Protective Effect of Diethyldithiocarbamate Pretreatment on Chromium (VI)-Induced Cytotoxicity and Lipid Peroxidation in Primary Cultures of Rat Hepatocytes

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(Received 18 April 1997/Accepted 12 August 1997)

ABSTRACT. Pretreatment of primary cultures of rat hepatocytes with sodium diethyldithiocarbamate (DDTC) for 15 min prior to exposure to $K_2Cr_2O_7$ resulted in a marked decrease in dichromate-induced cytotoxicity, as evaluated by the leakage of lactate dehydrogenase, and in lipid peroxidation, as monitored by malondialdehyde formation. In addition, pretreatment with DDTC attenuated the suppression of the level of vitamin E attributed to $K_2Cr_2O_7$. However, DDTC pretreatment had no effect on the cellular levels of glutathione or vitamin C or on the activity of the glutathione reductase, glutathione peroxidase, superoxide dismutase or alkaline phosphatase suppressed by dichromate. Under the same experimental conditions, cellular uptake or distribution of chromium was not affected by DDTC. These results indicate that the protective effect of DDTC on chromium (VI)-induced cytotoxicity as well as lipid peroxidation may be associated with the level of nonenzymatic antioxidants such as vitamin E. — **KEY WORDS:** chromium (VI), cytotoxicity, diethyldithiocarbamate, hepatocyte, lipid peroxidation.

J. Vet. Med. Sci. 60(1): 71–76, 1998

Chromium (VI) compounds are potent toxic and carcinogenic metals [6]. With respect to their toxicity, hepatic and renal toxicities have been reported in workers and animals exposed to chromium (VI) [19]. Chromium (VI) compounds induced also DNA damage *in vivo* [5] and in cultured cells [36] as well as inhibition of the activity of such enzymes as glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) in mammalian cells [34].

The biological effects of chromium (VI) are generally attributed to cellular uptake, since chromium (VI), in contrast to chromium (III), is easily taken into the cells by the sulfate anion transport system [7]. However, once internalized, chromium (VI) is reduced through reactive intermediates such as chromium (V) and (IV) to more stable chromium (III) by cellular reductants including glutathione, vitamins C and B2, as well as flavoenzymes [7]. Thus, the formation of Cr (III) or other intermediate oxidation states, in particular chromium (V), is believed to play a role in the biological effect of chromium (VI) compounds. Previous studies have shown that this process of reduction causes also the generation of active oxygen species [34]. For instance, not only chromium (VI)/(V) but also the chromium (III)/(II) redox couple serve as cyclic electron donors in a Fenton-like reaction to produce active oxygen species, resulting in induction of DNA damage [2]. In cultured cells, DNA strand breaks caused by chromium (VI) have been shown to be suppressed by a hydroxyl radical scavenger, 1,3-dimethyl-2-thiourea (DMTU) [45] and vitamin E, which decreased the cellular level of chromium (V) [35]. Since reactive oxygen species produce a number of toxic effects including DNA damage and lipid peroxidation, the toxicity of chromium (VI) may be

associated in part with the production of active oxygen.

Membrane-permeable metal-chelating agents such as deferoxamin (DFO) and o-phenanthroline (OP) have been reported to inhibit the DNA-strand breaks and cytotoxicity induced by hydrogen peroxide in cultured mammalian cells [8]. It is well known that these chelators form a complex with intracellular transition metals such as iron, preventing the occurrence of Fenton-type reaction of hydroxyl radical and protecting cells from the genetic and cytotoxic action of hydrogen peroxide [8]. With respect to chromium, recent studies showed that OP and DFO inhibited chromium (VI)-induced DNA breaks, cytotoxicity and lipid peroxidation in cultured cells [37, 41] and that these chelators suppressed the formation of *in vitro* chromium (V) and chromium (V)-mediated hydroxyl radical [28, 37].

Diethyldithiocarbamate (DDTC) a metal chelator is used for the selective determination of chromium (VI) in natural waters as a combining agent [31]. Dithiocarbamates have a variety of pharmacological actions and characteristics, namely antioxidation [22], immunoregulation [23], disinfection [25], radioprotection, radiosensitization [18] and ulcerogenicity [24]. With respect to metal toxicity, it has been shown that DDTC can reduce the systemic signs produced by lead [10], cadmium [11, 14, 47] and mercury [25] intoxication in animals. Interestingly, DDTC has been shown to inhibit lipid peroxidation induced by cadmium [15], carbon tetrachloride [21], ADP/Fe³⁺ [16] or cisplatin [30]. Furthermore, this chelator has also been shown to be an efficient inhibitor of Fe²⁺- and Cu²⁺-catalyzed hydroxyl radical formation, also protecting the cells from this toxic radical [46].

