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Dithiolthione-induced alterations in hepatic glutathione and related enzymes in male mice

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Dithiolthiones (DTT) are a group of naturally occurring compounds found in cruciferous vegetables [1, 2] that can apparently alter the metabolism and toxicity of selected hepatotoxins. Ansher *et al.* [3] demonstrated that the dithiolthiones, oltipraz (OTP) [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] and anethol dithiolthione (ADT) [3-(*p*-methoxyphenyl)-1,2-dithiol-3-thione], protect female mice from acute hepatotoxicity induced by acetaminophen or carbon tetrachloride. In these studies, the decreases in hepatic glutathione levels and glutathione-*S*-transferase activities and the increases in lethality and serum enzyme activities observed after CCl_4 or acetaminophen treatment were ameliorated by pretreatment with either OTP or ADT [3]. Studies to determine the effect of OTP or ADT on hepatic glutathione levels were not reported.

The purpose of the present study was to characterize the effects of ADT or OTP on hepatic glutathione status and on the activities of those enzymes altering glutathione homeostasis.

Methods

Male, CF-1 mice (15–20 g) were obtained from Sasco, Inc. (Omaha, NE) and allowed to acclimate for 1 week prior to use. Purina (no. 5012) rodent chow and tap water were available *ad lib*. OTP and ADT were donated by Dr. T. A. Lawson (Eppley Institute, Omaha, NE) and Dr. G. Jolles (Rhone-Poulenc Sante, Paris, France). Both compounds were used without further purification.

Mice received either OTP or ADT (4.5 mmoles/kg) or vehicle [25% glycerol (v/v) in 1% carboxymethylcellulose] by gavage at 96 and 48 hr prior to being killed.

All experiments were begun between 7:00 and 9:00 a.m. to eliminate variation due to circadian periodicity [4, 5]. The mice were killed by cervical dislocation, and the livers were excised immediately.

Reduced (GSH) and oxidized (GSSG) glutathione were measured in the acid soluble portion of liver homogenates by enzymatic conjugation with 1-chloro-2,4-dinitrobenzene [6].

Liver cytosolic fractions were prepared by differential centrifugation in 0.1 M Tris (pH 7.5). The following enzymic activities were measured using the cytosolic fraction (105,000 g_{max} supernatant): (1) glutathione reductase (GSSG-R), by the method of Racker [7]; (2) γ-glutamylcysteine synthetase (γ-GCS) by release of inorganic phosphate (P_i) due to ATP hydrolysis [8]; (3) glutathione peroxidase, by the method of Paglia and Valentine [9] measuring the catalysis of inorganic (hydrogen peroxide) and organic (cumene hydroperoxide) peroxides; and (4) glutathione-*S*-transferases (GSH-*S*-Tx) for five substrates [1-chloro-2,4-dinitrobenzene (CDNB); 1,2-dichloro-4-nitrobenzene (DCNB); *p*-nitrobenzyl chloride (NBC); bromosulfophthalein (BSP); or 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (ENPP)] by the method of Habig *et al.* [10]. Protein concentrations were measured by the method of Lowry *et al.* [11].

Data were analyzed by a one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test where appropriate [12]. The acceptable level for statistical significance was $P < 0.05$.

Results and discussion

Administration of the dithiolthiones, OTP and ADT, did not produce any changes in body weight, whereas relative liver weight (expressed as a proportion of body weight) was increased by DTT. No change in hepatic protein content was observed (Table 1).

Hepatic GSH levels were increased by both OTP (146%) and ADT (132%), whereas GSSG was essentially unchanged. The ratio of GSSG/GSH was relatively unchanged, suggesting that the overall oxidative status of glutathione was unaffected by DTT treatment (Table 2). The activities of enzymes which affect the cellular balance of GSH were altered by DTT treatment. Both γ-GCS and GSSG-R activities were increased markedly by ADT and OTP. Elevations in the activities of these enzymes, following DTT, imply an enzymatic correction of a previous perturbation in GSSG/GSH ratios. The increase in GCS activity is indicative of a greater rate of GSH synthesis and may account, in part, for elevated GSH levels. GSSG-R, likewise, would contribute to an increase in GSH content as well as maintaining physiologically low GSSG levels. Thus, DTT treatment may cause a transient shift in the oxidative state of GSH.

DTT treatment increased GSH-*S*-Tx activities toward four (CDNB, NBC, DCNB, and BSP) of the five substrates examined (Fig. 1). The lack of effect of DTT on GSH-*S*-Tx activity toward the epoxide substrate, ENPP, is indicative of a rather specific increase of the GSH-*S*-Tx isozymes. GSH-*S*-Tx catalyzed conjugation of electrophilic metabolites is generally recognized as a detoxication process.

