Studies on the Antinephritic Effect of Plant Components (4): Reduction of Protein Excretion by Berberine and Coptisine in Rats with Original-Type Anti-GBM Nephritis

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ABSTRACT — The present study was conducted to investigate the antinephritic effects of berberine and coptisine, which are contained in Coptidis rhizoma, on original-type anti-GBM nephritis in rats. Berberine and coptisine at the doses of 0.5, 1.0 and 5.0 mg/kg/day, i.p. were effective in inhibiting urinary protein excretion, elevation of serum cholesterol and creatinine contents as well as glomerular histopathological changes. In addition, berberine at 20 mg/kg/day, p.o. also inhibited urinary protein excretion throughout the experimental periods. Berberine and coptisine inhibited platelet aggregation in both in vitro and in vivo assays, and berberine inhibited the decline of renal blood flow. Although berberine inhibited an increase in thromboxane B2 formation, it increased the formation of 6-keto-prostaglandin F1α in platelets and isolated glomeruli. These results indicate that the antinephritic effects of berberine and coptisine may be partly due to antiplatelet action and improved renal hemodynamics via changing prostanoid synthesis.

Keywords: Nephritis, Proteinuria, Berberine, Coptisine, Coptidis rhizoma

It is well-known that Coptidis rhizoma, which is extracted from Coptidis japonica MAKINO (Ranunculaceae), has been used as a bitter stomachic drug in Japanese-Chinese herbal medicine prescriptions. Coptidis rhizoma has also been prescribed for the purpose of providing antiphlogistic, analgesic and sedative action. However, it remains unclear which of the components composing Coptidis rhizoma exhibit the pharmacological actions. Recently, Ohtsuka et al. (1) reported on the effects of constituents in the rhizomes of Coptidis japonica MAKINO by means of several inflammatory models. They demonstrated that the principal inhibitory elements acting against an inflammatory response such as granulation tissue formation were berberine and coptisine.

We have reported that TJ-8014, a new Japanese herbal medicine (2–6), and Coptidis rhizoma extract, one of eight crude drugs which constitute TJ-8014 (7), were effective in inhibiting the urinary protein excretion as well as glomerular histopathological changes in several renal diseases. However, it is unclear which components of TJ-8014 have the antinephritic action.

Therefore, to clarify the antinephritic effect of berberine and coptisine, we investigated the effects of berberine and coptisine on original-type anti-GBM nephritis in rats in comparison with the effects of dipyridamole. In the second experiment, the effects of berberine and coptisine on platelet aggregation and renal blood flow of nephritic control rats were examined to elucidate the antinephritic mechanisms of these components. In the third experiment, we investigated the effects of berberine and coptisine on thromboxane A2 (TXA2) and prostaglandin I2 (PGI2) formations in platelets and isolated glomeruli to determine the mechanisms by which these drugs exert their antiplatelet action and improve renal blood flow.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley strain SPF rats, weighing approx. 160 g (Nihon SLC, Shizuoka), were used in the experiments. These animals were housed in an air-conditioned room at 23 ± 1°C during the experimental period.
Drugs

Drugs used were purified berberine and coptidine (Tsumura Co., Ltd., Tokyo) and dipyridamole (Boehringer Ingelheim, Germany). Figure 1 indicates the chemical structures of berberine and coptisine. The purity (more than 98%) of these components was confirmed by HPLC and the following HPLC conditions were used: Column: Wakosil 5C18, 4.6 mm I.D. × 150 mm; Eluent: 0.1 M tartaric acid + 4 mM octane sulfonate : AcCN = 6 : 4. Berberine and coptisine were dissolved in distilled water or saline for both the in vitro and in vivo tests.

Fig. 1. Chemical structures of berberine and coptisine.

Induction of original-type anti-GBM nephritis

The rats were divided into 4 groups, so that the average body weight in each group was the same level. Original-type anti-GBM nephritis was induced in rats by injecting them with 0.70 ml of rabbit anti-rat GBM serum (anti-GBM serum) in the tail vein, as described previously (8).

