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Hexavalent chromium-induced apoptosis of granulosa cells involves selective sub-cellular translocation of Bcl-2 members, ERK1/2 and p53

Sakhila K. Banu[a,*], **Jone A. Stanley**[a], **JeHoon Lee**[a], **Sam D. Stephen**[a], **Joe A. Arosh**[a], **Patricia B. Hoyer**[b], and **Robert C. Burghardt**[a]

[a] Department of Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA

[b] Department of Physiology, University of Arizona, Tucson, AZ 85724-5051, USA

Abstract

Hexavalent chromium (CrVI) has been widely used in industries throughout the world. Increased usage of CrVI and atmospheric emission of CrVI from catalytic converters of automobiles, and its improper disposal causes various health hazards including female infertility. Recently we have reported that lactational exposure to CrVI induced a delay/arrest in follicular development at the secondary follicular stage. In order to investigate the underlying mechanism, primary cultures of rat granulosa cells were treated with 10 μM potassium dichromate (CrVI) for 12 and 24 h, with or without vitamin C pre-treatment for 24 h. The effects of CrVI on intrinsic apoptotic pathway(s) were investigated. Our data indicated that CrVI: (i) induced DNA fragmentation and increased apoptosis, (ii) increased cytochrome *c* release from the mitochondria to cytosol, (iii) downregulated anti-apoptotic Bcl-2, Bcl-XL, HSP70 and HSP90; upregulated pro-apoptotic BAX and BAD, (iv) altered translocation of Bcl-2, Bcl-XL, BAX, BAD, HSP70 and HSP90 to the mitochondria, (v) upregulated p-ERK and p-JNK, and selectively translocated p-ERK to the mitochondria and nucleus, (vi) activated caspase-3 and PARP, and (vii) increased phosphorylation of p53 at ser-6, ser-9, ser-15, ser-20, ser-37, ser-46 and ser-392, increased p53 transcriptional activation, and downregulated MDM-2. Vitamin C pre-treatment mitigated CrVI effects on apoptosis and related pathways. Our study, for the first time provides a clear insight into the effect of CrVI on multiple pathways that lead to apoptosis of granulosa cells which could be mitigated by vitamin C.

Keywords

Chromium; Ovary; Apoptosis; p53; Bcl-2

Introduction

Chromium (Cr) exists in a series of oxidation states from −2 to +6 valences; the most important stable states are elemental metal (0), trivalent (CrIII) and hexavalent (CrVI) compounds (Barceloux, 1999; Shi et al., 2004; Zhitkovich, 2005; Valko et al., 2006). CrVI is commonly used in numerous industrial processes and as emission or erosion byproducts

*Corresponding author at: Department of Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA. Fax: +1 979 847 8981. skbanu@cvm.tamu.edu (S.K. Banu).

Conflict of interest statement
None.

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of Cr-based catalytic converters, asbestos brake linings, cement dust, as well as in tobacco and food additives (Nriagu, 1988). Non-occupational sources of CrVI include contaminated soil, air and water (O'Brien et al., 2003). Occupational exposure to Cr is found among approximately half a million industrial workers in the United States and several millions worldwide. Significant contamination with CrVI has been found in approximately 30% of the drinking water in California (Salnikow and Zhitkovich, 2008). According to Environmental Working Group (EWG) water utility tests from 48,000 communities in 42 states (Sutton, 2010), at least 74 million people in nearly 7000 communities drink tap water polluted with CrVI. The USEPA has set a legal limit in tap water for total chromium of 100 ppb. However, chromium levels in the drinking water measured by EWG shows that total chromium is 1700 times higher than California's proposed public health goal for CrVI (Sutton, 2010). This disparity could indicate significant cancer risk and other health hazard for communities drinking chromium-containing tap water. The deposition of CrVI wastes in landfills and waterways by chromate industries affects millions of people residing in the vicinity of dangerously polluted sites who drink Cr containing water (BlacksmithInstitute, 2007). CrVI causes dermatitis, skin, lung and throat cancers, and infertility. Increased incidences of birth and developmental defects among children living around tanneries and chrome and leather industries are clearly evident in the developing world (BlacksmithInstitute, 2007).

Women working in Cr industries and living around Cr contaminated areas experience abnormal menses (Makarov and Shimtova, 1978), postnatal hemorrhage and birth complications with high levels of chromium in blood and urine (Shmitova, 1978, 1980). Cr is transported to offspring through milk in lactating women exposed to CrVI (Barceloux, 1999). CrVI can traverse the placental barrier in rodents (Tipton and Shafer, 1964; Barceloux, 1999). Within the pregnant uterus, CrVI alters early development and hatching of blastocysts (Jacquet and Draye, 1982), decreases the number of implantation sites and viable fetuses (Junaid et al., 1996; Kamath et al., 1997; Kanojia et al., 1998), produces embryotoxic and fetotoxic effects, and increases conceptus resorption in rodents (Junaid et al., 1996). Cr exposure through drinking water impairs ovarian follicular maturation and differentiation and promotes follicular atresia (Murthy et al., 1996), delays puberty, lengthens interestrus intervals and reduces number of ovulation (Kanojia et al., 1998) in rodents.

CrVI can escape from primary contact organs and blood erythrocytes and reach different organs (Barceloux, 1999; Dayan and Paine, 2001). In biological systems, after entry into cells, CrVI is rapidly detoxified/reduced to CrIII by an intracellular defensive reductant system that includes ascorbate (vitamin C), glutathione (GSH) and cysteine (Valko et al., 2005, 2006). CrIII is also a very popular nutritional supplement consumed by many people (Kirpnick-Sobol et al., 2006). Exposing yeast and mice via drinking water to CrVI and CrIII significantly increased the frequency of DNA deletions. Surprisingly, CrIII is a more potent inducer of DNA deletions than CrVI once CrIII is absorbed (Kirpnick-Sobol et al., 2006). Thus, both the environmental contaminant CrVI and the nutritional supplement CrIII increase DNA deletions in vitro and in vivo, when ingested via drinking water. Vitamin C accounts for ~80% of CrVI metabolism in target tissues such as lung, liver and kidney, being the fastest reducer of CrVI *in vitro* (Zhitkovich, 2005; Zhitkovich et al., 2005). Unlike rodents, human beings are unable to synthesize L-ascorbic acid because of their deficiency in t-gulono-g-lactone oxidase, the enzyme catalyzing the terminal step in L-ascorbic acid biosynthesis (Nishikimi et al., 1994). Therefore, the potential risk for CrVI exposure in humans might be more severe than what is reported in rodent models.

We have recently reported that lactational exposure to CrVI decreased primordial, primary, secondary, and antral follicles and thus delayed follicular development, decreased

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steroidogenesis, extended estrous cycle and pubertal onset in postnatal rat ovaries. Vitamin C supplementation protects ovary from these deleterious effects of CrVI (Banu et al., 2008a). However, the specific mechanism(s) responsible for CrVI-induced follicular arrest/atresia on follicular development are not yet understood. Follicular granulosa cell apoptosis or follicular atresia governs follicular growth and development in the ovary (Hirshfield, 1997; Hoyer, 2005). Metal toxins including CrVI and cadmium alter programmed granulosa cell death and follicular apoptosis (Blankenship et al., 1997; Matsuda-Minehata et al., 2006). In metal-induced apoptosis, mitochondria are reported to be the most pertinent target (Rana, 2008). Both mitochondrial damage and genotoxic effects determine the fate of CrVI-exposed cells to either growth arrest or apoptosis (Ye et al., 1999).

Therefore, we hypothesize that CrVI induces follicular atresia through apoptosis of granulosa cells by activating multiple cell signaling pathways. The objectives of the present study were to: (i) determine the effects of CrVI on activation of intrinsic apoptotic pathways and suppression of cell survival pathways in primary cultures of granulosa cells; (ii) understand the involvement of p53 and MAP-kinases in granulosa cell apoptosis; and (iii) evaluate the mitigative effects of vitamin C on CrVI-induced changes on the molecular end-points in granulosa cell apoptosis. Our results for the first time reveal that CrVI induces apoptosis of granulosa cells through activation of mitochondria-mediated intrinsic pathways, suppression of AKT pathways, and phosphorylation / activation of p53 through sustained and delayed activation of ERK1/2 pathways. Vitamin C partially mitigated these adverse effects of CrVI and protects granulosa cells from apoptosis.

Materials and methods

Chemicals

The reagents used in this study were purchased from the following suppliers: Antibiotic-antimycotic, Trypsin-EDTA (Invitrogen Life Technologies Inc., Carlsbad, CA); fetal bovine serum (Hyclone, Logan, UT); and tissue culture dishes and plates (Corning Inc., Corning, NY); potassium dichromate ($K_2Cr_2O_7$) and ascorbate (Sigma-Aldrich, St. Louis, MO); The other chemicals used were molecular biologic grade available from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). Antibodies were from Cell Signaling Technology (Danvers, MA).

