3-Monoglucuronyl-Glycyrrhetinic Acid Is a Substrate of Organic Anion Transporters Expressed in Tubular Epithelial Cells and Plays Important Roles in Licorice-Induced Pseudoaldosteronism by Inhibiting 11β-Hydroxysteroid Dehydrogenase 2

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Received November 14, 2011; accepted April 25, 2012

ABSTRACT
Licorice (glycyrrhiza root) has been used as a herbal medicine worldwide with its main active constituent being glycyrrhizin (GL). Licorice sometimes causes adverse effects such as inducing pseudoaldosteronism by inhibiting type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) caused by glycyrrhetic acid (GA), a major metabolite of GL. In this study we compared the inhibitory effects of GA, GL, and 3-monoglucuronoyl-glycyrrhetinic acid (3MGA), another metabolite of GL, on 11β-HSD2 activity by using microsomes and rat kidney tissue slices. GA, 3MGA, and GL inhibited 11β-HSD2 in rat kidney microsomes, with IC50 values of 0.32, 0.26, and 2.2 μM, respectively. However, the inhibitory activity of these compounds was reduced markedly in the slices, in a medium containing 5% bovine serum albumin. Assays using human embryonic kidney 293 cells with transient transformation in active ingredients of licorice. Licorice is obtained from the roots of Glycyrrhiza glabra or Glycyrrhiza uralensis and is prescribed frequently in herbal formulas of Japanese traditional kampo medicine to treat a variety of diseases. GL is also used as a chemical drug to treat chronic hepatitis, gastric ulcers, and allergic diseases in Japan and Europe (Arase et al., 1997; van Rossum et al., 1998; Kumada, 2002) and as a natural sweetener in various foods.

It is well known that licorice and products containing GL sometimes induce adverse effects such as peripheral edema, hypokalemia, and hypertension, named licorice-induced pseudoaldosteronism (Conn et al., 1968). This condition is considered to result from GA, a major metabolite of GL, inhibiting
type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) in renal tubular epithelial cells and resulting in an elevation in cortisol levels, a potent agonist of mineralocorticoid receptors, ultimately leading to increased sodium retention and potassium excretion (Stewart et al., 1987).

In our previous report, we showed that damage in the liver to multidrug resistant-association protein (Mrp) 2, a transporter involved in bile excretion of GL and its metabolites, led to an increase in plasma and urine concentrations of 3-monoglucuronyl-glycyrrhetinic acid (3MGA), another metabolite of GL (Fig. 1) (Makino et al., 2008). In contrast, urinary excretion of GA was very low regardless of its high plasma concentration when GL was administered orally to rats (Makino et al., 2008). In human beings GA is also excreted at very low levels in the urine (Ploeger et al., 2001). Another study reported that the plasma concentration of 3MGA was significantly higher after treatment with GL for longer than 4 weeks in patients with chronic hepatitis and hypokalemia than those with normal potassium levels. However, the plasma GA concentration did not differ between the two groups (Kato et al., 1995). These results attracted attention on the possible role of 3MGA in licorice-induced pseudoaldosteronism.

In the present investigation, we compared the inhibitory effects of GL, 3MGA, and GA on 11β-HSD2 activity in vitro by using rat kidney microsomes and in situ by using rat kidney slices. We also examined the uptake of GA and 3MGA in rat kidney slices. Finally, we evaluated the uptake of GA and 3MGA in cells expressing human anion transporters. We show here that 3MGA plays a crucial role by inhibiting 11β-HSD2 in rat renal epithelial cells, whereas GA does not.

**Materials and Methods**

**Animals.** Wistar rats, SD rats, and Eisai hyperbilirubinemic rats (EHBRs) were purchased from Japan SLC (Hamamatsu, Japan). EHBRs are known to express dysfunctional Mrp2 as a result of a point mutation in the open reading frame (Ito et al., 1997). The animals received food and water ad libitum under controlled temperature (25°C), humidity, and lighting (12-h light, 12-h dark) conditions. The experimental procedures were approved by the Animal Care Committee at the Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care.

**Reagents.** GL was purchased from Calbiochem (San Diego, CA), and GA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 3MGA, cortisone, cortisol, dexamethasone, p-hydroxybenzoic acid n-butyl ester (PHB), carboxymethylcellulose (CMC), penicillin, and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). Subtilisin, Dulbecco’s modified Eagle’s medium, poly-l-lysine, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Fetal bovine serum, human kidney total RNA, pCl-neo mammalian expression vector, Hilly Max reagent with a cationic liposome, and [glycyl-1-14C]-p-aminomipiric acid (PAH; 55 mCi/mmol) were supplied by Invitrogen (Carlsbad, CA), Clontech Laboratories (Mountain View, CA), Promega (Madison, WI), Dojindo Laboratories (Kumamoto, Japan), and American Radiolabeled Chemicals (St. Louis, MO), respectively. [1,2,6,7-3H] cortisol (79.3 Ci/mmol) and [6,7-3H] estrone sulfate (ES; 45.6 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). All other chemicals were analytical grade or the highest grade available.

