Inhibitory Effects of Glycyrrhetic Acid and Its Related Compounds on 3α-Hydroxysteroid Dehydrogenase of Rat Liver Cytosol

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Glycyrrhetic acid (GA), aglycone of glycyrrhizin (GL), inhibited potently ($I_{50}=7\times10^{-6}$ M) and non-competitively the activity of NAD(P)$^+$-linked 3α-hydroxysteroid dehydrogenase of rat liver cytosol. The inhibition was slightly weaker than that of indomethacin, a potent anti-inflammatory agent, but stronger than that of dexamethasone, another anti-inflammatory agent. GL, GA monoglucuronide, and 3-epi-glycyrrhetic acid also inhibited this enzyme activity, but did so less effectively ($I_{50}=5$—8×10^{-5} M). Carbenoxolone (GA 3-hemisuccinate) and 3-keto-glycyrrhetic acid showed potent inhibitory effects similar to GA, and 18α-GA showed the most powerful inhibition of the activity.

Keywords glycyrrhetic acid; 18α-glycyrrhetic acid; glycyrrhizin; 3α-hydroxysteroid dehydrogenase; inhibition

Introduction

Glycyrrhizin (GL), an active component of licorice, *Glycyrrhiza glabra*, is ingested orally as a component in oriental medicine and as a sweetener. GL shows various pharmacological effects including a steroid-like action,[1] anti-viral action[2] and interferon-inducing activity,[3] but has a side effect such as pseudoaldosteronism.[4,5] On the other hand, GL is hydrolyzed to 18β-glycyrrhetic acid (GA), an aglycone of GL, and then transformed to 3-epi-18β-glycyrrhetic acid (3-epiGA) *via* 3-keto-18β-glycyrrhetic acid (3-ketoGA) by human intestinal bacteria,[6-10] though GL is not hydrolyzed to GA, but to GA monoglucuronide, by human liver β-D-glucuronidase.[11] Moreover, when GL is administered orally to human beings, GL is not detected in their sera but GA is detected.[12] Accordingly, when GL is administered orally, GA, not GL, seems to be absorbed after the hydrolysis of GL to GA in the intestine. Although some effects of GL are explained by the mineral corticoid-mimetic activity of GL and GA,[1] the action-mechanisms of GL and GA are not yet clear. To clarify the mechanisms, effects of the agents on some steroid-metabolizing enzymes such as 5α-reductase,[13,14] 5β-reductase,[13,14] 3α-hydroxysteroid dehydrogenase (3α-HSD),[14] 3β-hydroxysteroid dehydrogenase (3β-HSD)[14] and 11β-hydroxysteroid dehydrogenase (11β-HSD)[15] were studied. GA inhibited potently the activities of 5β-reductase, 3β-HSD and 11β-HSD, and it was postulated that the inhibition of 5β-reductase and 11β-HSD resulted in the steroid-like action and the pseudoaldosteronism.

Penning and Talalay reported that a good correlation was found between the concentrations of anti-inflammatory agents required to inhibit 3α-HSD and human anti-inflammatory doses.[16,17] However, Latif *et al.* reported that GA did not inhibit the 3α-HSD of rat liver,[14] though GA is an effective anti-inflammatory agent.[18-20] In this paper, we studied the effects of GA and its derivatives on the 3α-HSD of rat liver cytosol.

Materials and Methods

Animals and Preparation of Liver Cytosol Wistar-strain male rats, 8—12 weeks old, were purchased from Shizuoka Laboratory Animal Center. After sacrifice of the rats by decapitation, livers perfused with saline were homogenized in 4 volumes of 0.15 M KCl solution, and then centrifuged at $10000\times g$ for 20 min. The supernatant was further centrifuged at $105000\times g$ for 90 min to yield the cytosol fraction. The cytosol fraction was further fractionated with 45—70% saturation of ammonium sulfate and then dialyzed against 20 mM potassium phosphate buffer (pH 7.2) as described by Smithgall and Penning.[21] The specific activity of the preparation was 43 nmol/min·mg as NAD$^+$-dependent androsterone (oxidized form of nicotinamide adenine dinucleotide-dependent 5α-androstan-3α-ol-17-one)-oxidizing activity.

