Toxicology xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Mechanisms of chromium (VI)-induced apoptosis in anterior pituitary cells

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ARTICLE INFO

Article history:
Received 15 February 2008
Received in revised form 10 April 2008
Accepted 15 April 2008
Available online xxx

Keywords:
Hexavalent chromium
Pituitary
Oxidative stress

ABSTRACT

Hexavalent chromium (Cr (VI)) is a highly toxic metal. Exposure to Cr (VI) compounds may affect reproductive functions. Due to the importance of anterior pituitary hormones on reproductive physiology we have studied the effects of Cr (VI) on anterior pituitary. We previously demonstrated that, after *in vivo* Cr (VI) administration, Cr accumulates in the pituitary gland and affects prolactin secretion. *In vitro*, Cr (VI) causes apoptosis in anterior pituitary cells due to oxidative stress generation. To better understand the mechanisms involved in Cr (VI)-induced apoptosis we studied: (a) whether Cr (VI) affects the intracellular antioxidant response and (b) which of the apoptotic factors participates in Cr (VI) effect. Our results show that Cr (VI) treatment induces a decrease in catalase and glutathione peroxidase (GPx) activity but does not modify glutathione reductase (GR) activity. Cr (VI) exposure causes an increase of GSH levels. p53 and Bax mRNA are also upregulated by the metal. Pifithrin α, a p53 transcriptional inhibitor, increases Cr (VI) cytotoxicity, suggesting a role of p53 as a survival molecule. The antioxidant *N*-acetyl-cysteine (NAC) could prevent Bax mRNA increase and caspase 3 activation, confirming that Cr (VI)-induced apoptosis involves oxidative stress generation.

1. Introduction

Chromium (Cr) is ubiquitous in the environment, occurring principally in trivalent and hexavalent forms. While Cr (III) is an essential nutrient, Cr (VI) is highly toxic and a strong oxidizing agent produced mainly by anthropogenic sources (O'Brien et al., 2003; Zayed and Terry, 2003). Like all metals, Cr is non-biodegradable and persists in the environment. As a result, it has become a widespread environmental contaminant (Costa, 2003; IARC, 1990; Zayed and Terry, 2003).

Occupational exposure to Cr (VI) compounds is associated with several adverse effects on health such as lung toxicity and bronchial asthma, and it also causes nephro- and hepatotoxicity (Bright et al., 1997; Costa, 1997; Dartsch et al., 1998; IARC, 1990). Besides, it has been shown to affect several other tissues and organs such as the brain (Costa, 1997; Travacio et al., 2000). Different studies have reported alterations on reproductive function as a result of Cr (VI) or Cr (VI) compounds exposure (Acharya et al., 2006; Bonde, 1990; Kumar, 2004; Murthy et al., 1996; Sutherland et al., 2000). Even though the metal could affect directly the reproductive organs, it is also possible that Cr effects on anterior pituitary contribute to the reproductive toxicity. We have previously demonstrated that after *in vivo* administration of Cr (VI), Cr accumulates in the pituitary and affects prolactin secretion (Quinteros et al., 2007). Cr (VI) was also cytotoxic for anterior pituitary cells in culture, an effect due to apoptosis and oxidative stress generation (Quinteros et al., 2007).

At physiological pH, Cr (VI) exists as a chromate oxyanion and can readily cross cell membranes through the sulfate anion transport system (O'Brien et al., 2003). Once inside the cell, Cr (VI) is reduced to the ultimate reduced form Cr (III). During this process, the reactive chromium intermediates Cr (V) and Cr (IV), and diverse reactive oxygen species (ROS) are generated. Cr (VI) exposure generates oxidative stress in many systems (Harris and Shi, 2003; Pulido and Parrish, 2003). Oxidative stress results from an imbalance between the antioxidant defense systems and ROS generation. The most abundant antioxidant in the cell is glutathione, which is a strong antioxidant by acting directly in the detoxification of ROS (Schafer and Buettner, 2001; Wu et al., 2004). It is also important in maintaining the cellular redox balance and participates in one of the most important defense systems against oxidative stress (Schafer and Buettner, 2001; ~~Wu,~~ 2004). The enzymes involved in the glutathione antioxidant defense system are glutathione peroxidase (GPx) and glutathione reductase (GR) which detoxify hydrogen peroxide in a reaction couple to glutathione redox cycling. Among the enzymatic defense, catalase also plays a fundamental role by reducing hydrogen peroxide to water and oxygen (Scandalios, 2005; Valko et al., 2006; Yang et al., 2006).

It is known that ROS also serve as signal transduction messengers and so participate in different signaling cascades. Excess ROS generation may lead to the activation of several oxidative stress

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0300-483X/$ – see front matter © 2008 Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2008.04.012

response transcription factors such as p53 (Adler et al., 1999; Haupt et al., 2003). Many studies confirmed that Cr (VI) treatment cause p53 activation and highlighted the importance of ROS in its activation and in the apoptotic process. It is also accepted that Cr (VI)-induced apoptosis can be independent of p53 (O'Brien et al., 2003; Pulido and Parrish, 2003; Wang et al., 2000; Ye et al., 1999).

The mechanism by which Cr (VI) induces apoptosis and oxidative stress may vary among cell types. At the pituitary level, we demonstrated that Cr (VI)-induced apoptosis involved caspase 3 activation. Cr (VI) caused an early increase of ROS levels and treatment with an antioxidant prevented the cytotoxicity of the metal. Oxidative stress generation is, therefore, a key event leading to cell death in response to Cr (VI). Taking into account these previous results, the aim of the present study was to investigate: (a) whether Cr (VI) affects the intracellular antioxidant response and (b) the mechanisms involved in Cr (VI)-induced apoptosis in anterior pituitary cells in culture.

