Inhibitory Effect of Glycyrrhetic Acid on Testosterone Production in Rat Gonads

KENJI SAKAMOTO^{1,2)} AND KATSUMI WAKABAYASHI²⁾

¹⁾*Tsumura Research Institute for Pharmacology.
Ami, Inashiki, Ibaraki 300-11, Japan*

²⁾*Hormone Assay Center, Institute of Endocrinology
Gunma University. Maebashi, Gunma 371, Japan*

Abstract

We studied the effects of shakuyaku-kanzo-toh (a Chinese herbal medicine) and its components on testosterone production by rat gonads. We used paeoniflorin as a main component of shakuyaku (*paeoniae radix*), glycyrrhizin as a main component of kanzo (*glycyrrhizae radix*) and glycyrrhetic acid as a main metabolite of glycyrrhizin. Oral administration of shakuyaku-kanzo-toh, glycyrrhizin, and glycyrrhetic acid decreased *in vitro* basal testosterone production in Leydig cells by LH stimulation. Glycyrrhizin and glycyrrhetic acid caused a significant decrease in testosterone production with an accumulation of 17 α -hydroxyprogesterone when incubated with isolated Leydig cells, while paeoniflorin showed no such effect. The inhibitory effect of glycyrrhetic acid was far more potent than that of glycyrrhizin, causing about 90% inhibition at 10 μ g/ml. Glycyrrhizin and glycyrrhetic acid did not change the cyclic AMP or progesterone level in the Leydig cells. When ¹⁴C-labeled androstenedione was incubated with microsomal fraction of testicular or ovarian tissue, glycyrrhizin and glycyrrhetic acid inhibited the conversion of androstenedione to testosterone, indicating that these compounds inhibit the activity of 17 β -hydroxysteroid dehydrogenase (EC. 1. 1. 1. 64). The ED⁵⁰ of glycyrrhetic acid was about 4 μ M.

The serum concentration of testosterone is sometimes high in anovulatory or infertile women, and there has been no effective treatment for such patients. Recently shakuyaku-kanzo-toh was incidentally found to be effective on such patients, reducing serum testosterone and making pregnancy possible (Yaginuma *et al.*, 1983), though its mechanism remained unknown. Originally, this prescription was used to suppress neu-

rogenic contraction. Maeda *et al.* (1983) found that this prescription is effective in inhibiting the acetylcholine release from the cholinergic nerve. Early reports indicated that liquorice (*glycyrrhizae radix*) extract had a deoxycorticosterone-like activity (Revers, 1946, Molhuysen, 1950) and that glycyrrhizin had a corticoidlike action by inhibiting the metabolism of corticoids. Kumagai *et al.* (1957, 1964, 1966, 1967) reported its anti-estrogenic action. Glycyrrhizin is known to be metabolized to glycyrrhetic acid in

the intestine (Hattori *et al.*, 1983).

In the present report, we intended to clarify the mechanism of the action of this herbal prescription on steroid metabolism by paying attention on the pathway of testosterone production in rat gonads.

Materials and Methods

Animals

Seven to eight-week-old and adult rats of Wistar strain of both sexes were purchased from Charles River Japan Inc. The animals were maintained under standardized conditions of light (lights on 05:00~19:00), temperature ($23 \pm 2^\circ\text{C}$), and humidity (55%), with free access to food (Oriental MF) and water.

Chemicals and Hormones

Extracts of kanzo and shakuyaku-kanzo-toh were prepared by Tsumura Laboratory. Glycyrrhizin and glycyrrhetic acid (Tokyo-kasei Co. Ltd.) and paeoniflorin (extracted by Tsumura Laboratory) were used in the present experiments as possible active components and the metabolite of the prescription. The chemical structures of these materials are shown in Fig. 1. All the compounds were first dissolved in a small volume of ethanol and diluted with PBS (0.01 M sodium phosphate and 0.14 M NaCl, pH 7.5) to the target concentrations. Bovine serum albumin (BSA) fraction V was purchased from Armour pharmaceutical Company. NIADDK rat LH-RP-2 and hCG were supplied by the National Hormones and Pituitary Program (Baltimore,

Md., U.S.A.). Porcine LH was purchased from UCB-Bioproducts, Inc. (The biological potency of this porcine LH is $\times 0.91$ compared with NIADDK rat LH-RP-2). Labeled steroids, 4- ^{14}C androstenedione (52.0 mCi/mmol, NEN) and 4- ^{14}C testosterone (58.0 mCi/mmol, Amersham) were dissolved in one drop of propylene glycol, and further diluted with Sol. A consisting of 0.25 M sucrose, 2 mM MgCl_2 , 20 mM Tris HCl, pH 7.5. All other chemicals were purchased from commercial sources.

