

Beneficial effect of *Sparassis crispa* on stroke through activation of Akt/eNOS pathway in brain of SHRSP

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Abstract *Sparassis crispa* (*S. crispa*) is a mushroom used as a natural medicine that recently became cultivatable in Japan. In this study, we investigated not only the preventive effects of *S. crispa* against stroke and hypertension in stroke-prone spontaneously hypertensive rats (SHRSP) but also the mechanism involved by using studies of the cerebral cortex at a young age. Six-week-old male SHRSP were divided into 2 groups, a control group and an *S. crispa* group administered 1.5% *S. crispa* in feed, and we then observed their survival. In addition, rats of the same age were treated with 1.5% *S. crispa* for 4 weeks and we measured body weight, blood pressure, blood flow from the tail, NO_x production, and the levels of expression of several proteins in the cerebral cortex by western blot analysis. Our results showed that the *S. crispa* group had a delayed incidence of stroke and death and significantly decreased blood pressure and increased blood flow after the administration. Moreover, the quantity of urinary excretion and the nitrate/nitrite concentration in cerebral tissue were higher than those of control SHRSP rats. In the cerebral cortex, phosphor-eNOS (Ser1177) and phosphor-Akt

(Ser473) in *S. crispa*-treated SHRSP were increased compared with those of control SHRSP rats. In conclusion, *S. crispa* could ameliorate cerebrovascular endothelial dysfunction by promoting recovery of Akt-dependent eNOS phosphorylation and increasing NO production in the cerebral cortex. *S. crispa* may be useful for preventing stroke and hypertension.

Keywords Akt/eNOS · Hypertension · SHRSP · *Sparassis crispa* · Stroke

Introduction

One of the main causes of stroke is hypertension. In patients with long-term hypertension, functional modifications in the cerebrovascular system have also been found [1]. Therefore, it is important to avoid high blood pressure as a preventative measure. Hypertensive drug therapy is an easy recently developed approach that can reduce blood pressure by the administration of an antihypertensive agent. However, there is a risk of side effects and the treatment lasts for a long time. This explains the utility of blood pressure control by selection of appropriate food for which there are few side effects.

The medical and pharmacological effects of mushrooms have been shown to vary greatly in terms of antihypertension, antitumor, and antidiabetic properties as well as those against various other diseases [2]. Some mushrooms have been used in Chinese medicine and folk medicine for a long time. Recently published reports have demonstrated that various kinds of mushrooms have beneficial effects on blood pressure [3–6]. *Sparassis crispa* (*S. crispa*) is an edible mushroom that has recently been cultivated in Japan. It contains a remarkably high concentration of beta-glucan,

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which exhibits antitumor [7] and antidiabetic [8] activities. However, there have not yet been any detailed studies of the effects of *S. crista* on hypertension and stroke.

Stroke-prone spontaneously hypertensive rats (SHRSP), a substrain of SHR created by selective breeding, have become a model for basic research on hypertension-associated cerebrovascular injury, because this model (SHRSP) presents with marked elevation of blood pressure and almost all of these animals develop stroke. Numerous nutritional and pathological studies using SHRSP have revealed that various foods are protective against hypertension and stroke [9, 10].

The present study investigated whether *S. crista* exhibits preventive effects against hypertension and stroke and the mechanisms behind such effects by using SHRSP.

Methods

Animals

Study 1

Six-week-old male SPF (specific pathogen free) stroke-prone spontaneously hypertensive rats (SHRSP) were purchased from Japan SLC (Shizuoka, Japan). All rats were housed in a climate-controlled (temperature 22–24°C, humidity 40–60%) light-regulated room with a 12-h light and dark cycle. SHRSP were separated into 2 groups ($n = 8–9$): a control group (not treated) and an *S. crista* group (administered 1.5% *S. crista*, which was supplied by Katsuragi Industry Co., Ltd., Japan). All groups consumed an SP diet (Funahashi Co., Tokyo, Japan) and had free access to rat chow and water throughout the experiment. The survival rate of the rats was determined. Autopsies were conducted on the day of death and all rats were checked for stroke at the brain. All procedures were carried out in accordance with the guiding principles for the care and use of animals in the field of physiological sciences established by the Physiological Society of Japan and the guidelines for the care and use of animals set by Mukogawa University.

