Protective Effects of an Extract of Young Radish (Raphanus sativus L) Cultivated with Sulfur (Sulfur-Radish Extract) and of Sulforaphane on Carbon Tetrachloride-Induced Hepatotoxicity

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The protective effects of an extract of young radish (Raphanus sativus L) cultivated with sulfur (sulfur-radish extract) and of sulforaphane, an isothiocyanate, on carbon tetrachloride (CCl₄)-induced liver injury were observed in mice. CCl₄ produced a marked increase in the serum level of alanine aminotransferase (ALT), primed lipid peroxidation, and resulted in intense necrosis due to oxidative stress. Oral administration of the sulfur-radish extract and of sulforaphane after CCl₄-induced liver injury both decreased the serum level of ALT, reduced the necrotic zones, inhibited lipid peroxidation, and induced phase 2 enzymes without affecting cytochrome P450-2E1 (CYP2E1). These results suggest that the administration of the sulfur-radish extract and of sulforaphane may partially prevent CCl₄-induced hepatotoxicity, possibly by indirectly acting as an antioxidant by improving the detoxification system.

Key words: phase 2 enzyme; radish; sulfur; sulforaphane; CCl₄-induced hepatotoxicity

Oxidative stress is one of the mechanisms involved in the acute hepatotoxicity induced by carbon tetrachloride (CCl₄). CCl₄ undergoes biotransformation by hepatic microsomal cytochrome P450 to produce the hepatotoxic metabolites and trichloromethyl free radicals, CCl₃ and CCl₃OO. These free radicals may interact with cellular proteins and membrane lipids to cause peroxidation of the membrane lipids, ultimately leading to cell necrosis. When the production of free radicals exceeds the capacity of an endogenous cellular antioxidant system, significant cellular injury may result. Although treatment with antioxidants has been demonstrated to reduce hepatotoxicity by decreasing CCl₄-induced liver damage, such protection may not be completely effective, especially during increased oxidative stress. Nonetheless, the development of methods for enhancing endogenous cellular defense has resulted in widespread human consumption of plant-based antioxidants such as ascorbic acid, tocopherols, carotenoids, and polyphenols.

An alternative and more effective strategy for combating the toxicity of reactive oxygen species (ROS) has recently emerged through the study of inducers of phase 2 detoxification enzymes. Sulforaphane, an isothiocyanate isolated from broccoli, has been identified as the most potent naturally occurring inducer of phase 2 enzymes to date. Isothiocyanates, which are released upon chewing or macerating certain cruciferous plants, are a metabolite of glucosinolates which are thioglycoside compounds contained in cruciferous plants. Myrosinase, which is released from a different cellular compartment, hydrolyzes glucosinolate-producing isothiocyanates as well as other products. A substantial amount of glucosinolates is present in a wide variety of cruciferous dietary vegetables. The major effect of isothiocyanates is to activate phase 2 detoxification enzymes and increase the glutathione level. Indeed, the induction of quinone reductase (QR), glutathione S-transferase (GST), and glutathione reductase by isothiocyanates has been demonstrated in several cell lines, including murine hepatoma, a BPrc1 p450-deficient mutant, and human adult retinal pigment epithelial cells, as well as in mouse liver, stomach, small intestine, and lung. One such isothiocyanate, sulforaphane, cannot react directly with free radicals or ROS; its function as an antioxidant is secondary to its role as a phase 2 enzyme inducer. Protection against oxidative damage by sulforaphane has also been detected in the form of enhanced cell survival after treatment with oxidants.

In the present study, a sulfur-radish extract and sulforaphane were separately administered to reduce CCl₄-induced liver injury in mice. Twenty-four hours

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after the oral administration of CCl₄, the liver and blood of mice were evaluated to determine whether the animals had been protected against hepatotoxicity.

Materials and Methods

Materials. Carbon tetrachloride (CCl₄), sulforaphane, PMSF, aprotinin, leupeptin, Trizma base, BSA, FAD, NADH, 2,6-dichloroindophenol (DCP), Tween 20, 1-chloro-2,4-dinitrobenzene (CDNB), thiourea, 2,4-dinitrophenylhydrazine, ascorbic acid, 2-thiobarbituric acid (TBA), 1-butanol, p-nitrophenol (PNP), NADPH, and dicumarol were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). A serum alanine aminotransferase (ALT) determination kit was purchased from Asan Chemical Co. (Kyunggi-do, Korea). Young radishes were cultivated in soil in the presence of sulfur (1,818 g/m³) at the Agricultural Institute of Kyungsang Namdo (Jinjoo, Korea).

