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Luteolin inhibits Cr(VI)-induced malignant cell transformation of human lung epithelial cells by targeting ROS mediated multiple cell signaling pathways

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Abstract

Hexavalent chromium [Cr(VI)] is a well-known human carcinogen associated with the incidence of lung cancer. Inhibition of metal induced carcinogenesis by a dietary antioxidant is a novel approach. Luteolin, a natural dietary flavonoid found in fruits and vegetables, possesses potent antioxidant and anti-inflammatory activity. We found that short term exposure of human bronchial epithelial cells (BEAS-2B) to Cr(VI) (5 μM) showed a drastic increase in ROS generation, NADPH oxidase (NOX) activation, lipid peroxidation, and glutathione depletion, which were significantly inhibited by the treatment with luteolin in a dose dependent manner. Treatment with luteolin decreased AP-1, HIF-1α, COX-2, and iNOS promoter activity induced by Cr(VI) in BEAS-2B cells. In addition, luteolin protected BEAS-2B cells from malignant transformation induced by chronic Cr(VI) exposure. Moreover, luteolin also inhibited the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) and VEGF in chronic Cr(VI) exposed BEAS-2B cells. Western blot analysis showed that luteolin inhibited multiple gene products linked to survival (Akt, Fak, Bcl-2, Bcl-xL), inflammation (MAPK, NF-κB, COX-2, STAT-3, iNOS,

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TNF-α) and angiogenesis (HIF-1α, VEGF, MMP-9) in chronic Cr(VI) exposed BEAS-2B cells. Nude mice injected with BEAS-2B cells chronically exposed to Cr(VI) in the presence of luteolin showed reduced tumor incidence compared to Cr(VI) alone treated group. Overexpression of catalase (CAT) or SOD2, eliminated Cr(VI)-induced malignant transformation. Overall, our results indicate that luteolin protects BEAS-2B cells from Cr(VI)-induced carcinogenesis by scavenging ROS and modulating multiple cell signaling mechanisms that are linked to ROS. Luteolin, therefore, serves as a potential chemopreventive agent against Cr(VI)-induced carcinogenesis.

Keywords

Hexavalent chromium; Luteolin; Carcinogenesis; Inflammation; Angiogenesis

Introduction

Hexavalent chromium [Cr(VI)] is recognized as a human carcinogen associated with an increased risk of lung cancer. Exposure to Cr(VI) induces DNA damage, cell morphological changes and malignant transformation in human lung epithelial cells (Ding *et al.*, 2013). Epidemiological studies have reported that the lung cancer morbidity rate is 20 times higher in ex-chromate workers than for non-smokers (Nakagawa *et al.*, 1984). Overproduction of reactive oxygen species (ROS) has been suggested to play a major role in Cr(VI) carcinogenesis (O'Brien *et al.*, 2003; Wang *et al.*, 2011). Reports suggested that ROS plays an important role in inflammation (Khodr and Khalil, 2001; Barbieri *et al.*, 2003), angiogenesis (Ushio-Fukai and Alexander, 2004) and malignant cell survival (Fruehauf and Meyskens, 2007). NADPH oxidase (NOX) is one of the major sources of cellular ROS, and previous reports from our laboratory implicate the role of NOX in Cr(VI)-induced ROS generation and carcinogenesis (Wang *et al.*, 2011). In addition, chronic Cr(VI) exposure has been shown to induce lipid peroxidation (LPO) and decrease glutathione (GSH) (Anand, 2005; Thompson *et al.*, 2011) in *in vivo*.

Chronic inflammation is associated with lung carcinogenesis. Repetitive exposure to Cr(VI) results in persistent inflammation, and such an inflammatory microenvironment can further promote lung carcinogenesis (Beaver *et al.*, 2009a; Beaver *et al.*, 2009b). Cyclooxygenase-2 (COX-2) is a key enzyme in the conversion of arachidonic acid to prostanoids, and its activation is associated with inflammation and carcinogenesis (Hakozaki *et al.*, 2014). Elevated COX-2 expression has been demonstrated in Cr(VI)-exposed cultured cells (Zuo *et al.*, 2012; Son *et al.*, 2013). Pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) are involved in several pathological processes (Bouraoui *et al.*, 2008). Upregulation of these cytokines was closely linked to chronic inflammation and lung cancer (Azad *et al.*, 2008; Pratheesh Kumar and Kuttan, 2009; Pine *et al.*, 2011; Pratheeshkumar and Kuttan, 2011a; Pratheeshkumar *et al.*, 2011). STAT3 is linked to inflammation-associated oncogenic transformation, by promoting pro-oncogenic inflammatory pathways, including nuclear factor-κB (NF-κB) and interleukin-6 (IL-6) (Yu *et al.*, 2009). Previous studies demonstrated a prolonged STAT3 activation and transactivation of IL-6, with Cr(VI) exposure in human

epithelial cells (O'hara *et al.*, 2007). Inducible nitric oxide synthase (iNOS), one of the three isoforms of nitric oxide synthase, catalyzes the oxidative deamination of l-arginine to produce citrulline and nitric oxide (Chen *et al.*, 2005). Aberrant or excessive expression of iNOS leads to an accumulation of nitric oxide, which can participate in cancer development (Weiming *et al.*, 2002; Chen *et al.*, 2005). Cr(VI) exposure also leads to activation of mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK)1/2, p38, and extracellular-signal regulated kinase (ERK)1/2 (Gao *et al.*, 2002; Wakeman *et al.*, 2005; Chen *et al.*, 2009).

The nuclear transcription factor NF-κB controls a number of genes involved in inflammatory responses, cell cycle progression, inhibition of apoptosis and cell adhesion, and thus plays a critical role in promoting carcinogenesis and cancer progression (Okamoto *et al.*, 2007). Altered activation of NF-κB has been reported in many tumors (Perkins, 1997). Exposure to Cr(VI) caused NF-κB activation in Jurkat cells (Shi *et al.*, 1999) and human bronchial epithelial cells (Zuo *et al.*, 2012). Hypoxia inducible factor 1 α (HIF-1α) plays a crucial role in coordinating the cellular response to oxygen stress conditions (Semenza, 2003; Vaupel, 2004). During hypoxic stress, HIF-1α activates the transcription of a variety of genes involved in the process of promoting malignant cell survival and carcinogenesis (Simiantonaki *et al.*, 2008b). Vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis by increasing the vascular permeability and proliferation of vascular endothelial cells. In addition, there is a signi cant correlation between HIF-1α and VEGF expression (Blancher *et al.*, 2000). HIF-1α is upregulated in response to hypoxia and accompanied by a significant increase in the production of VEGF (Simiantonaki *et al.*, 2008b). Previous studies has shown that exposure to Cr(VI) induces HIF-1α and VEGF expression through the production of ROS in human prostate carcinoma cells (Gao *et al.*, 2002).

Cancer prevention with naturally occurring dietary agents has gained immense interest because of their safety, low toxicity, and general availability (Pratheeshkumar *et al.*, 2012c). Flavonoids are a group of important naturally occurring polyphenolic compounds with a wide range of biological effects (Zheng *et al.*, 2014). Luteolin (3′,4′,5,7-tetrahydroxyflavone, Fig. 1A) is a common dietary flavonoid found in fruits, vegetables, and medicinal herbs with recognized anti-oxidant, anti-inflammatory and anticancer activities (Harris *et al.*, 2006; Tang *et al.*, 2011; Zhang *et al.*, 2013; Zhang *et al.*, 2014). Recently we have shown that luteolin could inhibit human prostate tumor growth by suppressing vascular endothelial growth factor receptor 2-mediated angiogenesis (Pratheeshkumar *et al.*, 2012b).

In this study, we investigated the protective effect of luteolin on Cr(VI)-induced malignant transformation of human bronchial epithelial cells (BEAS-2B) and with a focus on the key molecular events involved. We found that treatment of luteolin resulted in a significant inhibition of Cr(VI)-induced (i) ROS generation, NOX activation, lipid peroxidation, and glutathione depletion; (ii) malignant transformation; (iii) pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α); and multiple gene products linked to (iv) cell survival (Erk½, p38, JNK, Akt, Fak, Bcl-2, and Bcl-xL); (v) activation of NF-κB and IKKα, and degradation of IκBα; (vi) inflammation (COX-2, STAT-3, iNOS, TNF-α); and (vii) angiogenesis (HIF-1α, VEGF, MMP-9) in human bronchial epithelial cells.

