Prooxidant Cytotoxicity of Chromate in Mammalian Cells: The Opposite Roles of DT-Diaphorase and Glutathione Reductase

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The geno- and cytotoxicity of chromate, an important environmental pollutant, is partly attributed to the flavoenzyme-catalyzed reduction with the concomitant formation of reactive oxygen species. The aim of this work was to characterize the role of NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2) and glutathione reductase (GR, EC 1.6.4.2) in the mammalian cell cytotoxicity of chromate, which was evidenced controversially so far. The chromate reductase activity of NQO1 was higher than that of GR, but lower than that of lipoamide dehydrogenase (EC 1.6.4.3), ferredoxin:$NADP^+$ reductase (EC 1.18.1.2), and NADPH: cytochrome P-450 reductase (EC 1.6.2.4). The reduction of chromate by NQO1 was accompanied by the formation of reactive oxygen species. The concentration of chromate for 50% survival of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) during a 24-h incubation was (22 ± 4) μM. The cytotoxicity was partly prevented by desferrioxamine, the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine and by an inhibitor of NQO1, dicumarol, and potentiated by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates GR. The NADPH-dependent chromate reduction by digitonin-permeabilized FLK cells was partly inhibited by dicumarol and not affected by BCNU. Taken together, these data indicate that the oxidative stress-type cytotoxicity of chromate in FLK cells may be partly attributed to its reduction by NQO1, but not by GR. The effect of BCNU on the chromate cytotoxicity may indicate that the general antioxidant action of reduced glutathione is more important than its prooxidant activities arising from the reactions with chromate.

Key words: Chromate, DT-Diaphorase, Glutathione Reductase

Introduction

Chromate (Cr^{6+}, CrO_4^{2-}) is an important toxic environmental pollutant due to the wide use of chromium compounds in industries such as tanning, corrosion control, plating, pigment manufacture, and nuclear energetics. Inside the cell, chromate is reduced to Cr^{3+} by ascorbate and reduced glutathione (GSH), with an intermediate formation of Cr^{5+} and Cr^{4+} (Stearns and Wetterhahn, 1994; Lay and Levina, 1998). The concomitant formation of reactive oxygen species, including the participation of Cr^{5+} and, possibly, Cr^{4+} in the Fenton reaction (Krepkiy *et al.,* 2003) and the formation of Cr^{5+}-peroxo intermediates (Pattison *et al.,* 2001), is the important factor in causing cellular damage by chromate (Stearns and Wetterhahn, 1994; Lay and Levina, 1998; Vasant *et al.,* 2003, and references therein). Chromate is also reduced by the flavoenzymes glutathione reductase (GR), lipoamide dehydrogenase (LipDH), ferredoxin: $NADP^+$ reductase (FNR) (Shi and Dalal, 1990), NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase) (De Flora *et al.,* 1985), NADPH:cytochrome P-450 reductase (P-450R) and other microsomal enzymes (Jannetto *et al.,* 2001, and references therein), nitric oxide synthase (NOS) (Porter *et al.,* 2005), the complexes of mitochondrial electron transport chain (Rossi and Wetterhahn, 1989), flavin reductase and NfsA nitroreductase of *Escherichia coli*, and several flavoenzymes of *E. coli* and pseudomonads named chromate re-

Abbreviations: FNR, ferredoxin:$NADP^+$ reductase; P-450R, NADPH:cytochrome P-450 reductase; NQO1, DT-diaphorase; GR, glutathione reductase; GSH, reduced glutathione; LipDH, lipoamide dehydrogenase; NOS, nitric oxide synthase; SOD, superoxide dismutase; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; cL_{50}, concentration of compound for 50% cell survival; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea.

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ductases (Puzon *et al.,* 2002; Ackerley *et al.,* 2004a, b). However, the mechanisms of chromate reduction and concomitant reactive oxygen species formation by flavoenzymes, and their impact on the chromate cytotoxicity are insufficiently understood so far. Because of the slow reaction rates, the kinetic and mechanistic data are almost absent, except the recent studies of NOS (Porter *et al.,* 2005), and bacterial chromate- and nitroreductases (Puzon *et al.,* 2002; Ackerley *et al.,* 2004a, b).

