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The involvement of Nrf2 in the protective effects of diallyl disulfide on carbon tetrachloride-induced hepatic oxidative damage and inflammatory response in rats

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ABSTRACT

This study investigated the potential effect of diallyl disulfide (DADS) against carbon tetrachloride (CCl_4)-induced oxidative hepatic damage and inflammatory response in rat liver. DADS at doses of 50 and 100 mg/kg/day was administered orally once daily for 5 days, prior to CCl_4 administration. Pretreatment with DADS attenuated CCl_4-induced elevated serum transaminase activities and histopathological alterations in liver. It prevented the hepatocellular apoptotic changes with induction of Bcl-2-associated X (Bax), cytochrome *c*, and caspase-3 caused by CCl_4. An increase in the nuclear translocation of nuclear factor-kappaB (NF-κB) and phosphorylation of I kappaB alpha (IκBα) was observed in the livers of CCl_4-treated rats that coincided with induction of inflammatory mediators or cytokines. In contrast, DADS inhibited NF-κB translocation and IκBα phosphorylation, and that subsequently decreased inflammatory mediators. Furthermore, DADS prevented CCl_4-induced depletion of cytosolic nuclear factor E2-related factor 2 (Nrf2) and suppression of nuclear translocation of Nrf2, which, in turn, up-regulated phase II/antioxidant enzyme activities. Taken together, these results demonstrate that DADS increases the expression of phase II/antioxidant enzymes and simultaneously decreases the expression of inflammatory mediators in CCl_4-induced liver injury. These findings indicate that DADS induces antioxidant defense mechanism by activating Nrf2 pathway and reduces inflammatory response by inhibiting NF-κB activation.

1. Introduction

Carbon tetrachloride (CCl_4) is widely used in animal models to induce acute liver injury and to evaluate the protective effects of drugs against such injury because CCl_4-induced liver damage is regarded as the analog of liver damage caused by a variety of hepatotoxins (Reyes-Gordillo et al., 2007; Rudnicki et al., 2007). Hepatotoxicity caused by CCl_4 is thought to be mediated by at least two sequential processes. The initial phase involves reductive dehalogenation by the cytochrome P450 isoenzymes (CYPs) system in the microsomal compartment of the liver, into the highly reactive trichloromethyl free radical, which initiates lipid peroxidation and leads to hepatocellular membrane damage (Lee and Jeong, 2002; Recknagel et al., 1989; Wong et al., 1998). The second phase implicates the release of inflammatory mediators from activated hepatic macrophages, which are thought to potentiate CCl_4-induced hepatic injury (Badger et al., 1996; el Sisi et al., 1993). During the deteriorating phase, induction of inflammatory mediators including tumor

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; ARE, antioxidant response element; AST, aspartate aminotransferase; Bax, Bcl-2-associated X; CCl_4, carbon tetrachloride; Cox-2, cyclooxygenase-2; CYPs, cytochrome P450 isoenzymes; DADS, diallyl disulfide; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSTα, glutathione *S*-transferase alpha; H&E, hematoxylin and eosin; HO-1, heme oxygenase-1; IκBα, I kappaB alpha; IKK, IκB kinase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; Keap1, Kelch like-ECH-associated protein 1; MDA, malondialdehyde; NF-κB, nuclear factor-kappaB; NQO1, NAD(P)H quinine oxidoreductase; Nrf2, nuclear factor E2-related factor 2; SOD, superoxide dismutase; RT-PCR, real-time polymerase chain reaction; p-IκBα, phosphor-I kappa B alpha; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (Cox-2) through nuclear factor-kappaB (NF-κB) activation occurs in the liver of rats after CCl_4 treatment. Ongoing production of inflammatory mediators regulated by NF-κB is believed to aggravate CCl_4-induced liver injury (Son et al., 2007). According to some reports, several herbal medicines and dietary compounds that possess antioxidant activities and inhibit activation of NF-κB could protect the liver against oxidative damage and inflammation caused by CCl_4 (Domitrovic et al., 2012; Jeong, 1999; Rahman et al., 2006; Surh et al., 2001).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the cap "n" collar basic region-leucine zipper transcription factor that protects a variety of tissues and cells against reactive oxygen species (ROS) through antioxidant response element (ARE)-mediated induction of diverse antioxidant and phase II detoxifying enzymes, including heme oxygenase-1 (HO-1), NAD(P)H quinine oxidoreductase 1 (NQO1), glutathione *S*-transferase (GST), and glutamate-cysteine ligase (Lee and Johnson, 2004; Nguyen et al., 2003). Under basal condition, Nrf2-dependent transcription is suppressed by the negative regulator Kelch like-ECH-associated protein 1 (Keap1). Upon stimulation, Nrf2 escapes Keap1-mediated repression and is translocated from the cytosol to the nucleus, subsequently binds to ARE, resulting in up-regulation of antioxidant or phase II enzymes that confer cellular protection against oxidative stress damage and inflammation (Chen et al., 2006; Dhakshinamoorthy and Jaiswal, 2001; Kim et al., 2010).