Materials and methods

Antibodies and chemicals

Luteolin (>99% pure) was purchased from Sigma (St. Louis, MO, USA), dissolved in DMSO, aliquoted, and stored at −20°C. Potassium dichromate ($K_2Cr_2O_7$) was obtained from Sigma-Aldrich (St. Louis, MO). Dichlorodihydrofluoresceine acetate (DCFDA) and dihydroethidium (DHE) were obtained from Molecular Probes (Eugene, OR). Lipofectamine 2000 was purchased from Invitrogen Corporation (Carlsbad, CA). Antibodies specific for p-P38, P38, p-AKT, AKT, p-FAK, FAK, p-STAT3, STAT3, Bcl-2, Bcl-xL, iNOS were obtained from cell signaling Technology (Beverly, MA). The primary antibodies specific for p-ERK, ERK, p-JNK, JNK, NF-κB/p65, IKKα, IκBα, COX-2, TNF-α, MMP-9, VEGF, HIF-1-α, β-actin and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Glutathione (GSH) assay kit was obtained from Cayman Chemical (Ann Arbor, MI). Assay kit for thiobarbituric acid reactive substances (TBARS) was purchased from BioAssay Systems (Hayward, CA, USA). Luciferase assay kit was obtained from Promega (Madison, WI).

Cell lines and cell culture

BEAS-2B (Human bronchial epithelial cell line), obtained from the American Type Culture Collection (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. $K_2Cr_2O_7$ was used for Cr (VI) treatment. For short-term exposure, cells at 80–90% confluence, experienced an overnight incubation with DMEM containing 0.1% FBS and Cr(VI) indicated. In some experiments, cells were pretreated with inhibitors for 2 h and then exposed to Cr (VI). For chronic exposure to Cr (VI), the cells were continuously cultured in growth medium with Cr (VI) as indicated for 6 months.

ELISA kits

Highly specific quantitative 'sand wich' Elisa kits for human IL-1β, IL-6, IL-8, and TNF-α were purchased from BioLegend, Inc. (CA, USA) and the ELISA kit for VEGF was purchased from RayBiotech (GA, USA).

Plasmid constructs and transfection

CAT-Myc-DDK-tagged plasmid was purchased from Origene (Rockville, MD). The SOD2-EGFP-tagged plasmid was obtained from Addgene (Cambridge, MA). Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, BEAS-2B cells were seeded in 6-well culture plates; when approximately 50% confluent, cells were transfected with 4 μg plasmid. Cell clones resistant to G418 were isolated and overexpression of SOD2 and CAT protein production were confirmed by immunoblot as described previously (Wang *et al.*, 2011).

HIF-1α luciferase reporter (HIF-1α-Luc) was purchased from Panomics (Fremont, CA) and AP1–luciferase (AP1-Luc) plasmid was from Stratagene (Santa Clara, CA, USA). Other luciferase reporter plasmids (COX-2-Luc, and iNOS-Luc) were kindly provided by Dr.

Chuanshu Huang (Li *et al.*, 2006; Ouyang *et al.*, 2007) from Nelson Institute of Environmental Medicine, New York University School of Medicine, NY. Transfection experiments were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell viability assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide (MTT) assay. Active mitochondrial dehydrogenases in living cells metabolize MTT to a purple formazan dye, which is measured photometrically at 570 nm using a spectrophotometer as described previously (Pratheeshkumar and Kuttan, 2011c).

Clonogenic assay

BEAS-2B cells (10^5 cells) seeded into each well of a 6-well plate, were to attach overnight. After the indicated exposure, cells were collected by trypsinization, three hundred cells were then reseeded into each of three dishes (60 mm diameter), and grown for 10 days. The cells were fixed with 2% formalin for 10 min, stained with 0.5% crystal violet and counted.

Intracellular ROS determination

Cells were washed once with warm PBS and incubated with 10 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate ethyl ester (CM-H_2DCFDA; Molecular Probes) or 5 μmol/L dihydroethidium (DHE; Molecular Probes), respectively, in warm PBS for 40 minutes. Cells were subsequently harvested with trypsin, washed twice with cold PBS, and analyzed by fluorescence-activated cell sorting (FACS Calibur, BD Biosciences). The fluorescence intensity of DCF was measured at an excitation wavelength of 492 nm and an emission wave length of 517 nm. The fluorescence intensity of DHE was measured at an excitation wavelength of 535 nm and an emission wavelength of 610 nm.

NOX activity assay

NOX activity was measured by the lucigenin enhanced chemiluminescence method as described previously (Wang *et al.*, 2011). Briefly, cells were harvested and homogenized by sonication in cold lysis buffer (20 mM KH_2PO_4, pH 7.0, 1 mM EGTA, 1mM phenyl methyl sulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Centrifugation (800 × g at 4°C for 10 min) removed unbroken cells and debris, and used immediately. To start the assay, 100-μl aliquots of homogenates were added to 900 μl of 50 mM phosphate buffer, pH 7.0, containing 1mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM 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.

Glutathione assay

Harvested cells were sonicated in cold buffer (50 mM Phosphate, pH 6–7, containing 1 mM EDTA), then centrifuged at 10,000 g for 15 min at 4 °C. Total GSH content in the supernatants was determined by using the Cayman's GSH assay kit (Cayman Chemical Co., Ann Arbor, MI) following the manufacturer's instruction.

TBARS assay

Lipid peroxidation was determined using a QuantChrom^{TM} TBARS assay kit. Cells sonicated in ice cold PBS, were subjected to centrifugation at 12,000 g for 15 min at 4°C, and the supernatant assayed according to the manufacturer's instructions. Reacted samples were added to wells of a 96 well plate and measured spectrophotometrically at 532 nm using MDA as a standard.

Luciferase reporter assay

BEAS-2B cells transfected with the luciferase reporter constructs were seeded into 24-well plates (5×10^4/well), maintained until 80–90% confluent, and then subjected to various treatments. Cellular lysates were subjected to a luciferase reporter assay (Promega) using a luminometer (Wallac 1420 Victor 2 multilabel counter system; PerkinElmer, Waltham, MA, USA) as described previously (Ding *et al.*, 2006). The reported results are expressed as relative activity normalized to the luciferase activity in the control cells without treatment.

Anchorage-independent colony growth assay

Soft agar colony formation assay was performed as described previously (Wang *et al.*, 2011). BEAS-2B cells were cultured in DMEM supplemented with 10% FBS containing Cr(VI) (0 or 0.5 μM) with or without luteolin (1 and 2 μM) for 6 months. After 6-month treatment, cells were harvested, and 1×10^4cells 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 for four weeks, and colonies greater than 0.1 mm in diameter were scored by microscopic examination.

ELISA assays for VEGF and pro-inflammatory cytokines

BEAS-2B cells were cultured in DMEM supplemented with 10% FBS containing Cr (VI) (0 or 0.5 μM) with or without luteolin (1 and 2 μM) for 6 months. Subsequently, the culture media was collected and used to estimate the levels of VEGF and pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α using commercially available ELISA kits according to the manufacturers' recommendation.

ELISA for NF-KB/p65 and STAT3

NF-κB/p65 and STAT3 were quantitatively analyzed using the Trans^{AM} ELISA kit (Active Motif, Carlsbad, CA), following the manufacturer's protocol. For this assay, the nuclear extracts of cell samples from various treatment groups were prepared using the Nuclear Extraction kit (Active Motif) according to the manufacturer's direction. Absorbance was recorded at 450 nm with reference taken at 650 nm. The assay was done in duplicate and the results are expressed as the percentage absorbance of control group.

Western blot analyses

Cells lysates were prepared in ice-cold RIPA buffer (Sigma-Aldrich) with freshly added protease inhibitor cocktail. The lysate was then centrifuged at 12 000 g for 10 min at 4°C and the supernatant (total cell lysate) was collected, aliquoted and stored at −80°C. Nuclear and cytoplasmic extracts were prepared using a extraction kit from Thermo Scientific

(Rockford, IL) according to the manufacturer's protocol. The protein concentration was determined using Coomassie Protein Assay Reagent (Thermo, Rockford, IL). Approximately 40 μg cellular proteins were separated through 6%–12% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Nonspecific binding was blocked with 5% fat-free dry milk in 1X Tris-buffered saline (TBS) and the membrane incubated with antibody, as indicated. Protein bands, detected with horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD), were visualized with enhanced chemiluminescence reagent (Perkin Elmer, Boston, MA).

Tumorigenesis studies

Athymic nude mice (NU/NU, 6–8 weeks old; Charles River), housed in a pathogen-free room in the animal facilities at the Chandler Medical Center, University of Kentucky, were handled according to the Institutional Animal Care and Use (IACUC). Cells (2×10^6 cells per mouse) from different treatments were resuspended in serum-free medium with matrigel basement membrane matrix (BD Biosciences) at a 1:1 ratio (total volume=100 μl) and subcutaneously injected into the flanks of nude mice. Mice were monitored daily for tumor appearance, and tumor volume was measured every 3 days for 30 days. Tumor volume was determined using a Vernier caliper, following the formula of $A\times B^2\times 0.52$, where A is the longest diameter of tumor and B is the shortest diameter. At the end of the experiment, mice were sacrificed and the tumors excised and snap frozen.

