Distribution of Enzymes Involved in the Metabolism of Glycyrrhizin in Various Organs of Rat

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Glycyrrhizin (GL) was hydrolyzed to glycyrrhetic acid (GA), glycyrrhetic acid mono-β-D-glucuronide (GAMG) or both by glucuronidas in various organs of rat. GL, β-D-glucuronidase I, hydrolyzing GL to GA; GAMG β-D-glucuronidase, hydrolyzing GAMG to GA; and 3α-hydroxyglycyrrhetinate (3α-hydroxyGA) dehydrogenase, oxidizing 3α-hydroxyGA to 3-oxo-GA were found in the organs of this animal. GL, β-D-glucuronidase II was distributed in the lysosomal fraction of all organs except brain; 3α-hydroxyGA dehydrogenase was distributed in the microsomal fraction of the liver; but other enzymes were distributed in the nuclear, lysosomal, microsomal and soluble fractions of a variety of organs. GL, β-D-glucuronidase I, GL, β-D-glucuronidase II and GAMG β-D-glucuronidase activities in a mixture of lysosomes and microsomes of rat liver exhibited different patterns on hydroxyapatite column chromatography. These results showed the metabolic pathways of GL to be of two types: a β-D-glucuronidase hydrolyzing GL to GA, and the other consisting of two different β-D-glucuronidases hydrolyzing GL to GAMG and GAMG to GA.

Key words: glycyrrhizin; glycyrrhetic acid; glycyrrhetic acid mono-β-D-glucuronide; β-D-glucuronidase

An important liquorice among traditional herb medicines, glycyrrhiza glabra L. contains glycyrrhizin (GL) as a main component. Many examinations on GL have been reported and it has been shown to have antiviral activity(1) and interferon inducing activity(2). Also, glycyrrhetic acid (GA), an aglycone of GL, has pronounced anti-inflammatory properties.(3)

GL is hydrolyzed to GA and the intermediate glycyrrhetic acid mono-glucuronide (GAMG) is the same as GA, which is transformed to 3α-hydroxyglycyrrhetic acid (3α-hydroxyGA) through 3-oxo-glycyrrhetic acid (3-oxo-GA) by rat(4) and human(5) intestinal bacteria. The enzymes responsible for the metabolism of GL, GL, β-D-glucuronidase, hydrolyzing GL to GA; GAMG β-D-glucuronidase, hydrolyzing GAMG to GA; 3β-hydroxysteroid dehydrogenase, oxidoreduction between GA and 3-oxo-GA; and 3α-hydroxyGA dehydrogenase, oxidoreduction between 3α-hydroxyGA and 3-oxo-GA(3-6) were found in Escherichia coli, GL, Rumonococcus sp. POI-3 and Clostridium innocuum ES24-6, respectively. Moreover, Akaō et al.(7-12) demonstrated the existence of GA dehydrogenase, oxidoreduction between GA and 3-oxo-GA in rat liver microsomes; lysosomal β-D-glucuronidase, hydrolyzing GL to GAMG in rat; and hydroxylation of GA, 3-oxo-GA and 3α-hydroxyGA in rat liver microsomes.

This paper reports the distribution of enzymes involved in metabolizing GL in various organs of the rat and the new discovery of β-D-glucuronidase activities in a mixture of lysosomal fraction and microsomal fraction of rat liver using hydroxyapatite column chromatography.

MATERIALS AND METHODS

Chemicals GL monoammonium salt, GAMG and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo, Wako Pure Chemical Industries, Ltd., Osaka, and NacalaiTesque Inc., Kyoto, respectively. 3-Oxo-GA and 3α-hydroxyGA were prepared as described by Hattori et al.(9) All other reagents were of the best quality commercially available.