Evaluation of antinephritic effects of the drugs

Berberine and coptisine were given at the doses of 0.5, 1.0 and 5.0 mg/kg/day, i.p. and at 10.0 and 20.0 mg/kg/day, p.o. from the day of anti-GBM serum injection (the 0 day) to the 11th day. The effect of dipyridamole as a positive control drug was also examined. One group of nephritic rats served as the nephritic control and was given i.p. or p.o. only the vehicle. In addition, the urinary protein and plasma creatinine contents and histopathological parameters in the kidneys of the drug-treated group were compared with those of the control group.

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 collection, 0.4 ml of blood was drawn from the tail vein of each of the conscious animals with a disposable microsyringe and put into a tube containing 4.5 μl mol of EDTA-2Na. The blood was centrifuged at 5,000 rpm at 4°C to obtain serum for the determination of cholesterol and creatinine contents.

Determinations of urinary protein excretion, serum cholesterol and creatinine contents

The urinary protein content was determined by the method of Kingsbury et al. (9) and expressed as mg/24 hr. The cholesterol content was determined in accordance with the method of Allin et al. (10) and expressed as mg/dl. The serum creatinine content was measured by using a kit for creatinine determination (Kainos creatinine, Kainos Tokyo) and expressed as mg/dl.

Morphological studies

For light microscopic study, kidneys were dehydrated and fixed by immersing the tissues stepwise into low to high concentrations of ethyl 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. The number of nuclei (hypercellularity) and the incidence of adhesion to Bowman’s capsule of capillary walls (adhesion) in the glomeruli were observed under light microscopy. To assess the hypercellularity, the number of nuclei was counted and expressed as the number per equatorial cross section in 10 glomeruli/animal. To assess the adhesion, fifty glomeruli per section were observed, and the incidence of the adhesion was calculated. All the above experiments were performed “blindly” on coded sections.

The effects of berberine and coptisine on platelet aggregation and renal blood flow

Platelet aggregation was measured with a whole blood aggregometer (Chlonolog Co., Ltd., Tokyo) as reported previously (11). To test the in vitro effects of test drugs on platelet aggregation, blood was taken into a disposable syringe containing 0.25 ml of 3.18% sodium citrate in the amount of 2.5 ml from the renal vein of a normal rat under pentobarbital anesthesia 30 mg/kg, i.p.). Five hundred microliters of the blood was mixed with 495 μl of the solution of berberine, coptisine or dipyridamole dissolved with 0.9% NaCl to various concentrations in cuvettes. After incubating the mixture for 5 min at 37°C, collagen (10 μg/ml) was added to the incubated mixture, and platelet aggrega-
tion was then measured. The platelet aggregation was expressed as the resistance rate between two electrodes. To evaluate the in vivo effects of test drugs, berberine and coptisine were given p.o. at a dose of 10 and 20 mg/kg to each group of 5 rats at 2 hr after the anti-GBM serum injection. Likewise, indomethacin at the dose of 2.5 mg/kg, p.o. was also administered. The control group of 5 rats was given p.o. the distilled water instead of test drugs. The animals in the drug-treated and control groups had blood taken from their renal veins 2 hr after treatment with the respective drugs. The platelet aggregation was measured as in the in vitro assay.

The renal blood flow was determined by hydrogen gas clearance methods (Biomedical Science Co., Ltd., Model RBF-2) 4 hr after anti-GBM serum injection. The drugs were given 2 hr before RBF measurements.