Histology and Immunohistochemistry

Frozen tumor sections (5 μm) were hydrated in phosphate buffered saline (PBS), and non-specific binding sites blocked with 10% horse serum in PBS, then processed according to Vectastain ABC Kit protocol (Vector Laboratories, Burlingame, CA). Briefly, the sections were incubated with rabbit anti-CD31 (1:100; Novus Biologicals Inc, Littleton, CO), mouse anti-CD34 (1:100; BD Pharmingen Inc, San Diego, CA) and rabbit anti-Ki67 (1:100, Abcam, Cambridge, MA) antibodies for 2 h at room temperature, washed and then incubated with biotinylated secondary antibody for 45 min followed by incubation with ABC reagent. After washing in PBS, sections were developed in DAB solution until the desired staining intensity was achieved. Finally, the sections were counterstained with hematoxylin.

Statistical analysis

The values presented are means ± SD. One-way analysis of variance (ANOVA) was used for statistical analysis. $p<0.05$ was considered significantly different.

Results

Luteolin inhibits Cr (VI)-induced cell viability loss in culture

The effect of luteolin on Cr (VI)-induced cytotoxicity was determined by MTT assay (Fig 1B). In BEAS-2B cells, Cr(VI) at 5 μM showed a drastic decrease (53 %) in cell viability and treatment of luteolin (10 and 20 μM) significantly ameliorates the Cr(VI)-induced cell viability loss in a dose dependent manner. Luteolin (20 μM) alone was found to be non-toxic

towards BEAS-2B cells (Fig 1B). The above result was further confirmed by clonogenic assay (Fig 1C). Cr(VI) at 2 and 4 μM significantly decreased the colony number, 52 % and 73 % respectively, whereas treatment with luteolin (20 μM) inhibited the adverse effect of Cr(VI) by increasing colony number (Fig 1D).

Luteolin inhibits Cr(VI)-induced ROS generation in BEAS-2B cells

Cr(VI)-induced ROS production was quantified by flow cytometry using the fluorescent probes DCFDA and DHE. Cr(VI) exposure dramatically stimulated $O_2^{\cdot-}$ and H_2O_2 generation as indicated by an increase of DHE (Fig. 2A–B) and DCFDA (Fig. 2D–E) fluorescence intensity respectively in BEAS-2B cells compared to untreated control cells. DHE signal was increased by Cr(VI) and LY83853 ($O_2^{\cdot-}$ donor) and inhibited by MnTMPyP, cell-permeable SOD mimetic ($O_2^{\cdot-}$ scavenger) (Fig. 2C). Similarly, DCF signal was increased by Cr(VI) and H_2O_2 and inhibited by CAT (H_2O_2 scavenger) (Fig. 2F). The fluorescence intensity stimulated by Cr(VI) was also abolished by APO, a NOX inhibitor. Pretreatment with luteolin (5, 10 and 20 μM) significantly decreased the Cr(VI)-induced $O_2^{\cdot-}$ (Fig. 2A–B) and H_2O_2 generation (Fig. 2D–E). Taken together, the results suggested that luteolin was able to inhibit Cr(VI)-induced $O_2^{\cdot-}$ and H_2O_2 generation in BEAS-2B cells.

Luteolin inhibits Cr(VI)-induced NADPH Oxidase activity in BEAS-2B cells

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important source of Cr(VI)-induced ROS production (Wang *et al.*, 2011). To examine the effect of luteolin on Cr(VI)-induced NADPH oxidase, we measured NOX activity (Fig. 3A). BEAS-2B cells exposed to 5 μM Cr(VI) caused a time-dependent increase in NOX activation whereas co-treatment with luteolin significantly ($p<0.05$) reduced this activation.

Luteolin inhibits Cr(VI)-induced lipid peroxidation and glutathione depletion in BEAS-2B cells

Malondialdehyde (MDA) is a byproduct of lipid peroxidation (LPO) and is one of the biomarkers of increased oxidative stress (Bartsch and Nair, 2004). The effect of luteolin on Cr(VI)-induced lipid peroxidation is shown in Figure 3B. Lipid peroxidation was significantly higher in Cr(VI) (5 μM) exposed BEAS-2B cells compared with the control ($p<0.05$). Treatment with luteolin reduced Cr (VI)-induced accumulation of MDA in a concentration-dependent manner. Luteolin at 10 and 20 μM significantly attenuated lipid peroxidation in Cr(VI)-exposed BEAS-2B cells ($p<0.05$; Fig. 3B).

Glutathione (GSH) is the most abundant non-enzymatic antioxidant and plays a protective role in cells under oxidative stress (Reliene and Schiestl, 2006). Cr(VI) exposure drastically reduced the glutathione level in BEAS-2B cells compared to untreated control (Fig. 3C). However, treatment with luteolin significantly ($p<0.05$) attenuated the Cr(VI)-induced GSH depletion in BEAS-2B cells.

Luteolin inhibits Cr(VI)-induced AP-1, HIF-1α, Cox-2, and iNOS dependent transactivation in BEAS-2B cells

Previous studies have shown that AP-1, HIF-1α, Cox-2, and iNOS play an important role in carcinogenesis (Gao *et al.*, 2002; Li *et al.*, 2006; Farinati *et al.*, 2010; Zuo *et al.*, 2012). We investigated the effect of luteolin on Cr(VI)-induced transactivation of AP-1, HIF-1α, Cox-2, and iNOS using a luciferase reporter assay in BEAS-2B cells. Exposure to Cr(VI) at 5 μM showed a marked increase in AP-1, HIF-1α, Cox-2, and iNOS promoter activity. Treatment with luteolin at 5, 10 and 20 μM produced a dose-dependent decrease in AP-1, HIF-1α, Cox-2, and iNOS promoter activity induced by Cr(VI) in BEAS-2B cells (Fig 4, A–D).

Luteolin inhibits malignant transformation induced by chronic Cr(VI) exposure in BEAS-2B cells

Previous reports demonstrate that chronic Cr(VI) exposure to BEAS-2B cells results in malignant transformation as assessed by increased cell proliferation, and anchorage independent growth in soft agar (Wang *et al.*, 2011). Malignant transformation was assessed by anchorage-independent growth in soft agar (Carney *et al.*, 1980). Continuous exposure of BEAS-2B cells to 0.5 μM Cr(VI) for 6 months induced marked increase in size and number of colonies compared to untreated control (Fig. 5A). However, co-treatment of luteolin (1 and 2 μM) with Cr(VI) significantly ($p<0.05$) decreased anchorage-independent growth in soft agar. Similar result were observed with the colony formation assay; Cr(VI) exposure dramatically increased the number of colonies and co-treatment of luteolin with Cr(VI) significantly ($p<0.05$) reduced the colony number in a dose-dependent manner (Fig. 5B).

Luteolin inhibits VEGF and pro-inflammatory cytokine production induced by chronic Cr(VI) exposure in BEAS-2B cells

The effect of luteolin on production of pro-inflammatory cytokines after chronic Cr(VI) treatment was investigated and are shown in Figure 6(A–D). Chronic Cr(VI) exposure resulted in elevated secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8 in BEAS-2B cells. The co-treatment of luteolin with Cr(VI) caused a dose-dependent and statistically significant ($p<0.05$) decrease in the production of TNF-α (Fig. 6A), IL-6 (Fig. 6B), IL-1β (Fig. 6C), and IL-8 (Fig. 6D) in BEAS-2B cells in culture.

Luteolin inhibits mediators of inflammation that are induced by chronic Cr(VI) exposure in BEAS-2B cells

Inflammation is implicated in Cr(VI)-induced human lung cancer development (Zuo *et al.*, 2012). Repetitive exposure to Cr(VI) increases the expression of mediators of inflammation as shown from the increased protein expressions of COX-2, STAT-3, TNF-α, and iNOS in Western blots (Fig. 6E). However, luteolin co-treatment with Cr(VI) drastically reduced the protein expressions of COX-2, STAT-3, TNF-α, and iNOS when compared to Cr(VI) treated cells. Luteolin alone did not produce any change in COX-2, STAT-3, TNF-α, and iNOS protein expression when compared to untreated BEAS-2B cells (Fig. 6E). Moreover, treatment of luteolin significantly ($p<0.05$) reduced the Cr(VI)-induced STAT-3 activation, which confirms the above results (Fig. 6F).

Luteolin inhibits phosphorylation of MAPK proteins induced by chronic Cr(VI) exposure in BEAS-2B cells

Previous studies have shown that Cr(VI)-induced oxidative stress is implicated in the activation of MAPK, a protein that is widely acknowledged as playing an important role in carcinogenesis (Samet *et al.*, 1998; Gao *et al.*, 2002; Wang Jr *et al.*, 2010). Therefore, we studied the effect of luteolin on activation of the MAPK family proteins (ERK1/2, p38 and JNK1 2) that had been induced by chronic Cr(VI) exposure using Western blot analysis (Fig. 7A). The results indicate that chronic Cr(VI) exposure in BEAS-2B cells caused an increase in the phosphorylation of ERK1/2, p38 and JNK1 2 proteins. But co-treatment with luteolin markedly reduced the Cr(VI)-mediated phosphorylation of ERK1/2, p38 and JNK1 2 proteins when compared to Cr(VI) treatment alone. Treatment of luteolin alone did not produce any change in MAPK family proteins in BEAS-2B cells. Furthermore, the total amount of ERK1/2, JNK, and p38 proteins remained unchanged in each treatment group (Fig. 7A).

