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Pharmacokinetics of 6-shogaol, a pungent ingredient of Zingiberis Rhizoma, and the anti-inflammatory activity of its metabolite, 6-paradol

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Abstract

This study examined the pharmacokinetics and pharmacological activities of 6-shoganol, a pungent ingredient of Zingiberis Rhizoma, and its metabolite, 6-paradol. The concentrations of 6-shogaol and 6-paradol in rat plasma determined by LC/MS/MS reached their maximum values (Cmax) at 5 minutes after oral administration of 6-shogaol (10 mg/kg). Both 6-shogaol and 6-paradol were eliminated from the plasma within 2 hours after injection. The plasma concentration of 6-paradol, the metabolite, was about 4 times higher than that of 6-shogaol at all points during blood sampling. Next, pharmacological activities of 6-shogaol and 6-paradol were studied. *In vitro* experiment revealed that the cyclooxygenase-2-inhibitory activity of 6-paradol was about 6 times stronger than that of 6-shogaol. *In vivo* experiments, 6-paradol demonstrated significantly stronger anti-inflammatory, analgesic, and antipyretic activities compared to 6-shogaol. These results suggest that 6-shogaol in Zingiberis Rhizoma is metabolized rapidly to 6-paradol and that 6-paradol is the main compound having anti-inflammatory activity.

Key words 6-shogaol, 6-paradol, cyclooxygenase-2, anti-inflammatory activity.

Introduction

Zingiberis Rhizoma (*Zingiber officinale* Roscoe) is a perennial plant native to India and South East Asia. It is called Shokyo (Zingiberis rhizome) and Kankyo (Zingiberis siccatum rhizome) and is an important constituent of a large number of recipes in Kampo medicine, including Shoseiryuto and Kakkonto, having anti-inflammatory effects. Of the components contained in Zingiberis Rhizoma, 6-shogaol, the pungent component, is present only in minute amounts in fresh Zingiberis Rhizoma. It is produced by dehydration procession from 6-gingerol,[1)] thus increased in amount, and constitutes the main component of Kankyo besides gingerols.

To date, 6-shogaol has been reported to have antipyretic and analgesic activities,[2)] anti-inflammatory activities such as inhibition of cyclooxygenase-2 (COX-2),[3)] to promoting digestion activities by peristaltic movement,[2,4)] and central depressant actions such as suppression of motor activity[2)] and prolongation of hexobarbital-induced sleep.[2)] The main metabolite of 6-shogaol, 6-paradol, has also been found to have antimicrobial activity[5)] and activator protein 1 activation.[6)]

To elucidate the pharmacokinetics of these components have been conducted, the pharmacokinetics of 6-gingerol were studied by compartment model analysis

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of plasma concentration profile following intravenous administration in rats, and by elucidating the plasma protein binding ratio,[7] by elucidating the changes in pharmacokinetic parameters after caudal vein injection in rats with carbon tetrachloride-induced hepatic disorder,[8] and by examining metabolites in rat urine and bile.

On the other hand, *in vitro* metabolism experiments of 6-shogaol have revealed paradol compounds as reductive metabolites in the liver and kidney of mice, rats, and humans.[10-12] Chen reported that 6-paradol and other metabolic compounds were detected from a fecal sample after administration of 6-shogaol by oral gavage in mice.[13] Recently, the presence of glucuronate and sulfate conjugates has been reported as metabolites following administration of ginger in humans.[14,15] While there are a large number of experiments concerning the pharmacokinetics of 6-gingerol, 6-shogaol has been studied more in *in vitro* metabolism experiments, and not many *in vivo* studies have been conducted.

Our previous experiment showed that 6-shogaol is metabolized almost completely and excreted into the bile after oral administration in rats.[16] However, we have not yet clarified the pharmacokinetics of its metabolite, 6-paradol. In the present study, we developed a method for quantitative determination of 6-shogaol and its putative main metabolite, 6-paradol using LC/MS/MS, and studied the pharmacokinetics of 6-shogaol and 6-paradol following oral administration of 6-shogaol in rats. Anti-oxidative activity[17] and anticancer activity[17,18] are known as the pharmacological effects of 6-paradol, but there has been almost no report about anti-inflammatory activity of 6-paradol. Therefore, 6-shogaol and 6-paradol were compared in relation to their anti-inflammatory activity in mice and rats.