Preparation of Subcellular Fractions Fresh organs, i.e., brain, lung, heart, kidney, liver, spleen and gastrointestinal tract, obtained from three 7 week old male rats of the Wistar strain were used for the preparation of enzymes without storage. The stomach, duodenum, small intestine, cecum and colon were dissected from one another. The duodenum was cut at 7 cm from the stomach. The small intestine was cut into two parts of equal length, the upper small intestine and lower small intestine. The homogenates of twelve kinds of the organs in 0.25 M sucrose were separated into nuclear, mitochondrial, lysosomal, microsomal and soluble fractions using a modification of the method of Imai et al.(13)

Assay Methods for Enzyme Activities The assay mixture for the hydrolyzing activities contained 100 μmol of GL or GAMG, 50 to 200 μl of the enzyme solution and 0.1 M acetate buffer (pH 5.0) in a final volume of 0.5 ml. The oxidizing activities were measured as described earlier(11). The mixture was incubated at 37°C for 20 to 30 min, and the reaction was stopped by the addition of 1 ml HCl. First, GA, 3-oxo-GA and 3α-hydroxyGA were separated on a TLC plate (Merck, silica gel 60 F-254, layer thickness 0.25 mm) with a solvent system of chloroform–petroleum ether–acetic acid (5:5:1, v/v) and secondarily, GL and GAMG were separated with a solvent system of acetic acid–n-butanol–1,2-dichloroethane–H2O (4:1:4:1,v/v). Method quantifying the products was previously described.4,5 Protein was determined by the method of Lowry et al.(4)

Assay Methods for the Metabolism of GL and GAMG An assay mixture containing 67 μmol of GL or GAMG, 1.3 mmol of NADP+ and NADPH, 5 mg of protein and 0.1 M acetate buffer (pH 5.0) in a final volume of 1.5 ml was incubated at 37°C. The mixture was changed to alkaline by the addition of 1.8 ml of 0.1 M potassium phosphate buffer (pH 9.0) at 60 min for the reductive reaction, and was also changed to acid by the addition of 1.35 ml of 0.1 M acetate buffer (pH 5.0) at 120 min for the oxidative reaction. Sampling was accomplished using an indicated concentration of

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substrate every 20 min. The quantity of products metabolized from GL and GAMG was determined by the procedure described above.

RESULTS

Localization of Enzyme Activities Relating to the Metabolism of GL in Various Organs of Rat Three kinds of activities hydrolyzing GL to GA, GL to GAMG and GAMG to GA and other activities oxidizing GA to 3-oxo-GA and 3α-hydroxyGA to 3-oxo-GA relating to the metabolism of GL were measured using the homogenates of rat organs (Table 1). Activity hydrolyzing GL to GA was localized in the intestinal tract (cecum and colon), brain, heart, lung, spleen, liver and kidney; this enzyme activity was the highest in the brain. Also, activity hydrolyzing GL to GAMG was localized in the organs except the brain and this enzyme may act in the spleen and liver showing a particularly high activity. Activity hydrolyzing GAMG to GA was at a similar low level in several organs, while activity hydrolyzing GL to GAMG was higher than those hydrolyzing GL and GAMG to GA. Activity oxidizing GA to 3-oxo-GA was localized in the intestinal tract (upper small intestine and lower small intestine), brain, heart, lung and liver at a low rate, and that oxidizing 3α-hydroxyGA to 3-oxo-GA was localized only in the liver. As a matter of interest, the liver had five different enzymes involved in the metabolism of GL. These results showed that GL-hydrolyzing enzymes, hydrolytic enzyme(s) of GL to GA, GL to GAMG or both, exist in various organs of rat.

Subcellular Distribution of Enzyme Activities Involved in the Metabolism of GL in Various Rat Organs Figures 1A, B, C, D and E show the distribution of enzyme activities involved in the metabolism of GL in subcellular fractions.