In the present study, we using primary cultures of rat hepatocytes, to find examined whether DDTC has an effect on chromium (VI)-induced cytotoxicity and lipid

peroxidation. To understand the protective mechanism of DDTC, we further investigated cellular uptake and distribution of Cr, and cellular levels of antioxidants including glutathione and vitamins C and E as well as the activities of such enzymes as GR, GSH-Px, SOD, CAT, and alkaline phosphatase (ALPase) in the cells treated with chromium (VI).

Our results demonstrate that DDTC can diminish chromium (VI)-caused cytotoxicity, lipid peroxidation and vitamin E depletion in intact cultured cells.

MATERIALS AND METHODS

Chemicals: $K_2Cr_2O_7$ and sodium *N, N*-diethyl-dithiocarbamate trihydrate were obtained from Kanto Chemical Co., Inc. (Tokyo).

Cell Culture: Hepatocytes from male Wistar strain rats (200–300 g of body weight) were isolated and cultured according to the procedure described [39]. Twenty hours after plating, DDTC was added to a complete medium (Williams' E medium containing 5% heat-inactivated calf serum). Fifteen minutes after addition of DDTC, the cells were rinsed twice with a salts-glucose medium {SGM; 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid buffer (pH 7.2) with 100 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; and 5 mM glucose} to eliminate reaction of DDTC and chromium (VI) at extracellular sites, and the cells were then treated with $K_2Cr_2O_7$ at 37°C in this maintenance medium. DDTC and $K_2Cr_2O_7$ were freshly prepared in SGM before use. Under the same experimental conditions, we observed preliminarily that the acceleration of lipid peroxidation and cell injury were caused significantly 8 to 10 hr after treatment with 500 μM $K_2Cr_2O_7$. Furthermore, the depressions of antioxidants such as GSH and vitamin C in hepatocytes were also observed 2 to 4 hr after treatment with this metal. According to these results, the treatment with chromium (VI) of hepatocytes pretreated with DDTC varied as follows: to assay cytotoxicity and lipid peroxidation, cells were treated for 8 hr with 0.25 to 500 μM $K_2Cr_2O_7$ in SGM; to assay antioxidants, enzymes, and chromium content, cells were treated for 4 hr with 500 μM $K_2Cr_2O_7$ in SGM.

Cytotoxicity: Following the treatment of hepatocytes with $K_2Cr_2O_7$ for 8 hr, cytotoxicity was estimated from the leakage of lactate dehydrogenase (LDH) from cells, as previously described [39].

Assay for lipid peroxidation, antioxidants and enzymes: Following treatment of hepatocytes with $K_2Cr_2O_7$ for 8 hr, the cultured cells were removed from the dishes by scraping, were resuspended in ice-cold phosphate-buffered saline (PBS) and sonicated with a Handy Sonic Model UR-20P (Tomy Seiko Co., Ltd.). They were pulsed for 1 min at setting 8, and the homogenate obtained was used for the estimation of lipid peroxidation as monitored by malondialdehyde (MDA) formation [4]. For assay for antioxidants and enzymes, the cultured cells were treated with $K_2Cr_2O_7$ for 4 hr. The homogenate obtained as

described above was divided into two parts: one part was used to assay GSH as nonprotein sulfhydryls [3], vitamin C (ascorbic acid) [13], vitamin E [44] and alkaline phosphatase (ALPase) [29], and the other part of the homogenate was centrifuged at $10,000 \times g$ for 20 min. The resulting supernatant was used to assay the activities of the cytosolic enzymes including GR [32], GSH-Px [12], SOD [48] and CAT [1]. Proteins were measured by the method of Lowry *et al.* [20] with bovine serum albumin as the standard.

Estimation of chromium content: Following treatment with $K_2Cr_2O_7$ for 4 hr, the hepatocytes were washed twice with ice-cold PBS and dislodged from the dishes by scraping. The cells were then homogenized in ice-cold phosphate buffer-KCl solution (0.15 M KCl in 0.05 M Na_2HPO_4 buffer, pH 6.8) [43]. The procedure of Edwards *et al.* [9] was used to prepare nuclear, mitochondrial and post-mitochondrial supernatant fractions. Chromium was estimated with an atomic absorption spectrophotometer (Shimadzu AA-630-01) following wet ashing of the samples with concentrated nitric acid [39].

