0 or 2μM) for 24 h. After Cr(VI) stimulus, the expression of NOX1 or NOX3 was determined with immunoblotting. (C) The effect of shRNA on $p47^{phox}$ expression in Beas-2B cells. Beas-2B cells were transfected with pSUPER (vector), pSUPER-$p47^{phox}$-138, pSUPER-$p47^{phox}$-579, or pSUPER-$p47^{phox}$-1062 as described under the ''Materials and Methods'' section. Forty-eight hours after transfection, the expression of $p47^{phox}$ was determined with immunoblotting. The NOX activity was measured by lucigenin chemiluminescence assay as described previously. (D) The inhibition of pSUPER-$p47^{phox}$-579 on Cr(VI)-induced ROS generation. Beas-2B cells were transfected with pSUPER or pSUPER-$p47^{phox}$-579 as described under the ''Materials and Methods'' section. Forty-eight hours after transfection, the cells were exposed to Cr(VI) (0 or 2μM) for 6 h and then were labeled with DCFDA (10μM) as described under the ''Materials and Methods'' section. For graph 3A, 3C, and 3D, the data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control cells. $^{\#}p < 0.05$, statistically significant difference from Cr(VI)-treated cells.

transfected with SOD1 (SOD1_Beas-2B), SOD2 (SOD2_Beas-2B), CAT (CAT_Beas-2B), pSUPER-$p47^{phox}$-579 (shRNA-$p47^{phox}$_Beas-2B), or their corresponding vectors (Con_Beas-2B) and then continuously exposed to 0.25μM Cr(VI) for 3 months. As shown in Figures 5C and 5D, overexpression of SOD or CAT or knockdown of $p47^{phox}$ greatly decreased cell proliferation and the colony number and size in soft agar, suggesting that ROS and NOX indeed play important roles in malignant transformation of Beas-2B cells induced by chronic exposure of low dose of Cr(VI). To confirm the above findings, we evaluated *in vivo* tumorigenicity in mice. After 3-month exposure with or without Cr(VI), SOD1_Beas-2B, SOD2_Beas-2B, CAT_Beas-2B, shRNA-$p47^{phox}$_Beas-2B, and Con_Beas-2B were injected to nude mice. Six weeks after injection, tumor formation was evaluated. Cr(VI)-exposed SOD1_Beas-2B,

