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Response of antioxidant defense system to chromium (VI)-induced cytotoxicity in human diploid cells

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Abstract The aim of this study is to establish antioxidant indicators of chromium toxicity in fetal human lung fibroblasts (HLF). The results obtained corroborate and develop our earlier observation of low-dose and long-term action of Cr(VI) on human cells in culture. In the case of a nontoxic chromium dose, temporary oxidative stress is overcome by increased activity of the antioxidant system with correlation to cell cycle re-entry. The toxic concentrations misbalance the cell antioxidant defense systems and cause irreversible growth arrest and massive cell death by apoptosis. Sub-toxicity is defined as toxicity stretched in time. The activity of GPx (glutathione peroxidase) is proposed as a biomarker of oxidative stress caused by Cr(VI), and the GR (glutathione reductase) inhibition is considered as a marker of the toxicity developed under the complex Cr(VI) action. In HLF cells the glutathione dependent defense system is the first system destroyed in response to toxic chromium action. Only the balance between SOD (superoxide dismutase) and H_2O_2 degrading enzymes (catalase and GPx), should play an important role in the fate of a cell, not individual enzymes.

Keywords Chromium (VI) · Antioxidant enzymes · Cell cycle · ROS · Apoptosis

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Introduction

The exposure of cells to environmental oxidants such as UV and ionizing radiation, heavy metals, redox active chemicals, hypoxia and hyperoxia increases reactive oxygen species (ROS) production which shifts cell redox status to a more oxidized state known as an oxidative stress. ROS are capable of causing direct damage effect or acting as critical intermediate signaling molecules leading to diverse biological consequences (Costa and Klein 2006; Dewhirst et al. 2008; Leonard et al. 2004). Toxic stress resistance or adaptation of cells to shift in cell redox status could be characterized by the response of the intracellular antioxidant defense system (Mates et al. 2008). The modulation of antioxidant enzymes

in cultured cells in response to the action of Cr(VI)-mediated toxic effect could be used as a model to establish crucial biomarkers of cell adaptation or resistance to toxicants.

Toxicity of Cr(VI) has been demonstrated for various human cells, such as gastric mucosa cells, peripheral blood lymphocytes (Trzeciak et al. 2000), human lymphoblast cell line (Jurcat cells) (Shi et al. 1999), and human peripheral blood mononuclear cells (Bagchi et al. 2001). The major cellular targets of Cr(VI) toxicity are lung epithelial cells and lung fibroblasts, however human Cr(VI) intoxication is also associated with hepatotoxicity, nephrotoxicity, cardiotoxicity and immunotoxicity (Pourahmad and O'Brien 2001). Many in vitro studies indicate that when Cr(VI) has contact with biological fluids and tissues, reduction to Cr(III) occurs rapidly due to the presence of reducing agents that keep the body in homeostasis. But in spite of the high reduction capacity of blood plasma, the toxic effect of Cr(VI) still takes place, i.e. the elevated concentration of chromium is observed in peripheral lymphocytes of chrome-plating workers, which correlates with increased DNA strand breaks (Gambelunghe et al. 2003).

During the Cr(VI) reduction within a cell, a wide spectra of ROS, such as superoxide, hydrogen peroxide and hydroxyl radicals, are produced (Ye et al. 1999). The cellular toxicity of Cr(VI) is initiated by oxidative stress, resulting in the excess formation of ROS.

Cr(VI) inside a cell acts as a multipotent agent, and any biomacromolecules can be its targets. Cr(VI) can produce DNA damage from either an oxidative pathway or a metal-binding pathway that results in a wide variety of DNA lesions. Cr(VI) can inhibit DNA replication and repair (Holmes et al. 2008), alter gene expression (Dubrovskaya and Wetterhahn 1998), activate stress-response pathways (Chuang et al. 2000; Kim and Yurkow 1996; Leonard et al. 2004; Ye et al. 1999), trigger transient or terminal growth arrest and apoptosis (Carlisle et al. 2000; Pritchard et al. 2001a; Rana 2008; Ye et al. 1999). Cells treated with Cr(VI) exhibit apoptotic features, depending on cell line, dose and exposure time: 300 μM Cr(VI) after 3 h of exposure causes apoptosis in 24 h in human lung tumor A549 cells (Ye et al. 1999), a 24 h exposure to 12.5 μM Cr(VI) is apoptogenic for chronic myelogenous leukemic K562 cells, and for cultured J774A.1 murine macrophage cells 0.6 μM Cr(VI) is toxic after 48 h (Bagchi et al. 2001).

In cells, the toxic capacity of Cr(VI) can be decreased by antioxidant defense systems, poised against the oxidative assault, which can neutralize the ROS generated by Cr(VI) action.

The cellular protective mechanisms against ROS consist of multiple enzymatic [catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx)] and non-enzymatic [α-tocopherol, ascorbic acid (AA), beta-carotene, cysteine and glutathione (GSH)] antioxidants.

