Effects of Soyasaponins on Lipid Peroxidation through the Secretion of Thyroid Hormones

Yasuko Ishii* and Hisayuki Tanizawa

*School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan; and bDepartment of Human Life Sciences, Hiroshima Jogakuin University; 4–13–1 Ushita-higashi, Hiroshima 732–0063, Japan. Received March 6, 2006; accepted May 15, 2006

We investigated how soyasaponins (SS), which had been isolated from soybeans (Glycine max Merrill, seeds), influenced lipid peroxidation. The in vivo reduction in hepatic lipid peroxidation in mice intraperitoneally injected with total soyasaponins (TSS) was comparable to that which has been observed for vitamin E (VE). However, TSS and its five main constituent saponins (I, II, III, A1, and A2) had a much weaker in vitro inhibitory effect on lipid peroxidation induced by NADPH in mouse liver microsomes than VE. Therefore, we were not able to explain the in vivo effect of SS on lipid peroxidation level through direct antioxidative effects. We also demonstrated that TSS increased the levels of serum thyroid hormones. The effect of serum thyroid hormones on in vitro lipid peroxidation was much stronger than that observed for VE. Furthermore, the effects of TSS on levels of serum thyroid hormones and lipid peroxidation were markedly decreased by propylthiouracil, an antithyroid drug. These results indicate that the effects of SS on lipid peroxidation levels appear to be mediated through the secretion of thyroid hormones.

Key words soyasaponin; soybean; lipid peroxidation; thyroid hormone

In ancient China, soybeans were recommended as a crude drug for the prevention of aging. As soybeans have many important nutritious components such as proteins, fats, carbohydrates and vitamin E (VE), long-term continuous intake of this food might effectively prevent senility. Over the last few years, research has focused on soybean constituents such as isoflavones, phytosterols, saponins, water- and fat-soluble vitamins, and minerals. We have shown that total soyasaponins (TSS) and the five main constituent glucuronide-saponins (soyasaponin I, II, III, A1 and A2) inhibit the in vivo Adriamycin (an antitumor anthracycline antibiotic)-induced increase of lipoperoxide levels in the myocardium of mice. These constituents are also responsible for a reduction of NADPH-induced lipid peroxidation (in vitro) in mice hepatic microsomes.

As lipid peroxidation is reported to be closely related to aging, soyasaponins (SS) may play an important role in the aging-prevention effects of soybeans through an antioxidative effect. However, the mechanism by which SS restrained lipid peroxidation has yet to be fully elucidated.

On the other hand, enlargement of the thyroid in rats fed soybeans was discovered by McCarrison. Goiters were also induced in iodine-deficient rats maintained on a soybean diet. Suwa et al. reported that goiter was prevented relatively easily when iodine was added to the diet, and that the goitrogen appeared not to be a protein or peptide-like substance, since the proteolytic digestion of soybean curd did not eliminate the goitrogenic property. Recently, Ikekda et al. have reported that excess soybean treatment and iodine deficiency synergistically interact, resulting in remarkable induction of thyroid hyperplasia in rats. Additionally, a high isoflavone diet did not induce goiter under iodine deficiency conditions. There have been a considerable number of similar reports that have shown that soybeans act on the thyroid gland; however, the causative agent responsible for goiter remains to be unknown.

Additionally, since Suwa and Nakano has reported that thyroxine (T4), which has a phenolic structure, possesses a potent antioxidant activity on iron-induced phospholipid peroxidation, we hypothesized that the effect of SS on lipid peroxidation levels might be mediated through thyroid hormones. Therefore, the purpose of the present study was to elucidate the mechanism of action of SS on lipid peroxidation by determining the effects of SS on thyroid hormone levels.

MATERIALS AND METHODS

Materials TSS and five related glucuronide-saponins (SSI, SSII, SSIII, SSA1 and SSA2), which were extracted from soybeans and refined according to method of Kitagawa et al., were supplied by Taisho Pharmaceutical Co., Ltd. The used soybean powder included 0.3% TSS. The content of each saponin (SSI, SSII, SSIII, SSA1 and SSA2) in the TSS was 62.1, 11.2, 5.3, 11.8 and 9.5%, respectively.