Radioimmunoassays

Radioimmunoassays for testosterone, progesterone and 17 α -hydroxyprogesterone were carried out with antisera obtained by immunizing rabbits with testosterone 3-O-CMO (carboxymethyloxime): BSA (Sigma T5771), progesterone 3-O-CMO: BSA (Sigma P-4778), and 17 α -hydroxyprogesterone 3-O-CMO: BSA (Sigma H-7010), and labeled steroids, testosterone 3-O-CMO-2- ^{125}I iodohistamine (Amersham IM128), progesterone 3-O-CMO-2- ^{125}I iodohistamine (Amersham IM 139), and 17 α -hydroxyprogesterone 3-O-CMO-2- ^{125}I iodohistamine (Amersham IM145), respectively. Radioimmunoassay of cyclic AMP was carried out with antiserum obtained by immunizing rabbits with 2'-O-succinyl cyclic AMP: BSA. This succinylated cyclic AMP: BSA was prepared by the method of Steiner (1979) with minor modifications and iodinated cyclic AMP (Adenosine 3', 5-cyclic phosphoric acid 2'-O-succinyl 3- ^{125}I iodotyrosine methyl ester) was purchased from Amersham (IM 106). All radioimmunoassays proceeded as follows; 100 μl of standard compounds dissolved in 1% BSA-PBS, 100 μl of radioactive compounds (about 15,000

R=H: Glycyrrhetic acid

R=2x Glucuronic acid: Glycyrrhizin

Paeoniflorin

Fig. 1. Chemical structure of glycyrrhizin, glycyrrhetic acid and paeoniflorin.

cpm) dissolved in 1% BSA-PBS, and 100 μ l of antiserum diluted at 1:10000~50000 with 1% normal rabbit serum-50 mM EDTA-PBS were mixed and incubated overnight at 4°C, then 200 μ l of anti rabbit IgG goat serum diluted at 1:100 with 3.5% polyethyleneglycol-50 mM EDTA-PBS was added and incubated for 3 hrs at 37°C. The reaction mixture was spun and precipitate was counted in a gamma counter (Aloka ARC 600). The cross reactivity of each steroid radioimmunoassay was less than 0.1% and the cross reactivity of the cyclic AMP radioimmunoassay for cyclic GMP, ATP and GTP was also less than 0.1%. We could obtain good standard curves for cyclic AMP and these steroids with high specificity and high sensitivity (assay limits were about 100 fg/tube for steroids and 50 fm/tube for cyclic AMP).

Oral administration of glycyrrhetic acid and other materials

We administered shakuyaku-kanzo-toh extract (90 mg/kg/day, which was twice the amount for human therapeutic use), kanzo extract (45 mg/kg/day), glycyrrhizin (4.5 mg/kg/day, 5% glycyrrhizin is contained in shakuyaku-kanzo-toh), and glycyrrhetic acid (4.5 mg/kg/day) to 8-week-old male Wistar rats (10 rats in a group) for 20 days. After the administration, their testes were removed and pooled, and Leydig cell suspensions were prepared as described below to examine testosterone production. To these cell suspensions porcine LH was added to examine the testosterone release due to LH stimulation.

