Effects of Glycyrrhizin and Glycyrrhetic Acid on the Growth, Glycyrrhizin β -D-Glucuronidase and 3 β -Hydroxysteroid Dehydrogenase of Human Intestinal Bacteria

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The peak of glycyrrhizin (GL) β -D-glucuronidase activity for *Ruminococcus* sp. PO1-3 and *Eubacterium* sp. GLH changed to 24 h from 12 h of culture and to 12 h from 48 h, respectively, at almost the same level by the addition of 1.0 mM GL. This enzyme activity was about 20-fold higher in *Eubacterium* sp. GLH than in *Ruminococcus* sp. PO1-3. GL β -D-glucuronidase activity of *Ruminococcus* sp. PO1-3 with *Eubacterium* sp. GLH and the intestinal flora showed a maximal peak at 12 h of culture in the presence and absence of 1.0 mM GL. This enzyme activity was about 2.5-fold higher in mixed bacteria than in intestinal flora. 3 β -Hydroxysteroid dehydrogenase activity of *Ruminococcus* sp. PO1-3 and *Ruminococcus* sp. PO1-3 with *Eubacterium* sp. GLH was suppressed greater in the presence of GL than without GL. Also, *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH, and a mixture of both and intestinal flora, metabolized 1.0 mM GL to glycyrrhetic acid (GA) in yields of about 10, 70, 40 and 100%, respectively, with 24 h culture. From the level of GL β -D-glucuronidase activity, it is considered that the metabolism of GL by intestinal flora is due to both enzymatic and non-enzymatic reactions. Moreover, GA at a concentration of 1.0 mM suppressed growth of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH, and the mixture of both and intestinal flora, which metabolized 1.0 mM GA to a negligible amount of 3-oxo-glycyrrhetic acid, indicating the accumulation of unchanged GA. GL β -D-glucuronidase activity of intestinal flora was enhanced by GA, which stimulated bacteria possessing particular this characteristic.

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

The root of liquorice, *Glycyrrhiza glabra* L. (Leguminosae), has been in medicinal preparations and used as one of the traditional herbs with healing properties, for over 2000 years. This root contains glycyrrhizin (GL) and glycyrrhetic acid (GA).^{1,2)} The effects of GL and GA on cell levels are reported as antiviral action, anti-inflammatory action, stimulation of growth, histamine synthesis inhibition and growth inhibition.^{3–9)} The metabolism of GL, including enzymes and metabolites, by human intestinal bacteria and by rat internal organs, is described in Chart 1.^{10–18)} Human intestinal bacteria metabolize GL to produce a remarkable amount of GA, indicating accumulation.¹²⁾ GL and GA administered orally and intravenously to humans may produce different actions to gastrointestinal bacteria existing in the digestive tract. Therefore, this paper reports about the characterization of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH, these mixed bacteria and intestinal flora metabolizing GL and GA, and the difference in effects of GL and GA on the growth of these intestinal bacteria.

MATERIALS AND METHODS

Chemicals GL monoammonium and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo and Nacalai Tesque Inc., Kyoto, respectively. GL and GA, were purified to one spot using TLC to remove impurities. 3-Oxo-glycyrrhetic acid (3-oxo-GA) was prepared as described previously.¹⁶⁾ GAM broth (trade name) is a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the highest quality commercially available.

Culture Conditions for Bacteria and Preparation of Enzyme Solution *Ruminococcus* sp. PO1-3¹⁵⁾ and *Eubacterium* sp. GLH¹²⁾ isolated from human feces, and a suspen-

sion of fresh human feces, containing intestinal flora, were precultured for 18, 42 and 18 h, respectively, in GAM medium at 37 °C under anaerobic conditions. Each preculture was added to 9 volumes of GAM medium in the presence and absence of GL or GA at a concentration of 1.0 mM. The bacteria were then cultured for 72 h in triplicate. Bacterial growth was monitored by measuring the transmission at 650 nm. The bacteria were collected by centrifugation at 6000 $g \times 10$ min at 4 °C, were washed 5 times with ethanol for the removal of GL, GA and their metabolites, and then were washed once with 20 mM potassium phosphate buffer (pH 7.2) for the removal of ethanol. The bacteria were suspended in the same buffer (1 ml) and were disrupted by ultrasonic vibration for 1 min in an ice bath. The sonicated bacteria were used as the enzyme solutions.