Glomerular isolation, preparation of platelets and eicosanoid generation assay

A group of five rats received berberine and coptisine, 10 and 20 mg/kg, p.o., 2 hr after anti-GBM serum injection. Effects of berberine and coptisine on prostanoïd formation in platelets and isolated glomeruli were determined 2 hr later. Four hours after i.v.-injection of antiserum, the animals were sacrificed under pentobarbital anesthesia, and the blood and kidneys were taken for isolation of platelets and glomeruli. The platelet-rich plasma was prepared by centrifuging the whole blood with 0.45 μM EDTA•0.9% NaCl solution. Then the platelets were washed three times by Krebs-Ringer HCO3⁻ buffer. The sample was diluted to a platelet concentration of 3 × 10⁶/mm³. Glomeruli were isolated by a modification of the differential sieving technique, aimed at optimizing glomerular viability (12). The isolated platelets and glomeruli were incubated in plastic tubes containing 2 ml of Krebs-Ringer HCO3⁻ buffer, pH 7.2, at 37°C for 30 min. After centrifugation of the tubes at 1,000 rpm for five minutes at 4°C, thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F₁α were measured using TXB₂[¹²⁵I] and 6-keto-prostaglandin F₁α[¹²⁵I] kits (New England Nuclear, Boston MA, USA).

Statistical analyses

The data represent the mean ± S.D. and the results were statistically evaluated by analysis of variance, Student's t-test and Mann-Whitney’s U-test. Inhibitory percentage was calculated as follows:

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\text{Inhibitory percentage (\%)} = \frac{(\text{Control} - \text{Test drugs})}{(\text{Control} - \text{Normal})} \times 100
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RESULTS

Effects of berberine and coptisine on original-type anti-GBM nephritis in rats

Urinary protein excretion (Figs. 2 and 3): After a single i.v.-injection of anti-GBM serum, the urinary protein excretion of nephritic control rats markedly increased over the 10-day observation periods. Berberine at doses of 0.5, 1.0 and 5.0 mg/kg/day, i.p. inhibited urinary protein excretion by 34%, 30% and 46%, respectively, by the 10th day after i.v.-injection of anti-GBM serum. Coptisine at doses of 0.5, 1.0 and 5.0 mg/kg/day, i.p. also inhibited excretion by 40%, 23% and 26%, respectively (Fig. 2). On the other hand, the rats treated with berberine at 20.0 mg/kg/day, p.o. had significantly less proteinuria than the nephritic control rats from the 1st day to the 10th day (Fig. 3). However, there was no significant difference in the excretion of protein between coptisine p.o.-treated and nephritic control rats throughout the experimental periods (Fig. 3).

Serum cholesterol and creatinine contents (Figs. 4 and 5): The serum cholesterol content in the nephritic control was markedly elevated on the 11th day. In contrast, berberine at 5.0 mg/kg/day, i.p. inhibited the elevation of the serum cholesterol level by 54%. In addition, coptisine at 0.5 mg and 1.0 mg/kg/day, i.p. also inhibited the elevation of serum cholesterol content by 38% and 24%, respectively (Fig. 4). However, the oral administrations of berberine, coptisine and dipyridamole (400 mg/kg/day, p.o.) did not inhibit the elevation of serum cholesterol content by the 11th day (Fig. 5). The elevation of serum creatinine was inhibited by berberine at 0.5, 1.0 and 5.0 mg/kg/day, i.p. by 62% to 102%; and coptisine at 0.5, 1.0 and 5.0 mg/kg/day, i.p. also inhibited the elevation of serum creatinine by 53%, 84% and 76%, respectively (Fig. 4). In addition, coptisine at 0.5 mg and 1.0 mg/kg/day, i.p. inhibited the elevation of serum creatinine by 53%, 84% and 76%, respectively (Fig. 4). In contrast, berberine at 5.0 mg/kg/day, i.p. inhibited the elevation of serum cholesterol level by 54%.

Morphological studies (Figs. 6, 7 and 8): Light microscopic examination of the glomeruli on the 11th day showed that the lesion in anti-GBM serum-treated rats exhibited severe adhesion of capillary walls to Bowman's capsule, mesangioloproliferative form, mesangial hypercellularity and mild crescent formation (Figs. 6, 7 and 8B). In contrast, the number of nuclei (hypercellularity) and adhesion in the glomeruli of rats treated with berberine at 1.0 or 5.0 mg/kg/day, i.p. or coptisine at 5.0 mg/kg/day, i.p. were less than those of the nephritic control (Figs. 6, 7 and 8E). Berberine at all doses, coptisine at 20.0 mg/kg/day, p.o. and dipyrida-
Fig. 2. Effects of berberine and coptisine administered intraperitoneally on urinary protein excretion in original-type anti-GBM nephritis in rats. Test drugs were given i.p., daily during the period from the day (the 0 day) of anti-GBM serum injection to the 11th day. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. * indicates a significant difference from the control at P < 0.05.