Luteolin inhibits chronic Cr(VI)-induced AKT, FAK, Bcl-2, and Bcl-xL in BEAS-2B cells

Chronic Cr(VI) exposure elevated the expression levels of AKT, FAK and the anti-apoptotic proteins Bcl-2, Bcl-xL in BEAS-2B cells. Luteolin decreased the protein expression of AKT, FAK, Bcl-2, and Bcl-xL in a dose-dependent manner (Fig. 7B).

Luteolin inhibits chronic Cr(VI)-induced activation of the NF-κB pathway in BEAS-2B cells

NF-κB/p65 is a downstream target of the MAPK signal transduction pathways. Our Western blot analysis indicated that chronic Cr(VI) exposure in BEAS-2B cells stimulated the activation and translocation of NF-κB/p65 to the nucleus when levels were compared to the untreated control. Treatment of luteolin markedly inhibited the Cr(VI)-induced NF-κB activation and nuclear translocation in a dose-dependent manner (Fig. 7C). Investigations to understand the mechanisms involved in this process were pursued. Previous studies showed that Cr(VI) exposure resulted in degradation of the IκBα protein with subsequent activation and translocation of NF-κB/p65 to the nucleus (Zuo *et al.*, 2012). IKK phosphorylates serine residues in IκBα and its degradation activates NF-κB. Chronic exposure to Cr(VI) also activates IKKα and has been shown to be essential for the degradation of IκBα. To study the inhibitory effect of luteolin on Cr(VI)-induced degradation of IκBα, we determined the cytoplasmic level of IκBα protein expression. Western blot analysis showed that treatment with luteolin suppressed the IκBα degradation (Fig. 7D). Western blot analysis also indicated that levels of activated IKKα were higher in cells chronically treated with Cr(VI); however, co-treatment of luteolin inhibited the levels of activated IKKα in cytosols (Fig. 7D). The inhibitory effect of luteolin on Cr(VI)-induced NF-κB activation was further confirmed using ELISA for NF-κB/p65 (Fig. 7E).

Luteolin inhibits angiogenic mediators induced by Cr(VI) in BEAS-2B cells

Expression of HIF-1α and VEGF are upregulated in many human cancers and are associated with treatment failure (Bos *et al.*, 2003; Koukourakis *et al.*, 2006; Goel and Mercurio, 2013). Previous studies demonstrated an elevation in HIF-1α and VEGF levels that was induced by Cr(VI) treatment (Gao *et al.*, 2002). Therefore, we investigated the effect of

luteolin on protein expressions of HIF-1α, HIF-1β, VEGF, and MMP-9 in BEAS-2B cells chronically exposed to Cr(VI) by Western blot, and VEGF level by ELISA (Fig. 8). The results show that protein expressions of HIF-1α, VEGF, and MMP-9 (Fig. 8A), and VEGF levels (Fig. 8B) were increased markedly with chronic Cr(VI) exposure, whereas the expression of HIF-1β protein was not altered. Treatment of luteolin markedly decreased the Cr(VI)-induced protein expressions of HIF-1α, VEGF, and MMP-9 and the VEGF level.

Luteolin inhibits growth of xenograft tumors in mice from cells chronically exposed to Cr(VI)

In this study, nude mice were injected subcutaneously with BEAS-2B cells that had been exposed to the indicated concentration of Cr(VI), with or without luteolin, for 6 months as displayed in Figure 9A. In mice injected with Cr(VI)-treated BEAS-2B cells we observed visible tumor formation that progressively increased over a 4-week period; no tumor growth was observed in animals injected with untreated cells (Fig. 9A). Mice injected with BEAS-2B cells exposed to luteolin along with Cr(VI) showed a reduced tumor incidence. BEAS-2B cells chronically exposed to luteolin alone did not produce any tumors in nude mice. Consistent with our in vitro findings above, luteolin markedly attenuated the growth of tumors produced by Cr(VI) exposure in a dose-dependent manner.

To extend these investigations, we performed immunohistochemical analysis on these murine tumors. We observed a large number of CD31, CD34, and Ki67 (Fig. 9B) positive cells in the Cr(VI) treated group, and fewer stained cells from tumors co-treated with Cr(VI) and luteolin. All of these observations indicate the antiangiogenic efficacy of luteolin *in vivo* and strongly support the *in vitro* studies outline above.

Overexpression of antioxidant enzymes attenuates Cr(VI)-induced carcinogenicity in BEAS-2B cells

To study the role of ROS in Cr(VI)-induced malignant transformation and tumorigenesis, BEAS-2B cells were generated that stably overexpress CAT, SOD2 or their corresponding vectors (Wang *et al.*, 2011). These stable cells were continuously exposed to 0.5 μM Cr(VI) for six months. As shown in Figures 10(A–B), overexpression of SOD or CAT prominently decreased colony number and size in soft agar (Fig. 10A). Similar results were obtained with the colony formation assay, (Fig. 10B), indicating that ROS plays a key role in malignant transformation of BEAS-2B cells induced by chronic Cr(VI) exposure. To confirm these findings, we evaluated in vivo tumorigenicity in mice. After a 6-month exposure with or without Cr(VI), BEAS-2B cells stably expressing CAT, SOD2, or vector were injected to nude mice. Four weeks after injection, tumor development was assessed. Cr(VI)-exposed BEAS-2B-CAT, and BEAS-2B-SOD2 formed tumors that were smaller than those of Cr(VI)-exposed BEAS-2B-vector cells (Fig. 10C).

Discussion

Chromium is a potent human mutagen and carcinogen (Cancer and Cancer, 1990). Chromate Cr(VI) compounds, widely used in industries, such as leather tanning and wood treatment, cause environmental pollution and health concerns worldwide (Cohen *et al.*, 1993; Costa,

1997). The capability of chromium to cause cancers has been known for more than a century, and numerous epidemiological studies have been performed on workers exposed to Cr(VI) to determine its carcinogenicity (Holmes *et al.*, 2008; Xia *et al.*, 2014). Occupational exposure to hexavalent chromium [Cr(VI)] has been associated with the development of several pathologies, notably lung cancer (Abreu *et al.*, 2014). Phytomedicines have traditionally played a major role in the management of human health and are still important for health care in many countries (Kuttan *et al.*, 2011). Chemoprevention by use of natural products has emerged as a promising medical approach to reduce the risk of cancer. Luteolin is a common dietary antioxidant flavonoid found in fruits, vegetables, and medicinal herbs (Pratheeshkumar *et al.*, 2012b). Inhibition of metal induced carcinogenesis by a dietary antioxidant is a novel approach. Studies have demonstrated that co-treatment with Epigallocatechin-3-gallate (EGCG), the major polyphenol present in green tea, protected BEAS-2B cells from Cr(VI)-induced cell death in a dose-dependent manner (Wu *et al.*, 2012).

Intracellular ROS are primarily generated through aerobic metabolism or through a specialized group of enzymes, known as the NADPH oxidases (Bedard and Krause, 2007). NADPH oxidase activity is associated with several characteristic features of cancer, including cellular transformation, cell proliferation, malignant cell survival, invasion, and metastasis (Maraldi *et al.*, 2009; Block and Gorin, 2012; Liu *et al.*, 2014). In particular, increases in NADPH oxidase activity are observed in human bronchial epithelial cells exposed to hexavalent chromium (Wang *et al.*, 2011). Cr(VI) treatment also caused a significant increase in lipid peroxidation and decreases in total glutathione. Taken together these findings indicate that Cr(VI) causes oxidative stress in human bronchial epithelial cells. Similar results have been observed in previous studies involving Cr(VI) exposure (Ning and Grant, 2000; Ahmad *et al.*, 2011). Our data demonstrate that treatment with luteolin significantly ($p<0.05$) attenuates acute Cr(VI)-induced ROS generation, NOX activation, lipid peroxidation, and glutathione depletion in BEAS-2B cells in a dose dependent manner. Furthermore, luteolin also decreased the chronic Cr(VI)-induced ROS generation (data not shown).

Inflammation is an immediate defensive mechanism or reaction to an injury, which may be caused by infection, chemical agents or physical trauma (Kuby, 1997). Inflammation can accelerate cancer and chronic inflammation and is regarded as an essential factor for the progression of the neoplastic process (Wiseman and Halliwell, 1996). Several cytokines such as Tumor necrosis factor α (TNF-α), Interleukin-6 (IL-6), Interlukin-1 (IL-1β), and Interleukin-8 (IL-8) play an important role in the inflammatory process (Pratheesh Kumar and Kuttan, 2009). High circulating levels of these pro-inflammatory cytokines were reported to be associated with lung cancer (Azad *et al.*, 2008; Pine *et al.*, 2011). As a part of this investigation, we studied the effect of luteolin on proinflammatory cytokine levels in BEAS-2B cells chronically exposed to Cr(VI). The results shows that luteolin significantly suppresses proinflammatory cytokine levels in BEAS-2B cells chronically exposed to Cr(VI).

