Hasty Effect on the Metabolism of Glycyrrhizin by Eubacterium sp. GLH with Ruminococcus sp. PO1-3 and Clostridium innocuum ES24-06 of Human Intestinal Bacteria

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Eubacterium sp. GLH with Ruminococcus sp. PO1-3 and Clostridium innocuum ES24-06 possessing enzymes involved in the metabolism of glycyrrhizin (GL) was cultured in GAM medium with and without 1.0 mm GL or 1.0 mm glycyrhrhetic acid (GA). GA (1.0 mm) enhanced 3α-hydroxyglycyrrhetinate (3α-hydroxyGA) dehydrogenase activity, GA (1.0 mm) suppressed 3α-hydroxyGA dehydrogenase activity, GL β-d-glucuronidase activity and the mixed bacterial growth, and GL and GA showed almost no change in a lower level of 3β-hydroxy-3α-oxo-glucuronidase partially purified from each bacterium was converted GL to 3α-hydroxyGA, producing metabolites of about 60% after 10 min of incubation. These mixed bacteria possessed high enzyme activities could produce the metabolites of GL in under one hour under conditions.

Key words glycyrrhizin; glycyrhrhetic acid; β-d-glucuronidase; intestinal bacteria

Almost all Kampo medicine contains the herb liquorice Glycyrrhiza glabra L. (Leguminosae), and is administered orally to human as a decoction. A major component of liquorice is glycyrrhizin (GL), which showed antiviral action. Its aglycon, glycyrhrhetic acid (GA) possessed pronounced anti-inflammatory properties.

GL is metabolized to GA directly and to GA through glycyrhrhetic acid mono-glucuronide (GAMG). GA is converted to 3α-hydroxyglycyrrhetic acid (3α-hydroxyGA) through 3α-oxo-glycyrhrhetic acid (3α-oxo-GA) by rat and human intestinal bacteria and rat internal organs (Chart 1). GL is hydrolyzed to GA by GL β-d-glucuronidase of Eubacterium sp. GLH and Ruminococcus sp. PO1-3 and GL β-d-glucuronidase I of rat liver, and to GAMG by GL β-d-glucuronidase II of rat liver. GAMG is hydrolyzed to GA by GAMG β-d-glucuronidase of Eubacterium sp. GLH and rat liver. Oxido-reductive reaction between GA and 3α-oxo-GA is 3β-hydroxy-3α-oxo-glucuronidase (3β-HSD) of Ruminococcus sp. PO1-3 and GA dehydrogenase of rat liver micosomes. Oxido-reductive reaction between 3α-oxo-GA and 3α-hydroxyGA is 3α-hydroxyGA dehydrogenase of Clostridium innocuum ES24-06 and rat liver.

By intestinal flora, GL is converted almost completely to metabolite GA and a negligible amount of metabolite 3α-hydroxyGA and 3α-oxo-GA in culture 24 h. However, GL administered orally to rat metabolized and its metabolite GA appeared in plasma at 30 min. It is interesting in the difference of these times about metabolite GA. The conditions enhancing the bacterial growth and the metabolism of GA and GA were studied to learn whether GL-metabolizing hours can be shortened or not, using Eubacterium sp. GLH with Ruminococcus sp. PO1-3 and C. innocuum ES24-06 possessing the enzymes involved in the metabolism of GL.

MATERIALS AND METHODS

Chemicals GL monoammonium and GA was purchased from Tokyo Kasei Kogyo Co., (Tokyo) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. 3-Oxo-GA and 3α-hydroxyGA were prepared as described. GAM medium is a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the highest quality commercially available.

