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Effect of glutathione depletion on formation of paramagnetic chromium in Chinese hamster V-79 cells

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Abstract

Incubation of Chinese hamster V-79 cells with either buthionine sulfoximine (BSO) or diethylmaleate (DEM) prior to exposure to Na_2CrO_4 resulted in a depletion of GSH. ESR study shows that the depletion of GSH by BSO caused an increase in the cellular level of Cr(V) intermediate without affecting the level of Cr(III) complex, whereas the levels of Cr(V) and (III) were both suppressed by the depletion of GSH by DEM. GSH depletion by DEM decreased cellular uptake of chromate more than that by BSO. Glutathione reductase activity in cells, which is capable of reducing Cr(VI), was unaffected by BSO, however a strong inhibition was observed in cells treated with DEM, indicating that DEM affects not only GSH levels but also Cr(VI)-reducing enzyme activity. Thus, the depletion of GSH in cells increases Cr(V) formation, based upon the effect of BSO. The role of intracellular GSH in Cr(VI) reduction is discussed.

Key words: Glutathione; Chromium; ESR; Chinese hamster V-79 cell

1. Introduction

Cr(VI) compounds are well known to be human carcinogens [1]. They have been shown to produce DNA single strand breaks and DNA–protein crosslinks and to inhibit the activity of enzymes such as glutathione reductase (GR) in mammalian cells [2–4]. In contrast to Cr(III), Cr(VI) actively enters cells by the sulfate anion transport system and is then believed to be reduced through reactive intermediates such as Cr(V) and (IV) to more stable Cr(III) by cellular reductants including glutathione (GSH), ascorbic acid, riboflavin and flavoenzymes including GR [2–4]. Thus, the generation of paramagnetic chromium may play an important role in the cellular damage caused by Cr(VI). For instance, Cr(V) complexes have been reported to induce DNA breakage and 8-hydroxy-2′-deoxyguanosine in vitro as well as mutation in bacterial systems, and recent in vitro studies indicated that not only Cr(VI)/(V) but also the Cr(III)/(II) redox couple serve as cyclical electron donors, in a Fenton-like reaction, to produce active oxygen species [5–8]. Among the biological reductants, GSH has been reported to be present normally in millimolar concentrations in intact cells, and the modification of cellular GSH in cultured cells has been shown to alter the levels of DNA damage caused by Cr(VI) [9]. Furthermore, ESR studies have shown that Cr(III) and Cr(V) complexes are formed during reduction of Cr(VI) by GSH in vitro, and the formation of Cr(V) in rat thymocytes and human red blood cells treated with Cr(VI) has been reported to be suppressed by thiol-reactive agents such as diethylmaleate (DEM) and *N*-ethylmaleimide, respectively [10,11]. However, none of these thiol depletors are specific for GSH alone, and the influence of GSH depletion by an agent such as buthionine sulfoximine (BSO) which can deplete the cellular store of GSH via competitive inhibition of γ glutamylcysteine synthetase [12], on the formation of paramagnetic chromium in intact cells has not been investigated.

In the present study, the effect of GSH depletion in Chinese hamster V-79 (V-79) cells by either BSO or DEM on the formation of paramagnetic chromium was investigated using ESR spectrometry. In addition, we examined cellular uptake of Cr(VI), as well as the activity of Cr(VI)-reducing flavoenzymes such as GR in these GSH-depleted cells, to understand differences in the effects of BSO and DEM on the formation of Cr(V) and Cr(III). The role of intracellular GSH in Cr(VI) reduction was discussed, based upon the effects of these depletors.

2. Materials and methods

$Na_2CrO_4 \cdot 4H_2O$ (purity 99.7%) and the radioisotope $Na_2{}^{51}CrO_4$ were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and DuPont-New England Nuclear, respectively.