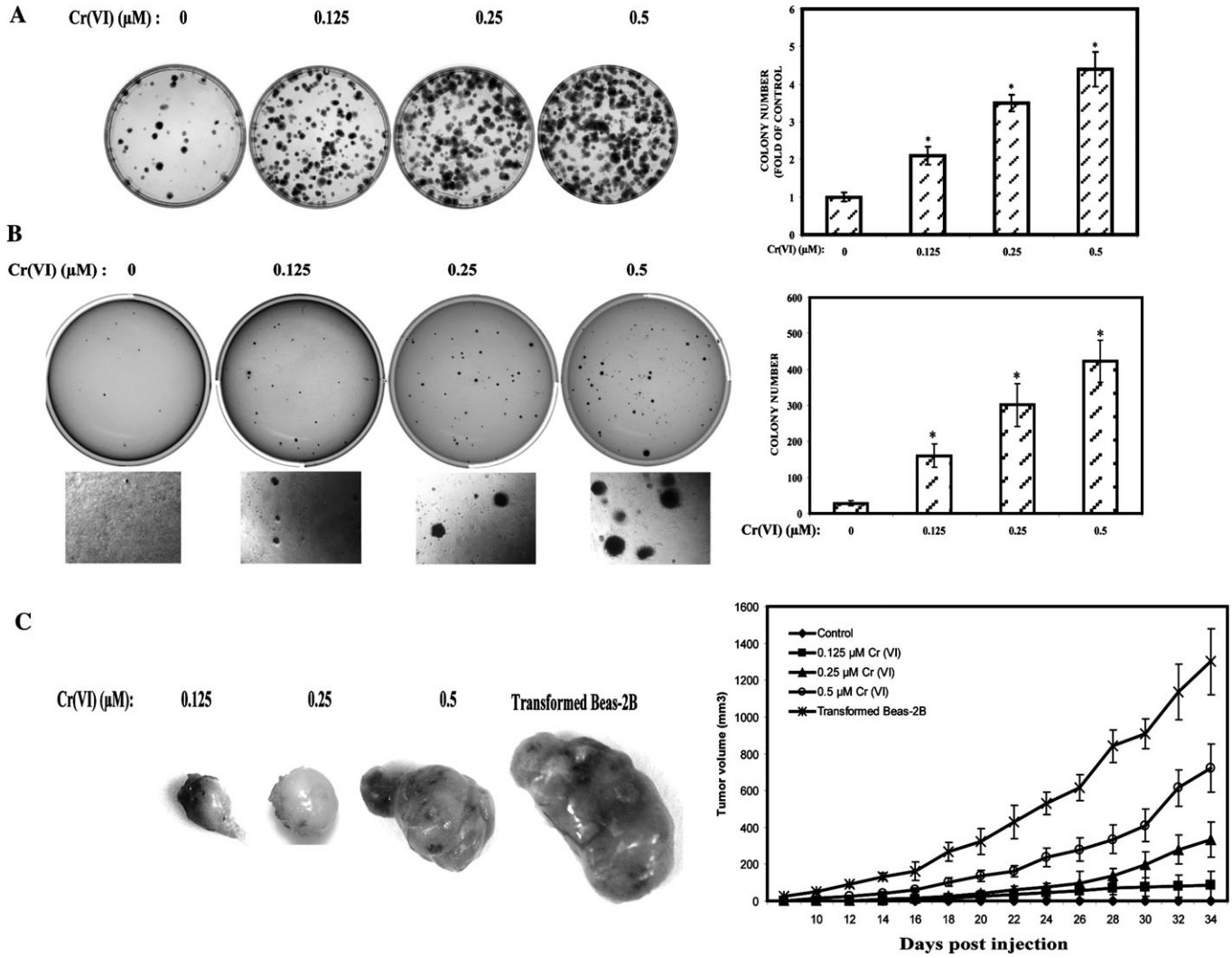

FIG. 4. The malignant transformation induced by chronic Cr(VI) exposure. (A) Clonogenic assay. Beas-2B cells were maintained in a medium containing (0 or indicated concentration of Cr(VI)) for 3 months. Then, cells were seeded at a density of 1×10^3 cells per 35-mm dish and cultured in DMEM plus 10% FBS at 37°C for 7–9 days. The colonies were stained with crystal violet. Colony numbers in the entire dish were counted. The data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control cells. (B) Soft agar assay. Beas-2B cells were maintained in a medium containing (0 or indicated concentration of Cr(VI)) for 3 months. Then, cells were seeded at a density of 5×10^3 cells per 35-mm dish and cultured in 0.35% soft agar in DMEM plus 10% FBS at 37°C for 4 weeks. The colonies were stained with 1 mg/ml iodonitrotetrazolium violet. Colony numbers in the entire dish were counted. The data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control cells. (C) Xenograft growth of tumors in nude mice. The transformed Beas-2B or Beas-2B cells exposed to indicated concentrations of Cr(VI) were injected sc (six mice per group) into 6-week-old male athymic mice. Mice were checked daily for tumor appearance, and tumor volume was measured every 3 days. The data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$, statistically significant difference from control groups.

SOD2_Beas-2B, CAT_Beas-2B, or shRNA-p47^{phox}_Beas-2B formed substantially smaller tumors than that the Cr(VI)-exposed Con_Beas-2B cells; the size of tumor decreased by 81.3, 86.5, 90.1, and 92.7%, respectively (Fig. 5E).

DISCUSSION

Cr(VI) is a well-defined human carcinogen, with lung as its primary target organ (Gibb *et al.*, 2000). Oxidative stress is a well-established mechanism in carcinogenesis induced by Cr(VI). However, the mechanisms by which Cr(VI) induces ROS generation is not clear yet. In the present study, we have demonstrated that NOX activation plays an important role in Cr(VI)-induced ROS generation in Beas-2B cells. Cr(VI) exposure increased NOX activity, the expression of several NOX members and subunits, and induced the phosphorylation and translocation of NOX subunit, p47^{phox}. Continuous exposure of Beas-2B to low doses of Cr(VI) (0.125, 0.25, and 0.5μM) resulted in cell malignant transformation with the characteristic of increasing cell proliferation, cell anchorage–independent growth in soft agar, and tumor growth in nude mice. It is likely that both