Spectrophotometer: The effect of DDTC on reduction of chromium (VI) during the reaction of 0.25 mM $K_2Cr_2O_7$ with 10 mM GSH in Tris-HCl buffer (pH 7.4) at room temperature was examined with a Hitachi U-2000A spectrophotometer. The level of chromium (VI) was estimated by measuring the absorbance at 372 nm [37].

Statistical analysis: All of the results are expressed as means and standard errors of four experiments. The differences between mean values of the data were evaluated by Student's *t*-test, and a *P* value smaller than 0.05 was considered to be statistically significant.

RESULTS

Cytotoxicity: Figure 1 shows the effects of the DDTC pretreatment on cytotoxicity, as evaluated by LDH leakage, in the hepatocytes treated with 125 to 500 μM $K_2Cr_2O_7$ for 8 hr. Pretreatment with 100–1,000 μM DDTC reduced significantly the dichromate-induced cytotoxicity. DDTC alone at 10 to 1,000 μM was noncytotoxic.

Lipid peroxidation: As shown in Fig. 2, the treatment with 125 to 500 μM $K_2Cr_2O_7$ alone caused a dose-dependent increase of MDA formation, as compared with the control. When cells were pretreated with DDTC (100–1,000 μM), however, there was a significant inhibition of the MDA formation caused by $K_2Cr_2O_7$.

Antioxidants and enzymes activity: Tables 1 and 2 show the effects of DDTC on the contents of nonenzymatic antioxidants such as GSH, vitamin C and vitamin E (Table 1), and activities of such antioxidant enzymes as GR, GSH-Px, SOD and CAT as well as ALPase a plasma membrane enzyme (Table 2) in the hepatocytes treated with 500 μM $K_2Cr_2O_7$ for 4 hr. As shown in Tables, the cellular levels of nonenzymatic antioxidants and the activities of the enzymes except CAT decreased upon treatment with $K_2Cr_2O_7$ alone. When cells were pretreated with 1,000 μM DDTC, there was a significant restoration of vitamin E levels suppressed

Fig. 1. Effects of DDTC on chromium (VI)-induced cytotoxicity. Hepatocytes were pretreated with DDTC (0: , 100: , 500: , 1,000 μM :) for 15 min in a complete medium and then treated with 125–500 μM $K_2Cr_2O_7$ for 8 hr in a salts-glucose medium. Following this treatment, cytotoxicity was evaluated from the leakage of LDH. Bars represent standard errors ($n=4$). *1, *2 $P<0.05$ compared to untreated values and chromate-treated values, respectively.

Fig. 2. Effects of DDTC on chromium (VI)-induced lipid peroxidation. Hepatocytes were pretreated with DDTC (0: , 100: , 500: , 1,000 μM :)

Table 1. Levels of nonenzymatic antioxidants in hepatocytes

DDTC (μM)	$K_2Cr_2O_7$ (μM)	Nonenzymatic antioxidant content		
		GSH ($\mu g/mg$ protein)	Vitamin C ($\mu g/mg$ protein)	Vitamin E (ng/mg protein)
0	0	9.3 ± 0.3	1.43 ± 0.06	98 ± 11
1,000	0	8.8 ± 0.4	$1.84 \pm 0.05^{*1}$	86 ± 5
0	500	$2.7 \pm 0.1^{*1}$	$0.44 \pm 0.03^{*1}$	$22 \pm 4^{*1}$
1,000	500	$2.8 \pm 0.1^{*1}$	$0.47 \pm 0.02^{*1}$	$67 \pm 5^{*1,*2}$

Cells were pretreated with DDTC for 15 min in a complete medium and then treated with $K_2Cr_2O_7$ for 4 hr in a salts-glucose medium. Each value is the mean $\pm$ SE ($n=4$). *1, *2 $P<0.05$ compared to untreated values and chromate-treated values, respectively.

by $K_2Cr_2O_7$. In contrast, no restorative effect of DDTC on GSH or vitamin C level, or GR, GSH-Px, SOD or ALPase activity was observed.

Chromium content and distribution: As shown in Table 3, the level of chromium in whole cell homogenates or subcellular fractions was not influenced by the pretreatment with 1,000 μM DDTC, demonstrating that this chelator had no effect on chromium uptake or subcellular distribution in the present study.