Superoxide generated by the mitochondria and other sources is converted to H_2O_2 and O_2 by SOD. Abundant catalase enzyme and peroxidases then convert H_2O_2 to H_2O and O_2. The antioxidant enzymes can be divided into two types: one that reacts with ROS and diminishes their level (SOD, catalase and peroxidases) and one [glutathione reductase (GR)] that restores reduced forms of non-enzymatic antioxidants (GSH). The disorder of cell redox status causes metabolic and cell proliferation dysfunction and/or cell death.

We have reported (Asatiani et al. 2004) that Cr(VI)-treated human epithelial-like L-41 cells died by apoptosis. The dose-dependent involvement of defense mechanisms in response to low level and long-term Cr(VI) treatment was analyzed in dynamic. The nontoxic chromium dose (2 μM) caused transient cell cycle and growth arrest that correlated with the increased activity of glutathione peroxidase—glutathione reductase antioxidant system. The toxic apoptogenic concentration (20 μM) destroyed the cell antioxidant defense systems, and caused irreversibly growth arrest and massive cell death via apoptosis. The particular marker of the toxic Cr(VI) action was the depletion of glutathione-dependent antioxidant defense system. Inhibition of GR was an important aspect of the Cr(VI) toxicity in the L-41 cells.

In the present study we continue to consider the cell antioxidant responses to Cr(VI) using fetal human lung fibroblasts (HLF) in culture. We performed a time course study of oxidative stress, cell cycle distortion and the behavior of the antioxidant enzymes in Cr(VI)-induced toxicity to determine if there is an association between oxidative stress, cell cycle distortion, and antioxidant enzymes, and if antioxidant enzymes Cu,Zn-SOD, Mn-SOD, GR, GPx and catalase respond in a coordinated way.

Materials and methods

Cell culture

The HLF cells (fetal human lung fibroblasts) were maintained as adherent cells in Dulbecco's modified Eagle's culture supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37°C in a 5% CO_2 incubator. Cells were harvested with trypsin (0.25%)/EDTA solution. In all experiments HLF cells were used in the range of 25–30 cell passages in culture.

Chromium treatment and viability assay

HLF cells were seeded at 3×10^4 cells per well in 200 μl culture medium in 96-well microtiter plates and cultured to 80% of confluence. Cr(VI) at 2, 5, 10, 15, 20, 25 and 30 μM was added as potassium chromate at 48 h of growth and the cells continued to grow for 24 and 48 h. Viability of chromium-exposed HLF cells was assessed by the ability of viable cells to convert the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) to a water-insoluble formazan dye, which is based on the activation of succinate dehydrogenase (Carmichael et al. 1987). The culture medium was removed and the colored precipitate was solubilized with DMSO (dimethyl sulfoxide). After 30 min, cell survival and background was determined by absorbance at 570 and 660 nm, respectively.

For detecting cell viability after 2 and 24 h of transient chromium action, the exposed cells were rinsed with phosphate-buffered saline and replaced in fresh medium without Cr(VI) up to 48 h prior to determining cell survival by MTT assay.

In these assays, four wells were usually examined for each concentration and time point.

Measurement of DNA content for cell cycle analysis

DNA content of propidium iodide (PI) stained cells was measured by flow cytometry using FACScan (Becton–Dickinson, USA) and the separation into phases of the cell cycle was based on the PI fluorescence according to the accepted method (Ormerod 2002). The DNA histogram showed the cell cycle distribution of the viable cells. The apoptotic cells should be observed as a distinct sub-G_1 peak of the hypodiploid DNA. All results are expressed as the mean percent cells from each cell cycle compartment of three experiments.

Measurement of ROS

The detection of ROS in living cells was carried out by the flow cytometry method using 2′-7′-dichlorofluorescein diacetate (DCFH-DA, Sigma). Cell-permeable DCFH-DA is oxidized in live cells to its fluorescent derivative 2′-7′-dichlorofluorescein (DCF) in the presence of ROS (predominantly hydrogen peroxide and partially $^{\bullet}OH$ and $^{\bullet}NO$ radicals). Accumulation of DCF was measured by an increase of fluorescence at 530 nm and the mean fluorescent intensity (MFI) was used for the estimation of intracellular ROS level. DCFH-DA was added (10 μM final concentration) to HLF cells (about 1×10^6 cells) and the mixture was incubated for 30 min at 37°C. Hydrogen peroxide (10 mM) was used as a positive control. After the incubation cells were subjected to flow cytometry analysis (FACScan, Becton–Dickinson). The elevated ROS level is proportional to an increase of the basal level of the probe (Curtin et al. 2002; Esposti 2002).

A flow cytometry method was used for quantitative measurement of superoxide anions with the fluorescent probe dihydroethidium (DHE). DHE is taken up by cells and in the presence of superoxide anion converted to ethidine, which intercalates into nuclear DNA. The degree of fluorescence is proportional to the superoxide anion amount (Carter et al. 1994; Pritchard et al. 2001b). 10 μM DHE was added per 0.5×10^6 cells in 1 ml PBS for 15 min at 37°C and analyzed by FACScan instrument (Becton–Dickinson, USA). Excitation of DHE was at 490 nm, emission was measured at 610 nm.

Glutathione-dependent antioxidant system

Glutathione reductase (GR) activity was measured by using the BIOXYTECH GR-340™ Assay (Oxis, USA), and glutathione peroxidase (GPx) activity was determined by using BIOXYTECH GPx-340 colorimetric assay for cellular GPx (Oxis, USA) according to the manufacturer instructions.

