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Rapeseed oil ingestion and exacerbation of hypertension-related conditions in stroke prone spontaneously hypertensive rats

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Abstract

Two groups of 20 stroke prone spontaneously hypertensive rats (SHRSP) at 5 weeks old were fed a diet containing 10 w/w% rapeseed (canola) oil or soybean oil as the only dietary fat, and given drinking water containing 1% NaCl. Life span of the canola oil group (62±2 days) was shorter than that of the soybean oil group (68±3 days). Stroke-related symptoms were observed in every animal, but the onset of those in the canola oil group, at 47±1 days after starting the administration was earlier than that in the soybean oil group, 52±2 days. Incidence of cerebral hemorrhage was similar in these groups, and no differences were found between lesions of organs in the groups. In another experiment, two groups of ten SHRSP at 5 weeks of age were fed the defatted diet and given canola oil or soybean oil by gavage at 10 w/w% of consumed food for 4 weeks without NaCl loading. After the 4-week administration, mean systolic blood pressure in the canola oil group and the soybean oil group were 233±2 and 223±0.3 mmHg, respectively. Phytosterol levels in both plasma and erythrocyte membranes reflected those contained in the oils ingested. Na^+, K^+-ATPase activities in the brain, heart and kidney were enhanced in the canola oil group. These results indicate that promotion of hypertension-related deterioration in organs is likely to have relevance to the short life span in the canola oil group. Enhanced Na^+, K^+-ATPase activity by phytosterols in the oil ingested may play a role in these changes.

Keywords: Rapeseed (canola) oil; Soybean oil; Stroke prone spontaneously hypertensive rat (SHRSP); Na^+, K^+-ATPase; Life span

1. Introduction

It has been reported that dietary intake of some vegetable oils, including rapeseed (canola) oil shortened the life span of stroke-prone sponta-

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neously hypertensive rats (SHRSP) when each oil was given as the major dietary fat (Shimokawa et al., 1988; Huang et al., 1996, 1997; Miyazaki et al., 1998; Ratnayake et al., 2000a,b). On the other hand, we found that blood pressure of SHRSP fed a diet containing 10% canola oil as the only dietary fat became higher than that of the animals given soybean oil (Naito et al., 2000c). Such a promotion of hypertension should facilitate the progress of vascular deterioration and might be one of causes for the shortened life span in SHRSP, since SHRSP is originally prone to cause vascular dysfunction including arteriosclerosis with aging (Tomita et al., 1977; Hayashi et al., 1978; Hazama and Sasahara, 1992). To ascertain whether or not the shortened life span is due to canola oil-specific lesions of organs, postmortem examination of the animals given canola oil should be rationally required in comparison with that of the animals given soybean oil, which is taken for one of the oils without life span shortening effect (Ratnayake et al., 2000a). Thus, in the present study, we aimed to confirm the shortening of life span of SHRSP by canola oil ingestion, and postmortem examination was done to find differences in the lesions of organs, if any, between the animals given canola oil and soybean oil.

Although causal substances for the shortening of life span are still unknown, Ratnayake et al. (2000b) speculated that cholesterol in biomembrane is replaced with phytosterols and the membrane becomes fragile when vegetable oils rich in phytosterols are ingested. Since such a change in the membrane may lead to early death of stroke in SHRSP through vascular injury, it is worthwhile to investigate the influence of rapeseed (canola) oil intake on biomembranes. From this point of view, Na^+, K^+-ATPase is a useful marker of changes in the membrane function, because this representative membrane-incorporated enzyme is affected by cholesterol content in the membranes (Claret et al., 1978) and cholesterol in the membranes can be replaced by phytosterols (Bruckdorfer et al., 1969; Vemuri and Philipson, 1989; Gray et al., 1997; Mora et al., 1999). Therefore, in the present study we also examined effects of canola oil ingestion on Na^+, K^+-ATPase activities of the heart, aorta, brain and kidney, and levels of phytosterols in plasma and erythrocytes of SHRSP.

2. Methods

2.1. Chemicals

Acetonitrile, bis(trimethylsilyl)trifluoroacetamide (BSTFA), brassicasterol, campesterol, chloroform, 5α-cholestane, ethylendiaminetetraacetate (EDTA), hexane, $MgCl_2$, methanol, KCl, pyridine, β-sitosterol, NaCl, stigmasterol and trimethylchlorosilane (TMCS) were purchased from Wako Pure Chemical (Osaka). Adenosine triphosphate tris salt (ATP), dithiothreitol, histidine, ouabain, sucrose and α-tocopherol were purchased from Sigma-Aldrich Japan (Tokyo). Lactate dehydrogenase, nicotinamide adenine dinucleotide, phosphoenolpyruvate, pyruvate kinase and sodium maleate were purchased from Roche Diagnostics (Mannheim). Heparin sodium, pentobarbital sodium and KOH were purchased from Novo Nordisk (Tokyo), Tokyo Kasei Kogyo (Tokyo) and Kanto Chemical (Tokyo), respectively. All other chemicals were of the purest grade commercially available.

2.2. Animals and breeding

For life span study 40 male SHRSP (Izm), 4 weeks old were purchased from Disease Model Cooperative Research Association (Kyoto) and acclimatized for 1 week. These rats were fed CRF-1 diet (Oriental Yeast, Tokyo) during the acclimation. Thereafter the animals were divided into two groups of 20 animals each, and fed purified powder diet of AIN-93 composition without fat (ingredients were as follows: 14.0000% casein; 0.1800% L-cystine; 50.5692% cornstarch; 15.5000% α-cornstarch; 10.0000% sucrose; 5.0000% cellurose powder; 3.5000% AIN-mineral mixture; 1.0000% AIN-93 vitamin mixture; 0.2500% choline hydrogen tartrate; 0.0008% *t*-butylhydroquinone, Oriental Yeast) mixed with 10 (w/w)% canola oil (Japan Oilseed Processors Association, Tokyo) or 10% (w/w) soybean oil (Japan Oilseed Processors Association) ad libitum.

Table 1
Fatty acid compositions (%) of soybean oil and canola oil

Fatty acid	Soybean oil	Canola oil
14:0 Myristic acid	0.1	0
16:0 Palmitic acid	11.0	4.0
16:1 Palmitooleic acid	0	0.2
18:0 Stearic acid	3.6	1.7
18:1 Oleic acid	23.4	58.8
18:2 Linoleic acid	54.0	21.4
18:3 Linolenic acid	7.0	11.3
20:0 Arachidic acid	0.3	0.5
20:1 Eicosaenoic acid	0.2	1.4
22:0 Behenic acid	0.4	0.3
22:1 Erucic acid	0	0.4

Ad libitum administration was suitable to avoid the stress of daily continual by-gavage administration. The soybean oil group was regarded as the control group, because the major component of fat in regular rat chow is soybean oil and soybean oil has been reported to have no life span shortening effect in SHRSP (Ratnayake et al., 2000a). Ingredients of the vegetable oils are shown in Table 1. α-Tocopherol was contained in soybean oil and canola oil at 10 mg/100 g and 22 mg/100 g, respectively (Japan Oilseed Processors Association). The content of α-tocopherol in each oil was adjusted to approximately 22 mg/100 g by adding α-tocopherol to soybean oil. The animals were allowed free access to the drinking water containing 1% NaCl. NaCl-loading was applied to accelerate the progress of hypertension (Sapirstein et al., 1950) for pronouncing the difference in effects of the treatments.

Also for the other study, 20 male SHRSP, 4 weeks old were purchased and acclimatized for 1 week. These rats were fed CRF-1 diet during the acclimation. Thereafter these animals were divided into two groups of ten animals each and fed purified powder diet of AIN-93 composition without fat. They were given canola oil or soybean oil by gavage at 10% of consumed food, once daily, for 4 weeks. For these animals NaCl loading was not carried out. These animals were examined for changes in blood pressure, phytosterol levels in plasma and erythrocyte membranes, and tissue Na^+, K^+-ATPase activity. In this experiment, by-gavage administration was preferable for controlling exactly the dosage of the oils, and NaCl-loading was best avoided when examining the effects of the oils on blood pressure and the enzyme.

All the animals were used following the instructions of the Committee for Ethical Usage of Experimental Animals in Hatano Research Institute.

2.3. Observation

General condition of all the animals was observed once a day. In the animals for the life span study, the following symptoms were regarded as signs of stroke, exophthalmus, hyperirritability, hyperkinesia, hyporesponsiveness motion, disturbance, tremor, convulsion, limb paralysis and sudden death (Okamoto et al., 1974; Nagaoka et al., 1976).

