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Diallyl Trisulfide Modulates Cell Viability and the Antioxidation and Detoxification Systems of Rat Primary Hepatocytes

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ABSTRACT This study investigated the effects of various concentrations of diallyl trisulfide (DATS) and incubation times on cell viability, glutathione (GSH) content, and GSH-related enzyme activity in rat primary hepatocytes. Isolated and cultured primary rat hepatocytes were used as an experimental model. Cells were treated with 0 (control), 0.025, 0.05, or 0.25 mmol/L DATS for 0, 4, 8, or 24 h. After 24 h of treatment, some cells were incubated in fresh medium without DATS for an additional 24 h (48-h incubations). Based on lactate dehydrogenase (LDH) leakage and morphological examination, hepatocytes treated with 0.025 mmol/L DATS did not differ from the control cells at 4, 8, 24, and 48 h of incubation. However, LDH leakage was higher than in the control cells (P < 0.05) when the hepatocytes were treated with 0.05 or 0.25 mmol/L DATS for 4 h or more. The intracellular GSH levels of hepatocytes treated with 0.025 or 0.05 mmol/L DATS were higher than those of the control cells (P < 0.05), whereas those treated with 0.25 mmol/L DATS did not differ. The activity of glutathione reductase (GRd) was higher than in the control cells at 24 h (P < 0.05) when the hepatocytes were treated with 0.025 mmol/L DATS. When the hepatocytes were treated with 0.25 mmol/L DATS, the activity of glutathione S-transferase (GST) was higher than in the control cells at 48 h (P < 0.05). In hepatocytes treated with 0.05 mmol/L DATS, the activity of GST and glutathione peroxidase (GPx) was higher than in the control cells (P < 0.05) at 24 and 48 h of incubation. The results indicate that 0.025 or 0.05 mmol/L DATS could enhance antioxidation and detoxification capabilities by increasing the intracellular GSH level and the activity of GPx, GRd, or GST in rat primary hepatocytes. However, 0.05 or 0.25 mmol/L DATS might adversely affect the viability of hepatocytes. J. Nutr. 134: 724–728, 2004.

KEY WORDS: • diallyl trisulfide • hepatocytes • cell viability • detoxification • antioxidation

Garlic (Allium sativum L.) is a commonly used foodstuff. Steam distillation is widely used to extract volatile organosulfur compounds (OSCs) from garlic, and the final oily product is known as garlic oil (GO). More than 20 OSCs have been identified in GO by gas chromatography (1). Among these, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are three major components. Garlic oil and its component OSCs are reported to benefit the hepatic antioxidant and detoxification systems and to elicit antibiotic, antiatherosclerotic, immunomodulatory, renoprotective, antimutagenic, and anticarcinogenic properties (2–10).

We previously demonstrated that 1 mmol/L DAS and 0.5 mmol/L DADS might enhance the detoxification and antioxidation capabilities of rat primary hepatocytes (11,12). The symmetrical compound DATS, which contains 2 allyl groups and 3 sulfur atoms, is similar to DAS and DADS in structure. Studies report that DATS shows antiplatelet activity by inhibiting platelet thromboxane formation (13) and inhibits cholesterol biosynthesis by inhibiting the activity of squalene monoxygenase (14). A previous study with mice suggested that treatment with DATS, DADS, and DAS increases both hepatic and forestomach glutathione S-transferase (GST) activity toward anti-7β,8α-dihydroxy-9α,10α-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene, which is the ultimate carcinogen of benzo(a)pyrene (BP), thereby preventing BP-induced cancer in mice (15). Furthermore, treatment with DADS and DATS, which are potent inhibitors of BP-induced forestomach tumorigenesis, greatly increases forestomach NAD(P)H:quinone oxidoreductase (NQO) activity in mice; DADS and DATS increase forestomach NQO activity much more than does DAS (16). In addition, 10 μmol/L DATS causes a marked and progressive increase in intracellular Ca2+ in A549 cells, and exposure to 1 μmol/L DATS for 24 h significantly induces apoptosis, as indicated by increased DNA fragmentation (17). Diallyl trisulfide also augments the activation of T cells and enhances the antitumor function of macrophages, suggesting that it may be useful in tumor therapy (18).

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3 Abbreviations used: BP, benzo(a)pyrene; BSA, bovine serum albumin; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; GO, garlic oil; GPx, glutathione peroxidase; GRd, glutathione reductase; GSH, glutathione; GST, glutathione S-transferase; LDH, lactate dehydrogenase; NQO, NAD(P)H:quinone oxidoreductase; OSC, organosulfur compound.

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724
However, the optimum concentration of DATS for the antioxidant and detoxification systems in rat primary hepatocytes is not known. Therefore, the present study investigated the effects of various concentrations of DATS and incubation times on cell viability, glutathione (GSH) content, and GSH-related enzyme activity in rat primary hepatocytes.