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Table 1. Enzyme Activities Involved in the Metabolism of GL in Rat Internal Organs

<table>
<thead>
<tr>
<th>Organ</th>
<th>GL→GA</th>
<th>GL→GAMG</th>
<th>GAMG→GA</th>
<th>GA→3-oxo-GA</th>
<th>3α-(OH)GA→3-oxo-GA</th>
<th>Protein (mg/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>N.D.</td>
<td>0.17</td>
<td>0.03</td>
<td>N.D.</td>
<td>N.D.</td>
<td>81.3</td>
</tr>
<tr>
<td>Duodenum</td>
<td>N.D.</td>
<td>0.23</td>
<td>0.04</td>
<td>N.D.</td>
<td>N.D.</td>
<td>113.6</td>
</tr>
<tr>
<td>Upper small intestine</td>
<td>N.D.</td>
<td>0.26</td>
<td>0.03</td>
<td>0.03</td>
<td>N.D.</td>
<td>101.0</td>
</tr>
<tr>
<td>Lower small intestine</td>
<td>N.D.</td>
<td>0.27</td>
<td>0.03</td>
<td>0.02</td>
<td>N.D.</td>
<td>81.3</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.06</td>
<td>0.35</td>
<td>0.19</td>
<td>N.D.</td>
<td>N.D.</td>
<td>99.0</td>
</tr>
<tr>
<td>Colon</td>
<td>0.02</td>
<td>0.42</td>
<td>0.05</td>
<td>N.D.</td>
<td>N.D.</td>
<td>96.2</td>
</tr>
<tr>
<td>Brain</td>
<td>0.25</td>
<td>N.D.</td>
<td>0.04</td>
<td>0.08</td>
<td>N.D.</td>
<td>113.6</td>
</tr>
<tr>
<td>Heart</td>
<td>0.04</td>
<td>0.07</td>
<td>0.04</td>
<td>0.05</td>
<td>N.D.</td>
<td>133.3</td>
</tr>
<tr>
<td>Lung</td>
<td>0.11</td>
<td>0.33</td>
<td>0.06</td>
<td>0.04</td>
<td>N.D.</td>
<td>158.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.10</td>
<td>0.79</td>
<td>0.12</td>
<td>0.18</td>
<td>0.02</td>
<td>90.1</td>
</tr>
<tr>
<td>Liver</td>
<td>0.04</td>
<td>0.69</td>
<td>0.09</td>
<td>0.18</td>
<td>0.02</td>
<td>59.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.03</td>
<td>0.18</td>
<td>0.04</td>
<td>N.D.</td>
<td>N.D.</td>
<td>54.3</td>
</tr>
</tbody>
</table>

a) 3α-HydroxyGA. N.D.: not detected.
Fig. 1
Fig. 1
from the organs of rat. Activity hydrolizing GL to GA was distributed in the nuclear fraction of the cecum, heart and lung, in the lysosomal fraction of the colon, brain and liver and in the microsomal fraction of the spleen and kidney (Fig. 1A). Activity hydrolizing GL to GAMG was found in the lysosomal fraction of the organs excepting the brain (Fig. 1B). Moreover, activity hydrolizing GAMG to GA was distributed in the nuclear fraction of the cecum, in the lysosomal fraction of the duodenum, upper small intestine, lower small intestine, colon, spleen, liver and kidney, in the microsomal fraction of the heart and lung and in the soluble fraction of the stomach and brain (Fig. 1C). The differences in the distribution of these hydrolizing activities suggest the existence of three different kinds of β-D-glucuronidase in the substrate species. Activity oxidizing GA to 3-oxo-GA was distributed in the lysosomal fraction of the upper small intestine and lower small intestine, and in the microsomal fraction of the brain, heart, lung, spleen and liver (Fig. 1D). Activity of oxido-reduction between 3α-hydroxyGA and 3-oxo-GA by C. innocuum ES24-06 of intestinal bacteria was only found in the microsomal fraction of liver (Fig. 1E).

Metabolism of GL and GAMG in the Homogenates

Figure 2 shows time course of the products obtained from GL or GAMG using the homogenates of the organs. GL was converted to GAMG by the homogenates of those except the brain possessing no enzyme hydrolizing GL to GAMG. The cecum, colon, spleen and liver accumulated a relatively large amount of GAMG and a small amount of GA. The gastrointestinal tract could metabolize GL to GA despite a remarkably small amount except for the stomach.

