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Carbon tetrachloride-induced changes in the activity of phase II drug-metabolizing enzyme in the liver of male rats: role of antioxidants

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Abstract

Glutathione S-transferases and glutathione play an important role in the detoxification of most toxic agents. In the present study, the protective effects of some antioxidants (L-ascorbic acid (AA), vitamin E (VE) or garlic) on carbon tetrachloride-induced changes in the activity of alanine amino transferase (ALT), aspartate amino transferase (AST), glutathione S-transferase (GST), and the level of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) were studied. The activities of ALT, and AST were assayed in plasma, whereas the activity of GST and the levels of GSH and TBARS were determined in the livers of rats. The current study included two experiments. In the first experiment, animals received single oral dose of CCl_4 (400 mg/kg body weight) after administration of AA (100 mg/kg b.w.), VE (100 mg/kg b.w.) or garlic (800 mg/kg b.w.) as single oral doses. In the second experiment, rats received repeated oral doses of antioxidants for 12 consecutive days followed by a single oral dose of CCl_4 on the 13th day and killed after that by 24 h. Treatment of male rats with CCl_4 significantly increased the activity of ALT and AST in plasma, and the levels of both GSH and TBARS in the liver. On the other hand, CCl_4 inhibited the activity of GST after single dose treatment. Single-dose treatments of rats with AA, VE or garlic prior to the administration of CCl_4 could not restore the alterations in the activity of ALT and AST caused by CCl_4 to the normal control level. However, repeated dose treatments with these agents restored such alterations to the normal level. We observed that VE is more effective than AA and garlic in restoring the inhibition of GST activity caused by CCl_4 to the normal level after single dose treatments. On the other hand, AA and VE are more effective than garlic in restoring the induced TBARS level caused by CCl_4 to the normal control level after repeated dose treatments. It has been observed that the tested antioxidants were able to antagonize the toxic effects of CCl_4, and such counteracting effects were more pronounced when they were administered as repeated doses prior to administration of CCl_4.

Keywords: Antioxidants; Glutathione; Glutathione S-transferase; Free radicals

Abbreviations: AA, ascorbic acid; ALT, alanine amino transferase; AST, aspartate amino transferase; GSH, glutathione; GST, glutathione S-transferase; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid reactive substances; VE, vitamin E.

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1. Introduction

Four families of glutathione S-transferase termed alpha (α), mu (μ), pi (π), and theta (θ) have been characterized. They have different but sometimes overlapping substrate specificities (Mannervik, 1985; Sato, 1989; Buetler, 1998). Conjugation of toxic metabolites with glutathione (GSH), catalyzed by glutathione S-transferases (GST), is one of the major pathways for the detoxification of toxic metabolites (Jakoby and Habig, 1980). Previous studies have shown that GSH and GST can reduce the covalent binding of epoxides of well-known carcinogens, e.g. benzo(a)pyrene and aflatoxin B_1, to DNA and consequently reduce hepatocarcinogenesis caused by these compounds (Lotlikar et al., 1980; Malaveill et al., 1980; Gopalan et al., 1992, 1994). Several dietary compounds have been demonstrated to reduce gastrointestinal cancer rates in both human and animals through induction of GST activity. For example, sulforaphane, indole-3-carbinol, D-limonene and relafen induced GSTα levels in small intestine and livers, GSTμ levels in stomach and small intestine, GSTπ levels in stomach, small and large intestines (Van Lieshout et al., 1998). The hepatic activity of GSTs and UDP-glucuronyl transferase were induced after administration of aqueous extracts of either green or black tea to rats as the sole drinking fluid for 4 weeks (Van Lieshout et al., 1998). The coffee specific diterpenses cafestol and kahweol have been reported to be anti-carcinogenic in several animal models due to induction of glutathione S-transferase π class (Schilter et al., 1996). On the other hand, many different chemical compounds, parasites, and dietary items were found to inhibit the activity of GST in the livers of mice and also in human bladder tissues (Sheweita and Mostafa, 1996; Sheweita et al., 1997, 1998, 2001; Sheweita, 1998, 2000). In addition, animals treated with acriflavine, a protein kinase c inhibitor, and allyl disulfide showed complete blockage of GST gene expression as early as 12 h of treatment (Kim et al., 1998). Analogues of glutathione preferentially inhibit GSTα, have less effect on μ isozymes, and finally have little effect on rat θ and π isozymes (Ebisawa and Deguchi, 1991; Ouwerkerk-Mahadevan et al., 1995).