2. Materials and methods

2.1. Drugs and reagents

Potassium dichromate ($K_2Cr_2O_7$) was purchased from Cicarelli, Argentina. Dihydrorhodamine123 (DHR123), and *N*-acetyl-cysteine (NAC) were purchased from Alexis, San Diego, CA, USA. L-Buthonine sulfoximine (BSO) and *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) were obtained from Sigma–Aldrich, St. Louis, MO, USA. Monochlorobimane (MCB) was purchased from Molecular Probes, Invitrogen, CA, USA. RNA isolation and RT-PCR reagents were obtained from Invitrogen, CA, USA. Reagents for cell culture were purchased from Gibco, Rockville, MD, USA; GenSA, Buenos Aires, Argentina and Sigma–Aldrich. All other drugs were obtained from Sigma–Aldrich.

2.2. Animals and cell culture

Adult male Wistar rats (250 g), kept on a 12-h light–dark cycle with controlled temperature (20–22 °C), were used. Food and water were supplied *ad libitum*. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were killed by decapitation and the anterior pituitary glands were removed.

Cells were obtained from the glands by enzymatic (trypsin/DNase) and mechanical dispersion (extrusion through a Pasteur pipette) as described previously (Velardez et al., 2004). In all cases the cells were cultured for 3 days (37 °C, 5% CO_2 in air) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 μl/ml MEM amino acids, 2 mM glutamine, 5.6 μg/ml amphotericin B, and 25 μg/ml gentamicin (DMEM-S-10% FBS). For caspase activity, flow cytometry studies and mRNA analysis, cells were seeded onto 24-well tissue culture plates (1×10^6 cells/well). For spectrophotometric studies, cells were seeded onto 24-well tissue culture plates (0.5×10^6 cells/well). For spectrofluorometric studies, cells were seeded onto 96-well culture plates (0.1×10^6 cells/well) and onto 24-well culture plates (0.5×10^6 cells/well).

2.3. Cell treatment

To study the effect of Cr (VI), cells were incubated for different time lapses in DMEM-S-10% FBS containing 10 μM Cr (VI).

To study the effect of NAC or BSO, cells were preincubated with 1 mM NAC or 30 μM BSO for 1 h. Then, cells were co-incubated with the metal plus NAC or BSO for the times indicated and once completed that time, the different assays were performed.

For cell activity assay, cells were preincubated with different concentrations of pifithrin α for 1 h. Then, cells were co-incubated with the metal plus pifithrin α for 9 h and once completed that time the medium was replaced by fresh medium without Cr (VI) to complete a total incubation time of 48 h.

2.4. Cell activity assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to determine cell activity as described previously (Velardez et al., 2004). In brief, cells were washed twice with Krebs Ringer bicarbonate buffer and then incubated with 110 μl of a MTT solution (500 μg/ml) for 4 h at 37 °C. After incubation, 90 μl of the medium was removed, 100 μl of 0.04 M HCl in isopropanol were added to each well, and the plate was gently shaken for 3 min. Optical density was determined at 600 nm in an ELISA plate reader. Cell activity was considered as an index of cell viability.

2.5. Caspase activity assay

Cells were washed three times with chilled PBS, then incubated with 75 μl of lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin and 500 μM phenylmethylsulfonyl fluoride, PMSF) for 30 min at 37 °C. Thereafter, the content from three wells was pooled and centrifuged at 20,000 × *g* for 20 min (4 °C). The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM dithiothreitol, 0.5 mM PMSF, 10% glycerol). Reaction began with addition of 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase 3 substrate) and caspase activity was measured by absorbance at 405 nm 1 h after incubating the mix at 37 °C. Caspase activity was expressed as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample) × 100.

2.6. Measurement of ROS

To measure ROS production, cells were loaded with 10 μM DHR123 20 min before the end of the treatment. Once finished, cells were resuspended by trypsinization in calcium- and magnesium-free Krebs buffer (10^6 cells/ml) and analyzed in a Becton Dickinson FACScalibur flow cytometer (ex λ: 488 nm, em λ: 535 nm, FL1). Immediately before the measurement, 10 μg/ml propidium iodide (PI) (ex λ: 488 nm, em λ: 585 nm, FL2) was added to each FACS tube in order to detect cells with disrupted plasma membrane, 10^4 cells were measured per treatment. Data were analyzed using WinMDI 2.8 software. In the control, plasma membrane integrity after trypsinization was always above 95%. Unviable cells (PI positive) were not used in the analysis.

2.7. Determination of antioxidant enzymes

To determine the activity of antioxidant enzymes, cells were rinsed with calcium- and magnesium-free Krebs buffer after the end of the treatment, resuspended by trypsinization in the same buffer, and centrifuged at 1000 × *g* for 10 min. The pellet (1×10^6 cells) was sonicated in 50 μl of lysis buffer (500 mM Hepes pH 7.4, 125 mM KCl, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM PMSF). Sonicates were centrifuged at 10,000 × *g* for 30 min (4 °C). The resulting supernatants were analyzed for GR, GPx, and catalase enzymatic activities using standard spectrophotometric assays. GR activity was measured by following the oxidation of NADPH at 340 nm for 3 min. The reaction mixture contained 50 mM phosphate buffer pH 7, 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. The activity of the enzyme was determined after addition of the sample and defined as nmoles NADPH oxidized/min/mg protein (value in control: 99.17 ± 6.98 nmoles NADPH oxidized/min/mg protein). GPx activity was measured by the oxidation of NADPH in a reaction coupled to GR. The reaction mixture contained 50 mM phosphate buffer pH 7, 1.5 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 mM GSH, and 0.2 U/ml GR. The reaction was started by the addition of 0.5 mM cumene hydroperoxide and the oxidation of NADPH was followed spectrophotometrically at 340 nm for 3 min. GPx enzymatic activity was defined as nmoles NADPH oxidized/min/mg protein (control value: 191.09 ± 25.43 nmoles NADPH oxidized/min/mg protein). Catalase activity was measured by the reduction velocity of 20 mM H_2O_2 in 50 mM phosphate buffer at 240 nm for 2 min. Catalase enzymatic activity was defined as μmoles H_2O_2 consumed/min/mg protein (control value: 7.00 ± 0.33 μmoles H_2O_2 consumed/min/mg protein).