**Protein Assay.** Protein concentrations in various samples were determined by using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA) with BSA as the calibration standard.

**Determination of In Vitro 11β-HSD2 Activity Using Rat Kidney Microsomes.** Assays were conducted as described by Diecher et al. (2000) with slight modifications. Male Wistar rats (7 weeks old) were sacrificed by using CO2 inhalation, and their kidneys were collected. After removal of the capsule, the kidneys were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 mM sucrose and 1 mM MgCl2. The homogenate was then centrifuged (10,000 g) at 4°C for 10 min, followed by further centrifugation of the supernatant at 40,000 g for 40 min. The pellet was suspended in a solution containing 50 mM Tris-HCl buffer/glycerol (2:1) to prepare a kidney microsome fraction. The microsome fraction (1.0 mg of protein) was then incubated at 37°C for 30 min in a reaction mixture containing 50 nM [1,2,6,7-3H] cortisol, 1 mM NAD+, and 0 to 12 μM GL, 3MGA, or GA in 250 μl of 0.1 M phosphate buffer, pH 6.0. The reaction was terminated by adding 250 μl of ethanol containing 1.25 mg of cortisol and 1.25 mg of cortisone. A 10-μl aliquot of the solution was spotted onto a silica gel 60 F254 plate (Merck, Whitehouse Station, NJ) and developed with chloroform/methanol (9:1). Spots corresponding to cortisone (Rf value, 0.43) were detected under UV light and scraped into a scintillation vial, and the radioactivity was measured with a liquid scintillation counter (Hitachi Aloka Medical, Tokyo, Japan).

**Determination of In Situ 11β-HSD2 Activity Using Rat Kidney Slices.** Male Wistar rats (9 weeks old) were sacrificed by using CO2 inhalation, followed by collection of their kidneys. After removal of the capsule, the kidneys were sliced at approximately 0.5-mm thickness in the frontal section of the organ, using a tissue slicer (Natsune Seisakusyo, Tokyo, Japan) and then cut at the renal hilus into two halves. The slices were preincubated at 37°C for 20 min in 24-well plates (BD Biosciences Discovery Labware, Bedford, MA) with 0.5 ml of the incubation medium (120 mM NaCl, 16.2 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, and 10 mM NaH2PO4/Na2HPO4, pH 7.4) with or without 5% BSA. The 3MGA and GA solutions were prepared by dissolving the compounds in incubation medium containing 5% BSA or preparing dimethyl sulfoxide (DMSO) solutions of 3MGA and GA (100 mM) diluted in the incubation medium. After removal of the preincubation medium, the slices were incubated at 37°C for 2 h with 0.5 ml of medium containing 3MGA and GA and with or without 5% BSA. The medium was then replaced with 0.5 ml of medium containing 50 μM cortisol in the same composition, and the slices were incubated further at 37°C for 30 min. A 100-μl aliquot of the medium was transferred into a tube containing 300 μl of dexamethasone solution (30 μg/ml in ethanol containing 0.5% acetic acid) and stored at -20°C until analysis. Cortisone concentrations were measured by using the LC/MS/MS system described below. The kidney slices were washed three times with 2 ml of ice-cold 0.15 mM phosphate-buffered saline (PBS; pH 7.2) and then homogenized with 600 μl of PBS by sonication. The homogenate was centrifuged at 14,000g for 5 min, and the protein concentration was determined. The activity of 11β-HSD2 in the kidney slices was calibrated by the
protein content of the slices. We confirmed that the linear trend in 11β-HSD2 activity under these experimental conditions depended on incubation time and the amount of kidney tissue protein.