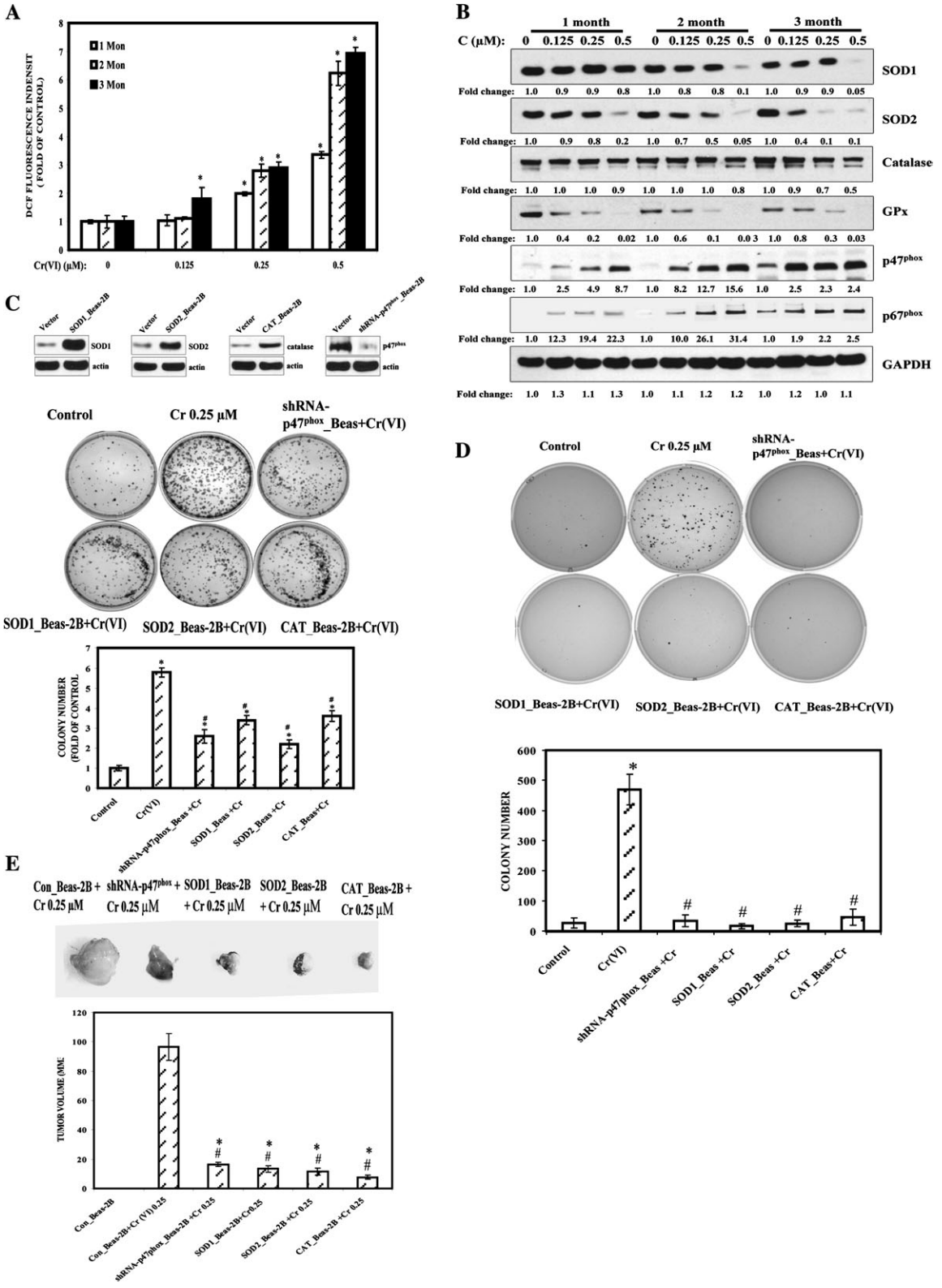

FIG. 5. Inhibition of NOX activation or overexpression of antioxidant enzymes blocked the Cr(VI)-induced malignant transformation. (A) ROS level during transformation induced by chronic Cr(VI) exposure. Beas-2B cells were maintained in a medium containing Cr(VI) (0 or indicated concentration of Cr(VI)) for specified times. After exposure, cells were labeled with DCFDA (10μM) as described under the ''Materials and Methods'' section. (B) The expression of NOX subunits ($p47^{phox}$ and $p67^{phox}$) and main antioxidant enzymes (SOD, CAT, or GPx) were determined with immunoblotting. (C and D) Inhibition of *in vitro* colony

upregulation of NOX and downregulation of main antioxidant enzymes contributed to the ROS generation induced by chronic Cr(VI) exposure. Inhibition of ROS overproduction by stable overexpression of antioxidant enzymes (SOD or CAT), or stable knockdown of $p47^{phox}$, suppressed the Cr(VI)-induced malignant transformation and tumor formation.

