Hepatoprotective Effect of 20(*S*)-Ginsenosides Rg3 and Its Metabolite 20(*S*)-Ginsenoside Rh2 on *tert*-Butyl Hydroperoxide-Induced Liver Injury

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To evaluate the hepatoprotective effect of Red Ginseng (RG), we isolated a main constituent 20(*S*)-ginsenoside Rg3 from RG, and its metabolite 20(*S*)-ginsenoside Rh2 by human intestinal microflora, and investigated their hepatoprotective activities in *tert*-butyl hydroperoxide (*t*-BHP)-induced hepatotoxicity of HepG2 cells and mice. When HepG2 cells were treated with *t*-BHP, its cytotoxicity was significantly increased. 20(*S*)-Ginsenoside Rh2 potently protected its cytotoxicity, but 20(*S*)-ginsenoside Rg3 weakly protected it. Intraperitoneally and orally administered 20(*S*)-ginsenoside Rh2 to *t*-BHP-injured mice significantly inhibited the increase of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Orally administered 20(*S*)-ginsenoside Rg3 also showed the inhibition against the increase of ALT and AST of *t*-BHP-induced mice. However, intraperitoneally administered 20(*S*)-ginsenoside Rg3 could not inhibit the elevation of serum ALT and AST activities. These results suggest that 20(*S*)-ginsenoside Rg3 a main component of RG may be a prodrug for hepatotoxicity.

Key words hepatotoxicity; *tert*-butyl hyperoxide; ginseng; 20(*S*)-ginsenoside Rg3; 20(*S*)-ginsenoside Rh2

Ginseng (the root of *Panax ginseng* C. A. Meyer, family Araliaceae) has been used as a herbal medicine in China, Korea, Japan and other Asian countries. Its major components are ginsenosides, which are glycosides with a dammarane skeleton.[1,2] When ginseng is steamed at 98—100 °C, it is called Red Ginseng (RG). Their main components are different: the former is a ginsenoside Rb1, and the latter is a ginsenoside Rg3.[3] These results suggest that the ginsenosides could be easily transformed under steaming. These ginsenosides have been reported to show various biological activities including anti-inflammatory,[4] antiallergic,[5,6] antitumor,[7—11] vascular relaxation,[12] and hepatoprotective activities.[13,14] To express these pharmacological actions, it is thought that ginseng saponins must be metabolized by human intestinal microflora after being taken orally.[7,15,16] For example, ginsenosides Rb1, Rb2 and Rc are metabolized to compound K by human intestinal microflora, and the ginsenoside Rg3 is metabolized to ginsenoside Rh2 by human intestinal microflora.[17] The transformed compound K and ginsenoside Rh2 induces an anti-metastatic or anti-carcinogenic effect.[8,11] However, the relationship between hepatoprotective effects of RG and the metabolism of its main constituent ginsenoside Rg3 have not been thoroughly studied.

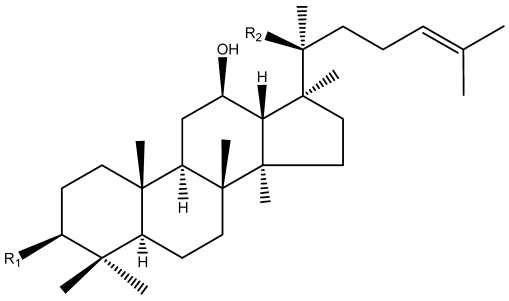
Therefore, we transformed 20(*S*)-ginsenoside Rg3 by human intestinal bacteria, isolated its metabolite 20(*S*)-ginsenoside Rh2, and investigated their hepatoprotective effects on HepG2 cells and mice injured by *tert*-butyl hydroperoxide (*t*-BHP).

MATERIALS AND METHODS

Materials Diagnostic kits for aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were purchased from Asan Pharmaceutical Co., Ltd. (Seoul, Korea). Silybin was purchased from Carl Roth (Karlsruhe, Germany). Minimum essential medium (MEM), fetal bovine serum (FBS) and antibiotics–antimycotics were obtained from Gibco BRL (N.Y., U.S.A.). *t*-BHP was purchased from Sigma Co. (St. Louis, MO, U.S.A.). The other chemicals were of analytical reagent grade.

20(*S*)-Ginsenoside Rg3 and its metabolite 20(*S*)-ginsenoside Rh2 (Fig. 1) were isolated according to the previous method.[18]

Culture of HepG2 Cells and Its Hepatoxicity Induction by *t*-BHP HepG2 cells (hepatocellular carcinoma cell line) donated from the Korean Cell Bank (Seoul, Korea) were cultured in MEM containing 10% FBS, 1% antibiotic-antimycotic solution, 1mM sodium pyruvate and 1.5 g/l sodium bicarbonate under 5% CO_2 at 37 °C. The protective effect of ginsenosides on HepG2 cells injured by *t*-BHP was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.[19] Briefly, HepG2 cells were dispensed into 96 well plates at the concentration of 1×10^4 cells per well. The test compounds were added into HepG2 cells, and preincubated for 2 h. Then the cultured media were replaced to the media containing *t*-BHP (100 μM), incubated for 3 h and then rinsed with phosphate-buffered saline. MTT reagent (0.25 mg/ml) was added into the cells, incubated for 1 h, and then added 100 μl of dimethylsulfoxide. Absorbance



	R_1	R_2
20(S)-Ginsenoside Rg3	–O-Glc-Glc	–OH
20(S)-Ginsenoside Rh2	–O-Glc	OH

Fig. 1. Structures of 20(*S*)-Ginsenosides Rg3 and Rh2

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at 540 nm was measured to estimate survived cells.