The animals were weighed at the day before starting the administration of canola oil or soybean oil, and thereafter, once a week. Water intake and food consumption for 24 h were measured once a week in the animals of the life span study.

2.4. Pathological examination

In the life span study postmortem examination was carried out as soon as possible when animals found dead. The organs, which showed macroscopically abnormal findings, that is, the brain, heart, lung and kidney were removed and immersed in 0.1 M phosphate buffer containing 10% formalin. The fixed tissues were embedded in paraffin and sectioned, stained with hematoxylin and eosin, and histologically examined under a microscope.

2.5. Measurement of blood pressure

In the animals of the 4-week administration study, systolic blood pressure was measured by tail cuff method using a sphygmomanometer (MK-1030, Muromachi, Tokyo) before and at the last week of the administration period. In brief, the rats were kept in the box warmed to 38 °C for 10 min and then placed in a holder. Systolic blood

pressure was measured as internal pressure of a latex cuff fixed around the tail of the animals. Systolic blood pressure of each animal at each measuring point was obtained as an average of three readings.

2.6. Measurement of sterols

Two hundred mg of each oil was dropped into 2.5 ml of 1 M KOH-methanol and 10 μg of 5α-cholestane was added as an internal standard. Sterols in the oil were converted to trimethylsilyl esters by adding 50 μl of BSTFA with 1% TMCS, 75 μl of dry pyridine and 75 μl of acetonitrile. Silylation was done at room temperature for 1 h. Cholesterol, brassicasterol, campesterol, stigmasterol and β-sitosterol were determined by a gas chromatograph (GC-17A, Shimadzu, Tokyo) with a flame ionization detector system. The column used was SUPERCO SAC-5 (30 mm × 0.25 mm i.d., 0.1 μm film thickness, Superco Japan, Tokyo). The oven was kept at 100 °C for 1.5 min and temperature was increased to 270 °C at a rate 30 °C/min, and then for 25 min at a rate of 6 °C/min. Temperature of the detector and injector was 310 °C. The flow-rate of the carrier gas (helium) was 60 ml/s at 80 kPa. Splitless injection with splitting ratio was 1:50. The injection volume was 1 μl.

After the 4-week administration period the animals were anesthetized with 50 mg/kg, iv, of pentobarbital sodium, and abdominal incision was performed. Blood was taken from the inferior vena cava using 1/10 volume of 3.8% citric acid solution as an anticoagulant, and centrifuged at 1500 × *g* for 15 min at 4 °C. Plasma was obtained and buffy coat was discarded. Then, erythrocytes in the sediment were washed 3 times by resuspending in the same volume of 0.9% NaCl solution followed by centrifugation at 2000 × *g* for 15 min at 4 °C. The erythrocyte suspension and barbital buffer were mixed at 1:20 (pH7.4), centrifuged at 20 000 × *g* for 40 min at 4 °C, and supernatant was removed. This procedure was repeated 3 times and erythrocyte membranes were obtained as the sediment. Four hundred μl of plasma and the erythrocyte membranes equivalent to those in 1 ml of blood were used for respective determination of sterols in the same manner as that mentioned above. An aliquot of erythrocyte membranes from each animal was lyophilized and weighed to obtain the enzyme activity per unit weight.

2.7. Na^+, K^+-ATPase (EC. 3. 6. 1. 3) activity assay

Following the blood sampling, the animals were killed by exanguination, and the brain, heart, aorta and kidneys were removed. The brain, heart, and kidneys from individual animals were separately put into 10 times the wet weight of each tissue of 0.25 M sucrose buffer containing 10^{-3} M EDTA and 10^{-5} M dithiothreitol, pH 7.0, and minced with a pair of scissors. The aortae were pooled for each group because the aortic tissue from each animal was not enough for individual assay. Then, the minced tissues were homogenized with an Ultra Turrax® (Janke & Kunkel, Staufen) at the maximal position for 20 s. The homogenate was centrifuged at 1000 × *g* for 20 min at 4 °C (Himac CR 5DL, Hitachi, Tokyo), and the supernatant was obtained. The pellet was resuspended in the buffer, homogenated and centrifuged at 1100 × *g* for 20 min at 4 °C, and the supernatant was mixed with the formerly obtained one. The mixed supernatant was centrifuged at 12 000 × *g* for 15 min at 4 °C, and the supernatant obtained was centrifuged again at 100 000 × *g* for 1 h at 4 °C (Himac CP75ß, Hitachi, Tokyo). The resulting pellet was resuspended in the buffer containing 10 mM sodium maleate, 1 mM EDTA (pH 7.0) and 10^{-5} M dithiothreitol and stored at −80 °C up to use.

The membranes were washed again by suspending followed by ultracentrifuge. The pellet was resuspended in the buffer and prepared to an adequate protein concentration. The protein concentration was determined by Lowry's method (Lowry et al., 1951) using a protein assay kit (Bio Rad Laboratories Japan, Tokyo).

Na^+, K^+-ATPase activity was measured by the coupled assay (Nørby, 1988). Briefly, 10 μl of 10 mg/ml pyruvate kinase, 10 μl of 1 mg/ml lactate dehydrogenase, 100 μl of 15 mg/ml phosphoenolpyruvate, 50 μl of 10.5 mg/ml nicotinamide adenine dinucleotide (reduced form), 300 μl of 30

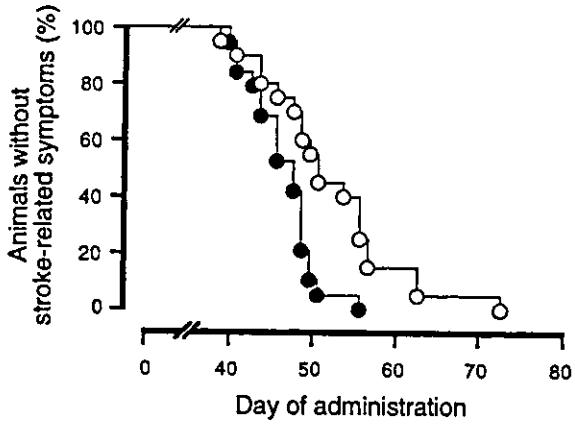

Fig. 1. Diminution curves of SHRSP without stroke-related symptoms fed diet containing 10% soybean oil or canola oil with 1% NaCl solution as drinking water. The onset of stroke was confirmed based on the appearance of stroke-related symptoms; exophthalmus, hyperirritability, hyperkinesia, hyporesponsiveness, motion disturbance, tremor, convulsion, limb paralysis and sudden death. Open and filled circles represent the values in the soybean oil group and the canola oil group, respectively. The curves are significantly different ($P < 0.01$, Log-rank test; Tarone–Ware test).

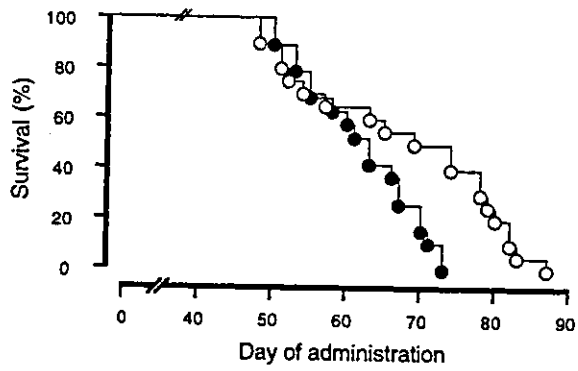

Fig. 2. Survival curves of SHRSP fed diet containing 10% soybean oil or canola oil with 1% NaCl solution as drinking water. Open and filled circles represent the values in the soybean oil group and the canola oil group, respectively. The curves are significantly different ($P < 0.05$, Log-rank test; Tarone–Ware test).

mM ATP, 500 μl of 180 mM histidine, 400 μl of 30 mM $MgCl_2$, 150 μl of 400 mM KCl, 150 μl of 2.6 M NaCl and 1000 μl of deionized water were mixed and 250 μl of 1 mM ouabain or deionized water was added (in total 3020 μl). Then 100 μl of the membrane suspension, 2–2.5 mg protein/ml, was added and change in absorbency at 340 nm, 37 °C was measured by a spectrophotometer (UVIDEC 610C, Nihon Bunko, Tokyo). Namely, the steady state values of dA_{340}/dt were measured in the absence and presence of ouabain and the difference between the values was obtained as ouabain-sensitive Na^+, K^+-ATPase activity. The measurement was carried out in triplicate.