Leydig cell preparations

The testes removed from male rats were decapsulated and placed in a culture bottle with 20 ml of cold PBS containing 25 mg of collagenase (Worthington, type I), 0.1% BSA, and incubated at 37°C for 10 min under oscillation at 100 cycles/min. After the incubation, the tissue was filtered through nylon gauze (0.5 mm mesh), and the filtrate was centrifuged at 200 $\times$ g for 10 min under refrigeration. The supernatant was decanted off, and the cell pellet was washed with cold medium 199 (Difco), then recentrifuged. The washed cells were suspended in 50 ml of medium 199 containing 0.1% BSA and placed in incubation wells (Corning, 24 wells per plate) and the population was adjusted to about one million Leydig cells/500 μ l medium. Viable Leydig cells were estimated by incubating 500 μ l of cell

suspension for 3 hrs at 35°C with 100 IU hCG dissolved in 100 μ l of medium 199, 1.6 mg of p-nitro blue tetrazolium in 400 μ l of PBS, 3.2 mg β -NAD in 200 μ l PBS, and 0.5 mg of dehydroepiandrosterone in 100 μ l propylene glycol. The cells stained purple were counted as viable cells.

Cyclic AMP and steroid hormone release from Leydig cells

In order to estimate the *in vitro* effects of glycyrrhizin, glycyrrhetic acid, paeoniflorin and LH on cyclic AMP and steroid release from Leydig cells, testicular cell preparations were treated according to the method by Dufau *et al.* (1974) with minor modification as follows. Five hundred μ l of cell suspension (1×10^6 cells) was placed in a well, and to it were added 50 μ l of 3-isobutyl-1-methylxanthine solution (Sigma, 0.4 mg/ml), 100 μ l of test material solution, add 350 μ l of 1% BSA PBS. The mixture was then incubated for 3 hrs at 35°C under an atmosphere of 95% O₂ and 5% CO₂ with constant oscillation at 100 cycles/min. The incubation was terminated by ice-cooling, and the media were transferred to centrifuge tubes and spun at 2,000 $\times$ g for 15 min. The supernatant fluids were assayed for cyclic AMP and steroid hormones by radioimmunoassay.

Preparation of microsomal fractions

Microsomal fractions were prepared from testes and ovaries obtained from 10 animals. Tissues were homogenized in 50 ml and 10 ml cold PBS for testes and ovaries, respectively, then centrifuged at 800 $\times$ g for 20 min. The supernatant fluids were centrifuged at 10,000 $\times$ g for 20 min, then were further centrifuged at 105,000 $\times$ g for 60 min. The pellets were suspended in 1 ml of Sol. A, and used as microsomal preparation.

Incubation of microsomal preparation with steroids

One hundred μ l of the microsomal preparation was placed in a test tube, and incubated for 0.5~3 hrs at 37°C with 100 μ l of NADPH solution (Sigma, 7.5 mg/ml Sol. A.), 100 μ l of a labeled steroid solution (0.2 μ Ci in Sol. A.), and 100 μ l of test material dissolved in Sol. A. Incubation was terminated by adding 500 μ l of cold ether and the tube was shaken to extract steroids. The ether extraction was repeated, and the ether layer was transferred to another tube

for evaporation. The residue was dissolved in 10 μ l of ethanol and applied on a thin layer silicagel chromatographic plate (Merck Art. 5553) and developed with chloroform:methanol (98:2). The radioactivity of the steroid spots on TLC plate was measured with a radiochromatoscanner (Aloka TLC-101). In other experiments, we also studied the inhibition of testosterone formation from androstenedione by glycyrrhetic acid. In this experiment we used unlabeled androstenedione (10 μ M) as a substrate in this incubation system. After 30 minutes' incubation with varied concentrations of glycyrrhetic acid, the converted testosterone was measured by radioimmunoassay. As the next experiment, we studied the influence of glycyrrhetic acid (200 μ M) on testosterone formation in various incubation periods and at various androstenedione concentrations.

Statistical Analyses

Comparison of means was carried out by means of Duncan's new multiple range test, Student's *t*-test, or the Cochran-Cox test, depending on the uniformity of variance.

Results

Response of Leydig cells to LH stimulation

In the first experiment, when NIH Rat-LH-RP-2 was added to normal rat Leydig cell suspension, as shown in Fig. 2, cyclic AMP, 17 α -hydroxyprogesterone, and testosterone were produced dose dependently. This indicated that Leydig cells were active and responsive to LH stimulation. We also used porcine LH as a stimulator, and we obtained the same results (date not shown).

