Studies on antinephritic effect of TJ-8014, a new Japanese herbal medicine (6) Effects on activities of reactive oxygen species-scavenging enzymes in original-type anti-GBM nephritis in rats

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(Received August 30, 1991. Accepted October 3, 1991.)

Abstract

In order to clarify the mechanisms of the antinephritic action of TJ-8014, we investigated the effects of this medicine on activities of reactive oxygen species (ROS)-scavenging enzymes, superoxide dismutase (SOD)-like enzyme, catalase and glutathione peroxidase (GSH-PX), in renal cortex of rats with original-type anti-glomerlar basement membrane (anti-GBM) nephritis. The SOD-like, catalase and GSH-PX activities in renal cortex from nephritic (control) rats were significantly lower than those from normal animals throughout the lst to the 10 th day after anti-GBM serum injection. TJ-8014 at 2.0 g/kg/day, *p.o.* significantly increased SOD-like, catalase and GSH-PX activities in renal cortex on the lst, 5 th and 10 th days. Of crude drugs which constitute TJ-8014, Bupleuri Radix and Coptidis Rhizoma significantly increased these three ROS-scavenging enzymes activities by giving *p.o.* at 1.0 g /kg once on the day after anti-GBM serum injection. SOD at 35,000 U/kg, *i.p.* and catalase at 110,000 U/kg, *i.p.* prevented the urinary protein excretion, while aminotriazole, at 500 mg/ kg, *i.p.* aggravated the nephritis. TJ-8014 (2.0 g/kg, *p.o.*) significantly increased three scavenging enzymes activities at 1.5 and 3 hr or at 1.5 hr after being given to normal rats. The increase in three scavenging enzymes activities by treatment with TJ-8014 (2.0 g/kg, *p.o.*) was completely inhibited by combination with cycloheximide or actinomycin D (5.0 mg/kg, *i.p.*). These results suggest that TJ-8014 may partly exert the antinephritic action by scavenging ROS generated in glomeruli via the increase in the synthesis of ROS-scavenging enzymes.

Key words nephritis, free radical scavengers, reactive oxygen species, Japanese herbal medicine, rats.

Introduction

We have already reported that TJ-8014, a new Japanese herbal medicine, has a beneficial effect on several experimental glomerulonephritis in rats [1-5] and the antinephritic effect of this medicine may be partly due to the antiplatelet action and the increasing action on corticosterone release from the adrenal glands.[1,2] However, little is known about other pharmacological actions related to the antinephritic effect of this medicine.

Recently, it has been shown that reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical ($OH\cdot$) may play an important role in mediating glomerular injury and proteinuria of various experimental glomerulonephritis.[6,7] Intraglomerular infiltration of 'macrophages and polmorpholeukocytes has been demonstrated in original-type anti-glomerular basement membrane (anti-GBM) nephritis which is induced in rats by a single *i.v.* injection of rabbit anti-rat GBM serum (anti-GBM serum).[8,9] The current study also demonstrates that intraglomerular macrophages and polymorphonuclear leukocytes in rats with nephritis and toxic renal injury produce a variety

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Journal of Medical and Pharmaceutical Society for WAKAN-YAKU 8, 125－134, 1991

of ROS.[10-12] These findings suggest that glomerular injuries and proteinuria in original-type anti-GBM nephritis in rats may be mediated by the ROS released from these intraglomerular inflammatory cells.

On the other hand, mammalian cells have been shown to possess ROS scavenging enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-PX) to protect the cells against toxic oxygen metabolites.[13, 14]

We previonsly reported that the ROS scavenging enzymes activities in renal cortex in original-type anti-GBM nephritis in rats were significantly decreased with the onset of protrinuria after anti-GBM serum injection. The dicrease also appered after treatment with catalase with an osmotic minipump from 3 hr before anti-GBM serum injection markedly prevented the development of proteinuria.[15] Our observation suggests that the diminished ability of SOD, catalase and GSH-PX to scavenge the ROS generated in glomeruli may be related to the development of proteinuria and glomerular injury in original-type anti-GBM nephritis in rats.