MATERIALS AND METHODS

Materials. Diallyl trisulfide was purchased from LKT Laboratories. Supplies of t-15 medium, fetal bovine serum (FBS), and penicillin/streptomycin solution were purchased from Gibco Laboratories. Bovine serum albumin (BSA), HEPES, β-NADH, β-NAD(P)H, SDS, 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane, GSH, oxidized glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), sodium azide (NaN₃), glutathione reductase (GRd), and iodoacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase (type I) and Percoll solution were purchased from Worthington Biochemical and Pharmacia LKB, respectively. Antiserums for the glutathione S-transferase (GST) Ya, Yb, and Yc isoforms were purchased from Biotrin.

Cell isolation and culture. Male Sprague-Dawley rats were purchased from the Taiwan National Animal Breeding and Research Center. At the age of 8 wk, the rats were used for hepatocyte isolation in the following experiments. The rats were housed in stainless steel grid cages with an artificial 12-h light–dark cycle, had free access to food (Laboratory Rodent Diet; PMI Feeds) and water, and were treated in compliance with the 1985 NRC guidelines (19).

Rat hepatocytes were prepared by collagenase perfusion as described by Berry and Friend (20), Bonney et al. (21) and our earlier report (11). The isolated hepatocytes (2.5 × 10⁶) were plated on a 60-mm collagen-precoated plastic tissue culture dish (Falcon Labware) and incubated in a humidified incubator (NUAIRE) at 37°C in an air atmosphere. At 4 h after plating, the culture medium was replaced with fresh medium containing 2.0 g/L of BSA instead of FBS. The medium was then changed daily at 20 h after plating.

Treatment. At 20 h after plating, cells (2.5 × 10⁶) were treated with 0, 0.025, 0.05 or 0.25 mmol/L DATS (final concentration) for 0, 4, 8, and 24 h. Furthermore, to investigate the potency of DATS, some cells treated for 24 h were then incubated in fresh medium without DATS for an additional 24 h, for a total of 48 h of incubation. At various time intervals, the reaction was stopped by removing the medium and washing with cold PBS. Cells were removed with a cell scraper for further analysis.

Cell viability. The effect of various concentrations of DATS on the viability of primary hepatocytes was evaluated by lactate dehydrogenase (LDH) leakage and microscopic examination. Intra- and extracellular LDH activity was assayed according to the method of Moldeus et al. (22). Photomicrographs were taken of the monolayer cultures for morphological examination, using a Nikon Diaphot 300 inverted-stage microscope equipped with phase contrast and camera attachments.

Analysis for GSH and its related enzymes. Samples for GSH assay were prepared by adding 1 mL of 1 mol/L perchloric acid containing 2 mmol/L 1,10-phenanthroline to each dish. The plates were scraped and the contents centrifuged at 10,000 × g for 10 min. The acid-soluble GSH in the hepatocellular supernatant was measured by HPLC (23). Gluthione peroxidase (GPx) activity was assayed spectrophotometrically, using hydrogen peroxide as the substrate (24). Gluthione reductase activity was assayed as described by Bellomo et al. (25). Gluthione S-transferase activity in hepatic cytosol was assayed according to the method of Habig et al. (26), using CDNB as the substrate. Protein concentration of the cells was assayed by the method of Lowry et al. (27).

Analysis by SDS-PAGE and Western blot. Levels of GST isozyme expression were assayed by SDS-PAGE and immunoblot analysis. Equal amounts of cytosol protein were treated with 10% polyacrylamide gel and electrophoresed as described by Laemmli (28). After electrophoresis, the proteins separated on SDS-polyacrylamide gel were transferred to polyvinylidene difluoride membrane. To block the nonspecific binding, the membrane was incubated overnight at 4°C with 5% skim milk, followed by a 30 min incubation at 37°C with an antiserum mixture containing antibodies for GST Ya, Yb, and Yc isoforms. Peroxidase-conjugated secondary antibody (Vector Laboratory) was used to detect the immunoreactive bands. The intensity of each immunoreactive band was quantified by an image analyzer (AlphaInnomet; Alpha Innotech) equipped with AlphaEase software (Alpha Innotech).

Statistical analysis. Statistical analysis was performed using SAS software (SAS Institute); ANOVA and Duncan’s multiple range test were used to identify significant differences among means (P < 0.05).

RESULTS

Cell viability. Lactate dehydrogenase leakage from cells incubated with 0.025 mmol/L DATS did not differ from that of the control cells during the 48-h incubation (Fig. 1). However, LDH leakage from cells incubated with 0.05 and 0.25 mmol/L DATS for 4, 8, 24, and 48 h was greater than from the control cells (P < 0.05). In addition, microscopic observation showed no changes in the morphology of hepatocytes treated with 0.025 mmol/L DATS, compared with that of the control cells (Fig. 2B, F, J, N), whereas hepatocytes incubated with 0.05 mmol/L (Fig. 2K, O) and 0.25 mmol/L DATS (Fig. 2L, P) for 24 and 48 h did show morphological changes.