Preparation of the sulfur-radish extract. Leaves of young radishes were washed with distilled water, suspended in 80% methanol, and disrupted for 5 min by an ice-chilled bead beater (Biospec Products, Bartleville, OK, USA). The homogenate was filtered through several layers of cheesecloth to remove the debris, and the filtrate was centrifuged at 10,000 × g for 40 min in a refrigerated centrifuge (T-324 with an A-8.24 rotor; Kontron Instruments, Zurich, Sweden). The supernatant was evaporated to remove methanol, dissolved in distilled water, and concentrated completely by ultrafiltration through an Amicon YM 10 membrane to distilled water, and concentrated completely by ultrafiltration through an Amicon YM 0.5 membrane to remove the high-molecular-weight products. The low-molecular-mass fractions were sterilized and dissolved for administration to mice. Each gram of the sulfur-radish extract yielded approximately 5 mg of an isothiocyanate-like compound by HPLC.

Animal experiments. Male C57BL/6 mice were obtained from Orient (Pusan, Korea). All animals were maintained under standard conditions (temperature of 24°C, light/darkness cycle of 12 h) with free access of food (standard Purina diet) and water. All procedures were conducted in accordance with the Guidelines for the Care and Use of laboratory Animals approved by the Animal Care Committee of the Immunomodulation Research Center, University of Ulsan, Ulsan, Korea. Acute liver injury was induced by a dose of CCl₄ (4 g/kg of body weight) dissolved in corn oil (1:1). The compound was initially dissolved in dimethyl sulfoxide and was further diluted with saline prior to its administration. The animals were sacrificed, and a blood sample was withdrawn from each by cardiac puncture. Plasma was separated from sodium citrate-treated whole blood by centrifugation at 1500 × g for 15 min at 4°C and was used to assay the serum ALT activity. The serum ALT level was measured with a spectrophotometric diagnostic kit (Asan Chemical Co., Kyunggi-do, Korea). The serum was stored at −80°C until needed for analysis. In addition, the liver was excised, a thin slice being preserved in buffered formalin to obtain histological sections; the rest was stored frozen for future assessment of the liver enzyme activities and lipid peroxidation.

Histological examination. Fresh liver tissue samples, which had been trimmed to a thickness of 2 mm, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. The fixed tissue samples were embedded in paraffin, sectioned, deparaffinized, and dehydrated by using standard techniques. The extent of CCl₄-induced necrosis was evaluated by assessing the morphologic changes in the liver sections stained with hematoxylin and eosin (H and E) by using standard techniques.

Hepatic lipid peroxidation. TBA-reactive substances (TBARS) in the liver homogenate was determined, using malondialdehyde (MDA) as a standard, for use as an index of lipid peroxidation. A plot of the absorbance at 535 nm (A₅₃₅) vs. [MDA] for a standard curve was constructed from the equation of A₅₃₅ = 0.64 [MDA, μg] + 0.005. One g of liver was homogenized in KCl (1.15%, w/v), and the homogenate was filtered through folded gauze. The liver homogenate was then mixed with H₃PO₄ (1%, v/v) and TBA (0.6%, w/v), and heated at 100°C for 45 min. An equal volume of 1-butanol was added to the reaction mixture, and the butanol phase was obtained by centrifugation at 400 × g for 10 min to determine A₅₃₅.

Phase 2 enzyme activity. Whole livers were washed, and sliced. The minced cells were homogenized for 5 min and left on ice for 10 min. The homogenate was then centrifuged at 13,000 × g for 30 min at 4°C. The resulting supernatant was transferred to a new tube, and the protein concentration was determined by a Bradford assay (Bio-Rad). The activity of cytosolic GST was determined with a spectrophotometer by incubating the liver extract with CDNB at 25°C. The reaction mixture contained 100 mM KPO₄ at pH 6.5, 1 mM glutathione, and 1 mM CDNB. The reaction was started by adding a cytosolic extract. QR was determined by measuring the reduction of 2,6-dichloroindophenol, the reaction mixture containing 25 mM Tris–HCl at pH 7.4, 60 μg of bovine serum albumin, 5 μM FAD, 0.2 mM NADH, 80 μM DCP, and 0.01% Tween 20 in 1 ml. The reaction was incubated for 5 min at room temperature and then terminated by adding 30 μM dicumarol. The absorbance
of the reaction mixture was read on a spectrophotometer at 600 nm.

**PNP hydroxylation activity.** The incubation system to assay the PNP hydroxylation activity consisted of 100 mM KPO$_4$ at pH 7.4 containing 0.5 mg of microsomal protein, 0.1 mM PNP, and 1 mM NADPH in a volume of 1 ml. After incubating the reaction at 37°C for 15 min, the reaction was stopped with TCA (5%, w/v). The absorbance was determined at 510 nm after neutralization.

**Statistical analysis.** Each value is presented as the mean ± SEM. Student’s t-test (unpaired) was used to evaluate the differences between samples of interest and the corresponding controls. $P < 0.05$ was considered statistically significant.