In the present study, we examined the effects of TJ-8014 and each crude drug which constitutes TJ-8014 on activities of three kinds of ROS-scavenging enzymes, SOD, catalase and GSH-PX in renal cortex in original-type anti-GBM nephritis in rats.

Furthermore, in order to clarify whether or not TJ-8014 increases the synthesis of these ROS-scavenging enzymes, we examined the effect of TJ-8014 in combination with cycloheximide or actinomycin D, a protein synthesis inhibitor, on activities of ROS-scavenging enzymes in renal cortex of normal rats.

Materials and Methods

Animals: Male Sprague-Dawley strain SPF rats, weighing approx. 200 g (NIHON SLC, Shizuoka), 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 lyopilized extract] (Tsumura Co, Ltd., Tokyo), eight crude drugs which constitute TJ-8014 [lyophilized extracts] (Tsumura Co, Ltd., Tokyo) (Table I), dipyridamole (Boehringer Ingelhem, Germany), corticosterone, Cu, Zn-SOD [Bovine liver], catalase [Bovine liver], cycloheximide and actinomycin D (Sigma Chemical Co., ST. Louis, Mo).

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

Crude drugs		Contents 1)
Bupleuri Radix	(Saiko)	7.0 g
Pinelliae Tuber	(Hange)	5.0 g
Glycyrrhizae Radix	(Kanzou)	2.0 g
Scutellariae Radix	(Ougon)	3.0 g
Ginseng Radix	(Ninjin)	3.0 g
Coptidis Rhizoma	(Ouren)	1.0 g
Hoelen	(Bukuryou)	3.0 g
Zizyphi Fructus	(Taisou)	3.0 g

The amounts of each crude drug required to prepare 4.5 g of TJ-8014.

Table II Effect of TJ-8014 on lipid peroxide content in renal cortex in original-type anti-GBM nephritis.

Groups	Malondialdehyde concentration (nMole/mg of protein)
Normal	1.4±0.32
Control	4.5±0.81###
TJ-8014 (2.0 g/kg/day, *p.o.*)	1.7±0.38***

The lipid peroxide content was determined on the 10 th day after *i.v.* injection of anti-GBM serum. ### indicates a significant difference from the normal at $p<0.001$. *** indicates a significant difference from the control at $p<0.001$.

Effects of TJ-8014 and other test drugs on activities of ROS-scavenging enzymes in renal cortex or glomeruli of nephritic rats: Original-type anti-GBM nephritis was induced in rats by injecting with 0.75 ml of rabbit anti-rat GBM serum (anti-GBM serum) into the tail vein, as described previously.[16] The 24 hr-urine samples after anti-GBM serum injection were 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. TJ-8014 was given *p.o.* daily in a volume of 1.0 ml/100 g of body weight from the day after anti-GBM serum injection (the lst day). The nephritic control group were given *p.o.* daily the vehicle (1 % gum arabic). TJ-8014-treated and respective control rats were sacrificed by perfusing both kidneys with 0.1 M phosphate buffer saline (PBS) under pentobarbital anesthesia at 1.5 hr after the last administration of the drug on the lst, 5 hr and 10 th days, respectively. Other test drugs were given *p.o.* once in a volume of 1.0 ml/100 g of body weight on the day after anti-GBM serum injection. The kidneys from each test were drug-treated and control rats were taken after perfusing both kidneys with PBS at 1.5 hr after treatment with respective drug. The ROS scavenging enzymes activities in renal cortex or glomeruli were determined as mentioned below.