Garlic (*Allium sativum L.*) is a widely used flavoring agent and is a traditional medicine to control various diseases such as hyperlipidemia, microbial infection, and heart disease (Chen et al., 1998). It also possesses diverse biological activities, including anticarcinogenesis, antithrombosis, antiatherosclerotic, antidiabetic, antioxidant, and anti-inflammatory effects (Agarwal, 1996). Garlic oil contains more than 20 organosulfur compounds, which are believed to play a major role in the reported biological activities. Diallyl disulfide (DADS), a secondary component derived from garlic, has a potent antioxidant property (Sheen et al., 2001; Singh et al., 1998; Wu et al., 2002) and anti-inflammatory activity (Chang and Chen, 2005; Dirsch et al., 1998). This component also down-regulates the expression of numerous genes involved in hepatic oxidative stress and pro-inflammatory response (Chiang et al., 2006; Guyonnet et al., 1999; Keiss et al., 2003). A few reports have suggested the ameliorating effect of DADS against CCl_4-induced hepatotoxicity and lipopolysaccharide-stimulated inflammatory response *in vitro* model (Fukao et al., 2004; Liu et al., 2006). However, the mechanism by which DADS elicits hepatoprotective and antioxidant effects in association with Nrf2 is unclear. Therefore, the aim of the present study was to evaluate the protective effects of DADS on CCl_4-induced oxidative hepatic injury and inflammatory responses and to elucidate the mechanisms underlying these protective effects in rats.

2. Materials and methods

2.1. Animals and environmental conditions

Thirty male Sprague–Dawley rats aged 6 weeks were obtained from a specific pathogen-free colony at Samtako Co. (Osan, Republic of Korea) and used after 1 week of quarantine and acclimation. Two animals per cage were housed in a room maintained at a temperature of 23 ± 3 °C and a relative humidity of 50 ± 10% with artificial lighting from 08:00 to 20:00 and with 13–18 air changes per hour. Commercial rodent chow (Samyang Feed, Wonju, Republic of Korea) sterilized by radiation and sterilized tap water were available *ad libitum*. The Institutional Animal Care and Use Committee of Chonnam National University approved the protocols for the animal study, and the animals were cared for in accordance with the Guidelines for Animal Experiments of Chonnam National University.

2.2. Test chemicals and treatment

CCl_4 (CAS No. 56-23-5) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). DADS was purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan). All other chemicals were of the highest grade commercially available. Test chemicals were dissolved in corn oil, and were prepared immediately before treatment. The daily application volumes of CCl_4 (10 ml/kg body weight) and DADS (2 ml/kg body weight) were calculated based on the most recently recorded body weight of the individual animal. DADS was gavaged to rats once daily for 5 days at dose levels of 50 and 100 mg/kg/day, respectively. Three hours after the final DADS treatment, the rats were given a single oral dose of CCl_4 (2 ml/kg, 20% in corn oil) to induce liver injury (Lee et al., 2003). All animals were sacrificed 24 h after administration of CCl_4.

