Effect of Soybean Hypocotyl Extract on Lipid Peroxidation in GK Rats

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Summary  Vascular complications, as a consequence of atherosclerosis, are main causes of morbidity and mortality in patients with diabetes mellitus. There is increasing evidence that lipid peroxidation and oxidative modification of low density lipoprotein (LDL) is important in atherogenesis. In this study we investigated the effect of soybean hypocotyl extract (SHE), rich in isoflavones and saponins, on lipid peroxide (LPO) levels in liver, plasma and lipoproteins in GK diabetic rats, and its efficacy on the reduction of susceptibility of LDL and high density lipoprotein (HDL) to oxidation. The oxidative modification of LDL and HDL was determined with the lag time of copper ion-induced oxidation curve identified by the conjugated dienes. In SHE group which were fed diet containing 40 g/kg of SHE for 16 weeks, LPO levels in liver, plasma and HDL fraction were significantly decreased compared with the control group. The lag phage of LDL oxidation curve was prolonged noticeably by a mean of 27 min in SHE group as compared to the control group, indicating a reduced susceptibility to oxidation. The results suggest that intake of soybean hypocotyl extract might be useful for the prevention and treatment of diabetes mellitus and diabetes-associated diseases.

Key Words: soybean, hypocotyl, oxidation, lipoproteins, diabetic rats

Introduction

Soybeans contain valuable nutritional attributes. It has been found that the intake of soy foods is closely related to lowering the occurrences of chronic diseases [1–5]. There are many functional ingredients contained in soy foods such as soy protein, isoflavones, saponins, phytic acid, phytosterol, and phenolic acid [2]. Among them, isoflavones and saponins are most commonly noted, and the both seem to be concentrated in the soybean hypocotyl. Soyasaponins have drawn interest in recent years due to their potential multiple health-promoting properties including plasma cholesterol lowering [3], anticarcinogenic [4, 5], hepatoprotective [6], and antiviral activities [7]. Recent reports demonstrated that the in vivo reduction in hepatic lipid peroxidation in mice intra-peritoneally injected with soyasaponins was comparable to that which has been observed for α-tocopherol, and the effects of soyasaponins on lipid peroxidation levels appear to be mediated through the secretion of thyroid hormones [8]. Soy primarily contains 3 types of isoflavone glycosides: genistin, daidzin, and glycitin. The isoflavone aglycones, genistein, daidzein, and glycine, are structurally related to 17-estradiol, hence, they are often referred to as phytoestrogens [9]. Kerry and Abbey reported that genistein inhibits copper- and peroxy radical–mediated low density lipoprotein (LDL) oxidation when added to a cell-free oxidation system but not when incorporated into LDL
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Kapiotis et al. also reported that genistein, but not daidzein, inhibits copper-mediated LDL oxidation, as shown by the inhibition of thiobarbituric acid–reactive substances (TBARS) and the formation of hydroperoxides in cell-free systems [11]. In contrast with in vitro studies, data from interventional studies have failed to show an antioxidant effect from isoflavone supplements [12–14]. Several studies have suggested that soy intake aids against oxidative stress, as indicated on the basis of measurements of conjugated dienes in the LDL fraction [15, 16], lag time for copper-induced LDL oxidation [17, 18], and plasma concentrations of F2-isoprostanes [17]. However, results from other studies failed to support the antioxidant effects of soy [19, 20]. The potential decrease in oxidative stress has been ascribed to the isoflavone component of the soybeans. Soybeans and products derived from soybeans represent the major source of dietary isoflavones [21]. Compared with the major antioxidants in plasma (ascorbic acid, uric acid, α-tocopherol, β-carotene, and other carotenoids) [22, 23], the concentration of isoflavones in plasma is relatively low and can reach concentrations comparable with those of carotenoids, only after the consumption of meals containing soy products high in isoflavones or isoflavone supplements [17, 24]. One possibility for the discordant observations to date is that there is a potential independent effect of soy isoflavones, or the synergistic effect of isoflavones and other soy-derived phytochemicals. This possibility has not been adequately addressed. Therefore, the aim of this study was to evaluate the anti-oxidative effect of soy hypocotyl, rich in soyasaponins and isoflavones in GK diabetic rats in order to clarify the paradox.

Materials and Methods

Reagents and chemicals

Cholesterol (CH), phospholipid (PL) and triglyceride (TG) kits and Glucose B Test kit were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Lipid peroxide (LPO) test kit was obtained from Kyowa Medex Company Ltd. (Tokyo, Japan). Malondialdehyde (MDA) test kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Daidzein, daidzin, malonyldaidzin, glycitein, glycitin, malonylglycitin and malonylgenistin were purchased from Fujicco Company Ltd. (Kobe, Japan). Genistein and genistin were obtained from Extrasynthese (Genay, France).