Effects of Cu, Zn-SOD, catalase and TJ-8014 in the presence or absence of aminotriazole on urinary protein excretion in original-type anti-GBM nephritis in rats: TJ-8014 (2.0 g/kg, *p.o.*), Cu, Zn-SOD (35,000 U/kg, *i.p.*), catalase (110,000 U/kg, *i.p.*) or aminotiazole (500 mg/kg, *i.p.*) was given once on the day after anti-GBM serum injection. TJ-8014 (2.0 g/kg) was given *p.o.* immediately after pretreatment with aminotriazole (500 mg/kg, *i.p.*) on the day after anti-GBM injection. Urinary protein excretion were determined in accordance with the method of Kingsbury *et al.*[17]

Effects of TJ-8014 alone and in combination with cycloheximide or actinomycin D on activities of ROS-scavenging enzymes in renal cortex of normal rats: TJ-8014 was given *p.o.* to normal rats and both kidneys were taken at 1.5, 3 and 5 hr after the drug administration. The ROS-scavenging enzymes activities in renal cortex were determined as described below. TJ-8014 was given *p.o.* immediately after treatment of normal rats with cycloheximide or actinomycin D (5 mg/kg, *i.p.*). One hour and a half later, both kidneys were taken for the determination of ROS-scavenging enzymes activities in renal cortex.

Determination of activities of ROS-scavenging enzymes in renal cortex and glomeruli: Renal cortex was obtained by excluding the part of the medula from kidneys perfused with PBS and was minced with small scissors. Ten ml of PBS including 20 mM sucrose was added to the minced tissue and then homogenized with a teflon glass homogenizer. The homogenate was centrifuged at 3,000 rpm for 15 min. The supernatant was used for determination of activities of three kinds of ROS-scavenging enzymes, SOD-like enzyme,[18] catalase[19] and GSH-PX.[20] The assay method of this SOD-like activity was then modified to give better sensitivity and minimize interference by coexisting protein. Hydroxylamine or its *O*-sulfonic acid, xanthine oxidase, hypoxanthine, EDTA, and the sample were incubatad with or without KCN at pH 8.2, 37°C, for 30 min. Diazo dyefloming reagent was added and the absorption was measured at 550 nm. Moreover, glomeruli were isolated by passing renal cortex through mesh metal sieves and washing with ice-cold 0.9% NaCl solution according to rhe method previously reported.[21] The purity of the preparation was evaluated by counting glomeruli under light microscopy, and only preparation with > 90 % purity were used (Fig. 1). In addition, to confirm the contamination of blood cell into glomeruli, we observed the purity of glomeruli by the immunohistochemical method (peroxidase stain) after fixation with paraform-lysin-periodate. The

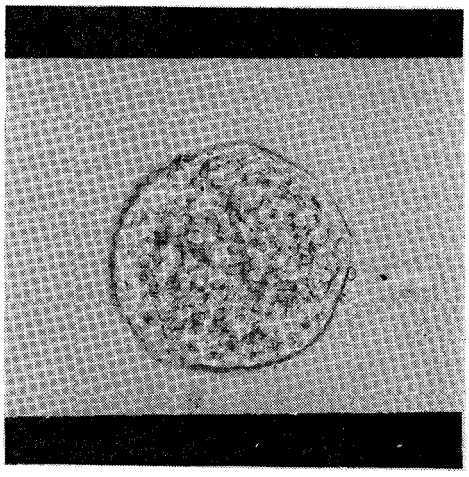

Fig. 1 Photograph of isolated glomeruli.
The glomeruli was completely separated from the tubules.