Study 2

Male SHRSP at 6 weeks of age were divided into 2 groups: a control group and an *S. crista* group administered 1.5% *S. crista* ($n = 8$ each). All rats were housed as in study 1. We measured daily water and food intakes of the rats. Body weight and blood pressure were measured every 2 weeks. Blood flow of venous microcirculation in the tail was measured before and after 4 weeks of treatment. At the conclusion of the 4-week treatment period, 24-h urine samples were collected by placing the rats in metabolic cages. Food was

withdrawn, but water was provided. Then, the animals were anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal, 100 mg/kg). The tissues were immediately harvested and cleaned for measurement of tissue weight, and brain was promptly frozen in liquid nitrogen for analysis. The samples were then stored at -80°C .

Blood pressure measurements

Systolic blood pressure (SBP) was measured by using a sphygmomanometer (UR-1000, Ueda Co., Chiba) using the tail-cuff method. An average of 3 measurements were taken as the initial mean SBP.

Blood flow measurements

The blood flow through the venous microcirculation in the tail was monitored by using a laser Doppler blood flow meter of the contact type (FLO-C1, Muromachi, Tokyo). Briefly, all rats were pre-warmed at 36°C and blood flow was measured while keeping the animal at 36°C on a holder. The rate of change in blood flow was calculated as follows: Rate of change = (blood flow before treatment/blood flow after treatment) $\times 100$.

Urinary and cerebral nitrate/nitrite assay

Samples of cerebral cortex were obtained by homogenization with twofold methanol followed by centrifugation at 3,000 rpm for 5 min; urinary samples were also centrifuged under the same conditions, and supernatants were then analyzed. Nitrate and nitrite (NO_x) concentrations in the urine and cerebral cortex were measured by the Griess method using an automated NO detector high-performance liquid chromatography system (ENO-20, Eicom, Kyoto) as described previously [11].

Primary antibodies used

Immunoblotting was performed with the following commercially available antibodies: anti-rabbit eNOS from Affinity BioReagents Inc. (golden); anti-rabbit phospho-eNOS (Ser1177), anti-rabbit Akt, anti-rabbit phospho-Akt (Ser473, Thr308), anti-rabbit mTOR, anti-rabbit phospho-mTOR (Ser2448), and anti-rabbit nNOS from Cell Signaling Technology (Beverly, MA). Anti-mouse β -actin and anti-rabbit iNOS were obtained from Sigma (St. Louis, MO, USA).

Western blot analysis

The cerebral cortex was homogenized with ice-cold homogenized buffer containing 50 mM Tris-HCl (pH 7.4),

100 mM NaCl, 1% Nonidet-P40, 0.25% Na deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 , 30 mM Na pyrophosphate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 0.02 g/mL trypsin inhibitor, 0.02 g/mL leupeptin, and 0.02 g/mL aprotinin. After incubation for 2 h, lysates were centrifuged at 15,000 rpm for 20 min and supernatants were isolated. Proteins were extracted by boiling the tissues in 0.5 mmol/l Tris/HCl, pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue, and 2-mercaptoethanol. The proteins (25 μg) were electrophoresed by using 7.5–12.5% SDS-PAGE gel at 100 V for 2 h. After fractionating, the proteins were transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Amersham Inc. Buckinghamshire) at 100 mA for 2 h. The membrane was blocked in Blocking One (Nacalai Tesque, Kyoto) for 20 min. After appropriate blocking, the blot was incubated with anti-iNOS (1:5,000), anti-nNOS, anti-eNOS, anti-phospho-eNOS (Ser1177), anti-Akt, anti-phospho-Akt (Ser473 and Thr308), anti-mTOR, and anti-phospho mTOR (1:1,000) in antibody solution 1 (Toyobo, Osaka) overnight. It was then washed with Tris-buffered saline (TTBS) and finally incubated for 1 h with a 1:5,000 dilution of anti-rabbit and mouse IgG–horseradish peroxidase. Detection was achieved by using an ECL kit (Amersham Inc. Buckinghamshire). β -actin was used as an internal control. The density of the bands was measured by using NIH Image.