In an in vitro antioxidant assay, Tg (3,3',5-triiodo-L-thyronine, sodium salt, 95%, Aldrich) and T4 (L-thyroxine, sodium salt, pentahydrate, Sigma) were dissolved in Tris HCl buffer solution containing a small amount of Tween 80, and saponins were emulsified instead of buffer solution. For the serum thyroid hormone (Tg and T4) assays, SPAC T3 RIA and SPAC T4 RIA kits were used (Daichichi Radioisotope Research Institute).

Experimental Animals Five-week-old male CDF1 mice with a mean weight of approximately 20—25 g were purchased from Japan SLC Inc., and kept under conventional conditions (12-h light/dark cycle, room temperature 25±1°C, humidity 50±5%) and fed a laboratory pellet chow (MF, Japan Oriental Yeast Co., Ltd.) and water ad libitum. In the in vivo experiment designed to examine the effects of SS lipid peroxidation in murine tissues, the mice were treated at 9:00 a.m. and sacrificed after a fixed time under ether anesthesia. All mice were handled in accordance with the Guiding Principles for the Care and Use of Experimental Animals at the University of Shizuoka.
Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-Dependent Lipid Peroxidation Test

NADPH-dependent lipid peroxidation in the microsomal fraction of mouse liver was assayed according to the methods of Svingen et al.\textsuperscript{(10)}

The TSS concentration was determined by the mean molecular weight of TSS, which was calculated from the molecular weight and constitution ratio of its five constituent saponins.

**Determination of Lipid Peroxide (LPO)** Determination of LPO in samples was carried out according to a modified Yagi method, which has been previously reported.\textsuperscript{(11)} The fluorophotometric method used for the LPO determinations in this experiment was based on the measurement of thiobarbituric acid-reactive substances (TBARS).

**Experimental Induction of Hypothyroidism** Hypothyroidism was induced experimentally according to the method of Katamine, who reported the technique in rats.\textsuperscript{(12)} Five-week-old male CDF\textsubscript{1} mice were given feed containing 10 mg of 6-n-propylthiouracil (6-PTU, Sigma) per 100 g for 10 d to cause hypothyroidism via inactivation of thyroid peroxidase (TPO).\textsuperscript{(13)}

**Statistical Analysis** Results are presented as mean ± standard error (S.E.M.). Statistical significance was compared between each treatment group and the corresponding control group by a Student’s t-test.

**RESULTS**

**Effect of SS on LPO Concentration in Tissues of Normal Mice** Hepatic LPO levels 3, 6, and 9 h after oral administration of TSS (250 mg/kg) were significantly ($p<0.001$) decreased compared to that of controls. LPO levels decreased to a minimum (ratio of reduction: 36%) at 6 h and recovered to the control level at 24 h (data not shown). No significant difference was apparent in the heart.

The relationship between the TSS dosage and hepatic LPO levels at 6 h after oral administration of TSS was examined. A significant reduction (14.9%, $p<0.01$) of LPO concentration was recognized at a dose of 100 mg/kg. The ratio of reduction rose to 300 mg/kg, with a RD$\textsubscript{50}$ (50% reducing dose) of 303.7 mg/kg (data not shown).

After intraperitoneal administration of TSS, a dose-dependent relationship was also observed between the TSS dosage (25, 50, 75, 100 mg/kg) and the LPO levels in the liver at 8 h (and in the heart at 3 h). The RD$\textsubscript{50}$ was 56.0 mg/kg (97.4 mg/kg) (data not shown). A significant reduction ($p<0.05$) for hepatic LPO concentration was evident 1 h after intraperitoneal injection of TSS (75 mg/kg). The reduction reached a maximum (67.1%) at 8 h, and recovered to the control level at 48 h (Fig. 1). In the heart, while LPO levels decreased significantly ($p<0.001$) at 3 h, the maximum ratio of reduction (40%) was not comparable to that which was observed in the liver, and after 12 h, there was no significant difference from controls (data not shown). In addition, at a dosage of 25 mg/kg, a significant reduction (29.3%, $p<0.05$) of hepatic LPO concentration was observed 8 h after TSS intraperitoneal injection.