Animals

Immature female Sprague–Dawley rats (22–25 days old) were euthanized by CO_2 asphyxiation followed by cervical dislocation and ovaries were collected in DMEM-F12 media. Animal use protocols were approved by the Animal Care and Use Committee of Texas A&M University and were in accordance with the standards established by Guiding Principles in the Use of Animals in Toxicology and Guidelines for the Care and Use of Experimental Animals by National Institute of Health.

Granulosa cell isolation and culture

Granulosa cells from 80 ovaries collected from 40 rats were harvested as described (Kayampilly and Menon, 2007). Briefly, 80 ovaries were cleared from the surrounding fat under a Stereo dissection microscope and punctured with 25-gauge needles. Cells were collected in phenol red free DMEM-F12 containing 0.2% BSA, 10 mM HEPES, and 6.8 mM EGTA, incubated for 15 min at 37 °C, and centrifuged for 5 min at 250*g*. The pellets were suspended in a solution containing 0.5 M sucrose, 0.2% BSA, and 1.8 mM EGTA in DMEM-F12 and incubated for 5 min. After incubation, the suspension was diluted with 3 vol DMEM-F12, centrifuged at 250*g*, and treated sequentially with trypsin (20 μg/ml) for 1 min, 300 μg/ml soybean trypsin inhibitor for 5 min, and DNase I (100 μg /ml) for 5 min at

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37 °C. The cells were washed with media and suspended in DMEM-F12. Cells obtained from the 80 ovaries were cultured in 18 (100 mm) dishes in DMEM-F12 supplemented with 20 mM HEPES (pH 7.4), 4 mM glutamine, 100 IU penicillin/ml, and 100 μg/ml streptomycin. To perform the CrVI in vitro experiments, these dishes were then divided into six groups with three dishes per group. Each group represents treatment as described below.

In vitro experimental design for CrVI treatment

At 70% confluency, cells were serum-starved for 24 h with or without vitamin C in the media, and divided into six treatment groups. *(1) Control*: cells were treated with media; *(2) CrVI-12 h*: cells were treated with 10 μM potassium dichromate for 12 h; *(3) CrVI-24* h: cells were treated with 10 μM potassium dichromate for 24 h; *(4) Vitamin C*: cells were treated with 1 mM ascorbate for 24 h; *(5) vitamin C+CrVI-12* h: cells were pre-treated with 1 mM ascorbate for 24 h and treated with 10 μM potassium dichromate for 12 h; *(6) vitamin C+CrVI-24* h: cells were pre-treated with 1 mM ascorbate for 24 h and treated with 10 μM potassium dichromate for 24 h. After the treatment, cells were harvested using 0.1% trypsin-EDTA and total RNA was isolated using TriZol (Banu et al., 2008). All treatments were performed in triplicates on the same day and each experiment was repeated three times on different days.

TUNEL assay

TUNEL assay was performed to assess apoptosis of granulosa cells as described (Banu et al., 2009). Briefly, non-adherent and adherent cells were harvested and resuspended at the concentration of 1×10^6 cells/ml. Nicks in DNA were determined by terminal deoxynucleotidyl transferase and 5-bromo-2′-deoxyuridine (BrdU) 5′-triphosphate labeling using an APOBrdU TUNEL assay kit (Molecular Probes Inc., Eugene, OR) as recommended by the manufacturer. Detection of BrdU incorporation at DNA break sites was achieved using Alexa Fluor 488 dye-labeled anti-BrdU antibody. Numbers of apoptotic cells were analyzed by flow cytometry (FACSCaliber; Becton Dickinson, San Jose, CA) using Cell Quest software.

Protein extraction and immunoblotting

After the CrVI treatment with or without vitamin C pre-treatment, total protein from granulosa cells was isolated and immunoblotting/western blotting was performed as we described previously (Banu et al., 2008b). Briefly, the cells were harvested using 1% Trypsin-EDTA and pelleted. The cell lysates were sonicated in sonication buffer which consisted of 20 mM Tris–HCl, 0.5 mM EDTA, 100 μM DEDTC, 1% Tween, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets: complete EDTA-free (1 tablet/50 ml) and PhosStop (1 tablet/10 ml). Sonication was performed using a Microson ultrasonic cell disruptor (Microsonix Incorporated, Farmingdale, NY). Protein concentration was determined using the Bradford method and a Bio-Rad Protein Assay kit. Protein samples (75 μg) were resolved using 7.5%, 10% or 12.5% SDS-PAGE. Chemiluminescent substrate was applied according to the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL). The blots were exposed to Blue X-Ray film and densitometry of autoradiograms was performed using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Cytosolic, mitochondrial and nuclear protein fractionation

Granulosa cells were harvested after CrVI treatment with or without vitamin C pretreatment. Cytoplasmic, mitochondrial and nuclear protein fractions were isolated using a Cell Fractionation kit from MitoSciences, (Eugene, OR) according to manufacturer’s instructions. The distribution of proteins between cytosolic (C), mitochondrial (M) and

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nuclear (N) fractions was calculated as percentage of the protein present in a fraction of the sum of the protein present in C and M fractions. For example, the determination of cytosolic cytochrome *c* is indicated by the formula below: cytochrome *c* fraction C (%)=100×cytochrome *c* fraction C/(cytochrome *c* fraction C+cytochrome *c* fraction M). Western blots of Rho-GD1, pyruvate dehydrogenase and histone H2B proteins were used as loading controls for cytosolic, mitochondrial and nuclear protein fractions, respectively.

p53 Luciferase reporter assay

The Cignal p53 Reporter kit (CCS-004L, SA Biosciences) was used to monitor the activity of the p53-regulated signal transduction pathway in granulosa cells treated with CrVI as per the protocol from the manufacturer. Briefly, granulosa cells were seeded at a density of 3×10^5 per well in 6-well plates and grown to ~60% confluency. For each well, a mixture of inducible p53-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct were cotransfected into the cells using the oligofectamine reagent (Invitrogen). After 24 h incubation with or without vitamin C (1.0 mM), cells were treated with CrVI (10 μM) for 24 h. The preparation of cell extracts and measurement of luciferase activities were carried out using the Dual-Luciferase Reporter Kit according to recommendations of the manufacturer (Promega). The firefly luciferase activity and Renilla luciferase activity were measured. Changes in firefly luciferase activity were calculated and plotted after normalization with changes in Renilla luciferase activity within the same sample.

Statistical analyses

All numerical data were subjected to one-way ANOVA to detect the effects of treatment and time interactions. Tukey–Kramer HSD test was used to adjust for multiple pair-wise comparisons of means. Least squares regression analysis was used to determine effects of treatment (Control, CrVI, Vitamin C, CrVI+ Vitamin C); time (CrVI 12 h, CrVI 24 h) and treatment×time interactions. Each value is the mean±SEM from 3 different plates per treatment, cultured using 80 ovaries collected from 40 immature rats. Similar results were obtained in three different experiments performed on three different days/time. $P<0.05$ was considered to be significant. Statistical analyses were performed using general linear models of Statistical Analysis System (SAS, Cary, NC).

Results

CrVI induced apoptosis of granulosa cells through intrinsic apoptotic pathway

Effects of CrVI on DNA fragmentation was measured using the TUNEL assay. Results indicated that CrVI induced DNA fragmentation and apoptosis of granulosa cells in a time-dependent manner ($P<0.05$) whereas pre-treatment of cells with vitamin C mitigated ($P<0.05$) the effect of CrVI (Fig. 1A). Release of cytochrome *c* from mitochondria into the cytosol and activation of caspase-3 and nuclear poly (ADP-ribose) polymerase (PARP) enzymes are important terminal events which promote apoptosis of cells (Jiang and Wang, 2004). Therefore we examined whether CrVI-induced apoptosis of granulosa cells is mediated through release of cytochrome *c* and activation of caspase-3. Results indicated that CrVI induced release of cytochrome *c* from the mitochondria into cytosol in a time-dependent manner. The increase of cytochrome *c* protein in the cytosol was negatively correlated ($r=-0.97$) with a concurrent decrease in the mitochondria whereas vitamin C prevented this translocation (Fig. 2A and B). Down-stream of cytochrome *c*, CrVI increased cleavage of caspase-3 and PARP proteins. Vitamin C mitigated the effect of CrVI on cleavage of caspase-3 and PARP (Fig. 2D–G). CrVI-induced apoptosis of granulosa cells was inhibited by a cytochrome *c* inhibitor (cyclosporine-A) or caspase-3 inhibitor (DEVD-

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fmk) (Fig. 1B). These results together indicate that CrVI induces apoptosis of granulosa cells through cytochrome *c* and caspase-3 dependent intrinsic apoptotic pathways.