2.8. Determination of reduced glutathione content ~~(MCB)~~

Intracellular level of glutathione (GSH) was determined using the GSH-sensitive probe monochlorobimane. After treatment, cells were incubated with the probe (100 μM) for 30 min at 37 °C and 5% CO_2. Then, the medium was removed, cells were rinsed twice with PBS 1×, lysated with 1% Nonidet P40-PBS 1×, and fluorescence was measured by spectrofluorometry (ex λ: 390 nm, em λ: 485 nm). Immediately after this, 10 μg/ml PI (ex λ: 488 nm, em λ: 600 nm) was added to each sample to assess the cellular DNA content. Basal fluorescence was determined from cells that were not incubated with the probe and was subtracted to all samples. Intracellular GSH content was expressed as (MCB fluorescence/PI fluorescence in treated sample)/(MCB fluorescence/PI fluorescence in control sample) × 100.

2.9. RNA isolation

Total RNA was isolated from cells using TRIzol® reagent. After isolation, total RNA from 4×10^6 cells of each treatment was spectrophotometrically quantified at 260 nm. 260/280 ratio was also determined.

2.10. RT and PCR reactions

First strand cDNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase. First, a 5-min incubation at 65 °C was performed for samples containing 2 μg RNA, 1 μl dNTP mix 10 mM and 1 μl Oligo dT 0.5 μg/μl in a final volume of 13 μl. The reverse transcription reaction was run at 37 °C for 50 min by adding 4 μl first strand buffer 5×, 2 μl DTT 5.5 mM and 1 μl M-MLV reverse transcriptase (3.125 U) to the anterior mixture. Reverse transcriptase was

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Table 1
Primers used for semi-quantitative RT-PCR assays

Gene	Primer	Product size
p53	Forward 5′-TTTGAGGTTCGTGTTTGTGC-3′ Reverse 5′-TTTTATGGCGGGACGTAGAC-3′	337 bp
bax	Forward 5′-CCGAGAGGTCTTCTTCCGGG-3′ Reverse 5′-TGGAAGAAGATGGGCTGAG-3′	317 bp
bcl-2	Forward 5′-CAAGCCGGGAGAACAGGGTA-3′ Reverse 5′-GCCTTCTTTGAGTTCGGTGGG-3′	450 bp
β-actin	Forward 5′-ACCACAGCTGAGAGGGAAATCG-3′ Reverse 5′-AGAGGTCTTTACGGATGTC AACG-3′	276 bp

inactivated by heating the samples at 70 °C for 15 min before the PCR reactions. To check for genomic contamination, the same procedure was performed on samples in a reaction solution lacking reverse transcriptase.

Specific primers for p53, Bax and Bcl-2 were designed using Primer3 software from Whitehead Institute (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Table 1. The specificity of the primers was confirmed by a BLAST software-assisted search of a non-redundant nucleotide sequence database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST). Actin was used as an endogenous control.

Samples were thermocycled for PCR amplification (Mastercycler, Eppendorf, Hamburg, Germany). The reaction mixture contained 2 μl cDNA, 10 μl Go Taq buffer 5×, 1 μl dNTP mix 10 mM, 1 μl of each primer 10 μM, 0.25 μl Go Taq polymerase (0.625 U) in a final volume of 50 μl. We utilized RT-PCR method to determine relative changes in mRNA expression. Reactions were subjected to a varying number (n = 20–40) of cycles of PCR amplification to find out the optimum cycle number within the linear range for PCR amplification. Amplified products collected at various cycles were analyzed by electrophoresis in 2% agarose-ethidium bromide gels. Reactions conditions for PCR were: for p53, 4 min at 94 °C followed by cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a last cycle of 10 min at 72 °C; for Bax, 2 min at 94 °C followed by cycles of 1 min at 94 °C, 1.5 min at 55 °C, 1.5 min at 72 °C and a last cycle of 10 min at 72 °C; for Bcl-2, 2 min at 94 °C followed by cycles of 1 min at 94 °C, 1.5 min at 60 °C, 1.5 min at 72 °C and a last cycle of 10 min at 72 °C; for β-actin, 4 min at 94 °C followed by cycles of 1 min at 94 °C, 1.5 min at 56 °C, 1.5 min at 72 °C and a last cycle of 10 min at 72 °C. The optimum cycle number resulted to be 24 cycles for p53 and 40 for Bax and Bcl-2.

2.11. Analysis of semi-quantitative PCR data

The intensity of PCR products signals was determined by digital image analysis using the Gel Pro Analyzer software (Media Cybernetics, LP, Silver Spring, MD) for Windows. To allow statistical comparison, p53, Bax and Bcl-2 levels were normalized to the value of the β-actin amplified band of each lane.

2.12. Protein determination

Protein content was assayed by the Bradford method (BioRad, Buenos Aires, Argentina) using bovine serum albumin as standard.

2.13. Statistical analysis

The results were expressed as mean ± S.E.M. and evaluated by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison *post hoc* test. Student's *t*-test, was also used depending on the experimental design. Differences between groups were considered significant if $p < 0.05$. Results were confirmed by at least three independent experiments.

3. Results

3.1. Effect of Cr (VI) on glutathione levels

Previously we have showed that NAC, a glutathione precursor, could prevent Cr (VI)-induced apoptosis in anterior pituitary cells (Quinteros et al., 2007). To address whether Cr (VI) toxicity involves changes in glutathione levels, intracellular GSH content was measured by a fluorometric method using the probe MCB. GSH levels remained unaltered at 2 h of Cr (VI) exposure and significantly increased at 4, 6 and 8 h (Fig. 1). These changes in GSH levels could be a result of an effect of Cr (VI) on GSH synthesis and/or on the antioxidant enzymes involved in GSH recycle.