Materials and Methods

Materials: Standard preparations of 6-shogaol and 6-paradol (purity ≥99%) (Figure 1) were supplied by Tsumura & Co. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals: Seven-week-old male SD rats (SPF) and male ddy mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and were used in the experiments after conditioning for a period of one week. The experiments were conducted in compliance with the rules regarding animal experiments set by Musashino University based on the law of humane treatment and management of animals, basic rules for keeping and managing animals, and basic policy for the conduct of animal experiments in research facilities.

Administration of 6-shogaol to rats: 6-shogaol was suspended in 2% Tween 80 in saline. Rats were given the suspension of 6-shogaol at a dose of 10 mg/kg by gavage under fasting condition, and blood was collected from the caudal vein using a heparinized syringe at 5, 15, 30, 60, and 120 minutes after administration. Plasma was separated by centrifugation at 4℃, 1,000g, for 10 minutes. The samples were stored at -20℃ until measurement of concentrations of 6-shogaol and 6-paradol by LC/MS/MS.

Measurement of concentrations of 6-shogaol and 6-paradol in rat plasma by LC/MS/MS: Standard solutions of 500, 100, 50, 10, 5, 1, and 0.5 ng/mL for the calibration curve were prepared by serially diluting 6-shogaol and 6-paradol with a mixture of methanol and 5 mmol/L ammonium acetate (1:1 vol/vol). A solution of ethyl 4-hydroxy-3-methoxycinnamate (10 ng/mL) was prepared as the internal standard (IS) solution by the same method. Then, 500 μL of purified water, 50 μL of each standard solution or plasma sample, 50 μL of the mixture of methanol and 5 mmol/L ammonium

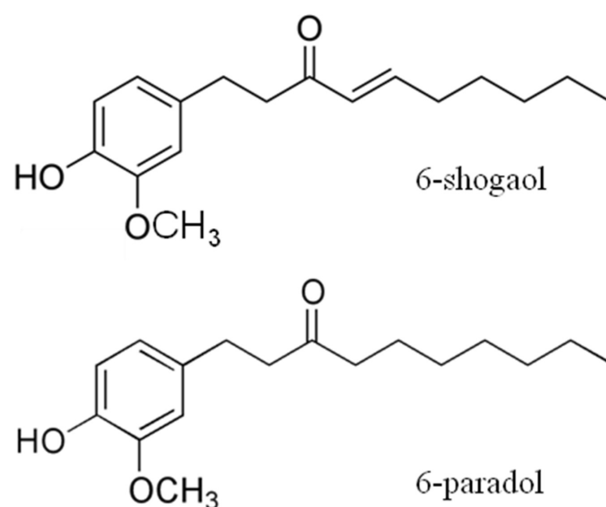

Figure 1 Chemical structures of 6-shogaol and 6-paradol.

acetate (1:1, vol/vol) and 50 μL of IS solution were loaded on an Oasis HLB cartridge column for solid phase extraction (Nihon Waters Co., Ltd., Tokyo, Japan) and eluted under vacuum. The column was washed with 1 mL of 10% methanol, and 1 mL of a mixture of cyclohexane and ethyl acetate (1:1, vol/vol) was used as the eluting solution. The eluate was evaporated to dryness under nitrogen, and the residue was dissolved in 150 μL of the mixture of methanol and 5 mmol/L ammonium acetate (1:1, vol/vol), and 20 μL of the solution was injected into the LC/MS/MS system.

HPLC of the LC/MS/MS analysis was carried out on an Agilent 1000 series (Agilent technologies, Inc., CA, USA) using an Atlantis dC18 column (2.1 mm I.D. × 50 mm, 3 μm, Nihon Waters) coupled with a 10 mm Atlantis dC18 guard column. Ammonium acetate (A) and methanol (B) were used as the mobile phase run at A:B=30:70 for 2 minutes A:B=30 to 10:70 to 90 for 2 to 5 min. The column temperature was 40℃, and the flow rate was 0.2 mL/min.