Statistical analysis

Data are expressed as the mean \pm SEM. Cumulative survival was analyzed for differences according to Kaplan–Meier followed by Wilcoxon test. Statistical analysis of the data was performed by Student's *t* test to determine the significance of differences. A *p* value of less than 0.05 was considered significant.

Results

Intakes of water and food, and volume of urine

Water and food intakes over the 4-week period of the study did not differ between the control group and the *S. crispa* group. In addition, no difference was observed in terms of the volume of urine between the two groups (Table 1).

Survival rate, blood pressure, and body weight

Kaplan–Meier survival curves revealed that rats in the *S. crispa* group had a significantly higher survival rate than rats in the control group (Fig. 1). Moreover, SHRSP rats treated with *S. crispa* showed significant inhibition of the rise in blood pressure after 2 and 4 weeks of oral administration

Table 1 Water, food intake and urinary extract volume at 4 weeks after administration

	Water intake (mL)	Food intake (g)	Urinary volume (mL)
Control	21.50 \pm 4.54	18.60 \pm 0.19	19.08 \pm 3.67
<i>S. crispa</i>	25.67 \pm 4.25	18.47 \pm 0.53	19.00 \pm 1.98

Data are means \pm SEM *n* = 6

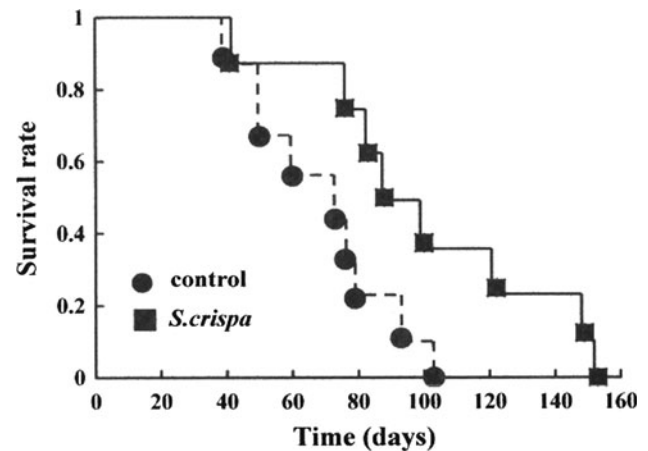


Fig. 1 Kaplan–Meier survival curves showing that SHRSP administered *S. crispa* had a significantly higher survival rate than the control group (Wilcoxon test)

(Fig. 2a). However, body weight did not differ between the SHRSP group and the *S. crispa* group (Fig. 2b).

Change in blood flow and NO production

The change in the amount of blood flow in venous microcirculation in the tail after treatment is shown in Fig. 3a. The administration of *S. crispa* significantly increased blood flow compared with that in the control group. In addition, 24-h urinary NO metabolite (nitrate and nitrite) excretion and concentration in the cerebral cortex in the *S. crispa* group was 2–3-fold higher than that in the control group (Fig. 3b, c). None of these changes reached significance, but these experimental data correspond to the blood pressure and blood flow data.

Measurement of tissue weight

Table 2 shows the ratio of tissue weight to body weight. There were no significant differences in this ratio between the control group and the *S. crispa* group at 4 weeks after administration.