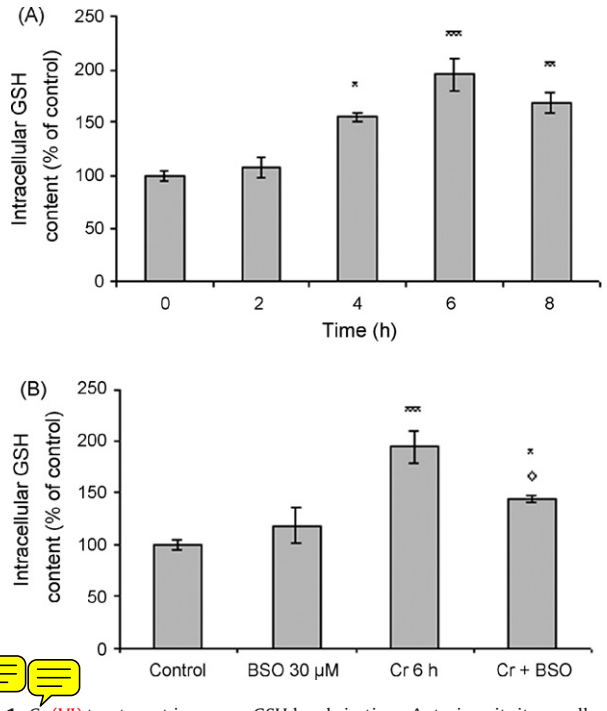

Fig. 1. Cr (VI) treatment increases GSH levels in time. Anterior pituitary cells were incubated with 10 μM Cr (VI) for different times and then, intracellular GSH content was measured by spectrofluorometry using the GSH-sensitive probe, MCB. PI was used to determine cellular DNA content in the sample. GSH content was expressed as (MCB fluorescence/PI fluorescence in treated sample)/(MCB fluorescence/PI fluorescence in control sample) × 100. Bars represent mean ± S.E.M., $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control without Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

3.2. Cr (VI) effect on GSH synthesis and the role of GSH in the detoxification of ROS

In order to investigate whether the increase in GSH levels was due to stimulation of its synthesis, cells were treated with BSO, an irreversible inhibitor of δ-glutamylcysteine synthetase, the rate-limiting enzyme of glutathione synthesis. Increased GSH levels at 6 h were significantly reduced by BSO treatment indicating that GSH synthesis is increased during Cr (VI) treatment (Fig. 2).

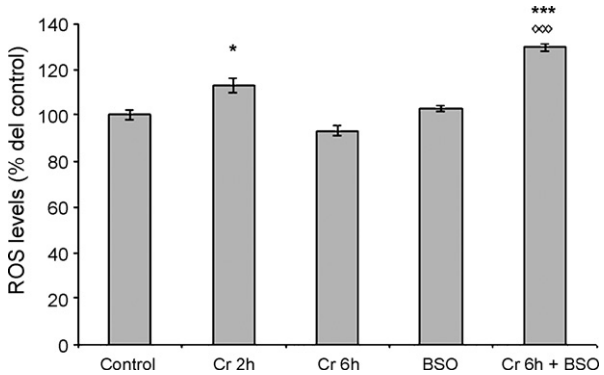

Fig. 2. BSO prevents GSH increase induced by Cr (VI). Anterior pituitary cells were pre- and co-treated with BSO (an inhibitor of GSH synthesis) plus 10 μM Cr (VI) for 6 h and then intracellular GSH levels were examined. GSH content was expressed as (MCB fluorescence/PI fluorescence in treated sample)/(MCB fluorescence/PI fluorescence in control sample) × 100. Bars represent mean ± S.E.M., $n = 3$. *$p < 0.05$, ***$p < 0.001$ vs. control without Cr (VI), ◇$p < 0.05$ vs. Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

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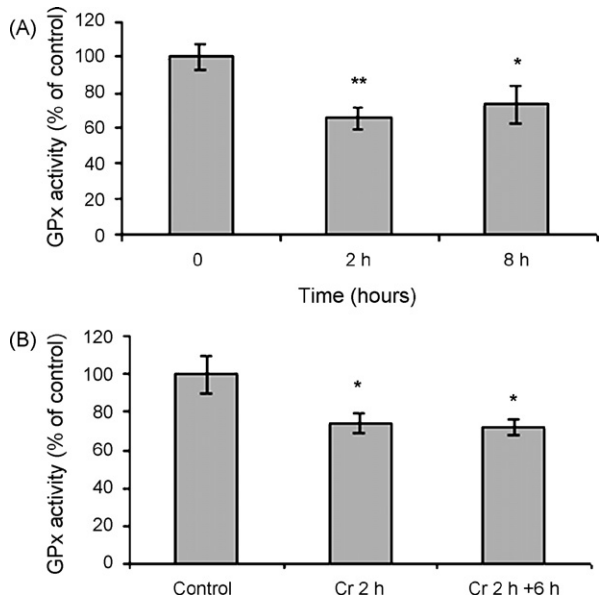

Fig. 3. Cr (VI) affects GPx enzymatic activity (A) but does not modify GR activity (B). Anterior pituitary cells were incubated with 10 μM Cr (VI) for the indicated times and the activity of the antioxidant enzymes was determined. GPx enzymatic activity (A). GR enzymatic activity (B). Data are expressed as percentage of control (nmoles NADPH oxidized/min/mg protein). Bars represent mean ± S.E.M., $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

Previous results indicated that ROS levels rose at 1 and 2 h of Cr (VI) exposure and that then they returned to control values (Quinteros et al., 2007). Knowing that GSH can itself act as a ROS scavenger, the role of GSH in the detoxification of ROS was studied. Cells were pretreated with or without BSO for 1 h and then treated with the metal in the presence or absence of BSO for 6 h. ROS levels were measured by detecting the fluorescent changes emitted by the oxidation of the probe DHR123 by flow cytometry. At 6 h of Cr (VI) exposure, ROS levels were similar to control but in the presence of BSO, a markedly increase of ROS was observed (Fluorescence Intensity, % of control, Cr 2 h: 114 ± 3.2%*, Cr 6 h: 88.2 ± 2.1%, BSO 30 μM: 102.8 ± 1.3%, Cr 6 h + BSO: 129.6 ± 1.3%$^{***\Delta\Delta\Delta}$, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control; $^{\Delta\Delta\Delta}p < 0.001$ vs. Cr (VI), $n = 3$, data representative of three individual experiments. ANOVA followed by Student–Newman–Keuls test). This result demonstrates that the increase in GSH synthesis during Cr (VI) exposure plays an important role in the detoxification of ROS.