2.8. Statistics

The differences between group mean values were evaluated by Student's t-test for unpaired observations. Log-rank test and Tarone–Ware test were applied for the analysis of the difference between the survival curves for two groups. The diminution curves of animals without symptoms for two groups also were compared in the same manner. Between group difference was considered to be significant when P value was less than 0.05.

3. Results

3.1. Stroke-related signs and life span

In the soybean oil group the first animal with stroke-related symptoms was found at the 39th day of administration period, and the onset of symptoms in the last animal was at the 73rd day. In the canola oil group the first animal with the symptoms was found at the 40th day, and the last animal, at the 56th day. The course of the increase in the number of animals with the symptoms, that is, progressive decrease in number of the normal animals was significantly rapid in the canola oil group ($P < 0.01$, Log-rank test and Tarone–Ware test) (Fig. 1). The mean durations from the start of administration to the onset of the symptoms were 52 ± 2 days ($N = 20$) and 47 ± 1 days ($N = 19$) ($P < 0.05$, t-test) for the soybean oil group and the canola oil group, respectively. One animal in the canola oil group was removed because of a decrease in body weight due to unsatisfactory water supply. Three animals in the soybean oil group died suddenly without any other abnormal signs. Mean survival time after starting adminis-

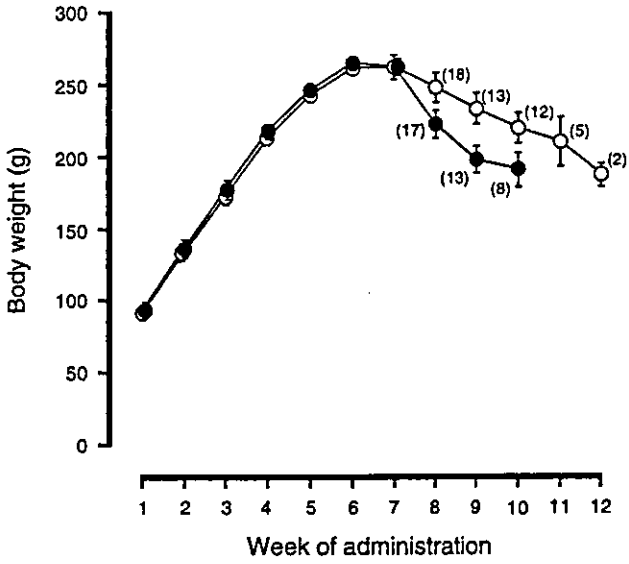

Fig. 3. Body weight changes in SHRSP fed diet containing 10% soybean oil or canola oil with 1% NaCl solution as drinking water. Open and filled circles represent the values in the soybean oil group and the canola oil group, respectively. Symbols and bars represent means and S.E. for 20 animals unless otherwise stated in parentheses.

tration, 62±2 days in the canola oil group appeared to be shorter than that, 68±3 days in the soybean oil group. The analyses of the survival curves showed significantly short life span for the canola oil group compared with that for the soybean oil group ($P < 0.05$, Log-rank test and Tarone–Ware test) (Fig. 2).

3.2. Body weight, food intake and water intake

Body weight of the animals increased steadily until the 6th week of administration (the animals were 10 weeks old at the 6th week), and then decreased gradually (Fig. 3). There was no difference in the body weight gain between the groups. Food consumption in the soybean oil group was between 13.5±0.4 and 17.7±0.4 g/day until the 6th week of administration, and that in the canola oil group was similar, 14.1±0.4 and 17.8±0.4 g/day. Food consumption started to decrease from the 7th week. Water intake increased from 25.1±1.4 to 48.9±2.3 g/day in the soybean oil until the 6th week, and from 25.8±2.0 to 53.3±1.8 g/day in the canola oil group. Water intake tended to decrease with large deviations from the 7th week.

3.3. Pathology of the brain, heart, aorta and kidney

Cerebral hemorrhage was found in 11 out of 20 animals in the soybean oil group and 12 out of 20 animals in the canola oil group. Enlargement of the heart was found in seven animals in the soybean oil group and six animals in the canola oil group. In these hearts pale colored areas were found suggesting necrosis due to ischemia. Seven animals in the soybean oil group and nine animals in the canola oil group had swelling kidney. Pulmonary edema was found in seven animals of each group. There were macroscopically no abnormal findings in other organs of the animals in both groups.

In histological examination, severe lesions were found in the organs, which showed abnormalities in the necropsy, that is, the brain, heart and kidney. However, the degrees of the lesions in these organs were not different when the soybean oil group and the canola oil group were compared (Fig. 4). The lesions observed were as follows. In the brain there were ventricular dilation, astrocytic gliosis and microcystic or cystic degeneration in the white matter, fibrosis and thickening of the intima and media of the arteriole in the subarch-

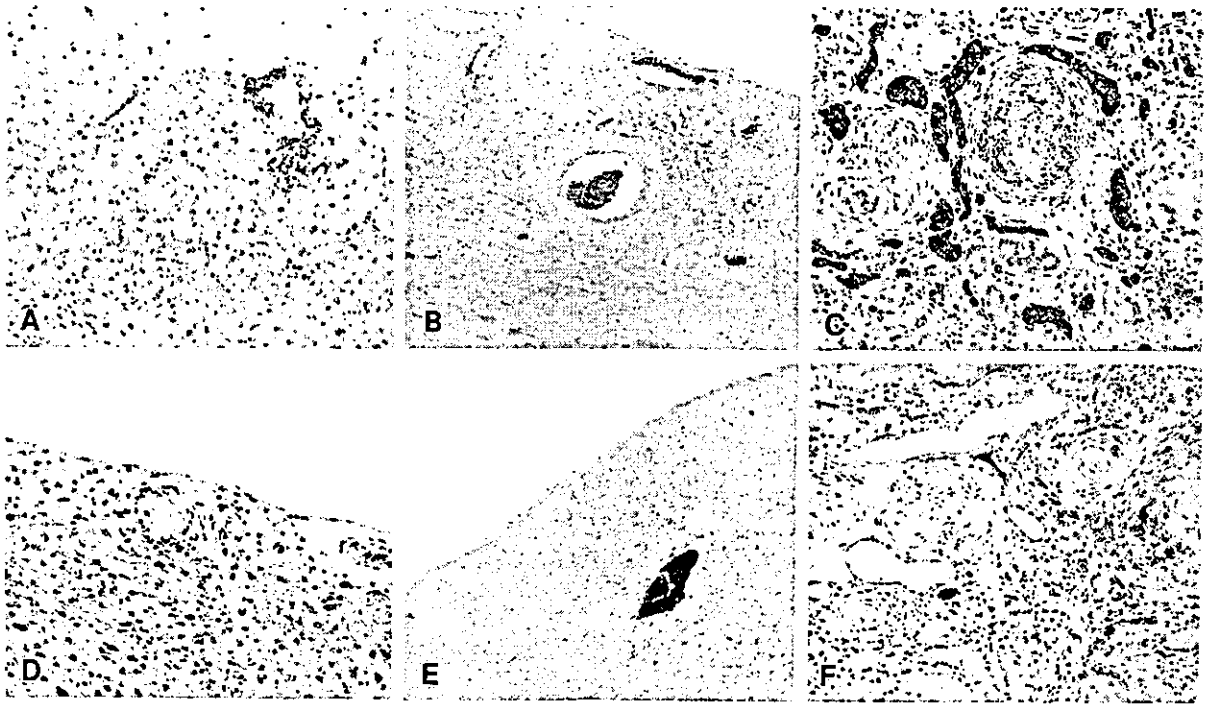

Fig. 4. Typical photomicrographs of the brains, hearts and kidneys of male SHRSP fed diet containing 10% soybean oil (A–C) or canola oil (D–F) with 1% NaCl solution as drinking water. Venous thrombus is observed in the cerebral cortex or in the subarachnoid space of the brain with moderate astrocytic gliosis (A and D, × 160). Fibrous thickening of the coronary artery with myocardial degeneration and/or fibrosis are observed in the left ventricle of the heart (B and E, × 60). Severe glomerular sclerosis, fibrous thickening of the ventricular wall manifested by whorl formation and dilatation of renal tubule are observed in the cortex of the kidney (C and F, × 60).

