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Evaluation of hepatotoxicity and oxidative stress in rats treated with *tert*-butyl hydroperoxide

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ABSTRACT

Although *tert*-butyl hydroperoxide (*t*-BHP) is commonly used to induce oxidative stress, little is known about the time- or dose-dependence of its oxidative effects. In this study, we examined hepatotoxicity and oxidative stress in male rats at various times (0–24 h) after *t*-BHP (0, 0.2, 0.5, 1 or 3 mmol/kg, ip) treatment. Serum hepatotoxicity parameters were increased from 2 h following 1 mmol/kg *t*-BHP and reached their maximum values at 8 h. Plasma malondialdehyde levels were maximally elevated by 62% at 0.5 h and returned to control levels by 4 h. Hepatic glutathione levels were decreased between 0.5 and 2 h, and hepatic glutathione disulfide levels were increased at 2 h. Interestingly, hepatic glutathione levels were increased at 24 h, which may be attributed to up-regulation of glutathione synthesis through induction of gamma-glutamylcysteine ligase expression. The elevation of hepatotoxic parameters and plasma MDA was observed from 0.5 to 1 mmol/kg *t*-BHP, respectively, in a dose-dependent manner. Considering that the maximal dose resulted in 20% lethality, 1 mmol/kg of *t*-BHP may be suitable for evaluating antioxidant activity of tested compounds. Our results provide essential information to characterize the *t*-BHP-induced oxidative stress and hepatotoxicity.

1. Introduction

In studies of the antioxidant effects of compounds of interest in cell and animal models, *tert*-butyl hydroperoxide (*t*-BHP) is commonly used as an inducer of oxidative stress. It is metabolized to free radical intermediates, such as *t*-butoxyl and -methyl radicals, by cytochrome P450 or hemoglobin (Hogberg et al., 1975; Minotti et al., 1986; Hix et al., 2000). These free radical intermediates can cause lipid peroxidation (Masaki et al., 1989), glutathione (GSH) depletion (Chance et al., 1979), hemolysis and oxidative denaturation of hemoglobin (Rice-Evans et al., 1985), permeabilization of cell membranes (Castilho et al., 1995), and DNA damage (Guidarelli et al., 1997). Alternatively, *t*-BHP can be rapidly converted to *t*-butyl alcohol by GSH peroxidase (Davies, 1989).

Many investigators have used *t*-BHP-treated rats to evaluate the antioxidant effects of various compounds (Wang et al., 2000; Liu et al., 2002; Yen et al., 2004; Kim et al., 2007; Hwang et al., 2008; Lee et al., 2008). In these experiments, oxidative liver injury is assessed by determining the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and the levels of malondialdehyde (MDA), GSH, and GSH disulfide (GSSG) at various intervals (18–24 h) after the administration of a single dose (0.1–2 mmol/kg) of *t*-BHP. However, little is known about the time- or dose-dependence of the oxidative stress induced by *t*-BHP.

In this investigation, we determined the time- and dose-dependence of *t*-BHP-induced toxicity and oxidative stress in rats. This knowledge is essential for evaluating the antioxidant activity of compounds of interest using *t*-BHP-induced oxidative stress models. In the present study, we measured markers of hepatotoxicity and oxidative stress in male rats 0–24 h after the intraperitoneal administration of 0–3 mmol/kg *t*-BHP. Liver injury was assessed by quantifying serum activities of ALT and sorbitol dehydrogenase (SDH), and oxidative stress was assessed by quantifying MDA, GSH, and GSSG levels and total oxyradical scavenging capacity (TOSC) against peroxyl radicals.