Cellular superoxide dismutase

The technique of SOD assay involves photoreduction of nitro blue tetrazolium (NBT) for the determination of activity of superoxide dismutase following native polyacrylamide gel electrophoresis. The protein corresponding to SOD can be then visualized as achromatic zones through the inhibition of NBT (Sigma) reduction via SOD (Steinman 1985). Achromatic bands were visualized for 50 μg protein equivalent. The positions of the two isozymes of SOD, Cu, Zn-SOD and Mn-SOD were identified by incubation of the cell lysate at 37° C with 2% (w/v) sodium dodecyl sulfate (SDS) for selective inactivation of Mn-SOD (Geller and Winge 1982).

Catalase activity

Catalase activity in the cell crude extract was determined by measuring the rate of H_2O_2 (10 mM) decomposition in 50 mM potassium phosphate buffer (pH 7.0), in the presence of the cell crude extract at 240 nm and 25°C, $\varepsilon_{H_2O_2} = 43.6\ M^{-1}\ cm^{-1}$ (Beers and Sizer 1952).

Preparation of crude cell extract

The catalase and SOD activities were investigated in cell crude extracts. Cells ($\sim 10^7$) were harvested by centrifugation (3,000 rpm, 5 min, 4°C), rinsed twice in 50 mM phosphate buffer, pH 7.8. The rinsed cells were resuspended in a definite volume of above-mentioned buffer 1:4 (w/v), sonicated five times for 10 s bursts (44 kHz), centrifuged (14,000 rpm, 20 min, 4°C), and the soluble extract was used as a sample. The cell crude extracts were standardized per microgram of total protein. Protein concentrations in the cell extract were determined using BCA (bicinchoninic acid) protein assay reagent (Pierce, USA).

Statistical analysis

Experiments were carried out at least in triplicate unless otherwise stated. All values were expressed as the mean ± SD and analyzed using one-way ANOVA with Scheffe's test. A *P*-value less than 0.05 were considered statistically significant.

Results and discussion

Inhibition of cell growth by Cr(VI)

Cell viability following chromium treatment depends on cell line, dose and exposure time, the degree of cell confluency and the number of cell passages in culture. The percentage of apoptotic cells in cultured human normal fibroblasts affected by the same chromium concentration was enhanced in the early passage fibroblasts (Pritchard et al. 2001a). The late passage fibroblasts are characterized by gradually increasing cell death, DNA fragmentation, mitochondrial dysfunction and appearance of apoptotic markers (caspase-3, cytochrome *c*) (Mammone et al. 2006). We have limited the passage number range of the studied cells to 25–30 cell passages.

The dose-dependent response of sub-confluent HLF cells to chromium action has been observed using MTT cytotoxicity assay. Cells were exposed to Cr(VI) in the concentration range from 2 to 30 μM for 24 and 48 h without medium replenishment. The character of Cr(VI) toxicity can be described by the exponential decay (first rate) (Fig. 1), and is dose- and time-dependent. 2 μM of Cr(VI) does not induce cytotoxicity during 24 h and decreases cell viability by 10% after 48 h. The cell culture exposed to 5 μM for 24 h and 48 h loses subsequently 10 and 15% of

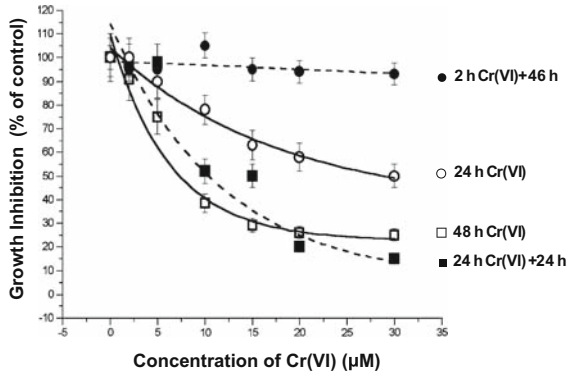

Fig. 1 Time- and dose-dependent Cr(VI)-induced cytotoxicity by MTT assessment. HLF cells were grown up to 80% of confluence prior to chromium treatment at the concentration range of 2–30 μM for 24 and 48 h (*solid lines*). To estimate the range and time of Cr(VI) reversible action HLF cells were treated by Cr(VI) for 2 and 24 h (*dash lines*), then the cultures were rinsed with PBS and replaced in fresh medium up to 48 h prior to determining cell survival. Controls consisted of untreated cells and were assigned as 100%

viable cell population. Incubation of the cells with 10 and 15 μM Cr(VI) decreases cell population by 20–35% after 24 h of chromium action and by 60–70% after 48 h. Cr(VI) above 20 μM causes significant decrease of the cell viability by 50 and 80% after 24 and 48 h, respectively.