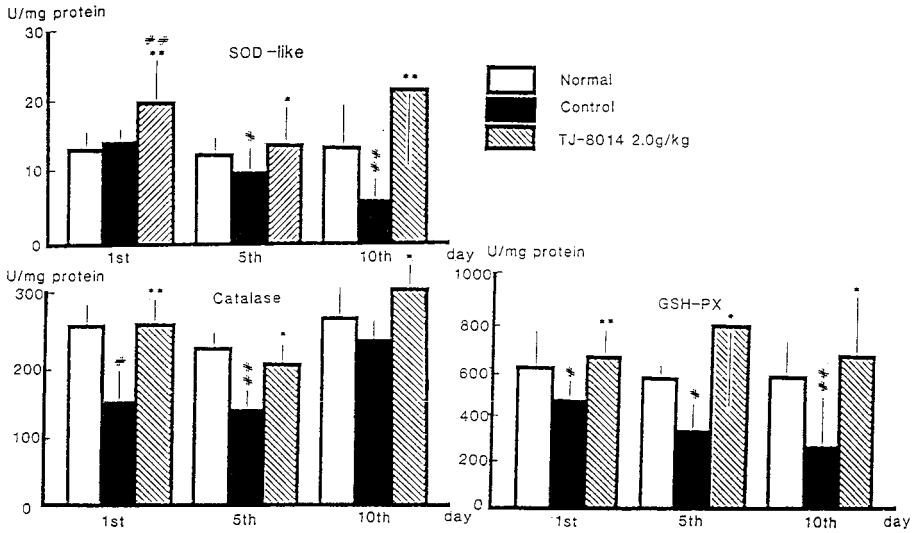

Fig. 2 Effects of TJ-8014 on activities of ROS-scavenging enzymes in renal cortex of rats with original-type anti-GBM nephritis.

TJ-8014 was given once daily from the day after anti-GBM serum injection (the lst day) to rats and the animals were sacrified at 1.5 hr after the last administration of the drug on the lst, 5 th and 10 th days, respectively, for determination of ROS-scavenging enzymes activities. Each column denotes the mean±S.D. of 5 rats. The number in parentheses indicates a percent increase which is derived from the following formula : (T-C)×100/C (C : Control, T : Test drugs). # and ## indicate a significant difference from normal at $p<0.05$ and 0.01, respectively. * and ** indicate a significant difference from control at $p<0.05$ and 0.01, respectively.

number of leukocyte and red blood cell were 1 to 2 cell/glomeruli. The glomeruli obtained were ultrasonically disintegrated for 10 min and then centrifuged at 3,000 rpm for 10 min. The supernatant was used for determination of SOD-like activity.

Lipidperoxide level : The lipidperoxide content in renal cortex was determined in accordance with the method of Yagi.[22]

Statistical analysis : 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.

Results

Effects of TJ-8014 and other test drugs on activities of ROS-scavenging enzymes in renal cortex or glomeruli of nephritic rats (Fig. 2 and 3)

The SOD-like activity in renal cortex taken from nephritic rats (control rats) was significantly decreased on the 5 th and 10 th days after anti-GBM serum injection, compared to that of normal rats, although it was not different from that of normal rats on the lst day (Fig. 2). TJ-8014 at 2.0 g/kg/day, *p.o.* significantly increased the SOD-like activity by 24 %, 35 % and 75 %, compared to the control on the lst, 5 th and 10 th days, respectively. Interestingly, on the lst day, the enzyme activity in rats given TJ-8014 was significantly higher than the activity in normal animals. Catalase and GSH-PX activities in renal cortex were also significantly decreased from the lst to 5 th day and through the experimental period of the lst to the 10 th day, respectively. TJ-8014 significantly increased the catalase activity by 82 %, 28 % and 32 %, and the GSH-PX activity by 41 %, 145 % and 141 % on the lst, 5 th and 10 th days, respectively.

When SOD activity was determined in glomeruli, the enzyme activity was also significantly lower in nephritic rats than in normal

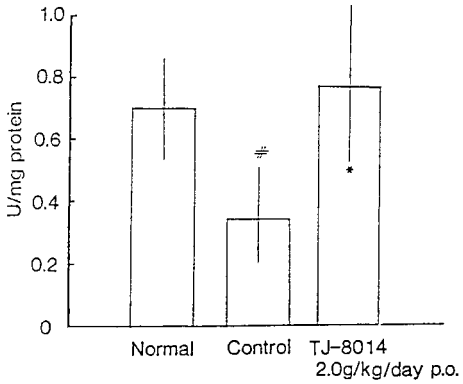

Fig. 3 Effect of TJ-8014 on SOD-like activity in glomeuli of rats with original-type anti-GBM nephritis. Tj-8014 was given once daily from the lst to the 10 th day to rats injected with anti-GBM serum and animals were sacrified at 1.5 hr after the last administration of the drug for determination of SOD-like activity. Each column denotes the mean±S.D. of 5 rats. # indicates a significant difference from the normal at 0.05. * indicates a significant difference from the control at $p<0.05$.

animals on the 10 th day after the anti-serum injection (Fig. 3). TJ-8014 (2.0 g/kg/day, *p.o.*) markedly increased the SOD-like activity in glomeruli as well as in renal cortex.