The roles of NQO1 and GR in mammalian cell cytotoxicity of chromate were studied because of their protection against the cytotoxicity of quinones and other redox cycling agents (O'Brien, 1991; Ollinger and Brunmark, 1991, and references therein). It has been reported that NQO1 may decrease chromate-induced DNA damage, presumably due to a two-electron reduction of Cr^{6+} into Cr^{4+} (De Flora *et al.,* 1985). However, the data of the mammalian cell cytotoxicity studies were controversial (Ning and Grant, 1999; Gunaratnam and Grant, 2001; Pourahmad *et al.,* 2005). It has been suggested that NQO1 may even lack chromate reductase activity (Aiyar *et al.,* 1992). The role of GR in chromate cytotoxicity is also presented controversially, because it is difficult to distinguish between the roles of direct and reduced glutathione-mediated chromate reduction by GR (Ning and Grant, 1999, 2000; Gunaratnam and Grant, 2001; Pourahmad *et al.,* 2005).

In this paper, we examined the chromate reduction by NQO1, GR, and other potentially important NAD(P)H-oxidizing flavoenzymes. We also demonstrated a contribution of NQO1 in chromate cytotoxicity in bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK). The direct reduction of chromate by GR apparently does not play an important role in its cytotoxicity, whereas the general antioxidant action of GSH seems to be more important than its prooxidant activities arising from the reactions with chromate.

Materials and Methods

The kinetic measurements were carried out spectrophotometrically using a Hitachi-557 spectrophotometer in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25 °C, unless specified otherwise. NADPH: cytochrome P-450 reductase (P-450R, EC 1.6.2.4) from pig liver was prepared as described by Yasukochi and Masters (1976), rat liver DT-diaphorase (NQO1, EC 1.6.99.2) was prepared as described by Prochaska (1988). Ferredoxin: $NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* was prepared as described by Pueyo and Gomez-Moreno (1991); it was a generous gift of Dr. M. Martinez-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). Human erythrocyte recombinant glutathione reductase (GR, EC 1.6.4.2) was a generous gift of Professor K. Becker (Gießen University, Germany). Pig heart lipoamide dehydrogenase (LipDH, EC 1.6.4.3, 200 U/mg) was obtained from Sigma. The enzyme concentrations were determined spectrophotometrically using ε_{460} = 22 mM^{-1} cm^{-1} (P-450R), ε_{459} = 9.4 mM^{-1} cm^{-1} (FNR), and ε_{460} = 11 mM^{-1} cm^{-1} (NQO1, GR, LipDH). The activity of P-450R using 50 μM cytochrome *c* as an electron acceptor (concentration of NADPH, 100 μM) was determined using $\Delta\varepsilon_{550}$ = 20 mM^{-1} cm^{-1}, and it was equal to 77 μmol mg^{-1} min^{-1}. The activity of FNR using 1 mM ferricyanide as electron acceptor (concentration of NADPH, 200 μM) was determined using $\Delta\varepsilon_{420}$ = 1.0 mM^{-1} cm^{-1}, and it was equal to 330 μmol mg^{-1} min^{-1}. The activity of NQO1 determined according to the rate of the menadione-mediated reduction of 50 μM cytochrome *c* in the presence of activators, 0.01% Tween 20 and 0.25 mg/ml bovine serum albumin (concentration of NADPH, 100 μM; concentration of menadione, 10 μM) was equal to 3300 μmol mg^{-1} min^{-1}. The activity of GR using 1.0 mM oxidized glutathione as an electron acceptor (concentration of NADPH, 100 μM), was determined using $\Delta\varepsilon_{340}$ = 6.2 mM^{-1} cm^{-1}, and it was equal to 240 μmol mg^{-1} min^{-1}. Other reagents were obtained from Sigma and used as received. The concentration of chromate was determined according to ε_{370} = 4.7 mM^{-1} cm^{-1} (Puzon *et al.,* 2002).The rates of flavoenzyme-catalyzed oxidation of 100 μM NAD(P)H by 100–500 μM chromate, corrected for the intrinsic NAD(P)H-oxidase activity of the enzymes, were determined according to $\Delta\varepsilon_{340}$ = 6.2 mM^{-1} cm^{-1}. Further, the reaction rates were corrected for the absorbance changes at 340 nm due to the chromate reduction (the details are given in Results and Discussion). The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of the reduction of chromate were calculated from the Lineweaver-Burk plots. k_{cat} is the number of NADPH molecules oxidized by the single active center of an enzyme per second.

