Inhibitory Effects of Soy Isoflavones on Cardiovascular Collagen Accumulation in Rats

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(Received August 23, 2006)

Summary Oxidative stress is a major cause of cardiovascular tissue fibrosis. We evaluated the effects of daily doses of soy isoflavones, genistein and daidzein on cardiovascular tissue fibrosis in Otsuka Long-Evans Tokushima Fatty (OLETF) diabetic rats and Long-Evans Tokushima Otsuka (LETO) non-diabetic rats as a severe or mild oxidative stress model, respectively. Glucose and lipid metabolisms did not improve with genistein or daidzein treatment. However, genistein decreased hydroxyproline concentrations in the heart. Hydroxyproline reductions as a result of genistein were mildly stronger than those of daidzein. Thus, genistein significantly suppressed the progression of myocardial fibrosis in LETO rats despite the insignificant changes in OLETF rats. Although a daily dosage of isoflavone was not sufficient to prevent tissue fibrosis under marked oxidative stress in the early stage of diabetes, isoflavones might promise significant clinical benefits by reducing oxidative stress in the heart during aging.

Key Words soy isoflavones, oxidative stress, tissue fibrosis, aging, diabetes mellitus

Isoflavone aglycones have a scavenging activity and antioxidant properties, and can inhibit lipoprotein oxidation and reduce the risk of cardiovascular disease (1–5). Vedavanam et al. demonstrated the dose-dependent inhibition of oxidative stress by genistein and daidzein (6). Hyperinsulinemia or insulin resistance during the prediabetic stage of type 2 diabetes mellitus (DM) is thought to be closely associated with oxidative stress and the accumulation of interstitial connective tissue and glycoproteins in the myocardial or aortic wall (7, 8). Collagen accumulation in the myocardial interstitium was seen to impair left ventricular diastolic properties, while accumulation in the medial layer of the aorta impaired wall elasticity (9, 10). Many factors are reported to be involved in tissue fibrosis or atherosclerosis, including vasoactive substances such as angiotensin II, mechanical stretches, growth factors, and cytokines (11–14). The purpose of the present study was to assess the in vivo antioxidant effects of daily doses of genistein and daidzein, and evaluate their inhibitory effects on collagen accumulation in the cardiovascular system of type 2 diabetic model (Otsuka Long-Evans Tokushima Fatty: OLETF) rats and non-diabetic (Long-Evans Tokushima Otsuka: LETO) rats.

MATERIALS AND METHODS

Animals. Thirty male OLETF and 30 male LETO rats (Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan), which originated from the same colony as the OLETF rats by selective mating but did not develop DM, were used as the experimental subjects. Starting age was 5 wk. All animals were maintained in the Animal Experiment Center of the Faculty of Agriculture Ehime University, in a pathogen-free facility under controlled temperature (23˚C) and humidity (5%) conditions with a 12-h artificial light and dark cycle. Animals were given free access to rat chow (MF, Oriental Yeast Co., Tokyo, Ltd., Japan) and tap water. All procedures were conducted in accordance with the institutional guidelines for animal research of Ehime University.

Experimental protocol. At 5 wk old, 10 OLETF and 10 LETO rats were randomly assigned to one of 3 experimental groups: 1) those given a AIN-93 control diet, 2) those given a diet supplemented with daidzein (0.2% w/w AIN-93 control diet) (Kikkoman, Tokyo, Japan), and 3) those given a diet supplemented with genistein (0.2% w/w AIN-93 control diet) (Kikkoman). The compositions of each diet appear in Table 1.