Enzyme Assay and Metabolites of GL and GA Assay mixture and the measurement of enzyme activities for hydrolysis of GL and oxidation of GA were described in the previous papers.^{12,15)} The reaction was stopped by the addition of 1 M HCl to the assay mixture and culture medium. The products and metabolites were extracted by ethyl acetate. GL, GA and 3-oxo-GA were chromatographed on TLC (Merck, silica gel 60F-254) with two solvent systems of chloroform–petroleum ether–acetic acid (5:5:1, v/v) and acetic acid–*n*-butanol–1,2-dichloroethane–H₂O (4:1:4:1, v/v). Quantity was measured with a TLC scanner using a calibration curve for authentic samples.

RESULTS

Enzyme Activities in the Growth of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3 with *Eubacterium* sp. GLH and Intestinal Flora in the

Human intestinal bacteria

Rat liver

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

Presence and Absence of GL or GA The growth of *Ruminococcus* sp. PO1-3 possessing GL β -D-glucuronidase and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) reached a maximum at 6 h of culture. GL (1.0 mM) added to the culture of this bacteria caused increased growth continuously during culture for 3 d, a maximal peak of GL β -D-glucuronidase activity at 24 h, from the 12 h culture time and 3 β -HSD activity was suppressed to a lower level. GA (1.0 mM) remarkably suppressed this bacterial growth and enzyme activities during the culture (Fig. 1A).

The growth of *Eubacterium* sp. GLH possessing GL β -D-glucuronidase reached a maximal stage at 24 h of culture. GL (1.0 mM) added to this bacteria increased slowly the growth during 3 d culture, and changed the maximal peak of GL β -D-glucuronidase activity to 24 h from 48 h. Although 1.0 mM GA suppressed this bacterial growth and GL β -D-glucuronidase activity, enzyme activity increased slowly from 12 h of culture (Fig. 1B).

The maximal stage of growth of *Ruminococcus* sp. PO1-3 cultured with *Eubacterium* sp. GLH changed to 12 h from 6 h of culture by addition of 1.0 mM GL. GL β -D-glucuronidase activity showed a maximal peak at 12 h culture in the presence and absence of 1.0 mM GL. This enzyme activity and the bacterial growth were about 4- and 2-fold higher, respectively, than those compared to without GL, and 3 β -HSD ac-

tivity showed the same suppression as that of *Ruminococcus* sp. PO1-3 alone. GA (1.0 mM) added to these mixed bacteria suppressed the growth, GL β -D-glucuronidase activity and 3 β -HSD activity (Fig. 1C). The characteristic of mixed bacteria of two species negated partially the appearance in that of single species.

GL (1.0 mM) added to intestinal flora enhanced GL β -D-glucuronidase activity, which showed two peaks at culture times of 12 and 48 h. GL β -D-glucuronidase activity in intestinal flora was about 4-fold higher than that in *Ruminococcus* sp. PO1-3 and was about 4.7-fold lower than that in *Eubacterium* sp. GLH alone and about 2.6-fold than that in *Ruminococcus* sp. PO1-3 with *Eubacterium* sp. GLH. It is clear that the enhancement of this enzyme activity by GL is due to the coexistence of bacteria, such as in the mixed bacteria and in the intestinal flora. GA (1.0 mM) added to intestinal flora suppressed growth, and GL β -D-glucuronidase activity was at a lower level up to 12 h of culture, and was higher than that in the absence of GA after 24 h, indicating the presence of different bacteria possessing GL β -D-glucuronidase stimulated by GA from *Ruminococcus* sp. PO1-3 and *Eubacterium* sp. GLH. Also, GA (1.0 mM) possessed activity for suppressing bacterial growth (Fig. 1D).

Metabolism of GL and GA by Intestinal Bacteria GL (1.0 mM) added to cultures of *Ruminococcus* sp. PO1-3, *Eu-*

Fig. 1. The Growth, GL β -D-Glucuronidase Activity and 3 β -HSD Activity of Intestinal Bacteria

(A) *Ruminococcus* sp. PO1-3, (B) *Eubacterium* sp. GLH, (C) mixture of both and (D) intestinal flora were cultured for 72 h in GAM medium (200 ml) with (Δ) and without ($\circ$) 1.0 mM GL or 1.0 mM GA. Samples were taken at each time. The enzyme activities were measured as described in Materials and Methods.