Culture of Bacteria and Preparation of Enzyme Solution Eubacterium sp. GLH, Ruminococcus sp. PO1-3 and C. innocuum ES24-06 isolated from human feces were cultured in GAM medium under an anaerobic condition as reported by Akao et al. Each precultured bacterium was mixed in equal volume. This mixed bacteria added to 9 volumes of GAM medium with and without 1.0 mm GL or 1.0 mm GA was cultured in triplicate for 4 h and 5 d. The sample cultured for 5 d. The bacteria were collected by centrifugation of 6000 g at 10 min at 4°C. GL, GA, and their metabolites adhering to bacteria were removed by washing with 4 ml of ethanol. The bacteria were washed with 20 mm potassium phosphate buffer (pH 7.2) to remove the ethanol and were suspended in the same buffer. The suspended bacteria were disrupted once by ultrasonic vibration for 1 min in an ice bath. The sonicated bacteria were used as an enzyme solution. Also, a bacterial mixture (100 ml) cultured for 4 h was collected by centrifugation of 6000 g at 10 min at 4°C, and was suspended in 3.0 ml of the same buffer. Sonicated bacteria (1.5 ml) was disrupted two times by ultrasonic vibration for 2 min in an ice bath, and whole bacteria (1.5 ml) were used as samples for examination. Bacterial growth was monitored by the transmission of 650 nm. The enzyme of bacteria was measured as activity in medium 1 ml.

Enzyme Assay and Determination of Metabolites The assay mixture contained 100 μmol of GL and 50 μl of en-
enzyme solution in 0.5 ml at a final volume of 0.1 ml acetate buffer (pH 5.0) for the measurement of GL hydrolyzing activity, and 100 μmol of GA or 3α-hydroxyGA, 800 μmol of NADP⁺ and 50 μl of enzyme solution in 0.5 ml at a final volume of 0.1 ml potassium phosphate buffer (pH 8.0) for GA and 3α-hydroxyGA oxidizing activities. The mixture was incubated at 37°C for 10 min. Enzyme reaction and medium cultured with GL or GA and bacteria (100 μl) were stopped by the addition of 1 ml HCl. Then, these products were extracted twice with 2 ml of ethyl acetate. After drying of the ethyl acetate solution, the residue was dissolved in a small amount of chloroform–methanol (1:1, v/v) solution and was mounted on TLC to separate GL, GA, 3-oxo-GA and 3α-hydroxyGA. TLC was performed on silica gel plate (Merck, silica gel 60F-254, layer thickness 0.25 mm). It was first chromatographed for GA, 3-oxo-GA and 3α-hydroxyGA with the solvent system of chloroform–petroleum ether–acetic acid (5:5:1, v/v) and secondly for GL with the solvent system of acetic acid-n-butanol-1,2-dichloroethane–H₂O (4:1:4:1, v/v). The quantity was measured with a TLC scanner.³

RESULTS

GL β-D-Glucuronidase Activity, 3β-HSD Activity, 3α-HydroxyGA Dehydrogenase Activity and Metabolites in the Growth of a Mixture of Eubacterium sp. GLH, Ruminococcus sp. PO1-3 and C. innocuum ES 24-06 with and without GL or GA. The main metabolite of GL by intestinal flora was GA, which accumulated in a remarkably large amount after 24 h of culture.¹³ Then, the effect of GL on the growth of these mixed bacteria including the enzyme activities was examined with that of GA (Fig. 1). The peak of GL β-D-glucuronidase activity changed to 12 h from 24 h at almost the same level by the addition of 1.0 mM GL or 1.0 mM GA. This enzyme activity and bacterial growth were suppressed by 1.0 mM GA. However, 3β-HSD activity in Ruminococcus sp. PO1-3 was lower against substrate glycyrrhetic acid, and the enzyme activity in these mixed bacteria with and without 1.0 mM GL or 1.0 mM GA was almost the same pattern at a lower level. The growth and 3α-hydroxyGA dehydrogenase activity in C. innocuum ES 24-06 reached the maximum at 4 h of culture.¹³ The peak of this enzyme activity was enhanced at between 6 and 24 h by 1.0 mM GL and was suppressed at 6 h by 1.0 mM GA. These showed that bacterial growth and the enzyme activity incidental to it increased in medium of the same volume, and thereafter the enzyme activity decreased rapidly. GL was metabolized to about 10% of GA and a negligible amount of 3-oxo-GA and 3α-hydroxyGA at 24 h of culture by these mixed bacteria (Fig. 1). The characteristic of bacteria of the three species was different from that in bacteria of multiple
species.⁷ Also, GA added to these mixed bacteria was metabolized to a negligible amount of 3-oxo-GA, but not 3α-hydroxyGA by the suppression of 3α-hydroxyGA dehydrogenase activity (Fig. 1).