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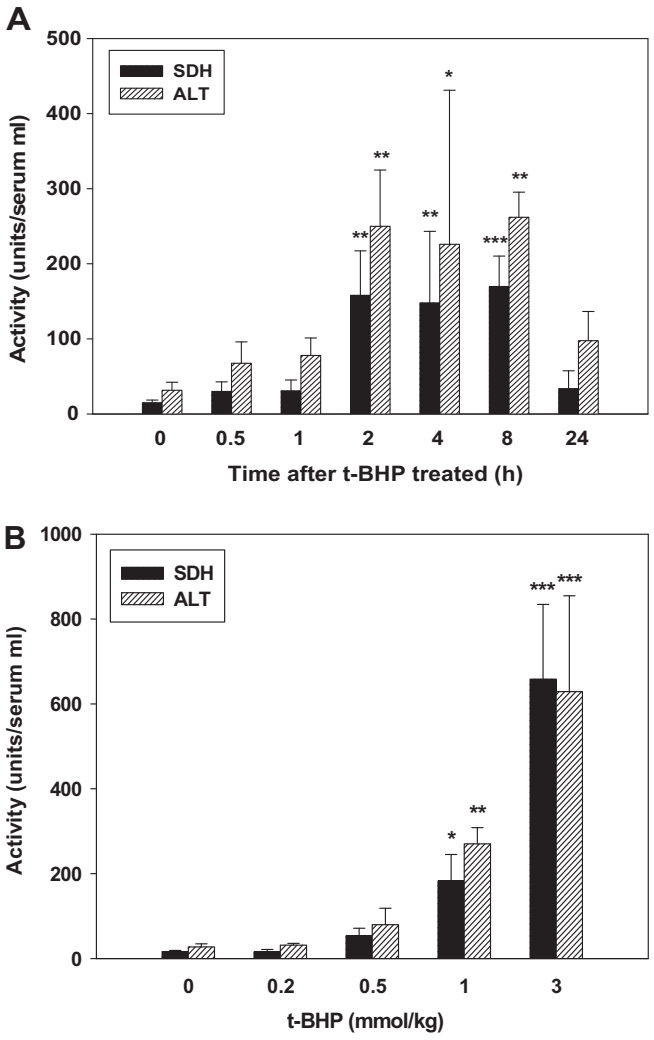

Fig. 1. Serum SDH and ALT activities in *t*-BHP-treated rats. (A) Rats were sacrificed 0, 0.5, 1, 2, 4, 8, and 24 h after the intraperitoneal administration of *t*-BHP (1 mmol/kg). Values shown are means ± SD for 4 or 5 rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to the control determined at $t = 0$ h (one-way ANOVA followed by Dunnett's test). (B) Rats were sacrificed 8 h after treatment with *t*-BHP at a dose of 0, 0.2, 0.5, 1, or 3 mmol/kg. Values shown are means ± SD for 3 or 4 rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to animals treated with saline only (one-way ANOVA followed by Dunnett's test).

2. Materials and methods

2.1. Animals and treatments

Male Sprague–Dawley rats (Samtako Bio Korea Inc., Osan, Korea) weighing 280–320 g were used in all experiments. Rats were housed in humidity- (55 ± 5%) and temperature-controlled (23 ± 1 °C) rooms with a 12-h/12-h light/dark cycle for at least 1 week before experimentation. Laboratory rat chow and tap water were available *ad libitum*. All procedures were approved by the Chungnam National University Committee for the Use and Care of Animals.

For time-course experiments, rats were sacrificed 0.5, 1, 2, 4, 8, or 24 h after the intraperitoneal administration of 1 mmol/kg *t*-BHP. For dose–response experiments, rats were sacrificed 8 h after the intraperitoneal administration of 0, 0.2, 0.5, 1, or 3 mmol/kg *t*-BHP. Control rats were treated with equivalent volumes of saline.

2.2. Chemicals

2-Thiobarbituric acid (TBA) and L-aspartic acid were purchased from ICN Biomedicals, Inc., (Aurora, OH, USA). Tetraethoxypropane, *t*-BHP, diethylenetriaminopentacetic acid, 2,6-ditertbutyl-4-methlyphenol, GSH, GSH reductase, 2,2′-azobisamidinopropane (ABAP), α-keto-γ-methiolbutyric acid (KMBA), β-NADPH, pyruvate, L-alanine, fructose, and 2-vinyl-pyrimidine were purchased from Sigma–Aldrich (St. Louis, MO, USA). An antibody against γ-glutamylcysteine ligase (GCL) catalytic subunit (GCLC) was purchased from NeoMarkers, Inc., (Fremont, CA, USA). All other chemicals and solvents were of reagent grade or better.