**Results**

CCl$_4$ was given intragastrically to each group of mice 1 h before administering the sulfur-radish extract or sulforaphane, and the serum level of ALT was subsequently determined. The administration of a single dose of CCl$_4$ resulted in a significant increase of the serum level of ALT due to liver injury. Treatment with the sulfur-radish extract (Fig. 1A) or sulforaphane (Fig. 1B) after CCl$_4$ dosing was significantly different from the group that was administered with CCl$_4$ alone (#, $P < 0.01$, n = 5; ###, $P < 0.001$, n = 5), whereas the level of the groups administered with the sulfur-radish extract and sulforaphane was not significantly different from oil-fed group.

**Fig. 1.** Effects of the Sulfur-Radish Extract (A) and Sulforaphane (B) on the CCl$_4$-Induced Increase in the Serum ALT Level in Mice.

The sulfur-radish extract (R; 0.2, 2 g/kg) and sulforaphane (S; 2.5, 25 mg/kg) were each separately administered 1 h after CCl$_4$ dosing (4 g/kg). Twenty-four hours after the CCl$_4$ administration, the serum ALT level was determined. Each result is presented as the mean ± SEM. The serum ALT level of the groups receiving the sulfur-radish extract (0.2, 2 g/kg) and sulforaphane (2.5, 25 mg/kg) after CCl$_4$ was significantly different from the group that was administered with CCl$_4$ alone (#, $P < 0.01$, n = 5; ###, $P < 0.001$, n = 5), whereas the level of the groups administered with the sulfur-radish extract and sulforaphane was not significantly different from oil-fed group.

**Fig. 2.** Effect of the Sulfur-Radish Extract (A) and Sulforaphane (B) on the CCl$_4$-Induced Increase of Liver TBARS in Mice.

The sulfur-radish extract (R; 0.2, 2 g/kg) and sulforaphane (S; 2.5, 25 mg/kg) were each separately administered 1 h after CCl$_4$ dosing (4 g/kg). Twenty-four hours after the CCl$_4$ administration, the animals were sacrificed, and the livers were harvested. The liver TBARS content was determined as described in the Materials and Methods section. Each result is presented as the mean ± SEM. There was no significant difference between the groups that were administered with the sulfur-radish extract (2 g/kg) or sulforaphane (2.5, 25 mg/kg) and the control group in the liver TBARS content. The liver TBARS content of the groups that were administered with the sulfur-radish extract (2 g/kg) and sulforaphane (2.5, 25 mg/kg) after CCl$_4$ dosing was significantly different from the group that was administered with CCl$_4$ alone (#, $P < 0.05$, n = 5; ##, $P < 0.01$, n = 5), whereas that of the groups administered with the sulfur-radish extract and sulforaphane was not significantly different from the oil-fed group.
observed from the sulfur-radish extract. We next determined the effect of orally administering the sulfur-radish extract or sulforaphane following CCl₄ administration on the induction and activities of the phase 2 enzymes, QR (Fig. 3A and B) and GST (Fig. 3C and D) in the liver. The groups treated with the sulfur-radish extract (0.2, 2 g/kg) or sulforaphane (25 mg/kg) after CCl₄ were significantly different from the group that was administered with CCl₄ alone (##, P < 0.01, n = 5). The liver GST activities of the groups that were administered with the sulfur-radish extract (0.2, 2 g/kg) or sulforaphane (25 mg/kg) after CCl₄ were significantly different from the group that was administered with CCl₄ alone (##, P < 0.01, n = 5; ###, P < 0.001, n = 5). The liver GST activities of the groups that were administered with sulfate (0.2, 2 g/kg) or with sulforaphane (25 mg/kg) after CCl₄ were significantly different from the group that was administered with CCl₄ alone (##, P < 0.01, n = 5; ###, P < 0.001, n = 5).

Fig. 3. Effects of the Sulfur-Radish Extract (A, C) and Sulforaphane (B, D) on the CCl₄-Induced Decreases of Liver Quinone Reductase (A, B) and GST (C, D) in Mouse Liver.