It is believed that ROS are produced via multiple processes, such as mitochondrial electron transport chain, NOX, nitric oxide synthase, and xanthine oxidase. Among these possibilities, recent attention has focused on NOX as a potential source of ROS production in many cell and tissue types, including lung tissue (Bocchino *et al*., 2010). Our results suggested that NOX is a major mediator of Cr(VI)-induced ROS generation although rotenone, a mitochondrial respiratory chain inhibitor, might partially inhibit generation of ROS in response to Cr(VI) (Fig. 3B). However, some studies reported that 5μM of Cr(VI) increased cellular nitric oxide (NO) level in Beas-2B cells (Azad *et al*., 2010). ROS have been previously shown to increase NO release in blood vessels and endothelial cell culture *in vitro* (Fulton *et al*., 1999). PI3K-Akt-eNOX signaling and NO synthesis are reduced in coronary vessels of $p47^{phox-/-}$ mice (Feng *et al*., 2010). One explanation for the observed differences in results is the differing concentration of Cr(VI) used. Two micromoles of Cr(VI) used in this study may not be able to induce obvious NO production. However, this is not likely to be the only contributing factor.

NOX family has seven members, NOX1 through NOX5 and Duox1 and Duox2, with the original $gp91^{phox}$ being NOX2 (Bocchino *et al*., 2010). The NOX consists of membrane-bound subunits, $gp91^{phox}$ and $p22^{phox}$, which form the flavocytochrome b_{558} complex, together with the cytosolic subunits $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ as well as small GTPase, Rac. Superoxide production is induced by assembly of the cytosolic and membrane-bound subunits, which is mediated through the serine phosphorylation of $p47^{phox}$ (Babior, 1999; Sigal *et al*., 2003). It has been demonstrated that the kinetics of $p47^{phox}$ serine phosphorylation paralleled that of NOX activation (Sigal *et al*., 2003). In this study, we found that Cr(VI) exposure increased the expression of several NOX members (NOX1, NOX2, NOX3, and NOX5) and subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$) in Beas-2B cells. Furthermore, the phosphorylation of $p47^{phox}$ and translocation of $p47^{phox}$ and $p67^{phox}$ from cytoplasm to membrane were also increased by Cr(VI) exposure. Because many studies have indicated that $p47^{phox}$ is an essential component used in conjunction with all the NOX isoforms, not just NOX2 (Taylor *et al*., 2006), we focused on $p47^{phox}$ regulation in this study to determine the role of NOX in Cr(VI)-induced ROS generation. A pharmacological inhibitor, APO or $p47^{phox}$ shRNA, blocked the increase of ROS production induced by Cr(VI). Because cellular redox homeostasis is maintained by the balance between ROS generation and elimination, increase in ROS generation or decrease in antioxidant capacity will shift the redox balance and result in an overall increase in the level of ROS. SOD, CAT, and GPx are important components of the antioxidant defense system. We found that short-term (within 48 h) exposure of Beas-2B cells to 2μM Cr(VI) had no effect on the expression of SOD, CAT, and GPx. Taken together, although other contribution in ROS generation cannot be ruled out, our results showed that NADPH-dependent oxidase system is a major source of Cr(VI)-induced ROS production.

Although NOX activity is necessary for cell signaling under physicological conditions, many studies have indicated that the NOX family of genes appears to be required for survival and growth of a subset of human cancer cells (Arnold *et al*., 2001; Fleury *et al*., 2002; Kamata, 2009; Lambeth, 2004; Ushio-Fukai and Nakamura, 2008). It is unclear whether NOX-dependent ROS generation is directly associated with Cr(VI)-induced carcinogenesis. Chronic exposure of Beas-2B cells to low dose of Cr(VI) (for up to 3 months) resulted in cell malignant transformation with the characteristic of cell anchorage-independent growth in soft agar in a dose-dependent manner. Injection of chronic Cr(VI)-exposed Beas-2B cells or transformed Beas-2B cells, which were obtained from the colony in soft agar, into nude mice led to tumor formation, whereas injection of passage-control Beas-2B cells did not induce tumor growth. Stable knockdown of $p47^{phox}$ using shRNA in Beas-2B cells dramatically decreased the number and size of colony in soft agar and tumor growth in nude mice, indicating an important role of NOX in the carcinogenesis induced by Cr(VI). However, we are aware of the limitation of this study due to the strong effect of ascorbate on Cr(VI) mutagenesis and genotoxicity. Ascorbate is reported a principal reducer of Cr(VI) *in vivo* (Salnikow and Zhitkovich, 2008). In contrast to millimolar levels of ascorbate in cells *in vivo*, cultured human and nonhepatic cells from other species contained at best about 50–60μM but usually much lower ascorbate concentrations, and they rely on thiols for Cr(VI) reduction (Salnikow and Zhitkovich, 2008). Supplement with cellular vitamin C increases chromate toxicity via a death