Effect of TJ-8014 on lipid peroxide content in renal cortex (Table II)

The lipid peroxide content in renal cortex of nephritic rats was markedly elevated on the 10 th day after *i.v.* injection of antiserum, compared with normal. TJ-8014 at 2.0 g/kg completely inhibited the elevation of lipid peroxide content.

Effects of crude drugs which constitute TJ-8014 (Fig. 4)

Both Bupleuri Radix (Saiko) and Coptidis Rhizoma (Ouren), given 1.0 g/kg, *p.o.* once on the day after anti-GBM serum injection, significantly increased activities of all three kinds of scavenging enzymes, SOD-like enzyme, catalase and GSH-PX, compared to control. Hoelen (Bukuryou) at 1.0 g/kg, *p.o.* apparently increased only catalase and GSH-PX activities. Glycyrrhizae Radix (Kanzou)

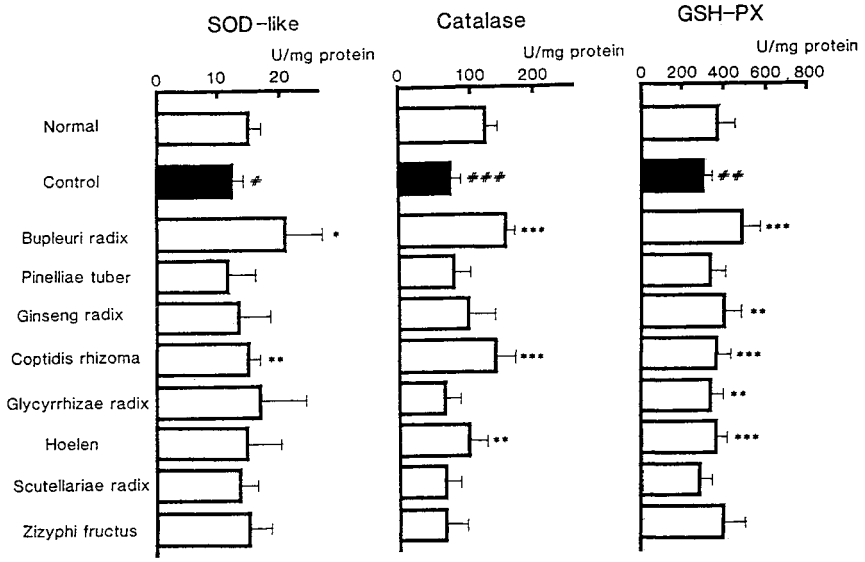

Fig. 4 Effect of crude drugs which constitute TJ-8014 on activities of ROS-scavenging enzymes in renal cortex of rats with original-type anti-GBM nephritis.

Each crude drug was given at *p.o.* 1.0 g/kg/day after anti-GBM serum injection to rats and the animals were sacrified at 1.5 hr after treatment with respective drug for determination of these enzymes activities. Each column denotes the mean±S.D. of 5 rats. #, ## and ### indicates a significant difference from normal at $p < 0.05$, 0.01 and 0.001, respectively. *, ** and *** indicate a significant difference from the control at $p<0.05$, 0.01 and 0.001, respectively.