To estimate the concentration range and time of reversible Cr(VI) action the treatment of HLF cells with Cr(VI) 2–30 μM Cr(VI) for 2 and 24 h was followed by cell growth in complete medium without chromium up to 48 h. The cell viability was not decreased at the early stage (2 h) of Cr(VI) action even for 30 μM Cr(VI). The effect of 5 μM Cr(VI) at 24 h was entirely reversed in 24 h after replacing the cultured cells in complete medium without chromium. Under these experimental conditions 10 and 15 μM Cr(VI) decreased in toxic effect by 15 and 20%. The cell exposure with toxic concentrations (above 20 μM) resulted in progressive cell death, indicating irreversible chromium action in 24 h.

The results show that chromium action on HLF cell culture could be separated into groups: nontoxic—2 and 5 μM Cr(VI), sub-toxic—10 and 15 μM Cr(VI) and toxic—above 20 μM Cr(VI). While entering a cell low concentration of Cr(VI) has no threshold (Liu et al. 2001). The cell culture system models all possibilities of Cr(VI) reduction, which exist under the physiological conditions, including the presence of extracellular reductants in the serum complemented media. The typical natural concentration of chromium reductant such as ascorbic acid (AA) in bovine serum is quite high—about 50–70 μM (Kleczkowski et al. 2005) and comparable with ascorbic acid in blood plasma (Carty et al. 2000). Generally, serum does not exceed 15% of a complete culture media, and the concentration of ascorbic acid comprises about 10 μM. On the other hand, serum could not be omitted from the media as it is a necessary component to support cell growth, and promote attachment of cells to the substrate. The question arises whether the concentration of AA in culture media supplemented with serum could contribute to the extracellular reduction of the complemented Cr(VI) and the way it could affect intracellular ROS level and activities of antioxidant defense system. As AA is highly permeable, one can suppose that the extracellular quantity of AA decreases during the experimental conditions and this results in a negligible participation of AA in the extracellular Cr(VI) reduction. But if we follow the consideration concerning the influence of AA/Cr(VI) ratio on the AA pro-oxidant versus antioxidant properties (Poljsak et al. 2005), we can suppose that in our case (low AA to Cr(VI) concentrations) the partial extracellular Cr(VI) reduction to Cr(V) with the appearance of ROS can not be excluded. However, it is difficult to separate inputs of intracellular Cr(VI), Cr(V) from penetrated Cr(V) reduction, and, likewise differentiating intracellularly generated or penetrated ROS because Cr(V) as well as Cr(VI), H_2O_2 and $O_2^{\cdot -}$ are highly permeable, and once in a cell they can affect the antioxidant defense system and ROS level.

Cr(VI) affects the cell cycle distribution and induces apoptosis

The cell cycle analysis for a single time-point gives basically static information detecting only disorders in the cell cycle phase distribution at stress conditions. The applied dynamic flow cytometry analysis provides the ability to follow the changes of the cell cycle phase distribution along with chromium exposure. The studied cell line was affected differently by the nontoxic (5 μM), sub-toxic (15 μM) and toxic (30 μM) doses of chromium, respectively, over 24 h (Fig. 2).

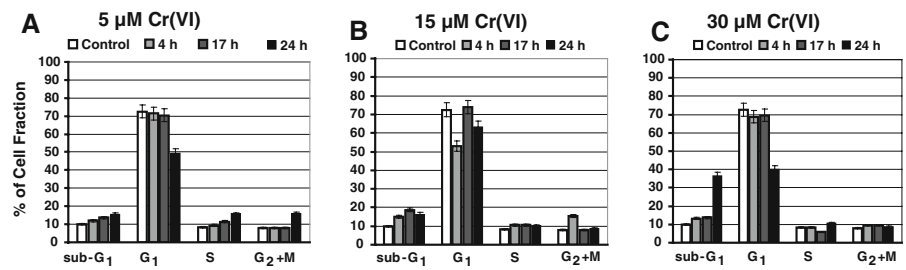

Fig. 2 Time- and dose-dependent effect of Cr(VI) exposure on cell cycle progression

Between 48 and 72 h of growth the control HLF cell population predominately consists of G_1 phase cells ($\sim$70%). The nontoxic 5 μM Cr(VI) had no influence on DNA histogram up to 17 h and caused the cell cycle re-entry at 24 h of Cr(VI) action, lowering the cell fraction in G_1 phase to 50% and increasing the cell fraction in S and $G_2 + M$ phase (Fig. 2a). Microscope analysis of cells grown on coverslips demonstrated that the mitotic index did not decrease by comparison with the control and was $\sim$12–15% M_i (data not shown).

The sub-toxic concentrations of chromium (15 μM) caused the cell cycle re-entry earlier (at 4 h) compared to the nontoxic concentration (Fig. 2b). The increase of the $G_2 + M$ cell fraction with the simultaneous decrease of the G_1 cell fraction at 4 h was followed by growth arrest predominantly in G_1 phase at 17 h, not affecting S and $G_2 + M$ phases. The growth arrest was also confirmed by the absence of the mitotic cells in the cell population as was demonstrated by microscope analysis (data not shown). The apoptotic sub-G_1 fraction increased insignificantly (not more 20%). The growth inhibition observed by the MTT assay at 24 h of chromium treatment (Fig. 1) may partly contribute to the detected growth arrest.