Effects of GL and GA on Resting Stage of Mixed Bacteria and Their Metabolism GL (1.0 mM) added to culture during 24 and 48 h led to a linear elevation of GL β-D-glucuronidase activity by stimulating the growth of Ruminococcus sp. PO1-3, Eubacterium sp. GLH or both (Figs. 1 and 2A) and was metabolized to GA, 3-oxo-GA and 3α-hydroxyGA, in an amount very similar to those cultured 48 h with 1.0 mm GL and bacteria (Fig. 2C). GA added after the bacterial growth showed almost no effect on the growth including the enzyme activity during culture (Fig. 2B), and was metabolized to 3α-hydroxyGA (Fig. 2D). Thus, the metabolites by mixed bacteria reaching a maximal stage can be produced rapidly for high enzyme activities.

Metabolism of GL and GA by Mass of Mixed Bacteria The mixed bacteria reaching a maximal stage and high enzyme activity after 4 h of culture were collected, and whole bacteria and sonicated bacteria were used for the metabolic examination of GL and GA. This was done to confirm whether or not 1.0 mM GL or 1.0 mM GA with or without NADPH, NADPH or both added to mixed bacteria affected the production of metabolites. Whole bacteria and sonicated bacteria converted GL to GA, 3-oxo-GA and 3α-hydroxyGA, and converted GA to 3-oxo-GA and 3α-hydroxyGA by the addition of NADPH, NADPH or both at 180 min of incubation (Figs. 3A, B, C and D). The metabolite 3-oxo-GA was obtained from 1.0 mM GL in a yield of about 20% at 180 min incubation and 1.0 mM GA in a yield of about 20% at 30 min incubation by whole bacteria, and 1.0 mM GL in yields of 20 to 60% at 180 min incubation and 1.0 mM GA in yields of about 20 to 50% at 120 min incubation by sonicated bacteria (Fig. 3). These results showed that the metabolite 3-oxo-GA produced from 1.0 mM GL and 1.0 mM GA could be obtained in relatively large amount by mass of these mixed bacteria in a relatively short period of culture.

Degradation of GL by GL β-D-Glucuronidase with 3β-HSD and 3α-HydroxyGA Dehydrogenase GL (1.0 mM) was incubated with a mixture of GL β-D-glucuronidase in Eubacterium sp. GLH, 3β-HSD in Ruminococcus sp. PO-13 and 3α-hydroxyGA dehydrogenase in C. innocuum ES24-06 partially purified and was converted to GA in a yield of about
50%, 3-oxo-GA in a yield of about 6% and 3α-hydroxyGA in a yield of about 3% at 10 min of incubation. 3β-HSD activity was 4- to 5-fold higher than GL β-glucuronidase activity and about 20-fold higher than 3α-hydroxyGA dehydrogenase activity (Fig. 4). It was thus found that bacteria existing under the same condition as the enzyme reaction can produce metabolites of GL within a short period.

**DISCUSSION**

The extract of liquorice has been used in the treatment of peptic ulcer for many years\(^1\) and has GL and GA as its main components. Administered orally to rats these components can be metabolized by intestinal bacteria possessing the metabolic pathway of GL and existing in the gastrointestinal tract (Figs. 1, 2 and 3).\(^3\) Peptic ulcerations, gastric and duodenal ulcers, were cured by a histamine H\(_2\)-receptor antagonist,\(^2\) suppression of secretion of excess acid,\(^3\) protect in gastric mucosa of ulcerative tissue\(^6\) and administration of anti-inflammatory drug such as aspirin, deglycyrrhizinised liquorice (DGL), GA and carbenoxolone.\(^6\)–\(^21\)

Revers noticed that patients of gastric ulcer had taken liquorice juice, which has a marked healing influence under controlled conditions. This extract is believed to be the glycoside, GL, which did not work as well.\(^1\) The reason is the absence of GL, suggesting the presence of GA in this extract. Hydrolysis of GL yields the pentacyclic triterpenoid, GA. The pharmacological properties of this preparation have been investigated extensively in animals and have been shown to be anti-inflammatory and to affect wound healing.\(^5\) GA used in healing is thus the same component as the metabolite of GL produced by intestinal bacteria (Figs. 1 and 2) and rat internal organs.\(^3\)–\(^5\) The same effect of these drugs can be expected in anti-inflammatory action on the same metabolic pathway. However, the treatment of peptic ulcer used GA and DGL, not GL.\(^17\)–\(^19\),\(^22\)