bacterium sp. GLH, a mixture of both and intestinal flora was metabolized to GA in yields of about 10, 70, 40 and 100% at 24 h, respectively (Fig. 2). These yields coincided well with the ratio of GL β -D-glucuronidase activity of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH and the mixture of both (Figs. 1 and 2). However, the intestinal flora showing lower GL β -D-glucuronidase activity than mixed bacteria, metabolized GL almost completely to GA by both enzymatic and non-enzymatic reactions. *Ruminococcus* sp. PO1-3, *Ruminococcus* sp. PO1-3 with *Eubacterium* sp. GLH and intestinal flora produced a negligible amount of 3-oxo-GA metabolite from 1.0 mM GL and 1.0 mM GA (Fig. 2). This is probably due to a lower level of 3 β -HSD activity and a faster rate for the reductive reaction of 3-oxo-GA to GA.¹⁵⁾ Intestinal flora could produce 3 α -hydroxyGA from GL and GA because of the presence of bacteria possessing 3 α -hydroxyGA dehydrogenase.¹⁷⁾

DISCUSSION

Liquorice is a medicine contained in most Kampo medicines. GL and GA are the main components of its extract.^{1,2)}

Oral administration of these components directly effects intestinal bacteria. The effect GL or GA on the growth and enzyme activities of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH, a mixture of, or intestinal flora were examined (Figs. 1 and 2). Exposure of GL to *Ruminococcus* sp. PO1-3 possessing GL β -D-glucuronidase and 3 β -HSD enhanced the growth and delayed the maximal peak of GL β -D-glucuronidase activity. Exposure of GL to *Eubacterium* sp. GLH possessing GL β -D-glucuronidase marginally enhanced growth and hastened the maximal peak of enzyme activity. GL β -D-glucuronidase activity of *Eubacterium* sp. GLH was about 20-fold higher than that of *Ruminococcus* sp. PO1-3. It was considered that the growth and enzyme activities of the mixed culture of these bacteria would be half of the characteristic values of each bacterium. However, exposure of GL to the mixed culture enhanced the growth about 2-fold and GL β -D-glucuronidase activity about 3.7-fold, and suppressed 3 β -HSD activity compared with the culture without GL. The patterns of growth and 3 β -HSD activity with or without GL were very similar, and GL enhanced GL β -D-glucuronidase activity about 2-fold. GL β -D-glucuronidase activity of mixed bacteria was about 2.5-fold higher than that of intestinal

Fig. 2. The Metabolism of GL and GA by Intestinal Bacteria

Ruminococcus sp. PO1-3, *Eubacterium* sp. GLH, a mixture of both and intestinal flora were cultured for 72 h in GAM medium containing 1.0 mM GL or 1.0 mM GA. Sample was obtained from the same bottle as those in Fig. 1. GL (○), GA (●), 3-oxo-GA (▲) and 3 α -hydroxyGA (△).

nal flora. The result of determination of enzyme activities coincided well with the amount of GA metabolite; about 10% in *Ruminococcus* sp. PO1-3, about 70% in *Eubacterium* sp. GLH and about 40% in mixed bacteria, produced from 1.0 mM GL (Fig. 2). Low GL β -D-glucuronidase activity of intestinal flora converted 1.0 mM GL almost completely to GA. It is considered that bacteria causing non-enzymatic hydrolysis of the glucuronide of GL exist in intestinal flora.

GA suppressed the growth and enzyme activity of *Ruminococcus* sp. PO1-3, *Eubacterium* sp. GLH and mixtures of these bacteria. However, exposure of GA to intestinal flora suppressed the growth and enhanced GL β -D-glucuronidase activity slowly from a 24 h culture, indicating the presence of bacteria stimulated by GA. GA was metabolized only to a negligible amount of 3-oxo-GA by these intestinal bacteria, and GA remained unchanged. Moreover, the production of GA by internal organs was converted from GL through glycyrrhetic acid mono-glucuronide (GAMG). GL and GAMG were converted to a remarkable amount of GAMG, a large amount of GA and a trace of 3-oxo-GA, respectively, by 1 h incubation of the enzymatic reaction by rat liver.¹¹⁾ Further, intravenous administration of GA to rats produced a residue in plasma and excretion of unchanged GA was below 1% and hydrolysate was about 14 to 16% in bile at 24 h,¹⁹⁾ in a similar manner to GA absorbed from the intestinal tract.²⁰⁾ The enzymes involved in the metabolism of GA in rat liver are GA dehydrogenase, 3 α -hydroxyglycyrrhetinate dehydrogenase and 22 α - and 24-hydroxylating enzymes.^{11,17,21)} Thus, it is considered that metabolism of GA leads to the active component.

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