Assay of Enzyme Activities The initial velocity of 3α-HSD activity was determined spectrophotometrically at 340 nm ($\varepsilon=6270\,M^{-1}cm^{-1}$ for reduced form of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH)) and at 25 °C as reported by Smithgall and Penning.[21] The oxidation of androsterone was measured as follows. The reaction mixture contained 0.1 μmol of the steroid in 10—20 μl of acetonitrile, 1 μmol of NAD$^+$ and the enzyme preparation in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.2). 5β-Androstan-3α-ol-17-one in place of androsterone or NADP$^+$ in place of NAD$^+$ was also added to the reaction mixture. The reduction of 5β-androstan-17β-ol-3-one was measured in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.2) containing 0.1 μmol of the steroid in 10—20 μl of acetonitrile, 0.3 μmol of NADPH and the enzyme preparation. 5α-Androstan-17β-ol-3-one in place of 5β-derivative or NADH in place of NADPH was also added. Varying concentrations of inhibitors were added in 5—20 μl of methanol or acetonitrile. Control velocity without inhibitors was determined in the presence of corresponding quantities of organic solvent. I_{50} values were obtained from linear-regression lines as the final concentrations of inhibitors that provide 50% inhibition.

Alcohol dehydrogenase[22] and 17β-hydroxysteroid dehydrogenase[23] activities were determined spectrophotometrically by measuring an increase of NADH at 340 nm in a 1.0 ml-system at 25 °C using 0.1 M ethanol and 0.1 mM testosterone (4-androsten-17β-ol-3-one) as the substrates, respectively, and using rat liver cytosol (15 and 0.32 nmol/min·mg, respectively) as the enzyme source.

Chemicals GL monoammonium salt was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and purified as described in our previous paper.[6] GA was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Nacalai Tesque Inc. (Kyoto), and purified by repeated crystallization. 3-EpiGA and 3-ketoGA were prepared according to the method reported previously.[6] 18α-Glycyrrhetic acid (18α-GA) was given by Maruzen Kasei Co. (Onomichi). 18β-Glycyrrhetyl mono-β-D-glucuronide (GAMG, 1-(18β-glycyrrhet-3-yl)-β-D-glucopyranuroic acid) was donated by Dr. M. Kanaoka (Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University). Carbenoxolone (18β-glycyrrhetyl-3-hemisuccinate) disodium salt, androsterone, 5β-androstan-3α-ol-17-one, 5α-androstan-17β-ol-3-one, 5β-androstan-17β-ol-3-one, and indomethacin were purchased from Sigma Chemical Co. (MO, U.S.A.). Testosterone was purchased from Nacalai Tesque Inc. (Kyoto). Dexamethasone was purchased from Wako Pure Chemical Industries Ltd. (Osaka). All other reagents were of the best commercial quality available.

Results

Inhibition of 3α-HSD Activity by GA Figure 1 shows the effects of GA on androsterone-oxidizing and 5β-androstan-17β-ol-3-one-reducing activities of rat liver cytosol. Both activities were inhibited in the presence of 0.1 mM GA, which was revealed by detecting the products