3.3. GPx and GR activity in response to Cr (VI) exposure

The other possibility is that the changes in GSH levels could be a result of an effect of Cr (VI) on the antioxidant enzymes involved in its regeneration. To study this, GPx and GR activity were measured. The activity of GPx and GR was determined by standard spectrophotometric methods after 2 and 8 h of Cr (VI) exposure. GPx activity decreased at both times tested while GR activity did not change suggesting a differential effect of the metal on both enzymes (Fig. 3A and B).

As GPx activity remained low after 8 h of exposure, we examined if it could be a consequence of a persistent effect of the metal. Cells were incubated with Cr (VI) for 2 h and then with medium without the metal for an additional 6 h. GPx activity remained low even after 6 h of Cr (VI) removal confirming a persistent effect of Cr (VI) on enzyme activity (GPx activity, % of control, Cr 2 h: 74.2 ± 5.3%*, Cr 8 h: 67 ± 9%*, Cr 2 h + 6 h without Cr: 71.9 ± 4%*, $^{*}p < 0.05$, $n = 3$, data representative of 3 individual experiments. ANOVA followed by Student–Newman–Keuls test).

3.4. Cr (VI) effect on catalase activity

Catalase acts as a primary hydrogen peroxide detoxifying enzyme (Matés et al., 1999; Scandalios, 2005). To further evaluate the effect of Cr (VI) on cellular enzymatic antioxidants and its role in oxidative stress generation, the activity of catalase was measured. Cells were exposed to Cr (VI) for 2 and 8 h and enzyme activity was measured spectrophotometrically. Catalase activity significantly decreased at 2 h and tended to return to control values at 8 h (Fig. 4).

3.5. Effect of NAC on Cr (VI)-induced ROS increase

Previously, we observed that the antioxidant NAC could prevent Cr (VI) toxicity on anterior pituitary cells in culture. To confirm that this effect is associated with its ability to scavenge ROS, intracellular ROS levels were examined by flow cytometry using DHR123 as a probe. As we previously showed, Cr (VI) treatment produced an increase in ROS levels at 2 h of exposure. Cells pretreated with NAC and then treated with Cr (VI) in the presence of NAC exhibited a marked decrease of ROS levels (Fluorescence Intensity, % of control, Cr 2 h: 128.0 ± 9.0%*, NAC 1 mM: 100.5 ± 4.8%, Cr 2 h + NAC: 108.4 ± 8.0% $^{\Delta}$, $^{*}p < 0.05$ vs. control; $^{\Delta}p < 0.05$ vs. Cr (VI), $n = 3$, data representative of three individual experiments. ANOVA followed by Student–Newman–Keuls test). It is known that NAC could reduce Cr (VI). If this happens in the extracellular environment there could be less Cr (VI) available to enter the cells. Based on this possibility and to confirm that NAC was preventing Cr (VI) cytotoxicity by its intracellular antioxidant effects, we evaluate the effect of Cr (VI) on ROS

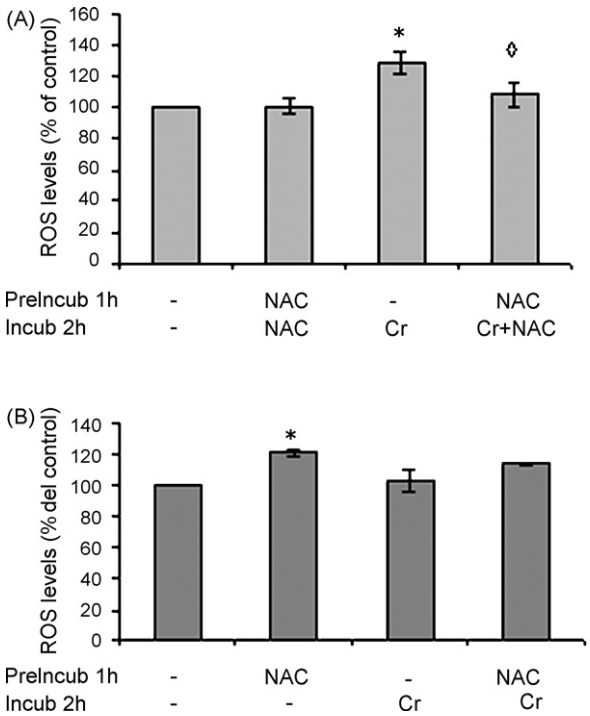

Fig. 4. Cr (VI) affects catalase activity. Anterior pituitary cells were incubated with 10 μM Cr (VI) for the indicated times and the activity of catalase was determined. Data are expressed as percentage of control (nmoles NADPH oxidized/min/mg protein). Bars represent mean ± S.E.M., $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without Cr (VI), $^{\circ}p < 0.05$ vs. Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

