Differences in the Metabolism of Glycyrrhizin, Glycyrrhetic Acid and Glycyrrhetic Acid Monoglucuronide by Human Intestinal Flora

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Glycyrrhizin (1.0 mM GL), glycyrrhetic acid (1.0 mM GA) and glycyrrhetic acid monoglucuronide (1.0 mM GAMG), as well as a combination of all components added to medium at the start of growth and at the maximal stage of intestinal flora were cultured for 24 and 12 h, respectively. GL alone enhanced GL β -D-glucuronidase activity about 2.7- to 6.8-fold and was metabolized to between 55 and almost 100% GA. GAMG alone was metabolized to almost 100% GA by GAMG β -D-glucuronidase activity. Intestinal flora grown to a maximal stage converted GL to about 15% GA and GAMG to about 13% GA at almost 0 h. GL in combined GL and GA was consumed about 20% at 12 h and about 100% at 24 h under different culture conditions. Metabolite GA and unchanged GA were metabolized to a negligible amount of 3-oxoglycyrrhetic acid, 3 α -hydroxyglycyrrhetic acid (3 α -hydroxyGA) or both by 3 β -hydroxysteroid dehydrogenase and 3 α -hydroxyGA dehydrogenase activities. Combined GL and GAMG consumed about 90% and 100% GAMG at 24 h and 12 h, respectively, regardless of culture conditions, and the consumption of GL was non-existent or negligible. Consumption of combined GL, GA and GAMG was similar to that of both combined GL and GA and combined GL and GAMG.

It was found that intestinal flora can metabolize GL alone, but does not readily metabolize GL when present among its metabolites containing GL.

Key words glycyrrhizin; β -D-glucuronidase; intestinal bacteria; glycyrrhetic acid; glycyrrhetic acid monoglucuronide

Almost all Kampo medicines contain liquorice in the form of *Glycyrrhiza glabra* L. Paeoniflorin and liquorice components show anti-inflammatory actions against gastric disease when used in combination.¹⁾ The extract of liquorice includes glycyrrhizin (GL) and glycyrrhetic acid (GA),^{2,3)} both of which possess anti-inflammatory effect.^{4,5)} GL is metabolized by human intestinal bacteria (Chart 1), rat intestinal bacteria and rat liver.^{6—13)} Moreover, the 22 α - and 24-positions of metabolites GA, 3-oxoglycyrrhetic acid (3-oxoGA) and 3 α -hydroxyglycyrrhetic acid (3 α -hydroxyGA) are hydroxylated by rat liver microsomes.¹⁴⁾

Notably, GL is hydrolyzed to glycyrrhetic acid monoglucuronide (GAMG) during its metabolism by human and rat livers.^{12,15)} Both GL and GAMG are excreted into the intestinal tract through the bile duct and are metabolized to GA by GL β -D-glucuronidase and GAMG β -D-glucuronidase of *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3 and human intestinal flora.^{11,13)} However, the combination of GL and GAMG is detected following the consumption of GAMG, but not GL.¹⁶⁾ Also, it is unknown whether GL in GL and GA, the extract of liquorice, is metabolized by intestinal flora or not. Thus, metabolism of GL, GA, GAMG and a combination of these components was examined by following their metabolites and related enzyme activities under controlled culture conditions using human intestinal flora.

MATERIALS AND METHODS

Chemicals GL monoammonium salt, GA and GAMG were purchased from Tokyo Kasei Kogyo Co., (Tokyo, Japan), Nacalai Tesque Inc., (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd., (Osaka, Japan), respectively. All three components were purified to a single spot using TLC (Merck, silica gel 60 F-254, layer thickness 0.25 mm). In addition, 3-oxoGA and 3 α -hydroxyGA were prepared as described.¹⁷⁾ GAM broth (a trade name) was a product of Nis-

Human intestinal bacteria

Chart 1. Metabolism of GL and GAMG by Human Intestinal Bacteria

sui Seiyaku Co., (Tokyo, Japan). All other reagents were of the highest commercial grade available.

Culture Method and Preparation of Enzyme Solution

A suspension of fresh human feces (51-year old female) containing intestinal bacteria was precultured at 37°C under anaerobic conditions. To the preculture, 9 volumes of GAM medium with or without GL, GA, GAMG and a combination of these mixed components were added at a concentration of 1.0 mM. The samples were then cultured for 12 or 24 h. Bacteria were collected by centrifugation at 6000 *g* for 10 min at 4°C. GL, GA, GAMG and their metabolites were removed by washing for 5 cycles with ethanol, which was removed from the bacteria by washing with 20 mM potassium phosphate buffer (pH 7.2). The bacteria were suspended in the same buffer and disrupted for 1 cycle by ultrasonic vibration for 1 min in an ice bath. The resultant sonicated bacteria were used as an enzyme solution. Bacterial growth was monitored by the turbidity of bacteria at 650 nm (Shimadzu UV-160 A, Kyoto).