**Uptake of 3MGA and GA by Rat Kidney Slices.** Rat kidney slices were preincubated at either 4 or 37°C for 15 min in 24-well plates with 0.5 ml of the incubation medium containing 5% BSA. 3MGA and GA were dissolved in this incubation medium at a concentration of 4 µM. After removal of the preincubation medium, 0.5 ml of medium containing 3MGA or GA, either precooled on ice or prewarmed at 37°C, were added to each well, followed by incubation of the slices at 4 or 37°C for 30, 60, and 120 min. After removal of the medium, the slices were washed three times with 2 ml of ice-cold PBS and homogenized with 600 µl of PBS by sonication. The homogenate was centrifuged at 14,000 g for 5 min, and 100 µl of aliquot of the supernatant was then mixed with 20 µl of subtilisin (9.1 U/ml) and incubated at 37°C for 30 min. A 300-µl aliquot of the PHB solution (3 µg/ml in ethanol containing 0.5% acetic acid) was added to the supernatant to stop protein digestion and stored at −20°C until analysis. The concentrations of 3MGA and GA in the samples were measured by using a LC/MS/MS system. The protein concentration of the supernatant was measured, and uptake of 3MGA and GA was calibrated against the protein content of the slices.

**Uptake of 3MGA and GA by Cells Expressing Organic Anion Transporters.** Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycmin at 37°C in 5% CO2. For the transport assay, HEK293 cells were seeded in poly-l-lysine-coated 24-well plates (1.5 × 105 cells/well) and incubated for 24 h. cDNAs encoding human organic acid transporter (OAT) 1 and OAT3 inserted into pH19 were generously supplied by Prof. Mitsuura Sugawara (Hokkaido University, Sapporo, Japan), and then subcloned into a pCI-neo mammalian expression vector. The open reading frame coding for human organic-acid-transporting peptide (OATP) 4C1 was amplified from human kidney total RNA by a reverse transcription-polymerase chain reaction method using polymerase chain reaction primers based on sequences in the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank DNA databases under accession number BC152989, and then subcloned into a pCI-neo mammalian expression vector. The constructs were transfected into HEK293 cells by using Hilly Max reagent and a Bank of Japan/European Bioinformatics Institute/GenBank DNA database.

**Oral Administration of GA in Rats.** Male SD rats or EHBRs (9 weeks old) were anesthetized by an intraperitoneal injection of urethane (Sigma; 1 g/kg), and their jugular veins were exposed. GA suspended in 0.5% CMC was then mixed with 20 µl of subtilisin (0.91 U/ml) and incubated at 37°C for 30 min, followed by the addition of 70 µl of PHB solution (3 µg/ml in ethanol containing 0.5% acetic acid) and storage at −20°C until analysis. The concentration of 3MGA and GA in plasma or urine was measured by using a LC/MS/MS system. The activity of 11β-HSD2 in the kidney microsomes was measured radiometrically.

**LC/MS/MS Analysis.** Concentrations of 3MGA, GA, and cortisone were measured by using a LC/MS/MS system (Quattro Premier XE; Waters, Milford, MA). The mass spectrometer used an electrospray ionization source in the positive ion mode with multiple reaction monitoring. The analytical column was an Inertil ODS-3, 2.1 i.d. × 100 mm, 3 µm (GL Sciences Inc., Tokyo, Japan). The mobile phase was delivered by using a linear gradient elution system, 0.5% AcOH (A)/acetomitrile containing 0.5% AcOH (B), at a flow rate of 0.2 ml/min, with the following gradient profile: 48% B (0–1 min), increasing from 48 to 80% (1–2 min), increasing from 80 to 98% (2–5 min), and 98% (5–6.5 min). Isocratic elution using 48% B at a flow rate of 0.2 ml/min was used for cortisone and dexamethasone. The transitions (precursor to daughter) monitored and retention times were 647.4 to 453.5 m/z for 3MGA (3.8 min), 471.3 to 91.0 m/z for GA (5.8 min), 195.2 to 139.0 m/z for PHB (3.9 min), 361.3 to 163.2 m/z for cortisone (2.4 min), and 393.2 to 373.5 m/z for dexamethasone (3.4 min). PHB was used as an internal standard for the measurement of 3MGA and GA, and dexamethasone was used as cortisone. Standard samples for 3MGA, GA, and cortisone were prepared in rat plasma, urine, kidney homogenate, or water depending on the target samples. Linear regression over the concentration range of 6.4 nM to 50 µM for 3MGA and GA and 0.08 to 10 µM for cortisone was examined by using the peak-area ratio of the compounds to their internal standards and the least-squares method (r2 > 0.98).