Chromium (VI) reduction: To find whether DDTC interferes with the reaction of chromium (VI) and GSH, we examined the reduction of chromium (VI) using a spectrophotometer. As shown in Fig. 3, the reduction of chromium (VI) was inhibited in the presence of 0.5–2.0 mM DDTC in a concentration-dependent manner. DDTC alone (1 mM) did not affect the reduction of chromium (VI) in the absence of GSH (data not shown).

DISCUSSION

The results of the present study with primary cultures of rat hepatocytes demonstrate that pretreatment with DDTC reduces chromium (VI)-induced cytotoxicity (Fig. 1). Additionally, dichromate-induced lipid peroxidation in the hepatocytes was also inhibited by this chelator (Fig. 2). These results indicate that DDTC has a suppressive effect on chromium (VI)-induced cytotoxicity and oxidative damages such as lipid peroxidation in intact cells. The possibility that the effects of DDTC observed in the present study resulted from the inhibition of cellular uptake of this metal was considered. However, since the cellular levels of chromium in the whole cell homogenates or in the subcellular fraction were not affected by pretreatment with DDTC (Table 3), such a possibility is unlikely.

Previous studies demonstrated the important roles of

for 15 min in a complete medium, and then treated with 125–500 μM $K_2Cr_2O_7$ for 8 hr in a salts-glucose medium. Following this treatment, lipid peroxidation as monitored by malonaldehyde (MDA) formation was estimated. Bars represent standard errors ($n=4$). *1, *2 $P<0.05$ compared to untreated values and chromate-treated values, respectively

Table 2. Levels of antioxidant enzymes and plasma membrane enzyme in hepatocytes

DDTC (μ M)	K ₂ Cr ₂ O ₇ (μ M)	GR (nmole NADPH oxidized/min/ mg protein)	GSH-Px (EU ^a /mg protein)	SOD (% inhibition ^b / mg protein)	CAT (nmol H ₂ O ₂ reduced/min/ mg protein)	Alpase (nmols <i>p</i> -NP/ mg protein/h)
0	0	15.3 $\pm$ 1.5	2.3 $\pm$ 0.2	25.3 $\pm$ 0.7	56 $\pm$ 2	22 $\pm$ 1.5
1,000	0	17.7 $\pm$ 0.7	2.4 $\pm$ 0.4	21.8 $\pm$ 1.1	58 $\pm$ 2	21 $\pm$ 1.3
0	500	4.8 $\pm$ 1.4 ^{*1}	1.2 $\pm$ 0.4 ^{*1}	20.0 $\pm$ 0.7 ^{*1}	59 $\pm$ 3	11 $\pm$ 0.8 ^{*1}
1,000	500	3.6 $\pm$ 0.2 ^{*1}	1.3 $\pm$ 0.3 ^{*1}	22.0 $\pm$ 1.2 ^{*1}	55 $\pm$ 1	9 $\pm$ 0.3 ^{*1,*2}

Cells were pretreated with DDTC for 15 min in a complete medium and then treated with K₂Cr₂O₇ for 4 hr in a salts-glucose medium. Each value is mean $\pm$ SE (n=4). a) An enzyme unit of activity was defined as a decrease in log [GSH] of 0.001 per min after the decrease in log [GSH] per min of the nonenzymatic reaction was subtracted [12]. b) Percentage inhibition in the reduction rate of nitroblue tetrazolium. *1, *2 *p*<0.05 compared to untreated values and chromate-treated values, respectively.

Table 3. Distribution of chromium in subcellular fractions of hepatocytes

DDTC (μ M)	K ₂ Cr ₂ O ₇ (μ M)	Homogenates (μ g Cr/mg protein)	Subcellular fractions Cr (%)		
			Nuclear	Mitochondrial	Post-mitochondrial supernatant
0	500	4.2 $\pm$ 0.2	85.2 $\pm$ 0.4	2.4 $\pm$ 0.1	11.9 $\pm$ 0.5
1,000	500	4.0 $\pm$ 0.1	87.0 $\pm$ 0.8	2.7 $\pm$ 0.4	10.4 $\pm$ 0.6

Cells were pretreated with DDTC for 15 min in a complete medium and then treated with K₂Cr₂O₇ for 4 hr in a salts-glucose medium. Each value is the mean $\pm$ SE (n=4)