Carbon tetrachloride has been widely used to elicit experimental liver damage. CCl_4-induced liver damage has been thought to depend on the formation of reactive intermediates such as trichloromethyl free radical produced by cytochrome P450 mixed function oxidase system (Recknagel et al., 1989), and further converted to a peroxy radical (Slater, 1987). These free radicals react with polyunsaturated fatty acids to propagate a chain reaction leading to lipid peroxidation or bind covalently to lipids and proteins, resulting in destruction of membranes (Slater, 1987; Recknagel et al., 1989). Peroxidation of the polyunsaturated fatty acids in biological membranes is believed to be the mechanism of toxicity for some chemicals such as carbon tetrachloride, ethanol, yellow phosphorus and orotic acid (DiLuzio, 1973; Recknagel et al., 1989). In addition to lipid peroxidation, carbon tetrachloride inhibited the activity of adenosine triphosphatase (ATPase), acetylcholine esterase, and glucose-6-phosphatase in rats (Gonzalez et al., 1996; El-Demerdash et al., 1999), and increased the activity of lactate dehydrogenase (El-Demerdash et al., 1999).

It has been reported that ascorbic acid and vitamin E act as antioxidants (Geetanjali et al., 1993; Guha et al., 1993; Meydani, 1995). Vitamin E is residing mainly in the cell membranes and thus helping to maintain membrane stability (Baker et al., 1996). The role of vitamin C as an antioxidant in the prevention and cure of diseases that result from free radicals has been of considerable interest and controversy lately (Barinaga, 1991). It minimizes the genotoxic effect not only of pesticides (Hoda and Sinha, 1991) but also other xenobiotics. Garlic (*Allium sativum*) is widely used as a part of diet and as a medicine (Al-Bekairi et al., 1990). In addition, garlic had a high antioxidant capacity against peroxy radicals (Cao et al., 1996) and contained active components lowering the levels of lipid and cholesterol (Myung et al., 1982). Considering the multifaceted role-played by vitamin C, vitamin E, and garlic, the present study was, therefore, conducted to investigate the changes in the activities of ALT, AST, GST, and the levels of GSH, and TBARS to evaluate the protective effect of these antioxidants against the toxicity of CCl_4.

2. Materials and methods

2.1. Chemicals

Ascorbic acid (AA) as sodium salt, glutathione, bis-(3-carboxy-4-nitophenyl)-disulfide, thiobarbituric acid, and 1-chloro-2,4-dinitrobenzene were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Garlic powder was obtained from El-Nasr Company for Drying, Alexandria, Egypt. Vitamin E (DL α-tocopherol acetate) was purchased from F. Hoffman–La Roche Ltd, Switzerland. Carbon tetrachloride was obtained from Merck, Germany.

2.2. Animals and administration schedule of CCl_4 and antioxidants

Male Sprague–Dawley rats weighing 130–150 g were used throughout the study. The local ethics committee approved the design of the experiments, and the protocol conforms to the guidelines of the NIH. Animals were housed seven animals per cage where food and water were provided ad libitum. Table 1 summarizes the protocol for administration of CCl_4 and antioxidants. Briefly, in single-dose experiment, carbon tetrachloride was administered directly as a single dose after administration of AA, VE, or garlic (Table 1). In repeated-dose experiment, AA, VE, or garlic were administered for 12 consecutive days and CCl_4 was administered on the 13th day as a single dose. Rats in both single- and repeated-dose experiments were killed after 24 h of CCl_4 administration. The control animals received corn oil as vehicle. The number of animals in each treatment was five rats.

2.3. Enzymes assay

In both experiments, at the designated time point, animals were sacrificed by cervical dislocation. Freshly removed livers were washed and homogenized in 0.1 M potassium phosphate buffer, pH 7.6. The homogenates were centrifuged at $12000 \times g$ for 20 min at 4 °C. The supernatants were used for estimation of glutathione S-transferase activity, glutathione levels, and thiobarbituric acid reactive substances. Glutathione (GSH) levels were estimated in the supernatant of liver tissue homogenates according to the method of Mitchell et al. (1973), using sulfosalicylic acid for protein precipitation and bis-(3-carboxy-4-nitrophenyl)-disulfide for color development. Glutathione S-transferase activity was assayed according to the method of Habig et

Table 1
The protocol for administration of CCl_4 and antioxidants to male rats

Treatment	Experimental groups[a]															
	Single dose experiment (24 h)[b]								Repeated-dose experiment (12 consecutive days)[c]							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Corn oil (1 ml/kg)	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
AA (mg/kg)	–	–	100	–	–	100	–	–	–	–	100	–	–	100	–	–
VE (mg/kg)	–	–	–	100	–	–	100	–	–	–	–	100	–	–	100	–
Garlic (mg/kg)	–	–	–	–	800	–	–	800	–	–	–	–	800	–	–	800
CCl_4 (mg/kg)	–	400	–	–	–	400	400	400	–	400	–	–	–	400	400	400

[a] The number of animals in each group was five rats.