Tandem mass spectrometry was performed using an API4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, CA, USA) under the following conditions: ion source: electrospray ionization (ESI), voltage setting: -4500 V, negative multiple reaction monitoring mode. The monitored ions were m/z 275.1 →139.0 (Q1→Q3) for 6-shogaol, *m/z*=277.0→141.3 (Q1 →Q3) for 6-paradol and *m/z*=220.9→205.7 (Q1→Q3) for IS. Analysis of the data was performed using Analyst 1.4 software (Applied Biosystems/MDS SCIEX). The recovery ratios of 6-shogaol and 6-paradol from rat plasma were 87.0% and 80.9%, respectively. The limit of quantitation of the present method was 0.5 ng/mL. Pharmacokinetic parameters such as maximum drug concentration in plasma (Cmax) and time to maximum drug concentration in plasma (Tmax) were determined using WinNolin (Ver. 5.2) from the plasma drug concentration values.

Measurement of COX-1 and COX-2 inhibition: Inhibitory activities of 6-shogaol, 6-paradol, and indomethacin against COX-1 and COX-2 were evaluated using the Colormetric COX (ovine) inhibitor Screening Assay Kit (Cayman Chemical Co., USA). The activity of COX without addition of the sample was taken as 100%.

Anti-inflammatory activity on carrageenan-induced hind paw edema: Male SD rats were given, by gavage, 6-shogaol suspended in 2% Tween 80 in saline (100 mg/kg), 6-paradol suspended in 2% Tween 80 in saline (100 mg/kg), or purified water (control) at a dose of 2 mL/kg. After 30 minutes, 0.1 mL of 1% aqueous solution of carrageenan was injected subcutaneously into the right hind paw, and the hind-paw volume was measured at 0.5, 1, 2, and 5 hours using 7140 Plethysmometer (UGO Basile, Italy). The hind-paw volume at 30 minutes prior to administration was taken as 100%.

Analgesic activity on acetic acid-induced writhing response: Male ddy mice weighing 30 to 33 g were fasted for 18 hours and given oral administration of a suspension of 6-shogaol, 6-paradol, or aminopyrine in 2% Tween 80 (100 mg/kg) at a dose of 0.1 mL/10 g body weight. After 10 minutes, the animals were given intraperitoneal administration of 0.1 mL/10 g body weight of 0.7% acetic acid. Starting at 10 minutes after injection of 0.7% acetic acid, the number of writhing responses during the following 10 minutes was recorded. Animals in the control group were given 0.1 mL/10 g body weight of 2% Tween 80 by the oral route.

Antipyretic activity on yeast-induced fever in mice: Male ddy mice aged 8 to 11 weeks were fasted for 18 hours and then given a subcutaneous injection of 20% yeast suspension in physiological saline in the back. The rectal temperature measured after 18 hours using Thermalert TH-5 (Physitemp Instruments, Inc., USA) was used as the reference body temperature. At 19 hours after yeast administration, the animals were given oral administration of 0.1 mL/10 g body weight of 6-shogaol suspension in 2% Tween 80 or 6-paradol suspension in 2% Tween 80, corresponding to doses of 70 mg/kg and 140 mg/kg, or aminopyrine corresponding to a dose of 100 mg/kg. The rectal temperature was measured at 30, 60, and 120 minutes after drug administration. Animals in the control group were given an oral dose of 0.1 mL/10 g body weight of 2% Tween 80.

Test of significance: The data are expressed as mean ± SE. Statistical significance was tested with the Dunnett's test.

Results

Measurement of concentrations of 6-shogaol and 6-paradol in rat plasma by LC/MS/MS: A sample preparation method using an OASIS HLB cartridge column for solid phase extraction was established, and conditions for LC/MS/MS to isolate and detect 6-shogaol and 6-paradol using IS were optimized (Figure 2). In this system, good calibration curves were obtained in the range of 0.5 to 500 ng/mL.

The dose of 6-shogaol administered to rats was established as 10 mg/kg based on the result of a study with ^{14}C-6-shogaol in which dose dependency was observed for the range of 3 to 30 mg/kg.[16] Changes in plasma concentrations of 6-shogaol and 6-paradol following an oral dose of 6-shogaol are shown in Figure 3. The Cmax of 6-shogaol in plasma (13.7 ± 5.2 ng/mL) was reached at 5 minutes after administration and 6-shogaol was eliminated from the plasma within 2 hours after administration. The Cmax of 6-paradol in plasma (43.7 ± 16.3 ng/mL) was reached also at 5 minutes after administration of 6-shogaol. The plasma concentration of 6-paradol was about 4 times higher than that of 6-shogaol at all points in time during blood sampling.

