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Hepatoprotective Effect of Lactic Acid Bacteria, Inhibitors of β-Glucuronidase Production Against Intestinal Microflora

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The hepatoprotective activity of lactic acid bacteria (*Lactobacillus brevis* HY7401, *Lactobacillus acidophilus* CSG and *Bifidobacterium longum* HY8001), which inhibited β-glucuronidase productivity of intestinal microflora, on *t*-BHP- or CCl_4-induced hepatotoxicity of mice were evaluated. These oral administration of lactic acid bacteria lowered β-glucuronidase production of intestinal microflora as well as *Escherichia coli* HGU-3. When lactic acid bacteria at a dose of 0.5 or 2 g (wet weight)/kg was orally administered on CCl_4-induced liver injury in mice, these bacteria significantly inhibited the increase of plasma alanine transferase and aspartate transferase activities by 17~57% and 57~66% of the CCl_4 control group, respectively. These lactic acid bacteria also showed the potent hepatoprotective effect against *t*-BHP-induced liver injury in mice. The inhibitory effects of these lactic acid bacteria were more potent than that of dimethyl diphenyl bicarboxylate (DDB), which have been used as a commercial hepatoprotective agent. Among these lactic acid bacteria, *L. acidophilus* CSG exhibited the most potent hepatoprotective effect. Based on these findings, we insist that an inhibitor of β-glucuronidase production in intestine, such as lactic acid bacteria, may be hepatoprotective.

Key words: Hepatoprotective, Lactic acid bacteria, β-Glucuronidase inhibitor, Intestinal bacteria, *t*-BHP, CCl_4

INTRODUCTION

β-Glucuronidase has been found in animals, plants and bacteria (Stahl and Fishman, 1983). This enzyme does not catalyze the hydrolysis of β-glucuronide conjugates of exogenous and endogenous compounds produced in the body, such as benzo[a]pyrene glucuronides, but also transforms their conjugates to toxic compounds (Stahl and Fishman, 1983; Dutton, 1980).

In the tissue of mammals, β-glucuronidase has been shown to be a typical lysosomal enzyme through subcellular fractionation study (Stahl and Fishman, 1983). Pineda *et al.* (1959) demonstrated that liver damage caused an increase of β-glucuronidase in blood, and Mill *et al.* and Levy *et al.* reported that liver cancer could be related to this enzyme (Mills and Smith, 1951; Levy *et al.*, 1948; Mills *et al.*, 1953). We found that silymarin, a commercial crude drug used as a hepatoprotective, and glycyrrhizin, which is a main component of hepatoprotective herbal medicine, not only inhibited β-glucuronidase *in vitro* and *in vivo,* but also improved CCl_4-induced hepatotoxicity (Kim *et al.*, 1994; Shim *et al.*, 2000).

The exogenous and endogenous compounds detoxified in liver, such as benzo[a]pyrene glucuronides and bisphenol A glucuronides, are excreted in intestine via bile duct, β-glucuronidase of intestinal microflora catalyze retoxification of these compounds and causes colon cancer (Hill and Crowther, 1971; Kinoshita and Gelvoin, 1978; Sakamoto *et al.*, 2002; Goldin and Gorbach, 1976). Furthermore, the hydrolyzed glucuronate conjugates are reabsorbed into blood and can cause liver injury (Kinoshita and Gelvoin, 1978; Sakamoto *et al.*, 2002). Kim *et al.* reported that the inhibition of the enzyme production reduced the promotion of mouse colitis and colon cancer induced by chemicals (Kim *et al.*, 1995a, 1995b). However, the effect of intesteinal bacterial β-glucuronidase inhibitors against liver injury has not been thoroughly studied.

As part of our continuing search for bacterial hepatoprotective agents, we screened lactic acid bacteria as a β-glucuronidase production inhibitor, and investigate its

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hepatoprotective activity on *t*-BHP- or CCl_4-induced hepatotoxified mice.