[b] In single-dose experiment, groups 1, 2, 3, 4, and 5 received corn oil as vehicle,CCl_4, AA, VE, and garlic respectively, whereas groups 6, 7, and 8 received a single dose of CCl_4 directly after administration of antioxidants.

[c] In repeated-dose experiment, groups 1, 3, 4, and 5 received corn oil as vehicle, AA, VE, and garlic respectively for 12 consecutive days, whereas groups 6, 7, and 8 received single dose of CCl_4 on the 13th day after administration of antioxidants. In addition, group 2 received single dose of CCl_4 on the 13th day. All animals group in either single- or repeated-dose experiments were killed after 24 h of CCl_4 administration.

al. (1974). The incubation mixture contained 30 μg protein of the liver supernatant fraction, 0.5 ml of glutathione (0.5 mM); 0.1 M sodium phosphate buffer, pH 7.3. After preincubation at 37 °C for 5 min the reaction was initiated by adding 50 μl of 1-chloro-2,4-dinitrobenzene (CDNB) (0.5 mM) in the incubation mixture and incubated at 37 °C for another 5 min. Trichloroacetic acid solution (0.2 ml, 33%) was added to terminate the reaction. After centrifugation, the CDNB conjugate was measured in the supernatant at 340 nm. Calculations were made using a molar extinction coefficient of 9.6 $mM^{-1} cm^{-1}$. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of CDNB conjugate/mg protein/min under the assay conditions. Thiobarbituric acid-reactive substances (TBARS) were measured as described by Tappel and Zalkin (1959). The color intensity of the reactants (TBARS) was measured at 532 nm. An extinction coefficient of 156000 $M^{-1} cm^{-1}$ was used for calculation. The protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard. Monitoring of serum amino transferases activity was carried out in the plasma according to the instructions of the commercially available kits (Boehringer Mannheim GmbH, Germany).

2.4. Statistical analysis

Data analyses were conducted using General Linear Model Procedure of Statistical Analysis System (1995), and the difference between means was compared using least-squares difference (LSD) at 0.05 significant level (Steel and Torrie, 1980).

3. Results and discussion

Glutathione and glutathione S-transferase aid in the protection of cells from the lethal effects of toxic and carcinogenic compounds (Ketterer, 1988). Inducers of GSTs are generally considered as protective compounds against carcinogens. One of the hypotheses explaining the mechanism of chemopreventive activity of antioxidants against carcinogens is that they activate detoxification system such as glutathione S-transferase (Hatono et al., 1996). The human diet contains many compounds that inhibit various steps of the carcinogenic process (Wattenberg, 1985; Nijhoff and Peters, 1994; Hatono et al., 1996). In agreement with these observations, single- and repeated-dose treatments of male rats with AA, VE or garlic induced the activity of glutathione S-transferase relative to CCl_4-treated group (Tables 2 and 3). This induction could be one of the protective mechanisms of AA, VE, or garlic against the toxic metabolites of CCl_4. Supporting this suggestion, it has been found that VE and AA reduced the toxicity and mortality caused in rats by cigarette smoking and aflatoxin B_1 (Chow et al., 1984; Netke et al., 1997). Also, consumption of garlic powder significantly decreased the in vivo binding of 7,12-dimethylbenzo(a)anthracene to mammary DNA and consequently depressed mammary tumors (Lin et al., 1992). Diallyl disulfide, a component of garlic oil, exerted an inhibitory effect in colon and renal carcinogenesis in rats treated with carcinogens (Takahashi et al., 1992). Moreover, ascorbic acid and α-tocopherol acetate reduced the mutagenic effects of some heterocyclic amines (Edenharder et al., 1999).

