Studies on Antinephritic Effect of TJ-8014, a New Japanese Herbal Medicine, and Its Mechanisms (2): Effect on the Release of Corticosterone from Adrenal Glands

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Abstract—In order to elucidate the mechanisms of the antinephritic action of TJ-8014, the effect of this drug on corticosterone release from the adrenal cortex was investigated by using normal rats and rats with original-type anti-GBM nephritis. When the serum corticosterone level was determined 5 hr after test drugs were given p.o. to normal rats, TJ-8014 at 0.5 and 2.0 g/kg significantly elevated the hormone level by 48% and 74%, respectively. Of the crude drugs that constitute TJ-8014, Bupleuri radix (SAIKO) and Glycyrrhizae radix (KANZOU) at 1.0 g/kg also significantly elevated the serum level. When TJ-8014 was given p.o. daily from the next day of anti-GBM serum injection to the 15th day, 2.0 g/kg/day of the drug inhibited the urinary protein excretion. In addition, TJ-8014 (2.0 g/kg/day) inhibited the decrease in the serum and adrenal corticosterone levels induced by nephritis. When corticosterone at 10 mg/kg was given s.c. daily from the next day of the anti-serum injection to the 10th day, it not only reduced proteinuria, but also inhibited glomerular histopathological changes. In contrast, metyrapone, a corticosterone synthetase inhibitor, at 100 mg/kg x 2/day, p.o., aggravated the nephritis. These results suggest that the antinephritic action of TJ-8014 may be partly due to the enhancement of the synthesis or release of corticosterone from the adrenal cortex.

We previously reported that TJ-8014, a new Japanese herbal medicine, was effective on the urinary protein as well as glomerular histopathological changes in anti-glomerular basement membrane (anti-GBM) nephritis in rats, and antiplatelet action was one of the mechanisms of the antinephritic action of this medicine (1). However, the details of other mechanisms involved in the antinephritic action of this herbal medicine still remained unclear.

Glucocorticoids are one of the agents most widely used for treatment of glomerulonephritis in the clinical situation. We demonstrated that dexamethasone, a steroid, showed a beneficial effect on anti-GBM nephritis in rats (2, 3).

In the recent year, a combination of Japanese herbal medicines, Chai-Ling-Tang (Sairei-to in Japanese) or Xio-Chai-Hu-Tang (Syo-saiko-to in Japanese) and a steroid has often been used to inhibit the side effects of the steroid and to potentiate various pharmacological actions of the steroid (4–6). It has been indicated that saikosaponin-a and saikosaponin-d, which are included in TJ-8014 and these herbal medicines, are both able to elevate serum corticosterone levels in rats (7).

Therefore, the aim of the present study was to clarify whether or not TJ-8014 stimulates the release of glucocorticoids from adrenal glands as the mechanism for the antinephritic action of this medicine. For this purpose, we investigated the effects of TJ-8014 and each crude drug in the composition of TJ-8014, on...
the corticosterone levels in serum and adrenal glands in normal rats and rats with original-type anti-GBM nephritis. Furthermore, an experiment was carried out by using this nephritic model to elucidate if corticosterone has antinephritic action and if metyrapone, a corticosterone synthetase inhibitor, makes the disease worse.

Materials and Methods

Animals: Male Sprague-Dawley strain SPF rats, weighing approx. 200 g (Shizuoka Laboratory Animal Center), were used in the experiment. These animals were housed in an air-conditioned room at 23±1°C during the experimental period.

Drugs: Drugs used were TJ-8014 [a lyophilized extract] (Tsumura Co., Ltd., Tokyo) and eight crude drugs that constitute TJ-8014 [lyophilized extracts] (Tsumura Co., Ltd., Tokyo), corticosterone (Sigma Chemical Co., St. Louis, MO) and metyrapone (Ciba Gaigy, Hyogo). Compositions of TJ-8014 are shown in Table 1. These drugs were suspended in 1% gum arabic.

Effects of TJ-8014 and each crude drug contained in TJ-8014 on serum corticosterone in normal rats: The serum was obtained by centrifugation of whole blood collected by decapitation of the animals at 5 hr after each test drug was given to the rats p.o. The serum corticosterone level was assayed as described below.