Effect of oral administration of glycyrrhetic acid and other materials on *in vitro* testosterone production

Fig. 3 shows testosterone release from Leydig cells obtained from the rats, which had received sample materials orally for 20 days, in response to LH stimulation. Response was strongly inhibited in the

Fig. 2. Response of Leydig cells to LH stimulation. Leydig cells were incubated in the presence of LH, and released cyclic AMP (●), 17 α -hydroxyprogesterone (▲), and testosterone (■) were measured by radioimmunoassay.

shakuyaku-kanzo-toh, glycyrrhizin, and glycyrrhetic acid treated groups compared with the control group. The response of kanzo extract-treated group was also less compared with control. Basal testosterone release without LH stimulation was inhibited by about 40% in all the treated groups. From this experiment, we chose paeoniflorin (a component of shakuyaku), glycyrrhizin (a component of kanzo), and glycyrrhetic acid (a main metabolite of glycyrrhizin) for the next experiment.

In vitro effects of paeoniflorin, glycyrrhizin and glycyrrhetic acid on cyclic AMP and steroid hormone release from Leydig cells

There was no significant change in cyclic AMP and progesterone release in the presence of sample materials (Fig. 4a, 4b). The

accumulation of 17 α -hydroxyprogesterone in the medium was increased by glycyrrhizin (at 100 μ g/ml; $P < 0.05$ vs control) and glycyrrhetic acid (at 100 μ g/ml; $P < 0.001$ vs control). Paeoniflorin had no effect on the production of 17 α -hydroxyprogesterone (Fig. 4c). As shown in Fig. 4d, testosterone production was strongly inhibited by 10 μ g/ml of glycyrrhetic acid ($P < 0.001$ vs control).

Effects of paeoniflorin, glycyrrhizin and glycyrrhetic acid on the conversion of 14 C-androstenedione to testosterone by microsomal fractions

As shown in Fig. 5a, when the radioisotope labeled androstenedione was incubated as a substrate with ovarian microsomal fraction for 1 hour, the radioactivity was found in testosterone in the control. Gly-

Fig. 3. *In vitro* testosterone release in response to LH stimulation from Leydig cells of the animals received oral administration of kanzo extract (○, 45 mg/kg/day), shakuyaku-kanzo-toh (△, 90 mg/kg/day), glycyrrhizin (◇, 4.5 mg/kg/day), glycyrrhetic acid (◆, 4.5 mg/kg/day), and control (▲, distilled water), for 20 days. The values are the mean of 3 wells experiments.

Fig. 4. *In vitro* effects of glycyrrhetic acid, glycyrrhizin, and paeoniflorin on cyclic AMP, and steroid hormone release from Leydig cells. Cyclic AMP and steroid hormones were measured by radio-

immunoassays. Bars represent the mean $\pm$ SEM. for 3 different wells. (*: $P < 0.05$; **: $P < 0.001$ vs control.)

cyrrhizin and paeoniflorin showed little effect on testosterone formation (Fig. 5b, 5c). On the other hand, the testosterone formation was strongly inhibited by glycyrrhetic acid (Fig. 5d). These results indicated that glycyrrhetic acid inhibited the activity of 17 β -hydroxysteroid dehydrogenase which converts androstenedione to testosterone. The same results were obtained with testicular microsomal fraction (data not shown).

Effects of glycyrrhetic acid on the conversion of unlabeled androstenedione to testosterone by microsomal fractions

Fig. 6 shows the concentration-dependent effect of glycyrrhetic acid on the conversion of unlabeled androstenedione to testosterone with testicular microsomal fraction. A linear mode of inhibition was observed when the amount of testosterone formed was plotted against a glycyrrhetic acid logarithmic concentration scale. The

Fig. 5. Effects of glycyrrhizin, paeoniflorin, and glycyrrhetic acid on the conversion of ^{14}C -androstenedione to testosterone by ovarian microsomal fraction. One hundred μg of each material was added to the incubation tubes. Steroids were separated by TLC. O: origin, F: front. A and T indicate the positions of androstenedione and testosterone spots, respectively. (a: control, b: glycyrrhizin 100 μg , c: paeoniflorin 100 μg , d: glycyrrhetic acid 100 μg).

conversion was inhibited by a very low concentration of glycyrrhetic acid, 50% inhibition being obtained at about $4\ \mu\text{M}$. Fig. 7 shows that the conversion of androstenedione to testosterone depends on the incubation period and substrate con-

centration. In the presence of $200\ \mu\text{M}$ of glycyrrhetic acid, the conversion was inhibited almost completely. A slight increase in the conversion was observed with the increase in the substrate concentration.

