COMPARATIVE STUDIES OF THE STEREISOMERS OF GLYCERYRHERINIC ACID ON ANTI-INFLAMMATORY ACTIVITIES

SAKAE AMAGAYA,ETSUKO SUGISHITA, YUKIO OGHARA,* SUSUMU OGAWA, KENZO OKADA AND TATSUO AIZAWA**

Faculty of Pharmaceutical Sciences, Nagoya City University,* 3-1, Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan and Maruzen Kasei Corporation,** 14703–10, Koto-cho, Onomichi, Hiroshima, 722, Japan

(Received June 8, 1984)

Anti-inflammatory activity of the stereoisomers, 18α and 18β-glyceryrheticinic acid (18α and 18β-GA), obtained from Glycyrrhizae Radix, was investigated by using carrageenan-induced edema in mice and 18α-GA was found to be more active than 18β-GA. Therefore, to clarify the difference of action of 18α and 18β-GA, the inhibitory effects of both stereoisomers on the cotton pellet granuloma formation in adrenalectomized rats and on the reduction of steroidal compounds by Δ⁴-5β-reductase in the microsome fraction of rat liver were investigated. 18α-GA, 30 mg/kg p.o., showed the similar anti-granulomatous action in normal and adrenalectomized rats. On the other hand, 18β-GA, 30 mg/kg p.o., which exhibited the inhibitory effects in normal rats, showed no activity in adrenalectomized rats. More than 50% of inhibitory effects of 18α and 18β-GA on the 5β-reduction of testosterone and cortisol were recognized by an equimolar ratio of steroids to 18α or 18β-GA. From these results, the activity of 18α-GA is similar to that of glucocorticoid and the difference of action between 18α-GA and 18β-GA could be explained by its stereochemical structure of D/E trans conformation. In addition to the glucocorticoid action, 18α-GA inhibited the inactivation of endogenous glucocorticoid in liver, which is also recognized by the application of 18β-GA.

Keywords — carrageenan-induced edema; cotton pellet granuloma; Δ⁴-5β-reductase; adrenalectomized rat; 18α-glyceryrheticinic acid; 18β-glyceryrheticinic acid

INTRODUCTION
Glycyrrhizae Radix (Japanese name: Kanzo), the root of Glycyrrhiza glabra L. (Leguminosae), has been used medicinally, mainly as a demulcent and sweetening agent. It has been elucidated that glycyrrhizin (GL), one of the main components of Glycyrrhizae Radix, has anti-inflammatory action and its activity was mainly attributable to the action of its aglycone, glyceryrheticinic acid (GA). Molhysan et al.,¹ Takahashi et al.,₂ and Finney et al.³ reported the anti-inflammatory activity of GA using laboratory methods. GA of which activities were reported was the 18β-form or the mixture of 18α and 18β-form. There have been no publications on the anti-inflammatory activity of pure 18α-form because of its small content in Glycyrrhizae Radix and its difficulty to separate with each other. The difference of the stereo structure between 18α-GA (trans conformation of D/E ring) and 18β-GA (cis conformation of D/E ring) is shown in Fig. 1. Our previous paper⁴ showed that 18α-GA is more active than 18β-GA on anti-inflammatory activity. Thus, we have examined the effects of both stereoisomers on hypothalamus-adrenocortical gland axis using adrenalectomized rats and on endogenous glucocorticoid metabolism in liver using rat's liver microsome fraction to explain the difference of the action of 18α-GA and 18β-GA.

MATERIALS AND METHODS
Animals — Male ddY mice, 6 weeks old, or male wistar rats, 6 weeks old, were purchased
from Shizuoka Laboratory Animal Center. Experimental animals were housed in a constant environment and fed food and water *ad libitum*. The room lights were turned off between 20:00 and 8:00.

**Reagents** — 18α-GA and 18β-GA were purified in Maruzen Kasei Co. Prednisolone and Tween-80 were purchased from Nakarai Chem. Co. λ-Carrageenan was purchased from Sigma Chem. Co.

**Anti-inflammatory Activity on λ-Carrageenan-Induced Hind Foot Edema in Mice** — λ-Carrageenan (1.0% in physiological solution, 0.05 ml) was injected s.c. under the plantar surface of right hind paw of mouse 1 h after the oral administration of drugs. 18α-GA, 18β-GA and prednisolone were suspended in a 5.0% Tween-80 solution. Increase in the foot volume was measured as described before. Anti-edema effects of the test drugs were expressed in terms of percent inhibition of the foot edema in drug treated group compared with the foot edema in the control group treated with the vehicle.

