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COMMUNICATIONS

Intestinal bacterial hydrolysis is indispensable to absorption of 18β-glycyrrhetic acid after oral administration of glycyrrhizin in rats

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Abstract—Gnotobiote rats were prepared by infecting germ-free rats with *Eubacterium* sp. strain GLH, a human intestinal bacterium capable of hydrolysing glycyrrhizin to 18β-glycyrrhetic acid. Their faeces and caecal contents showed glycyrrhizin-hydrolysing activities (31·7 and 31·3 pmol min^{-1} (mg protein)$^{-1}$, respectively) similar to those (81·0 and 39·9 pmol min^{-1} (mg protein)$^{-1}$, respectively) of conventional rats, although there was no detectable activity in germ-free rats. When glycyrrhizin (100 mg kg^{-1}) was orally administered to conventional, germ-free and gnotobiote rats, no glycyrrhizin could be detected in plasma 4 or 17 h after the administration, using EIA and HPLC assays. Plasma 18β-glycyrrhetic acid was not detected 4 or 17 h after the administration of glycyrrhizin to germ-free rats nor could this compound be detected in caecal contents or in the faeces. However, 18β-glycyrrhetic acid (0·6–2·6 nmol mL^{-1}) was detected in plasma of the conventional and the gnotobiote rats 4 and 17 h after the administration, and the caecal contents after 4 h and the cumulative faeces up to 17 h of the conventional and the gnotobiote rats contained considerable amounts of 18β-glycyrrhetic acid. These findings indicate that orally administered glycyrrhizin is poorly absorbed from the gut, but is hydrolysed to 18β-glycyrrhetic acid by intestinal bacteria such as *E.* sp. strain GLH, and the resulting 18β-glycyrrhetic acid is absorbed.

Glycyrrhizin, an active component of liquorice, *Glycyrrhiza glabra* L., is ingested orally as a component of oriental medicine and as a sweetener. It is hydrolysed to 18β-glycyrrhetic acid by human intestinal bacteria (Hattori et al 1983; Akao et al 1987). On the other hand, glycyrrhizin is not hydrolysed to 18β-glycyrrhetic acid by human liver β-D-glucuronidase (Akao et al 1991). Moreover, when glycyrrhizin is administered orally to man, glycyrrhizin is not detected in the serum but 18β-glycyrrhetic acid is detected (Nakano et al 1980). These results suggest that glycyrrhizin is not absorbed from the gastrointestinal tract, but 18β-glycyrrhetic acid produced by the hydrolysis of glycyrrhizin by intestinal bacterial enzymes is absorbed.

We have isolated *Eubacterium* sp. (strain GLH) capable of hydrolysing glycyrrhizin to 18β-glycyrrhetic acid from human faeces (Akao et al 1987). This strain produced both glycyrrhizin β-D-glucuronidase, a novel type of β-D-glucuronidase, and a common type of β-D-glucuronidase (EC 3.2.1.31).

In this paper, we present evidence that intestinal glycyrrhizin-hydrolysing bacteria are indispensable to the appearance of 18β-glycyrrhetic acid in the serum after oral administration of glycyrrhizin.

Materials and methods

Materials. Glycyrrhizin monoammonium and 18β-glycyrrhetic acid were purchased from Tokyo Kasei Co. (Tokyo, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively, and purified by repeated crystallization. Anti-glycyrrhizinyl-30-aminomethylcyclohexanoic acid-bovine serum albumin (BSA) serum and β-galactosidase-conjugated glycyrrhizin were prepared as described previously (Kanaoka et al 1983). Anti-18β-glycyrrhetyl-30-glycine-BSA serum and [3α-^{3}H]18β-glycyrrhetic acid were prepared as reported previously (Kanaoka et al 1981, 1988). 4-Methylumbelliferyl-β-D-galactoside was purchased from Sigma Chemicals Co. (St Louis, MO, USA). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). General anaerobic medium (GAM) was a product of Nissui Seiyaku Co. (Tokyo, Japan).