The culture of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was grown and maintained in Eagle's medium supplemented with 10% fetal bovine serum at 37 °C as described previously (Nemeikaitė and Čėnas, 1993). In the cytotoxicity experiments, cells ($3.0 \cdot 10^4$/ml) were grown in the presence of various amounts of chromate for 24 h, and counted using a hematocytometer with the viability determined by the exclusion of Trypan blue. Before the count, the cells were trypsinized. For the studies of chromate reduction, the trypsinized cells were washed twice by 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA, suspended to the final concentration 10^6/ml, and permeabilized by 0.2 mg/ml digitonin. The reactions were performed at 37 °C in the presence of 200 μM chromate and NADPH regeneration system, 20 μM NADPH, 10 mM glucose-6-phosphate, and 10 U/ml glucose-6-phosphate dehydrogenase. The extent of chromate reduction was monitored at 370 nm.

Results and Discussion

In this study, we examined the reduction of chromate by the NAD(P)H-oxidizing flavoenzymes P-450R, NQO1, GR, and LipDH, which may be important in the mammalian cell cytotoxicity of chromate, and a model enzyme, FNR. Because it has been hypothesized that the initial two-electron reduction of chromate to Cr^{4+} may contribute to the chromate detoxification, whereas the initial single-electron reduction with the transient formation of Cr^{5+} may contribute to its cytotoxicity (Ackerley *et al.,* 2004a, b), the enzymes were selected according to their electron-transfer properties: P-450R and FNR catalyze the single-electron reduction of quinones (Iyanagi and Yamazaki, 1969), whereas NQO1 catalyzes the two-electron reduction (O'Brien, 1991; Anusevičius *et al.*, 2002), and LipDH and GR catalyze the mixed single- and two-electron reduction (Čėnas *et al.*, 1989; Vienožinskis *et al.*, 1990).

In the presence of NADPH regeneration system, FNR reduced excess chromate, as it is evident by a gradual decrease in absorbance at 370 nm (Fig. 1). The same although slower spectral changes were observed using NQO1 (data not shown). In the absence of flavoenzymes, the reduction rates were slower at least by one order of magnitude. The spectral changes (Fig. 1) were analogous to those observed in the nitric oxide synthase (NOS)-catalyzed chromate reduction (Porter *et al.*, 2005). They were not accompanied by the absorbance increase at 450–500 nm, which may indicate

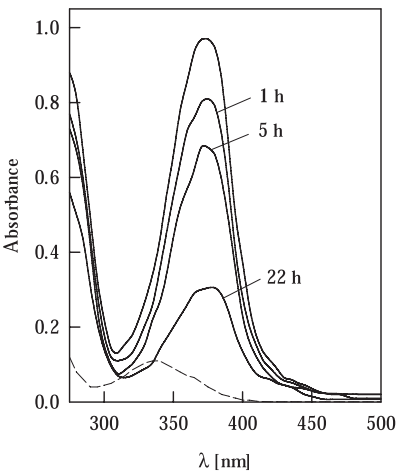

Fig. 1. The reduction of 200 μM chromate by 50 nM ferredoxin:$NADP^+$ reductase in the presence of NADPH regeneration system at 25 °C. The dashed line shows the absorbance of the reaction mixture in the absence of chromate.

Table I. The intrinsic NAD(P)H-oxidase activities and chromate reductase activities of NAD(P)H-oxidizing flavoenzymes at pH 7.0 and 25 °C. The catalytic (k_{cat}) and bimolecular rate (k_{cat}/K_m) constants of reduction of chromate were obtained after the correction of the reaction rates for the intrinsic NAD(P)H:oxididase activities, as described in the text. The reactions of NQO1 were studied in the absence of the activators.

Enzyme	NAD(P)H:	Chromate reductase	
	oxidase [s^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_m [M^{-1} s^{-1}]
NADPH:cytochrome P-450 reductase	0.11 ± 0.01	1.8 ± 0.1	$(3.8 \pm 0.2) \cdot 10^4$
Ferredoxin:$NADP^+$ reductase	0.15 ± 0.01	1.0 ± 0.1	$(3.0 \pm 0.2) \cdot 10^3$
Lipoamide dehydrogenase	0.12 ± 0.01	0.28 ± 0.04	$(9.0 \pm 1.0) \cdot 10^2$
DT-diaphorase	0.015 ± 0.002	0.16 ± 0.02	$(1.0 \pm 0.1) \cdot 10^2$
Glutathione reductase	0.025 ± 0.005	> 0.03	≥ 20