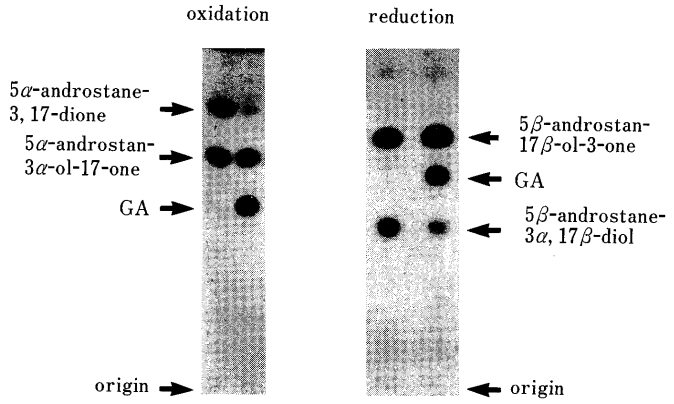

Fig. 1. Effect of GA on Oxidation of Androsterone (5α-Androstan-3α-ol-17-one) and Reduction of 5β-Androstan-17β-ol-3-one

The reaction mixture was the same as described in Materials and Methods except for the addition of 1.0×10^{-4} M GA as an inhibitor on the right lane of both plates. After incubating at 37 °C, the reaction was stopped by adding 1 N HCl, and then extracted with ethyl acetate. Ethyl acetate phase was evaporated and chromatographed on a silica gel plate (Merck, 60F-254, layer thickness 0.25 mm) with a solvent system of chloroform–petroleum ether–acetic acid (5 : 5 : 1, v/v). After developing, the substrates and their products were detected on the silica gel plates by spraying with phosphomolybdic acid–acetic acid–H_2SO_4 solution (1 g : 20 ml : 5 ml) and heating for 5—10 min at 150 °C.

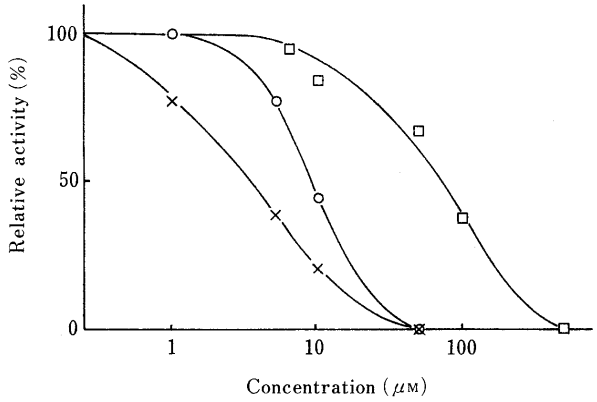

Fig. 2. Inhibition of NAD^+-Dependent Androsterone-Oxidizing Activity by GA, Indomethacin and Dexamethasone

○—○, GA; ×—×, indomethacin; □—□, dexamethasone.

on thin layer plates. The oxidizing activity was inhibited more strongly than the reducing activity. Furthermore, androsterone-oxidizing activity with NAD^+ was measured spectrophotometrically using a 45—70% saturated ammonium sulfate fraction as the enzyme preparation. Indomethacin, GA and dexamethasone inhibited dose-dependently the activity as shown in Fig. 2, and their I_{50} values were 4×10^{-6}, 7×10^{-6} and 8×10^{-5} M, respectively. The I_{50} values of indomethacin and dexamethasone are almost compatible with those reported by Penning and Talalay.[16] Thus, GA showed an inhibitory effect on 3α-HSD slightly less than the inhibitory effect of indomethacin, the most potent inhibitor of 3α-HSD.[16,17] The I_{50} value of GA on 5β-androstan-17β-ol-3-one-reducing activity with NADPH was also determined to be 3×10^{-5} M by spectrophotometric assay. The I_{50} values for the oxidizing and reducing activities in combination of 5α-androstan-3α-ol-17-one, 5β-androstan-3α-ol-17-one, 5α-androstan-17β-ol-3-one or 5β-androstan-17β-ol-3-one as a substrate with NAD^+, $NADP^+$, NADH or NADPH as a cofactor are shown in Table I. GA was less inhibitory on the reducing activities than on the oxidizing activities, and

Table I. I_{50} Values of GA against 3α-HSD for Various Steroids with Both Nicotinamide Nucleotides