The toxic 30 μM Cr(VI) did not affect cell cycle distribution during 17 h of permanent chromium treatment and sharply increased the apoptotic sub-G_1 fraction (about 35%) and lowered the cell number in G_1 phase to 40% only at 24 h. The resultant decrease of G_1 cell fraction is the consequence of the massive cell apoptosis developed at 24 h of chromium exposure (Fig. 2c).

Cell population was analyzed by fluorescent microscopy after staining with DNA-specific fluorescent dyes [acridine orange (AO) and ethidium bromide (EB)] for the detection of apoptotic cells (Diaz et al. 1999). Apoptotic nuclei are characterized by highly condensed chromatin. The data of the morphological analysis of Cr(VI) on HLF cells in the range from 5 to 30 μM Cr(VI) action at 24 h are presented in Fig. 3.

The results show, that a narrow range of chromium concentrations initiated diverse effects in HLF cell culture such as, induction of the cell cycle re-entry (nontoxic concentration of 5 μM), growth arrest (sub-toxic concentration of 15 μM) and apoptosis (toxic concentration of 30 μM).

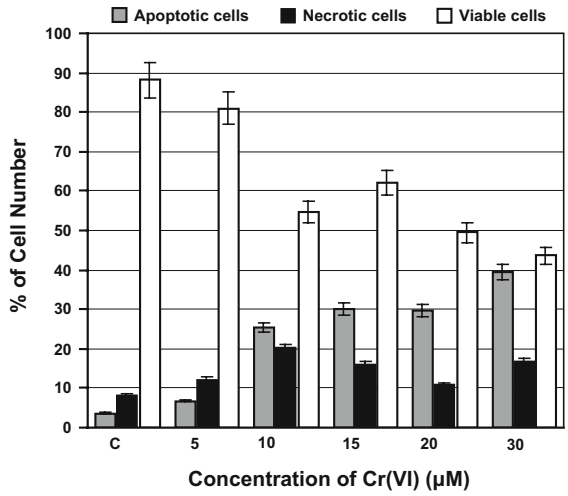

Fig. 3 Effect of Cr(VI) on cell survival after treatment of HLF cells with various chromium concentrations for 24 h determined by morphological analysis

ROS in HLF cells during Cr(VI) action

The intracellular redox environment may influence cell cycle progression (Conour et al. 2004; Noda et al. 2001). It was hypothesized that ROS could contribute to cell cycle progression, and a late-G_1 phase checkpoint was proposed after transition across the growth-factor-dependent G_1 restriction point that was coordinating cellular ROS production with cell population transition from G_1 to S phase (Havens et al. 2006). There is evidence that fluctuation in the cellular redox state contributes in the cell cycle regulatory pathways (Mennon and Goswami 2007; Sarsour et al. 2008).

The nontoxic, sub-toxic and toxic doses of Cr(VI) initially increased ROS production to a comparable level (above twofold) at 2 h of chromium action (Fig. 4a). The nontoxic concentration caused the reestablishment of ROS level to the control value at 24 h. The ROS level had decreased at 24 h under sub-toxic and toxic concentrations. The decrease of ROS level under these concentrations correlated with the greater loss of cell viability and apoptosis. The decrease of the ROS level at the late stages of apoptosis was also observed in the case of Fas-induced apoptosis (Aronis et al. 2003; Shen and Pervaiz 2006).

Superoxide anion production detected by DHE (Fig. 4b) was enhanced at 2 h of chromium action. The extent of the increase was more pronounced for

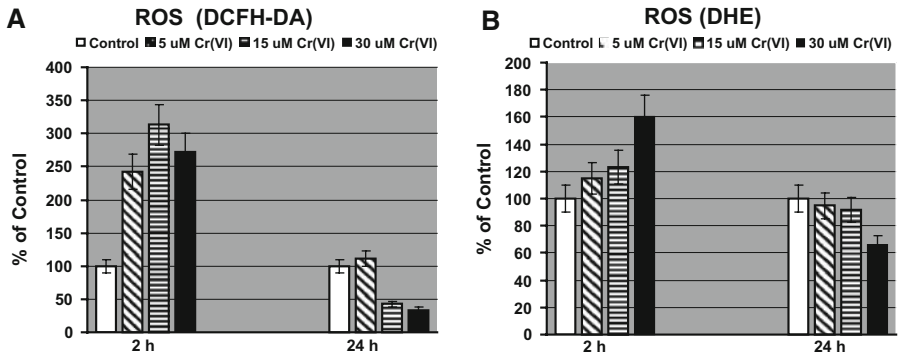

Fig. 4 ROS level in control untreated and Cr(VI)-treated HLF cells

the toxic concentration. The nontoxic and sub-toxic concentrations restored superoxide anion level to control value at 24 h. Only toxic concentration lowered superoxide anion production.

Cr(VI) induces the oxidative stress initially during the first hours of cell treatment. Later intracellular ROS scavengers are able to overcome it. The nature of the functioning of antioxidants should determine the cell susceptibility to oxidative stress and chromium toxicity.

Glutathione-dependent antioxidant system in HLF cells during Cr(VI) action

The glutathione peroxidase—glutathione reductase system is generally accepted as crucial in modulating ROS level. Changes in the glutathione-dependent system may work as a primary defense against cellular redox imbalance (Haddad et al. 2000; Harlan et al. 1984). Figure 5a, b represents the activity of glutathione-dependent antioxidant enzymes under 5, 15 and 30 μM of Cr(VI) action.