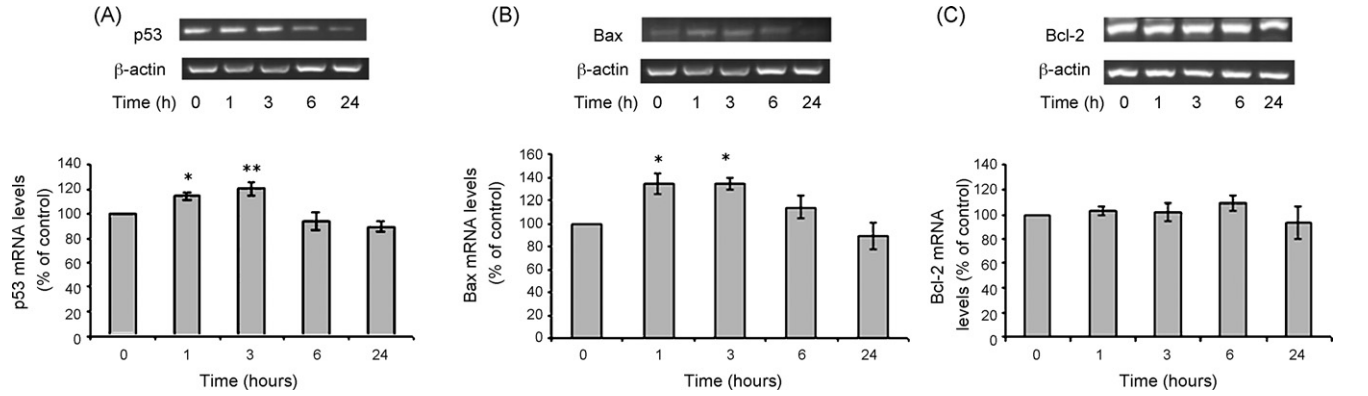

Fig. 5. Cr (VI) alters the expression of p53 and Bax but does not modify Bcl-2 expression. Anterior pituitary cells were incubated with 10 μM Cr (VI) for the indicated times. p53, Bax and Bcl-2 mRNA levels were determined by semi-quantitative RT-PCR assay. *Upper figures*: representative blots from one experiment. *Bottom figures*: average densitometric values. The expression levels were normalized to that of β-actin and expressed as the percentage of control level. Each value represents mean ± S.E.M. of three independent experiments, $^{*}p < 0.05$ vs. control without Cr (VI). ANOVA followed by Dunnett's test.

levels when NAC was present only during the pretreatment period. Cells loaded or not with NAC for 1 h were then exposed to Cr (VI) for 2 h. The presence of NAC only during 1 h of pretreatment prevented the increase of ROS observed after 2 h Cr (VI) exposure ~~(Fluorescence Intensity, % of control, Cr 2 h: 121.0 ± 2.0%*, NAC 1 mM, 1 h: 102.5 ± 7.6%, Cr 2 h + NAC: 113.5 ± 0.8%, *$p < 0.05$ vs. control, $n = 3$, data representative of three individual experiments. ANOVA followed by Student–Newman–Keuls test).~~ These results indicate that ~~pretreatment with NAC is enough to decrease ROS levels under Cr (VI) exposure.~~

3.6. *Effect of Cr (VI) on apoptotic and anti-apoptotic markers: caspase 3, p53 and Bcl-2 family proteins*

We had previously shown that Cr (VI)-induced apoptosis by a mechanism that involves caspase 3 activation. To further evaluate the events leading to cell death in response to Cr (VI) exposure, mRNA levels of p53, Bax and Bcl-2 were evaluated. Cells were exposed to the metal for 1, 3, 6 and 24 h and mRNA levels were measured by RT-PCR. p53 and Bax mRNA levels significantly increased at 1 and 3 h of exposure, while Bcl-2 levels did not change at any time tested (Fig. 5).

Since the antioxidant NAC was able to prevent Cr (VI)-induced apoptosis, we next examined whether caspase 3 activation and increased p53 and Bax mRNA expression could also be prevented by this antioxidant.

To evaluate caspase 3 activation, cells were treated with or without NAC for 1 h and then with Cr (VI) or Cr (VI) plus NAC for 18 h, time at which a high increase in its activity was observed in previous experiments. Caspase 3 activity, determined by a colorimetric method, increased in cells exposed to Cr (VI) for 18 h and NAC treatment completely prevented this increase (Fig. 6). The effect of NAC on Cr-induced increase of p53 and Bax mRNA expression was evaluated by incubating the cells with or without NAC for 1 h and then with Cr (VI) or Cr (VI) plus NAC for another hour. As shown in Fig. 7, the increase in Bax mRNA levels after Cr exposure was prevented by NAC.

In regards to p53 expression, as the antioxidant *per se* decreased p53 mRNA levels it was not possible to asses its effects on Cr (VI)-treated cells (data not shown). Instead, we evaluated whether pifithrin α, an inhibitor of p53 transcriptional activity, could prevent Cr (VI) cytotoxicity. Cell treatment and cell activity determination were conducted as described in Section 2. Treatment with ~~pifithrin~~ α increased Cr (VI) cytotoxicity suggesting that p53

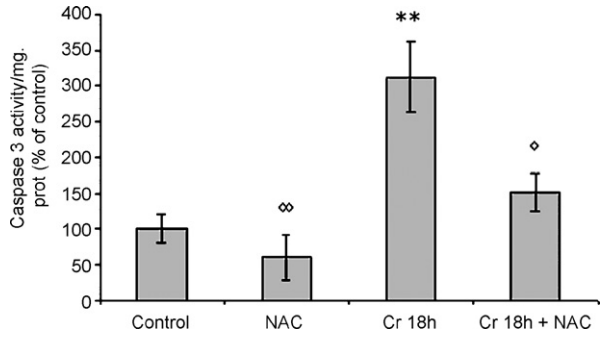

Fig. 6. NAC treatment prevents caspase 3 activation induced by Cr (VI). Anterior pituitary cells were incubated with 10 μM Cr (VI) for 18 h with or without NAC. Caspase 3 activity was analyzed by a specific colorimetric method. Data are expressed as percentage of control ± S.E.M., $n = 6$. $^{**}p < 0.01$ vs. control without Cr (VI), $^{\diamond\diamond}p < 0.01$ vs. Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