MATERIALS AND METHODS

Materials

Carbon tetrachloride, *tert*-butyl hydroperoxide (*t*-BHP) and thiobarbituric acid (TBA) were purchased from Sigma Co. (U.S.A.). Diagnostic kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Asan Pharmaceutical Co., Ltd (Korea). Diphenyl dimethyl bicarboxylate (DDB) was kindly donated from Dr. N. J. Kim, Kyung Hee East-West Medical Research Institute, Kyung Hee University. General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., ltd (Japan). Other chemicals used in this study were of analytical reagent grade.

Escherchia coli HGU-3 and lactic acid bacteria [*Lactobacillus brevis* HY7401 (**7**), *Lactobacillus acidophilus* CSG (**C**) and *Bifidobacterium longum* HY8001 (**8**)] previously isolated were used (Kim *et al.*, 1994; Shim *et al.*, 2000).

Cocultivation of lactic acid bacteria with *E. coli* HGU-3

The bacteria were cultured at 37°C for 20 h in GAM broth. The fresh cultured lactic acid bacteria (1×10^5) and *E. coli* HGU-3 (1×10^5) were inoculated in 10 mL of GAM broth, cultured at 37°C for 20 h, collected at 5000 × g for 20 min, washed with saline, suspended in 50 mM phosphate buffer, pH 7.0 and used as a bacterial enzyme source.

Animals

Mice (ICR, male, 20-22 g) were supplied from Orient Co., ltd (Korea), and were maintained on pellet food (Orient Co., ltd, Korea) and tap water. Five mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animals Care (NIH publication no. 85-23, revised 1985).

To evaluate the protective effect of lactic acid bacteria on liver injury of mice induced by CCl_4, animals were orally treated with lactic acid bacteria (suspended in 1% CMC-Na) at dose of 0.5 g/kg and 2 g/kg, and DDB (suspended in 1% CMC-Na) at dose of 200 mg/kg. Animals of control group were given with saline (0.2 mL/20 g) instead of the sample. The samples were treated three times (once per day). Animals were orally treated with 0.025 mL CCl_4/kg dissolved in 0.5% v/v in olive oil 1 h after the final sample administration (or intraperitoneally treated with 1.5 mmol *t*-BHP/kg 1 h after the final sample administration). Blood samples were collected 24 h after CCl_4 administration (or 18 h after *t*-BHP administration) by cardiac puncture under ether anesthesia and serum was obtained by centrifugation (2000 × g, 15 min).

Enzyme activity assay

The stool (0.5 g) was suspended in 4.5 mL cold phosphate buffered saline (PBS), centrifuged at 5000 × g for 30 min, suspended in cold PBS, and used as a stool enzyme solution. Enzyme activities were measured according to our previous method (Kim *et al.*, 1995c). The reaction mixture for β-glucuronidase activity assay (total volume of 500 μL) containing 100 μL of 2 mM ρ-nitrophenyl β-D-glucuronide, 380 μL of 0.2 M NaOH-glucuronide buffer (pH 9.0), and 100 μL of enzyme solution was incubated at 37°C for 1 h. The reaction was stopped by the addition of 500 μL 0.5 N NaOH, and the absorbance was measured at 405 nm.

The reaction mixture for alkaline phosphatase activity assay (total volume of 500 μL) containing 20 μL of 10 mM ρ-nitrophenyl phosphate, 380 μL of 0.1 M phosphate buffer (pH 7.0), and 100 μL of enzyme solution was incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 mL 0.5 N NaOH, and the absorbance was measured at 405 nm.

Activities of alanine transferase (ALT), and aspartate transferase (AST) in serum were analyzed according to manufactures procedure (Asan diagnostic kits) (Reitman and Frankel, 1957).

Statistics

All the data were expressed all mean ± standard deviation and statistical significance was determined using student *t*-test.

RESULTS

Inhibitory effect of lactic acid bacteria on β-glucuronidase productivity of *E. coli* HGU-3

During the screening program to discover hepatoprotective agents for human intestinal bacteria, we have been used *E. coli* HGU-3 as a β-glucuronidase producer of human intestinal microflora. Therefore, we anaerobically cocultured some lactic acid bacteria (*L. brevis* HY7401, *L. acidophilus* CSG and *B. longum* HY8001) with *E. coli* HGU-3 in GAM, and measured the inhibitory activity of lactic acid bacteria on β-glucuronidase production of *E. coli* HGU-3 (Fig. 1). Among tested bacteria, *L. acidophilus* CSG potently inhibited β-glucuronidase production. However, these bacteria did not directly inhibit β-glucuronidase activity (data not shown).