Fig. 3. Effects of berberine and coptisine administered orally on urinary protein excretion in original-type anti-GBM nephritis in rats. Test drugs were given p.o., daily during the period from the day (the 0 day) of anti-GBM serum injection to the 11th day. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. * and ** indicate significant differences from the control at P < 0.05 and 0.01, respectively.
Fig. 4. Effects of berberine and coptisine administered intraperitoneally on serum cholesterol and creatinine contents in original-type anti-GBM nephritis in rats. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. *, ** and *** indicate significant differences from the control at $P < 0.05$, $0.01$ and $0.001$, respectively.

Fig. 5. Effects of berberine and coptisine administered orally on serum cholesterol and creatinine contents in original-type anti-GBM nephritis in rats. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. * and ** indicate significant differences from the control at $P < 0.05$ and $0.01$, respectively. Dip: Dipyr-
Fig. 6. Effects of berberine and coptisine administered intraperitoneally on hypercellularity and adhesion in glomeruli of original-type anti-GBM nephritic rats. The histopathological parameters in glomeruli were detected on the 11th day. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicate inhibitory percentage. *, ** and *** indicate significant differences from the control at P < 0.05, 0.01 and 0.001, respectively.

Fig. 7. Effects of berberine and coptisine administered orally on hypercellularity and adhesion in glomeruli of original-type anti-GBM nephritic rats. The histopathological parameters in glomeruli were detected on the 11th day. Each column denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. * and ** indicate significant differences from the control at P < 0.05 and 0.01, respectively. Dip: Dipyridamole.
mole also inhibited hypercellularity and the incidence of adhesion by 50% to 89% (Figs. 6 and 7; Fig. 8: C, D, F).

Fig. 8. Light micrographs of glomeruli from rats of the normal group (A); control group (B); group given dipyridamole, 400 mg/kg/day, p.o. (C); group given coptisine, 20 mg/kg/day, p.o. (D); group given berberine, 5.0 mg/kg/day, i.p. (E); group given berberine, 20 mg/kg/day, p.o. (F). The drug-treated rats were examined on the 11th day after i.v.-injection of anti-GBM serum (Hematoxyline eosin stain). B shows an advanced lesion with mesangial proliferation, adhesion of capillary wall to Bowman's capsule, hypercellularity and mild crescent formation. Note that the histological lesions in the group treated with berberine at 20 mg/kg/day, p.o. (F) are markedly less than those of the control.

Effects of berberine, coptisine and dipyridamole on platelet aggregation (Fig. 9)

The in vitro assay (not shown in the figures or the tables): The IC50 value for berberine and coptidine was 185 and 312 μM/ml, respectively. On the other hand, the IC50 value for dipyridamole was 271 μM/ml.

The in vivo assay (Fig. 9): The normal rate of platelet aggregation was 5.0 ± 2.2 %. The nephritic control at 4 hr after i.v.-injection of antisera showed a significant increase in platelet aggregation (9.5 ± 3.3; * and ** indicate significant differences from the control at P < 0.05 and 0.01, respectively. Indo.: Indomethacin.

Fig. 9. Effects of berberine and coptisine administered orally on platelet aggregation in original-type anti-GBM nephritis in rats. The platelet aggregation was determined at 4 hr after injection of anti-GBM serum. The number in parentheses indicates inhibitory percentage.
In contrast, berberine (10 and 20.0 mg/kg, p.o.) and coptisine (20.0 mg/kg, p.o.) also inhibited platelet aggregation by 131, 104 and 104%, respectively. Indomethacin at 2.5 mg/kg, p.o. also inhibited the increase of platelet aggregation by 82%. Dipyridamole also completely inhibited it (5.5 ± 3.0 vs. control, P < 0.05; not shown in Fig. 9).