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Bacterial strains. Eubacterium sp. strain GLH and *Streptococcus faecalis* were isolated from human faeces (Akao et al 1987, 1988) and maintained in GAM broth. *E.* sp. strain GLH and *S. faecalis* were cultured in GAM broth for 48 and 24 h, respectively, just before use.

Animals, treatments and sampling. Male Wistar rats (5 weeks old) and male Wistar germ-free (WAJIC) rats (6 weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan). Conventional rats were maintained for one week before use and then kept individually in metabolic cages for the experiments; water and standard laboratory food (CE-2, Clea Japan, Inc.) were freely available during the experiments. Germ-free rats were individually maintained in metabolic cages under germ-free conditions and autoclaved water and germ-free CE-2 were freely available. Seven germ-free rats were infected to make the gnotobiote rats with *E.* sp. strain GLH (2 mL medium) and *S. faecalis* (1 mL medium) on the first day and with *E.* sp. GLH (2 mL medium) on the fifth day to ascertain and to establish the gnotobiote rats. Glycyrrhizin was orally administered on the seventh day. Glycyrrhizin (100 mg kg^{-1}) was orally administered to three groups of conventional, germ-free, and gnotobiote rats. Fresh faeces were obtained to measure the enzyme activities just before administration. Cumulative faeces were collected 4 and 17 h after the administration. Blood from the abdominal vein and contents of the gastrointestine were taken under pentobarbitone anaesthesia, 4 and 17 h after the administration. Plasma was prepared by centrifugation of heparinized blood.

Enzyme immunoassay (EIA) and radioimmunoassay (RIA). EIA of glycyrrhizin and RIA of 18β-glycyrrhetic acid were carried out using plasma without extraction according to the methods reported previously (Kanaoka et al 1983, 1988). The measurable range of glycyrrhizin and 18β-glycyrrhetic acid was 0·5–80 μg mL^{-1} for EIA and 0·016–80 μg mL^{-1} for RIA, respectively.

High-performance liquid chromatography (HPLC). Glycyrrhizin in plasma (50 μL) was extracted with methanol (250 μL) containing carbamazepine (2 μg mL^{-1}) as an internal standard. The methanol phase (150 μL) was evaporated to dryness under vacuum. The dried sample was dissolved in 250 μL 35% acetonitrile in 3·5 mM H_3PO_4 and 50 μL of the solution was applied to an STR-ODS II column (Shimadzu Techno Research, 150 × 4·6 i.d. mm) operated at 40°C. The mobile phase was 35% acetonitrile in 3·5 mM H_3PO_4 and the flow rate was 1·6 mL min^{-1}. Glycyrrhizin was monitored at a wavelength of 249 nm. 18β-Glycyrrhetic acid in plasma (100 μL) was extracted twice with ether (5 mL) after addition of 150 μL salt solution, 25 μL 18β-glycyrrhetyl acetate (22·78 μg mL^{-1} in ethanol) as an internal standard and 500 μL 0·1 M NaH_2PO_4 (pH 3·0). The ether phase was dried with nitrogen and then dissolved in 400 μL

chloroform. The solution was applied to a 3-mL Bond Elut Si column (Analytical International, USA), which had been washed with 2 mL chloroform:methanol (7:3, v/v), 2 mL ethyl acetate and 4 mL chloroform. Analyses were eluted with 1·6 mL chloroform:methanol (7:3) after washing with 5 mL ethyl acetate. The dried eluate was dissolved in 0·5 mL acetonitrile and then extracted with 1 mL n-hexane to remove neutral lipid. The dried acetonitrile phase was dissolved in 200 μL HPLC mobile phase and 50 μL of this solution was applied to a Cosmosil 5C18-300 column (Nacalai Tesque, Inc., Kyoto, 150 × 4·6 mm i.d.). 18β-Glycyrrhetic acid was eluted with methanol:2% acetic acid (80:20, v/v) at 1·1 mL min^{-1} at 45°C, and monitored at a wavelength of 254 nm. The measurable range was 0·04–4 μg mL^{-1} plasma.