The nontoxic 5 μM Cr(VI) caused gradual increase of GPx activity to the maximal level at 24 h. Further GPx activity decreased to the control value at 48 h (Fig. 5a). For the considered time-points (2–48 h) the GR activity (Fig. 5b) decreased, then increased and even surpassed the corresponding control value at 24 and 48 h. The simultaneous increase of both GPx and GR activities (5 μM, 24 h) could be an indication of the intensification of oxidative stress and successful functioning of the glutathione cycle enzymes, which resulted in the normalization of ROS level. It seems that the intracellular antioxidant defense system overcomes the initial oxidative stress connected with the action of the nontoxic (5 μM) chromium concentration as it recovers over 48 h.

The cell treatment with the sub-toxic chromium concentration [15 μM Cr(VI)] revealed the coordinated character of GPx and GR changes at 2 and 4 h. These alterations pointed to the coordinated functioning of the glutathione defense antioxidant cycle during the first hours of chromium treatment. As the action of 15 μM Cr(VI) proceeds to 24 h GPx activity continues to increase, but the GR activity falls sharply. The absence of GR activity could reflect the irreversible disruption of the glutathione cycle that was paralleled with the decreased cell viability and growth arrest at the whole cell population level. Therefore the toxic effect of 15 μM Cr(VI) was time-dependent and developed at 24 h. This time-dependent enhanced toxicity of 15 μM Cr(VI) could be associated with the involvement of GR in the intracellular chromium reduction that affected the glutathione cycle and irreversibly inhibited it.

The transient increase of oxidative stress with simultaneous appropriate function of glutathione-cycle enzymes in the cases of nontoxic concentration (24 h) and the early effect of sub-toxic concentration (4 h) correspond to the cell cycle re-entry (Fig. 2).

Regarding 24 h as the critical point for the sub-toxic 15 μM Cr(VI) concentration, we additionally examined the glutathione-dependent enzyme system at 10 μM Cr(VI). This concentration is also defined as sub-toxic for the studied system, as it causes irreversible inhibition of cell viability (Fig. 1) during prolonged exposure. At this concentration the GPx activity is also increased as compared to the control (Insert to Fig. 5a), but GR activity is characterized by its high level (Insert to Fig. 5b). Later, at 36 h, GR is

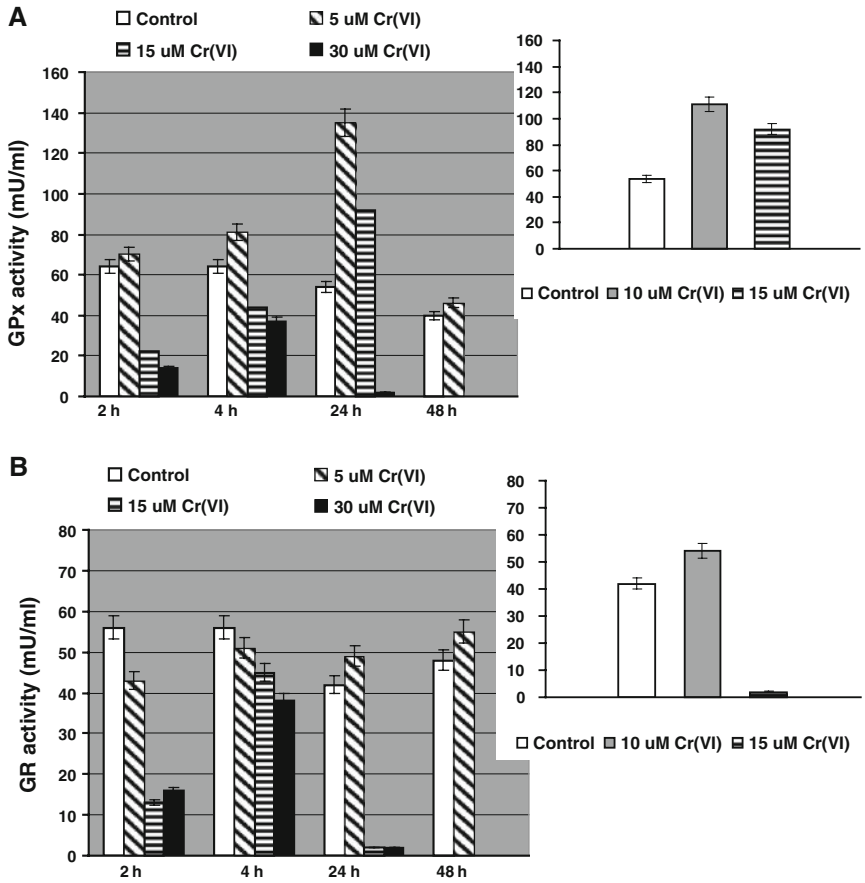

Fig. 5 Glutathione-dependent antioxidant system in control untreated and Cr(VI)-treated HLF cells. Inserts are GPx and GR activity at 24 h of sub-toxic 10 and 15 μM Cr(VI) action

inhibited and as a result collapse of glutathione cycle occurs (data not shown).