**Effects of berberine and coptisine on renal blood flow (Fig. 10)**

The normal renal blood flow in the cortex (1 mm) was 97.2 ± 6.0 ml/min/100 g tissue weight. The mean value renal blood flow in anti-GBM nephritic rats, which was examined 4 hr after anti-GBM serum injection, was significantly lower than that of normal rats (84.6 ± 5.9 ml/min/100 mg tissue weight, P < 0.05 vs. normal). Figure 9 shows that the nephritic rats given berberine at 20.0 mg/kg, p.o. had a significant increase in renal blood flow, by 119%, compared with the control, whereas in the nephritic rats given coptisine, renal blood flow was not different from that of the nephritic control. Indomethacin significantly reduced renal blood flow by 34%.

**Effects of berberine and coptisine on TXB2 and 6-keto-prostaglandin F1α formation in isolated glomeruli and platelets (Fig. 11)**

Platelets and glomeruli TXA2 and prostaglandin F1α synthesis was measured in terms of the generation of immunoreactive TXB2 and 6-keto-prostaglandin F1α, in the supernatant of isolated platelets or glomeruli. Platelet (Normal: 378.0 ± 161.5 pg/10⁴ platelet/30 min incubation) and glomerular (Normal: 63.6 ± 5.4 pg/mg protein/30 min incubation) TXB2 production in rats studies 4 hr after i.v.-injection of anti-GBM serum was increased 15- and 20-fold as shown in Fig. 11. In contrast, berberine at 10.0 and 20.0 mg/kg, p.o. significantly reduced the formation of TXB2 in platelets and glomeruli. Platelet (Normal: 602.2 ± 254.9 pg/10⁴ platelet/30 min incubation) and glomerular (Normal: 220.6 ± 43.5 pg/mg protein/30 min incubation) 6-keto-prostaglandin F1α formation in rats with anti-GBM nephritis was also significantly higher than that of the normal rats (3 to 5 fold). The platelet and glomeruli formations of 6-keto-prostaglandin F1α in berberine (20.0 mg/kg, p.o.) -treated rats were significantly increased.

**DISCUSSION**

The present results showed that although the rats treated with anti-GBM serum exhibited greater proteinuria than that of normal rats, berberine and coptisine markedly inhibited urinary protein excretion, the elevations of serum cholesterol and creatinine content levels, and histopathological changes in the glomeruli, when these were measured on the 11th day.

Currently, it is generally believed that the alterations in coagulation and platelet aggregation in the glomeruli play an important role in the development and progression of various renal diseases (13-17). We (18) and Poelstra et al. (19) produced increased platelet aggregability in rats with anti-GBM serum injections. In addition, we previously found that dipyridamole, an anti-platelet agent, inhibited urinary protein excretion in rats with anti-GBM nephritis (20). An increased platelet aggregation, which is induced by immune complex or collagen on injured glomerular capillary walls may release many proinflammatory substances, including vasoactive amines, cationic proteins, TXA2 and proteases from the adhered platelets. These released substances released may then cause not only platelet aggregation but also vasoconstriction, which leads to the reduction of renal hemodynamics. Several studies have suggested that platelets, perhaps via the release of these products, may mediate changes in capillary wall permeability and renal functions (16, 17).