V-79 cells were grown as previously described [13]. BSO (Sigma) was added to the complete growth medium at the time cells were plated. After 24 h treatment, logarithmically growing cells were rinsed twice with salts-glucose medium (50 mM Hepes (pH 7.2) containing 10 mM

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NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM glucose), and the cells were then treated for 2 h with Na_2CrO_4 at 37°C in this maintenance medium. In the case of DEM experiments, logarithmically growing V-79 cells were pretreated for 2 h with 1 mM DEM in the complete growth medium and then treated with Na_2CrO_4 for 2 h in salts-glucose medium. The total glutathione (oxidized and reduced) and GR activity in cells were determined as previously described [14,15], and the enzyme activity was expressed as nmol of NADPH oxidized/min/mg protein [15]. The formation of paramagnetic chromium was examined in cells directly by ESR spectrometry as previously described [13]. Briefly, 40×10^6 cells were placed into an ESR tube and were rapidly frozen in liquid nitrogen. ESR measurements were made at temperatures of 153K using a JES-FE3X spectrometer with a 100-kHz field modulation, 8 mW of microwave power, and a 4.0-G modulation amplitude. Cellular uptake of chromate was also estimated by radioisotope ^{51}Cr analysis as detailed in [13].

3. Results and discussion

We first examined the effect of treatment with BSO (1–25 μM) for 24 h or with 1 mM DEM for 2 h on the content of GSH in V-79 cells. Treatment with either BSO or DEM alone did not alter cell growth in V-79 cells (data not shown). As shown in Table 1, treatment of cells with 1, 5 and 10 μM BSO caused approximately 40, 85 and 90% depletion of cellular GSH level, respectively, and 25 μM BSO or 1 mM DEM treatment resulted in a complete depletion (> 98%) of GSH.

Table 1 also shows the influence of GSH depletion by BSO or DEM on the ESR signal intensity of Cr(V) and Cr(III) complexes in V-79 cells treated with 200 μM Na_2CrO_4, and their ESR spectra were shown in Figs. 1 and 2. No significant ESR signal was obtained from cells incubated in the absence of Na_2CrO_4 or in the presence of depletors alone (data not shown). The ESR signal intensity due to Cr(V) (g = 1.989) in cells was not altered by a 40% depletion of GSH caused by 1 μM BSO (Fig. 1b) compared to that observed in unpretreated Cr(VI)-treated cells (Fig. 1a, Table 1). However, as shown in Fig. 1c–e, when the depletion of GSH exceeded 85% in cells pretreated with 5, 10 and 25 μM BSO, the signal intensity of Cr(V) increased up to 170% of the control (Table 1). In contrast, as was in the case of previous studies using Thiol-reactive agents [10,11], 1 mM DEM pretreatment resulted in a decrease of Cr(V) signal intensity (Fig. 1f, Table 1). With respect to Cr(III) formation, the intensity of the broad ESR signal due to Cr(III) (g = 2.03, ΔA = 700–800 G) was not affected by BSO (Fig. 2a–e), however, the intensity of Cr(III) was reduced to about 60% of the control level by 1 mM DEM (Fig. 2f, Table 1). These results indicated that the depletion of GSH by BSO caused an increase of Cr(VO) formation without affecting Cr(III) level whereas the depletion by DEM decreased both paramagnetic chromium species in V-79 cells.

To clarify whether these depletors exerted their effect by altering the cellular uptake of chromate, the intracellular levels of this metal were also examined, as shown in Table 1. Pretreatment with BSO (5, 10 and 25 μM) caused small but significant decreases (14, 22 and 23%) in the cellular level of chromium, and 1 mM DEM pretreatment resulted in an approximately 50% reduction in cellular levels of this metal.

Since the cellular reduction of Cr(VI) has been reported to be catalyzed by flavoenzymes such as GR [2–4], we also investigated the influence of these depletors on GR activity in V-79 cells. Results showed that no significant alteration of enzyme activity was observed in cells treated with 25 μM BSO alone, however, the activity was strongly suppressed by 1 mM DEM (control, 15.4 ± 1.7 (S.D.); 25 μM BSO, 14.6 ± 1.1; 1 mM DEM, 2.5 ± 0.2 nmol/min/mg protein, n = 4).