The marked increases in GSH-*S*-Tx activities in conjunction with the increased GSH levels suggest that DTT

Table 1. Effects of dithiolthiones on physiological and hepatic variables

	Body weight (g)	Liver weight (g/100 g body wt)	Protein concentration (mg/g wet liver)
Control	25 ± 1*	6.0 ± 0.1*	104 ± 3*
OTP	24 ± 1*	9.0 ± 1.0†	99 ± 4*
ADT	24 ± 1*	7.9 ± 0.4†	116 ± 9*

Male, CF-1 mice received either OTP or ADT (4.5 mmoles/kg, p.o.) at 96 and 48 hr prior to being killed.

*, † Data represent the mean ± SE for five to six animals and were analyzed by ANOVA followed by Duncan's Multiple Range Test. Values not sharing a common superscript within columns are significantly different ($P < 0.05$).

Table 2. Effects of dithiolthiones on hepatic glutathione and related enzymes

	GSH (μmoles/g)	GSSG (μmoles/g)	GSSG-R (nmoles NADPH oxidized/ min/mg protein)	γ-GCS (nmoles P_i released/ min/mg protein)	GSH-Px (nmoles NADPH oxidized/ min/mg protein)	
					H_2O_2	Cumene-OOH
Control	9.7 ± 0.3*	0.5 ± 0.1*	113.0 ± 8*	68 ± 2*	688 ± 61*	695 ± 67*
OTP	14.2 ± 0.9†	0.7 ± 0.2*	258 ± 9†	109 ± 5†	422 ± 51†	619 ± 55†
ADT	12.8 ± 0.9†	0.9 ± 0.2*	189 ± 11‡	87 ± 6‡	519 ± 49†	777 ± 52†

Male, CF-1 mice received either OTP or ADT (4.5 mmoles/kg, p.o.) at 96 and 48 hr prior to being killed.
*-‡ Data represent the mean ± SE for five to six animals and were analyzed by ANOVA followed by Duncan's Multiple Range Test. Values not sharing a common superscript within columns are significantly different ($P < 0.05$).

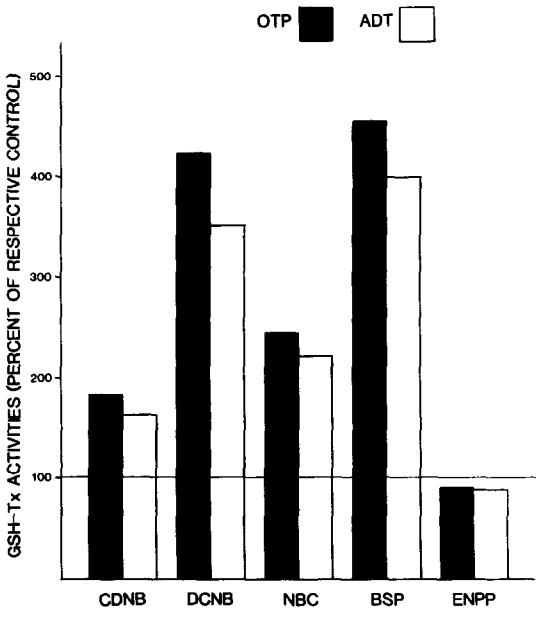

Fig. 1. Effect of DTT treatment on GSH-*S*-Tx activities. Male CF-1 mice received either 4.5 moles of ADT or OTP/kg (p.o.) or vehicle only at 96 and 48 hr prior to being killed. GSH-*S*-Tx activities are presented as percent of control for each substrate. Statistical analysis (ANOVA followed by Duncan's test, $P < 0.05$) was performed using the original data and is expressed as nmoles product formed/min/mg protein. All substrate conjugations in DTT-treated mice, except ENPP, were significantly greater than vehicle-treated animals. Control GSH-*S*-Tx activities were: CDNB, 4076 ± 275; DCNB, 122 ± 7; NBC, 396 ± 28; BSP, 18 ± 1; and ENPP, 258 ± 13.

pretreatment may increase GSH-*S*-Tx-mediated conjugation of electrophilic agents and, thus, produce protective effects against some hepatotoxicants [13].

Decreases in GSH-Px activities were unexpected, considering the marked increases in GSH and GSH-*S*-Tx activities. This is very interesting since some GSH-*S*-Tx are reported to possess GSH-Px activity [14]. These results provide additional evidence that DTT effects possess specificity in the mouse liver.

In summary, OTP and ADT administration produced marked increases in hepatic GSH levels and GCS, GSSG-R, and GSH-*S*-Tx activities. GSH-Px activities were decreased. These changes could alter the toxicity of compounds conjugated with GSH.