Various types of NOS protein expression

To investigate whether expressions of synthases for NO, which are vasodilators, showed any differences between

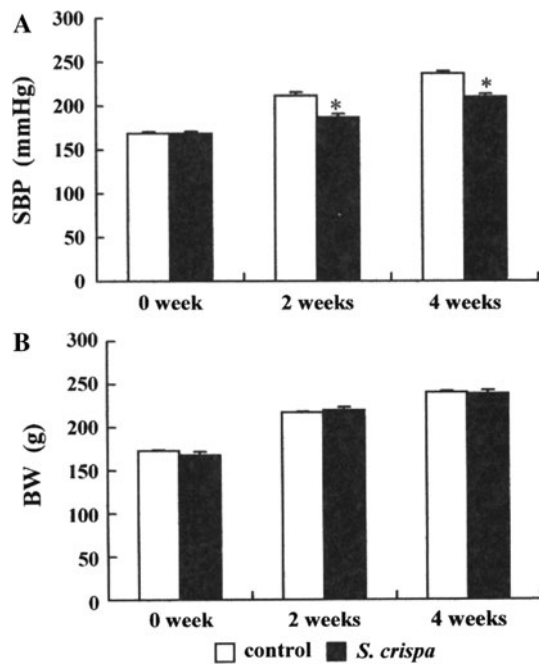


Fig. 2 Effect of *S. crispa* on systolic blood pressure and body weight: control group ($n = 8$) and *S. crispa* group ($n = 8$). All values are the means \pm SEM; * $p < 0.01$ versus control group (Student's t test)

the control group and the *S. crispa* group, we used antibodies to measure iNOS, nNOS, and eNOS. We determined that SHRSP receiving *S. crispa* did not show any changes in these protein levels (Fig. 4).

Effects of phosphorylation of eNOS via PI3 K/Akt pathway

The level of phosphorylation of eNOS at Ser1177 in the cerebral cortex of the *S. crispa* group was significantly higher than that in the control group, as determined by western blotting with phosphospecific antibody (Fig. 5d). Because activation of the serine/threonine kinase Akt phosphorylates and activates eNOS, which in turn induces NO production and vasorelaxation, the phosphorylation of Akt was also studied. The results of western blot analysis demonstrated that cerebral cortex expression of Akt phosphorylated at Ser473 was significantly induced in the *S. crispa* group compared with that in the control group (Fig. 5b). Immunoblot analysis with antibodies against total Akt showed equal protein levels in all groups, thus demonstrating that the induced amount of phosphor-Akt (Ser473) was not due to increased expression of Akt (Fig. 5a). In addition, there was no difference in Thr308 between the two groups (Fig. 5c).

Effects of phosphorylation of mTOR

Although mTOR is located downstream of Akt, the ratio of phosphor-mTOR (Ser2448) to total mTOR did not differ