CrVI altered expression of Bcl-2, Bcl-XL, Bax, Bad, HSP70 and HSP90 proteins in granulosa cells

The Bcl-2 family proteins (Bcl-2, Bcl-XL Bad, and Bax) constitute critical components to regulate activation of intrinsic apoptotic pathways by governing mitochondrial membrane permeabilization and subsequent release of cytochrome *c* (Danial and Korsmeyer, 2004). In addition to Bcl-2 family, heat shock proteins HSP70 or HSP90 are anti-apoptotic by preventing mitochondrial membrane permeabilization (Joly et al., 2010; Stankiewicz et al., 2005; Bivik et al., 2007). Down-regulation or inhibition of HSP70 or HSP90 protein is sufficient to sensitize a cell for apoptosis (Lanneau et al., 2007; Kang et al., 2007; Neckers et al., 2007). Therefore, we studied the effects of CrVI on expression of antiapoptotic proteins Bcl-2 and Bcl-XL, proapoptotic proteins Bax and Bad, as well as HSP70 and HSP90 proteins. Results indicated that CrVI decreased ($P<0.05$) expression of Bcl-2 and Bcl-XL proteins, increased ($P<0.05$) total Bad and Bax proteins, and decreased ($P<0.05$) HSP70 and HSP90 proteins. Vitamin C mitigated effects of CrVI on the levels of Bcl-2, Bcl-XL, Bax and Bad, HSP70 and 90 proteins temporally at 12 and 24 h. (Fig. 3A and B). In the absence of apoptotic stimuli, Bad protein is phosphorylated at serine 112 and 136 by MAPK and AKT pathways. Phosphorylated Bad proteins bind with 14-3-3 proteins and are sequestered in the cytosol (Zha et al., 1996). Dephosphorylation of Bad is important for its translocation into mitochondria to initiate intrinsic apoptotic pathways. Therefore, we determined the effect of CrVI on phosphorylation of Bad 112 and 136. Results indicated that CrVI decreased ($P<0.05$) phosphorylation of Bad protein at ser-112 (p-Bad-112) and ser-136 (pBad-136) within 24 h, and interestingly, vitamin C mitigated CrVI effects on phosphorylation of Bad protein. Together, these results indicate that CrVI decreases expression of antiapoptotic proteins and increases expression of pro-apoptotic proteins in granulosa cells.

CrVI altered mitochondrial translocation of BAX, BAD, HSP70 and HSP90 in granulosa cells

Translocation of Bax and Bad proteins from cytoplasm into mitochondria is critical to execute apoptotic cell death in response to oxidative stress and DNA damage (Youle and Strasser, 2008). Therefore the effect of CrVI on the translocation of Bax and Bad was studied. In control cells, Bcl-2 and Bcl-XL proteins were abundantly localized in the mitochondria, CrVI decreased ($P<0.05$) mitochondrial levels of Bcl-2 and Bcl-XL proteins and vitamin C partially mitigated the effects of CrVI on Bcl-2 but not Bcl-XL protein. CrVI increased ($P<0.05$) translocation of Bax and Bad from the cytosol to the mitochondria and vitamin C mitigated ($P<0.05$) the effect of CrVI on the translocation of Bax and Bad to mitochondria. Once dephosphorylated, Bad translocates to the mitochondria and interacts with Bcl-2 or Bcl-XL proteins to neutralize their activity (Rapp et al., 2007). Therefore, the effect of CrVI on translocation of Bad was studied. In control cells, as expected, p-Bad-112 and p-Bad-136 proteins are sequestered ($P<0.05$) in the cytosol but not in the mitochondria. CrVI decreased ($P<0.05$) phosphorylation p-BAD-112 and p-BAD-136 in the cytosol, and vitamin C mitigated this effect. HSP-70 and HSP-90 are localized in the cytosol and mitochondria of control cells. CrVI decreased ($P<0.05$) cytosolic and mitochondrial expression of HSP70 and mitochondrial HSP90 proteins; and vitamin C mitigated the effect of CrVI on HSP90 but not on HSP70 proteins (Fig. 4A and B). These results together indicate that CrVI translocates Bax and Bad proteins from the cytosol into mitochondria in granulosa cells.

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CrVI increased phosphorylation of ERK and JNK, and decreased PI3K/AKT in granulosa cells

MAP-kinase and AKT pathways are the major intracellular cell survival pathways and are associated with expression of Bcl-2 and Bcl-XL proteins and phosphorylation of Bad protein. Therefore, we determined the effects of CrVI on phosphorylation of MAP-kinases and AKT. Our data showed that CrVI increased (P<0.05) phosphorylation of ERK1/2 at 12 h and 24 h, increased JNK at 24 h. However, CrVI decreased (P<0.05) AKT at 12 h and 24 h and did not alter p38MAPK. Vitamin C inhibited (P<0.05) effects of CrVI on phosphorylation of ERK1/2 protein. Furthermore, vitamin C mitigated effects of CrVI by decreasing (P<0.05) phosphorylation of JNK and increasing (P<0.05) phosphorylation of AKT (Fig. 5A–E). These results together indicate that CrVI suppresses the AKT pathway but activates the EKR1/2 pathway in granulosa cells.

CrVI increased phosphorylation of p53 at multiple serine sites and decreased MDM-2 expression

DNA damage promotes phosphorylation and subsequent stabilization of p53 leading to apoptotic cell death (Meek, 2009). Apoptosis induced by oxidative stress involves p53 phosphorylation at ser-6, ser-9, ser-15, ser-20, ser-37, ser-46 and ser-392 in various cells (Meek, 1998b; Dumaz and Meek, 1999; Jardine et al., 1999; Brooks and Gu, 2003; Meek and Anderson, 2009). MDM2 promotes p53 degradation through an ubiquitin-dependent pathway (Moll and Petrenko, 2003). However, effects of CrVI on p53 phosphorylation resulting in apoptosis of granulosa cells are not understood. Therefore, this study attempted to understand the effect of CrVI on the phosphorylation of p53 at various serine sites. Interestingly, CrVI increased levels of total p53 protein in granulosa cells at 12 h and 24 h and induced phosphorylation of p53 protein at ser-9 and ser-392 at 12 h and 24 h and at ser-6, ser-15, ser-20, ser-37 and ser-46 at 24 h. Interestingly, vitamin C mitigated effects of CrVI on expression and phosphorylation of p53 protein at ser-6, ser-9, ser-15, ser-20, ser-37, ser-46 and ser-392. MDM-2 protein was abundantly expressed in untreated granulosa cells and CrVI decreased (P<0.05) its expression level in a time-dependent manner at 12 h and 24 h. Vitamin C mitigated CrVI-induced effects on expression of MDM-2 protein in granulosa cells (Fig. 6A and B). These results together indicate that CrVI phosphorylates p53 protein at multiple serine sites in granulosa cells.

CrVI increased mitochondrial translocation of p53 in granulosa cells

Recent studies have shown that translocation of p53 from cytosol to the mitochondria is important for its interactions with antioxidants and apoptotic proteins (Pani et al., 2004; Siu et al., 2009; Galluzzi et al., 2010; Holley et al., 2010a,b,c). Mitochondrial translocation of p53 triggers a rapid proapoptotic response (Erster and Moll, 2004). Therefore, we determined whether CrVI induces translocation of p53 protein from cytosol to the mitochondria. CrVI increased accumulation of phosphorylated p53 protein at ser-9, ser-15, ser-20, ser-37, ser-46 and ser-392 in the mitochondria compared to cytosol. Vitamin C mitigated effects of CrVI on translocation of p53 to the mitochondria (Fig. 7A and B). These results indicate that CrVI induces translocation of p53 protein from cytosol to the mitochondria.

CrVI increased p53 transcriptional activity through ERK1/2 pathway

MAP-kinases have been identified as upstream kinases that activate p53 phosphorylation in several cell types (Meek et al., 1997; Milczarek et al., 1997; Meek, 1998a). In Fig. 5, we have shown that CrVI increases phosphorylation of ERK1/2 and JNK proteins. Therefore, we determined interaction between p53 and ERK1/2 or JNK. Transcriptional activity of p53 was measured in cells exposed to increasing concentrations of CrVI (0–200 μM). Results

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showed a dose-dependent increase (P<0.05) in p53 transcriptional activity and that reached (P<0.05) maximal levels at 50 μM CrVI. Vitamin C pre-treatment mitigated (P<0.05) the effect of CrVI on p53 transcriptional activity even at the highest dose (200 μM) of CrVI used (Fig. 8A). To determine whether ERK1/2 or JNK is involved in the activation of p53 and apoptosis, granulosa cells were treated with ERK1/2 inhibitor (U0126) or JNK inhibitor (SP600125) in the presence or absence of CrVI, and p53 transcriptional activity and apoptosis were measured. Results indicated that inhibition of ERK1/2 decreased (P<0.05) p53 activity and apoptosis (Fig. 8B and C). Inhibition of JNK did not inhibit p53 transcriptional activity but decreased apoptosis. These results indicate that CrVI activates p53 through ERK1/2 pathway in granulosa cells.