Thin layer chromatography (TLC). TLC using silica gel plates (Merck 60F-254, thickness 0·25 mm) was carried out to determine 18β-glycyrrhetic acid in faeces and intestinal contents. A sample was suspended in 5–10 vol water with a Waring blender and an aliquot (200 μL) was twice extracted with ethyl acetate (2 mL) after acidification with 1 M HCl. The ethyl acetate phase was dried in a rotary evaporator and the residue dissolved in a small amount of methanol. The prepared plate was developed with a solvent system of chloroform: petroleum ether: acetic acid (5:5:1, v/v/v). 18β-Glycyrrhetic acid was detected on the plate under UV light, and quantified using Shimadzu Chromatoscanner CS-910 ($\lambda s = 250$ nm, $\lambda r = 400$ nm).

Enzyme assay. Faecal and intestinal bacterial suspensions were sonicated to obtain maximal activities and used as enzyme sources.

β-D-Glucuronidase activity using *p*-nitrophenyl-β-D-glucuronide as a substrate and glycyrrhizin-hydrolysing activity were measured as reported previously (Akao et al 1987). β-D-Glucosidase activity for *p*-nitrophenyl-β-D-glucopyranoside was measured as follows: the assay mixture contained 25 μmol *p*-nitrophenyl-β-D-glucopyranoside, an enzyme solution and 100 mM potassium phosphate buffer (pH 6·5) in a final volume of 1·0 mL. The mixture was incubated for 10–30 min at 37°C and the reaction was stopped by adding 0·25 mL 5% Na_2CO_3. The supernatant solution after centrifugation at 3000 rev min^{-1} was measured at a wavelength of 405 nm. The reaction rate was calculated using a calibration line for *p*-nitrophenol.

Determination of protein. Protein was determined by the Lowry method using BSA as the standard (Lowry et al 1951).

Results and discussion

Enzyme activities of fresh faeces and caecal contents. Faeces and caecal contents of germ-free rats showed no β-D-glucosidase, β-D-glucuronidase or glycyrrhizin hydrolase activity (Table 1). On the other hand, faecal and caecal glycyrrhizin-hydrolysing activities of gnotobiote rats, which were infected with *E.* sp. strain GLH and *S. faecalis* from human faeces, were found and were similar to those of conventional rats (Table 1), although both β-D-glucosidase and β-D-glucuronidase activities of the gnotobiote rats were remarkably lower (one-tenth and one-fifth, respectively) than those of the conventional rats. As *S. faecalis*, which does not produce any β-D-glucuronidases for *p*-nitrophenyl-β-D-glucuronide and glycyrrhizin (Akao et al 1988), was infected to keep the *E.* sp. strain GLH alive, these results indicate that a human intestinal bacterium, *E.* sp. strain GLH, producing glycyrrhizin-specific β-D-glucuronidase (Akao et al 1987) inhabits the intestinal tract of the gnotobiote rats. Moreover, the finding that ratios of glycyrrhizin-hydrolysing activity to β-D-glucosidase and β-D-glucuronidase activities were much higher in the gnotobiote rats in comparison with those in the conventional rats suggests an important role of *E.* sp. strain GLH in glycyrrhizin metabolism in human intestine.

18β-Glycyrrhetic acid in faeces and caecal contents after oral administration of glycyrrhizin. The results (Table 2) suggest that orally administered glycyrrhizin is hydrolysed to 18β-glycyrrhetic acid within 4 h in rat intestinal tract and then 18β-glycyrrhetic acid is excreted in the faeces. The low recovery in the gnotobiote

Table 1. Activities of β-glucosidase, β-glucuronidase and glycyrrhizin hydrolase of faeces and caecal contents in conventional, germ-free and gnotobiote rats.

	β-Glucosidase (nmol min^{-1} mg^{-1})		β-Glucuronidase (nmol min^{-1} mg^{-1})		Glycyrrhizin hydrolase (pmol min^{-1} mg^{-1})	
	Faeces	Caecum	Faeces	Caecum	Faeces	Caecum
Conventional	17·1 ± 3·0	7·48 ± 1·46	10·8 ± 1·2	7·39 ± 0·94	81·0 ± 12·3	39·9 ± 17·9
Germ-free	ND	ND	ND	ND	ND	ND
Gnotobiote	0·670 ± 0·120	0·800 ± 0·130	1·68 ± 0·28	2·40 ± 0·41	31·7 ± 3·7	31·3 ± 11·8

ND: not detected. Means ± s.e. are given (n = 6 except for 7 gnotobiote rats).