Cell GST concentration. Hepatocytes treated with 0.025 and 0.05 mmol/L DATS for 4 h or more had higher intracellular GST levels than the control cells (70–80 and 30–40%, respectively; P < 0.05; Fig. 3). Conversely, intracellular GST levels of hepatocytes treated with 0.25 mmol/L DATS did not differ from those of the control cells at any incubation time.

Cell GPx, GRd, and GST activity. The level of GPx activity in hepatocytes treated with 0.025 mmol/L DATS...
tended to be greater than in the control cells at 24 and 48 h (P = 0.1), and was greater in cells treated with 0.05 mmol/L DATS (P < 0.05; Table 1). Cells treated with 0.25 mmol/L DATS did not differ from untreated hepatocytes.

The level of GRd activity in hepatocytes treated with 0.025 mmol/L DATS was greater than in the control cells at 24 h (P < 0.05; Table 1). Cells treated with 0.25 mmol/L DATS did not differ from untreated hepatocytes.

The level of GST activity in hepatocytes treated with 0.025 or 0.05 mmol/L DATS was greater than in the control cells at 24 and 48 h (P < 0.05; Table 1). Cells treated with 0.25 mmol/L DATS did not differ from untreated hepatocytes.

**Cell GST isozyme expression.** The band intensity of GST Ya, Yb, and Yc expression (Fig. 4), quantified by an image analyzer (Table 2), showed that the expression of all 3 GST isozymes was greater in hepatocytes treated with 0.025, 0.05, and 0.25 mmol/L DATS than in the control cells at 24 and 48 h (P < 0.05). The expression of GST was greatest in hepatocytes treated with 0.05 mmol/L DATS for 48 h. The expression levels of GST Ya, Yb, and Yc in hepatocytes treated with 0.025, 0.05, and 0.25 mmol/L DATS were ~1.9–3.0, 2.5–4.4, and 2.1–4.0 times the control values at 24 h and 2.0–3.9, 2.4–4.8, and 2.0–4.0 times the control values at 48 h.

**DISCUSSION**

Isolated and cultured rat primary hepatocytes provide a useful model for studies of liver cell function in vitro (29). Cell viability was determined by LDH leakage assay and microscopic examination. The LDH leakage assay is both sensitive and specific as an indicator of plasma membrane damage. Results indicated that incubation with 0.025 mmol/L DATS for 48 h did not affect the viability of hepatocytes, but treatment with 0.05 and 0.25 mmol/L DATS for 24 h increased LDH leakage (P < 0.05). However, after 24 h of incubation, LDH leakage decreased when the culture medium was replaced with fresh medium without DATS.

These results indicated that the viability of hepatocytes might recover in the absence of treatment with higher concentrations of DATS. Hepatocyte LDH leakage was dose-dependent on the DATS concentration. However, treatment with 0.05 and 0.25 mmol/L DATS caused marked morphological changes in hepatocytes after 24 and 48 h of incubation.
Microscopic examination showed close correlation with the LDH leakage data. The organosulfur compounds DAS, DADS, and DATS are the major components of garlic oil (1). The LDH leakage data from the present study indicate that the cytotoxicity of DATS is much higher than that of DAS and DADS, in contrast to the data published by Sheen et al. (11,12). The present results support the data published by Sundaram and Milner (30), which showed that DAS, DADS, and DATS markedly inhibited the growth of canine mammary tumor cells (CMT-13). The effectiveness of DAS, DADS, and DATS was related to the number of sulfur atoms and was ranked in the following order: DATS > DADS > DAS. Diallyl trisulfide is more reactive with the thiol group of cysteine than DADS (31).

The selective toxicity of DATS to lung neoplastic liver cells. The selective toxicity of DATS to lung neoplastic cells. The selective toxicity of DATS to lung neoplastic cells was related to the number of sulfur atoms and was ranked in the following order: DATS > DADS > DAS. Diallyl trisulfide is more reactive with the thiol group of cysteine than DADS (31).

24 0 8.0 5.1 8.8
0.025 15.5 12.5 18.5
0.05 24.2 20.1 30.0
0.25 24.3 22.1 34.9
48 0 8.5 5.8 9.9
0.025 17.7 13.7 20.2
0.05 33.0 27.6 39.7
0.25 26.6 24.9 38.8

1 Values are means ± SD, n = 3. Means in a column at a time without a common letter differ, P < 0.05.
2 After incubating with DATS for 24 h, some cultures were incubated in fresh medium for another 24 h (48-h cultures).
LITERATURE CITED