Thiol-reactive agents such as DEM are well known to react with SH groups of proteins. Previous studies have shown that DEM has many metabolic effects including inhibition of cell division, respiration, and glycolysis [16]. In fact, the present results showed that cellular treatment

Table 1
GSH depletion by BSO or DEM and their effects on the formation of paramagnetic chromium and chromate uptake of V-79 cells

Pretreatment (μM)	GSH (nmol/mg protein)	Na_2CrO_4 (μM)	ESR signal intensity (%) Cr(V)	Cr(III)	Cellular uptake of ^{51}Cr (nmol/10^6 cells)
–	15.81 ± 1.92	200	100	100	6.36 ± 0.53
BSO, 1	9.92 ± 1.98	200	104 ± 3	96 ± 8	–
5	2.26 ± 0.26	200	128 ± 8	98 ± 18	5.44 ± 0.20*
10	1.09 ± 0.07	200	146 ± 9	95 ± 20	4.99 ± 0.30*
25	0.17 ± 0.02	200	167 ± 22	81 ± 15	4.92 ± 0.51*
DEM, 1000	0.10 ± 0.01	200	72 ± 14	59 ± 15	3.47 ± 0.13*

Cells were pretreated with BSO (1–25 μM) for 24 h or with 1 mM DEM for 2 h in growth medium and then cellular levels of GSH were measured. In addition, cells pretreated with BSO or DEM were treated for 2 h with 200 μM Na_2CrO_4 in salts-glucose medium. Following Na_2CrO_4 treatment, ESR spectra of paramagnetic chromium and ^{51}Cr uptake of cells were measured as described in section 2. Each value is the mean ± S.D. ($n \geq 3$). *$P < 0.05$ compared to unpretreated – 200 μM Na_2CrO_4 treated cells (Student's t-test).

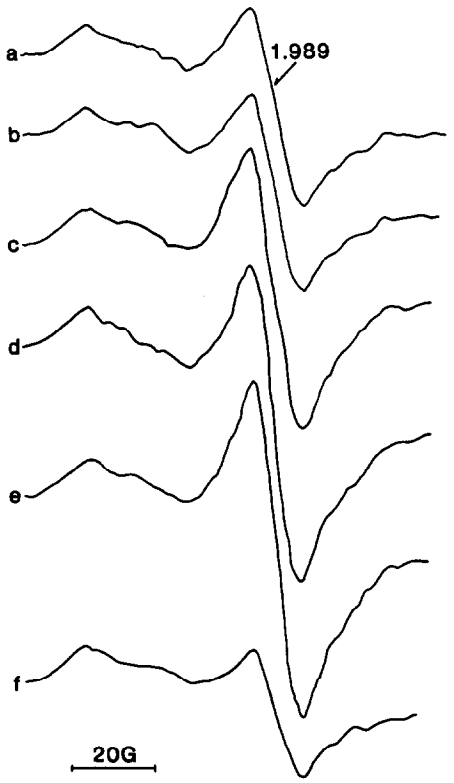

Fig. 1. ESR spectra of Cr(V) complex at 153K. Cells were pretreated with 0 (a), 1 (b), 5 (c), 10 (d) and 25 μM (e) BSO for 24 h or with 1 mM DEM (f) for 2 h, and then treated for 2 h in salts-glucose medium with 200 μM Na_2CrO_4. Following treatment, an ESR signal was obtained from the cells.

with DEM caused a strong inhibition of chromate-reducing GR activity, indicating that the suppression of Cr(III) and (V) by DEM might be associated not only with the decrease of cellular levels of GSH but also with the inhibition of chromate-reducing SH enzymes and/or protein. In addition, it is possible that DEM reacting with the SH group of the sulfate transport system inhibits chromate uptake in cells. In contrast to DEM, treatment with BSO did not affect the activity of Cr(VI)-reducing GR in V-79 cells. Another study has also shown that complete GSH depletion by BSO could not sensitize the cells to the toxicity of paraquat as a herbicide, but that this toxicity was enhanced after complete GSH depletion by DEM, possibly due to the binding of DEM to protein [17]. Although a small but significant inhibition (< 23%) of chromium uptake was observed in BSO-treated cells, in contrast to those results with DEM, the present results observed with BSO, in which the depletion of GSH caused an increase of Cr(V), may be primarily due to the effect of intracellular GSH depletion on the formation of paramagnetic chromium in V-79 cells.