Animals Mice (ICR, male, 20—25 g) were supplied from the Orient Charles River Co., Ltd. (Seoul, Korea), and were maintained on pellet food (Orient Charles River Co., Seoul, Korea) and tap water. Five mice in each group were used. Animals were orally or intraperitoneally administered with ginsenoside Rg3, ginsenoside Rh2 or silybin suspended in 6% cremophore. Control group was given with saline (0.1 ml/20 g) instead of the sample compounds. Orally administered samples were treated three times (once per day) or intraperitoneally administered ones were treated once. Animals were intraperitoneally treated with 1.5 mmol *t*-BHP/kg 24 h after the final sample administration. Blood samples were collected 18 h after *t*-BHP administration by cardiac puncture under ether anesthesia and serum was obtained by centrifugation (1000×***g***, 15 min).

Enzyme Activity Assay Activities of ALT and AST in serum were analyzed according to manufacture's procedure (Asan diagnostic kits).[20]

Statistics All the data were expressed mean±standard deviation and statistical significance was determined by the one way ANOVA followed by Student–Newman–Keuls test.

RESULT

Protective Effect of 20(*S*)-Ginsenosides Rg3 and Rh2 on HepG2 Cells Toxicified by *t*-BHP The hepatoprotective effect of RG was reported.[13] To understand what an active compound in RG is against liver injury, we isolated a main component 20(*S*)-ginsenoside Rg3 for RG and its metabolite 20(*S*)-ginsenoside Rh2 by human intestinal microflora and investigated their protective effects on cytotoxicity of HepG2 induced by *t*-BHP (Fig. 2). When *t*-BHP was treated on HepG2 cells, cell viability was dose-dependently decreased. *t*-BHP at a concentration of 100 μM was decreased to 61.3% of normal control group. The pretreatment of 20(*S*)-ginsenoside Rh2 on HepG2 cells significantly inhibited *t*-BHP-induced cytotoxicity. 20(*S*)-Ginsenoside Rh2 at a concentration of 1 mM protected the *t*-BHP-induced cytotoxicity of HepG2 to 92.4% of control group. The protective effect of 20(*S*)-ginsenoside Rh2 was stronger than that of silybin, a commercial agent, which protected the *t*-BHP-induced cytotoxicity of HepG2 to 72.3% of control group at a concentration of 1 mM. However, the 20(*S*)-ginsenoside Rh2 was less effective at the concentration of 10 mM against *t*-BHP-induced cytotoxicity. The 20(*S*)-ginsenoside Rh2 did not show cytotoxicity at a concentration of less than 10 mM. However, 20(*S*)-ginsenoside Rg3 did not exhibit the protective effect against the cytotoxicity.

Hepatoprotective Effect of 20(*S*)-Ginsenosides Rg3 and Rh2 in *t*-BHP-Induced Liver Injury of Mice 20(*S*)-Ginsenoside Rh2 showed the potent protective activity against cytotoxicity of *t*-BHP. Therefore, the hepatoprotective effect of 20(*S*)-ginsenosides Rg3 and Rh2 was investigated in liver-injured mice by *t*-BHP (Table 1). When *t*-BHP was intraperitoneally injected into mice, serum ALT and AST levels were significantly increased, compared to those of normal control group. The reference agent silybin (100 mg/kg) inhibited the increases of serum ALT and AST levels to 63% and 82% of control group treated with *t*-BHP alone, respectively. Orally administered 20(*S*)-ginsenosides Rg3 and Rh2 both potently inhibited the increase of serum ALT and AST levels induced by *t*-BHP treatment. Intraperitoneally administered 20(*S*)-ginsenoside Rh2 also inhibited serum ALT and AST levels by 67% and 71% of the *t*-BHP control group, respectively (Table 2). However, intraperitoneally administered 20(*S*)-ginsenoside Rg3 did not inhibit the increase of serum ALT and AST.