**Cotton Pellet Granuloma in Rats** — According to the method described by Meier *et al.*, and Kumagai *et al.*, experiments were carried out. All experiments were repeated twice. Cotton rolls (Kawamoto Co., Ltd.) were cut into small segments so that each piece weighed 45—50 mg. After sterilization in an autoclave, four cotton rolls were bilaterally implanted subcutaneously into the dorsal area of normal and adrenalectomized rats. 18α-GA, 18β-GA and prednisolone, suspended in a 5.0% Tween-80 solution, were administered through stomach tubes once a day for 7 d after the implantation of the cotton pellets. After 8 d, the rats were killed and the pellets were carefully removed from surrounding tissues and weighed after being dried over night at 65°C. The rate of the granuloma formation was calculated as follows:

\[
\frac{\text{(dry wt of granuloma)} - \text{(initial wt of cotton pellet)}}{\text{(initial wt of cotton pellet)}} \times 100
\]

---

**FIG. 1. Structure of 18α-Glycyrrhetinic Acid, 18β-Glycyrrhetinic Acid and Prednisolone**

- prenisolone
- 18α-glycyrrhetinic acid
- 18β-glycyrrhetinic acid
Competitive Effects on 5β-Reduction of Steroid — According to the method described by Tamura et al., experimental results were carried out. Three rats were sacrificed by decapitation at 10:00 a.m. The livers were perfused with saline and placed in ice-cold 0.25 M sucrose solution containing 1 mM ethylenediamine tetra acetic acid (EDTA). The livers were blotted and then homogenized in 0.25 M sucrose-1 mM EDTA solution. After preparing a 50% homogenate, it was spun at 10000 × g for 20 min. The supernatant was centrifuged at 105000 × g to yield the soluble fractions. 5β-Reductase activity was measured by incubating steroids (0.17 μmol, in 0.05 ml methanol) with 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.2, 2 mg of nicotinamide adenine dinucleotide phosphate (NADPH), 0.2 ml of soluble fractions and water to bring the final volume up to 2.5 ml. Immediately after the components were mixed thoroughly, 1 ml of aliquots were removed and extracted with 5 ml of methylene chloride. The remaining 1.5 ml was incubated in shaking water bath at 37 °C for 20 min. After finishing the reaction, 1 ml of aliquots was extracted with methylene chloride. The extracted aliquots were centrifuged and after the aqueous phase and the coagulated protein were removed, the optical density of the solvent at 240 nm was measured. The difference between the initial and the 20 min reading represented the amount of substrate which had disappeared.

RESULTS

λ-Carrageenan-Induced Hind Foot Edema in Mice

Anti-inflammatory effects of 18α-GA and 18β-GA on λ-carrageenan induced edema in the mouse hind paw were investigated and the results are shown in Fig. 2. Anti-edema action of 18α-GA was more active than that of 18β-GA in the range of oral doses from 3 to 30 mg/kg.

Cotton Pellet Granuloma in Rats

The inhibitory effects of 18α-GA, 30.0 mg/kg p.o., 18β-GA, 30.0 mg/kg p.o., and prednisolone, 5.0 and 30.0 mg/kg p.o., per day for 7 d, on the granuloma formation in normal and adrenalectomized rats were investigated. The results are summarized in Table I. In normal rats, inhibitory effects of about 43% on an average with prednisolone, 5.0 mg/kg p.o., and more than 60% on an average with prednisolone, 30 mg/kg p.o., were recognized. 18α-GA, 30.0 mg/kg p.o., showed the inhibition of 51% on an average (p<0.01 in Exp. I and II). But the inhibitory effect of 18β-GA, 30.0 mg/kg p.o., was about 28% on an average (p<0.05 in Exp. I and p<0.01 in Exp. II). The activity of 18β-GA was less potent comparing with that of 18α-GA (p<0.01 in Exp. I and p<0.05 in Exp. II).

In adrenalectomized rats, inhibitory effects of about 29% on an average with prednisolone, 5.0 mg/kg p.o., and about 52% on an average with prednisolone, 30.0 mg/kg p.o., were recognized. 18α-GA, 30.0 mg/kg p.o., caused significant inhibition of about 30% on an average (p<0.05 in Exp. I and p<0.01 in Exp. II) and it was similar to that of prednisolone, 5.0 mg/kg p.o. In contrast, 18β-GA, 30.0 mg/kg p.o., showed no significant inhibition in both experiments. On the other hand, the increase in body weight for 7 d had no difference between the control group

![Graph showing inhibitory effects of 18α-Glycyrrhetinic Acid and 18β-Glycyrrhetinic Acid on the Swelling of Mouse Hind Paw Induced by λ-Carrageenan (1.0%, 0.05 ml)](image)

Δ --- Δ : 18α-glycyrrhetinic acid,
□ --- □ : 18-glycyrrhetinic acid,
○ --- ○ : prednisolone.