Baseline and weekly body weights were measured, and heart rate and blood pressure (tail-cuff method: sphygmomanometer PS-100 Rick, Riken Kikaihatsu, Tokyo, Japan) were measured at the baseline and at 15 wk old. After obtaining these measurements, the OLETF and LETO rats were anesthetized with sodium

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pentobarbital. Blood samples were obtained from the thoracic aorta after which the heart and thoracic aorta were excised and weighed. The thoracic aorta was consequently separated into 2 samples and the heart and thoracic aorta sample were frozen in liquid nitrogen, and stored at −80°C until required for hydroxyproline measurements. The other aorta sample was used for histopathological examination. Plasma concentrations of glucose (Hexokinase method), insulin (IRMA method), glycoalbumin (Enzyme method), total cholesterol (COD-POD method), and triglyceride (GK-GPO method) were measured using the methods in parenthesis.

**Histopathological examinations.** The excised aorta were immediately fixed in 10% phosphate-buffered formalin solution and embedded in paraffin. Four-micrometer-thick sections were cut from each sample stained with hematoxylin-eosin and viewed under a light microscope. Images were recorded directly through a 3-CCD camera (Olympus IX70 and CS 220, Tokyo, Japan). The intima-medial thickness on the hematoxylin-eosin stained aorta images at ×200 magnification were measured using NIH image analyzing software.

**Measurement of hydroxyproline.** Half the heart specimens were dried and hydrolyzed in 6 n HCl at 110°C for 24 h after which they were dried and reconstituted in 3 mL H2O. The hydrolysates were then mixed with ethanol and chloramines T solution and oxidized for 20 min at room temperature. The resultant oxidized product was reacted with p-dimethylaminobenzaldehyde in ethanol and H2SO4 solution at 60°C for 15 min. Chrophrophores in the heart specimen were quantitated spectrophotometrically at 572 nm against a hydroxyproline standard curve, and the ratio of total hydroxyproline content against weight was calculated.

**Statistical analysis.** Data are expressed as mean±standard deviation (SD). The comparisons between control and the other group were made using Scheffe’s.

### Table 1. The compositions of each diet.

| Ingredient                  | Diet  | C     | D1   | G2   |
|-----------------------------|-------|-------|------|------|
| Casein1                     |       | 200   | 200  | 200  |
| Soybean oil                 |       | 70    | 70   | 70   |
| AlN93 mineral mixture       |       | 35    | 35   | 35   |
| AlN93 vitamin mixture       |       | 10    | 10   | 10   |
| Carbohydrate                |       | 100   | 100  | 100  |
| *α*-Cornstarch              |       | 532   | 531.85 | 531.85 |
| Cellulose                   |       | 50    | 50   | 50   |
| L-Cystine                   |       | 3     | 3    | 3    |
| Daidzein10                  |       | —     | 0.15 | —    |
| Genistein10                 |       | —     | 0.15 | —    |

1D, which contains daidzein of 200 mg/kg diet.
2G, which contains genistein of 200 mg/kg diet.
3Edible lactic casein, 80 mesh, purchased from New Zealand Daily Board, Wellington, New Zealand.
4Provided by Ajinomoto-seiyu, Tokyo, Japan.
5According to AIN93.
6Granulated sugar, purchased from Nippon Beet Sugar Manufacturing, Tokyo, Japan.
7Gelatinized starch, provided by Nihon Shokuhinn Kako, Shizuoka, Japan.
8Provided by DANISCO CULTOR, Tokyo, Japan.
9Purchased from Nacalai Tesque, Kyoto, Japan.
10Provided by Kikkoman, Chiba, Japan.

### Table 2. Effects of daidzein and genistein treatments on the hemodynamics, body weight and heart weight of diabetic and nondiabetic rats.1