In addition to inflammation, a variety of biological mediators have been implicated in cell transformation and/or tumor growth. COX-2 plays an important role in the development of

various types of cancer, including lung cancer (Şahin *et al.*, 2009); drugs targeting this enzyme have achieved widespread clinical use (Bertagnolli, 2007). Nitric oxide has been implicated in the induction of neoplastic cell transformation (Mordan *et al.*, 1993; Robertson *et al.*, 1996), and also with tumor angiogenesis by stimulating VEGF production (Konopka *et al.*, 2001; Kisley *et al.*, 2002). Expression of iNOS is regulated by transcription factors, including NF-κB (Stoner *et al.*, 1989), AP-1 (Lee *et al.*, 2003), and by the signal transducer and activator of transcription 1α (STAT1) (Tedeschi *et al.*, 2003). In the present study, we observed that Cr(VI) exposure resulted in the activation of AP-1, COX-2, iNOS, and HIF-1 α, and is consistent with previously published studies showing that Cr(VI) exposure leads to the activation these proteins in an oxidative-stress–dependent manner (Gao *et al.*, 2002; Li *et al.*, 2006; Yao *et al.*, 2008; Zuo *et al.*, 2012). Treatment with luteolin markedly reduced the Cr(VI)-induced trans-activation of AP-1, COX-2, iNOS, and HIF-1 α in BEAS-2B cells.

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor activated in response to cytokines and growth factors, and plays critical role in various biological activities including cell proliferation, migration, and carcinogenesis (Macias *et al.*, 2013). STAT3 is constitutively activated in cells transformed by the oncogenes v-Src and v-Abl, and in a variety of human cancers, including hematologic, pancreas, breast, head and neck, and prostate cancer (Bowman *et al.*, 2000; Macias *et al.*, 2013). Moreover, STAT3 is upregulated in both premalignant tumors (papillomas) and squamous cell carcinomas of mouse skin induced by topical treatment with DMBA + TPA (Chan *et al.*, 2004). Cr(VI)-induced transactivation of STAT3 has been reported in human airway epithelial cells (O'hara *et al.*, 2007). Cr(VI) exposure in BEAS-2B cells increased Stat3 activation and phosphorylation whereas treatment of luteolin suppressed them. MAPKs are made up of three family members that include extracellular-signal-related protein kinases (ERKs), stress-activated c-JUN N-terminal (JNKs/SAPs) and p38 kinases (Einspahr *et al.*, 2008). Previous studies have shown that Cr(VI) markedly induces MAP kinase pathways in CL3 cells and is positively correlated with oxidative stress (Chuang *et al.*, 2000). Antioxidants have been shown to attenuate the activation of MAPK signaling, indicating that MAPK signaling pathway is an important target of oxidative stress (Ballif and Blenis, 2001; Katiyar *et al.*, 2001; Sharma *et al.*, 2007). Luteolin remarkably downregulated the Cr(VI) induced MAPK signaling in BEAS-2B cells. Chronic exposure to Cr(VI) increased anti-apoptotic proteins such as Bcl-2 and Bcl-XL, leading to apoptotic resistance and accumulation of genetically damaged cells (unpublished data). In our study luteolin dramatically suppressed the initiation of Cr(VI)-induced malignant transformation thereby attenuating the up regulation of anti-apoptotic proteins.

The transcription factor nuclear factor-κB (NF-κB) has been implicated in cancer development due to its ability to upregulate the expression of genes with pro-oncogenic functions (Pratheeshkumar *et al.*, 2012a). NF-κB is inactive when cytoplasmic, and bound to the inhibitory proteins IκBs. Upon activation, IκB becomes phosphorylated, a process that targets it for ubiquitination and degradation by proteasomes. This activation results the rapid translocation of NF-κB to the nucleus, where it binds to κB binding sites in the promoter region of target genes, and induces the transcription of pro-inflammatory mediators, including iNOS, COX-2, TNF-α, IL-6 and others (Perkins, 1997; Pratheeshkumar and

Kuttan, 2011c; Wang *et al.*, 2012). Previous studies have shown that NF-κB activation occurs in responses to several environmental carcinogens (Ding *et al.*, 2006; Ouyang *et al.*, 2007). In addition, p65 was required for Cr(VI)-induced NF-κB activation and COX-2 expression (Zuo *et al.*, 2012). Several chemopreventive phytochemicals have been shown to inhibit COX-2 and iNOS expression by blocking NF-κB activation (Singh and Aggarwal, 1995; Lyss *et al.*, 1997; Surh *et al.*, 2001). It has been documented that NF-κB is a downstream target of the MAPK signal transduction pathway, and that activation of NF-κB plays crucial roles in inflammation, cellular proliferation, and induction of cancers (Sharma *et al.*, 2007). The present study shows that co-treatment of luteolin markedly decreased the Cr(VI)-induced degradation of IκBα and activation of NF-κB/p65 in BEAS-2B cells.

Increased expression of matrix metalloproteinases (MMPs) has been strongly implicated in tumor growth, invasion, and metastasis (Sounni *et al.*, 2003; Pratheeshkumar and Kuttan, 2011a). Among diverse proteolytic enzymes, gelatinases such as MMP-2 and MMP-9 play a key role in degrading ECM components surrounding tumour tissue (Davidson *et al.*, 2003). Accordingly, inhibition of angiogenesis is an attractive approach to treat cancer (Carmeliet and Jain, 2000). Hypoxia-inducible factor 1 alpha (HIF-1alpha) is involved in processes promoting carcinogenesis of many tumors (Simiantonaki *et al.*, 2008a). In addition, HIF-1α is overexpressed in 70% of human cancers and their metastases. One of the genes up-regulated by HIF-1 is VEGF, a potent mediator of angiogenesis that enhances endothelial cell survival and invasion and also induces vasodilatation (Pratheeshkumar and Kuttan, 2011b). Previous studies have demonstrated the elevated expressions of HIF-1α, VEGF and MMP-9 by Cr(VI) exposure (Gao *et al.*, 2002; Son *et al.*, 2013). In our present study chronic Cr(VI) exposure elevated the expressions of HIF-1α, VEGF and MMP-9 and these were markedly suppressed by the treatment of luteolin in BEAS-2B cells.

In summary, our findings show that luteolin inhibited Cr(VI)-induced ROS generation, NOX activation, lipid peroxidation, and glutathione depletion in a dose dependent manner. Moreover, treatment with luteolin decreased AP-1, HIF-1α, COX-2, and iNOS promoter activity induced by Cr(VI) in BEAS-2B cells. Luteolin protected normal human bronchial epithelium from malignant transformation, production of pro-inflammatory cytokines and VEGF induced by chronic Cr(VI) exposure. Stable overexpression of SOD2, or CAT in BEAS-2B cells attenuated the colony and tumor formation induced by chronic Cr(VI) exposure, suggesting an important role for ROS in Cr(VI) induced carcinogenesis. Western blot analysis showed that luteolin inhibited multiple gene products linked to malignant cell survival (Akt, Fak, Bcl-2, Bcl-xL), inflammation (MAPK, NF-κB, COX-2, STAT-3, iNOS, TNF-α) and angiogenesis (HIF-1α, VEGF, MMP-9) in BEAS-2B cells after chronic Cr(VI) exposure (Fig. 11). Nude mice injected with BEAS-2B cells chronically exposed to Cr(VI) in the presence of luteolin showed reduced tumor incidence compared to Cr(VI) treatment alone. Overall, our results indicate that luteolin protects BEAS-2B cells from Cr(VI)-induced carcinogenesis by scavenging ROS and modulating multiple cell signaling mechanisms that are linked to ROS. Luteolin, therefore, serves as a potential chemopreventive agent against Cr(VI) induced carcinogenesis.

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ABBREVIATIONS

COX-2	cyclooxygenase-2
IL	interleukin
TNF-α	tumor necrosis factor-α
iNOS	inducible nitric oxide synthase
AP-1	activator protein 1
HIF-1α	Hypoxia-inducible factor 1-alpha
VEGF	Vascular endothelial growth factor
MMP-9	Matrix metalloproteinase-9
STAT-3	signal transducer and activator of transcription 3

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Highlights

- Luteolin inhibited Cr(VI)-induced oxidative stress.
- Luteolin inhibited chronic Cr(VI)-induced malignant transformation.
- Luteolin inhibited chronic Cr(VI)-induced inflammation.
- Luteolin inhibited chronic Cr(VI)-induced angiogenesis.