The sulfur-radish extract (R; 0.2, 2 g/kg) and sulforaphane (S; 2.5, 25 mg/kg) were each separately administered 1 h after CCl₄ dosing (4 g/kg). Twenty-four hours after the CCl₄ administration, the animals were sacrificed, and their livers were harvested. Each liver extract was prepared as described in the Material and Methods section, and the protein concentration was determined by a Bradford assay. QR was determined by measuring the reduction of 2,6-dichloroindophenol at 600 nm. Each result is the mean ± SEM. There was a significant difference between the groups that were administered with the sulfur-radish extract (0.2, 2 g/kg) or sulforaphane (25 mg/kg) and the control group in the liver QR activity (**, P < 0.01, n = 5). The liver QR activities of the groups that were administered with the sulfur-radish extract (0.2, 2 g/kg) or sulforaphane (2.5, 25 mg/kg) after CCl₄ were significantly different from the group that was administered with CCl₄ alone (#, P < 0.05, n = 5; ##, P < 0.01, n = 5; ###, P < 0.001, n = 5). The activity of cytosolic GST was determined at 340 nm by the formation of CDNB conjugates. The reaction was started by the addition of the cytosolic extract. There was a significant difference between the groups that were administered with the sulfur-radish extract (0.2, 2 g/kg) or sulforaphane (25 mg/kg) and the control group in their liver GST activities (*, P < 0.05, n = 5; ***, P < 0.001, n = 5). The liver GST activities of the groups that were administered with the extract of young radish cultivated with sulfur (0.2, 2 g/kg) or with sulforaphane (25 mg/kg) after CCl₄ were significantly different from the group that was administered with CCl₄ alone (##, P < 0.01, n = 5; ###, P < 0.001, n = 5).

Discussion

We have demonstrated that both sulfur-radish extract and sulforaphane reduced the CCl₄-induced hepatotoxicity in mice. An oral administration of the sulfur-radish extract or sulforaphane obviously reduced the liver injury from a histological examination together with a significant improvement in the biochemical parameters for liver injury already observed.
with an amelioration of the biochemical parameters for liver injury. The treatment lowered the number of lipid droplets and necrotic zones in the hepatocytes, both of which were eminently increased after the ingestion of CCl₄. Each treatment also prevented the CCl₄ effects on lipid peroxidation and induced the phase 2 enzymes, QR and GST. The administration of CCl₄ lowered the level of QR, but not that of GST. CCl₄ requires activation by liver cytochrome P450 to produce free radicals (CCl₃ and CCl₃OO), and these trichloromethyl free radicals can react with sulfhydryl groups; subsequently covalent binding of the free radicals to cellular proteins is considered to be an initial step in a chain of events that eventually leads to membrane lipid peroxidation and finally to necrosis. Although several isoforms of cytochrome P450 can react with CCl₄, an altered level of P450 2E1 has been reported to affect the susceptibility to hepatic injury by CCl₄. The generated free radicals initiate and promote the propagation of lipid peroxidation which can be detected by the liver TBARS content. The liver TBARS content in the CCl₄-induced mice was increased, and an oral administration of the sulfur-radish extract or sulforaphane reduced the severity of lipid peroxidation. Treatment with the sulfur-radish extract or sulforaphane alone did not significantly change the liver TBARS content or cytochrome P450 2E1 level. Based on these results, the induction of phase 2 enzymes by the sulfur-radish extract or sulforaphane could act directly or indirectly to restrain CCl₄-induced hepatotoxicity.

Our previous results have demonstrated that a sulfur-radish extract contained isothiocyanate-like compounds from an HPLC analysis (approximately 5.0 mg/g of the sulfur-radish extract; 1.9 mg/g of the radish extract) and induced quinone reductase-inducing activity in Hepa 1c1c cells, although we have not yet elucidated the structure of the isothiocyanate-like compounds. We have also shown that a sulfur-radish extract protected against the pulmonary colonization induced by B16-F10 melanoma cells with the concomitant induction of phase 2 enzymes. Sulforaphane, a naturally occurring isothiocyanate, has been shown to be a potent inducer of antioxidant-response element (ARE)-regulated genes such as phase 2 enzymes in cultured cells and mouse tissues in vivo, and a modulator of nuclear transcription factor erythroid 2p45-related factor 2 (Nrf2). In addition, sulforaphane directly binds Kelch-like ECH-associated protein 1 (KEAP1) which sequesters Nrf2 in cytoplasm and prevents translocation to the
The chemopreventive activity of sulforaphane has been diminished in Nrf2-deficient mice, indicating that the Nrf2-ARE signaling pathway played a role in the action of sulforaphane. Phase 2 enzymes perform their protective functions not only by inactivating the electrophiles produced in a cellular environment, but also through their antioxidative activities. Fruits, vegetables, and several herbs have been shown to be rich sources of chemopreventive agents that can enhance the activities of toxicant-metabolizing enzymes and to bind with toxicants, thereby reducing their effective critical concentration. Chemopreventive agents also act as antioxidants and counteract the increased amount of oxidants generated by toxicants. The collective action of both antioxidants and such phase 2 enzymes as GST and QR, besides acting on small non-enzymatic water-soluble biomolecules, is to protect against the adverse effects of oxidants. Sulforaphane has also shown to have a protective effect on CCl₄-induced hepatotoxicity. Thus, it is reasonable to assume that the increased activities of GST and QR in the liver played a critical role in relation to the observed CCl₄-induced hepatotoxicity in mice treated with the sulfur-radish extract or sulforaphane. Our study bolsters the validity of the prediction that chemical compounds that induce phase 2 enzymes are promising candidates for chemoprotection from various oxidants.

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