To evaluate whether there is an inhibitory effect of berberine and coptisine on proteinuria, presumably due to the fact that berberine and coptisine improve the hyperaggregability of platelets and increase renal
hemodynamics within the glomeruli, we studied the platelet aggregation and renal blood flow in the cortex of rats with anti-GBM nephritis treated with berberine and coptisine. We found in a preliminary test that the platelet aggregation in rats with anti-GBM nephritis for the 10 days of the experimental period was highest at 4 hr after anti-GBM serum injection, so we determined the effects of berberine and coptisine on platelet aggregation at 4 hr after i.v.-injection of antiserum. The results in the present study indicated that berberine at 10.0 and 20.0 mg/kg, p.o. and coptisine at 10.0 mg/kg, p.o. markedly inhibited the increase of platelet aggregation, yielding a level similar to that of the normal rats. Moreover, the rats with anti-GBM nephritis at 4 hr after anti-GBM serum injection had less renal blood flow, whereas oral administration of berberine at 20.0 mg/kg, by preventing the urinary protein excretion, prevented the decrease in the renal blood flow in anti-GBM nephritic rats. These results indicate that the antiplatelet action and the increase in reduced renal blood flow produced by berberine may contribute to the antinephritic effect of the components.

Recently, several laboratories have shown alterations of glomerular prostanoid formation in models of glomerulonephritis (12, 21–23). They reported that the major AA metabolite of nephritic glomeruli was TXA2. TXA2 released from platelets or glomeruli is a potent vasoconstrictor and induces platelet activation. Badr et al. (24) confirmed that the infusion of platelet activation factor (PAF) into glomeruli induced the reduction of renal blood flow, and its effect was mediated via the secondary release of TXA2. In addition, We (18, 25) and Lianos et al. (21) demonstrated that the increased glomerular TXA2 production was responsible for acute reduction in renal plasma flow and glomerular filtration rate, as pretreatment of the rats with thromboxane

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**Fig. 11.** Effects of berberine and coptisine administered orally on platelet and glomerular TXB2 and 6-keto-prostaglandin F1α formation in original-type anti-GBM nephritis in rats. The platelet and glomerular TXB2 and 6-keto-prostaglandin F1α formations at 4 hr after injection of anti-GBM serum. * and ** indicate significant differences from the control at P < 0.05 and 0.01, respectively. Indo.: Indomethacin.
synthetase inhibitors inhibited the increment in glomerular TXA2 and prevented reduction in renal function and platelet hyperaggregability in nephrotoxic nephritis in rats. In can be concluded that TXA2, released from platelets or glomeruli, plays an important role via alteration of platelet aggregation and hemodynamics in the development of glomerular disease. In contrast, prostaglandins I2 and E2 are vasodilators and potent inhibitors of platelet aggregation (26, 27). Administration of pharmacological amounts of these prostaglandins have been shown to have a beneficial effect in several animal models of immune glomerulonephritis (28, 29). Accordingly, it is postulated from these findings that these alterations in glomerular and platelet prostanooid formation may exert either protective or deleterious effects on glomerular diseases.

To further examine the mechanisms responsible for the inhibitory effects of berberine and coptisine on the increased platelet aggregation and decreased renal blood flow, we studied the effect of these components on the formation of TXB2 and 6-keto-prostaglandin F1α, the stable breakdown product of TXA2 or prostaglandin I2, from platelets and isolated glomeruli. TXB2 formation in platelets and glomeruli of the nephritic control at 4 hr after antiserum injection was 15-20 times higher than that of the normal animals, while 6-keto-prostaglandin F1α formation was only approx. 2 times higher. On the other hand, no changes in prostaglandin E2 formation in rats with anti-GBM nephritis have been found compared to normal rats (data not shown). It seems that the action of TXA2 may be superior to that of prostaglandin I2 in the development of anti-GBM nephritis. The administration of berberine at doses suppressing platelet aggregation and increasing renal blood flow inhibited TXB2 formation; and in contrast, it increased 6-keto-prostaglandin F1α formation in platelets and glomeruli. These results suggest that berberine prevents platelet hyperaggregability and a decrease in renal blood flow by inhibiting the synthesis of TXA2 and promoting the synthesis of prostaglandin I2 in glomeruli.

In the present study, the coptisine administered intraperitoneally was more effective, compared with that by oral administration, although both administrations of berberine were effective. The discrepancy of the anti-nephritic effect remains unclear. We consider that the absorption from the digestive organ or the rate of their metabolisms in the liver may be associated with a structural difference between bererine and coptisine, leading to the different kinetics of these components.

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