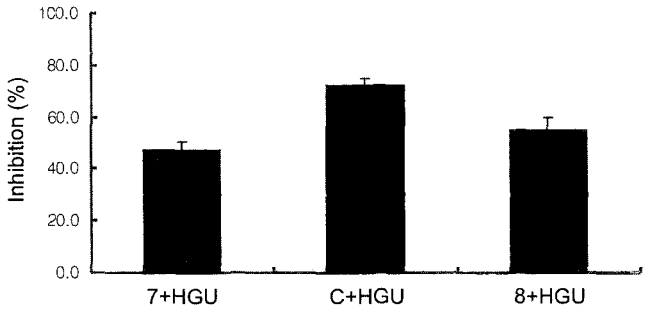

Fig. 1. The inhibitory effect of lactic acid bacteria on β-glucuronidase productivity of *E. coli* HGU-3. 7, *L. brevis* HY7401; C, *L. acidophilus* CSG; 8, *B. longum* HY8001; HGU, *E. coli* HGU-3.

Hepatoprotective effect of lactic acid bacteria, inhibitors of β-glucuronidase productivity of *E. coli* HGU-3, on CCl_4-induced liver injury in mice

To investigate the relationship between β-glucuronidase production inhibitors and their hepatoprotective activity, their hepatoprotective effects were measured on CCl_4-induced liver injury in mice (Fig. 2). When CCl_4 was orally treated into mice, serum ALT and AST levels were significantly increased, compared to normal control group. When commercial hepatoprotective DDB (200 mg/kg), which is transformed to an antioxidant drug in liver (Wang, 1984), was orally treated on CCl_4 control group, serum ALT and AST levels were decreased by 58 and 39%, compared to CCl_4 control group, respectively.

Lactic acid bacteria inhibited the increase of serum ALT and AST levels induced by CCl_4 treatment. *L. brevis* HY7401, *L. acidophilus* CSG and *B. longum* HY8001 at a dose of 0.5 g/kg inhibited serum ALT and AST levels by 17, 57 and 49%, and 57, 66 and 62% of the CCl_4 control group, respectively. The hepatoprotective activity of *L. acidophilus* CSG was more potent than that of DDB.

Hepatoprotective effect of lactic acid bacteria on *t*-BHP-induced liver injury in mice

Lactic acid bacteria showed the potent protective activity for CCl_4-induced liver injury in mice. Therefore, the hepatoprotective effect of lactic acid bacteria was also investigated in liver injured mice induced by *t*-BHP (Fig. 3). When *t*-BHP was intraperitoneally treated into mice, it significantly increased serum ALT and AST levels, compared to those of normal control group. The reference agent DDB (200 mg/kg) inhibited the increased serum ALT and AST levels to 28% and 39% of control group treated with *t*-BHP alone, respectively. Orally administered lactic acid