Biogastrone-acid (GA, 100 mg) and biogastrone-disodium salt (carbenoxolone, 100 mg) were given to patients with gastric and duodenal ulcer by barium meal. The ulcer crater of duodenal ulcer patients disappeared radiologically was practically the same in both the biogastrone and the control groups (5/28 and 3/14). In contrast, the ulcer of gastric ulcer patients healed radiologically in 37% (11/30), whereas it healed in only 5% (1/20) of the control. Biogastrone-sodium salt and acid promoted the healing of gastric ulcers by the reduction of inflammation locally in the gastric mucosa.\(^17\) This GA was not metabolized to most other metabolites by intestinal bacteria (Figs. 1, 2D and 3). One difference in the organs
forming these ulcers is the presence of bile components in the duodenum. GL added after the exposure of various bile acids to intestinal bacteria was metabolized to GA, which was about 2- to 3-fold higher than that metabolized by the exposure of GL alone to intestinal bacteria. However, it appeared that gastric ulcer and duodenal ulcer were not cured by the same medicine. Combining taurodeoxycholic acid and aspirin-induced rat gastric mucosal damage caused a significant increase in the number of lesions. DGL with aspirin greatly diminished this damage. These gastric ulcers were formed by the examination, not duodenal ulcer. From these events, a potential ulcer commonly occurs in the duodenum where taurodeoxycholic acid in bile is present. The disappearance of duodenal mucosal damage may be needed to change the component of bile. Moreover, DGL in the absence of GL is an extract of liquorice containing GA. The ulcer niche of gastric ulcer patients by the administration of DGL cured 78% containing patients (44%) disappeared the crater radiologically. The reduction in size of the ulcer niche of the placebo group was 34%, and ulcer disappearance occurred in 6%. The crater of gastric ulcer patients was eliminated by the anti-inflammatory action of GA which is a component of liquorice. DGL and GA showed the same healing effect in rat and human with gastric ulcer. Dehpour et al. have reported the effect of liquorice containing GL on aspirin-induced gastric ulcer in rats. Aspirin coated with liquorice and highly glycyrrhizic liquorice reduced the number and size of ulcers, the ulcer index from 1.5 ± 0.12 to 0.5 ± 0.12 and to 0.7 ± 0.12, respectively, and the incidence from 96% to 46% and to 62%, respectively. It is found that the extract of liquorice can be used as medicine for the healing of gastric ulcer. Its effect is greater in GA than in GL because of the metabolism of GL. The active component can be produced by the same metabolic pathway from GL and GA (Figs. 2 and 3). On the other hand, GL administered orally to rat appeared as GL and metabolite GA at 30 min in plasma. This time was reduced to the metabolic time of GL by intestinal bacteria (Figs. 1, 2, 3 and 4). GL metabolizing time can be shortened under prepared conditions. Thus, GL or GA added to a resting stage of intestinal bacteria including high
enzyme activities rapidly produced metabolite GA or 3-oxo-GA, respectively (Fig. 2). Whole bacteria and sonicated bacteria possessing higher prepared enzyme activities converted GL to a relatively large amount of GA and 3-oxo-GA and a negligible amount of 3α-hydroxy-GA in a short period, and GA to a remarkably large amount of uncharged GA and a small amount of 3-oxo-GA and 3α-hydroxy-GA at between 60 and 180 min in the presence of NADP + and NADPH (Fig. 3). Surprisingly, intestinal bacteria with high enzyme activi-

ties under an prepared condition can metabolize GL to a small amount of 3α-hydroxy-GA through GA and 3-oxo-GA at 10 min culture (Fig. 4). Moreover, GL metabolized continuously (Fig. 2B), and GA and 3-oxo-GA showed high affinity in 22α- and 24-hydroxylation of rat liver microsomes.25) GA or 3-oxo-GA being the metabolite of GL may be nearer to active component than GL.

REFERENCES