On the other hand, CCl_4 treatment as a single dose inhibited the activity of glutathione S-transferase in the livers of rats (Table 2). In agreement with the present study, it has been found that carbon tetrachloride and deoxycholic acid decreased the activity of GST in cultured rat hepatocytes (Lee et al., 1991). This inhibition could potentiate the toxicity of CCl_4 upon the liver and probably other organs.

Glutathione is an important cellular factor influencing the effectiveness of a variety of chemotherapeutic and alkylating agents; organs with low GSH levels are more susceptible to the action of these agents, whereas those with high GSH levels are expected to be more protected and therefore resistant (Hamilton et al., 1985). In accordance with these observations, the mechanism of VE protection against the toxicity of CCl_4 might be due to enhancing of GSH level since single- and repeated-dose treatments of rats with VE markedly induced the levels of GSH (Tables 2

Table 2
Influence of AA, VE or garlic after single-dose treatments prior to the administration of carbon tetrachloride on the activity of amino transferases (ALT and AST), glutathione S-transferase, and the levels of glutathione/TBARS in the liver of male rats

Parameters	Treatments							
	Control	CCl_4	AA	VE	Garlic	AA + CCl_4	VE + CCl_4	Garlic + CCl_4
ALT	31.5 ± 0.48^{E}	131.5 ± 7.3^{B}	31.0 ± 2.35^{E}	61.5 ± 4.0^{C}	42.0 ± 1.9^{D}	153.0 ± 4.1^{A}	137.0 ± 1.5^{B}	135.0 ± 1.2^{B}
AST	27.8 ± 3.0^{CD}	184.0 ± 13.0^{B}	21.5 ± 2.4^{D}	26.0 ± 3.4^{D}	51.7 ± 6.7^{C}	203.1 ± 13.2^{AB}	198.0 ± 9.5^{B}	226.0 ± 12.3^{A}
GSH	8.7 ± 1.0^{CD}	13.5 ± 1.1^{A}	13.5 ± 0.8^{A}	14.3 ± 0.7^{A}	10.5 ± 1.0^{BC}	12.2 ± 0.6^{AB}	7.6 ± 0.54^{D}	9.0 ± 0.9^{CD}
GST	0.7 ± 0.02^{A}	0.55 ± 0.01^{CD}	0.7 ± 0.01^{AB}	0.72 ± 0.05^{A}	0.6 ± 0.06^{BC}	0.57 ± 0.02^{CD}	0.65 ± 0.01^{ABC}	0.48 ± 0.02^{D}
TBARS	0.150 ± 0.02^{B}	0.8 ± 0.021^{E}	0.14 ± 0.01^{B}	0.03 ± 0.002^{D}	0.1 ± 0.01^{BC}	0.22 ± 0.04^{A}	0.04 ± 0.01^{CD}	0.12 ± 0.02^{B}

Note: means with different superscript letters differ significantly ($P < 0.05$). Values are the mean ± SE of five male rats of each treatment. ALT, AST activity expressed as international unit (IU), which is defined as the amount of the enzyme that under defined assay conditions would catalyze 1 mol of substrate/min/liter. GSH value was expressed as μM GSH/g. liver. GST activity was expressed as unit/mg protein. TBARS level expressed as μM/g liver.

Table 3
Influence of AA, VE or garlic after repeated-dose treatments prior to the administration of carbon tetrachloride on the activity of amino transferases (ALT and AST), glutathione S-transferase, and the levels of glutathione/TBARS in the liver of male rats

Parameters	Treatments							
	Control	CCl_4	AA	VE	Garlic	AA + CCl_4	VE + CCl_4	Garlic + CCl_4
ALT	38.5 ± 4.2^{C}	132.8 ± 4.6^{A}	33.3 ± 3.8^{C}	31.0 ± 2.2^{C}	28.0 ± 3.25^{C}	116.6 ± 9.9^{B}	43.0 ± 5.7^{C}	116.8 ± 6.0^{B}
AST	15.5 ± 1.2^{CDE}	95.0 ± 10.3^{A}	18.1 ± 2.5^{CD}	16.7 ± 0.84^{DE}	15.0 ± 0.24^{E}	77.0 ± 5.2^{B}	12.6 ± 0.8^{CDE}	22.4 ± 3.0^{F}
GSH	7.7 ± 0.6^{C}	13.5 ± 1.11^{A}	7.5 ± 0.3^{C}	9.6 ± 0.54^{B}	8.6 ± 0.3^{BC}	8.5 ± 0.6^{BC}	11.5 ± 0.7^{A}	8.2 ± 0.6^{BC}
GST	0.8 ± 0.03^{AB}	0.5 ± 0.04^{D}	0.7 ± 0.03^{C}	0.9 ± 0.05^{AB}	0.7 ± 0.02^{BC}	0.6 ± 0.03^{CD}	0.9 ± 0.08^{A}	0.88 ± 0.1^{AB}
TBARS	0.11 ± 0.03^{CD}	0.8 ± 0.08^{A}	0.1 ± 0.02^{CD}	0.04 ± 0.003^{E}	0.1 ± 0.01^{CD}	0.13 ± 0.009^{CD}	0.02 ± 0.003^{E}	0.2 ± 0.01^{B}