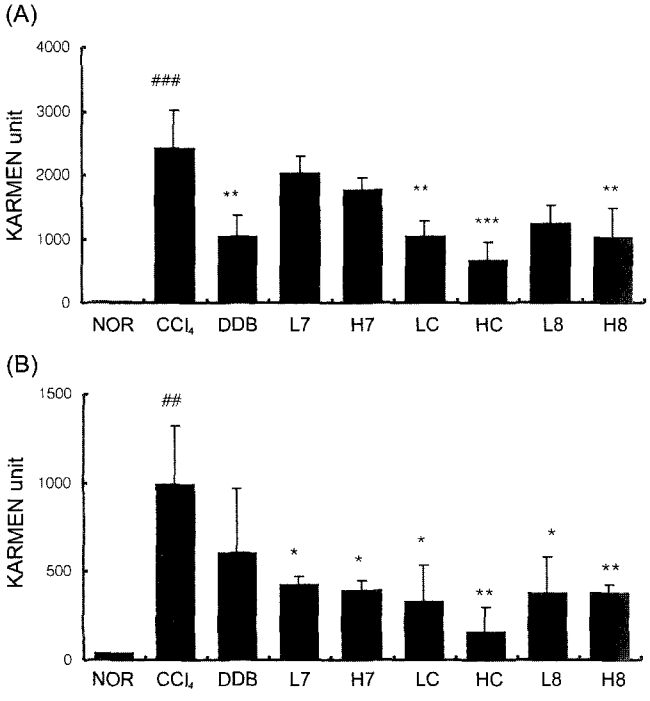

Fig. 2. The protective effect of lactic acid bacteria on CCl_4-injured liver injury in mice. (A), ALT; (B), AST. L7, 0.5 g/kg *L. brevis* HY7401; H7, 2 g/kg *L. brevis* HY7401; LC, 0.5 g/kg *L. acidophilus* CSG; HC, 2 g/kg *L. acidophilus* CSG; L8, 0.5 g/kg *B. longum* HY8001; H8, 2 g/kg *B. longum* HY8001. #Significantly different form normal group (#, $p<0.05$; ##, $p<0.01$). *Significantly different form normal group (*, $p<0.05$; **, $p<0.01$).

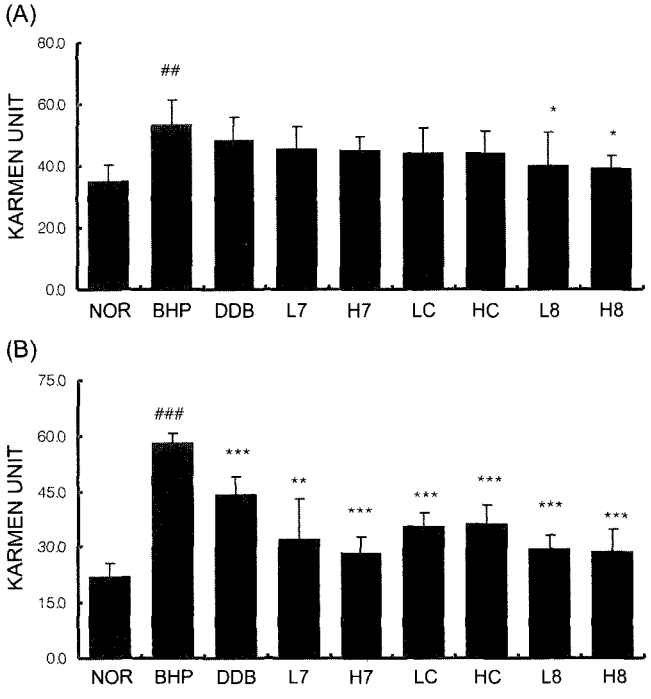

Fig. 3. The protective effect of lactic acid bacteria on *t*-BHP-injured liver injury in mice. (A), ALT; (B) AST. L7, 0.5 g/kg *L. brevis* HY7401; H7, 2 g/kg *L. brevis* HY7401; LC, 0.5 g/kg *L. acidophilus* CSG; HC, 2 g/kg *L. acidophilus* CSG; L8, 0.5 g/kg *B. longum* HY8001; H8, 2 g/kg *Bifidobacterium longum* HY8001. #Significantly different form normal group (#, $p<0.05$; ##, $p<0.001$). *Significantly different form normal group (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

bacteria all potently inhibited the increase of serum ALT and AST levels induced by *t*-BHP treatment. *L. brevis* HY7401, *L. acidophilus* CSG and *B. longum* HY8001 at a dose of 0.5 g/kg inhibited serum ALT and AST levels by 40, 48, and 73%, and 47, 62, and 79% of the *t*-BHP control group, respectively. The hepatoprotective activity of *L. acidophilus* CSG was more potent than that of DDB.

Effect of orally administered lactic acid bacteria in intestinal bacterial enzyme activites in mice

To evaluate the inhibitory effect of lactic acid bacteria on the harmful enzyme β-glucuronidase activity of intestinal bacteria, 0.5% and 1% lactic acid bacteria-contained diets were fed for 4 weeks in mice and some enzyme activities were measured. Fecal β-glucuronidase activities of lactic acid bacteria-treated groups were significantly decreased, compared to that of normal control group (Table I). However, the difference of fecal alkaline phosphate activity between groups treated with and without lactic acid bacteria was not significant (Table II).