the formation of Cr^{4+} species ($\varepsilon_{460} \sim 1.6$ мМ$^{-1}$ cm^{-1}; Lay and Levina, 1998). Thus, because of the negligible absorbance of Cr^{3+} species at 350–600 nm (Lay and Levina, 1998; Puzon *et al.,* 2002), one may suppose that Cr^{3+} is the final product of the reactions. Probably, the initial formation of Cr^{5+} or Cr^{4+} did not significantly influence the net formation of Cr^{3+}, because both Cr^{5+} and Cr^{4+} species are disproportionating finally forming Cr^{6+} and Cr^{3+} (Lay and Levina, 1998).

The kinetic parameters of chromate reduction were determined as follows: (i) The chromate disappearance rate was calculated from the absorbance changes at 410 nm using $\Delta\varepsilon_{410} = 1.08$ мМ$^{-1}$ cm^{-1} (Fig. 1), which was consistent with the previously used $\Delta\varepsilon_{405} = 1.25$ мМ$^{-1}$ cm^{-1} (Aiyar *et al.,* 1992); and (ii) the obtained rate was used for the correction of the NAD(P)H oxidation rate determined at 340 nm, using the calculated value of $\Delta\varepsilon_{340}$ of chromate, 2.12 мМ$^{-1}$ cm^{-1} (Fig. 1). The obtained k_{cat} and k_{cat}/K_m of the reactions are given in Table I. One may note that chromate is an extremely poor substrate for all the enzymes investigated, *e.g.,* its k_{cat} of reduction by GR, LipDH, and NQO1 is close or below 0.1% of the disulfide or quinone substrate reduction rate (Čėnas *et al.,* 1989; Vienožinskis *et al.,* 1990; Anusevičius *et al.,* 2002). However, in terms of k_{cat}/K_m the reactivity of NQO1 is higher than that of GR. For all the enzymes investigated, the calculated ratios between the rates of NAD(P)H oxidation and chromate disappearance were almost the same, 2.0–2.1, being slightly above that expected for the stoichiometric formation of Cr^{3+} from chromate, 1.5. On the other hand, the ratios 1.8–2.2 mol NAD(P)H/mol chromate were reported for the reactions of bacterial chromate reductases (Ackerley *et al.,* 2004a).

Because the formation of the reactive oxygen species during the reduction of chromate by GR is well documented (Shi and Dalal, 1990), we attempted to assess whether these phenomena are characteristic for the reaction of NQO1. One should note that the pathways of the chromate-induced generation of superoxide and H_2O_2 by flavoenzymes are not completely understood, most probably, due to the low chromate reductase activity, which in turn is close to the intrinsic NAD(P)H-oxidase activity of flavoenzymes. A recent study of the single-electron transferring NOS (Porter *et al.,* 2005) revealed that: (i) Chromate increased the formation of $O_2^{-\cdot}$ by NOS, although the mechanism of this reaction remains unclear; (ii) NADPH-chromate reductase activity of NOS was stimulated by the metal ion chelator desferrioxamine, which decreased the steady-state level of the transiently formed Cr^{5+} and presumably inhibited the Fenton reaction:

$$Cr^{5+} + H_2O_2 \rightarrow Cr^{6+} + OH^{\cdot} + OH^{-}; \qquad (1)$$

and (iii) superoxide dismutase (SOD) increased the steady-state level of Cr^{5+} due to the inhibition of its reduction by superoxide (Porter *et al.,* 2005):