Substrate	Cofactor	I_{50} of GA (μM)
5α-Androstan-3α-ol-17-one	NAD^+	7
5β-Androstan-3α-ol-17-one	NAD^+	6
	$NADP^+$	29
5α-Androstan-17β-ol-3-one	NADPH	60
5β-Androstan-17β-ol-3-one	NADH	16
	NADPH	30

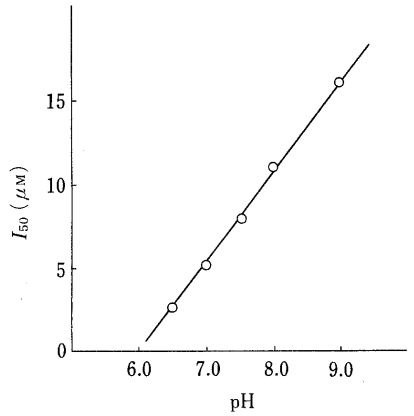

Fig. 3. Effect of pH on the Inhibition by GA of Androsterone-Oxidizing Activity by 3α-HSD

NAD^+-linked oxidation rates of androsterone were measured at various pHs as described in Materials and Methods. The I_{50} value of GA was obtained at each pH.

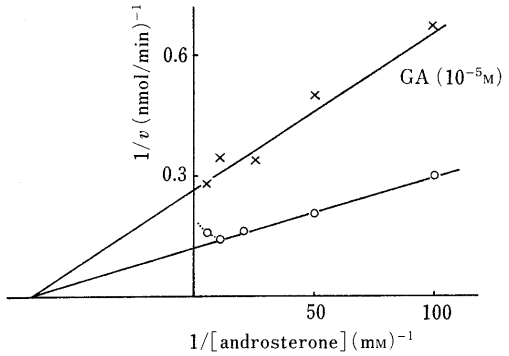

Fig. 4. Non-competitive Inhibition by GA of NAD^+-Dependent Androsterone-Oxidizing Activity by 3α-HSD

less inhibitory on the activities with $NADP^+$ and NADPH than on those with NAD^+ and NADH. The I_{50} values of GA were dependent on the pH of the assay mixture. Thus, the I_{50} values for NAD^+-dependent androsterone-oxidation varied from 2×10^{-6} M at pH 6.5 to 2×10^{-5} M at pH 9.0, resembling the results obtained with non-steroidal anti-inflammatory carboxylic acids.[17] A Lineweaver–Burk plot indicated that GA was a non-competitive inhibitor against the NAD^+-linked oxidation of androsterone (Fig. 4).

On the other hand, GA (10^{-4} M) did not have any effect on the enzyme activities of NAD^+-dependent 17β-hydroxysteroid and NAD^+-dependent alcohol dehydrogenases of rat liver cytosol (data not shown).

Effects of GL and GA Derivatives on NAD^+-Dependent 3α-HSD Activity GL and GAMG had less inhibitory effects ($I_{50} = 5 \times 10^{-5}$ M) on NAD^+-linked androsterone-

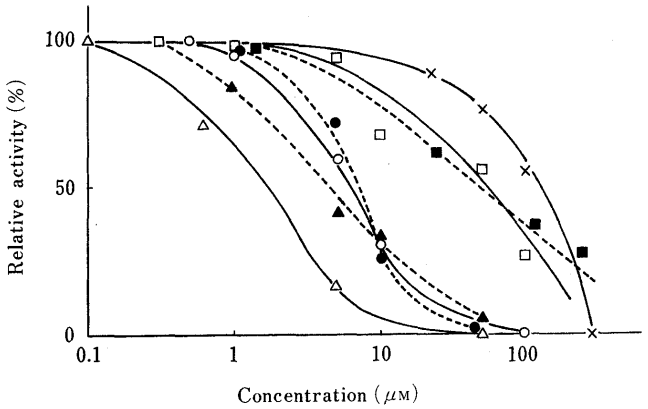

Fig. 5. Inhibition by GA and the Related Compounds of Androsterone-Oxidizing Activity by 3α-HSD