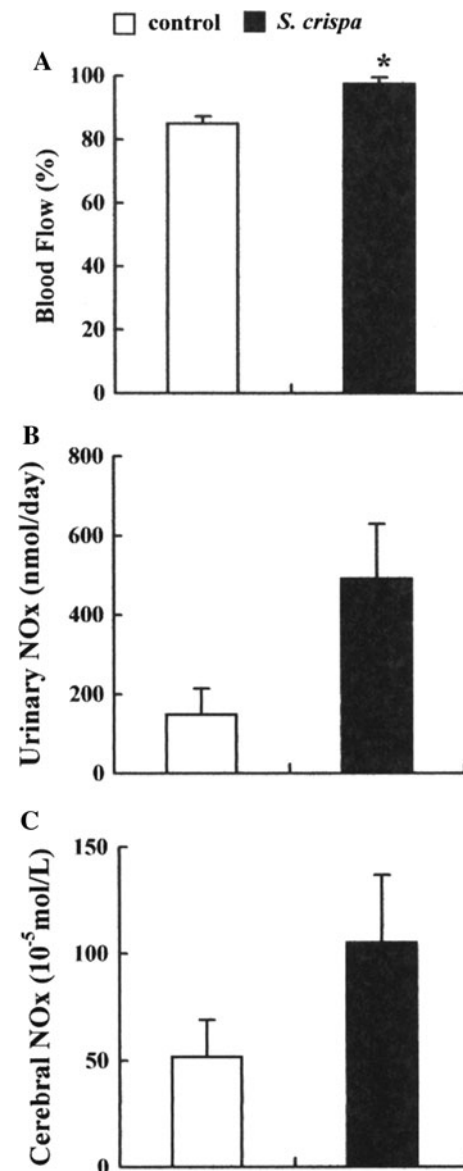


Fig. 3 **a** Measurement of blood flow in the tail of rats in the control group ($n = 8$) and the *S. crispa* group ($n = 8$) before and after administration. The rate of change in blood flow is expressed as a percentage relative to the value before treatment. **b** Urinary nitrate/nitrite (NO_x) excretion and **c** cerebral nitrate/nitrite (NO_x) concentration at 4 weeks after administration. Control group ($n = 4$) and *S. crispa* group ($n = 4$). Data are means \pm SEM; * $p < 0.01$ versus control group (Student's t test)

between the control group and the *S. crispa* group (Fig. 5e).

Discussion

The major findings of the present study are as follows: (1) The administration of *Sparassis crispa* (*S. crispa*) to SHRSP from a young age improves survival and reduces

Table 2 Effects of *S. crispa* on tissue weight (g/100 g body weight)

Tissues	Control (n = 6)	<i>S. crispa</i> (n = 6)
Brain	0.60 ± 0.04	0.57 ± 0.02
Heart	0.47 ± 0.04	0.44 ± 0.03
Spleen	0.22 ± 0.01	0.22 ± 0.01
Liver	3.39 ± 0.12	3.18 ± 0.10
Kidney	0.85 ± 0.03	0.82 ± 0.03
Adrenal gland	0.02 ± 0.00	0.02 ± 0.00
Testis	1.20 ± 0.07	1.14 ± 0.04

Values are means ± SEM

the elevation of blood pressure. (2) The mechanism for this action was improvement of endothelial dysfunction via increases in NO production with activation of the Akt/eNOS signaling pathway on vessel cerebral cortex.

The SHRSP is a model of essential hypertension in humans, and was developed from SHR demonstrating abnormalities of the renin-angiotensin system, catecholamines, vasopressin, and vasoactive intestinal peptide [12]. It is well known that blood pressure in SHRSP is already significantly higher than that in WKY, a normal blood pressure rat, at a young age and that almost all SHRSP develop stroke. Moreover, a significant decrease in regional cerebral blood flow in SHRSP frontal cortex at 6 weeks of age compared with that in WKY was observed [13]. The data presented in our survival studies clearly showed that *S. crispa* protected SHRSP against stroke death. Furthermore, the administration of *S. crispa* was associated with an inhibition of elevated blood pressure and a restoration of impaired peripheral blood flow, which indicates that *S. crispa* may improve vascular function including that in brain blood vessels.

Nitric oxide (NO) plays an important role in the modulation of vascular tone and structure. In animal models of

hypertension and patients with hypertension, nitric oxide (NO) production is abnormal, leading to hypertensive vascular lesion formation [14]. NO in the brain inhibits sympathetic nerve activity [15, 16] or directly affects vasodilatation via a cGMP mechanism, thereby reducing blood pressure [17–19]. The present study showed that *S. crispa* induced a higher level of NO_x in the cerebral cortex compared with that in the control group.

It is well known that NO is generated by nitric oxide synthases (NOSs), which have three distinct isoforms. NO from endothelial NOS (eNOS) when present in vessel endothelial cells is known to regulate vascular tone, while neuronal NOS (nNOS) is involved in neural signaling, and inducible NOS (iNOS) modulates immune function [20–22]. Our study suggests that none of the types of NOS showed any differences in expression between the control group and the *S. crispa* group at 4 weeks after administration. In particular, of all NOS types, NO generation from eNOS would initially limit stroke and brain injury by inducing vasodilation [23]. We previously reported that eNOS activity plays an important role in vasodilation, rather than eNOS expression, at a younger age in the SHRSP strain. The enzymatic activity of eNOS is regulated by phosphorylation at Ser1177, and one of the kinases that mediates phosphorylation of eNOS is Akt, which has an activation process involving two phosphorylation sites, Ser473 and Thr308, by phosphoinositide-dependent protein kinases [24]. Our previous study also demonstrated that phosphorylation of eNOS and Akt was decreased in the cerebral cortex of SHRSP compared with that in WKY. Interestingly, *S. crispa* restored eNOS phosphorylation, which was markedly impaired in the cerebral cortex of SHRSP, and this improvement was associated with the recovery of Akt, especially at the site of Ser473 phosphorylation. Enhanced Akt/eNOS signaling upon the