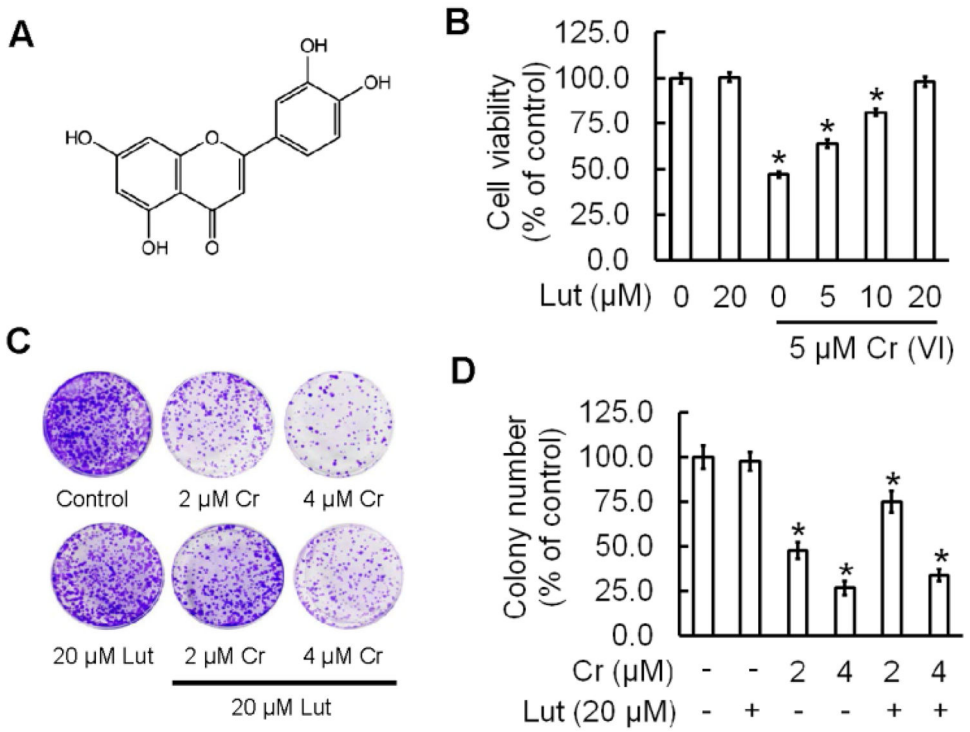

Figure 1.

Luteolin inhibits Cr (VI)-induced cytotoxicity. (A) Chemical structure of luteolin (B) Cell viability, determined by MTT, was assessed in BEAS-2B cells treated with Cr(VI) (5 μM) for 24 h in the presence of luteolin (0, 5, 10, 20 μM). (C–D) BEAS-2B cells were treated with 2 μM or 4 μM Cr(VI) with or without 20 μM luteolin for 48 h, reseeded and cultured in drug free medium for an additional 7 days and stained with crystal violet. The data are expressed as the mean ± SD of three independent experiments.*($p<0.05$).

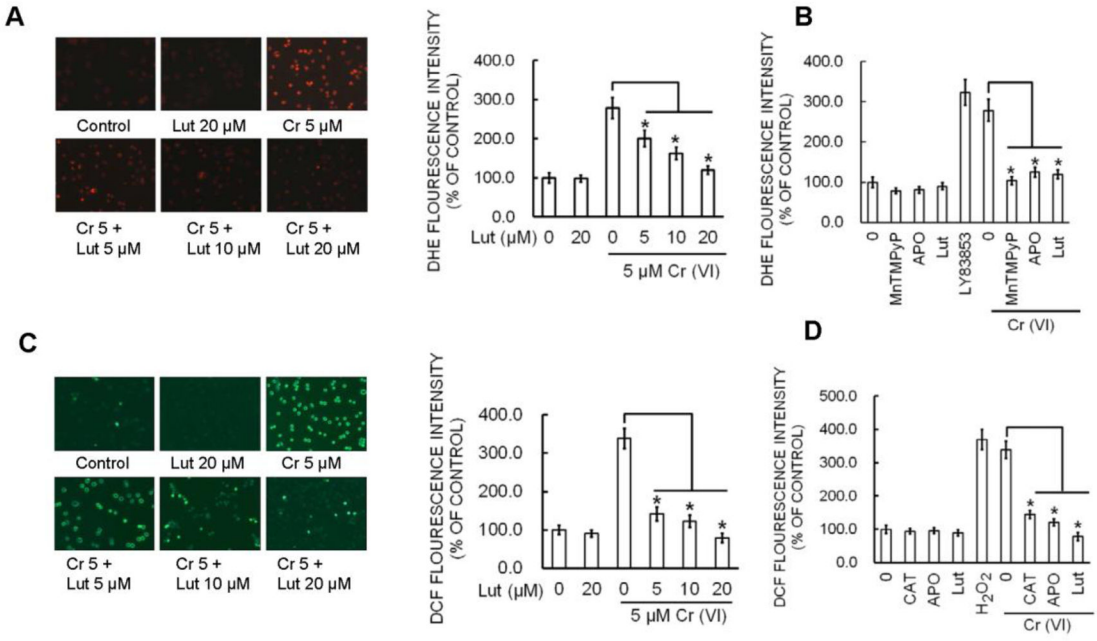

Figure 2.

Luteolin inhibits Cr(VI)-induced ROS generation. (A–B) BEAS-2B cells were exposed to Cr(VI) (0 or 5 μM) with or without luteolin (0, 5, 10, 20 μM) for 12 h and then were labeled with DHE (5 μM) or (D–E) DCFDA (10 μM). Images were taken with fluorescence microscopy and fluorescent intensity determined by flow cytometry. *($p<0.05$). BEAS-2B cells were exposed to Cr(VI) (0 or 5 μM) or were pretreated with SOD (500 U/ml), CAT (1000 U/ml), or APO (50 μM) for 2 h followed by Cr(VI) (5 μM) treatment for 6 h and then were labeled with (C) DHE (5 μM) or (F) DCFDA (10 μM) as described previously. LY83853 (10 μM) and H_2O_2 (0.1 mM) were used as positive controls for DHE and DCF measurements, respectively. The data are expressed as mean ± SD of three independent experiments. *$p<0.05$, statistically significant difference from control cells.

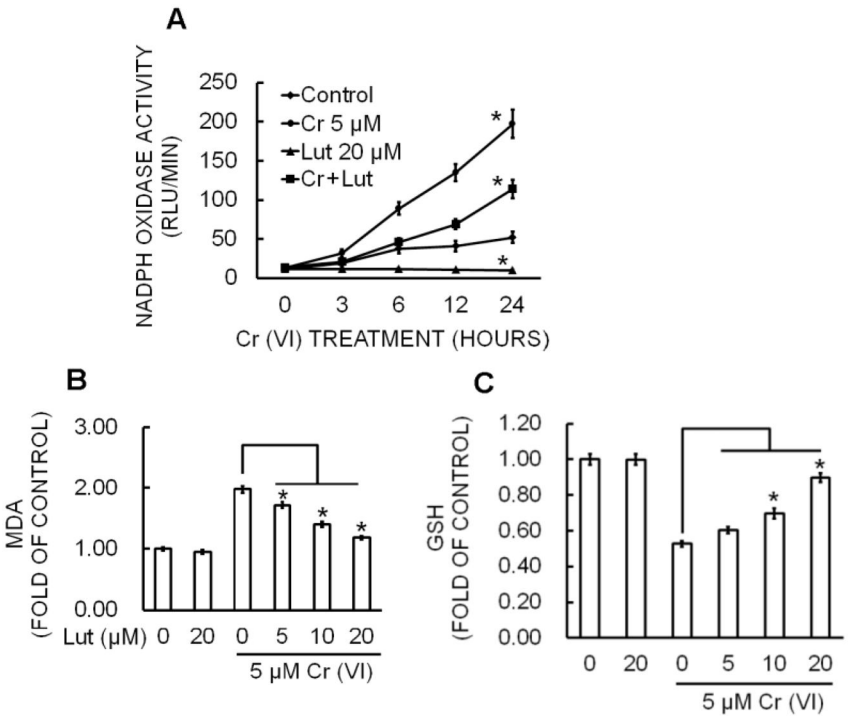

Figure 3.

Luteolin inhibits Cr(VI)-induced NOX activation, Lipid Peroxidation, Glutathione depletion in BEAS-2B cells. (A) NOX activity was measured by the lucigenin chemiluminescence assay with Cr(VI) (0 or 5 μM) in the presence of luteolin (0, 5, 10, 20 μM) for indicated times. The data are expressed as the mean ± SD of three independent experiments. *$p<0.05$, statistically significant difference from control cells. BEAS-2B cells were treated with Cr(VI) (5 μM) for 24 h in the presence of luteolin (0, 5, 10, 20 μM). Cell lysates were prepared by sonication in ice cold PBS and was used for the determination of oxidative stress in terms of (B) lipid peroxidation and (C) glutathione depletion. The data are expressed as the mean ± SD of three independent experiments. *$p<0.05$, statistically significant difference from control cells.