The changes of GPx and GR activities under the toxic 30 μM Cr(VI) are similar to the sub-toxic concentration effect at 2 and 4 h. The prolonged action of 30 μM Cr(VI) to 24 h inhibits entirely both GPx and GR activities, thus the disruption of the glutathione-dependent antioxidant defense system takes place.

Consideration of GR as the marker of Cr(VI) toxicity in the studied system expands the number of the cell types, for which the accompaniment of Cr(VI) toxicity with GR inhibition has been reported. The chromate-caused inhibition of GR has been observed in erythrocytes (Koutras et al. 1965), fibroblasts (Sugiyama et al. 1991), osteoblasts (Ning and Grant 2000) and hepatocytes (Gunarantnam and Grant 2004). Recently the complete inhibition of GR activity by 10 μM Cr(VI) action has been shown in the J744.1 murine macrophage cell line (Lalaouni et al. 2007). The mechanism of the toxic action of Cr(VI) on GR is proposed as arising from its participation in one- and/or two-electron transference and reduction of Cr(V) to Cr(IV) and/or Cr(III) (Bal and Kasparzak 2002). Generated toxic metabolites irreversibly inhibit GR activity. Thus GR under chromium action has a dual function as an antioxidant, restoring the GSH pool, and as a pro-oxidant, reducing intracellular chromium, and as a result participating in ROS generation.

The complicated character of the alteration in the glutathione-dependent defense enzyme activities in the studied system testifies to their involvement both in oxidative stress and in chromium toxicity. The nontoxic concentration initiates the general antioxidant reactions, revealed by the progressive coordinated increase of GPx and GR at the early stage of Cr(VI) action and reaching the control level at 48 h of Cr(VI) action. The toxic concentration also causes the coordinated response of GPx and GR followed by total inhibition at 24 h. The time-dependent behavior of GPx at sub-toxic concentration is very much the

same as the activity of GPx at nontoxic Cr(VI) concentration when the oxidative stress takes place. The time-dependent changes of GR activity at sub-toxic Cr(VI) concentration is similar to the GR behavior when the toxic conditions are developing under 30 μM of Cr(VI). The glutathione-dependent defense system at the sub-toxic concentration reveals general antioxidant activity at 2 and 4 h of Cr(VI) action and pro-oxidant activities at 24 h, expressed in the GPx/GR imbalance. Thus the GR depletion can be considered as one of the markers of toxicity and GPx as a marker of oxidative stress caused by Cr(VI).

ROS and antioxidant enzymes

Antioxidant enzymes predominate in the antioxidant defenses to protect against ROS. The antioxidant enzymes in the enzymatic antioxidant pathway influence each other and their ratio can define a cell physiology. Overexpression of Cu, Zn-SOD in human glioma cells changes endogenous GPx and catalase activities. Depending on the Cu, Zn-SOD/GPx value the cell clones could more or less suppress the malignant phenotype (Zhang et al. 2002). The complex relation between the two antioxidant enzymes Mn-SOD and catalase and cell death by apoptosis is summarized in (Kahl et al. 2004).

In the studied system the rise in the $O_2^{\cdot -}$ radical level (Fig. 4b) correlates with the increase of activity of both SODs under action of all examined Cr(VI) concentrations at the early stage (Fig. 6). After 24 h of Cr(VI) action the tendency of $O_2^{\cdot -}$ radical reduction also correlates with the changes in SOD activities. The reaction product of $O_2^{\cdot -}$ dismutation via SOD action is H_2O_2, therefore were expected simultaneous changes of H_2O_2 level under examined Cr(VI) concentrations. Indeed, the rise in $O_2^{\cdot -}$ radical level at 2 h contributes in the increase of H_2O_2 level (Fig. 4), although the changes of H_2O_2 quantity are pronounced strongly.

Catalase shares H_2O_2 as the substrate with GPx, although GPx alone can react with lipids and other organic peroxides. That is why GPx is a major source of protection against low level oxidative stress (low level of H_2O_2), whereas catalase becomes more significant in severe oxidative stress (Halliwell and Gutteridge 1999).

At the early stage (2 h) of Cr(VI) action (with a sharp increase in H_2O_2 level) nontoxic concentration