2.3. Experimental groups and dose selection

Thirty healthy male rats were randomly assigned to five experimental groups: (1) vehicle control, (2) DADS, (3) CCl_4, (4) CCl_4 + DADS 50, and (5) CCl_4 + DADS 100 ($n = 6$ per group). The effective doses of DADS were based on previous studies (Guyonnet et al., 1999; Kalayarasan et al., 2009; Wu et al., 2002).

2.4. Necropsy and serum biochemical analysis

All treated animals were euthanized by carbon dioxide inhalation for blood sample collection 24 h after administration of CCl_4 on the scheduled termination day (test day 6). Blood samples were drawn from the posterior vena cava and serum samples were collected by centrifugation at 800×*g* for 10 min within 1 h after collection and stored at −80 °C before analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with an autoanalyzer (Dri-chem 4000i, Fujifilm Co., Tokyo, Japan).

2.5. Histopathological examination

A portion of liver was dissected and fixed in 10% neutral buffered formalin solution for 2 weeks. The remaining livers were frozen quickly in dry ice and stored at −80 °C for biochemical analysis. The fixed tissues were processed routinely, and were embedded in paraffin, sectioned to 4 μm thickness, deparaffinized, and rehydrated using standard techniques. The extent of CCl_4-induced liver injury and the protective effects of DADS were evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin (H&E). All observations were made manually with a light microscope with 5×, 10×, 20×, and 40× objective lenses and a 100× oil immersion lens in a totally blinded manner. The following variables were used for assessment of histological changes of the liver: (1) hepatocyte degeneration/necrosis; (2) fatty changes; (3) inflammatory cell infiltration; and (4) congestion.

2.6. Determination of lipid peroxidation and, reduced glutathione (GSH), and antioxidant enzymes

A portion of frozen liver was homogenized in a glass-Teflon homogenizer with 50 mM phosphate buffer (pH 7.4) to obtain 1:9 (w/v) whole homogenate. The homogenates were then centrifuged at 11,000×*g* for 15 min at 4 °C to discard any cell debris, and the supernatant was used to measure malondialdehyde (MDA) and GSH concentrations. Concentration of MDA was assayed by monitoring thiobarbituric acid reactive substance formation using the method of Berton et al. (1998). GSH content was measured by the method of Moron et al. (1979). Antioxidant enzyme activities, including catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and GST were also determined using commercial assay kits (Cayman Chemical, Ann Arbor, MI, USA). Total protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.7. Hepatic cytoplasmic and nuclear protein isolation

A frozen liver sample was cut into small pieces and washed in ice-cold (10 mM Tris–HCl, pH 7.4). Samples were homogenized in a glass–Teflon homogenizer with a suitable hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 Mm EGTA) containing protease inhibitor cocktail and dithiothretiol as a reducing agent for lysing the cell membranes. The lysate was incubated on ice for 15 min and added NP-40 to a final concentration of 0.5%, and then centrifuged at 250×*g* for 15 min. The supernatant (cytosol fraction) was removed and stored at −80 °C for subsequent analysis. The pellet containing the nuclear fraction was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail) and vigorously vortexed for 15 min on ice. The nuclear suspension was centrifuged at 16,000×*g* for 30 min. The supernatant (nuclear fraction) was stored at −80 °C for Western blot analysis.

2.8. Western blotting analysis

Equal amounts of proteins (50 μg/well) from each sample were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Whatman, Maidenstone, UK), and blocked in blocking buffer (150 mM NaCl in 10 mM Tris, pH 7.5 containing 5% non-fat dry milk) for 1 h at room temperature. The membranes were incubated with primary rabbit