From Table 1 and Fig. 2A, it is believed that after oral administration of GL to human and rat, unchanged GL absorbed from the gastrointestinal tract is metabolized to GAMG in the epithelial cells of the same tract. GAMG is transferred to various organs including the liver, where the GAMG hydrolizing enzyme exists and which metabolizes transferred GAMG. Substrate GAMG was therefore metabolized to determine the amount of metabolite GA. GAMG was converted to GA slowly by the homogenates of various organs, indicating that activity of this enzyme is relatively low (Table 1 and Fig. 2B). Only the liver possessed GA- and 3α-hydroxyGA-oxidizing enzymes (Table 1), and converted GAMG to a small amount of 3-oxo-GA through GA, but not to 3α-hydroxyGA; as the reason, 3-oxo-GA was not accumulated for reductive activity is faster than oxidative activity. These results indicated that the enzymes involved in the metabolism of GL in the organs in rats accumulated GAMG derived from GL and GA derived from GAMG.

Separation of Hydrolizing Activities Involved in the GL Metabolism in a Mixture of Lysosome and Microsome Fractions from Rat Liver by Hydroxyapatite Column Chromatography

Enzymes hydrolizing GL to GAMG and GAMG to GA showed different peaks in the fractions of 22 and 21, respectively. Although activity hydrolizing GL to GA showed minor peak in each of fractions 8, 22 and 30, this activity had a large peak in fraction 36, indicat-
Fig. 2. Time Course of the Metabolism of GL and GAMG in Homogenates from the Organs of Rat

(A) Metabolism of GL. (B) Metabolism of GAMG. The reaction mixture was changed to the alkaline side for the reductive reaction by the addition of 0.1 M potassium phosphate buffer (pH 9.0) after 60 min and to the acid side for the oxidative reaction by the addition of 0.1 M acetate buffer (pH 5.0) after 120 min. Metabolites were chromatographed with authentic samples and were quantified as described in Materials and Methods. Symbols: GL(△), GAMG(△), GA(O) and 3-oxo-GA(●).
Fig. 3. Separation of Hydrolyzing Activities in the Mixture of Lyosome and Microsome Fractions from Rat Liver by Hydroxyapatite Column Chromatography

A mixture of lyosome and microsome fractions from rat liver was treated by sonication. The supernatant solution (27 ml) obtained by centrifugation (16000 g, 90 min at 4°C) was applied to a column (1.8×11 cm) of hydroxyapatite previously equilibrated with 5 mM potassium phosphate buffer (pH 7.2) containing 0.2 mM EDTA. The column was washed with the same buffer and GL- and GAMG-hydrolyzing activities were eluted using a linear gradient of two steps of 5 to 200 mM and 200 to 400 mM potassium phosphate buffer (pH 7.2). The activities hydrolyzing GL to GA(△), GL to GAMG(○) and GAMG to GA(●) were measured as described in Materials and Methods and also in all fractions of 18 to 28. Protein was measured at the absorbance of 280 nm.

Fig. 4. TLC of Hydrolyzing Activities

Enzyme solutions separated by hydroxyapatite column chromatography using a mixture of microsomes and lysosomes of rat liver as shown in Fig. 3, fractions 20 to 27 (A and B) and 34 to 44 (C and D), were used to measure the enzyme activities hydrolyzing GL to GA, GL to GAMG and GAMG to GA, the reaction solution containing 0.1 M acetate buffer (pH 5.0), 200 μl of enzyme solution and substrate GL (A and C) or GAMG (B and D) was incubated at 37°C for 20 min. The photograph was taken under UV of 254 nm.