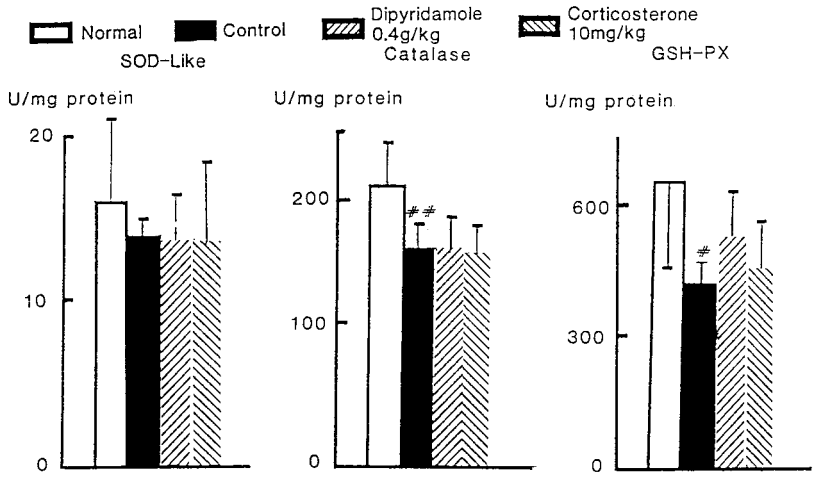

Fig. 5 Effects of dipyridamole and corticosterone on activities of ROS-scavenging enzymes in renal cortex of rats with original-type anti-GBM nephritis. Dipyridamole or corticosterone was given on the day after anti-GBM serum injection to rats and the animals were sacrified at 1.5 hr after the drug administration. Each column denotes the mean±S.D. of 5 rats. # and ## indicate a significant difference from the normal at $p<0.05$ and 0.01, respectively.

and Ginseng Radix (Ninjin) at 1.0 g/kg, *p.o.* increased only GSH-PX activity.

Effects of dipyridamole and corticosterone (Fig. 5)

Adminstration of dipyridamole (0.4 g/kg, *p.o.*) or corticosterone (10 mg/kg, *s.c.*) on the day after anti-GBM serum injection did not affect any ROS-scavenging enzyme activies.

Effects of Cu, Zn-SOD, catalase and TJ-8014 in presence or absence of aminotriazole on urinary protein excretion in original-type anti-GBM nephritis in rats (Fig. 6)

On the lst day after *i.v.* injection of anti-GBM serum, Cu, Zn-SOD at 3.500 U/kg, *i.p.*, catalase at 110,000 U/kg, *i.p.* and TJ-8014 at 2.0 g/kg, *p.o.* prevented the urinary orotein excretion by 60 % to 70 %, compared with nephritic control. In contrast to the effects of these agents, aminotriazole at 500 mg/kg, *i.p.* increased the urinary protein excretion. In addition, the decrease in the urinary protein excretion by TJ-8014 (2.0 g/kg, *p.o.*) was inhibited by combination with aminotriazole (500 mg/kg, *i.p.*).

Effect of TJ-8014 alone and in combination with cycloheximide or actinomycin D on activities of ROS-scavenging enzymes in renal cortex of normal rats

TJ-8014 alone (Fig. 7): TJ-8014 at 2.0 g/kg, *p.o.* significantly increased SOD-like activity at 1.5 and 3 hr after giving to normal rats (Fig. 7). Catalase and GSH-PX activities were significantly increased only at 1.5 hr after treatment with this medicine.

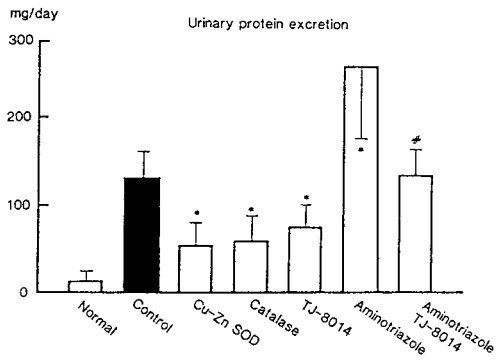

Fig. 6 Effects of SOD, catalase and aminotriazole, TJ-8014 in presence or absence of aminotriazole on urinary protein excretion in original-type anti-GBM nephritis. Each column denotes the mean±S.D. of 5 rats. * indicates a significant difference from the control at $p<0.05$. # indicate a significant difference from TJ-8014-treated at $p<0.05$.