**Statistics.** Statistical analysis of the data included repeated one-way analysis of variance and Bonferroni-type multiple t test for comparison of multiple groups for multiple data and Student’s t test for comparison of two independent groups. The analyses were con-
Values were 2.2 in inhibition in a dose-dependent manner (Fig. 2). The IC50 for rat kidney microsomes showed all three compounds caused inhibitory effects of GA, 3MGA, and GA on 11β-HSD2 activity in situ were estimated (Fig. 3A). A probability value of less than 0.05 was considered to be statistically significant. Inhibitory Effects of GL, 3MGA, and GA on 11β-HSD2 Activity in Rat Kidney Microsomes. Comparison of inhibitory effects of GA, 3MGA, and GA on 11β-HSD2 activity in rat kidney microsomes showed all three compounds caused inhibition in a dose-dependent manner (Fig. 2). The IC50 values were 2.2 μM for GL, 0.26 μM for 3MGA, and 0.32 μM for GA.

Inhibitory Effects of GL, 3MGA, and GA on 11β-HSD2 Activities in Rat Kidney Slices. Inhibitory effects of GL, 3MGA, and GA on 11β-HSD2 activity in situ were estimated by measuring the formation of cortisol from exogenous cortisol in rat kidney slices. When the slices were preincubated with 4 μM GA for 2 h in albumin-free conditions, the production of cortisol from cortisol decreased to approximately 6% of that measured in control medium. Preincubation of the slices with 4 μM 3MGA and GL caused a small and insignificant decrease in cortisone production, to approximately 75% of the level seen in controls (Fig. 3A). In contrast, neither GL, 3MGA, nor GA at concentrations up to 20 μM had an inhibitory effect on 11β-HSD2 activity in slices incubated with 5% BSA (data not shown). At a concentration of 100 μM, both 3MGA and GA, but not GL, caused a small decrease in cortisone production to approximately 80% of that measured in controls (Fig. 3B).

Uptake of 3MGA and GA by Rat Kidney Slices. The uptake of 3MGA and GA by rat kidney slices was monitored in the presence of BSA. When incubated at 4°C the amount of 3MGA in the slices increased for 60 min and then reached a plateau, whereas at 37°C the amount of 3MGA tended to continue to increase for 120 min (Fig. 4A). In contrast, the amount of GA in kidney slices reached a maximum regardless of the incubation temperature, with uptake profiles at 37 and 4°C completely overlapping (Fig. 4B). Uptake of 3MGA and GA by Cells Expressing Organic Anion Transporters. HEK293 cells were transfected with a plasmid containing the open reading frame of OAT1, OAT3, or OATP4C1, and the expressions of these transporters were functionally confirmed by measuring the uptakes of their substrates (Fig. 5, A, C, and E). The uptake of 3MGA by HEK293 cells transiently expressing OAT1, OAT3, or OATP4C1 was significantly greater than in mock-transfected cells (Figs. 5, B, D, and F and 6A). However, the uptake profile of GA by cells expressing the transporters was overlapping with that in mock cells (Fig. 6B). Figure 6C shows the concentration dependence of 3MGA uptake by HEK293 cells expressing organic anion transporters. The initial velocity data were visualized by Eadie-Hofstee plots (Fig. 6D), with the apparent Km values (mean ± S.E. of triplicate measurements) of 3MGA being 49.0 ± 18.3 μM for OAT1, 30.1 ± 2.9 μM for OAT3, and 41.8 ± 4.8 μM for OATP4C1.

Effects of GA and Its Metabolites on 11β-HSD2 in Rats. GA was administered orally at a dosage of 50 mg/kg to SD rats or EHBRs under anesthesia, and changes in plasma concentrations of GA and 3MGA were compared. In SD rats, the plasma GA concentration reached a peak at 90 min and was almost eliminated 12 h after administration. 3MGA was not detected in the SD rats (Fig. 7A). In EHBRs, plasma clearance of GA was slower than in SD rats, with plasma 3MGA concentration continuing to increase after administration (Fig. 7B). We then orally administered 50 mg/kg of GA to SD rats and EHBRs every 12 h for 16 days. On day 16, the plasma 3MGA concentration in EHBRs was significantly higher than in the SD rats, although plasma GA concentrations were similar in the two strains (Fig. 8A). Consistent with 3MGA and GA concentrations in plasma, the urinary...
excretion of 3MGA in EHBRs was significantly higher than in SD rats, whereas the urinary excretion of GA was not different in the two strains (Fig. 8B). The ratio of urinary excretion of GA over 24 h to the oral dosage was approximately 0.00033% in SD rats and 0.00071% in the EHBRs. We also showed that 11β-HSD2 activity in kidney microsomes of EHBRs treated with GA was significantly lower than in control EHBRs, whereas activity in SD rats treated with GA was not significantly different from control SD rats (Fig. 7C). Protein levels of 11β-HSD2 in kidney microsomes measured by Western blot analysis showed no differences between the four groups (data not shown). Although the activity of 11β-HSD2 in kidney microsomes was suppressed markedly by GA treatment in EHBRs, there was no significant difference in plasma potassium levels between any of the experimental groups (Fig. 7D). Urinary sodium levels were also unaffected by GA treatment (data not shown).