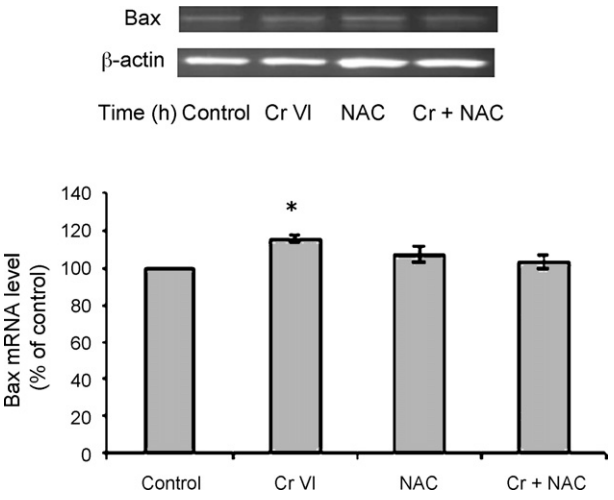

Fig. 7. NAC treatment prevents increased expression of Bax after Cr (VI) exposure. Anterior pituitary cells were incubated with 10 μM Cr (VI) for 1 h with or without NAC. Bax mRNA levels were determined by semi-quantitative RT-PCR assay. *Upper figure*: a representative blot from one experiment. *Bottom figure*: average densitometric values. The expression levels were normalized to that of β-actin and expressed as the percentage of control level. Each value represents mean ± S.E.M. of three independent experiments. $^{*}p < 0.05$ vs. control without Cr (VI). ANOVA followed by Student–Newman–Keuls test.

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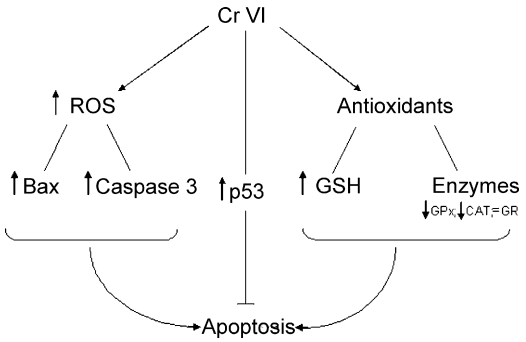

Fig. 8. Pifithrin α, a transcriptional inhibitor of p53, increases Cr (VI) cytotoxicity. Anterior pituitary cells were incubated with 10 μM Cr (VI) for 9 h and with or without different concentrations of pifithrin α (PFT). After that time, fresh medium without the metal was added to complete 48 h of incubation. Cell activity, considered an index of cell viability, was measured by MTT assay. Values represent means ± S.E.M., $n = 8$. $^{***}p < 0.001$ vs. control without Cr (VI), $^{\diamond}p < 0.05$, $^{\diamond\diamond}p < 0.01$ vs. Cr (VI). ANOVA followed by Student–Newman–Keuls test. Results are representative of three independent experiments.

transcriptional activity is not involved in the process of apoptosis induced by the metal. Moreover, this result point up to a role of p53 in a survival mechanism (Fig. 8).

4. Discussion

Within cells, ROS can be generated from endogenous and/or exogenous sources. When an imbalance between ROS levels and antioxidants occurs, oxidative stress is generated (Scandalios, 2005; Valko et al., 2006). We have previously shown that Cr (VI) exposure caused apoptosis in anterior pituitary cells. Oxidative stress generation was an important event leading to cell death given that Cr (VI) caused an early increase of ROS, and two antioxidants with different specificity such as TROLOX®, which acts detoxifying lipid radicals, and NAC, an antioxidant *per se* and precursor of glutathione, could prevent Cr (VI) cytotoxicity (Quinteros et al., 2007). In the present work, we investigated the response of antioxidant defense systems against Cr (VI)-induced oxidative stress and the mechanisms involved in Cr (VI)-induced apoptosis in anterior pituitary cells in culture.

Among the non-enzymatic antioxidants in cells, GSH is the most abundant. It has been shown that GSH depletion lead to cell death in numerous systems and that GSH increase may serve as a protective mechanism (Chandra et al., 2000; O'Brien et al., 2003; Wu et al., 2004). Here, we did not found modifications in GSH content at 2 h of Cr (VI) exposure when ROS levels were high but increased over that time, concomitantly with ROS decrease. Synthesis *de novo* was partially responsible for the increase of GSH. Since blockade of GSH synthesis resulted in the maintenance of the high levels of ROS at 6 h it could be suggested that GSH participates in the detoxification of ROS. A balance between its use and its synthesis could explain the fact that no rise of GSH content was observed at 2 h. GR and GPx are implicated in GSH regeneration (Matés et al., 1999; Yang et al., 2006). GR activity did not vary at 2 and 8 h of Cr (VI) exposure while GPx activity was reduced at those times. This enzyme retained some of its activity suggesting that GSH regeneration could still be happening though to a lesser extent. Cr (VI) generally depletes GSH in many systems (Lalaouni et al., 2007; O'Brien et al., 2003; Subramanian et al., 2006). On the contrary, in our study Cr (VI) increases GSH content which may act as a protective molecule. However, in spite of GSH being in a process of continuous regeneration and synthesis, its levels would not be enough to overcome the excess of ROS generated, as observed after 2 h of Cr (VI) exposure.

To further assess whether other antioxidant components of the cell were affected by Cr (VI), catalase activity was evaluated. The activity of this enzyme decreased at 2 h of Cr (VI) exposure. At 8 h catalase activity tended to restore to control values. It is known that the activity of the antioxidant enzymes may vary according to the nature and the degree of the insult. Several reports showed reduced catalase and GPx activity after treatment with Cr (VI) in different cells types (Asatiani et al., 2004; O'Brien et al., 2003; Pedraza-Chaverri et al., 2005; Travacio et al., 2000). In the present study, reduction of GPx activity at 2 h did not restore after 6 h of metal-free treatment, indicating that there could exist some long lasting mechanism of inhibition of the enzyme. In addition, Cr (VI) differential effects on catalase and GPx activities have also been reported in other systems (Asatiani et al., 2004; Pedraza-Chaverri et al., 2005; Travacio et al., 2000). The decreased in catalase and GPx activity could add another component to explain the early elevated ROS levels, since they are both crucial enzymes involved in the detoxification of ROS.