Preparation and characterization of test extract

Soybean seeds (Glycine max (L.) Merrill) were obtained from the National Institute of Agrobiological Resources of Japan. Soybean hypocotyl was shaken with 50% ethanol overnight and the solution was filtered. The filtrate was spray-dried; the powder was then dissolved in 10% ethanol and applied to ODS column (YMC, ODS-A60-S150, 5 cm × 74 cm). The column was then eluted step-wisely with 10%, 30%, 50%, 80% and 100% ethanol. Each fraction was collected, evaporated to dryness under vacuum, and finally lyophilized. Fractions were analyzed with thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) before further application. The 10% fraction consisted of soybean oligosaccharides; 30%, 50% and 80% fractions consisted of soybean isoflavones and soyasaponins. Therefore, soybean hypocotyl extract (SHE) was the mixture of 30%, 50% and 80% fractions in this experiment. The composition of SHE was as follows (mg/g). Isoflavone: malonyldaidzin 63.2, malonylglycitin 27.5, malonylgenistin 19.1, daidzin 29.3, glycitin 10.4, genistin 6.3, daidzein 12.7, glycitein 3.6, genistein 2.9; saponin: Aa 147.9, Ae 316.4, Ba 20.3, Bb 81.5, Bd 15.7, Be 30.3, ag 21.9, βg 73.8.

To guarantee reproducibility of pharmacological experiments, isoflavone and saponin contents of fractions and SHE were analyzed with TLC and HPLC before further application. For analysis of isoflavones and saponins, each fraction was dissolved in methanol and filtered through a 0.45 μm filter (Millipore). TLC was performed on silica gel (Merck, Kieselgel 60 F-254) with a lower phase of chloroform-methanol-water (65:35:10, v/v). Spots were visualized first by UV-monitoring at 210 nm for saponins and 260 nm for isoflavones, then by heating at 120°C for 10 min after spraying with 10% sulfuric acid. HPLC was carried out as follows. HPLC instrument: Waters 600E system; column: YMC-packed ODS-AM-303 column (5 μm, 4.6 mm × 50 mm); flow rate: 1.0 ml/min; UV-detection: absorption at 210 nm and 260 nm for saponins and isoflavones, respectively; mobile phase: 40% acetonitrile containing 1% trifluoroacetic acid for the detection of saponins, a linear gradient of acetonitrile from 15% to 35% containing 1% trifluoroacetic acid for the detection of isoflavones.

Experimental animals

Six-week-old male GK/Jcl type 2 diabetic rats with a mean weight of approximately 92 g were purchased from Japan Clea Inc. Twenty GK rats were randomly assigned to two groups: the control and SHE group. The control group was fed on a standard laboratory chow; while SHE group was fed a standard diet containing 40 g/kg of SHE for 16 weeks. The composition of the control diet was as follows (g/kg). Casein 250, corn starch 300, sucrose 250, cellulose powder 50, corn oil 100, mineral mixture 35, vitamin mixture 10, methionine 3, choline bitartrate 2. The composition of the mineral mixture was according to AIN-93G MX and that of the vitamin mixture to AIN-93G VX [25]. SHE was added to the diet at the expense of starch. Experimental animals were housed in an air-conditioned room at 25°C,
under a natural light-dark regimen. Rats were allowed free access to powdered feed and water that was supplied through an automatic watering system ad libitum. All experiment was carried out in accordance with the recommendations of the Institutional Animal Care and Use of Committee of Yanbian University.

**Determination of MDA in liver homogenate**

At the end of the experimental period, rats were sacrificed and the liver was excised, rinsed with chilled 0.2 M phosphate buffer (pH 7.2). The liver (1 g) were homogenized in 0.2 M phosphate buffer saline (pH 7.2) in an ice bath and the homogenate was centrifuged at 145 × g for 10 min at 4°C. The supernatant was used for assay of MDA with the thiobarbituric acid method with the MDA test kit.

**Determination of blood biochemical parameters**

The blood sample were collected in the test tube containing EDTA after an overnight fast at the beginning and at the end of the 16-week period, and plasma was immediately separated by centrifugation at 1000 × g for 15 min at 4°C. Blood lipids and glucose and other parameters were detected with a biochemical autoanalyser (Hitachi 736-15, HITACHI, Japan) with respective commercial test kits. LPO were determined enzymatically, using a kit according to the manufacturer’s instruction. MDA content, a degrading product of lipid peroxidation known as thiobarbituric acid–reactive substances (TBARS), was determined according to the thiobarbituric acid method using a MDA test kit.

**Preparation of lipoproteins and determination of lipid**

The very low density lipoprotein (VLDL), low density lipoprotein(LDL), high density lipoprotein (HDL) were isolated by sequential ultracentrifugation by a modified method of Havel et al. [26] in a HITACHI himac cpα 100 ultracentrifuge. Following ultracentrifugation of HDL, the bottom fraction was taken to represent very high density fraction with a density greater than 1.21 (VHDL, also called lipoprotein-deficient fraction). TG, CH and LPO levels in lipoprotein were determined by enzymatic methods using commercial test kits; protein was measured according to the modified Lowry procedure of Markwell et al. [27]. All the data were normalized for the original volume of blood plasma.