Table 2
Sterols in soybean oil and canola oil (mg/100 g oil)

	Cholesterol	Brassicasterol	Campesterol	Stigmasterol	β-Sitosterol	Total phytosterol
Soybean oil	1	4	61	53	168	286
Canola oil	2	73	250	2	380	705

noid space, neuropil infiltration and acidophilic atrophic petrosal nerve cells in the gray matter and small vacuole in the perineuronal space. In the heart, there were thickening of the media and intima, proliferation of the endothelial cells in the arterioles and small arteries and fibrin thrombus and fibrinoid degeneration, basophilic glanular deposition, fibrinous degeneration and neutrophil infiltration in the myocardium. In the kidney fibrin thrombus and thickening in the media and intima of the interlobar artery, fibrinoid deposition in the glomerular capillary lumen, and tubular regeneration. Beside the lesions in these organs, edema, hemorrhage and macrophage infiltration were found in the lung.

3.4. Blood pressure

Mean systolic blood pressures before starting administration were 184±4 mmHg in the soybean oil group and 180±4 mmHg in the canola oil group. At the 4th week during the administration

Table 3
Sterol levels in plasma of SHRSP orally administered soybean oil or canola oil at 10% of consumed diet for 4 weeks

Group		Cholesterol	Brassicasterol	Campesterol	Stigmasterol	β-Sitosterol	Total phytosterol
Soybean oil	Mean	1850	n.d.	18.2	1.17	21.7	41.1
	±S.E.	129		0.980	0.164	1.48	2.46
Canola oil	Mean	1790	n.d.	45.2***	n.d.	34.5***	79.6***
	±S.E.	46.1		1.63		1.02	2.61

Values are means±S.E. (nmol/ml) of ten animals. n.d. < 0.5 nmol/ml.
*** $P < 0.001$, significantly different from the values of the soybean oil group (unpaired t-test).

Table 4
Sterol levels in erythrocyte membrane of SHRSP orally administered soybean oil or canola oil at 10% of consumed diet for 4 weeks

Group		Cholesterol	Brassicasterol	Campesterol	Stigmasterol	β-Sitosterol	Total phytosterol
Soybean oil	Mean	414	n.d.	2.79	0.255	2.19	5.23
	±S.E.	38.7		0.184	0.0242	0.163	0.354
Canola oil	Mean	415	0.135	6.49***	n.d.	3.09***	9.72***
	±S.E.	9.77	0.0376	0.173		0.0899	0.264

Values are means±S.E. (nmol/mg erythrocyte membranes) of ten animals. n.d. < 0.1 nmol/mg erythrocyte membranes.
*** $P < 0.001$, significantly different from the values of the soybean oil group (unpaired t-test).

period mean systolic blood pressures in the canola oil group was 233±2 mmHg and that in the soybean oil group was 223±3 mmHg ($P < 0.05$, t-test).

3.5. Sterols in oil ingested, plasma and erythrocytes

Sterols identified in soybean oil and canola oil are represented in Table 2. Brassicasterol was found proper to canola oil and stigmasterol was proper to soybean oil. Levels of campesterol and β-sitosterol in canola oil were approximately 4 and 2 times higher, respectively, than those in soybean oil. The total amount of these sterols in canola oil was 2.5-fold of that in soybean oil.

Plasma levels of phytosterols suggested different kinetics in the absorption and distribution of the phytosterols. Brassicasterol and stigmasterol appeared to be only slightly absorbed or distributed in plasma. On the other hand, reflecting the amounts contained in the oils, campesterol and β-sitosterol were found in plasma from the animals of both groups. Plasma levels of campesterol and β-sitosterol in the canola oil group was 2.5 and 1.6 times higher, respectively, than those in the soybean oil group. In parallel with the increase in phytosterols the plasma level of cholesterol in the canola oil group tended to be lower than that in the soybean oil group (Table 3).

Phytosterol concentrations in erythrocyte membranes reflected those in the oils. In the canola oil group the level of campesterol was about 2.3 times higher than that in the soybean oil group and β-sitosterol was about 1.4 times higher (Table 4).

Table 5
Effects of orally administered soybean oil or canola oil at 10% of consumed diet for 4 weeks on Na^+, K^+-ATPase activities

	Soybean oil	Canola oil
Brain	63.9±6.9	92.4±7.7*
Heart	39.4±8.4	80.1±17.5*
Kidney	19.1±3.0	34.0±6.5*
Aorta	72.7	95.2

Values are means±S.E. (nmol/mg protein per min) of ten samples, except aorta. Values of aorta represent the activities obtained from pooling samples.
* $P < 0.05$, significantly different from the values of soybean oil group (unpaired t-test).

3.6. Na^+, K^+-ATPase (EC. 3. 6. 1. 3) activities in organs

Ouabain-sensitive Na^+, K^+-ATPase activities in the membranes from the brain, heart and kidney in the canola oil group were enhanced significantly compared with those in the soybean oil group ($P < 0.05$, t-test) (Table 5). The activity of the pooled membranes from the aortae in the canola oil group also tended to be enhanced compared with that in the soybean oil group.

4. Discussion

The statistical analyses of survival curves demonstrated significantly shortened life span of the canola oil group compared with that of the soybean oil group. This finding supports the results of previous studies from other laboratories (Huang et al., 1996, 1997; Miyazaki et al., 1998; Ratnayake et al., 2000a,b). During the observation period, all the animals showed stroke-related symptoms and the onset of the symptoms was found earlier in the canola oil group. However, the incidences of pathologically identified stroke by necropsy were comparable in these groups (11 and 12 out of 20 animals each in the soybean oil group and the canola oil group, respectively). In both groups macroscopic observation revealed abnormal findings also in the heart, kidney and lung in addition to the brain, and histology of each of the organs showed severe lesions. However, the severity of the lesions in any of the organs was similar between the groups. These results suggest that the shortening of life span is not due to canola oil-specific lesions in the organs but to the exacerbation by canola oil of the progressive deterioration in these organs that is associated with hereditary hypertension in SHRSP. As mentioned above, most of the animals were supposed to have a fit of stroke. However, severe lesions in other organs than the brain suggested that stroke was not necessarily sole, direct cause of death. Therefore, some mortal dysfunction in the heart, lung and kidney might also be a cause of the death in several animals.

In the second experiment, systolic blood pressure of the animals in the canola oil group became higher than that in the soybean oil group after the 4-week administration. This supports previous results from our group in SHRSP (Naito et al., 2000c), SHR and WKY rats (Naito et al., 2000a,b) where the elevation of blood pressure had nothing to do with the salt load, and suggests that some ingredients in canola oil increase blood pressure of the rat. About the effect on blood pressure, however, other investigators reported that rapeseed (canola) oil-induced shortening of life span of SHRSP was not accompanied with change in blood pressure (Huang et al., 1996, 1997; Miyazaki et al., 1998; Ratnayake et al., 2000a,b). The reason for this discrepancy is unknown, but at least the elevation of blood pressure which we found seems to have some relevance to the shortened life span in the canola oil group.

The levels of campesterol and β-sitosterol were found higher in both plasma and erythrocyte membranes in the canola oil group than those the soybean oil group. These levels reflected the relative content of the sterols in the oil ingested. It has been reported that the cholesterol content is inversely proportional to the membrane fluidity and the activity of Na^+, K^+-ATPase in human erythrocyte membranes (Lijnen and Petrov, 1995), umbilical venous endothelial cells (Mayol et al., 1999), bovine kidney (Yeagle et al., 1988) and rabbit cardiac muscle cells (Gray et al., 1997). On the other hand, cholesterol deficiency (Claret et al., 1978) or replacement by phytosterols of cholesterol molecules may increase ion permeability and activate Na^+, K^+-ATPase in biomembranes (Bruckdorfer et al., 1969; Vemuri and Philipson, 1989). Therefore, it is possible that the increased campesterol and β-sitosterol in membranes affect membrane-bound functional molecules such as Na^+, K^+-ATPase. Actually in the present study, Na^+, K^+-ATPase was found activated in the brain, heart, aorta and kidney in the canola oil group compared to those in the soybean oil group. Although we did not measure phytosterol levels in these organs, an increase in phytosterol levels in these organs was suggested by the campesterol and β-sitosterol levels in erythrocyte membranes.