CrVI treatment leads to persistent nuclear and mitochondrial translocation of activated ERK1/2 in granulosa cells

ERK has been traditionally viewed as a mitogenic factor; however, sustained and/or delayed activation of ERK is associated with apoptosis (Kulich and Chu, 2001; Kulich and Chu, 2003); (Stadheim et al., 2001; Brantley-Finley et al., 2003). Besides the important mitogenic activity of ERK1/2 in the nucleus, ERK1/2 is localized in the mitochondria and plays roles in cell survival/apoptosis (Dagda et al., 2008; Poderoso et al., 2008). Therefore, we determined translocation of total (t-ERK1/2) and phosphorylated or active (p-ERK1/2) forms of ERK1/2 in the cytosol, mitochondria and nucleus. Results indicated that t-ERK1/2 protein is constitutively expressed in the cytosol and mitochondria and not in the nucleus; the expression level of t-ERK1/2 protein was lower in the mitochondria compared to that in cytosol; and p-ERK1/2 was not detected in cytosol, mitochondria or nucleus in untreated granulosa cells. CrVI treatment did not alter levels of t-ERK1/2 proteins in cytosol, mitochondria, and nucleus (Fig. 9A and C). By contrast, CrVI increased (P<0.05) translocation of p-ERK1/2 from cytosol into the mitochondria and nucleus. Vitamin C pretreatment mitigated effects of CrVI and prevented translocation of pERK1/2 from cytosol into the mitochondria and nucleus (Fig. 9B and D). These results indicate that CrVI accelerates selective translocation of active ERK1/2 into nucleus in granulosa cells.

Discussion

Lactational exposure to CrVI during the postnatal days 1–21 decreased development of antral follicles and arrested follicular development at the secondary follicular stage in rat (Banu et al., 2008b; Samuel et al., 2010). The underlying molecular and cellular mechanisms that regulate CrVI-induced follicular atresia/apoptosis are not known. Results of the present study for the first time showed that CrVI induces apoptosis of granulosa cells through multiple mechanisms.

Bcl-2 family members Bcl-2, Bcl-XL, Bax and Bad proteins are the key mediators of intrinsic apoptotic pathway. In addition, HSP70 protects the cells against apoptosis by inhibiting translocation of BAX protein from the cytosol to the mitochondria, release of cytochrome *c* from the mitochondria into the cytosol, and activation of caspase-3 and PARP proteins (Stankiewicz et al., 2005; Bivik et al., 2007; Joly et al., 2010). HSP90 protein located in the mitochondria regulates mitochondrial membrane permeabilization and release of cytochrome *c* (Kang et al., 2007; Neckers et al., 2007). Results of the present study indicated that CrVI decreased expression of antiapoptotic and cell survival proteins Bcl-2, Bcl-XL, HSP70 and HSP90 proteins, translocated BAX and BAD proteins from cytosol to the mitochondria, increased mitochondrial membrane permeability, facilitated the release of cytochrome *c*, and activated caspase-3 and PARP proteins, and thus induced apoptosis of granulosa cells. These results suggest that CrVI attenuates antiapoptotic pathways in order to stabilize pro-apoptotic members to execute apoptosis of granulosa cells.

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The fate of cells to die or survive depends on balance between survival and apoptosis signaling (Matsuzawa et al., 2002). Further, expression of Bcl-2 and Bcl-XL proteins are regulated by MAPK, JNK and AKT pathways (Matsuzawa et al., 2002). Therefore, we determined effects of CrVI on ERK1/2, AKT, p38MAPK, and JNK pathways in granulosa cells. Interestingly, CrVI inhibited phosphorylation of AKT proteins, and in contrast, increased phosphorylation of ERK1/2 and JNK proteins, and did not alter activation of p38MAPK protein. ERK1/2 pathways are mainly associated with mitogenesis and cell survival (Meloche and Pouyssegur, 2007). Inactive ERKs are bound to anchoring proteins in resting cells, mostly confined to the cytosol. Upon phosphorylation, ERK becomes active, translocates to the nucleus, and activates transcription of several proteins (Lidke et al., 2010). Interestingly, recent findings have documented a role for delayed and sustained ERK activation in apoptosis (Gladys et al., 1999; Stanciu and DeFranco, 2002). ERK can be activated often in the same cell type by pro-survival factors and toxic/apoptotic stimuli (Hetman et al., 1999) and thus ERK activation alone may not be predictive of subsequent cellular survival responses (Stanciu et al., 2000; Stanciu and DeFranco, 2002). It has been shown that activated JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax (Tsuruta et al., 2004). It is evident from the present data that CrVI activates ERK1/2 temporally in a delayed and sustained manner and activates JNK in granulosa cells.

DNA damage promotes phosphorylation and subsequent stabilization of p53 and leads to apoptosis (Meek, 2009). Phosphorylation of p53 protein at one serine residue is not sufficient to induce apoptosis, whereas phosphorylation at multiple serine residues is required (Kurihara et al., 2007). Site specific phosphorylation of p53 is induced by activation of diverse cell signaling pathways and DNA damage (Kurihara et al., 2007). Phosphorylation of p53 at ser-392 is required for p53-mediated growth arrest (Cox and Meek, 2010). Phosphorylation of p53 at ser-15 can be induced by oxidative stress (Long et al., 2007), H_2O_2 (Verschoor et al., 2010), and ionization (Sluss et al., 2010) and UV irradiation (Milne et al., 1995). In addition, function of p53 is regulated by its negative regulator MDM2 (Shieh et al., 1997). In order to understand the role of p53 in CrVI-induced apoptosis of granulosa cells we determined phosphorylation of p53 protein at multiple serine sites and expression of MDM2 protein. Our results indicate that CrVI increased phosphorylation of p53 protein at ser-6, ser-9, ser-15, ser-20, ser-37, ser-46 and ser-392 and decreased expression of MDM-2 protein in granulosa cells in a time-dependent manner. These results suggest that CrVI increases p53 phosphorylation at multiple serine sites, decreases its interaction with its negative regulator MDM2 and thereby stabilizes p53 and promotes apoptosis of granulosa cells.

One of the interesting findings of the present study is that CrVI selectively translocated active p53 protein into mitochondria in granulosa cells. p53-mediated cell death is primarily routed through the mitochondrial pathways (Schuler and Green, 2001) which require translocation of p53 protein into mitochondria (Zhao et al., 2005). Recent studies showed translocation of p53 from the cytosol to mitochondria and its association with antioxidants and apoptotic proteins (Pani et al., 2004; Siu et al., 2009; Galluzzi et al., 2010; Holley et al., 2010a,b,c). Mitochondrial translocation of p53 triggers a rapid pro-apoptotic response (Erster and Moll, 2004). After translocation into mitochondria, p53 protein could interact with endogenous antiapoptotic Bcl-XL and/or Bcl-2 protein, induce oligomerization of Bak protein, increase permeabilization of the outer mitochondrial membrane in order to facilitate cytochrome *c* release (Mihara et al., 2003), or interact with MnSOD and inhibit its ability to scavenge free radicals (Holley et al., 2010a). The results of the present study along with available information suggest that p53 could play a central role in CrVI-induced apoptosis by inhibiting association or balance between pro-and anti-apoptotic proteins.

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p53 regulates transcription of several genes (Meek, 1998a) that regulate cell cycle, growth arrest, and apoptosis (Agarwal et al., 1998; Giaccia and Kastan, 1998). However, cell signaling associated with phosphorylation of p53 is complex and largely unknown. Our data indicated that CrVI activated ERK1/2 and JNK pathways. Therefore, we tested whether the inhibition of ERK1/2 or JNK decreases CrVI-induced p53 transcriptional activity and apoptosis. Our data showed that inhibition of ERK1/2 decreased CrVI-induced apoptosis of granulosa cells through suppression of transcriptional activity of p53. By contrast, inhibition of JNK did not decrease transcriptional activity of p53 although it decreased apoptosis of granulosa cells. These results indicate that ERK1/2 might be a potential upstream kinase that activates p53 and mediates CrVI-induced apoptosis of granulosa cells through p53.

ERK1/2 proteins are localized in several microenvironments of mitochondria and regulate survival or apoptosis of cells or modulate steroid synthesis (Dagda et al., 2008; Poderoso et al., 2008). Phosphorylation of p53 by ERK1/2 is important for doxorubicin-induced p53 activation and cell death (Yeh et al., 2004). Therefore, we hypothesized that CrVI translocates active ERK1/2 proteins into mitochondria in addition to nucleus in granulosa cells. Our results indicated that CrVI translocated active ERK1/2 proteins not only into the nucleus but also to the mitochondria. The present study indicates that CrVI translocates active p53 protein into mitochondria. Based on these data, we propose that sustained activation of ERK1/2 by CrVI could phosphorylate p53, which in turn, interacts with other mitochondrial proteins of cell survival pathways and or antioxidants, and thus promotes apoptosis. In addition, CrVI translocates active ERK1/2 to the nucleus in granulosa cells and induces apoptosis. This finding is consistent with other evidence that prolonged nuclear retention of activated ERK promotes cell death (Stanciu et al., 2000; Stanciu and DeFranco, 2002). Moreover, association of ERK1/2 activation with granulosa cell apoptosis in the present study supports the recent finding that ERK1/2 is not essential for the active proliferation of granulosa cells from preovulatory follicles; rather ERK1/2 plays an essential role to cease granulosa cell proliferation and to initiate the terminal differentiation response to LH in preovulatory follicles (Fan et al., 2009).