Fig. 6. Effects of glycyrrhetic acid on the conversion of unlabeled androstenedione to testosterone by testicular microsomal fraction. Converted testosterone was measured by radioimmunoassay. Details are described in Materials and Methods.

Fig. 7. Time and concentration dependent conversion of androstenedione to testosterone and the inhibitory effect of glycyrrhetic acid ($200\ \mu\text{M}$). Converted testosterone was measured by radioimmunoassay. ($\square$, $\triangle$, $\circ$): 0.5, 1.0, 3.0 hrs' incubation, respectively, without glycyrrhetic acid; ($\blacksquare$, $\blacktriangle$, $\bullet$): 0.5, 1.0, 3.0 hrs' incubation, respectively, with $200\ \mu\text{M}$ of glycyrrhetic acid).

Discussion

The present study shows that glycyrrhetic acid, a metabolite of glycyrrhizin, the main component of kanzo extract, has a strong inhibitory effect on testosterone production in rat gonads. Glycyrrhetic acid acts on 17β -hydroxysteroid dehydrogenase (EC. 1.1.1.64) and inhibits the conversion of androstenedione to testosterone. *In vitro* experiments using ovarian microsomal preparation indicated that the conversion of ^{14}C -androstenedione to testosterone was almost completely inhibited by $100\text{ }\mu\text{g/ml}$ of glycyrrhetic acid (Fig. 5d). The compound was also effective on Leydig cells where $10\text{ }\mu\text{g/ml}$ caused strong inhibition (Fig. 4d). The original component of kanzo, glycyrrhizin was not effective in this *in vitro* system, though it reduced the testosterone producing ability of Leydig cells when administered orally, suggesting that glycyrrhizin acted after being metabolized to glycyrrhetic acid. Paeoniflorin, a component of shakuyaku, did not have any effect on testosterone production. The structural similarity of glycyrrhetic acid to steroids suggests that glycyrrhetic acid exerts its inhibitory effect through competition with steroids. In the study with testicular microsomal fraction, the inhibitory effect depended on the concentration of glycyrrhetic acid (Fig. 6), and when the amount of androstenedione added to the incubation system was increased, its conversion to testosterone showed a tendency to increase (Fig. 7). Kumagi *et al.* pointed out that the structural similarity of glycyrrhizin to estrogen might be related to anti-estrogenic action (Kumagi *et al.*, 1967). Kumagai *et al.* (1966) indicated the glycyrrhetic acid inhibited 5β -reductase activity on the metabolism of corticosteroids in rat liver *in vitro*, and suggested that the suppression of 5β -reductase activity by glycyrrhetic acid administration might delay the

clearance of corticosteroids and prolong the biological half life of cortisol resulting in the synergism of corticoids.

In our experiments, 17α -hydroxyprogesterone was accumulated with *in vitro* treatment of Leydig cells with higher doses of glycyrrhizin and glycyrrhetic acid. Kumagi *et al.* (1957) also observed the increase in plasma 17-OHCS after the administration of glycyrrhizin. The mechanism of the accumulation of 17α -hydroxyprogesterone is not clear. In the Leydig cell incubation system the accumulation was caused by $100\text{ }\mu\text{g/ml}$ of glycyrrhetic acid, and to a lesser degree, by $100\text{ }\mu\text{g/ml}$ of glycyrrhizin. Glycyrrhetic acid, at $10\text{ }\mu\text{g/ml}$, reduced the testosterone level in the medium appreciably, and the level was far less than that obtained with $100\text{ }\mu\text{g/ml}$ of glycyrrhizin. However, with $10\text{ }\mu\text{g/ml}$ of glycyrrhetic acid, no significant accumulation of 17α -hydroxyprogesterone was observed. These facts indicate that the inhibition of 17β -hydroxysteroid dehydrogenase, which decreases testosterone production is not related to the accumulation of 17α -hydroxyprogesterone. At higher concentrations, glycyrrhetic acid and glycyrrhizin may have some influence on other steroid conversion enzymes, such as the activation of 17α -hydroxylase (EC. 1.14.99.9 steroid mono oxygenase) or the inhibition of C17-20 lyase. It is known that excess administration of glycyrrhizin may cause pseudoaldosteronism in the clinical field. This phenomenon had been explained by the fact that glycyrrhetic acid would delay the clearance of corticosteroids by inhibiting 5β -reductase in the liver. Our results presented another possible explanation, i. e. that glycyrrhetic acid accumulates 17α -hydroxyprogesterone which can be a starting material for corticosteroids.