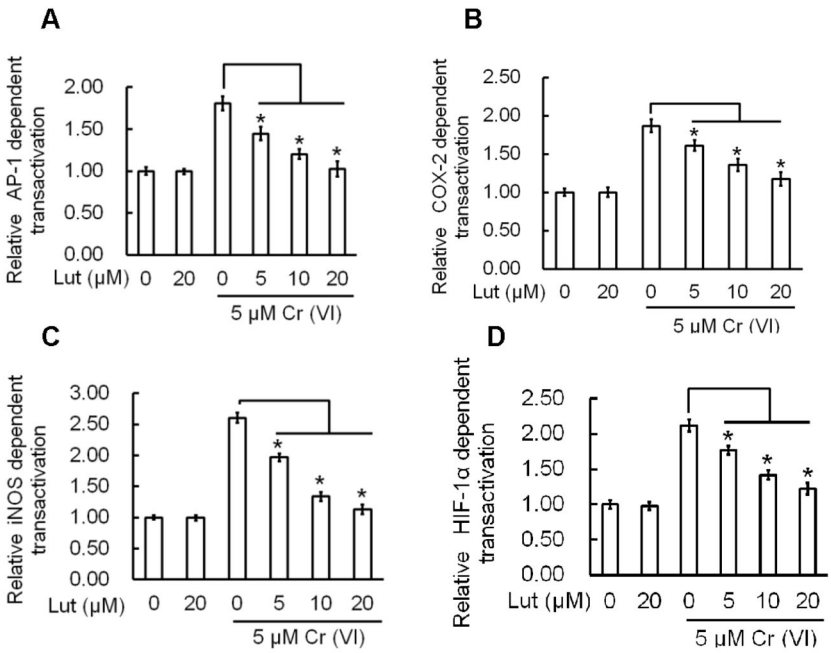

Figure 4.

Luteolin inhibits Cr(VI)-induced AP-1, Cox-2, iNOS, HIF-1α, dependent transactivation in BEAS-2B cells. BEAS-2B cells transfected with the luciferase reporter constructs were treated with Cr(VI) (5 μM) for 24 h in the presence of luteolin (0, 5, 10, 20 μM). Cellular lysates were assessed for luciferase reporter activity as described previously. The results are expressed as relative activity normalized to the luciferase activity in the control cells without treatment. The data are expressed as the mean ± SD of three independent experiments. *$p<0.05$, statistically significant difference from control cells.

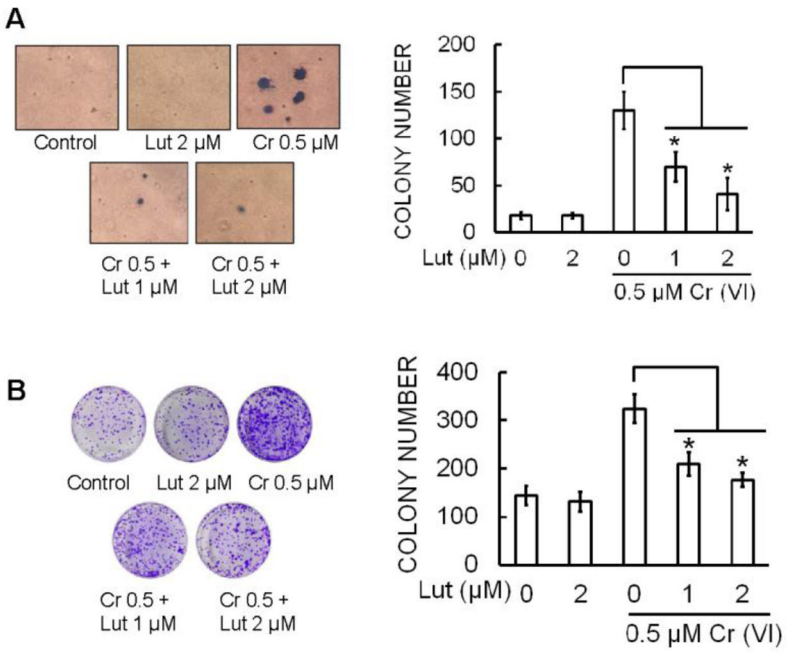

Figure 5.

Luteolin inhibits chronic Cr(VI)-induced malignant transformation. BEAS-2B cells were maintained in a medium containing Cr(VI) (0 or 0.5 μM) with or without luteolin (1 and 2 μM) for 6 months. (A) Cells were cultured in 0.35% soft agar for 5 weeks. Colony numbers in the entire dish were counted. (B) Cells cultured in drug free medium for an additional 7 days and stained with crystal violet. Colony numbers in the entire dish were counted. The data are expressed as the mean ± SD of three independent experiments. *($p<0.05$).

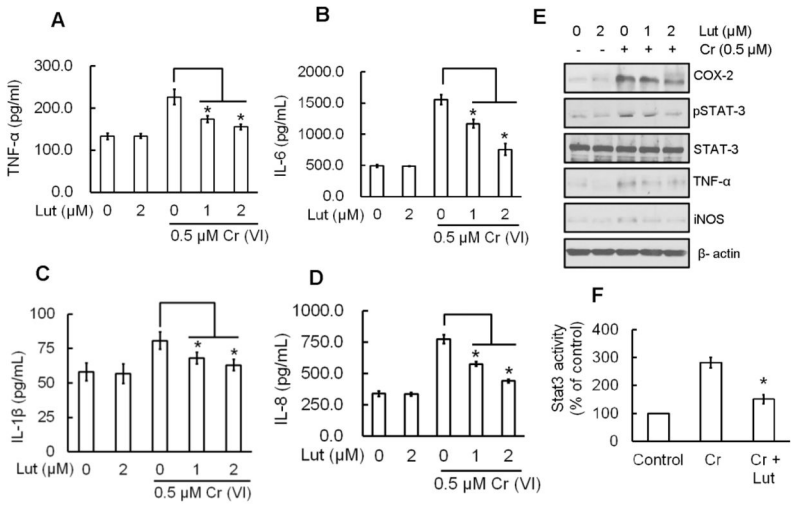

Figure 6.

Luteolin inhibits mediators of inflammation induced by chronic Cr(VI) exposure in BEAS-2B cells. BEAS-2B cells were treated as described in Fig. 5. Culture medium was collected to estimate the (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-8 levels using commercially available ELISA kits according to manufacturer's recommendation.(E) Total cell lysates were prepared for Western blot analysis using specific antibodies against COX-2, STAT3, iNOS, TNF-α and β-actin. (F) STAT3 activity was measured in the nuclear fraction of cell lysates by ELISA following the manufacturer's protocol. The data are expressed as the mean ± SD of three independent experiments. Significant difference compared to Cr(VI) alone, *$p<0.05$.

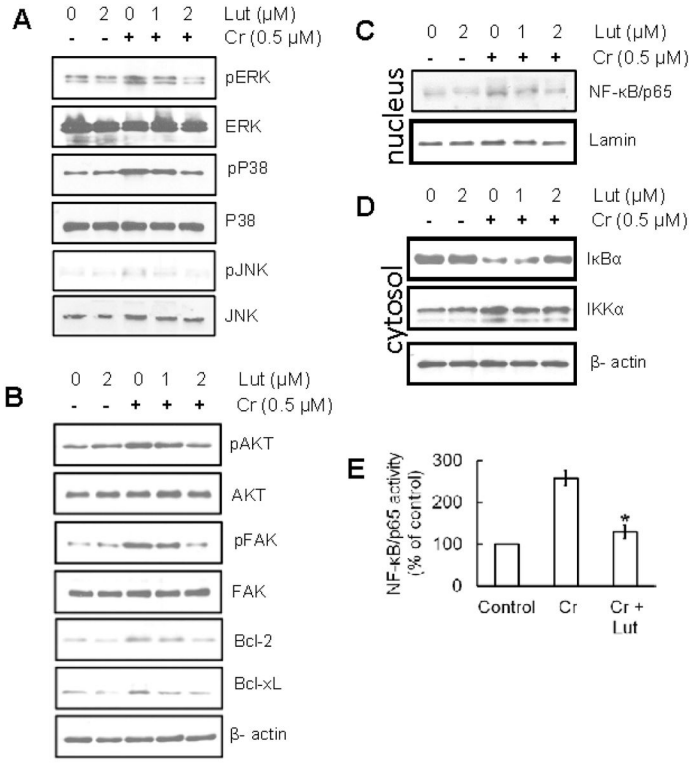

Figure 7.