Ratnayake et al. (2000a) pointed out that an increased fragility in vascular membranes, which may be due to the cholesterol deficiency by the replacement with phytosterols, can be a cause of early onset of cerebral breeding in SHRSP given canola oil. In the previous study, we found in SHRSP given canola oil for 4 weeks that erythrocytes became intolerant to low osmotic pressure (Naito et al., 2000d) and Na^+, K^+-ATPase activity in the aorta was enhanced compared to those in the animals given soybean oil (Naito et al., 2000c). Thus, the activation of Na^+, K^+-ATPase in the canola oil group may be due to increased intracellular Na^+ by increased Na^+ permeability through the deteriorated membrane. On the other hand, if the Na^+, K^+-ATPase activation is due to a direct effect by phytosterols, the enhanced activity of the enzyme facilitates Na^+ reabsorption in the renal tubules and causes the retention of Na^+ and water that may lead to an increased body fluid. The injured biomembranes or the facilitated Na^+ reabsorption by membrane-incorporated phytosterols may play a role in early onset of stroke in the canola oil group.

SHRSP possesses a strain-specific metabolic pathway of cholesterol and therefore tends to deposit phytosterols in the body (Ikeda et al., 2001). Cholesterol levels in plasma and erythrocytes are substantially lower than those in WKY rats (Yamori et al., 1980). The cholesterol depletion enhanced ion permeability in SHRSP erythrocytes (Yamori et al., 1984), and it is possible that Na^+, K^+-ATPase activity secondarily increases to maintain the ion content in the membrane. These strain specific characteristics might also lead to pronounced and fatal effects in SHRSP with some vegetable oils. The deterioration of organs by canola oil ingestion probably occurs also in rats of other strains, but it may not be as serious as that in SHRSP. Previously we found that SHR fed purified powder diet containing 10% canola oil as the only dietary fat for 26 weeks showed lesions in the heart and kidney that were somewhat severer than those found in the animals given soybean oil (Naito et al., 2000a). In the same study, we obtained similar results in WKY rats, though the lesions found in both groups of WKY rats were far less in incidence and degree than those in SHR, the strain from which SHRSP has been derived.

Thus far, phytosterols in canola oil appear to be candidates for the causal molecules for shortening life span in SHRSP. The adverse effect in rats of phytosterols also was supposed in corn oil (Ratnayake et al., 2000b). However, it has been reported that certain vegetable oils shortened the life span of SHRSP despite smaller amounts of phytosterols in the oils than those in canola oil or corn oil (Ratnayake et al., 2000b). Moreover, several precise studies from another laboratory (Huang et al., 1996, 1997; Miyazaki et al., 1998) have been showing that shortening of life span is not due to fatty acid composition, sulfur compounds or sterols, but may be due to unknown substances. Therefore, research for other substances than phytosterols might be still rationally required to elucidate the cause of the shortening of life span.

In summary, we confirmed in the present study that SHRSP given canola oil as the only dietary fat shows shortened life span as compared with animals given soybean oil. The promotion of hypertension-related deterioration of organs appears to be one of the underlying mechanisms. Enhanced Na^+, K^+-ATPase activity and facilitated elevation of blood pressure may have relevance to the exacerbation of the deterioration of organs. Although the causal substances have not been identified yet, phytosterols in the oil ingested appear to be candidates, which play a role in the shortening of life span.

Acknowledgements

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Cholesteryl Glucoside-induced Protection against Gastric Ulcer

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ABSTRACT. **The cytoprotective effect of heat shock proteins (HSPs) promises new therapeutic modalities for medical treatment. We examined the anti-ulcer effect of cholesteryl glucoside (1-*O*-cholesteryl-β-D-glucopyranoside, CG) on cold-restraint stress-induced gastric ulcer in rats, in terms of its correlative ability to activate heat shock factor (HSF) and to induce HSP70. Rapid induction of CG occurred in animal tissues, especially in stomach, after exposure to stress, indicating that this glycolipid might act as an anti-stress, lipid mediator involved in the very early stages of stress-induced signal transduction. Orally administered CG apparently showed anti-ulcer activity in rats *via* HSF activation and HSP70 induction. When compared with geranylgeranylacetone (GGA), the well known as an effective, synthetic anti-ulcer agent, CG proved to have the same level of strength on ulcer inhibition. GGA caused CG and HSP70 induction in gastric mucosa, indicating that GGA induced HSP70 *via* CG production. CG thus might be useful for medical treatment of stress-induced diseases, and as an anti-stress supplement for daily diet.**

Key words: steryl glucoside/stress response/heat shock protein/heat shock factor/anti-stress reagent

A wide range of toxic conditions induces heat shock proteins (HSPs) (Lindquist *et al.*, 1988). HSP70, a family of proteins known to enhance cell survival following exposure to a variety of stress conditions (Li *et al.*, 1991; Mosser *et al.*, 1992; Gabai *et al.*, 1995; Simon *et al.*, 1995), functions as a molecular chaperone in refolding denatured polypeptides (Gething *et al.*, 1992; Hartl *et al.*, 1996). Overexpression of HSP70 reduces stress-induced denaturation and aggregation of certain proteins (Kampinga *et al.*, 1995; Kabakov *et al.*, 1995); thus, the refolding and anti-aggregating activities of HSP70 determine its role in cytoprotection against some stresses (Georgopoulos *et al.*, 1993; Kampinga *et al.*, 1993). Overexpression of HSP70 prevents stress-induced apoptosis (Jaattela *et al.*, 1992), which in turn prevents the activation of the stress-inducible protein kinase, JNK (Gabai *et al.*, 1997), a mediator of apoptotic cell death (Verheij *et al.*, 1996; Xia *et al.*, 1995; Frisch *et al.*, 1996; Chen *et al.*, 1996). The production of HSP is transcriptionally regulated by the activation of heat shock transcription factor (HSF), that binds to the heat shock promoter element, HSE (Li *et al.*, 1991). HSF1, 2, 3, and 4 are now known to compose HSF family in higher eukaryotes (Robinson *et al.*, 1991; Shultz *et al.*, 1991; Nakai *et al.*, 1993; Nakai *et al.*, 1997), and HSF1 has been shown to be a mediator of stress-induced transcription of HSP genes (Morimoto *et al.*, 1992).

Previously, we found that some forms of stress, such as heat shock, starvation, and high salt, rapidly induced steryl glucoside (SG) in mold cells (Murakami-Murofushi *et al.*, 1997) and human fibroblasts (Murakami-Murofushi *et al.*, 1997; Kunimoto *et al.*, 2000). In a true slime mold, *Physarum polycephalum,* induction of poriferasteryl glucoside (PG) was followed by activation of a novel protein kinase (Maruya *et al.*, 1997) and HSP production (Murakami-Murofushi *et al.*, 1997; Shimada *et al.*, 1992). In human fibroblasts, heat shock rapidly induced cholesteryl

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Abbreviations: HSP, heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; SG, steryl glucoside; CG, cholesteryl glucoside; PG, poriferasteryl glucoside; UI, ulcer index; GGA, geranylgeranylacetone.

glucoside (1-*O*-cholesteryl-β-D-glucopyranoside, CG), a member of the SG family, and subsequent HSF activation and HSP induction occurred (Kunimoto *et al.*, 2002). As glycosylation of membrane sterol might be a very early and crucial step in the stress-induced signal transduction, we hypothesized that SG functions as a crucial lipid mediator in the process of stress responses. Recently, we have reported that CG itself induced HSF activation and following HSP70 production when CG was added to a culture of human fibroblasts (Kunimoto *et al.*, 2002).

In this report, we showed that CG strongly inhibits gastric ulcer formation in rats under cold-restraint stress, and that this effect is likely mediated by HSP induction. Also we compared the effect of CG on ulcer formation and HSP70 induction with that of a popular effective anti-ulcer agent, geranylgeranylacetone (GGA).