In the present study, vitamin C exhibited a selective and time-dependent molecular intervention of CrVI effects in several signaling pathways that lead to granulosa cell apoptosis. Vitamin C was more effective in mitigating CrVI effects at 12 h of treatment compared to 24 h in most of the end-points studied. This suggests that with short-time (12 h) CrVI exposure, the cells may still retain DNA repair machinery and operational survival signals. However, after 24 h of CrVI treatment, the DNA damage may have exceeded native DNA repair mechanisms so that vitamin C can not rescue granulosa cells from apoptosis. In conclusion, the novel findings of the present study are that CrVI: (i) decreased expression or activity of Bcl-2, Bcl-XL, and AKT proteins; (ii) increased activation and mitochondrial translocation of pro-apoptotic BAD, BAX, (iii) increased sustained activation of ERK1/2 and its sub-cellular translocation into nucleus and mitochondria; (iv) increased phosphorylation of p53 at multiple serine sites and thereby induced apoptosis of granulosa cells. Vitamin C partially mitigated the adverse effects of CrVI on granulosa cells; therefore, vitamin C could be a potential intervention to prevent or reduce the toxic effects of CrVI on the ovary to preserve the fertility.

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Abbreviations

CrVI	Hexavalent chromium
CrIII	Trivalent chromium
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BAX	BCL2-associated X protein
BAD	Bcl-xL/ Bcl-2-Associated Death Promoter
HSP70/HSP90	Heat shock proteins-70 and -90
MAP Kinase	Mitogen-activated protein (MAP) kinase
ERK	Extracellular-signal-regulated kinase
JNK	c-Jun N-terminal kinase
PARP	Poly (ADP-ribose) polymerase

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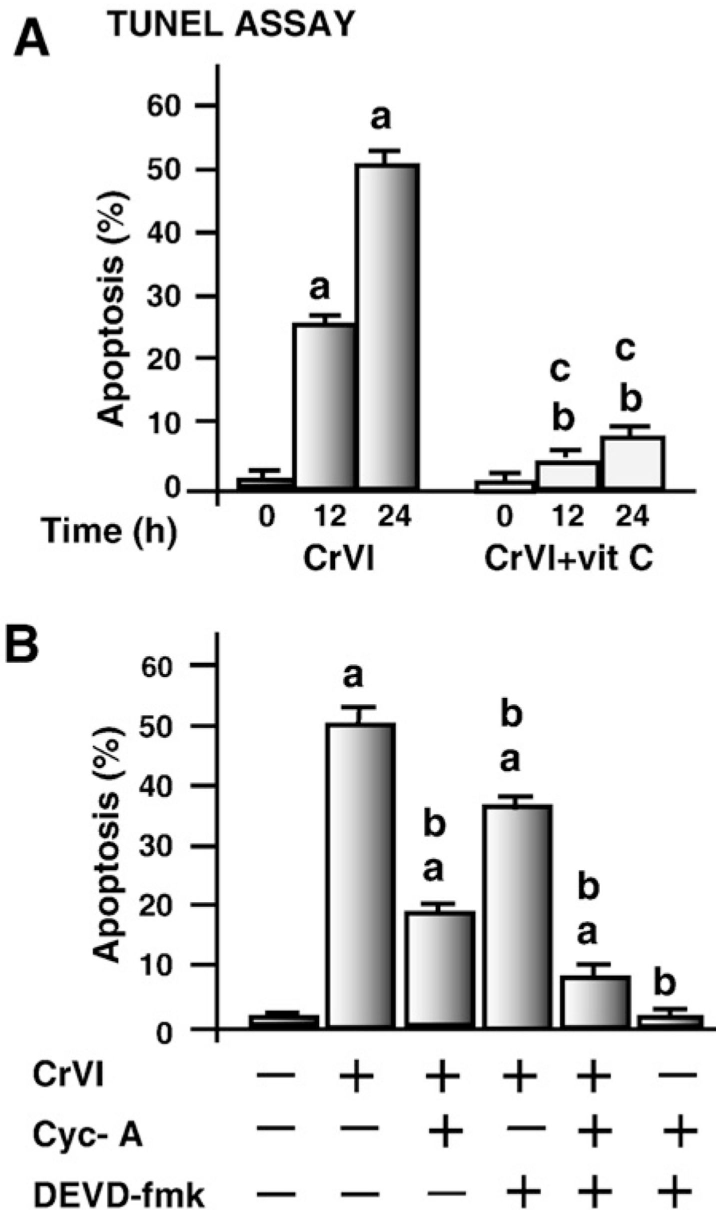

Fig. 1.

Effect of CrVI on apoptosis of granulosa cells. Apoptosis was assayed by TUNEL assay as described in Materials and methods. Cells were pre-treated with or without vitamin C and treated with CrVI (10 μM) for 0 h, 12 h and 24 h period and harvested and processed for TUNEL assay. Each value is the mean±SEM of three experiments, $P<0.05$. A, Effect of CrVI on apoptosis. a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI+Vitamin C-treatment, 0 h *vs* 12 h or 24 h; c: CrVI (0 h or 12 h or 24 h) *vs* CrVI+Vitamin C (0 h or 12 h or 24 h; B, Effects of cytochrome *c* inhibitor cyclosporine A and/or caspase-3 inhibitor DEVD-fmk on apoptosis. Cells were serum deprived and pre-treated with or without cyclosporine A (cyc A) (5 μM) and/or DEVD-fmk (50 μM) for 1 h, and then treated with CrVI (10 μM) for a 24 h period, and processed for TUNEL assay. a: Control *vs* CrVI-treatment with cyclosporine A and/or DEVD-fmk; b: CrVI *vs* CrVI+cyclosporine A and/or DEVD-fmk.

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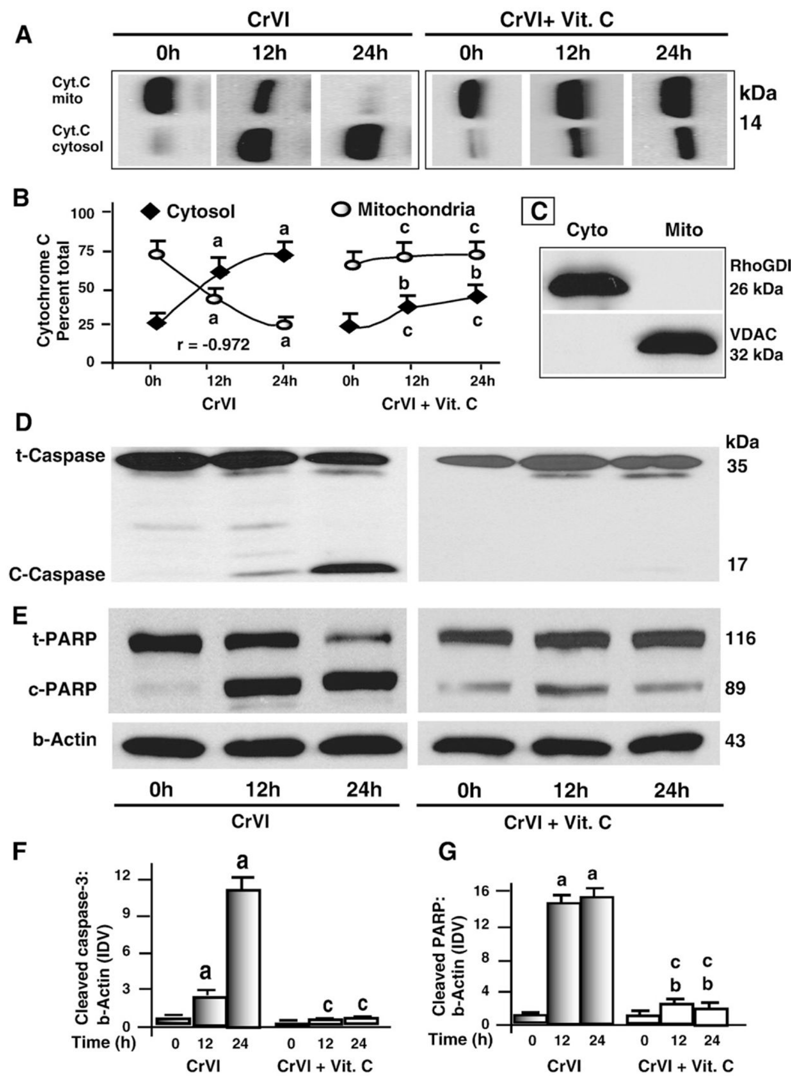

Fig. 2.