Reagents were administered orally 1 h before the injection of λ-carrageenan. Each point represents the mean of 6 male mice. Vertical bars indicate S.E.M.
and GA treated group in normal and adrenalectomized rats.

**Competitive Effects on 5β-Reduction of Steroid**

Effects of 18α-GA and 18β-GA on 5β-reduction of testosterone and cortisol using Δ4-5β-reductase in microsome fraction of rat liver were investigated and the results were shown in Table II. According to the molar ratio of steroids to 18α-GA, 50:1, 25:1, 10:1, 5:1, 1:1 and 1:2, 18α-GA apparently decreased 5β-reductase activity on the metabolism of testosterone and cortisol in dose-dependent manner. More than 50% of inhibitory effects were demonstrated by an equimolar ratio of steroids to 18α-GA or 18β-GA. The inhibition by a molar ratio of steroids to 18α-GA of 1:2 was nearly 90% (p<0.01 in both Exp.). On the other hand, nearly 70% inhibition (p<0.01 in both Exp.) was demonstrated by a molar ratio of steroids to 18β-GA of 1:2.

**DISCUSSION**

Anti-inflammatory activity of GA was examined focusing on the stereochemical difference between 18α and 18β form. In carrageenan-induced edema, 18α-GA exhibited more intense inhibition than 18β-GA. It is supposed that the increase of the anti-inflammatory activity of 18α-GA is due to the planar conformation of molecular which is derived from the D/E trans binding (Fig. 1). This conformation could nearly fix the C-30 carboxyl group of 18α-GA within the environment of the C-20 carboxyl group and the C-21 hydroxyl group of prednisolone. So, 18α-GA could possess the affinity with the receptor of prednisolone in a target cell. Furthermore, 18α-GA could inhibit the metabolism of the endogenous corticoid in liver and increase its

**TABLE I. Effects of 18α and 18β-Glycyrrhetinic Acid on the Granuloma Formation and Body Weight in Rats**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Dose (mg/kg/d)</th>
<th>Number of rats</th>
<th>Granuloma (mg/cotton mg)</th>
<th>Increase in body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp. I</td>
<td>Exp. II</td>
<td>Exp. I</td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>1.42 ± 0.04</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5.0</td>
<td>5</td>
<td>5</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>5</td>
<td>4</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>18α-Glycyrrhetinic acid</td>
<td>30.0</td>
<td>5</td>
<td>5</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>18β-Glycyrrhetinic acid</td>
<td>30.0</td>
<td>5</td>
<td>5</td>
<td>1.06 ± 0.10</td>
</tr>
<tr>
<td>Adrenalectomized rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>8</td>
<td>5</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5.0</td>
<td>8</td>
<td>5</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>8</td>
<td>5</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>18α-Glycyrrhetinic acid</td>
<td>30.0</td>
<td>8</td>
<td>4</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>18β-Glycyrrhetinic acid</td>
<td>30.0</td>
<td>8</td>
<td>4</td>
<td>0.95 ± 0.08</td>
</tr>
</tbody>
</table>

Each value is the mean ± S.E.M. a) p < 0.05, b) p < 0.01 vs. control
Reagents were administered orally for 7 d.
level in blood. To explain the hypothesis mentioned above, the differences of the steroidal action of two stereoisomers, 18α-GA and 18β-GA, were investigated.

At first, we investigated whether these isomer's action was participated in hypothalamus-adrenocortical gland axis or not using cotton pellet granuloma test in rats. 18α-GA was observed to have an intense inhibition of granuloma formation in adrenalectomized rats as well as in normal rats. In contrast, 18β-GA which inhibited the granuloma formation in normal rats with significant manner showed no inhibition in adrenalectomized rats. We reported\textsuperscript{b)} that