[Images of TLC results are shown, indicating various bands of GL, GA, GAMG, and GA at different fractions.]

ing a very different profile from the activities hydrolyzing GL to GAMG and GAMG to GA (Fig. 3). Figure 4 shows differences in hydrolyses of GL to GA, GL to GAMG and GAMG to GA. The amount of GA converted from GL is out of all proportion to that of GA converted from GAMG shown in fractions 21 to 22 and fractions 36 to 38, and that of GAMG converted from GL shown in fractions 21 to 24 and 34 to 44. This is seen in Fig. 1, which depicts the differences in distribution of these three kinds of hydrolyzing activities. These results suggest that rat liver possesses the enzymes defined as GL β-D-glucuronidase I (hydrolyzing GL to GA); GL β-D-glucuronidase II (hydrolyzing GL to GAMG); and GAMG β-D-glucuronidase (hydrolyzing GAMG to GA). It thus seems to include two pathways in the hydrolysis of GL to GA in rat liver. One involves direct hydrolysis of GL to GA and the other hydrolysis of GL to GA through GAMG as an intermediate (Fig. 5).
DISCUSSION

Pseudoaldosteronism simulating hyperaldosteronism occurred hypertension and hypokalemia by the surplus secretion of aldosterone was induced by long-term ingestion of liquorice containing GL or carbexoxolone being a chemical derivative of liquorice and a drug for peptic ulcer.\(^{15,10}\) Although patients have signs of mineralocorticoid excess, they have low circulating levels of aldosterone.\(^{15}\) The only source of mineralocorticoid (aldosterone) and glucocorticoid (cortisol in human and corticosterone in rat) exists in the adrenal cortex. Aldosterone was bound to the receptors of aldosterone and mineralocorticoid. The cloned and expressed mineralocorticoid receptor showed high and equivalent affinity for aldosterone, cortisol and corticosterone, confirming the presence of mineralocorticoid receptor in rat kidney and hippocampus.\(^{17}\) These three components are like structures possessing 3-ketonic group, \(\Delta^2\)-group and 11\(\beta\)-hydroxyl group in a steroid skeletal structure. At least seven enzymes, 3\(\alpha\)- and 3\(\beta\)-hydroxy-\(\Delta^2\)-steroid dehydrogenases, \(\Delta^2\)-5\(\alpha\)- and \(\Delta^2\)-5\(\beta\)-reductases, 11\(\beta\)-hydroxysteroid dehydrogenase (11\(\beta\)-HSD) and 11\(\beta\)- and 18-hydroxylases are believed to be responsible for metabolizing GL in the human body. 3\(\alpha\)- and 3\(\beta\)-Hydroxy-\(\Delta^2\)-steroid dehydrogenases, however, have not yet been characterized.

The substrate of 11\(\beta\)-HSD is cortisol and corticosterone, but not aldosterone. The 11\(\beta\)-hydroxyl group of aldosterone is protected by cyclization with the aldehyde group at C18, establishing that this 18-oxocorticosterone 11,18-hemiacetal was obtained from beef adrenal extract.\(^{18}\) This is not determined as being an enzymatic reaction, not is it clear whether or not oxidative reaction of 11\(\beta\)-hydroxyl group of aldosterone by 11\(\beta\)-HSD occurs. In rat, 11\(\beta\)-HSD was distributed in the liver, kidney, testis, lung, heart, colon and brain (Figs. 2, 3 and 4). GA and 3-oxo-GA being metabolites of GL and carbexoxolone inhibited the oxidation of corticosterone to 11\(\beta\)-corticoesterone by classic 11\(\beta\)-HSD in rat liver.\(^{20}\) This makes possible the accumulation of cortisol and corticosterone. This may be one cause of the pseudoaldosteronism syndrome evidenced by a decrease of aldosterone by inhibition occurring on the same metabolic pathway.

Although corticosterone can be completely converted to 18-hydroxy cortisol and aldosterone by 11\(\beta\)- and 18-hydroxylases, one enzyme obtained from porcine adrenal, the yields of aldosterone are not high. Also, metyrapone is an inhibitor of 11\(\beta\)- and 18-hydroxylases in metabolic pathway of corticosterone to aldosterone, indicating that the metabolism of corticosterone inhibits and itself accumulates, but not aldosterone.\(^{22}\) From these results, GL and its metabolite lead to the increase of corticosterone and the decrease of aldosterone may have an action like metyrapone.