Time-course effect of CrVI on cytochrome *c* release from mitochondria, cleavage of caspase-3 and PARP in granulosa cells. Cells were pre-treated with or without vitamin C for 24 h and treated with CrVI (10 μM) for 0 h, 12 h and 24 h period. Protein (50 μg of either total protein or mitochondrial or cytosolic fraction) from each sample was subjected to western blot analysis as described in Materials and methods. A, Western blots of cytochrome *c*; B, Quantification of cytochrome *c* in cytosol and mitochondria with time; *C*, Western blots of mitochondria-specific voltage-dependent anion channel (VDAC) and cytosol-specific RhoGDI in mitochondrial and cytosolic protein fractions. Western blots of *D* total (t) and cleaved (c) caspase-3; and *E* total (t) and cleaved (c) PARP, representative blots are shown. Quantification of average protein levels of *F* caspase-3 and *G* PARP from three individual experiments is shown. Each value is the mean±SEM of three experiments, $P<0.05$; a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI+Vitamin C-treatment, 0 h *vs* 12 h or 24 h; c: CrVI (0 h or 12 h or 24 h) *vs* CrVI+Vitamin C (0 h or 12 h or 24 h). Cyt.C—cytochrome *c*; Vit.C—Vitamin C; mito—mitochondrial protein fraction; cyto—cytosolic protein fraction; t-PARP—total PARP; c-PARP—cleaved PARP; t-caspase-3—total caspase-3; c-caspase-3—cleaved caspase-3.

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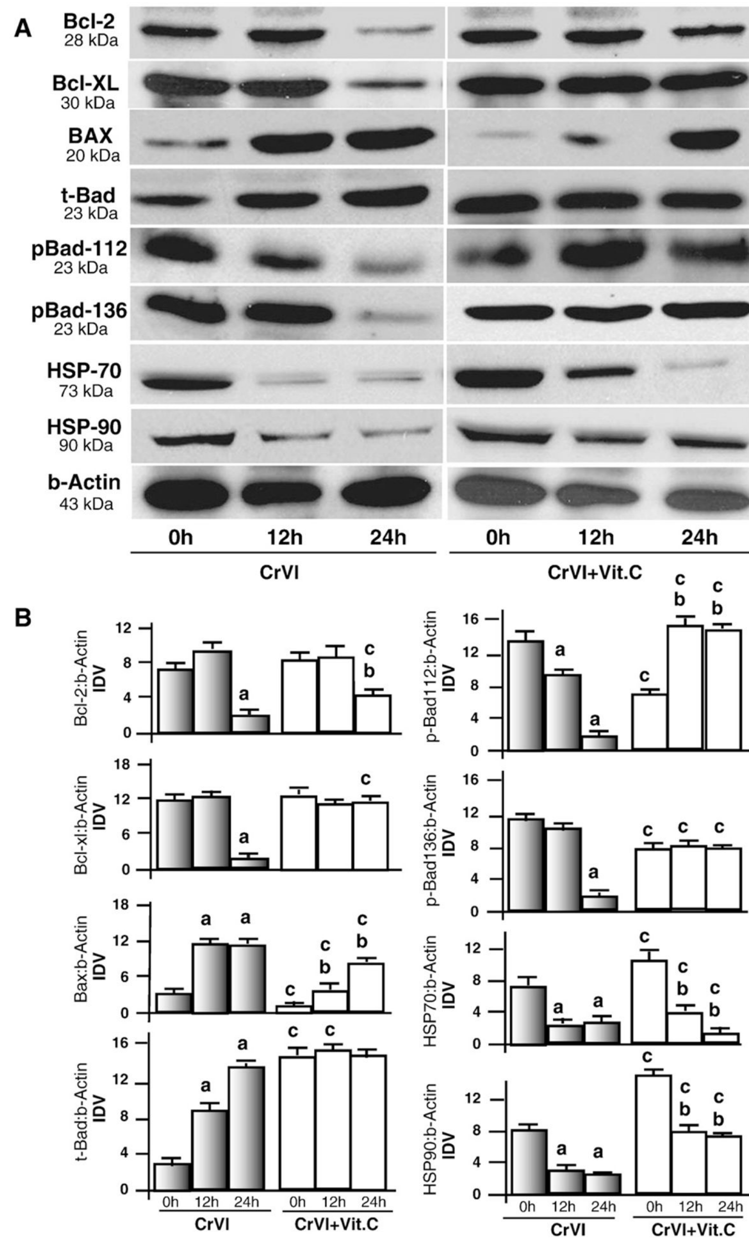

Fig. 3.

Effect of CrVI on Bcl-2 family proteins and heat shock proteins 70 and 90 in granulosa cells. Cells were pre-treated with or without vitamin C for 24 h and treated with CrVI (10 μM) for 0 h, 12 h and 24 h period. Western blots were performed with 75 μg of protein, as described in Materials and methods. A, Representative western blots of Bcl-2, Bcl-XL, BAX, total Bad (t-Bad), phosphorylated Bad (pBad-112, pBad-136), HSP-70, HSP-90, and b-actin. B, Histograms of Integrated Density Value (IDV) for each protein, normalized to beta-actin. Each value is the mean±SEM of three experiments, $P<0.05$; a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI+Vitamin C-treatment, 0 h *vs* 12 h or 24 h; c: CrVI (0 h or 12 h or 24 h) *vs* CrVI+Vitamin C (0 h or 12 h or 24 h).

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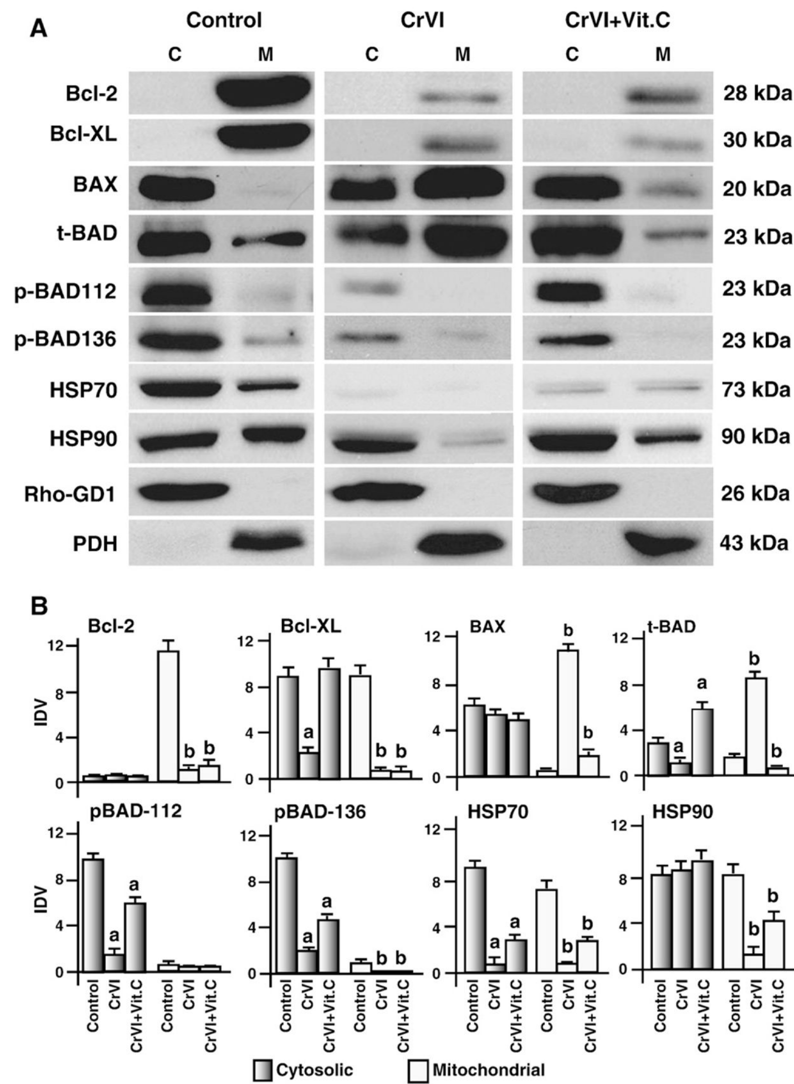

Fig. 4.

Effect of CrVI on translocation of Bcl-2 family proteins and heat shock proteins 70, and 90 in the mitochondria and cytosol. Cells were pre-treated with or without vitamin C for 24 h and treated with CrVI (10 μM) for 0 h, 12 h and 24 h. Western blots were performed with 75 μg of protein, as described in Materials and methods. A, Representative western blots of Bcl-2, Bcl-XL, BAX, total Bad (t-Bad), phosphorylated Bad (pBad-112, pBad-136), HSP-70 and HSP-90 in the cytosolic and mitochondrial protein fractions. M- mitochondrial protein fraction; C-cytosolic protein fraction. B, Histograms of Integrated Density Value (IDV) for each protein. Each value is the mean±SEM of three experiments, $P<0.05$; a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI+Vitamin C-treatment, 0 h *vs* 12 h or 24 h. Vit.C—Vitamin C.