2.3. Measurement of serum SDH and ALT activities

Blood was sampled from the abdominal aorta of rats under light ether anesthesia. Blood samples were allowed to clot at room temperature and then centrifuged to separate the serum. Serum SDH activity was determined using a modification of the method of Gerlach (1983). Serum ALT activity was determined using a modification of the method of Reitman and Frankel (1957).

2.4. Histopathology

The liver tissues from three rats of each group were, respectively, excised 8 h after intraperitoneal treatment with 0.2, 0.5, 1, and 3 mmol/kg *t*-BHP or 2, 4, and 24 h after 1 mmol/kg *t*-BHP. The liver tissues were fixed in 10% buffered neutral formalin. After routine tissue processing, the liver tissues were embedded in a low-melting-point paraffin. Tissue sections, 3 μm in thickness, were stained with hematoxylin and eosin and then mounted using DPX mountant, followed by microscopic examination under light microscope (Olympus CX41RF, Olympus Co., Tokyo, Japan).

2.5. Lipid peroxidation

MDA levels in liver and plasma were measured using high-performance liquid chromatography (HPLC). Liver samples were homogenized with a 3 × volume of cold 1.15% KCl. Aliquots (30 μL) of liver homogenate or plasma were mixed with 300 μL of 0.2% TBA in a 2 M sodium acetate buffer (pH 3.5) containing 1 mmol/kg diethylenetriaminopentacetic acid and 3 μL of 0.5% 2,6-ditertbutyl-4-methlyphenol in 96% ethanol and incubated at 95 °C for 45 min. After cooling on ice, the samples were centrifuged (10,000*g*, 5 min), and the supernatant fractions were injected into an HPLC instrument equipped with a fluorescence detector (RF-10AxL, Shimadzu, Japan) and a 4.6 × 150-mm stainless steel column (Inertsil ODS-3; GL Science Inc., Tokyo, Japan). The mobile phase was 40% methanol and 60% 50 mM sodium phosphate buffer (pH 7.0). The MDA–TBA complex was eluted at a flow rate of 1.3 mL/min and monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm. The concentration of MDA was calculated from a calibration curve prepared using tetraethoxypropane. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.6. TOSC assay

TOSC was measured using an assay based on the ethylene-yielding reaction of KMBA with oxyradicals (Winston et al., 1998). Peroxyl radicals were generated by thermal decomposition of 20 mM ABAP at 35 °C in 100 mM potassium phosphate buffer (pH 7.4). Reactions were carried out in 20-mL rubber septa-sealed vials in a final volume of 1 mL and were initiated by the injection of 100 μL of 200 mM ABAP in water directly through the rubber septum. One-milliliter syringe injector samples were taken from the headspace of the reaction vials at indicated intervals during the course of the reaction and analyzed for ethylene production by analysis on a Shimadzu-2010 (Shimadzu Co., Kyoto, Japan) gas chromatograph equipped with a Supelco SPB-1 capillary column (30 m × 0.32 mm × 0.25 mm) and a flame ionization detector. Total ethylene formation was quantified from the area under the kinetic curve.

The oven, injection, and flame ionization detector temperatures were 60, 180, and 180 °C, respectively. High-purity helium was used as the carrier gas at a flow rate of 30 mL/min. TOSC values were quantified using the equation $TOSC = 100 - (SA/CA \times 100)$, where SA and CA are the integrated ethylene peak areas obtained from the sample and control reactions, respectively. Specific TOSC values were calculated by dividing the experimental TOSC values by the volumes of plasma used.

2.7. Measurement of GSH and GSSG levels

Liver samples were homogenized in a 3 × volume of cold 1.15% KCl solution. The homogenates were then mixed with an equal volume of 12% perchloric acid solution and centrifuged. The resulting supernatant fractions were assayed for GSH and GSSG according to the method of Griffith (1980).

2.8. Immunoblot analysis of GCLC and GCL activity

Liver samples were homogenized in a 3 × volume of cold buffer consisting of 0.154 M KCl, 50 mM Tris–HCl (pH 7.4), and 1 mM EDTA. All subsequent steps were performed at 0–4 °C. The homogenates were centrifuged at 10,000*g* for 20 min. The resulting supernatant fractions were removed and then centrifuged at 100,000*g* for 60 min. The resulting supernatant (cytosolic) fractions were then used as samples