| Group  | Mean BP (mmHg) | Heart rate (beats/min) | Body weight (g) | Heart weight (g) | Heart/body weight (mg/g) |
|--------|----------------|------------------------|------------------|------------------|--------------------------|
| 5 wk old OLETF |                  |                        |                  |                  |                          |
| Control | 96±8            | 450±26                 | 327±17           |                  |                          |
| Daidzein| 92±9            | 428±18                 | 326±18           |                  |                          |
| Genistein| 98±13           | 444±19                 | 325±21           |                  |                          |
| LETO   |                  |                        |                  |                  |                          |
| Control | 104±9           | 456±40                 | 273±14           |                  |                          |
| Daidzein| 99±7            | 445±32                 | 270±14           |                  |                          |
| Genistein| 97±7            | 445±23                 | 276±11           |                  |                          |
| 15 wk old OLETF |                  |                        |                  |                  |                          |
| Control | 91±5            | 378±16                 | 550±40           | 1.31±0.13        | 2.39±0.20                |
| Daidzein| 88±7            | 373±18                 | 536±29           | 1.36±0.15        | 2.54±0.24                |
| Genistein| 88±7            | 384±15                 | 546±38           | 1.37±0.16        | 2.52±0.25                |
| LETO   |                  |                        |                  |                  |                          |
| Control | 73±4            | 397±17                 | 455±18           | 1.25±0.09        | 2.76±0.23                |
| Daidzein| 79±8            | 398±21                 | 455±32           | 1.25±0.10        | 2.74±0.16                |
| Genistein| 73±10           | 395±28                 | 460±10           | 1.28±0.08        | 2.78±0.14                |

1 Each value represents the means±standard deviation (SD), n=6.
multiple comparison procedure for multiple comparison analysis following analysis of variance. All statistical tests were done with StatView (SAS Institute Inc., Cary, NC). Statistical significance was defined as \( p < 0.05 \).

RESULTS

Blood pressure, heart rate, and body and heart weights

No significant differences in systolic blood pressure or heart rate were observed at any age. The body weights of the OLETF rats were significantly greater than those of the LETO rats. No significant differences in body weight were observed between the OLETF and LETO rats in any of the 3 study groups (standard diet, supplemented diet with daidzein, or supplemented diet with genistein) (Table 2).

Glucose and lipid metabolism

At 15 wk old, impaired glucose and lipid metabolisms were observed in the OLETF groups. No significant differences in blood glucose, insulin, plasma cholesterol, or triglyceride and glycoalbumine levels were observed among the 3 OLETF groups (Table 3).

| Group | Total cholesterol (mmol/L) | Triglyceride (mmol/L) | Insulin (mU/mL) | Glucose (mg/dL) | Glycoalbumine (%) |
|-------|--------------------------|----------------------|-----------------|-----------------|------------------|
| OLETF | Daidzein 5.00±0.45       | 4.00±1.86            | 12.1±0.12       | 177.6±27.4      | 21.7±2.6         |
|       | Genistein 4.57±0.68      | 3.42±1.78            | 1.15±0.06       | 199.5±29.4      | 19.5±1.5         |
|       | Control 3.70±0.27        | 1.43±0.78            | 1.04±0.06       | 154.4±18.4      | 10.5±1.0         |
| LETO  | Daidzein 3.73±0.24       | 1.10±0.47            | 1.03±0.05       | 158.7±6.2       | 9.8±0.9          |
|       | Genistein 3.80±0.19      | 0.98±0.34            | 1.05±0.07       | 167.5±12.0      | 9.7±1.3          |

\(^1\) Each value represents the means±standard deviation (SD), \( n = 6. \)

Hydroxyproline and aortic wall thickness

At 15 wk old, hydroxyproline levels/weight of the heart decreased significantly in the genistein-treated LETO rats, but showed no significant changes among the 3 OLETF groups. Data are expressed as the mean±SD, \( n = 10. \) Asterisks (*) indicate a significant difference in comparison with the control group (\( p < 0.05 \)).

**DISCUSSION**

LETO and OLETF rats were used as models for early stages of aging or diabetes mellitus, respectively. Under both conditions, oxidative stress was a key factor in tissue fibrosis or damage, and was affected by the antioxidant potency of the dietary components. Although the mechanisms associated with the insignificant differences in hydroxyproline concentrations of the heart and aorta between untreated LETO and untreated OLETF rats were unclear in the present study, cardiovascular tissue fibrosis was mildly suppressed by soy isoflavones.