GA is a potent inhibitor of cytosolic \(\Delta^4\)-5\(\beta\)-reductase activity, which reduces aldosterone to 5\(\beta\)-dihydroaldosterone and does not affect microsomal \(\Delta^4\)-5\(\alpha\)-reductase activity, which reduces aldosterone to 5\(\alpha\)-dihydroaldosterone in rat liver.\(^{23}\) This \(\Delta^4\)-5\(\beta\)-reductase is relatively nonspecific to the steroid substrates in rat liver homogenates, indicating that this is the enzyme metabolizing cortisol and corticosterone. That is, there is an accumulation of cortisol and cortisone.

GL absorbed after oral administration\(^{24,25}\) is then hydrolyzed to a large amount of GAMG and a small amount of GA in the gastrointestinal tract and various organs, and directly to a remarkably small amount of GA in brain lacking GL \(\beta\)-glucuronidase II (Fig. 2A), where enzymatic metabolism occurs (Table 1 and Figs. 2, 3 and 4). Fuggersberger and Franz\(^{26}\) reported that GL is hydrolyzed to GA through GAMG by \(\beta\)-glucuronidase of bovine liver with incubation of 37°C for 48 h. Hydrolytic products GAMG and GA were accumulated with 24 h of incubation and GA alone with 48 h
of incubation. GA may have been due to a bacteria such as *Escherichia coli* possessing GAMG β-o-glucuronidase, multiplying by incubation at 37 °C for 48 h. Also, GAMG was detected at concentrations of 3.18—1.3 μg/g of guinea pig liver after intravenous administration of GL, but GA was not detected. Although the method of administration may be different (i.e., intravenous or oral), the same metabolite may be circulated enterohepatically. The metabolic enzyme of GAMG was distributed in various organs although at a low rate (Table 1). The metabolite GA and 3-oxo-GA were obtained depending on the conditions (Fig. 2) in amounts equal to the amount of administered GL for the organ producing the enzymes. The inhibitory action in the activities of 11β-HSD and Δ⁴-5β-reductase by GA and 3-oxo-GA was potentially. The inhibition causes cortisol in human and corticosteron in rat to accumulate gradually and continuously and to be bound to mineralocorticoid receptors in excess, causing a syndrome simulating hyperaldosteronism.

A remarkably amount of GAMG excreted in the bile reaches intestinal bacteria, *Eubacterium* species, *E. coli*, *Ruminococcus* sp. POI-3 and *Clostridium* species possessing the GAMG-hydrolyzing enzyme. This activity was higher than the GL-hydrolyzing activity. Also, intestinal flora derived from human feces converted GAMG to GA in about 30% yield after 2 h of culture and slowly converted GL to GA. Therefore, GAMG converted by the organs and GL administered orally can be metabolized to GA, 3-oxo-GA and 3α-hydroxyGA by human and rat intestinal bacteria (Fig. 2B) living in the gastrointestinal tract. 11β-HSD present in the liver, kidney and colon meets the GA absorbed from the gastrointestinal tract, which inhibits the activity. The administration of licorice containing GL to human thus is associated with a disturbance in the metabolism of cortisol and a significant rise in urinary free cortisol, despite there being no change in plasma levels. As an example, corticosterone and cortisol showed very significant antinatriuretic and kaliuretic properties following pretreatment with carbamoxolone in the adrenalectomized rat.

GA and GAMG, 3-oxo-GA and 3α-hydroxyGA (not determined) appeared in the plasma after administration of GL to rat and human is metabolized from GL and metabolite GAMG by both the organs and intestinal bacteria (Fig. 2). Proof of this led to the conclusion that an excessive amount of cortisol and corticosterone which are accumulated as a result of inhibition in the liver, lung, heart, colon and brain producing the enzymes (Table 1) and metabolite GA involved in the metabolism of GL, 11β-HSD, Δ⁴-5β-reductase and unknown enzyme whether the enzyme activities are high cause pseudoaldosteronism by bound to mineralocorticoid receptor.

REFERENCES