Table 2. 18β-Glycyrrhetic acid in caecum and faeces of conventional, germ-free and gnotobiote rats after oral administration of glycyrrhizin.

	18β-Glycyrrhetic acid (μmol)			
	Caecum		Faeces	
	4 h	17 h	4 h	17 h
Conventional	2·56 ± 0·24	0·107 ± 0·107	0·068 ± 0·058	10·7 ± 2·2
Germ-free	ND	ND	ND	ND
Gnotobiote	1·13 ± 0·79	ND	ND	4·08 ± 0·49

ND: not detected. Means ± s.e. are given (n = 3 except for 4 gnotobiote rats after 17 h). 18β-Glycyrrhetic acid was determined in caecal contents at 4 and 17 h, and in cumulative faeces until 4 and 17 h after oral administration of glycyrrhizin (100 mg kg^{-1}).

Table 3. Plasma 18β-glycyrrhetic acid in conventional, germ-free and gnotobiote rats 4 and 17 h after oral administration of glycyrrhizin.

	18β-Glycyrrhetic acid (nmol mL^{-1})			
	4 h		17 h	
	RIA	HPLC	RIA	HPLC
Conventional	1·99 ± 0·31	2·29 ± 0·36	0·767 ± 0·237	0·600 ± 0·240
Germ-free	ND	ND	ND	ND
Gnotobiote	2·12 ± 1·03	2·58 ± 1·33	1·24 ± 0·53	1·70 ± 0·72

ND: not detected. Means ± s.e. are given (n = 3 except for 4 gnotobiote rats after 17 h).

rats as 18β-glycyrrhetic acid may be due to better absorption of 18β-glycyrrhetic acid from intestine in the gnotobiote rats, which is suggested from a higher plasma concentration of 18β-glycyrrhetic acid in the gnotobiote rats than in the conventional rats (Table 3).

Although glycyrrhizin was detected in the cumulative faeces and the caecal contents of all groups of rats, quantitative data could not be obtained because of difficulty of complete recovery in the extraction step.

Plasma glycyrrhizin and 18β-glycyrrhetic acid concentrations after oral administration of glycyrrhizin. Glycyrrhizin was not detected in all the plasma of conventional, germ-free or gnotobiote rats 4 or 17 h after oral administration of glycyrrhizin by either EIA or HPLC (data not shown). Accordingly, glycyrrhizin itself seems not to be absorbed from the gut.

On the other hand, plasma 18β-glycyrrhetic acid (0·6–2·6 nmol mL^{-1}) was detected in the conventional and gnotobiote rats 4 and 17 h after the oral administration of glycyrrhizin (Table 3). RIA and HPLC assay results were similar. These findings are compatible with the amounts of 18β-glycyrrhetic acid found in the caecal contents at 4 and 17 h (Table 2). However, no 18β-glycyrrhetic acid was detected in plasma of the germ-free rats at either time after the oral administration of glycyrrhizin, by either assay method, as would be expected from the lack of glycyrrhetic acid in the caecal contents and faeces of the germ-free rats throughout the experiment (Table 2).

From the present results, it is concluded that intestinal bacteria such as *E.* sp. strain GLH producing glycyrrhizin β-D-glucuronidase are necessary for the appearance of 18β-glycyrrhetic acid in rat plasma after oral administration of glycyrrhizin. Thus, orally administered glycyrrhizin is first hydrolysed to 18β-glycyrrhetic acid by bacteria in the lower parts of intestine and thereafter 18β-glycyrrhetic acid, not glycyrrhizin, is absorbed from the intestine. Accordingly, when glycyrrhizin is orally administered, its pharmacological and other side-effects seem to be caused by the resulting 18β-glycyrrhetic acid, which is known to inhibit 11β-hydroxysteroid dehydrogenase causing pseudoaldosteronism (Monder et al 1989).

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