\begin{table}[h]
\centering
\begin{tabular}{ llcccc c c}
\hline
Substrate & Additive & Molar ratio of substrate/additive & Decrease in absorbance ($\times 10^{-2}$) & Inhibitory percent \\
\hline
Testosterone & 18α-Glycyrrhetic acid & 1/0 & 9.16±0.32 & 0 \\
 & & 1/2 & 0.24±0.07\textsuperscript{b)} & 97.4 \\
 & & 1/1 & 3.04±0.16\textsuperscript{b)} & 66.8 \\
 & & 5/1 & 3.65±0.19\textsuperscript{b)} & 60.2 \\
 & & 10/1 & 4.33±0.71\textsuperscript{b)} & 52.7 \\
 & & 25/1 & 6.26±0.37\textsuperscript{b)} & 31.7 \\
 & & 50/1 & 7.53±1.04 & 17.8 \\
18β-Glycyrrhetic acid & & 1/0 & 13.05±0.30 & 0 \\
 & & 1/2 & 3.58±0.46\textsuperscript{b)} & 72.6 \\
 & & 1/1 & 6.09±1.22\textsuperscript{b)} & 53.3 \\
 & & 5/1 & 7.79±0.88\textsuperscript{b)} & 40.3 \\
 & & 10/1 & 9.95±0.66\textsuperscript{b)} & 23.8 \\
 & & 25/1 & 10.81±0.47\textsuperscript{b)} & 17.2 \\
 & & 50/1 & 10.64±0.44\textsuperscript{b)} & 18.5 \\
Cortisol & 18α-Glycyrrhetic acid & 1/0 & 14.76±1.09 & 0 \\
 & & 1/2 & 0.45±0.59\textsuperscript{b)} & 92.6 \\
 & & 1/1 & 4.83±0.99\textsuperscript{b)} & 67.3 \\
 & & 5/1 & 5.86±0.39\textsuperscript{b)} & 60.3 \\
 & & 10/1 & 7.03±0.80\textsuperscript{b)} & 52.4 \\
 & & 25/1 & 8.94±0.43\textsuperscript{b)} & 39.4 \\
 & & 50/1 & 11.66±0.78\textsuperscript{a)} & 21.0 \\
18β-Glycyrrhetic acid & & 1/0 & 8.39±0.78 & 0 \\
 & & 1/2 & 2.03±0.35\textsuperscript{b)} & 75.8 \\
 & & 1/1 & 3.83±0.73\textsuperscript{b)} & 54.4 \\
 & & 5/1 & 5.65±1.04 & 32.7 \\
 & & 10/1 & 6.83±0.61 & 18.6 \\
 & & 25/1 & 8.08±0.60 & 3.7 \\
 & & 50/1 & 10.00±1.78 & 19.2 \\
\hline
\end{tabular}
\caption{Inhibitory Effects of 18α and 18β-Glycyrrhetic Acid on 5β-Reduction of Testosterone and Cortisole Using Microsome Fraction of Rat Liver}
\end{table}

\textit{Content of substrates in each incubation study is constant (0.17 \textmu mol in 2.5 ml of sodium phosphate buffer) and that of additives (18α and 18β-glycyrrhetic acid) is changed. Decrease in absorbance at 240 nm shows the amount of disappeared substrate by the incubation for 20 min. Each value is the mean ± S.E.M. of 8 samples.} \textit{a)} \textit{p} < 0.05 \textit{and} \textit{b)} \textit{p} < 0.01 vs. control (substrate/additive; 1/0).
anti-inflammatory action of 18α-GA was blocked by messenger ribonucleic acid (m-RNA) synthesis inhibitor, actinomycin D, or protein synthesis inhibitor, cycloheximide. These results suggest that 18α-GA possesses steroidal action, having affinity with steroid receptor in a target cell. And this action may be explained by the stereochemical structure of D/E trans conformation. 18β-GA whose molecular conformation is derived from D/E cis binding have little steroidal action. Next, we examined the effects of 18α-GA and 18β-GA on the steroidal hormone metabolic enzyme in rat liver. Tamura et al. and Atherden reported that 18β-GA inhibited 5β-reduction of Δ4-3-ketosteroids by rat liver homogenates. And they inhibited 5β-reduction far more strongly than 5α-reduction. Bayliss also suggested a possibility that GA may act by blocking the enzyme systems which metabolize corticosteroids. In our experiments, 18α-GA and 18β-GA showed little effects on the 5α-reductase activity but they were inhibitors of 5β-reduction of testosterone and cortisol. The effect of 18β-GA was less potent than that of 18α-GA. But, Tamura et al. reported that 18β-GA showed more than 90% of inhibition by an equimolar ratio of testosterone or cortisol to 18β-GA. Judging from these data, it is supposed that the difference of the conformation of both stereoisomers does not show strong influence on the binding with metabolic enzyme.

From these results, the mechanism of 18α-GA in anti-inflammatory activity is presumed to be both steroidal action in a target cell and inhibition of metabolism of endogenous corticosteroid in liver. On the other hand, the mechanism of 18β-GA in anti-inflammatory action is mainly explained by the inhibition of metabolism of endogenous corticosteroid. But, our results show the indirect evidence for the contention that 18α-GA exerts glucocorticoid activity. To clarify the detail mechanism of action, competitive receptor binding study using 18α-GA might be carried out.

REFERENCES