Materials and Methods

Animals and cold-restraint stress

Female Sprague-Dawley rats (180–200 g) obtained from SLC Inc. (Shizuoka, Japan) were housed in an animal breeding room at 22°C with 55% humidity, and water and food were withheld for 3 h before experiment. For cold-restraint stress, rats were immobilized in separate compartments of the stress cage (Takagi *et al.*, 1968) and kept at 4°C according to Brodie and Valitski (Brodie and Valitski, 1963) with minor modifications by Murakami *et al.* (Murakami *et al.*, 1981; Murakami *et al.*, 1983). Animals were sacrificed by dislocation of cervical vertebrae after 2 h under stress condition, and their stomachs (n=4 in each group) were excised immediately. We injected the excised organ with 10 ml saline and immersed it in 5% neutral formalin for 5 min to fix the outer layer. An incision was made in the stomach along the greater curvature, and any lesions were examined under a dissecting microscope and measured with a scale. The sum of individual ulcer lengths was referred to as the ulcer index (UI, mm). CG and GGA (structure, Fig. 1a) were chemically synthesized according to Alivisatos *et al.* (Alivisatos *et al.*, 1981) and Murakami *et al.* (Murakami *et al.*, 1981; Murakami *et al.*, 1983), respectively. They were emulsified with 5% gum arabic and 0.6% Tween 80, administered orally at a dosage of 100 mg/kg 30 min before the application of stress. Data were assessed for significant differences by Student's t-test. For the analyses of HSF activation and HSP induction, animals were sacrificed at different periods, and their stomachs were excised immediately. The gastric mucosa were then washed with chilled PBS, cut into small pieces with fine scissors and then dissolved in a sample buffer or a mixture of chloroform-methanol to analyze proteins or lipids (Murakami-Murofushi *et al.*, 1997; Kunimoto *et al.*, 2000), respectively. Immunoblotting analysis was performed as described elsewhere (Kunimoto *et al.*, 2002).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were carried out in accordance with manufacture protocol of LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) with slight modifications as described elsewhere (Kunimoto *et al.*, 2002). Twenty μl of each reaction mix (1×binding buffer, 25% (v/v) glycerol, 5 mM $MgCl_2$, 50 ng/μl poly (dI·dC), 0.05% NP-40, 100 fmol biotin-labeled HSE oligonucleotide duplexes (5'-CTAGAAGCTTCTAGAAGCT-TCTAG-3') (Genset, Kyoto, Japan), 150 mM KCl, buffer C extract containing 10 μg of protein) was incubated at 25°C for 1 h. The optical density of the biotin-bands was computer analyzed by NIH Image 1.61 Software (NIH).

Analysis of the expression of mRNA of HSP70

To measure the transcribed mRNA for inducible HSP70, semi-quantitative RT-PCR was performed according to Maloyan *et al.* (Maloyan *et al.*, 1999) with minor modifications. Ablated gastric mucosa was added with Stabilization Reagent (Qiagen, Hilden, Germany) and stored at 4°C overnight. Total RNA was extracted with Isogen (Nippongene, Tokyo Japan) in accordance with manufacture protocol and treated with DNase (DNA-free, Ambion, Austin, TX) in the presence of RNase inhibitor, SUPERase (Ambion), and then used for experiments. A quantity of 0.5 μg of total RNA was reverse transcribed in a 20 μl of reaction mixture containing 1 μM of oligo(dT_{20}) as a primer, 0.5 mM of each dNTP, 20 units of RNase inhibitor, 4 units of Omniscript RT according to Omniscript Reverse Transcriptase protocol (Qiagen). DNA oligonucleotide primers for inducible HSP70 were selected from the published HSP72 gene nucleotide sequence (Longo *et al.*, 1993). The sense primer was based on the sequence number 546-567, 5'-GCTGAC-CAAGATGAAGGAGATC-3', and the antisense number 1015-1034, 5'-GAGTCGATCTCCAGGCTGGC-3'. For internal control, actin amplification was performed with the 5'-GAGACCT-TCAACACCCCAGCC-3' (sense primer) and 5'-GGCCATCTCT-TGCTCGAAGTC-3' (antisense primer). A number of studies (e.g., McCully *et al.*, 1995) have already proven that hyperthermia does not affect the steady-state level of the mRNA of this housekeeping gene. PCR conditions were performed according to Maloyan *et al.* (Maloyan *et al.*, 1999). For the PCR, 2 μl (for HSP70 amplification) or 0.2 μl (for actin amplification) of the cDNA mixture were added to 20 μl of a master mix containing 200 μM of each dNTP, 0.4 μM of each HSP70 primer or 0.2 μM of each actin primer, 1 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The reaction was performed under the following conditions: an annealing temperature of 64°C, cycle number 43 for Hsp70 amplification, and anealing temperature of 62°C, cycle number 30 for actin amplification. PCR products were separated on 2% agarose gel and stained with ethidium bromide. Stained gels were photographed under ultraviolet illumination using Polaroid film. Optical density of the bands was computer analyzed by NIH Image 1.61 Software. The relative intensity of bands for the relevant mRNA was correlated with the relative intensity of the internal control, actin.

Lipid extraction and TLC analysis

After cold-restraint stress, rats were lightly anesthetized with ether,

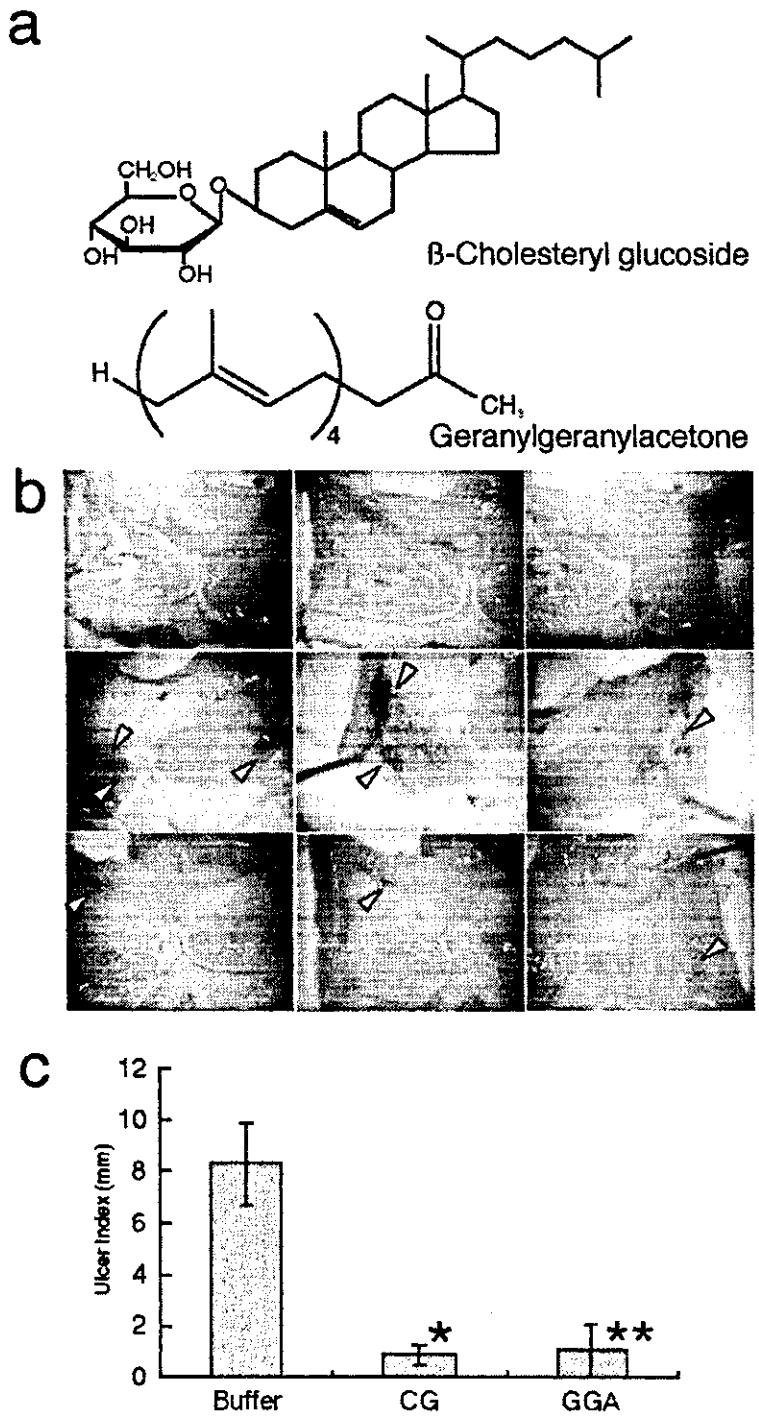

Fig. 1. Inhibition of cold-restraint stress-induced gastric ulcer by CG. (*a*) Structures of CG and GGA. (*b, c*) Test compounds were administered orally at a dose of 100 mg/kg, 30 min before the exposure to the stress. Each value represents the inhibitory rate (mean±S.E.) as compared to the corresponding control index ($^{*}P<0.05$ and $^{**}P<0.01$% vs. control). Four rats were used in each group. (*b*) Upper row, normal rat's gastric mucosa; middle row, gastric mucosa exposed to cold-restraint stress without CG; lower row, gastric mucosa exposed to cold-restraint stress after treatment with CG.