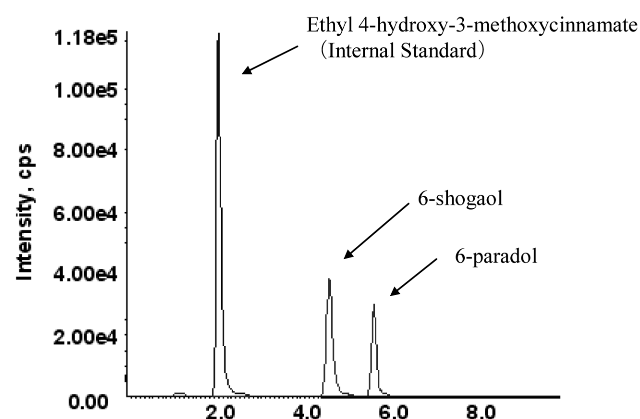

Figure 2 LC/MS/MS MRM chromatograms of Standard solution (6-shogaol, 6-paradol, I.S.; 10 ng/mL each).

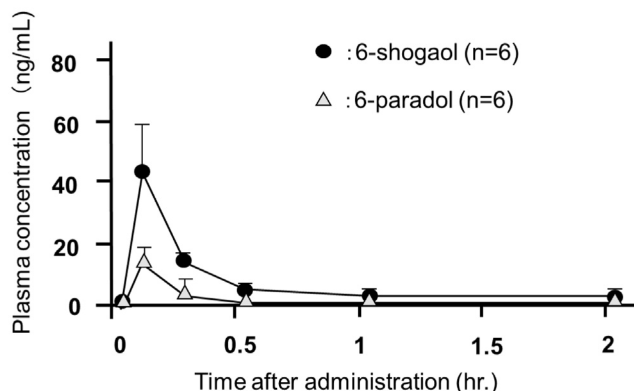

Figure 3 Plasma concentration of 6-shogaol and 6-paradol after oral administration of 6-shogaol (10 mg/kg) in rats.
Each value represents the mean ± SE. (n=6)

Inhibition of COX by 6-shogaol and 6-paradol: Inhibitory activities of 6-shogaol, 6-paradol and indomethacin (control) against COX were evaluated using an assay kit. Indomethacin showed the strongest inhibitory activity against COX-1, and 6-shogaol and 6-paradol showed weak inhibitory activities of similar magnitude (Table 1). Indomethacin showed the strongest inhibitory activity also on COX-2, and though slightly weaker than indomethacin, 6-paradol likewise strongly inhibited COX-2. The inhibitory activity of 6-shogaol on COX-2 was 6 times weaker than that of 6-paradol.

The COX-2 selectivity index (ratio of COX-1 IC_{50}/COX-2 IC_{50}) of indomethacin was 0.2, compared to an index of 2.1 for 6-shogaol and of 10 for 6-paradol. Of the three compounds, 6-paradol was found to inhibit COX-2 most selectively.

Table 1 IC_{50} of COX-1, COX-2, and ratio of COX-1 IC_{50}/COX-2 IC_{50}.

	COX-1 IC_{50} (μM)	COX-2 IC_{50} (μM)	Ratio of CoX-1 IC_{50}/COX-2 IC_{50}
6-shogaol	403.89	192.74	2.1
6-paradol	328.89	31.89	10.3
Indomethacin	2.69	12.55	0.2
Celecoxib[17]	82	6.8	12
Meloxicam[17]	37	6.1	6.1
Ibuprofen[17]	12	80	0.15

Data represent the mean ± S.E.M value (n=4-7)

Anti-inflammatory activity on carrageenan-induced paw edema: Subcutaneous injection of carrageenan is known to cause an increase in paw volume and edema.[19] Animals in the control group showed an increase in paw volume until 30 minutes after injection, and at 5 hours, the paw volume was about 1.4 times of that before injection. In animals that received a 6-shogaol injection, increase in paw volume was suppressed compared to that in the control animals; however, the activity was not significant. In animals that

received 6-paradol, on the other hand, significant suppression of paw volume increase compared to the control group was found at all points in time (Figure 4).