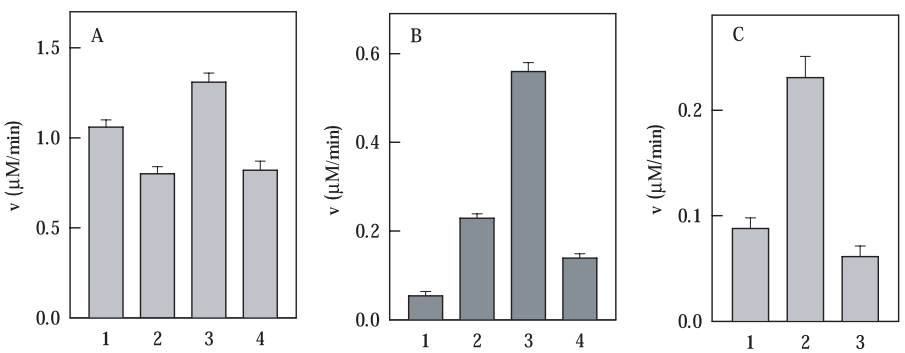

Fig. 2. The effects of desferrioxamine and SOD on chromate reductase reaction of NQO1. Concentration of NQO1, 84 нМ; concentration of NADPH, 50 μМ; concentration of chromate, 300 μМ; concentration of desferrioxamine, 500 μМ; concentration of SOD, 5 U/ml; 25 °C. (A) The rates of reduction of added 50 μМ cytochrome *c* by NQO1 and NADPH determined at 550 nm. Additions: 1, none; 2, SOD; 3, chromate; 4, chromate + SOD; $n = 3$; $p < 0.05$ for 3 against 1, 2, 4. (B) The rates of NADPH oxidation by NQO1 determined at 340 nm and corrected for a decrease ion chromate absorbance. Additions: 1, none; 2, chromate; 3, chromate + desferrioxamine; 4, chromate + SOD; $n = 3$; $p < 0.01$ for 3 against 1, 2, 4, $p < 0.05$ for 2 against 4. (C) The rates of chromate reduction by NQO1 and NADPH determined at 410 nm. Additions: 1, none; 2, desferrioxamine; 3, SOD; $n = 3$; $p < 0.02$ for 1 against 2, $p < 0.05$ for 1 against 3.

$$Cr^{5+} + O_2^{-\cdot} \rightarrow Cr^{4+} + O_2. \quad (2)$$

We found that the reduction of chromate by NQO1 is accompanied by the same events as the reactions of NOS: (i) Chromate stimulated the reduction of cytochrome *c* by NQO1 (Fig. 2A), whereas the presence of SOD decreased the reduction rate to the control level. It shows that chromate stimulates the $O_2^{-\cdot}$ generation by NQO1. (ii) Desferrioxamine stimulated the NADPH: chromate reductase reaction of NQO1, whereas SOD inhibited it (Figs. 2B, C). The similar effects of desferrioxamine and SOD were observed in the reactions of P-450R (Figs. 3A, B), and FNR and LipDH (data not shown). Thus, the reduction of chromate by the two-electron transferring NQO1 is accompanied by the prooxidant events, like the reactions of single-electron transferring flavoenzymes.

Next, we examined the cytotoxicity of chromate in FLK cells, which are characterized by the activities of NQO1, 250 nmol NADPH oxidized/(mg protein · min), P-450R, 43 nmol cytochrome *c* reduced/(mg protein · min), and GR, 53 nmol NADPH oxidized/(mg protein · min) (Nemeikaitė and Čėnas, 1993). The reduction of chromate by the digitonin-permeabilized FLK cells in the presence of the NADPH regeneration system was inhibited by an inhibitor of NQO1, dicumarol, and was not affected by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates GR (Ollinger and Brunmark, 1991) (Fig. 4A). The concentration of chromate for 50% survival of FLK cells (cL_{50}) during a 24-h incubation was (22 ± 4.0) μM. The prooxidant character of chromate cytotoxicity is evidenced by the partial protection by the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine (Ollinger and Brunmark, 1991) and by desferrioxamine, the latter preventing the Fenton reaction

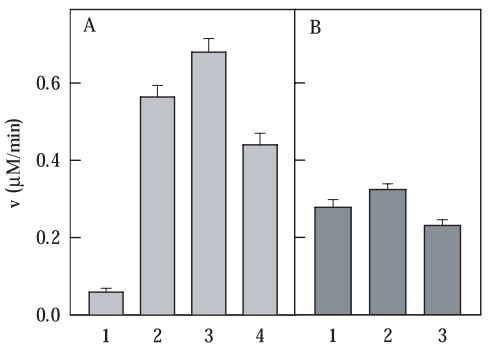

Fig. 3. The effects desferrioxamine and SOD on chromate reductase reaction of NADPH:cytochrome P-450 reductase (P-450R). Concentration of P-450R, 7 nM; concentration of other compounds the same as in Fig. 2. (A) The rates of NADPH oxidation by P-450R determined at 340 nm and corrected for a decrease ion chromate absorbance. Additions: 1, none; 2, chromate; 3, chromate + desferrioxamine; 4, chromate + SOD; $n = 3$; $p < 0.05$ for 2 against 3, 4. (B) The rates of chromate reduction by P-450R and NADPH determined at 410 nm. Additions: 1, none; 2, desferrioxamine; 3, SOD; $n = 3$; $p < 0.05$ for 1 against 2, 3.