Previous results of oral glucose tolerance testing revealed that the OLETF rats were prediabetic and that insulin resistance occurred at 10 to 20 wk old (9). In the present study, casual blood glucose and insulin levels were mildly elevated at 15 wk old in the OLETF rats fed with standard chow. These data regarding glucose
metabolism suggest that the OLETF rats in the present study were also in a stage of insulin resistance during which impairment of lipid metabolism is simultaneously apparent (9). An antioxidant, vitamin E, improved lipid and glucose metabolisms. Using a type 2 diabetes mouse model, Kaneto et al. demonstrated that apoptosis induced by oxidative stress causes reduced β-cell masses, and that antioxidant treatment (N-acetyl-L-cysteine) suppress apoptosis in pancreatic β-cells (15). Consequently, the exact reason for the improved lipid and glucose metabolism observed in the previous experiment remains unknown, but could have been the result of preserved β-cell function or the amelioration of insulin resistance through strong anti-oxidation (16).

Although the usefulness of antioxidants in type 2 diabetes treatment has been suggested, our results suggest improvements of neither glucose nor lipid metabolism (17). Anderson et al. examined the effects of soy protein intake on serum lipids in the meta-analysis of 38 controlled clinical trials, and found that soy protein consumption was associated with a decrease of 0.6 mmol per liter of total cholesterol concentration (18). In the present study, a decrease of 0.43 and 0.44 mmol per liter was observed in the OLETF rats with daidzein and genistein supplementations, respectively; however, this difference was not statistically significant. Both this study and Anderson’s study were performed with a daily dose of isoflavones or a daily intake of soy-beans. In the present study, it is unlikely that the 10 OLETF rats used in each study group was a sufficient number for statistical analysis. On the other hand, although mild antifibrotic effects of soy isoflavones were observed in the heart of LETO rats, the hydroxyproline concentrations in the OLETF rats did not decrease with daidzein or genistein administration. In the present study, 0.2% daidzein or genistein was used, which was considered a possible daily concentration. However, the antioxidant effects of daidzein or genistein at this dose were not only insufficient in preventing β-cell damage, but also for inhibiting myocardial fibrosis in OLETF rats.

The proliferation and migration of cultured smooth muscle cells as an insulin-induced responses, and a strong relationship between vascular smooth muscle cell proliferation and active oxygen species production has been demonstrated (19). Our in vivo study also demonstrated that the potent anti-oxidant activity of vitamin E sufficiently prevents cell proliferation, suggesting that oxidative stress is a relatively important cell proliferation mechanism. In addition to cell proliferation, our previous study demonstrated that collagen also significantly accumulates in the aortic media of OLETF rats (10). In the present study, a significant decreases in hydroxyproline concentrations in the heart, an indicator of collagen, was observed in genistein-treated LETO rats. A mild decrease in hydroxyproline as a result of genistein treatment was also shown in the aorta of LETO rats, but this was not significant. These genistein-induced decreases in collagen content and the inhibition of cell proliferation might result in a mild decrease in the intima-media wall thickness of the aorta in LETO rats; however, this observation was not statistically significant.

In the heart, decreased hydroxyproline concentrations were observed as a result of both daidzein and genistein treatment; however, this effect was more potent with genistein. Vedavanam et al. evaluated the concentration-dependent inhibition of glucose-induced LDL lipid peroxidation with isolated flavonoids, and demonstrated that the IC50 values of genistein and daidzein were 1.5 mM and 1.7 mM, respectively (6). This mildly more potent antioxidant effect of genistein compared to daidzein corresponds to the results of aortic and heart hydroxyproline contents seen in this study. The molecular structure of each soy isoflavone is involved in regulating its antioxidant activity (9).

In summary, our results demonstrate that genistein significantly suppresses the progression of myocardial fibrosis in LETO rats despite the insignificant results observed in OLETF rats. Although a daily dosage of isoflavone was not enough to prevent tissue fibrosis under marked oxidative stress conditions during early diabetes, isoflavone might promise significant clinical benefits by reducing oxidative stress during aging processes of the heart.

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