GA (○—○), 18α-GA (△—△), 3-ketoGA (●---●), 3-epiGA (×—×), carbenoxolone (▲---▲), GAMG (■---■), or GL (□—□) at various concentrations was added in the reaction mixture for NAD^+-linked androsterone-oxidizing activity.

oxidizing activity than GA, but carbenoxolone inhibited potently ($I_{50}=6\times10^{-6}$ M), similar to GA (Fig. 5). 3-KetoGA ($I_{50}=6\times10^{-6}$ M) and 3-epiGA ($I_{50}=8\times10^{-5}$ M), metabolites of GA by human intestinal bacteria, showed potent and weak inhibitions of the activity, respectively. 18α-GA, which is reported to have a potent anti-inflammatory effect,[24] also had a potent inhibitory effect ($I_{50}=1.4\times10^{-6}$ M), similar to indomethacin (Fig. 1). Thus, 18α-GA inhibited most potently 3α-HSD activity; and carbenoxolone, 3-ketoGA and GA also had potent inhibitory effects; but GL, GAMG and 3-epiGA were less inhibitory.

Discussion

Penning and Talalay proposed a good correlation between the anti-inflammatory effects of drugs and their inhibitory effects against 3α-HSD.[16] According to the present results, 18α-GA, GA and carbenoxolone, which have anti-inflammatory action,[18,19,24-26] potently inhibited 3α-HSD activity (Figs. 2 and 5). Moreover, 18α-GA inhibited the enzyme activity more potently than GA, parallel to the order of anti-inflammatory actions of these agents, where 18α-GA acts more potently than GA.[24,25] For example, 18α-GA at a daily dose of 3 mg/kg *p.o.* exhibited a similar inhibitory effect to GA at a daily dose of 30 mg/kg *p.o.* on the formation of cotton pellet granuloma in mice. Although Latif *et al.* reported that GA did not inhibit 3α-HSD activity,[14] the difference between their results and ours may be explained in that they observed the effects of GA only on the 5β-pregnan-18-al-11β,21-diol-3,20-dione-reducing activity of rat liver cytosol.

When GL was administered orally to human beings, GL was not detected in their sera but GA was detected.[12] Moreover, GL had less inhibitory activity against 3α-HSD (Fig. 5). Accordingly, GA absorbed after the hydrolysis of GL by intestinal bacteria appears to show the anti-inflammatory action. GAMG, which is found in the serum of a patient with pseudoaldosteronism[27] and is produced by lysosomal β-glucuronidase of human liver,[11] also had less inhibitory activity (Fig. 5). On the other hand, 3-ketoGA, derived from GA by 3β-HSD of human intestinal bacteria[9] and GA dehydrogenase of rat liver microsomes,[28] shows potent inhibition against 3α-HSD (Fig. 5); but 3-epiGA, transformed from 3-ketoGA by human intestinal bacteria,[6,10] has one-tenth less effect than GA and 3-ketoGA (Fig. 5). GA, produced from GL by human intestinal bacteria, may be inactivated by being further transformed to 3-epiGA *via* 3-ketoGA by human intestinal bacteria during enterohepatic circulation.

From the fact that 18α-GA acts more potently than GA in its anti-inflammatory effect[25] and in the inhibition of 3α-HSD (Fig. 5) and 5β-reductase,[25] the planar conformation of 18α-GA, close to the conformation of prednisolone,[25] may also be important for the pharmacological property and the enzyme inhibition.