formation in Beas-2B cells with overexpressed antioxidant enzymes or knockdown of $p47^{phox}$ in clonogenic assay and soft agar. Beas-2B cells were stable transfected with SOD1 (SOD1_Beas-2B), SOD2 (SOD2_Beas-2B), CAT (CAT_Beas-2B), shRNA-$p47^{phox}$ (shRNA-$p47^{phox}$_Beas-2B), or their corresponding vectors (Con_Beas-2B). Blots show the efficiency of stable overexpression of SOD1, SOD2, or CAT, as well as knockdown of $p47^{phox}$ in Beas-2B cells. After exposure of above stable cell lines with Cr(VI) (0 or 0.25μM) for 3 months, clonogenic assay and soft agar assay was performed as previously described. (E) Inhibition of *in vivo* tumor growth in nude mice with overexpressed antioxidant enzymes or knockdown of $p47^{phox}$. After Con_Beas-2B, SOD1_Beas-2B, SOD2_Beas-2B, CAT_Beas-2B, or shRNA-$p47^{phox}$_Beas-2B was exposed to Cr(VI) (0 or 0.25μM) for 3 months, xenograft growth of tumors in nude mice was performed as described previously. The data are expressed as the mean ± SE of three independent experiments. $^{*}p < 0.05$, statistically significant difference from control cells. $^{\#}p < 0.05$, statistically significant difference from Cr(VI)-treated cells.

program requiring mismatch repair but not p53 in human primary IMR90 fibroblasts and epithelial H460 cells (Reynolds and Zhitkovich, 2007). Although our *in vitro* model cannot mimic the exact physiological model of Cr(VI) metabolism, it still can provide some information to better understand the mechanism of carcinogenesis induced by Cr(VI).

The ROS level during chronic Cr(VI) exposure was increased in a dose- and time-dependent manner. Under physiological conditions, normal cells maintain redox homeostasis with a low level of basal ROS by controlling the balance between ROS generation (pro-oxidants) and elimination (antioxidant capacity). Besides Cr(VI)-induced NOX activation, it is of interest to note that the expression of the antioxidant enzymes, such as SOD, CAT, and GPx, were also decreased in response to chronic Cr(VI) exposure. Stable overexpression of SOD1, SOD2, or CAT in Beas-2B cells attenuated the colony and tumor formation induced by chronic Cr(VI) exposure, indicating the important role of ROS in carcinogenesis induced by Cr(VI). Our data are consistent with some earlier studies, which demonstrated that loss of SOD has been correlated with a cancer phenotype and that overexpression of SOD leads to reversion of transformation (Loven et al., 1984; Church et al., 1993; Yan et al., 1996). However, the ROS generation during transformation is very complicated, which may vary in the different stages of transformation. In our recent studies, the ROS level in transformed Beas-2B cells induced by Cr(VI), which is derived from colony in soft agar, is much lower than that in normal cells, indicating that ROS generation is in the dynamic regulation between before and after transformation.

In summary, our finding shows that NOX activation plays an important role in Cr(VI)-induced ROS generation in Beas-2B cells. Both short-term (2μM) and chronic low doses of Cr(VI) (0.125, 0.25, and 0.5μM) exposure increased ROS generation and the expression of NOX members and related subunits. Concomitantly, Cr(VI) induced phosphorylation of $p47^{phox}$ and membrane translocation of $p47^{phox}$ and $p67^{phox}$, which are required for NOX activation. Upregulation of NOX induced by chronic exposure to low doses of Cr(VI) is associated with downregulation of the main antioxidant enzymes, such as SOD and GPx. Chronic exposure to low doses of Cr(VI) resulted in cell malignant transformation with the characteristic of cell anchorage–independent growth in soft agar and tumor growth in nude mice. Inhibition of ROS overproduction by stable overexpression of antioxidant enzymes, such as SOD and CAT, or inhibition of NOX activation by stable knockdown of its subunit $p47^{phox}$ suppressed malignant transformation induced by Cr(VI) and tumor formation. Our studies provide insight into the underlying mechanisms of Cr(VI)-induced carcinogenesis and provide important information for therapeutic approaches.

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