Furthermore, LPO concentration in the liver of normal mice 8 h after intraperitoneal injection of the five main constituent saponins of TSS (75 mg/kg) was estimated in comparison to VE (75 mg/kg), which has a strong anti-oxidation action. In Fig. 2, results are shown as the reducing ratio (%) compared to the control group. The reducing ratio of TSS (67.1%) was comparable to that seen for VE (71.1%), and all of the SS that were tested caused a significant reduction in LPO concentration (reducing ratios: SS I, 52.9%; II, 41.6%; III, 55.2%; A\textsubscript{1}, 15.0%, and A\textsubscript{2} 15.8%). However, all ratios were lower than that which was observed for TSS. Moreover, SS I, II, and III, which are degraded to aglycon soyasapogenol B, reduced more strongly than SS A\textsubscript{1} and A\textsubscript{2}, which are degraded to the aglycon soyasapogenol A.

**Effect of SS on NADPH-Dependent Lipid Peroxidation in Mouse Liver Microsomes (in Vitro)** The influence of TSS on NADPH-dependent lipid peroxidation in mouse liver microsomes was compared with that of VE (75 mg/kg). Each sample inhibited the NADPH-induced increase of LPO in the mouse liver microsomes (Fig. 3). This inhibitory effect of TSS was observed at a concentration of 10 μM, but it was much weaker than that of VE, which demonstrated concentration-dependent inhibitory effects and an inhibition ratio of 100% at 50 μM. Furthermore, the effects of the five SS on NADPH-dependent LPO levels were examined. The IC\textsubscript{50} (50% inhibitory concentration) of each saponin (SSI, SSII, SSIII, SSA\textsubscript{1}, and SSA\textsubscript{2}) were 82.3, 60.2, 118.0, 71.1, 236.3 μM, respectively; hence, as was the case for TSS, these inhibitory effects were much weaker than that which was seen for VE (Fig. 3).
Time Course of Thyroid Hormone Concentration after TSS Intraperitoneal Injection in Normal Mice

The concentration of serum thyroid hormones (T3 and T4) was measured over time after intraperitoneal injection of TSS (75 mg/kg) in CDF1 mice. In the control group, the serum concentration of T3 and T4 decreased from 9:00 a.m. to noon, and then increased gradually, with full recovery by 9:00 a.m. the next day. The time course of the ratio of serum thyroid hormone concentrations in the TSS injected group to that in the corresponding control group (with the latter taken to be 100%) is shown in Fig. 4.

After TSS injection, significant increases were found for T3 serum concentrations after 3 h and for T4 after 1 h, with maximal concentrations of both T3 and T4 (about 127% and 149% of the corresponding control levels, respectively) observed at 3 h. No differences were observed at 24 h.

Effect of SS on Thyroid Hormones

Eight hours after intraperitoneal injection (75 mg/kg) of SS or VE, the concentrations of serum thyroid hormones (T3 and T4) were determined (Fig. 5). SS A1, SS A2, and VE did not differ from controls in terms of either T3 and T4. However, SSI, SSII, and TSS all induced a significant increase in T3 and T4, with the strongest induction noted for TSS.

Effect of TSS on Hypothyroid Mice

Mice given feed containing 6-PTU exhibited a significant decrease in serum concentration of T3 (p<0.01) and T4 (p<0.001) when compared to normal mice, indicating that the treated mice became hypothyroid. On the other hand, LPO levels increased significantly in the tissues of hypothyroid mice (liver: p<0.001; heart: p<0.05). While the weight of these mice increased significantly (p<0.01), there was no difference in the relative organ weight observed (data not shown).

Mice with established hypothyroidism were treated with TSS (75 mg/kg, i.p.), and serum thyroid hormone and hepatic LPO concentrations were examined. Three hours after injection, no increase was noted in the serum thyroid hormone concentration in hypothyroid mice. In contrast, there was a significant increase observed in normal mice (p<0.01) when compared to corresponding controls (Figs. 6A, B). Eight hours after injection of TSS, hepatic LPO did not decrease in hypothyroid mice, although it fell significantly in normal mice (p<0.001) as compared to corresponding controls (Fig. 6C).