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Immunochemical determination of microsomal epoxide hydrolase (preneoplastic antigen) in extrahepatic tissue

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An increase in microsomal epoxide hydrolase (mEH, EC 3.3.2.3) activity is a common response to acute and chronic treatments by a number of diverse xenobiotics [1, 2], whereas only a few or no compounds have been reported to increase the cytosolic and cholesterol EHs respectively [3–8]. Many of these compounds are carcinogens [9–11], and after chronic treatment the increase in mEH activity is greater in hepatic nodules [12–14]. Furthermore, an antigen found specific for preneoplastic and neoplastic liver nodules in rats (preneoplastic antigen) has been identified as mEH [15]. Recently, this preneoplastic antigen has been found to increase in sera of humans [16] and rats [17] with hepatocellular carcinoma. For these reasons there has been speculation that increases in liver and serum mEH activity may be a suitable marker for, and related to, the neoplastic event.

An important consideration in the relationship between the increase in mEH activity and neoplasia is the mechanism of, and tissue localization of, the increase in mEH. For a few carcinogens and promoters the acute increase in mEH activity recently has been associated with an increase in enzyme cotent (i.e. induction) [9, 11]. These studies have been limited to hepatic tissue and confined to genotoxic carcinogens and established promoters of liver cancer. Clofibrate is one of a number of compounds which share the ability to cause proliferation of hepatic and renal peroxisomes and a decrease in serum lipids. All peroxisome proliferators so tested have been found to cause hepatic cancer [18], whereas none has shown any direct genotoxic action [18, 19]. It has been shown recently that clofibrate and other peroxisome proliferators increase mEH activity in liver and kidney microsomes of rats and mice [4, 6, 7, 20–22]. We have now used a new non-competitive double-sandwich enzyme-linked immunosorbent assay (ELISA) to test whether the increase in mEH activity arose from an increase in enzyme content by a nongenotoxic carcinogen. In addition, we have studied the content of mEH in extrahepatic tissue and tested whether these tissues are also responsive to induction.

Male mice (Swiss–Webster, 25–30 g, Bantin–Kingman, Fremont, CA) and rats (Sprague–Dawley, 180–200 g, Charles Rivers, Wilmington, MA) were housed as previously described [22], and treated with clofibrate and phenobarbital (see Table 1). Animals were killed, and liver, kidney and testis microsomes were prepared as previously described [22]. Blood for serum assays was drawn from anesthetized rats by cardiac puncture. The rate of hydrolysis of *cis*-stilbene oxide in microsomes was determined by our previously described radiometric partition assay [23], and *cis*-stilbene oxide hydrolysis in sera and benzo[*a*]-pyrene-4,5-oxide hydrolysis in microsomes were determined by previously described thin-layer chromatography methods [16, 20].

Immunochemical determination of mEH was carried out using a non-competitive double-sandwich ELISA as described in more detail in the legend to Fig. 1. Rat liver microsomes were solubilized by treatment with deoxycholate and centrifuged, and the resulting supernatant fractions were standardized by comparison with the original antigen and used as working standards. Using deoxycholate-solubilized microsomes, the ELISA demonstrated a sensitivity of 1–2 ng EH (Fig. 1). The sensitivity of this non-competitive ELISA was a slight improvement over the 2–5 ng reported previously for our competitive ELISA [24] with the additional advantages that less antigen is required and the final measurement is an increase from background rather than a decrease from 100% absorbance. Inclusion of 0.2% Lubrol PX, used to disperse microsomal samples, affected the slope of the standard (Fig. 1). Lubrol PX was therefore also used during dilution of the standard. The specificity of the ELISA was demonstrated by the negative reactions when cytosolic EH (Fig. 1), cytochrome *c*, ovalbumin, or bovine serum albumin (not shown) were used in place of microsomes and from the negative reaction when preimmune rabbit sera was used as the second antisera (Fig. 2).

Microsomes from control and clofibrate-treated rats and mice gave positive reactions in the ELISA with slopes characteristic of the purified mEH or solubilized microsomes used as standards (Fig. 2). In liver microsomes from control and treated animals, the slope for mouse tissue was consistently 75–85% of rat tissue. A similar difference in slope was also noted with the competitive ELISA [24] and may indicate the immunochemical difference between the rat and mouse mEH. These representative plots also demonstrate greater amounts of liver mEH in rats than in mice and that the quantity of mEH was elevated in both species after clofibrate treatment (Fig. 2). The values for mouse mEH were determined from rat standards and must, therefore, only be considered estimates which depend upon the immunochemical similarity of mEH in the two species. Control male mice had 4.4 μg mEH/mg protein compared to 12.1 μg mEH/mg protein in male rats. The amounts of mEH in rat and mouse liver microsomes are in close agreement with our own [24] and other [9, 11] previous immunochemical determinations of mEH content. In mice,