Enzyme Assay and Determination of Metabolite Concentration The activities of GL β -D-glucuronidase, GAMG β -D-glucuronidase, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 3 α -hydroxyGA dehydrogenase were determined as described.^{7-9,12,13} Culture medium (5 ml) containing GL, GAMG and GA were mounted directly onto a TLC plate for the detection of metabolites. Metabolites 3-oxoGA and 3 α -hydroxyGA were extracted from the culture medium (100 μ l) as described.¹⁷ Then, GA, 3-oxoGA and 3 α -hydroxyGA were chromatographed with the solvent chloroform-petroleum ether-acetic acid (5:5:1, v/v) system, and GL and GAMG by a solvent system of acetic acid-*n*-butanol-1,2-

dichloroethane-H₂O (4:1:4:1, v/v). Quantity was measured using a TLC scanner ($\lambda_s=250$ nm, $\lambda_r=400$ nm, Shimadzu CS-910, Kyoto).¹⁷ The mean values for enzyme activity and metabolite concentration were determined based on triplicate experiments. These components and metabolites were detected by heating at over 100°C for 5 min on TLC plates sprayed with phosphomolybdic acid-acetic acid-H₂SO₄ (1 g:20 ml:5 ml) solution for identification.

RESULTS

Enzyme Activity of Intestinal Flora In intestinal flora cultured without the components, GAMG β -D-glucuronidase activity was 4- to 5-fold greater than GL β -D-glucuronidase activity. GAMG β -D-glucuronidase activity was enhanced with bacterial growth, and GL β -D-glucuronidase activity did not change at a lower level. The activities of 3 β -HSD and 3 α -hydroxyGA dehydrogenase are lower, indicating the slow growth of bacteria possessing these enzymes among intestinal flora (Fig. 1A). GL (1.0 mM) added to intestinal flora enhanced GL β -D-glucuronidase activity approximately 6.8-fold after 6 h and GAMG β -D-glucuronidase activity approximately 1.5-fold after 3 h (Fig. 1B). GL possessed an enhancing action on the enzyme activities of intestinal bacteria including *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 with or without *Clostridium innocuum* ES24-06.^{16,17} Whereas GA (1.0 mM) added to intestinal flora suppressed bacterial growth by approximately 40%, it enhanced GAMG β -D-glucuronidase activity about 2-fold and GL β -D-glucuronidase activity about 1.5-fold (Fig. 1C). This result showed that intestinal bacteria with GL and GAMG β -D-glu-

Fig. 1. Enzyme Activities of Intestinal Flora Cultured with or without GL, GA, GAMG and a Combination of These Components

Intestinal flora was cultured with or without 1.0 mM GL, 1.0 mM GA, 1.0 mM GAMG and a combination of these components under anaerobic conditions at 37°C. GL β -D-glucuronidase (○), 3 β -HSD (△), GAMG β -D-glucuronidase (●) and 3 α -hydroxyGA dehydrogenase (▲) activities and bacterial growth (---) were measured as described in Materials and Methods.

Fig. 2. Metabolism of GL, GA, GAMG and a Combination of These Components by Intestinal Flora

Intestinal flora was cultured with 1.0 mM GL, 1.0 mM GA, 1.0 mM GAMG and a combination of these components under anaerobic conditions at 37°C. Samples were taken from the same bottle as Fig. 1. GL (○), GA (●), GAMG (□), 3-oxoGA (△), 3α-hydroxyGA (△) and bacterial growth (---) were measured as described in Materials and Methods.

curonidases did not have a strong effect on GA. GAMG added to intestinal flora gradually enhanced bacterial growth to control levels at 18 h and GAMG β -D-glucuronidase activity about 1.6-fold at 6 h and clearly suppressed GL β -D-glucuronidase activity by about 70%, suggesting a positive suppressive effect of GAMG (Fig. 1D). Although the combination of GL and GA added to intestinal flora suppressed bacterial growth by about 30% and GAMG β -D-glucuronidase activity by about 80% up to 12 h, GL β -D-glucuronidase activity and GAMG β -D-glucuronidase activity increased about 2-fold and about 8-fold at 15 h, respectively (Fig. 1E). This result showed that intestinal bacteria possessing GL and GAMG β -D-glucuronidases were suppressed by the combined effect of GL and GA. Combined GL and GAMG added to intestinal flora strongly suppressed GAMG β -D-glucuronidase activity about 1/18-fold against control, indicating the synergistic effect of GL and GAMG (Fig. 1F). The action of combined GL and GAMG was quite different from that of combined GL and GA. Combined GL, GA and GAMG added to intestinal flora suppressed bacterial growth by about 70%, GL β -D-glucuronidase activity by about 70% and GAMG β -D-glucuronidase activity by about 95% relative to controls (Fig. 1G). GAMG in these combinations may show a stronger suppressive effect.