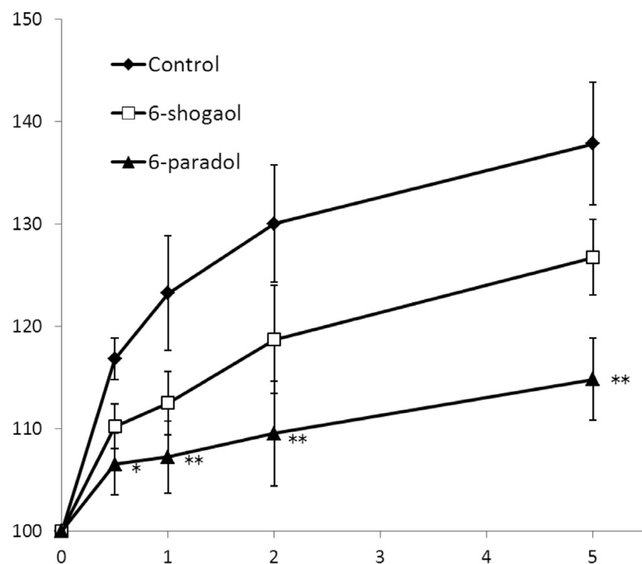

Figure 4 Effects of 6-shogaol and 6-paradol on rat hind edema induced by carrageenan. The hind-paw volume at 30 minutes prior to 6-shogaol and 6-paradol administration (100 mg/kg, p.o.) was taken as 100%. Data represent the mean ± SE. value (n=4-6)
*,**: Significantly different from the control at $p<0.05$, $p<0.01$

Analgesic activity on acetic acid-induced writhing response: Significant decrease in the number of writhing responses was observed in the 100 mg/kg 6-paradol group and in the aminopyrine group compared to the control group, while no decrease in writhing response was found in the 6-shogaol group (Figure 5). The analgesic activity of 6-paradol was not stronger than that of aminopyrine.

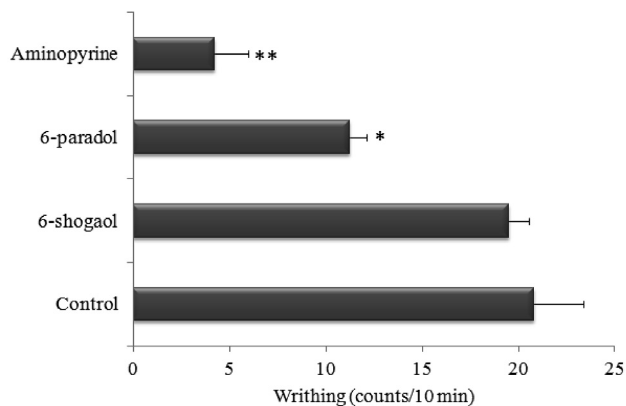

Figure 5 Analgesic activity of 6-shogaol and 6-paradol (100 mg/kg, p.o.) in writhing symptom of mice. Data represent the mean ± SE. value (n=4-6)
*,**: Significantly different from the control at $p<0.05$, $p<0.01$

Antipyretic activity on yeast-induced fever in mice: Figure 6 shows rectal temperatures at 0, 30, 60, and 120 minutes after each drug administration relative to the reference temperature at 18 hours after yeast administration. No significant change compared to the control group was found in animals treated with 70 mg/kg and 140 mg/kg of 6-shogaol. By contrast, significant decrease in rectal temperature was observed in animals treated with 6-paradol at 0 and 60 minutes after administration of 70 mg/kg and at 30 and 60 minutes after administration of 140 mg/kg. The rectal temperature in the 140 mg/kg 6-paradol group decreased by more than 2℃ within 60 minutes, and the antipyretic activity lasted for longer than 1 hour. The control drug aminopyrine caused significant decrease in rectal temperature by more than 4℃ at all time points of 30, 60, and 120 minutes after administration. No significant effect of 6-shogaol and 6-paradol on normal body temperature was observed (data not shown).

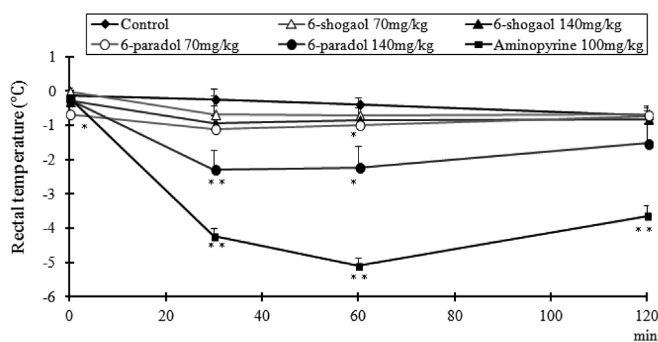

Figure 6 Antipyretic activity of 6-shogaol and 6-paradol (70mg/kg or 140mg/kg, p.o.) in the Brewer's yeast-febrile mice. Data represent the mean ± SE. value (n=7)
*,**: Significantly different from the control at $p<0.05$, $p<0.01$