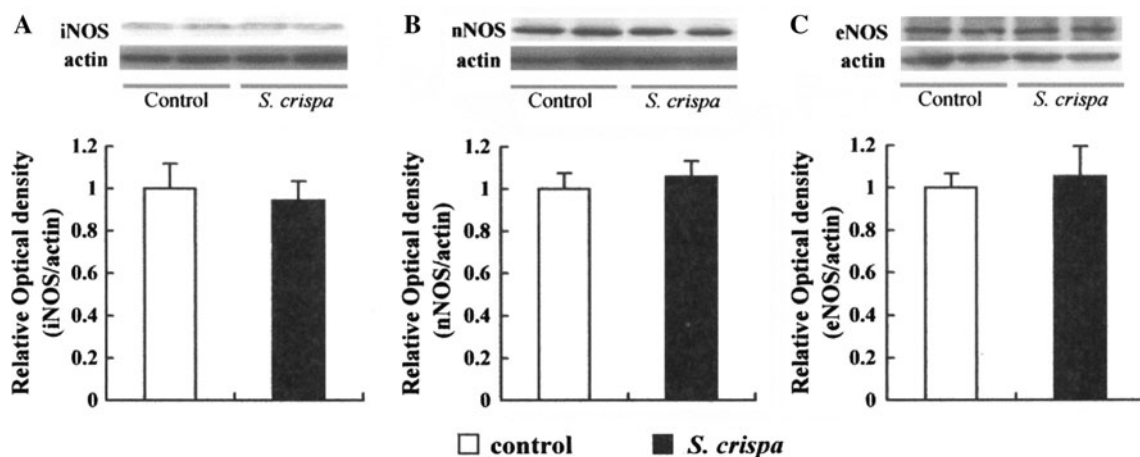


Fig. 4 Western blot analysis of the protein expression of iNOS (a), nNOS (b), and eNOS (c) in the cerebral cortex at 4 weeks after administration. β -actin served as protein quantity control in each

experiment. Control group (n = 4) and *S. crispa* group (n = 4). Data are means ± SEM (Student's *t* test)