Metabolism of GL, GA, GAMG and their Combination by Intestinal Flora The consumption of each component in combined GL, GA and GAMG was measured. GL was metabolized to about 100% GA for enhanced GL β -D-glucuronidase activity and a negligible amount of 3-oxoGA and 3α-hydroxyGA by 3 β -HSD and 3α-hydroxyGA dehydrogenase (Fig. 2A), indicating a reversible reaction.^{7,9)} Thus, GA

alone was metabolized to a negligible amount of 3-oxoGA (Fig. 2B). GAMG started the metabolism to GA from 3 h and was metabolized almost completely to GA at 15 h by strong GAMG β -D-glucuronidase activity and to a negligible amount of 3-oxoGA and 3α-hydroxyGA (Fig. 2C). GL in combined GL and GA was consumed from 9 h to almost 100% at 24 h, and metabolite GA was increased with unchanged 1.0 mM GA from 9 h and reached a concentration of 2 mM at 24 h. Metabolites 3-oxoGA and 3α-hydroxyGA were found at negligible amounts (Fig. 2D). The combination of GL and GAMG consumed GAMG from 3 h and about 90% GAMG at 24 h regardless of lower GAMG β -D-glucuronidase activity. The consumption of GL could not be determined during 24 h due to low GL β -D-glucuronidase activity. Metabolite GA was produced over 1.0 mM at 24 h (Fig. 2E). The patterns of consumption and the production of metabolites in the combination of GL, GA and GAMG were similar to those in combined GL and GAMG (Fig. 2F). The metabolic duration of the complex of drugs was influenced by the strength of the enzymatic activities metabolizing each drug.

Effects of GL, GA, GAMG and their Combination on Intestinal Flora The effects of GL, GA, GAMG and the combination added to intestinal flora cultured for 12 h were followed by enzyme activities. 3 β -HSD activity and 3α-hydroxyGA dehydrogenase activities were at a lower level regardless of culture conditions. GL enhanced GL β -D-glucuronidase activity by about 2.7-fold and showed no effect against GAMG β -D-glucuronidase activity (Fig. 3A). GA, GAMG and a combination of these components strongly suppressed GL and GAMG β -D-glucuronidase activities by

Fig. 3. Effects of GL, GA, GAMG and a Combination of These Components on Intestinal Flora Reached a Maximal Stage

Intestinal flora added to GAM medium was cultured for 12 h under anaerobic conditions at 37 °C. GL (1.0 mM), 1.0 mM GA, 1.0 mM GAMG and a combination of these components added to cultured medium were indicated by an arrow. Intestinal flora containing each component was cultured for 0 to 12 h under the same condition. Each effect was estimated by measurement of GL β -D-glucuronidase (○), 3 β -HSD (Δ), GAMG β -D-glucuronidase (●) and 3 α -hydroxyGA dehydrogenase (Δ) activities and bacterial growth (---).

Fig. 4. Metabolism of GL, GA, GAMG and a Combination of These Components by Intestinal Flora Reached a Maximal Stage

Intestinal flora added to GAM medium was cultured for 12 h under anaerobic conditions at 37 °C. GL (1.0 mM), 1.0 mM GA, 1.0 mM GAMG and a combination of these components added to cultured medium were metabolized for 0 to 12 h under the same condition. Metabolites of GL were measured from up to 3 h every 1 h to 12 h. Samples were taken from the same bottle as Fig. 3. GL (○), GA (●), GAMG (□), 3-oxoGA (Δ), 3 α -hydroxyGA (Δ) and bacterial growth (---) were measured as described in Materials and Methods.

about 50 to 96% (Figs. 3B, C, D, E and F). This may show the death of bacteria. GAMG suppressed GAMG β -D-glucuronidase activity more strongly than GA. GL, GAMG and GA differed in the number of glucuronides, 2, 1 and 0, respectively and their actions.