Luteolin inhibits MAPK, AKT, FAK, Bcl-2, Bcl-xL and NF-κB induced by chronic Cr(VI) exposure in BEAS-2B cells. (A) Luteolin inhibited Cr(VI)-induced MAPK signaling in in BEAS-2B cells. Cells were treated as described in Fig. 5 and lysates were prepared to determine the phosphorylated and total protein levels of ERK1/2, p38, and JNK by Western blot analysis, as described previously. (B) Western blot analysis demonstrating that luteolin inhibited Cr(VI)-induced AKT, FAK, Bcl-2, and Bcl-xL in BEAS-2B cells. (C–E) Western blot analysis demonstrating that luteolin inhibited Cr(VI)-induced activation of NF-κB/p65 and IKKα, and degradation of IκBα in in BEAS-2B cells. (C) nuclear translocation of NF-κB/p65 (D) activation of IKKα, or degradation of IκBα in cytosol. The relative intensities of each band after normalization for the levels of lamin/β-actin are shown under each blot. A representative blots from three independent experiments with identical observations, and equivalent protein loading was confirmed by probing stripped blots for β-actin as shown. (E) The activity of NF-κB in nuclear fraction of cell lysates was measured using ELISA following the manufacturer's protocol. The data are expressed as the mean ± SD of three independent experiments. Significant difference compared to Cr alone, *$p<0.05$.

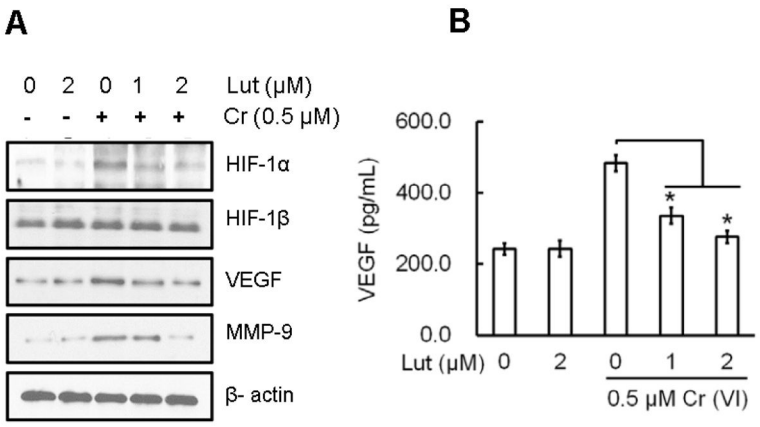

Figure 8.

Luteolin inhibits chronic Cr(VI)-induced angiogenic mediators in BEAS-2B cells BEAS-2B cells were treated as described in Fig. 5 and lysates prepared to determine the protein levels of HIF-1α, HIF-1β, VEGF, and MMP-9 by Western blot analysis. (E) Culture medium was collected to estimate VEGF levels using a commercially available ELISA kit according to manufacturer's recommendation. The data are expressed as the mean ± SD of three independent experiments. Significant difference compared to Cr alone, *$p<0.05$.

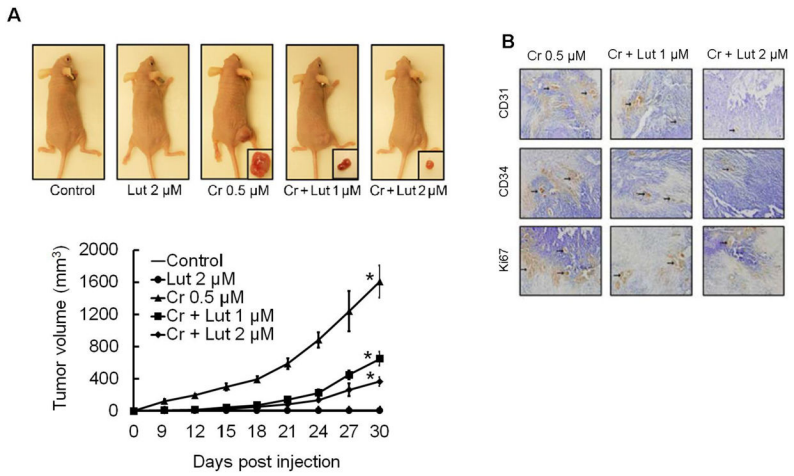

Figure 9.

Luteolin inhibits growth of xenograft tumors in mice chronically exposed to Cr(VI). Cells treated as indicated from different treatments were injected into the flanks of 6-week old athymic nude mice (2×10^6 cells per mouse) and checked daily for tumor appearance; tumor volume was measured every 3 days for 30 days. Tumor volume was determined by Vernier caliper, following the formula of $A\times B^2\times 0.52$, where A is the longest diameter of tumor and B is the shortest diameter. (A) Mice injected with BEAS-2B cells exposed to luteolin along with Cr(VI) showed reduced tumor incidence. (B) Angiogenic (CD31, CD34) and proliferation (Ki67) markers were decreased in tumors treated with both Cr(VI) and luteolin as evident from immunohistochemistry. Frozen tumour sections (5 μM thick) were subjected to immunoperoxidase staining (dark brown) to detect CD31, CD34, and Ki67 expression.

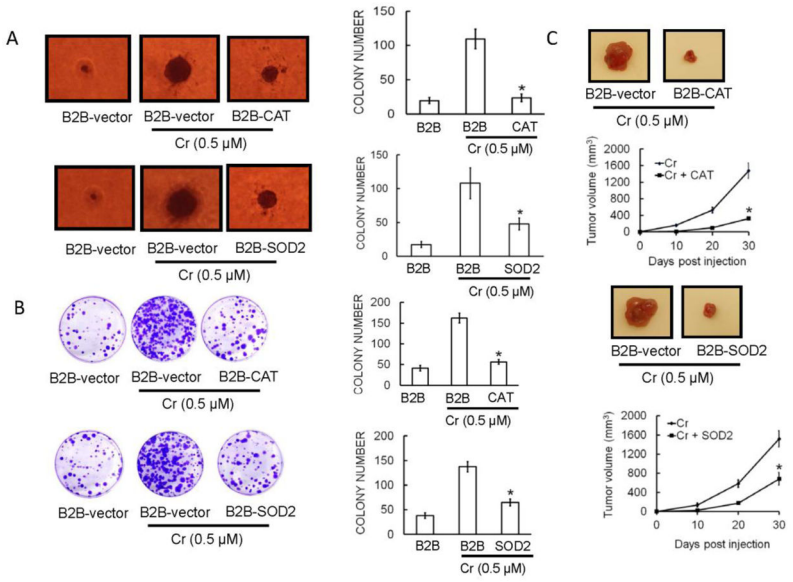

Figure 10.

Overexpression of antioxidant enzymes block Cr(VI)-induced malignant cell transformation and tumorigenesis in BEAS-2B cells. Inhibition of *in vitro* colony formation in BEAS-2B cells with overexpressed antioxidant enzymes is demonstrated in (A) soft agar and (B) clonogenic assay. BEAS-2B cells were stably transfected with CAT (BEAS-2B-CAT), SOD2 (BEAS-2B-SOD2), or their corresponding vectors (BEAS-2B-vectors) as controls. After exposure of above stable cell lines with Cr(VI) (0 or 0.5 μM) for 6 months, soft agar assay and clonogenic assay was performed as previously described. (C) Inhibition of in vivo tumor growth in nude mice with overexpressed antioxidant enzymes. After BEAS-2B-vector controls, BEAS-2B-CAT, and BEAS-2B-SOD2, cells were exposed to Cr(VI) (0 or 0.5 μM) for 6 months, xenograft growth of tumors in nude mice was performed as described previously. The data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$, statistically significant difference from Cr(VI)-treated cells.

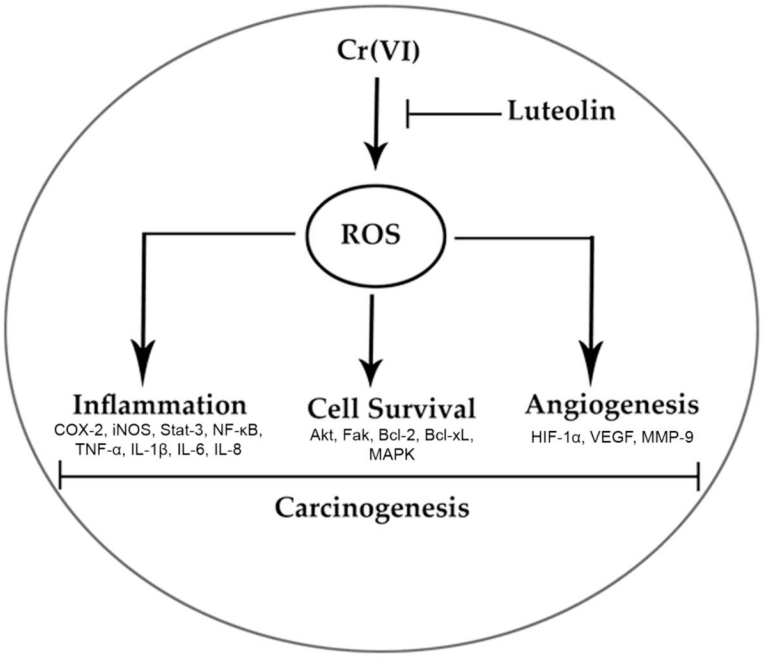

Figure 11.