Effect of TJ-8014 on corticosterone levels in original-type anti-GBM nephritis: Nephritis was induced in rats by injecting 0.75 ml of rabbit anti-rat GBM serum (anti-GBM serum) into their tail veins, as described previously (8). The 24 hr-urine after anti-GBM serum injection was collected, and the rats were then divided into groups of 5 animals, so that the average protein content in the 24 hr-urine samples of each group was at the same level. Test drugs were given to each group p.o. daily in a volume of 1.0 ml/100 g of body weight from the next day of anti-GBM serum injection (the 1st day) to the 15th day. One group of nephritic rats served as the nephritic control and was given p.o. only the vehicle (1% gum arabic) instead of drug solution. In addition, a non-treated (normal) group of 5 rats was used for comparison with the nephritic groups. On the 1st, 5th, 10th and 15th days, adrenal glands were taken immediately after blood was drawn. Urinary protein content and corticosterone levels in the serum and adrenal glands were determined as described below.

Effects of corticosterone and metyrapone on original-type anti-GBM nephritis: Rats were given daily corticosterone, s.c., or metyrapone, p.o., from the day after anti-GBM serum injection to the 10th day. The urinary protein excretion and histopathological parameters were determined as described below.

Urine and blood collections: The 24 hr-urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr. At the beginning of the urine collection, each animal received 8 ml of distilled water orally without feeding. The urine was then centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant was used for the determination of protein. Immediately after the urine collections, approx. 5 ml of blood was drawn from the carotid of each conscious animal between 9:00 and 10:00 a.m. by decapitation. The blood was centrifuged at

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Table 1. Compositions of crude drugs that constitute TJ-8014

| Crude drugs     | Contents* (g) |
|-----------------|---------------|
| Bupleuri radix  | 7             |
| Pinelliae tuber | 5             |
| Glycyrrhizae radix | 2         |
| Scutellariae radix | 3         |
| Ginseng radix   | 3             |
| Coptidis rhizoma | 1           |
| Holien          | 3             |
| Zizyphi fructus | 3             |

*The amount of each crude drug required to prepare 4.5 g of TJ-8014 extract.
5,000 rpm at 4°C to obtain serum for the determination of serum corticosterone.

**Determination of corticosterone in adrenal glands:** Adrenal glands were minced with small scissors, and 4 ml of Krebs Ringer buffer was then added to the minced tissues in accordance with the method of Kato et al. (9). After incubating the mixture for 60 min at 37°C, the mixture was centrifuged at 1,000 rpm for 5 min at 4°C. The supernatant was used for the determination of corticosterone level.

**Determinations of the urinary protein and corticosterone:** The urinary protein content was determined by the method of Kingsbury et al. (10) and expressed as mg/24 hr-urine. The corticosterone level was determined in accordance with Zinker and Bernstein (11). The Ex and Em wavelengths for corticosterone were determined at 470 and 530 nm, respectively, by using a spectrophotofluorometer, model FP-770 (Nihon Bunkou, Tokyo).

**Assessment of histopathological parameters:** For light microscopic study, kidneys were dehydrated and fixed by immersing the tissues stepwise into low to high concentrations of alcohol. The tissues were then embedded in paraffin and cut into 2–3 μm thick sections. The sections were stained with hematoxylin and eosin and Masson trichrome. Hypercellularity and adhesion to Bowman’s capsule of capillary walls (adhesion) in glomeruli were observed under a light microscopy. For assessing the hypercellularity, the number of nuclei was counted and expressed as the mean number per equatorial cross section in 10 glomeruli/animal. For assessing the adhesion, fifty glomeruli per section were observed, and the incidence of the adhesion was calculated. All the above experiments were performed “blind” on the coded sections.

**Statistical analysis:** The data represent the mean±S.D., and the results were statistically evaluated by analysis of variance, Student’s t-test and the Mann-Whitney’s U-test.