Comparison of TSS and Thyroid Hormones on NADPH-Dependent Lipid Peroxidation in Mouse Liver Microsomes (in Vitro)

The influence of TSS on NADPH-dependent lipid peroxidation in mouse liver microsomes was compared with that of T3 and T4 using VE (75 mg/kg) as a reference. All samples inhibited the NADPH-induced increase of LPO in the mouse liver microsomes (Fig. 7). The inhibitory effects of T3 and T4 were much stronger than that of VE. TSS, VE, T3 and T4 had IC50s of 87.8, 21.9, 10.9 and 5.2 μM, respectively.
DISCUSSION

The current study indicated that SS reduced LPO levels in normal murine tissues. Therefore, we further investigated the effects of TSS on lipid peroxidation in detail. When TSS was administered to normal mice both orally and intraperitoneally, a significantly decreased lipid peroxidation was observed in the mouse liver. It was important that the effects associated with an oral administration be confirmed, as soybeans are normally eaten (i.e., oral route) on a daily basis. However, we also injected SS intraperitoneally in order to more precisely evaluate the effects of TSS. The TSS-caused lipid peroxidation decrease in mice hearts was only significantly observed after an intraperitoneal injection. The reason why a difference is noted between these tissues is perhaps because the hyperoxidation reaction for which the lipids are the substrates, occurs to a greater degree in the liver versus the heart. Additionally, the main target organ of the thyroid hormones is the liver.

Massiot et al.\textsuperscript{14} have reported that soybeans contain soyasaponin IV (SS IV), which is soyasaponin conjugated with 2,3-dihydro-2,5-dihydroxy-6-methyl-4-H-pyrene-(DDMP), and that solutions of SS IV are slowly decomposed into SSI. Later studies have reported that various kinds of DDMP-conjugated SS are included in soybeans. However, these are unstable under heat in soaked conditions, resulting in conversion to their non-DDMP form.\textsuperscript{15} Since soybeans are generally consumed after heat treatment (cooking), it is most likely that SS ingestion will be mainly of the non-DDMP form. It appeared that the TSS-mediated \textit{in vivo} reduction of hepatic lipid peroxidation was caused by synergism of the constituent saponins, as the effect of each of the constituent saponins of TSS was weaker than that which was observed for TSS, and because additionally, the TSS that was used in this study did not included above-mentioned SS.

This \textit{in vivo} effect of TSS on lipid peroxidation was comparable to that of VE; however, the \textit{in vitro} inhibitory effect of TSS on lipid peroxidation that was induced by NADPH in mouse liver microsomes was weaker than that observed for VE. Therefore, the \textit{in vivo} effects of TSS cannot be explained as being due to a direct antioxidative effect.

We also investigated the relationship between TSS and thyroid hormone, which stimulates the basal metabolism of the organism. Thyroid hormones restrained NADPH-dependent lipid peroxidation in the mouse liver microsomes more strongly than VE, with TSS obviously elevating the levels of the serum thyroid hormones. Additionally, 6-PTU, an antithyroid drug, markedly decreased these effects of TSS. Therefore, this suggests that the effects of SS on lipid peroxidation levels might be mediated through the secretion of thyroid hormones. The actual mechanism responsible for the reduction of the tissue LPO via the thyroid hormones could be due to either a direct and/or indirect antioxidant action.

SS are classified into two major groups. Group A saponins are thought to be responsible for the undesirable bitter and astringent taste in the soy food product.\textsuperscript{16} The Group B saponins are thought to be responsible for the health-contributing activities of soyasaponins.\textsuperscript{17} Our study indicated that the \textit{in vivo} reducing effect in the hepatic lipid peroxidation of group B saponins (SS I, II, and III) was much stronger than that which was observed for group A saponins.
(SS A₁ and A₂), although both groups did not have a strong in vitro inhibitory effect together. Furthermore, only group B saponins induced a significant increase in thyroid hormone. These differences in actions for these groups provide further evidence that the thyroid hormone is responsible for mediating the effects of SS.

In this study, we were not able to elucidate whether TSS elevated the levels of serum thyroid hormones through direct action on the thyroid gland or via secretion of thyroid stimulating hormone. Further studies are planned that will hopefully be able to clarify both the mechanism and the pharmacokinetics of SS.

Acknowledgments We would like to thank Ms. Yoshie Osada for her excellent technical assistance in this study.

REFERENCES