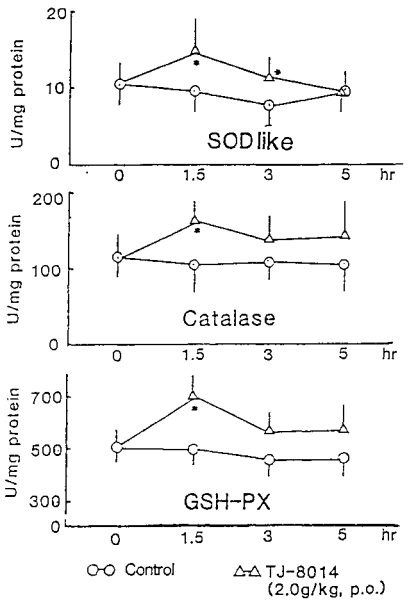

Fig. 7 Effects of TJ-8014 on activities of ROS-scavenging enzymes in renal cortex of normal rats. TJ-8014 was given to normal rats and the animals were sacrified at 0, 1.5, 3 and 5 hr after the drugs administration for determination of these enzyme activities. Each plot denotes the mean±S.D. of 5 rats. * indicates a significant difference from the control at $p<0.05$.

TJ-8014 in combination with cycloheximide or actinomycin D (Fig. 8): When SOD-like, catalase and GSH-PX activities were determined at 1.5 hr after giving to normol rats, the increase in these three enzymes activies by TJ-8014 (2.0 g/kg, *p.o.*) was completely inhibited by combination with either cycloheximide or actinomycin D (5.0 mg/kg, *i.p.*) (Fig. 7).

Discussion

The present study has demonstrated that TJ-8014, a new Japanese herbal medicine, elevates the activities of ROS-scavenging enzymes, SOD-like enzyme, catalase and GSH-PX in renal cortex or in glomeruli in rats with original-type anti-GBM nephritis and this medicine may partly exert the antinephritic action by scavenging cytotoxic ROS generated in glomeruli via the elevation of these radical-scavenging enzymes activities.

Intraglomerular infiltration of polymorphonuclear neutrophils and macrophages has been recognized in the acute (heterologous) and autolo-

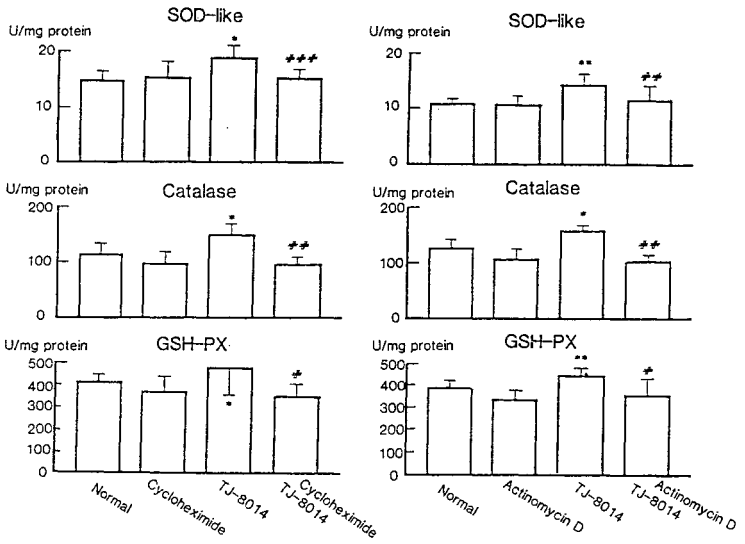

Fig. 8 Effects of cycloheximide or actinomycin D on increasing actions of TJ-8014 on activities of ROS-scavenging enzymes in renal cortex of normal rats. TJ-8014 was given *p.o.* at 2.0 g/kg immediately after cycloheximide or actinomycin D at 5 mg/kg, *i.p.* to normal rats and the animals were sacrified at 1.5 hr after the administration of TJ-8014 for determination of these scavengers. Each column denotes the mean±S.D. of 5 rats. * indicates a significant difference from respective control at $p<0.05$. #, ## and ### indicate a significant difference from TJ-8014-treated group at $p<0.05$, 0.01 and 0.001, respectively.

gous phases, respectively, in anti-GBM nephritis in rats and both inflammatory cells are thought to be important mediators to induce glomerular injury in both phases of this model.[5-7] Recently, it has been shown that these inflammatory cells infiltrated within glomeruli induce glomerular injury by generating the ROS which are directly cytotoxic to a variety of normal cells.[10 12] In addition, the role of ROS in the development of glomerular injury and proteinuria of experimental anti-GBM nephritis may be supported by the fact that Cu, Zn-SOD or catalase, ROS-scavenging enzymes, in the present experiment, markedly attenuated this nephritis, while aminotriazole, catalase inhibitor, aggravated the disease.