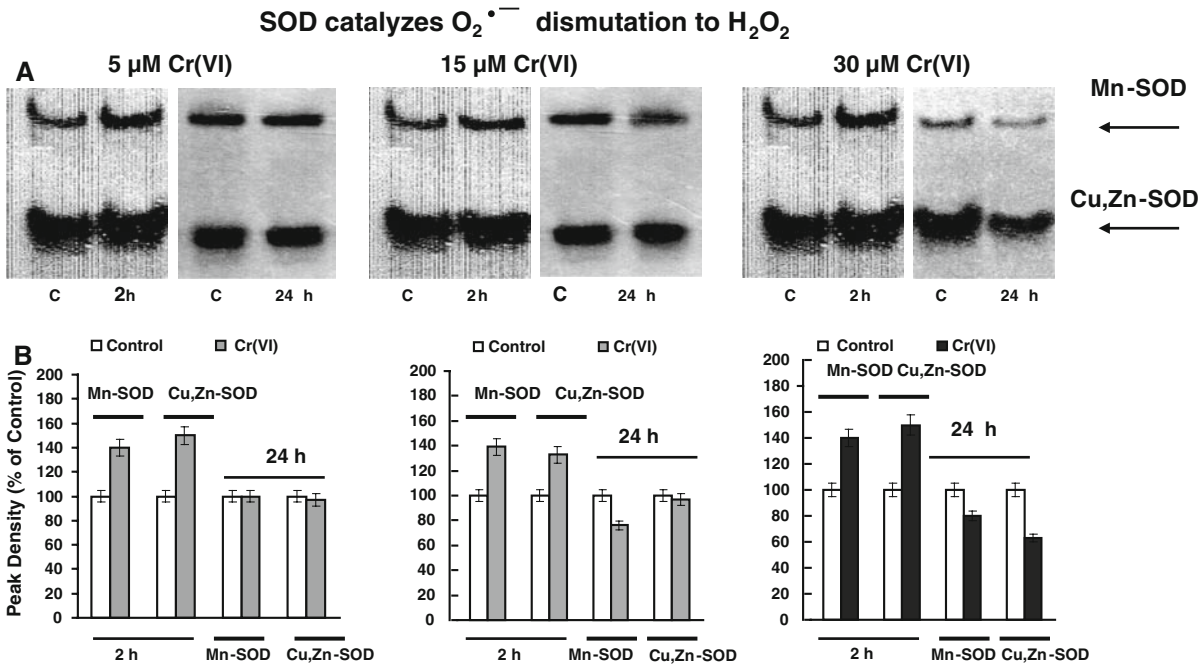

Fig. 6 SODs activity in control untreated (C) and Cr(VI)-treated HLF cells. The photochemical NBT method of SOD detection in 12.5% native gel. The achromatic zones of SOD positions in a gel are presented in the invert images (**a**). Quantification of the gel bands measuring the peak density (**b**)

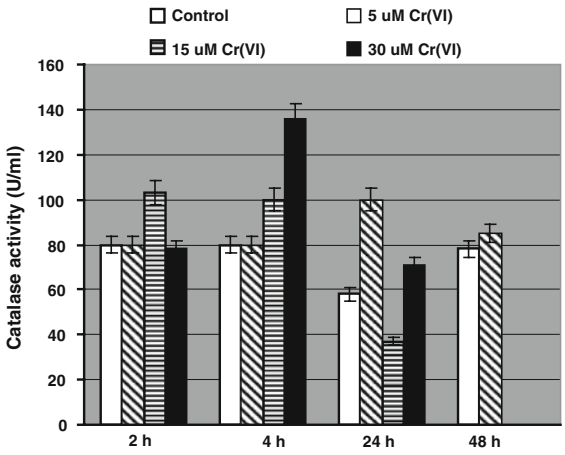

Fig. 7 Catalase activity in control untreated and Cr(VI)-treated HLF cells

did not cause an appreciable increase of catalase and GPx activities compared with corresponding controls, whereas under sub-toxic concentration increase of catalase activity corresponds to the sharp reduction of GPx activity, which becomes stronger at toxic concentration (30 μM Cr(VI), 4 h, Figs. 7, 5a). Under long-term action (24 h) of nontoxic 5 μM Cr(VI) the activities of catalase and GPx rise sharply, that is a probable cause of H_2O_2 level diminution up to the corresponding control level (Fig. 4a). The action of Cr(VI) toxic concentration at 24 h causes complete exhaustion of GPx, while catalase still functions. The subsequent examination of catalase and GPx activities under sub-toxic and toxic concentrations of Cr(VI) points to the interchangeable role of these enzymes.

The correlation between the activities of ROS–catalase–GPx–SOD, suggests that a balance between SOD and H_2O_2 degrading enzymes, but not individual enzymes, should play an important role in the fate of a cell.

Concluding remarks

Chromium affects all antioxidants in the enzymatic antioxidant pathways depending on the concentration range. The nontoxic Cr(VI) concentration (5 μM) activates glutathione-dependent antioxidant enzymes and SODs from the early beginning of its action in response to the increased ROS level, and when the oxidative stress is gradually overcome the activities are normalized to their control values. Sub-toxic concentrations (10 and 15 μM) separate the activity of glutathione-cycle enzymes in a time-dependent manner, revealing their antioxidant activities at the early stage of Cr(VI) action in response to the oxidative stress observed under these conditions. The imbalance of GPx/GR at the sub-toxic concentrations developed later points to the toxic pro-oxidant effect of Cr(VI) reduction, leading to Cr(III) formation and destruction of macromolecules. At the toxic concentration (30 μM) the glutathione-cycle enzymes are inhibited, as compared to the control enzyme activities, from the beginning of Cr(VI) action, then the system activates to an insignificant degree and after the prolonged Cr(VI) action becomes fully inhibited with the cells dying by means of apoptosis. By the time the SODs activity is decreased, the catalase activity is still high, however the remaining activity of the enzymes can not reverse the cell fate. Cell re-entry in the proliferative cycle at transient oxidative stress takes place only at the coordinated function of antioxidant enzymes.

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