Table I. The effect of lactic acid bacteria on fecal β-glucuronidase productivity

	Activity (μmol/h/g wet feces)		
	Lactic acid bacteria-treated period		
	0 week	2 week	4 week
Nontreated	2.75 ± 0.27	2.43 ± 0.30	2.41 ± 0.141
0.5% HY7401	2.45 ± 0.39	0.90 ± 0.19***	1.73 ± 0.62**
1% HY7401	2.29 ± 0.40	1.64 ± 0.52**	1.30 ± 0.60***
0.5% CSG	2.36 ± 0.16	1.85 ± 0.51*	1.29 ± 0.42***
1% CSG	2.44 ± 0.16	1.57 ± 0.37**	1.93 ± 0.25**
0.5% HY8001	2.56 ± 0.11	1.57 ± 0.63**	1.49 ± 0.40***
1% HY8001	2.28 ± 0.19*	1.39 ± 0.49***	1.56 ± 0.46***

Significantly different from normal group (, $p<0.05$; **, $p<0.01$; ***, $P<0.001$).

Table II. The effect of lactic acid bacteria on fecal alkaline phosphatase productivity

	Activity (μmol/h/g wet feces)		
	Lactic acid bacteria-treated period		
	0 week	2 week	4 week
Nontreated	1.49 ± 0.58	1.61 ± 0.28	1.71 ± 0.34
0.5% HY7401	1.60 ± 0.20	1.67 ± 0.37	1.64 ± 0.34
1% HY7401	1.73 ± 0.21	1.64 ± 0.37	1.57 ± 0.16
0.5% CSG	1.49 ± 0.53	1.77 ± 0.28	1.65 ± 0.27
1% CSG	1.25 ± 0.21	1.87 ± 0.45	1.31 ± 0.53
0.5% HY8001	1.25 ± 0.10	1.94 ± 0.12	1.57 ± 0.37
1% HY8001	1.49 ± 0.19	1.76 ± 0.37	1.98 ± 0.22

Significantly different from normal group (, $p<0.05$; **, $p<0.01$).

DISCUSSION

There have been many reports to prove the relationship between serum β-glucuronidase levels and hepatic diseases: Liver damage causes an increase of serum β-glucuronidase activity in blood and β-glucuronidase inhibitors, silymarin and glycyrrhizin, protected CCl_4-injured liver imjury in mice (Mills and Smith, 1951; Levy *et al.*, 1948; Mills *et al.*, 1953; Kim *et al.*, 1994; Shim *et al.*, 2000). However, the relationship between β-glucuronidase production of intestinal microflora and hepatotoxicity has not been thoroughly studied.

Therefore, we investigated the effect of lactic acid bacteria on the productivity of β-glucuronidase of intestinal bacteria. Lactic acid bacteria *L. brevis* HY7401, *L. acidophilus* CSG and *B. longum* HY8001 inhibited the productivity of *E. coli* HGU-3 β-glucuronidase. These lactic acid bacteria *in vivo* also inhibited the productivity of β-glucuronidase of intestinal microflora by the feeding of lactic acid bacteria for 4 weeks.

To evaluate hepatoprotective effect of these lactic acid bacteria inhibiting productivity of intestinal bacterial β-glucuronidase, we measured their effects on CCl_4- and *t*-BHP-induced hepatotoxicity of mice. The treatment with CCl_4 or *t*-BHP caused a significant increase in serum ALT and AST activities in mice. Oral pretreatment with lactic acid bacteria in mice dose-dependently protected CCl_4- and *t*-BHP-induced liver injury. The hepatoprotective effects of lactic acid bacteria are comparable to that of DDB, which is a representative hepatoprotective agent used in clinic (Wang, 1984) and exhibits the more potent hepatoprotective effect than silymarin in CCl_4-hepatotoxified rats (data not shown).

Based on these findings, an inhibitor of β-glucuronidase production in intestine may be hepatoprotective and lactic acid bacteria should be an effective agent against liver injury.

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