Apoptosis is a genetically regulated cellular suicide mechanism in which multiple signaling pathways are implicated (Kiechle and Zhang, 2002). Among them, oxidative stress is an important event which may affect different macromolecules and components of the cells, triggering the activation of several antioxidant response genes and mechanisms (Chandra, 2000; Kiechle and Zhang, 2002). Q2
We have previously shown that the antioxidant NAC could prevent Cr (VI) toxicity on anterior pituitary cells in culture. Here, we demonstrated that ROS elevation produced by the metal was also prevented by NAC. Concomitantly with the early increase of ROS, p53 mRNA levels rose at 1 and 3 h of Cr (VI) exposure. The p53 tumor suppressor gene is critically involved in cellular mechanisms such as programmed cell death, cell cycle arrest and senescence. Cellular stressors such as ROS can lead to the activation of the p53 protein which then, acting both in a transcriptional dependent or independent manner, can induce different responses (Haupt et al., Q3
2003; Moll et al., 2005; Vousden, 2006). There have been described two major pathways of Cr (VI)-induced apoptosis: p53-dependent or -independent pathways (Pulido and Parrish, 2003). In different cell types, Cr (VI)-activated p53 and ROS seem to be implicated in this fact (Harris and Shi, 2003; O'Brien et al., 2003; Wang et al., 2000; Ye et al., 1999). In the present study, we could not demonstrate a role of ROS in p53 mRNA elevation since the antioxidant used decreased p53 mRNA levels *per se*. Interestingly, by using a p53 transcriptional inhibitor, it was shown that the transcriptional activity of p53 might not be implicated in the process of Cr (VI)-induced apoptosis. Cells exposed to Cr (VI) plus the inhibitor exhibited an increased cytotoxicity. Therefore, it could be suggested that in our system p53 may be involved in a survival mechanism. p53 mRNA levels rose during the first hours of Cr (VI) exposure and then decreased to control levels. This result sustains a novel mechanism of Cr (VI) toxicity in which p53 may primary act trying to rescue cells from apoptosis, but as cell fate may be finally a result of a balance between pro- and anti-apoptotic molecules, cell death is inevitable. In agreement with this hypothesis, Bax mRNA levels increased at 1 and 3 h of Cr (VI) exposure. Interestingly, an antioxidant could prevent this effect suggesting that ROS are involved in its stimulation. Though p53 transactivation activity has been implicated in the mechanism of Cr (VI)-induced apoptosis in different cell types, in our system it seems that Bax activation could not be a result of p53 transactivation ability. p53 protein belongs to a family of transcription factors involved in stress responses and development. Among them, it has been shown that p73 induces apoptosis and that Bax could be a downstream target that participates in the apoptotic process (Levrero et al., 2000; Ramadan et al., 2005). Besides, it is known that different stimulus like DNA damage or ROS, acting via diverse molecules other than p53, might trigger

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Bax activation (Levrero et al., 2000; Melino et al., 2004; Ramadan et al., 2005). Though we cannot rule out the mechanism of Bax mRNA levels increase in our system, it is clear that, either directly or indirectly, ROS participate in this activation. Caspases constitute another essential component of the apoptotic process (Fan et al., 2005). The release of cytochrome *c* from the mitochondria to the cytosol allows the formation of the apoptosome which, in turn, activates effector caspases. This important pathway of caspase activation is regulated by the pro- and anti-apoptotic Bcl-2 family proteins, which either induce or prevent the permeabilization of the outer mitochondrial membrane (Fan et al., 2005). While Bax mRNA levels rose, anti-apoptotic Bcl-2 mRNA levels did not change in response to Cr (VI) exposure, reflecting a fine regulation of pro-apoptotic signals in the pathway of Cr (VI)-induced cell death. Additionally, caspase 3 activation was observed after 6 h of Cr (VI) exposure and was also prevented by NAC, thus supporting a mechanism in which ROS elevation activates a sequence of downstream events leading to cell death. In fact, we have previously reported that 3 h of Cr (VI) exposure were enough to commit anterior pituitary cells to death (Quinteros et al., 2007). Therefore, increased ROS and Bax mRNA levels, taking part early in time, might be the key components of the apoptotic signal of Cr (VI).

In conclusion, the present study shows that oxidative stress induced by Cr (VI) causes apoptosis in anterior pituitary cells in culture. As a protective mechanism against ROS, Cr (VI) stimulates GSH synthesis. However, this increase is not enough to rescue cells from apoptosis. Moreover, the reduction of the activity of catalase and GPx, two major antioxidant enzymes, may be fundamental in oxidative stress generation after Cr (VI) exposure. In relation to the mechanism of Cr (VI)-induced apoptosis, we demonstrated a novel mechanism by which p53 primary act as a survival molecule and Bax activation occurs independently of p53. The early increase of ROS levels seemed to be crucial in the progress of apoptosis since treatment with an antioxidant reversed not only the elevation of Bax mRNA levels and caspase 3 activation but also the cytotoxicity produced by Cr (VI). The results presented here give insight into the mechanism of Cr (VI)-induced apoptotic cell death in anterior pituitary cells. This response is similar but not identical to that observed in other cells, remarking the role of GSH synthesis and p53 activity in cell survival mechanisms. Considering the important role of anterior pituitary hormones in reproduction and normal physiology of the organisms these results are of great significance taking into account the metal accumulation in this gland and its effects on hormone release.

Acknowledgments

This work was supported by research Grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Universidad de Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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