**Results**

1. **Effects of TJ-8014 and eight crude drugs contained in TJ-8014 on serum corticosterone level in normal rats (Table 2)**

   When the serum corticosterone level was determined 5 hr after treatment with test drugs, TJ-8014 at 0.5 and 2.0 g/kg, p.o., significantly elevated the serum corticosterone by 48% and 74%, compared with the control, respectively. Of the crude drugs, Bupleuri radix (SAIKO) and Glycyrrhizae radix

| Groups               | Corticosterone level (μg/ml) | % stimulation |
|----------------------|------------------------------|---------------|
| Control              | 0.27±0.08                    |               |
| TJ-8014              |                              |               |
| 0.5 g/kg/day, p.o.   | 0.40±0.04*                   | 48            |
| 2.0 g/kg/day, p.o.   | 0.47±0.05***                 | 74            |
| Control              | 0.37±0.05                    |               |
| Bupleuri radix       | 0.47±0.07*                   | 28            |
| Pinelliae tuber      | 0.28±0.03                    |               |
| Ginseng radix        | 0.29±0.18                    |               |
| Glycyrrhizae radix   | 0.45±0.10*                   | 21            |
| Coptidis rhizoma     | 0.35±0.17                    |               |
| Scutellariae radix   | 0.30±0.11                    |               |
| Zizyphi fructus      | 0.40±0.08                    |               |
| Holen                | 0.29±0.11                    |               |

The serum corticosterone level was determined 5 hr after each test drug was given. The percent stimulation was calculated from the following formula:

\[
\frac{T-C}{C} \times 100 \quad (C: \text{Control, } T: \text{Test drug})
\]

* and ** indicate a significant difference from the control at P<0.05 and 0.001, respectively.
Fig. 1. Effects of TJ-8014 on urinary protein excretion in original-type anti-GBM nephritis in rats. Each plot denotes the mean±S.D. of 5 rats. The number in parentheses indicates the percent inhibition that is calculated from the following formula:

\[
\frac{C-T}{C-N} \times 100 \quad (C: \text{Control}, T: \text{Test drug}, N: \text{Normal})
\]

* and ** indicate a significant difference from the control at \( P<0.05 \) and 0.01, respectively.

(KANZOU) at 1.0 g/kg, p.o., also significantly increased the level by 28% and 21%, respectively.

2. Effect of TJ-8014 on urinary protein excretion and corticosterone levels in original-type anti-GBM nephritis in rats

Urinary protein excretion (Fig. 1): TJ-8014 at 2.0 g/kg/day, p.o., inhibited the urinary protein excretion by 45%–80% throughout the experimental periods of 5, 10 and 15 days. However, at 0.5 g/kg/day, p.o., it only tended to reduce the urinary protein.

Serum and adrenal corticosterone levels (Fig. 2): Although the serum corticosterone level was 0.4–0.5 \( \mu \text{g/ml} \) in normal rats for the 1st day to the 15th day, the serum corticosterone level of the nephritic control rats was approx. 80% lower than the normal level. TJ-8014 at 2.0 g/kg/day, p.o., inhibited the decrease in the serum corticosterone level by 50%–80% during the experimental periods. TJ-8014 at 0.5 g/kg/day, p.o., also significantly inhibited the decrease by 30% on the 10th day (Fig. 2a).

Throughout the experimental periods of the 1st to 10th day, the adrenal corticosterone level of the nephritic control was significantly lower (47%–90%) than that of the normal group. The decrease in the corticosterone level was inhibited by TJ-8014 (2.0 g/kg/day, p.o.) by 148%, 72% and 96%, respectively, on the 5th, 10th and 15th days (Fig. 2b).

3. Effects of corticosterone (Fig. 3) and metyrapone (Fig. 4) on original-type anti-GBM nephritis

Corticosterone at 10 mg/kg/day, s.c., significantly inhibited the urinary protein excretion by 48% on the 10th day (Fig. 3a). Moreover, light microscopic observation indicated that it significantly reduced the incidence of the adhesion and hypercellularity by 28% and 51%, respectively (Fig. 3b). In contrast to the effect of corticosterone, metyrapone at 100 mg/kg x 2/day, p.o., increased the urinary protein by 41% and 97%, respectively, on the 5th and 10th days (Fig. 4a). In addition, it increased the incidence of adhesion to capillary walls of Bowman's capsule and the hypercellularity by 110% and 30%, respectively (Fig. 4b).

Thus, corticosterone prevented the progression of the nephritis, while metyrapone made the disease worse.