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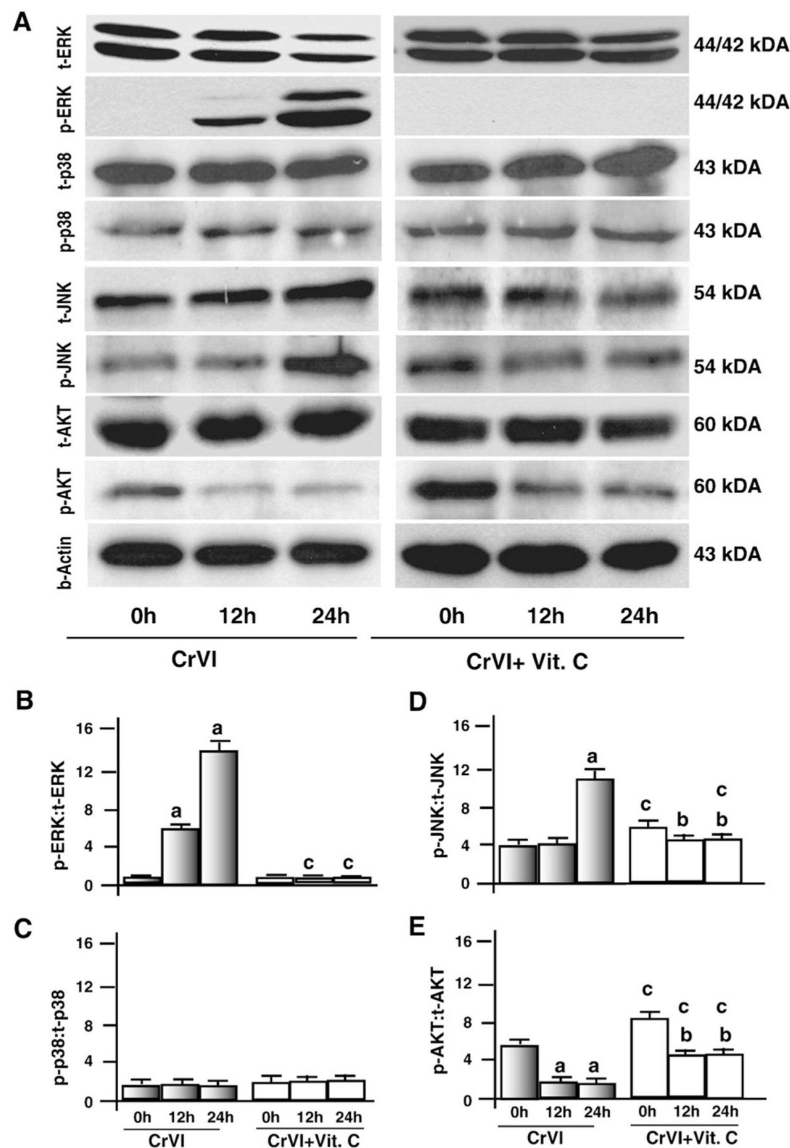

Fig. 5.

Effect of CrVI on phosphorylation of ERK1/2, p38, JNK and AKT. Cells were pre-treated with or without vitamin C for 24 h and treated with CrVI (10 μM) for 0 h, 12 h and 24 h. Western blots were performed with 75 μg of protein, as described in Materials and methods, and probed with the antibodies for total and phosphorylated forms of ERK, p38, JNK, and AKT/PI3K. A, Representative western blots of total and phosphorylated forms of p38, ERK, p38, JNK, and AKT/PI3K. B–E, Histograms showing the ratio between phosphorylated to total forms of each protein expressed as Integrated Density Value (IDV). Each value is mean ±SEM of three experiments, $P<0.05$. a: Control *vs* CrVI-treatment, 0 h vs 12 h or 24 h; b: Vitamin C *vs* CrVI+Vitamin C 12 h or 24 h; c: CrVI (0 h or 12 h or 24 h) *vs* CrVI+Vitamin C (0 h or 12 h or 24 h).

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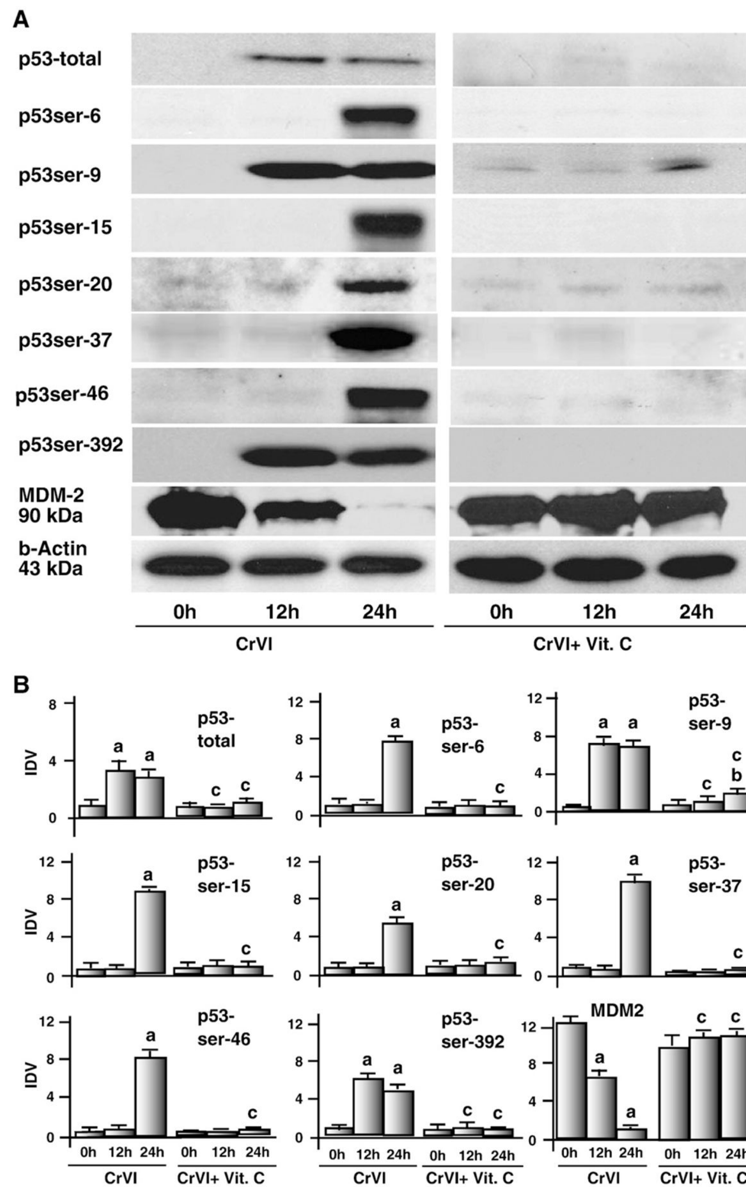

Fig. 6.
Effect of CrVI on total p53 protein, phosphorylation of p53 at ser-6, ser-15, ser-20, ser-37, ser-46, ser-392 and on MDM-2. Cells were pre-treated with or without vitamin C and treated with CrVI (10 μM) for 0 h, 12 h and 24 h. Western blots were performed with 75 μg of protein, as described in Materials and methods. A, Representative western blots of total p53 protein, phosphorylated p53 at ser-6, ser-15, ser-20, ser-37, ser-46, ser-392 and MDM-2. B, Histograms of Integrated Density Value (IDV) for each protein. Each value is the mean ±SEM of three experiments, $P<0.05$; a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI +Vitamin C-treatment, 0 h *vs* 12 h or 24 h; c: CrVI (0 h or 12 h or 24 h) *vs* CrVI+Vitamin C (0 h or 12 h or 24 h). Vit.C—Vitamin C.