Discussion

Ingestion of licorice sometimes causes pseudoaldosteronism (Conn et al., 1968), and the mechanism is considered to be based on inhibition of 11β-HSD2 in tubular epithelial cells by GA, whereas GL is thought to have no effect (Stewart et al., 1987). Monder et al. (1989) reported that GA had a 178-fold greater inhibitory activity on 11β-HSD1 (Ki value, 3.8 nM) compared with GL (Ki value, 675 nM) in rat liver microsomes. On the other hand, Kato et al. (1995) evaluated the inhibitory effects of GA and 3MGA on 11β-HSD2 by using rat kidney microsomes and showed an equivalent effect at a concentration of 1 μM, whereas GA had a marginally greater inhibitory effect than 3MGA at 0.1 μM concentration. The present study evaluated the inhibitory effects of GA, 3MGA, and GL in rat kidney microsomes and showed that GA and 3MGA had equivalent inhibitory effects on 11β-HSD2, with a
IC$_{50}$ of approximately 0.3 $\mu$M. In contrast, the IC$_{50}$ value of GL on 11$\beta$-HSD2 was approximately 7-fold higher than either GA or 3MGA. To the best of our knowledge, this is the first report comparing the inhibitory effects of GA, 3MGA, and GL on 11$\beta$-HSD2.

Because 11$\beta$-HSD2 is located in the cytoplasm of tubular epithelial cells (Odermatt and Kratschmar, 2012), to examine the inhibitory effects of these compounds in vivo it is necessary to determine the cell membrane transport systems for GA, 3MGA, and GL. When GA, 3MGA, or GL was dissolved in BSA-free medium containing DMSO, GA had a significant inhibitory effect on 11$\beta$-HSD2 in rat kidney slices at a concentration of 4 $\mu$M, but 3MGA and GL did not. That suggests that GA passes easily through cellular membranes because of its hydrophobic structure, resulting in a stronger inhibitory effect than either 3MGA or GL that has a hydrophilic sugar moiety in its chemical structure.

It is known that GA and GL bind efficiently to serum albumin (Ichikawa et al., 1985; Ishida et al., 1988, 1989), and the pharmacokinetics of GA and GL are affected by the albumin concentration (Koga et al., 2008). Because albumin-bound compounds cannot penetrate cellular membranes, the above results may not be reflected by in vivo events. When GA, 3MGA, and GL were dissolved in medium containing BSA, inhibition of 11$\beta$-HSD2 was considerably lower than in medium without BSA. Therefore, to predict the in vivo pharmacological action of GA, 3MGA, and GL it is necessary to consider the system used to penetrate the cell membrane and also binding to albumin.

Because 3MGA and GA both have a carboxyl group in their chemical structures, it is considered that these compounds may be substrates of organic anion transporters. The major transporters for organic anions found in the basolateral membrane of renal tubular epithelial cells are OAT1 (Hosoyamada et al., 1999), OAT2 (Sun et al., 2001), OAT3 (Motohashi et al., 2002), and OATP4C1 (Mikkaichi et al., 2004). We focused on OAT1, OAT3, and OATP4C1 because the expression level of OAT2 in kidney is much lower than those of OAT1 and OAT3 in rats (Kojima et al., 2002), and PAH-transporting capability of human OAT2 is negligible compared with those of OAT1 and OAT3 (Sun et al., 2001). Compared with uptake by mock cells, HEK293 cells express-
transported from the plasma into tubular epithelial cells where it inhibits 11β-HSD2. GA is not a substrate of these transporters and exists as an unbound portion of GA bound to plasma albumin that diffuses passively into the cells and inhibits 11β-HSD2. Therefore, 3MGA, which appears in the circulation when hepatic Mrp2 function is impaired, may have a major role in pseudoaldosteronism induced by the ingestion of licorice. To prevent pseudoaldosteronism after ingestion of kampo medicines containing licorice it may be useful to monitor plasma and urine 3MGA concentrations in patients.

Acknowledgments

We thank Professor Mitsu Sugawara (Laboratory of Pharmacokinetics, Faculty of Pharmaceutical Science, Graduate School of Hokkaido University) for providing plasmids containing the open reading frame of OAT1 and OAT3.

Authorship Contributions

Participated in research design: Makino, Ohtake, Inoue, and Mizukami.

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