In the present experiment, the SOD - like, catalase and GSH-PX activities in renal cortex were signicantly lower in nephritic rats than in normal animals through the experimental period of the lst to the 10 th day after anti-GBM serum injection. Recently, it has been reported that the SOD activity in an inflamed site is significantly decreased in experimental hepatitis[23] and patients with rheumatoid arthritis, too.[24] The mechanism by which the activities of these ROS-scavenging enzymes in renal cortex are decreased during the process of nephritis is unclear. Of ROS, superoxide anion radical is initially produced and it then in turn gives rise to other ROS by reacting with hydrogen peroxide such as hydroxyl radicals and singlet oxygen. In addition to the lysis of lysosomes and peroxidation of cell membrane lipids, superoxide anion may not activate the ROS - scavenging enzymes.[25] As another possible mechanism, it is postulated that the ability of cells to synthesize ROS - scavenging enzymes may be impaired by cell dysfunction, because the ROS can cause cell dysfunction and death by interaction with bilayer of cell membrane, lysosomes and nuclei.

In the present experiment, TJ-8014 significantly elevated the decreased activities of ROS-scavenging enzymes in renal cortex of nephritic rats. In general, it has been belived that the elevation of ROS-scavenging enzymes activities may be due to the results of the increase in ROS generation. However, in the present experiment, the lipid peroxide content in renal cortex of nephritic rats was increased as compared with that of normal animals and the increase in lipid peroxide content was significantly inhibited by treatment with TJ-8014, In addition, TJ-8014 elevated the activities of ROS scavenging enzymes of normal rats. Therefore, it is unlikely that TJ-8014 secondary elevates these scavenging enzymes activities by increasing the ROS formation, or the antinephritic action of TJ-8014 via other mechanisms.

Recently, Aoyagi *et al.*[26] reported that Bupleuri Radix (Saiko), one of the crude drugs which constitute TJ-8014, was able to dismute oxygen radicals generated by incubating puromycin aminonucleoside with isolated liver cells. We demonstrated that, of eight crude drugs which constitute TJ-8014, Coptidis Rhizoma (Ouren) in addition to Bupleuri Radix also elevated SOD-like, catalase and GSH-PX activies in renal cortex. This result suggests that these two crude drugs may contribute to the enhancing action of TJ-8014 on these scavenging enzymes activities.

The elevation of these scavenging enzymes activities by TJ-8014 was recognized in the renal cortex of normal rats as well as nephritic rats. Furthermore, the combination with cycloheximide or actinomycin D, a protein synthesis inhibitor, in normal rats, completely inhibited the elevation in these three enzymes activities by TJ-8014. Our results indicate that TJ-8014 directly promotes the synthesis of these enzymes protein in renal cortex.

In the present experiment, dipyridamole, an antipletelet agent, which had been proved to be effective against this nephritis,[27] did not affect ROS-scavenging enzymes activities in renal cortex of nephritic rats. Therefore, it is unlikely that the elevation of these enzymes activities by TJ-8014 is due to the result of the antinephritic action of this medicine.

We have already demonstrated that TJ-8014 enhances corticosterone release from adrenal glands.[2] However, at present, the treatment with corticosterone (10 mg/kg, *s.c.*) on the day after anti-GBM serum injection did not affect any ROS-scavenging enzymes activities in renal cortex.

Accordingly, TJ-8014 does not appear to enhance these scavenging enzymes activities via the increase in the release of the adrenal cortical hormone.

In summary, the present findings suggest that TJ-8014 may partly exert the antinephritic action by scavenging the ROS via the increase in the synthesis of ROS-scavenging enzymes in glomerular cells.

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