Yaginuma (1983) reported that the administration of shakuyaku-kanzo-toh to 10 infertile women with high serum testosterone and oligomenorrhea, lowered serum tes-

tosterone in all cases and 5 women conceived without any side effects. There are some supplemental reports supporting his results (Japanese reports). Results of the present study clarified the reason for the above effect of shakuyaku-kanzo-toh on patients with high serum testosterone. When a patient takes shakuyaku-kanzo-toh, glycyrrhizin in the kanzo extract is metabolized to glycyrrhetic acid in the intestine, and this glycyrrhetic acid acts on 17β -hydroxysteroid dehydrogenase to inhibit the conversion of androstenedione to testosterone, and normalizes the serum testosterone level, and hormonal homeostasis is recovered. In our oral administration experiments, where Leydig cells of the animals were incubated with different concentration of LH, pretreatment of the animals with shakuyaku-kanzo-toh extract, glycyrrhizin, and glycyrrhetic acid reduced the responsiveness of the Leydig cells to the LH stimulation. Kanzo extract also caused a reduction in the response especially when the concentration of LH was low. But the inhibitory effect of the higher concentration of LH was much less than the others, indicating possible interaction among the components of shakuyaku-kanzo-toh. Further studies will be necessary to clarify this.

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References

- Yaginuma, T. (1983). Relation of serum testosterone levels and oligomenorrhea. *Jap. J. Fer. Ster.* 28, (3), 386-389.
- Maeda, T., K. Shinozuka., K. Baba., M. Hayashi and E. Hayashi (1983). Effect of shakuyaku-kanzoh-toh, a prescription composed of shakuyaku (*Paeoniae Radix*) and kanzoh (*Glycyrrhiziae Radix*) on guinea pig ileum. *J. Pharm. Dyn.* 6, 153-160.
- Steiner, A. L. (1979). Cyclic AMP and cyclic GMP. In "Methods of Hormone Radioimmunoassay" (B. M. Jaffe and H. R. Behrman, eds.) pp. 3-17. Academic Press, New York.
- Hattori, M., T. Sakamoto, K. Kobayashi and T. Namba (1982). Metabolism of glycyrrhizin by human intestinal flora. *Planta Medica.* 48, 38-42.
- Molhuysen, J. A., J. Gerbrandy and L. A. Vries (1950). A liquorice extract with deoxycortone-like action. *Lancet* 23, 381-386.
- Dufau, M. L., C. Menderson and K. J. Catt (1976). A highly sensitive *in vitro* bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed Leydig cells. *J. Clin. Endocrinol. Metab.* 39, 610-613.
- Kumagai, A., S. Yano and M. Otomo (1957). Study on the corticoidlike action of glycyrrhizine and the mechanism of its action. *Endocrinol. Japon.* 4, 17-27.
- Kumagai, A., S. Yano, K. Takeuchi, K. Nishino, Y. Asanuma, N. Nanaboshi and Y. Yamamura (1964). An inhibitory effect of glycyrrhizin on the antigranulomatous action of cortisone. *Endocrinology* 74, 145-148.
- Kumagai, A., K. Nishino, M. Yamamoto, M. Nanaboshi and Y. Yamamura (1966). An inhibitory effect of glycyrrhizin on metabolic actions of cortisone. *Endocrinol. Japon.* 13, 416-419.
- Kumagai, A., K. Nishino, A. Shimomura, T. Kin and Y. Yamamura (1967). Effect of glycyrrhizin on estrogen action. *Endocrinol. Japon.* 14, 34-38.