Metabolism of GL, GA, GAMG and their Combination by Intestinal Flora Reached a Maximal Stage The duration of GL metabolism decreased from about 12 h to almost 0 h using intestinal bacteria of different growth.^{16,18)} On exposing GL, intestinal flora produced about 15% GA. The conversion rate of metabolite GA from GL was about 55% at 12 h, indicating the requirement of higher GL β -D-glucuronidase activity for GL metabolism (Fig. 4A). GA was converted to a negligible amount of 3-oxoGA and 3 α -hydroxyGA (Fig. 4B). On exposure of GAMG, the bacteria converted to about 13% GA. Metabolite GA was produced to almost 100% at 6 h (Fig. 4C). The consumption of combined GL and GA occurred in GA from the product of a negligible amount of metabolite 3-oxoGA and in about 20% GL at 12 h by low GL β -D-glucuronidase activity (Fig. 4D). Combined GL and GAMG occurred in the consumption of about 13% GAMG at almost 0 h and the pattern of GL consumption was similar to that of GL consumption in combined GL and GA (Fig. 4D). Metabolite GA was produced at about 13 to over 110% at 0 to 12 h (Fig. 4E). GL, GA and GAMG in combined GL, GA and GAMG were consumed from 3, 0 and 0 h, respectively (Fig. 4F). This result was similar to the consumption of each component in GA (Fig. 4B), combined GL and GA (Fig. 4D), and combined GL and GAMG (Fig. 4E). GL with GA, GAMG or both was not readily metabolized by intestinal flora.

DISCUSSION

When GL, the major extract of liquorice, was administered orally to humans (100 mg), metabolite GA without GL appeared in the plasma at 30 min.¹⁹⁾ This is too early to metabolize GL. This appearance time was demonstrated by the examination of human intestinal flora. GL with mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 and intestinal flora added to medium was metabolized to about 50%¹⁸⁾ and 100% and a negligible amount of 3-oxoGA and 3 α -hydroxyGA (Figs. 1B and 3A), respectively, at 24 h. However, intestinal flora grown to a maximal stage produced GL to about 15% metabolite GA at almost 0 h (Figs. 2A and 4A). This metabolic time coincided with its appearance in blood. Next, the present study considered which digestive tract contain intestinal bacteria for metabolizing GL after oral administration of GL.²⁰⁾ GL remains in the stomach, which is the first organ. The strong possibility exists that intestinal bacteria are involved in the metabolism of GL, and that they multiplied in the nutrient-rich human stomach, suggesting a derivation from food and the mouth. This has been observed in the stomach contents of rat.²⁰⁾ Thus, GL and GA are believed to be absorbed from the stomach. The results suggested that epithelial cells in the stomach of humans possess GL-hydrolyzing enzymes due to the lack of appearance of GL in human plasma. The consensus is that metabolites include GL transported to the liver. GL and GA were converted to GAMG (0.96 to 1.54 nmol/min/mg) and to unknown products by human liver.^{12,14)} GA and GAMG including GL were ex-

creted to intestinal tract through the bile duct and were exposed into the intestinal flora, which consumed about 13% GAMG among GL, GA and GAMG and produced about 13% GA at almost 0 h (Figs. 2F and 4F). GL metabolizes less readily than either GA or GAMG. Thus, I concluded that GL can be metabolized by intestinal flora during its first passage in the stomach. GA and metabolite GA produced a negligible amount of 3-oxoGA and 3 α -hydroxyGA regardless of culture conditions (Figs. 4B, D, E and F). However, GA with a glycine conjugate of bile acid added to *Ruminococcus* sp. PO1-3 of human intestinal bacteria enhanced the production of metabolite 3-oxoGA by 2- to 3-fold.²¹⁾ Intestinal bacteria can produce GL to a relatively large amount of 3-oxoGA in the duodenum. GA and GAMG with GL were enterohepatically circulated up to the point of consumption.

GL and GA are administered orally to humans as a decoction of Kampo medicines including liquorice. GL in these components is not readily metabolized by intestinal flora present in the stomach and took a comparatively long time to be metabolized by intestinal flora. Metabolite GA and unchanged GA were metabolized to 3-oxoGA and 3 α -hydroxyGA (Figs. 2D and 4D). This reflects the fact that oral administration (after meal) of Shosaiko-to extract granules (GL, GA, baicalin and baikalein) to humans reaches a maximal peak of GL at 2 h due to the absorption of unchanged GL and GA content in plasma increases slowly from 2 h due to the absorption of GA, indicating the absence of any consumption of GL in GL and GA.²²⁾ Thus, combined GL and GA is likely absorbed in the stomach (Figs. 2A and 4A). The consensus is that these components are transported to the liver. The metabolic system of combined GL and GA is believed to be the same as that of GL alone.

Fecal samples from both conventional flora rats and rats inoculated with a human flora exhibited an enzyme profile (β -glucosidase, β -glucuronidase, nitrate reductase and nitroreductase) similar to that of human feces,²³⁾ indicating the presence of intestinal bacteria possessing the enzymes involved in the metabolism of GL in rats²⁰⁾ and humans (Figs. 2A and 4A). It can be concluded that the administration of GL to the rat induces the same outcome as in humans.^{18,24,25)}

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