The present results with BSO showed that the levels of Cr(V) in GSH-depleted cells increased while the levels of Cr(III) remained unchanged. This may be explained, in part, by the different intracellular concentrations of Cr(III) and (V). As shown in Fig. 2, when the relative concentrations of Cr(V) and Cr(III) in cells were estimated by analyzing the areas under their ESR signals, the concentrations of Cr(III) were found to be about 30 times greater than those of Cr(V) [13], indicating that the levels of Cr(V) alteration in the present study were relatively small as compared to the levels of Cr(III). Similarly, pretreatment of V-79 cells with vitamin B_2 or E has been shown to modify the levels of Cr(V) without altering the levels of Cr(III) [2]. These results indicate that intracellular GSH may not be the major cellular reductant to form Cr(III), but that the formation of Cr(V) appears to be closely associated with intracellular GSH levels.

It is very interesting that the level of Cr(V) was increased in the > 85% GSH-depleted cells, but was not affected by the 40% depletion. Since BSO treatment decreased cellular uptake of chromate and had no effect on chromate-reducing GR activity, the increase of Cr(V) by the depletion is unlikely to be related to alterations of the metal uptake and chromate-reducing GR activity. It is possible that, in V-79 cells containing > 60% of total GSH, the concentration of GSH may be fully adequate for reducing Cr(VI) to Cr(III) without affecting Cr(V), however, in cells containing < 15% of total GSH, Cr(VI) could be reduced only to the Cr(V) level. This hypothesis is consistent with that of a previous study [19] and is

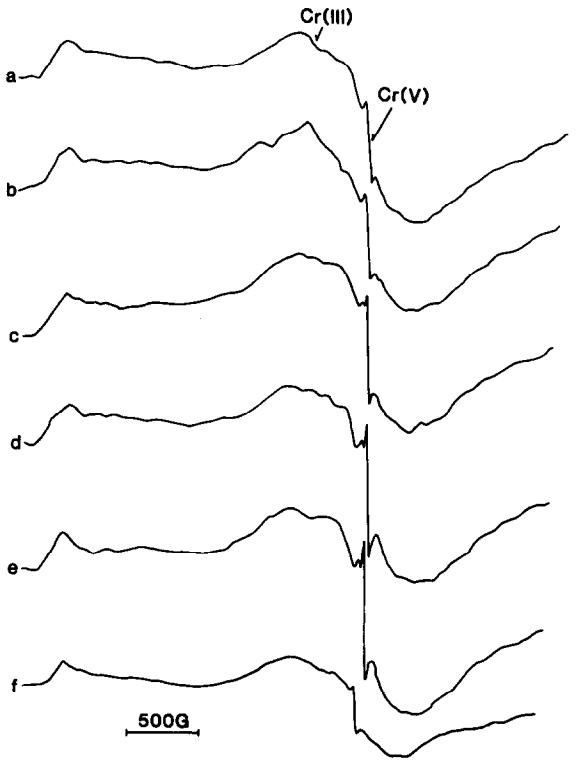

Fig. 2. ESR spectra of Cr(III) complex at 153K. Same samples and ESR settings as in Fig. 1 except for the field scan.

supported by a recent in vitro study on the reaction of Cr(VI) and GSH using ESR spectroscopy [18]. These results suggested that the concentrations of Cr(VI) and GSH inside cells may be critical factors in the formation of Cr(V) complex.

In conclusion, the present study showed that GSH depletion by BSO increased the formation of Cr(V) without altering the level of Cr(III) while the levels of both Cr(V) and Cr(III) were decreased after complete GSH depletion by DEM. These latter decreases might, however, be attributed not only to intracellular GSH but also to the binding of DEM to protein, because DEM was found to strongly inhibit cellular uptake of chromate as well as chromate-reducing flavoenzymes such as GR. Since Cr(V) in particular may play a key role in the induction of Cr(VI) toxicity, mutagenicity and DNA alteration [2–4], and since the molar ratio of Cr(VI) and GSH in cells may be associated with the induction of Cr(VI) damage, further study is needed to elucidate the precise role of intracellular GSH in the formation of paramagnetic chromium in a variety of tissues and cultured cells.

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