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References

1) A. Kumagai, S. Yano, and M. Otomo, *Endocrinol. Jpn.*, **4**, 17 (1957).
2) R. Pompei, O. Flore, M. A. Marccialis, A. Pani, and B. Loddo, *Nature* (London), **281**, 689 (1979).
3) N. Abe, T. Ebina, and N. Ishida, *Microbiol. Immunol.*, **26**, 535 (1982).
4) J. A. Molhuysen, J. Gerbrandy, L. A. deVries, J. C. deJong, J. B. Lenstra, K. P. Turner, and J. G. G. Borst, *Lancet*, **2**, 135 (1950).
5) J. W. Conn, D. R. Rovner, and E. L. Cohen, *J. Am. Med. Assoc.*, **205**, 80 (1968).
6) M. Hattori, T. Sakamoto, K. Kobashi, and T. Namba, *Planta Medica*, **48**, 38 (1983).
7) M. Hattori, T. Sakamoto, T. Yamagishi, K. Sakamoto, K. Konishi, K. Kobashi, and T. Namba, *Chem. Pharm Bull.*, **33**, 210 (1985).
8) T. Akao, T. Akao, and K. Kobashi, *Chem. Pharm. Bull.*, **35**, 705 (1987).
9) T. Akao, T. Akao, M. Hattori, T. Namba, and K. Kobashi, *J. Biochem.* (Tokyo), **99**, 1425 (1986).
10) T. Akao, T. Akao, M. Hattori, T. Namba, and K. Kobashi, *J. Biochem.* (Tokyo), **103**, 504 (1988).
11) T. Akao, T. Akao, M. Hattori, M. Kanaoka, K. Yamamoto, T. Namba, and K. Kobashi, *Biochem. Pharmacol.*, **41**, 1025 (1991).
12) N. Nakano, H. Kato, H. Suzuki, N. Nakao, S. Yano, and M. Kanaoka, *Yakuri To Chiryō*, **8**, 4171 (1980).
13) Y. Tamura, T. Nishikawa, K. Yamada, M. Yamamoto, and A. Kumagai, *Arzneim.-Forsch.*, **29**, 647 (1979).
14) S. A. Latif, T. J. Conca, and D. J. Morris, *Steroids*, **55**, 52 (1990).
15) C. Monder, P. M. Stewart, V. Lakshmi, R. Valentino, D. Burt, and C. R. W. Edwards, *Endocrinology*, **125**, 1046 (1989).
16) T. M. Penning and P. Talalay, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4504 (1983).
17) T. M. Penning, I. Mukharji, S. Barrows, and P. Talalay, *Biochem. J.*, **222**, 601 (1984).
18) R. S. H. Finney and G. F. Somers, *J. Pharm. Pharmacol.*, **10**, 613 (1958).
19) K. K. Tamgri, P. K. Seth, S. S. Parmar, and K. P. Bhargava, *Biochem. Pharmacol.*, **14**, 1277 (1965).
20) K. Takahashi, S. Shibata, S. Yano, M. Harada, H. Saito, Y. Tamura, and A. Kumagai, *Chem. Pharm. Bull.*, **28**, 3449 (1980).
21) T. E. Smithgall and T. M. Penning, *Biochem. Pharmacol.*, **34**, 831 (1985).
22) E. Racker, "Methods in Enzymology, "Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, 1955, pp. 500—503.
23) G. L. Endahl, C. D. Kochakian, and D. Hamm, *J. Biol. Chem.*, **235**, 2792 (1960).
24) E. Sugishita, S. Amagawa, and Y. Ogihara, *J. Pharmacobio-Dyn.*, **5**, 379 (1982).
25) S. Amagaya, E. Sugishita, Y. Ogihara, S. Ogawa, K. Okada, and T. Aizawa, *J. Pharmacobio-Dyn.*, **7**, 923 (1984).
26) R. S. H. Finney and A. L. Tárnoky, *J. Pharm. Pharmacol.*, **12**, 49 (1960).
27) M. Kanaoka, S. Yano, H. Kato, and T. Nakada, *Chem. Pharm. Bull.*, **34**, 4978 (1986).
28) T. Akao, T. Akao, and K. Kobashi, *Biochim. Biophys. Acta*, **1042**, 241 (1990).