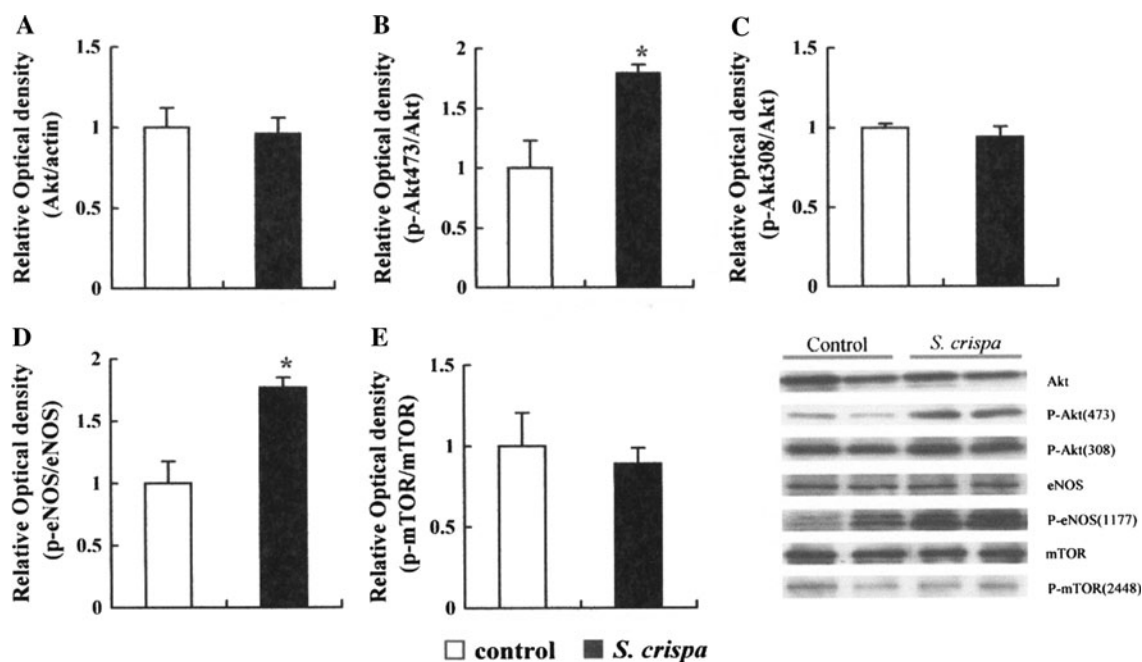


Fig. 5 Analysis of Akt/eNOS signaling by western blotting in cerebral cortex in SHRSP (control and *S. crista* treatment). **a** The total protein expression of Akt. Densitometric analysis of the phosphorylation level on Akt Ser473 (**b**), Akt Thr308 (**c**), eNOS

Ser1177 (**d**), and mTOR Ser2448 (**e**). Control group ($n = 4$) and *S. crista* group ($n = 4$). Data are means \pm SEM; * $p < 0.01$ versus control group (Student's *t* test)

administration of *S. crista* was associated with an inhibition of elevated blood pressure, restoration of impaired blood flow, and increased NO_x in the cerebral cortex.

In contrast to eNOS, the mammalian target of rapamycin (mTOR), which is also downstream of Akt, is an important molecule in the regulation of protein synthesis and cell growth, and mTOR activity also requires phosphorylation at Ser2448 by Akt. Many neurodegenerative diseases are characterized by neuronal death via apoptosis, and it is possible that modulation of mTOR activity may offer some protection against these effects [24]. In particular, diseases involving oxygen and nutrient deprivation, such as stroke, would be expected to be associated with inhibition of the mTOR pathway [25]. Moreover, previous studies have demonstrated that focal ischemic brain injury significantly decreases the levels of phosphorylated Akt and mTOR, and suppresses protein synthesis [26]. We expected activation of mTOR with the increase in phosphor-Akt due to the administration of *S. crista*; however, no such effect was observed. In brief, upon the administration of *S. crista*, there is only improvement of Akt/eNOS signaling without an effect on mTOR in SHRSP brain.

Many mushrooms have been reported to exhibit antihypertension effects. It has been reported that mushroom species such as *Lentinula edodes*, *Ganoderma lucidum*, *Grifola frondosa*, *Pleurotus cornucopiae*, and *Pleurotus nebrodensis* have antihypertension activities; moreover,

their mechanisms of action have been suggested to involve the decrease blood pressure by improvement in blood levels of triglyceride and cholesterol as well as kidney function and inhibition of angiotensin-converting enzymes [3, 4, 27, 28]. However, this is the first report to indicate an antihypertensive mechanism involving an improvement in endothelial signaling due to mushrooms.

In conclusion, long-term hypertension has been implicated in functional modifications in the cerebrovascular system and causes of stroke. Therefore, it is important to avoid high blood pressure as a preventative measure. *S. crista* can delay stroke through restoration of cerebrovascular endothelial dysfunction via enhancement of the Akt/eNOS/NO system in the cerebral cortex of SHRSP. This suggests that *S. crista* may have utility as a functional food for the prevention of hypertension and stroke; moreover, novel components from this mushroom may be discovered in the future.

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