Discussion

Corticosterone is one of the naturally occurring adrenal cortical steroids and is an important factor in the control of the pro-
Effect of TJ-8014 on Corticosterone

Fig. 2. Effects of TJ-8014 on serum (a) and adrenal (b) corticosterone levels in original-type anti-GBM nephritis in rats. * and ** indicate a significant difference from the normal at P<0.05 and 0.01, respectively. The number in parentheses indicates the percent inhibition that is calculated from the following formula:

\[ \frac{T-C}{N-C} \times 100 \]  

(C: Control, T: Test drug, N: Normal)

* and ** indicate a significant difference from the control at P<0.05 and 0.01, respectively.

gression of inflammatory responses. In the present study, it was demonstrated that TJ-8014, a new Japanese herbal medicine, elevated the serum corticosterone level in normal rats and inhibited the decrease in the serum corticosterone level as well as the increase in the urinary protein excretion during the process of original-type anti-GBM nephritis in rats. Furthermore, the adrenal corticosterone level was also markedly decreased during the process of the nephritis and TJ-8014 inhibited the decrease in the endogenous glucocorticoid. The decrease in the adrenal corticosterone level observed during the course of the disease may be due to the reduced production of corticosterone in the adrenal cortex, which leads to the decrease in the serum level of this hormone. These data also indicate that TJ-8014 protects the decreased ability of the adrenal cortex to produce corticosterone. The results obtained with normal rats suggest that TJ-8014 may promote the production of the cortical steroid in the adrenal cortex by the direct action of this drug to the adrenal cortex or through the action of this drug to hypothalamus-pituitary glands. We evaluated the effects of crude drugs that constitute TJ-8014 on serum corticosterone level in normal rats; all these drugs were used at a dose of 1.0 g/kg, p.o., a dose at which *bupleuri radix* (SAIKO), the main crude drug, showed anti-inflammatory action (12). Of the eight crude drugs, *bupleuri radix* (SAIKO) and
glycyrrhizae radix (KANZOU) significantly elevated the serum corticosterone level. Saikosaponins-a and -d and glycyrrhizin, chemical components that are contained in both crude drugs mentioned above, have been reported to promote the synthesis or secretion of corticosterone from the adrenal cortex in rats (13, 14). Therefore, it is
deduced from these findings and our results obtained with crude drugs that *bupleuri radix* and *glycyrrhizae radix* may mainly contribute to the increase in synthesis or release of corticosterone from the adrenal cortex by TJ-8014.

The next experiment was carried out to clarify if corticosterone has antinephritic action and if metyrapone, an adrenal cortical steroid synthetase inhibitor, in contrast, makes the disease worse. In the experiment, corticosterone was injected s.c. to the rats after anti-GBM serum injection at the dose of 10 mg/kg/day, which was the dose required to show nearly same level of serum corticosterone as when TJ-8014 at 2.0 g/kg/day was given p.o. to normal rats (data not shown). On the other hand, metyrapone was given p.o. at a dose of 100 mg/kg×2/day, because this dose could completely inhibit the serum corticosterone level for 8 hr in normal rats (data not shown). As a result, corticosterone was effective in inhibiting the urinary protein excretion as well as glomerular histopathological changes, while metyrapone aggravated the nephritis. The present results suggest that the antinephritic action of TJ-8014 may be at least partly due to the enhancement of corticosterone synthesis or release from the adrenal cortex.

Glucocorticoids are potent anti-inflammatory agents and have been widely used as the first choice drugs for the treatment of nephrotic syndrome in humans. Our previous study indicated that dexamethasone, a glucocorticoid, was markedly effective on anti-GBM nephritis in rats (1, 2). It is generally considered that glucocorticoids may inhibit the synthesis of arachidonate metabolites by interfering with phospholipase A2, which is the enzyme responsible for the release of arachidonic acid from membrane phospholipids via the synthesis of intracellular protein. In addition, it has been shown that the synthesis and release of the protein in kidneys are stimulated by glucocorticoids (15). Lionas et al. (16, 17) found the excess production of intraglomerular thromboxane A2 and 12-HETE, arachidonate metabolites, which are generated by the cyclooxygenase and lipoxygenase pathways, respectively, after the injection of anti-GBM serum to rats. The increased intraglomerular thromboxane A2 production may cause an acute fall in renal plasma flow and platelet aggregation (18, 19). On the other hand, 12-HETE, a chemotactic lipid, may play a role in the infiltration of neutrophils and monocytes in this nephritis. The above findings suggest that corticosterone, an endogenous glucocorticoid, may exert its antinephritic action by inhibiting the synthesis of arachidonate metabolites such as thromboxane A2 and 12-HETE.

It is deduced from our results and the above finding that TJ-8014 may exert antinephritic action by inhibiting the arachidonate metabolite synthesis via the enhancement of synthesis or release of adrenal cortical hormone.

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