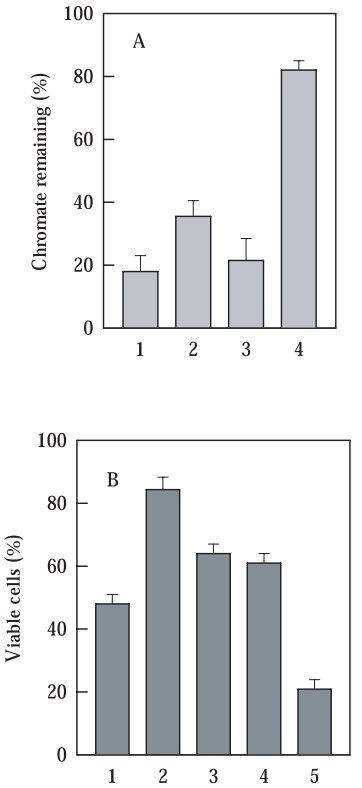

Fig. 4. (A) The reduction of 200 μM chromate by 10^6/ml digitonin-permeabilized FLK cells in the presence of NADPH regeneration system. The extent of chromate reduction was monitored according to a decrease in absorbance at 370 nm; reaction time, 24 h; 37 °C. Additions: 1, chromate; 2, chromate + 20 μM dicumarol; 3, chromate + 20 μM BCNU, 4, chromate in the absence of NADPH regeneration system; $n = 3$; $p < 0.05$ for 1 against 2. (B) Cytotoxicity of 25 μM chromate in FLK cells. Additions: 1, chromate; 2, chromate + 300 μM desferrioxamine; 3, chromate + 2.5 μM *N,N′*-diphenyl-*p*-phenylene diamine; 4, chromate + 20 μM dicumarol; 5, chromate + 20 μM 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). Cell viability in control experiments, (97 ± 2)%; desferrioxamine and BCNU decreased the cell viability by 1–2.5%; $n = 3$; $p < 0.05$ for 1 against 3, 4, $p < 0.02$ for 1 against 2, 5.

(Fig. 4B). It is important to note that dicumarol also protected against the cytotoxicity, whereas BCNU potentiated it (Fig. 4B). These data point to the participation of NQO1 in the prooxidant cytotoxicity of chromate in FLK cells, which is in line with the prooxidant events accompanying the chromate reduction by NQO1 (Figs. 2A–C), and its participation in chromate reduction by digitonin-permeabilized cells (Fig. 4A). In turn, the much lower chromate reductase activity of GR (Table I) is in line with the absence of the effect of BCNU on the chromate reduction by digitonin-permeabilized cells (Fig. 4A). Thus, most likely, the direct reduction by GR does not play an important role in chromate cytotoxicity in FLK cells. Because the inactivation of GR by BCNU causes the depletion of GSH (Ollinger and Brunmark, 1991), the effect of BCNU (Fig. 4B) may reflect the role of GSH-dependent processes. In our case it seems likely that the general antioxidant action of GSH is more important than its prooxidant action arising from the reactions with chromate (Lay and Levina, 1998).

It has been reported that dicumarol protected against the chromate cytotoxicity in osteoblasts (Ning and Grant, 1999). On the other hand, it potentiated or did not affect the chromate toxicity in hepatocytes (Gunaratnam and Grant, 2001; Pourahmad *et al.,* 2005). In turn, BCNU protected against the toxicity of chromate in osteoblasts and hepatocytes (Ning and Grant, 2000; Gunaratnam and Grant, 2001), or did not affect the hepatocyte cytotoxicity (Pourahmad *et al.,* 2005). In our opinion, this discrepancy may be partly caused by the the shorter incubation times, 3 h, and higher chromate concentrations, 0.1–1.0 mM, used in the hepatocyte cytotoxicity experiments (Gunaratnam and Grant, 2001; Pourahmad *et al.,* 2005). In this context, the longer incubation time and lower chromate concentration used in our studies may more closely resemble the natural conditions, and may more adequately reflect the roles of NQO1 and GR in the chronic intoxication by chromate.

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