Note: means with different superscript letters differ significantly ($P < 0.05$). Values are the mean ± SE of five male rats of each treatment. ALT, AST activity expressed as international unit (IU), which is defined as the amount of the enzyme that under defined assay conditions would catalyze 1 mol of substrate/min/liter. GSH value was expressed as μM GSH/g. liver. GST activity was expressed as unit/mg protein. TBARS level expressed as μM/g liver.

and 3). In agreement with the present study, it has been found that red blood cells glutathione level was significantly greater in rats fed 1500 ppm vitamin E than rats fed 0 or 100 ppm vitamin E (Lii et al., 1998). GSH level did not change after repeated dose treatments of rats for 23 weeks with garlic (Khanum et al., 1998), and this is in agreement with the present study since repeated dose treatments of rats with garlic for 12 consecutive days did not change GSH level (Table 3). However, pretreatment of rats with garlic as single- or repeated-dose treatments restored the induced GSH levels caused by CCl_4 to the normal control levels (Tables 2 and 3). Also, pretreatment of rats with either single dose of VE and repeated dose of AA restored the alteration in GSH to the normal level (Tables 2 and 3). Induction of GSH levels may be due to enhancing of GSH synthesizing enzymes such as γ-glutamylcysteine synthetase and GSH synthetase, which are key enzymes in the biosynthesis of glutathione (GSH) (Griffin, 1982; Seelig and Meister, 1985).

Carbon tetrachloride increased TBARS level after single-dose treatment (Table 2). Interestingly, pretreatment of rats with AA, VE or garlic as repeated doses prior to the administration of CCl_4 was found to reduce the induced level of TBARS caused by CCl_4 (Table 3). This recovery in TBARS level could be one of the protective mechanisms of AA, VE, or garlic against the

toxicity caused by CCl_4 and probably other toxic compounds. Single- and repeated-dose treatments of rats with vitamin E only was found to reduce the level of free radicals (thiobarbituric acid-reactive substances) below the normal levels (Tables 2 and 3). Supporting our present study, it has been reported that vitamin E prevented lipid peroxidation through reduction of free radical level (Kawai-Kobayashi and Yoshida, 1986; Meydani, 1995; Lii et al., 1998) and also exerted a protective effect on CCl_4-induced hepatic fibrosis (Britton and Bacon, 1994).

Serum enzymes including aspartate amino transferase (AST) and alanine amino transferase (ALT) are used in the evaluation of hepatic disorders. An increase in these enzyme activities reflects active liver damage. Inflammatory hepatocellular disorders result in extremely elevated transaminase levels (Fortson et al., 1985; Hultcrantz et al., 1986). In accordance with these findings, CCl_4 treatments has a significant role in the alteration of liver functions since the activity of AST and ALT were significantly higher than those of normal value (Table 2). In agreement with the present study, it has been found that treatment of mice with CCl_4 induced the activity of lactate dehydrogenase (LDH) which can be used as a marker for hepatotoxicity (Animesh et al., 1997). Single-dose treatments with AA, VE or garlic could not recover the induction of AST or ALT activities caused by CCl_4 to the normal control level (Table 2). However, repeated dose treatments of rats with VE or garlic restored the induction of AST caused by CCl_4 to the normal control level (Table 3). Only vitamin E is effective in restoring the activity of ALT activity to the normal level (Table 3). It is obvious that VE is more effective than AA and garlic in recovering the alteration in ALT activity to the normal level after repeated dose treatments (Table 3).

Carbon tetrachloride-induced changes in the activity of glutathione S-transferase, amino transferases (ALT and AST) and also in the levels of reduced glutathione and free radicals. It is concluded that AA, VE or garlic substantially suppressed CCl_4-induced hepatotoxicity especially after repeated-dose treatments.

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