Discussion

We have reported that most of 6-shogaol orally administered to rats is probably metabolized rapidly and excreted.[16] In the present study, a sensitive method for simultaneous quantitative determination of 6-shogaol and 6-paradol was developed using LC/MS/MS and the pharmacokinetics of 6-shogaol and its putative main metabolite, 6-paradol, in plasma after oral administration of shogaol in rats were analyzed. As a result, the Tmax of 6-shogaol and 6-paradol were 5 minutes. The concentration of 6-paradol in plasma was about 4 times higher than that of 6-shogaol all the time. Some reports

show that 6-shogaol was metabolized into 6-paradol in liver microsomes in *in vitro* experiments.[10-12] In this study, while we demonstrated that the 6-shogaol was absorbed within 5 minutes after administration, rapidly metabolized, and was converted to 6-paradol, the metabolic tissue of 6-shogaol into 6-paradol were not revealed. Therefore, in future it will be necessary to investigate further with a more detailed study on pharmacokinetics and metabolism of 6-shogaol into 6-paradol.

It is known that 6-shogaol inhibits COX and thus exhibits anti-inflammatory activity.[3] In the present study, the pharmacological activities of 6-shogaol and 6-paradol were compared in terms of their inhibitory activities against COX. The inhibitory activities on COX-1 of 6-shogaol and 6-paradol were over 120 times weaker than those of indomethacin; the inhibitory activity on COX-2 of 6-paradol, on the other hand, was 3 times weaker than that of indomethacin and 6 times stronger than that of 6-shogaol (Table 1). The index of selectivity for COX-2 (ratio of COX-1 IC_{50}/COX-2 IC_{50}) of 6-paradol was 10, compared to an index of 2.1 for 6-shogaol and of 0.2 for indomethacin. Van Breemen reported that the paradols showed the strongest binding potency to the active site of COX-2 followed by shogaols, and then the gingerols and gingerdiones, and suggested that the carbonyl group at carbon 3 was essential for binding to COX-2.[20] Our results are congruent with those reported by Van Breemen. Considering the selectivity index of (ratio of COX-1 IC_{50}/COX-2 IC_{50}) celecoxib and meloxicam, a selective COX-2 inhibitor, of about 12 and 6.1,[21] 6-paradol was shown to have a relatively strong selectivity for COX-2.

Next, we performed *in vivo* experiments of analgesic activity in acetic-acid induced writhing and antipyretic activity in mice with yeast-induced fever to study the anti-inflammatory activities of 6-shogaol and 6-paradol. Stronger anti-inflammatory activity was found with 6-paradol than with 6-shogaol. These results are considered to be attributable to the higher COX-2 selectivity of 6-paradol than 6-shogaol. Anti-inflammatory activity on carrageenan-induced paw edema was studied, and 6-paradol was shown to continuously and significantly suppressed edema starting at 30 minutes after administration. Formation of edema in response to carrageenan injection was bi-phasic, i.e., the first phase reached its peak and disappeared at about 30 minutes after injection and the second phase occurred after 1 hour after injection and persisted for a long period of time.[22] It is known that histamine and serotonin are involved in the first phase edema,[23] and that prostaglandins are involved in the second phase edema[24]; it is also suggested that the second phase edema is associated with various chemical mediators such as nitric oxide (NO).[25] Considering these facts, 6-paradol is likely to have other pharmacological effects besides inhibition of COX. With regards to 6-shogaol, inhibition of NO production in lipopolysaccharide-activated RAW cells has been reported.[26] In the present study, on the other hand, the inhibitory activity of 6-shogaol on carrageenan-induced edema was weaker than to that of 6-paradol; its association with the mechanism of inflammation must be studied in the future.

The present study suggested that 6-shogaol, the main component of Zingiberis Rhizoma, is metabolized to 6-paradol in the body and 6-paradol exhibits strong anti-inflammatory activity. The results indicate not only elucidation of the mechanism of action of Zingiberis Rhizoma but also suggest potential clinical application of 6-paradol with its anti-inflammatory activity. These anti-inflammatory effects of 6-shogaol and 6-paradol ought to contribute to the potencies of Kampo medicines including Zingiberis Rhizoma.

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