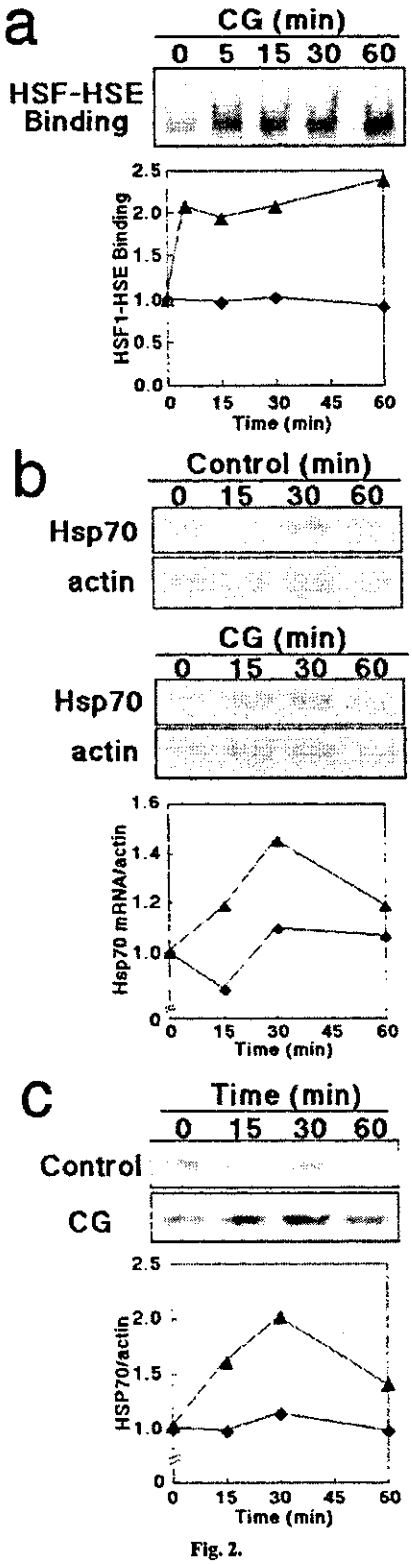

Fig. 2.

and the carotid artery severed to gather blood. Rats were then sacrificed by decapitation and each tissue sample was removed immediately, and added with Krebs-Henseleit buffer containing 118 mM NaCl, 47 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$-$7H_2O$, 25 mM $NaHCO_3$, and homogenized at 4°C. Only the liver was perfused with Krebs-Henseleit buffer to remove blood in tissue. Total lipid from these tissue homogenates was added to 2.5 vol. of methanol and 1.25 vol. of chloroform, mixed at room temperature for 2 min and left 10 min at room temperature. The homogenate was then added with 1.25 vol. of chloroform and PBS, and mixed. The lipid fraction of lower phase was pooled and evaporated, and then resuspended with a mixture of chloroform-methanol (1:1, v/v). Whole lipids were analyzed by TLC in solvent systems I and II: I, chloroform/methanol/water (70:30:4, v/v); II, hexane/ether/acetic acid (90:10:1, v/v). CG bands were visualized by spraying with 2% orcinol in 2N H_2SO_4 followed by heating, or by spraying with acidic ferric chloride solution (0.05% $FeCl_2$, 5% glacial acetic acid and 5% sulfuric acid), and the intensity of each visualized band was densitometrically determined by FLA-2000 (Fuji Film).

Results

Effects of CG and GGA on stress-induced gastric ulcer in rats

When animals were exposed to cold-restraint stress for 2 h, many superficial erosions of varying lengths occurred in the gastric fundic mucosa (Fig. 1b middle row). The mean ulcer index (UI) obtained in four animals exposed to such a stress was 8.3±1.6. Orally administered CG (structure, Fig. 1a) at a dosage of 100 mg/kg apparently prevented the occurrence of ulcer formation (Fig. 1b lower row). CG dosage level of 50–300 mg/kg was very effective and at these ranges CG showed no side effects on the animals. CG had a marked anti-ulcer effect, inhibiting ulcer formation by 90±5% ($p<0.05$, Fig. 1c). The effect of CG in inhibiting stress-induced gastric ulcer was compared to that of GGA (structure, Fig. 1a), which is a commercially available anti-ulcer drug (Murakami *et al.*, 1981; Murakami *et al.*, 1983). GGA at 100 mg/kg, which is reported as the optimal dosage (Murakami *et al.*, 1983; Alivisatos *et al.*, 1981), also inhibited ulcer formation by 87±13% ($p<0.01$, Fig. 1c). CG is thus a remarkable anti-ulcer agent and its effect is comparable with that of GGA.

Fig. 2. HSF activation and HSP induction by treatment of rats with CG. The substances at a dose of 100 mg/kg were administered to rats, and the activation of HSF1 and HSP70 induction was analyzed. (*a*) The binding of HSF-HSE was determined by EMSA, (*b*) induction of HSP70 mRNA was analysed by semi-quantitative RT-PCR, and (*c*) HSP70 protein expression was determined by SDS-PAGE followed by immunoblotting. The visualized bands were then densitometrically analyzed; values represent the relative intensity determined. Triangle and diamond represent with or without (control) CG, respectively. Data were determined in triplicate, and these curves are representative of typical pattern in several separate experiments.

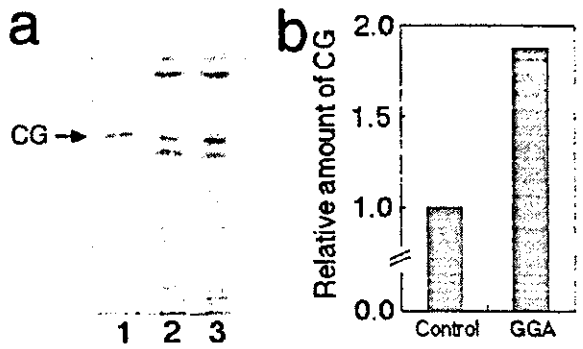

Fig. 3. CG induction by exposure of rats to GGA. Rats were exposed to GGA, and after the indicated treatment periods, they were sacrificed and gastric mucosa were excised; the lipids and proteins were then extracted and analyzed. (*a*) TLC analysis of whole lipids; lane 1, standard CG; lane 2, exposure of rats to buffer; lane 3, exposure of rats to GGA. (*b*) Visualized bands were densitometrically determined; values represent the relative intensity determined.

HSF activation and HSP induction by CG administration

To investigate whether the anti-ulcer effect of CG is mediated by HSP induction, CG was administered orally at a dose of 100 mg/kg, and at the indicated periods, rats were sacrificed. The gastric mucosa was then removed and analyzed for HSF activation by HSF1-HSE binding by electrophoretic mobility shift assay (Fig. 2a) and HSP production by expression of mRNA of HSP70 (Fig. 2b) and level of HSP70 protein (Fig. 2c). Five- to 15-min exposure of rat stomach to CG was sufficient to induce activation of HSF1, while 15- to 30-min exposure induced expression of mRNA of HSP70 and 15- to 30-min exposure produced HSP70 protein. These results indicate that CG's rapid induction of HSP70 might enhance HSP70's anti-ulcer activity.

Induction of CG in gastric mucosa by GGA treatment

The artificial compound GGA is an effective, commercially available anti-ulcer agent, and known to induce HSP70 in guinea pig gastric mucosa (Hirakawa *et al.*, 1996; Rokutan *et al.*, 2000). In order to determine any correlation between the artificial substance GGA and the naturally occurring substance CG, GGA was administered and the appearance of CG in the lipid fraction of the gastric mucosa was analyzed. As shown in Fig. 3, GGA apparently induced CG production wihin 15 min.