Fig. 3. Effects of DDTC on reduction of chromium (VI) during the reaction of 0.25 mM K₂Cr₂O₇ with 10 mM GSH in Tris-HCl buffer (pH 7.4) at room temperature. The level of chromium (VI) in the presence (0.5: Δ , 1.0: Δ , and 2.0 mM: $\circ$) or absence ($\bullet$) of DDTC was estimated by measuring absorbance at 372 nm.

in the induction of cell injury or DNA breaks with chromium (VI) [34]. For instance, metal chelators such as OP and DFO have been shown to inhibit the *in vitro* formation of chromium (V), leading to a decrease of chromium (VI)-induced DNA breaks in intact cells. However, these chelators did not affect the reduction of chromium (VI) [37, 41]. On the other hand, the present study showed that *in vitro* reduction of chromium (VI) in the presence of GSH was inhibited by the addition of DDTC, as examined with a spectrophotometer (Fig. 3). These results suggest that the protective mechanism of DDTC is associated with its ability to directly combine with chromium (VI) within the cells, resulting in inhibition of chromium (V) formation and chromium (V)-related hydroxyl radical generation induced during the reduction process of chromium (VI). Furthermore, chromium (VI) combined with DDTC within the cell may not elicit its toxic effect.

It is well known that the protection of cells from oxidative damage can be accomplished through nonenzymatic and enzymatic antioxidant systems [38]. Previous studies showed that these antioxidant systems were disrupted by chromium (VI) compounds [33, 34]. The present study also showed that chromium (VI) treatment resulted in decreased levels of nonenzymatic antioxidants such as GSH, vitamins C and E, and inhibited activities of enzymatic antioxidants such as GR, GSH-Px and SOD in cultured rat hepatocytes. Similar results were observed in previous studies [40–42]. In the present study, pretreatment with DDTC had no effect on the reduction of GSH or vitamin C level or on inhibition of GR, GSH-Px or SOD activity induced by chromium (VI). Since the levels of GSH in the hepatocytes were decreased

chromium (V) and chromium (V)-related hydroxyl radical

by treatment with nontoxic levels of chromium (VI) (data not shown), it was thought possible that depression in these antioxidants may have resulted by free state chromium (VI) within the cell. However, it is very interesting that pretreatment with DDTC did inhibit the suppression of vitamin E level induced by chromium (VI), and that pretreatment with DDTC alone led to higher levels of vitamin C than in untreated control cells (Table 1). Since DDTC is an effective inhibitor of Fe^{2+} - and Cu^{2+} -catalyzed hydroxyl radicals [46], it is considered that DDTC protects the consumption of these antioxidants by hydroxyl radicals catalyzed by chromium (V) as well as Fe^{2+} or Cu^{2+} . Concerning these antioxidants, our previous studies with cultured hepatocytes also showed that a metal chelator DFO had no influence on cellular levels of GSH and the activities of GR as well as SOD, however this chelator attenuated the suppression of cellular levels of vitamin C and vitamin E induced by chromium (VI), resulting in the suppression of chromium (VI)-induced DNA breaks, cytotoxicity and lipid peroxidation [41]. Similarly, treatment with vitamin E or melatonin inhibited chromium (VI)-induced cytotoxicity as well as lipid peroxidation, and normalized the levels of vitamins C and E suppressed by dichromate, without affecting such antioxidant enzymes as GSH-Px, SOD and CAT [40, 42]. These and the present study suggest that the protective effect of DDTC against chromium (VI)-induced cytotoxicity and lipid peroxidation may be related more to the levels of nonenzymatic antioxidants such as vitamin E than to the enzymatic antioxidant activity within cells.

It is well known that lipid peroxidation causes membrane damages [27] and that the inhibition of ALPase reflects selective damage to the plasma membrane [17]. In the present study, DDTC significantly reduced the lipid peroxidation induced by dichromate without reversing the inhibition of ALPase activity. These results suggest that the activity of this enzyme does not entirely depend on lipid peroxidation, and that DDTC protects membrane lipids but not membrane proteins disordered by chromium (VI).

In conclusion, pretreatment of primary cultures of rat hepatocytes with the metal chelator DDTC markedly decreased in the cytotoxicity and lipid peroxidation caused by chromium (VI), without affecting chromium uptake or cellular chromium distribution in cells, possibly through its ability to increase the cellular level of vitamin E and/or to directly combine with chromium (VI). These results suggest that the metal chelator DDTC is an effective chelating or an antioxidant agent for chromium (VI) compounds. Further investigation is necessary to determine whether DDTC has an effect on other kinds of chromate-induced oxidative damages such as DNA-single strand breaks in intact cells.

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