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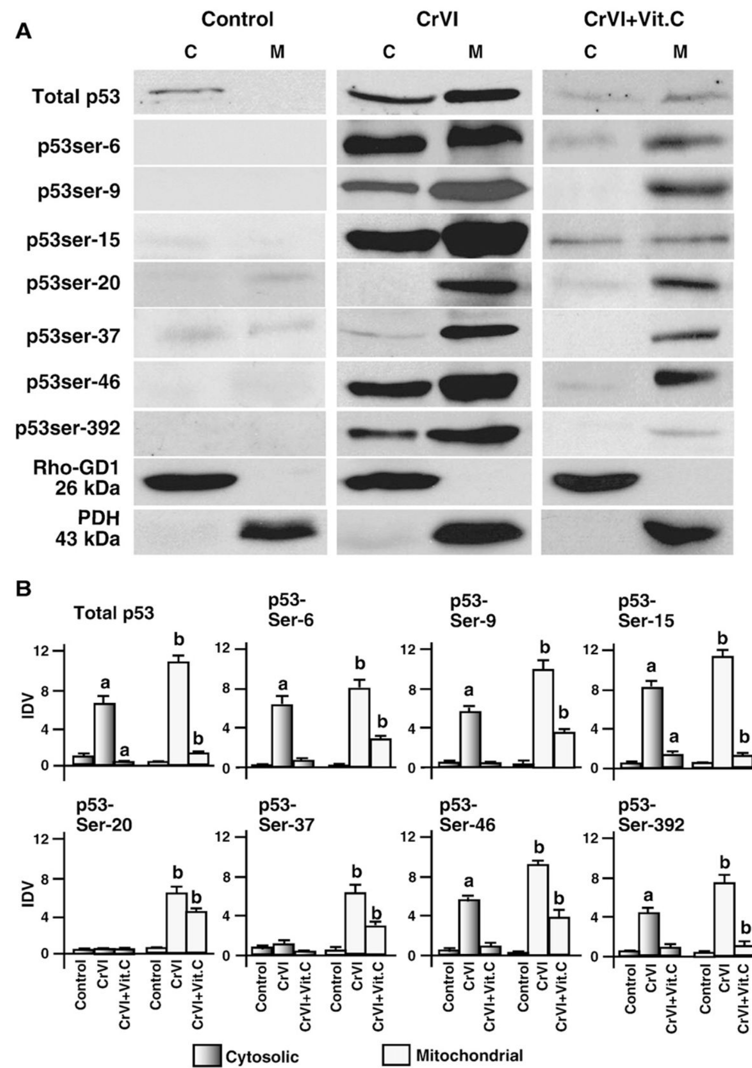

Fig. 7.

Effect of CrVI on translocation of total and phosphorylated p53 from cytosol to the mitochondria. Cells were pre-treated with or without vitamin C and treated with CrVI (10 μM) for 0 h, 12 h and 24 h. Western blots were performed with 75 μg of protein, as described in Materials and methods. A, Representative western blots of total p53 and phosphorylated p53 in the cytosolic and mitochondrial protein fractions. M-mitochondrial protein fraction; C-cytosolic protein fraction. B, Histograms of Integrated Density Value (IDV) for each protein. Each value is the mean±SEM of three experiments, $P<0.05$; a: CrVI-treatment, 0 h *vs* 12 h or 24 h; b: CrVI+Vitamin C-treatment, 0 h *vs* 12 h or 24 h. Vit.C—Vitamin C.

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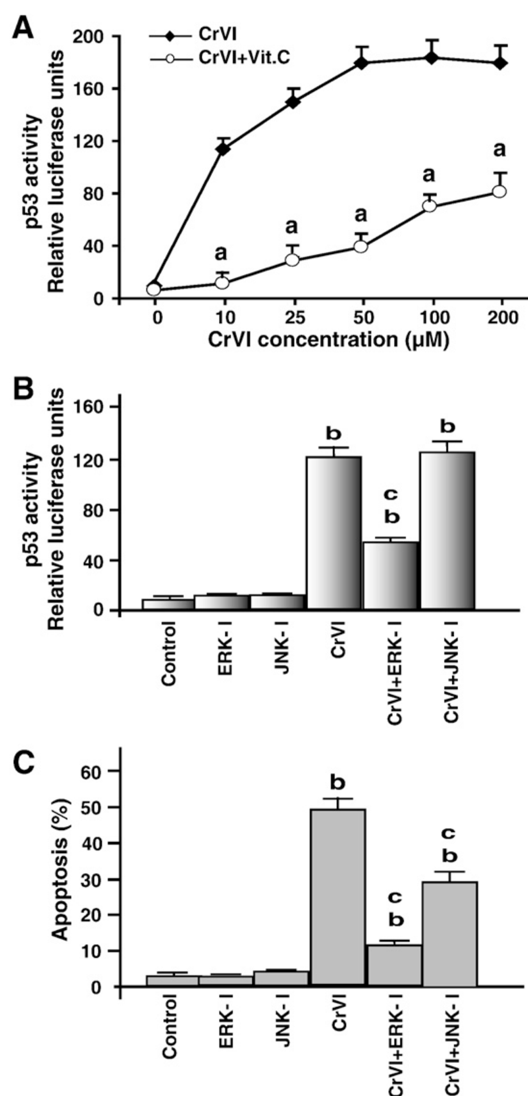

Fig. 8.

Effect of CrVI on the transcriptional activity of p53 through activation of ERK1/2. Cells were pre-treated with or without vitamin C for 24 h, and transfected with p53 reporter according to the manufacturer's protocol. After 24 h of transfection, cells were treated with CrVI (10 μM). Dual Luciferase assay was performed 24 h after treatment, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization. A, Effect of CrVI on p53 activity in the presence or absence of vitamin C; B, Effect of CrVI on p53 activity in the presence or absence of ERK-inhibitor and/ or JNK-inhibitor; C, Effect of CrVI on apoptosis in the presence or absence of ERK-inhibitor and/or JNK-inhibitor. Each value is the mean±SEM of three experiments, $P<0.05$. a: CrVI *vs* CrVI +Vitamin C-treatment. b: Control *vs* CrVI with or without ERK-I or JNK-I; c: CrVI *vs* CrVI +ERK-I or CrVI+JNK-I. ERK-I, ERK inhibitor; JNK-I, JNK inhibitor.

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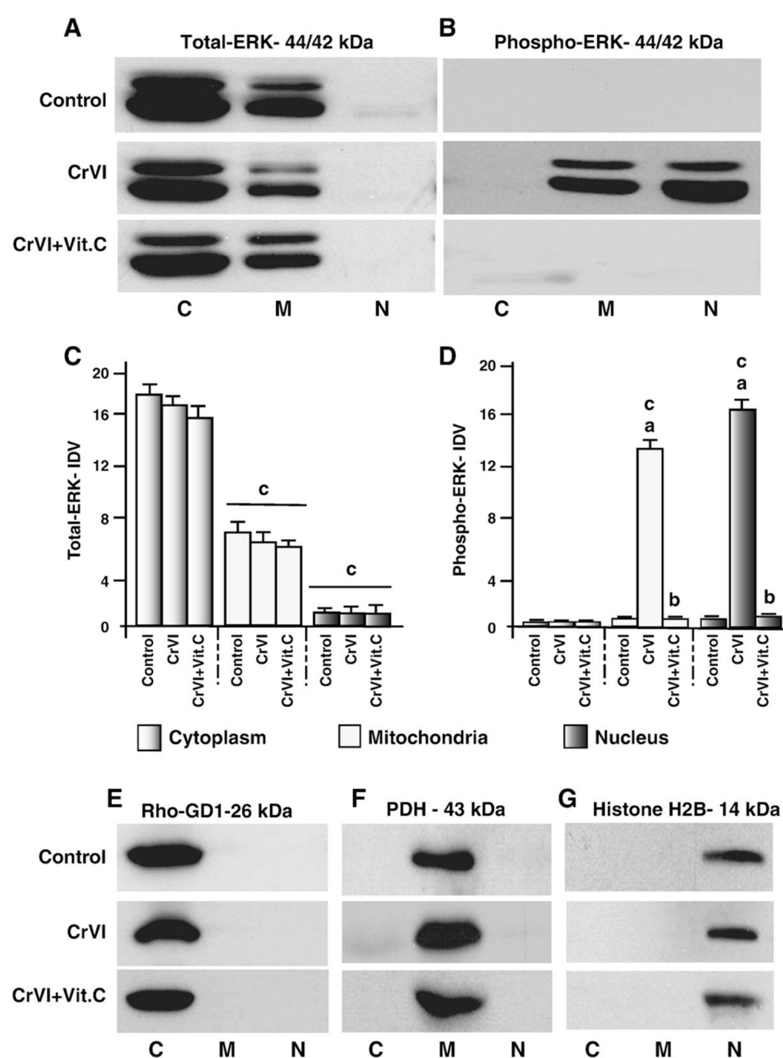

Fig. 9.

Effect of CrVI on translocation of total and phosphorylated ERK from cytosol to the mitochondria and the nucleus. Cells were pre-treated with or without vitamin C for 24 h and treated with CrVI (10 μM) for 0 h, 12 h and 24 h. Western blots were performed with 75 μg of protein, as described in Materials and methods. Representative western blots of A, total ERK (t-ERK); and B, phospho-ERK (p-ERK) in the cytosolic, mitochondrial and nuclear protein fractions. Histogram showing Integrated Density Value (IDV) of C, total ERK, and D, phospho-ERK for each protein fraction. M—mitochondrial protein fraction; C—cytosolic protein fraction; N—Nuclear protein fraction. Each value is mean±SEM of three experiments, $P<0.05$. a: Control *vs* CrVI treatment; b: CrVI vs CrVI+Vit.C; c: t-ERK or p-ERK in the cytosol *vs* mitochondria or nucleus. Rho-GD1—loading control for cytosolic protein; pyruvate dehydrogenase (PDH) —loading control for mitochondrial protein; histone H2B—loading control for nuclear protein.