Induction of CG by cold restraint stress in rat tissues

We investigated the efftect of cold restraint stress on CG

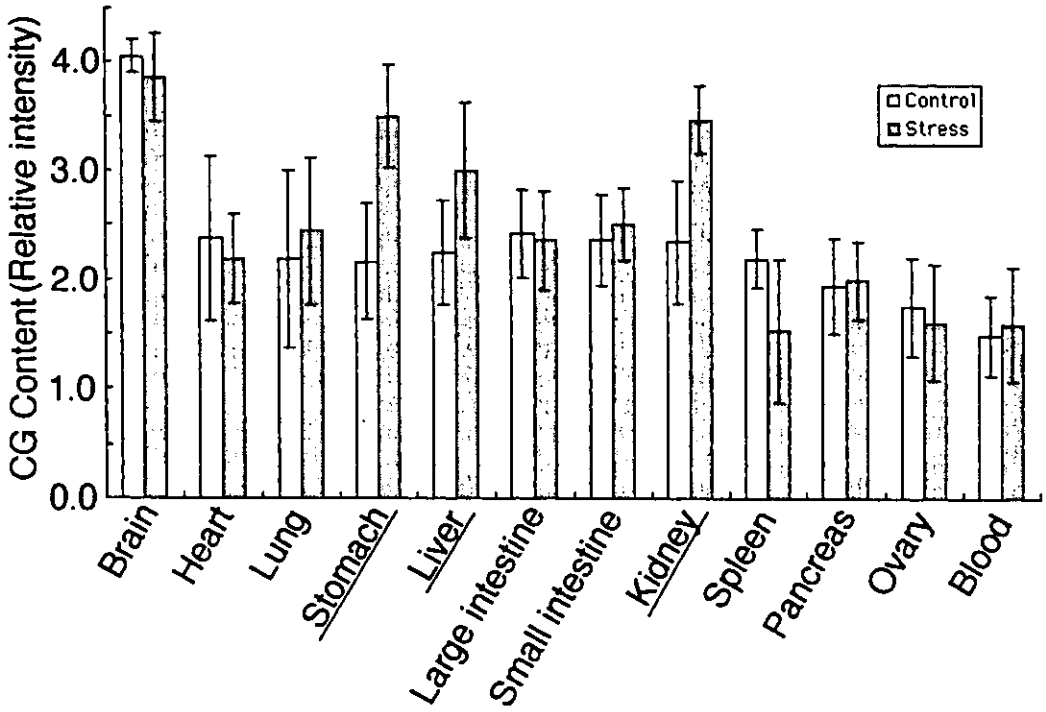

Fig. 4. Stress induced CG in rat tissues. Rats were immobilized in separate compartments of the stress cage and kept at 4°C. After 2 h under stress condition, their tissues were excised immediately, the lipids were extracted and analyzed by TLC. The visualized purple bands with both $FeCl_2$ and orcinol were then densitometrically analyzed; values represent the relative intensity to whole phospholipids determined.

level in rat tissues. As a result, CG was apparently induced in stomach, liver and kidney after the exposure of rats to cold restraint stress as shown in Fig. 4.

Induction of CG and following stress-responsive reactions by heat shock in excised stomach of rat

We exposed the excised stomach to heat shock at 42°C to examine the induction of CG, HSF1-HSE binding, mRNA of HSP70 and HSP protein in gastric mucosa (Fig. 5). Five- to 15-min after heat shock, the induction of CG and subsequently HSF1-HSE binding, expression of mRNA and protein of HSP70 were observed in rat stomach.

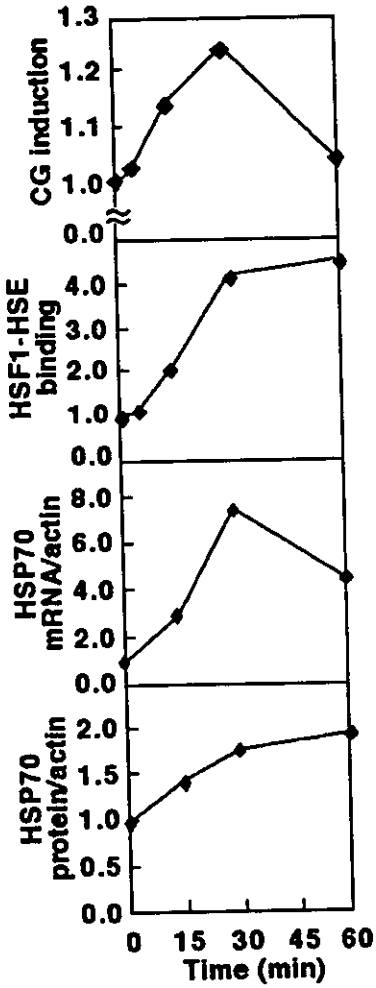

Fig. 5. Induction of CG and following stress-responsive reactions by heat shock in excised stomach of rats. Excised stomach was exposed to heat shock at 42°C to examine the induction of CG, HSF1-HSE binding, mRNA of HSP70, and HSP70 protein in gastric mucosa by TLC, EMSA, semi-quantitative RT-PCR, and immunoblotting, respectively. Visualized bands were densitometrically analyzed; values represent the relative intensity determined.

Discussion

We previously reported that rapid induction of SG occurs after the exposure of the cells of a wide range of organisms to heat shock (Murakami-Murofushi *et al.*, 1997; Kunimoto *et al.*, 2000), and suggested cholesteryl glucoside (CG) to be a mediator in the early stages of stress response to result in HSP production in human fibroblasts (Kunimoto *et al.*, 2002). In this report, we showed that orally administered CG caused stress response reactions in rat gastric mucosa that protected them against cold-restraint stress. As physiological stress leads to the depletion of gastric mucosa and secreted stomach acid inflicts injury on the gastric mucosal cells, such stress results in the rapid formation of gastric ulcer. Exogenously added CG helped the spontaneous, protective action in the mucosal cells under stressful conditions as shown in Results. Considering that CG was endogenously induced in rat tissues by cold restraint stress, it is suggested that when the stress is not so severe, the physiological and spontaneously occurring induction of CG might be sufficient to protect the gastric mucosa, but experimental cold-restraint stress might be too much for such auto-protective reactions to handle.

When the excised stomach was exposed to heat shock, the induction of CG, followed by HSF-HSE binding, HSP70 mRNA expression and HSP production were observed. We have already shown that the same phenomenon occurs in human fibroblasts under heat shock conditions (Kunimoto *et al.*, 2002). From these results, we propose the role of CG as a second messenger to induce HSP production in the very early stages of stress responsive signal transduction (Fig. 6).

In order to determine the regulatory mechanism of CG induction in response to environmental stress, we are attempting to purify the enzyme which catalyzes the synthesis of CG from UDP-glucose and cholesterol. We have already determined the existence of the enzyme in animal cells and tissues (Kunimoto *et al.*, 2002), and we have partially purified and characterized a *Physarum* enzyme (Murakami-Murofushi *et al.*, 1987; Murakami-Murofushi *et al.*, 1989).

Additionally, when the compound GGA, well known as an anti-ulcer agent, was administered, CG was rapidly induced in rat stomach. This suggests that the ability of GGA to induce HSP production may be mediated by CG induction.

As SG is a substance naturally occurring in a wide range of organisms (Murakami-Murofushi *et al.*, 1997; Kunimoto *et al.*, 2000) that protects the cells against environmental stress *via* HSP production, its correlate CG might be a safe and effective cytoprotective agent to use for medical treatment of gastric ulcer and other stress-induced diseases. It might also be useful in daily diet as an anti-stress supplement for those living in stressful, social environments.

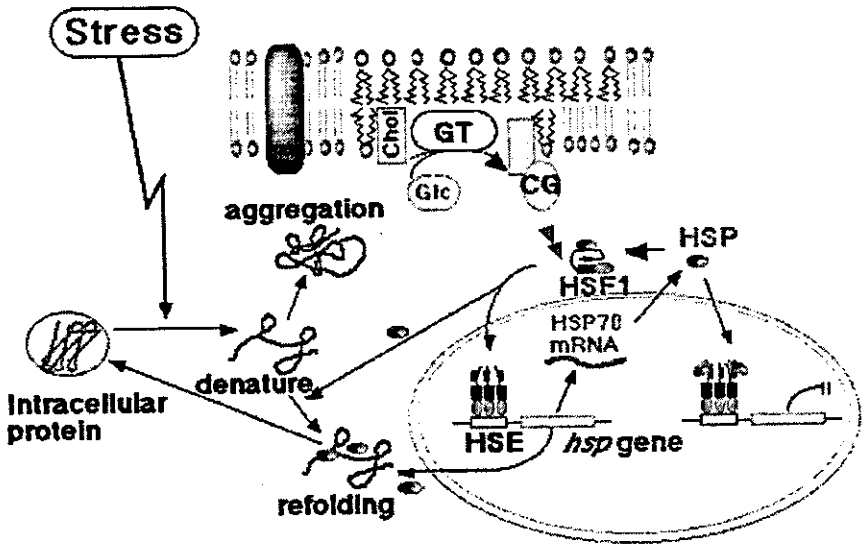

Fig. 6. Hypothetical representation of the role of CG as the second messenger to cause HSP production. Chol, cholesterol; Glc, glucose; CG, cholesteryl glucoside; GT, UDP-glucose:sterol glucosyltransferase.

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