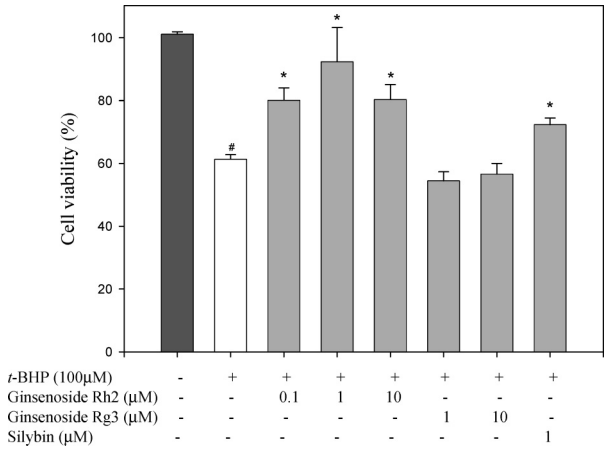

Fig. 2. Cytoprotective Effect of 20(*S*)-Ginsenosides Rg3 and Rh2 on *t*-BHP-Toxicified HepG2 Cells

Statistically significant compared with normal data ($p<0.05$). * Statistically significant compared with *t*-BHP control data ($p<0.05$).

Table 1. Preventive Effect of Orally Administered 20(*S*)-Ginsenosides Rg3 and Rh2 on *t*-BHP-Induced Hepatotoxicity in Mice

Group	*t*-BHP	Dose (mg/kg)	ALT (Karmen unit)	AST (Karmen unit)
Normal control	–	0	35.8±1.7	61.0±17.4
t-BHP control	+	0	128.3±19.3#	175.7±24.1#
Silybin	+	100	69.8±9.6*	82.0±13.1*
20(*S*)-Ginsenoside Rh2	+	25	93.0±13.4*	108.0±11.0*
	+	50	73.8±13.5*	87.7±9.3*
20(*S*)-Ginsenoside Rg3	+	25	107.5±20.3	85.3±24.8*
	+	50	84.8±31.4	82.3±39.7*

Statistically significant compared with normal data ($p<0.05$). * Statistically significant compared with *t*-BHP control data ($p<0.05$).

Table 2. Preventive Effect of Intraperitoneally Administered 20(*S*)-Ginsenosides Rg3 and Rh2 on *t*-BHP-Induced Hepatotoxicity in Mice

Group	*t*-BHP	Dose (mg/kg)	ALT (Karmen unit)	AST (Karmen unit)
Normal control	–	0	31.7±2.5	46.3±5.0
t-BHP control	+	0	180.8±38.5#	186.3±23.7#
Silybin	+	100	49.7±3.8*	70.7±15.0*
20(*S*)-Ginsenoside Rh2	+	12.5	90.7±37.4*	88.3±21.8*
	+	25	79.7±9.8*	87.0±25.9*
20(*S*)-Ginsenoside Rg3	+	12.5	177.5±45.0	156.7±32.4
	+	25	164.3±63.1	134.3±28.0

Statistically significant compared with normal data ($p<0.05$). * Statistically significant compared with *t*-BHP control data ($p<0.05$).

DISCUSSION

t-BHP can be metabolized to free radical intermediates by cytochrome P450 (hepatocytes) or hemoglobin (erythrocytes), which can subsequently initiate lipid peroxidation,[21]

affect cell integrity and form covalent bonds with cellular molecules resulting in cell injury.[22] *t*-BHP is known to cause lactate dehydrogenase and ALT leakage in hepatocyte cells.[23,24] Therefore, *t*-BHP has been used as a chemical inducer for the preparation of liver injured animal model.[25,26] We used *t*-BHP-hepatoxicified mouse model to evaluate the hepatoprotective effect of ginsenoside Rg3 isolated from RG, which exhibited the hepatoprotective effects in chemical-induced hepatoxicity.[13]

Therefore, to identify the active ginseng saponin of RG against liver injury, we measured hepatoprotective activities of 20(*S*)-ginsenoside Rg3, a main constituent of RG, and its metabolite 20(*S*)-ginsenoside Rh2 on liver-injured mice by *t*-BHP. *t*-BHP dose-dependently showed cytotoxicity against HepG2 cells. However, the pretreatment of 20(*S*)-ginsenoside Rh2 potently inhibited this cytotoxicity. Therefore, *in vivo* hepatoprotective effect of 20(*S*)-ginsenoside Rh2 on *t*-BHP-induced hepatotoxicified liver injury was investigated. Orally administered 20(*S*)-ginsenoside Rg3 showed the potent hepatoprotective effect. However, when 20(*S*)-ginsenoside Rg3 was intraperitoneally administered, it did not exhibit hepatoprotective effect. 20(*S*)-Ginsenoside Rh2 showed the hepatoprotective effect even if it was intraperitoneally administered. The protective effect of 20(*S*)-ginsenoside Rh2 was stronger than that of silybin, a commercial agent. However, 20(*S*)-ginsenoside Rh2 at the high dosage decreased the hepatoprotective activity (data not shown). Its results may be due to the cytotoxicity of 20(*S*)-ginsenoside Rh2.

Based on these findings, 20(*S*)-ginsenoside Rg3 may be metabolized to 20(*S*)-ginsenoside Rh2 in human intestine, when 20(*S*)-ginsenoside Rg3 or RG is orally administered, and the biotransformed 20(*S*)-ginsenoside Rh2 may protect liver injury.

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