**Copper ion-mediated oxidation of LDL and HDL**

The isolated LDL and HDL were incubated with pH 7.4, 10 mM PBS buffer for a total of 20 h at 4°C. Following dialysis, the protein concentration in lipoprotein fractions was determined by the modified Lowry procedure of Markwell et al. [27] and adjusted to 0.1 g/L. LDL and HDL oxidation was stimulated by the addition of copper sulfate in PBS to produce a final copper ion concentration of 5 μM. The formation of conjugated dienes (CD) was continuously monitored in a Shimadzu UV-1202 spectrophotometer at 234 nm over a period of 3 h. The indices of lipid peroxidation measured were lag time and oxidation rate. The length of the lag phase was defined as the lag time to the intercept of the tangent of the absorbance curve in the propagation phase with baseline. Propagation rate was expressed as the slope of the tangent (change in absorbance/min) [10, 28].

**Statistical analysis**

All the results were presented as mean ± SD. Student’s t test was performed using SPSS 10.0 software. Differences were considered statistically significant when p<0.05.

**Results**

Table 1 shows the levels of body weight (BW), blood glucose (BG), TG, CH and HDL-C at the beginning (0 week) and at the end of the experimental period (16 week). During the feeding period, BG levels and body weights increased and TG decreased in each group of all the rats. However, no differences in body weights and TG levels were observed in this experiment, when comparing SHE group with the control group. No changes were observed in the levels of total cholesterol or HDL-cholesterol in this experiment, when comparing SHE group with the control group. All the results are expressed as means ± SD of ten rats per group. *p<0.05, compared with control group.

| Period (week) | Group | BW (g) | BG (mM) | TG (mg/dl) | CH (mg/dl) | HDL-C (mg/dl) | LPO (μM) | MDA (μM) |
|--------------|-------|--------|---------|------------|------------|---------------|----------|----------|
| 0            | Control | 91 ± 5 | 5.8 ± 0.9 | 31.9 ± 3.3 | 112 ± 21 | 81.2 ± 11.9 | 7.0 ± 1.3 | 5.2 ± 2.1 |
| SHE          | 92 ± 5 | 5.8 ± 1.0 | 32.2 ± 3.2 | 112 ± 17 | 81.1 ± 12.0 | 7.1 ± 0.7 | 5.4 ± 1.2 |
| 16           | Control | 357 ± 11 | 14.3 ± 1.3 | 17.9 ± 3.1 | 107 ± 23 | 78.2 ± 14.3 | 11.2 ± 2.2 | 7.9 ± 2.4 |
| SHE          | 364 ± 13 | 11.4 ± 0.9* | 18.2 ± 3.5 | 109 ± 17 | 82.1 ± 11.9 | 8.1 ± 0.7* | 5.9 ± 1.8* |

The results are expressed as means ± SD of ten rats per group. *p<0.05, compared with control group.
creatinine (Cr), uric acid (UA), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were not modified by SHE treatment (Table 2).

The hepatic lipid peroxidation in terms of thiobarbituric acid-reactive substances was also measured as an index of oxidative damage to liver. Results show that the hepatic MDA level of SHE group [(3.2 ± 1.1) μmol/g protein] was significantly lower than that of control group [(4.9 ± 1.4) μmol/g protein]. Results confirm that SHE participates in the prevention of oxidative damage to both the plasma and liver.

Lipid peroxidation (LPO) was also studied in lipoprotein fractions at the end of the experiment. Results show that 16 weeks of SHE feeding significantly decreased HDL-LPO fractions at the end of the experiment. Results show that 16 weeks of SHE feeding significantly decreased HDL-LPO fractions at the end of the experiment.

The initial hydroperoxide contents in lipoprotein fractions at the beginning of copper ion-induced oxidation were expressed on a per mg cholesterol basis. *Absorbance units were converted to molar units of conjugated dienes (CD) using the molar extinction coefficient 29500 M⁻¹·cm⁻¹. † Probability (p) was calculated with Student’s t test using SPSS 10.0 software.

The results are expressed as means ± SD of ten rats per group. *P<0.05, compared with control group.

The isolated LDLs and HDLs were subjected to copper ion-induced oxidation. The results indicated a significant mean prolongation of the LDL lag phase by more than 27 min in SHE group as compared to control group after 16 weeks of SHE feeding (Table 4). The lag phase of HDL oxidation curve also increased, but insignificantly. There was also a significant decrease in the propagation rate of LDL but the accumulated LDL and HDL conjugated dienes were not significantly different between SHE and control animals during the incubation period (Table 4). When the lipid peroxide content in lipoprotein fractions was expressed on a per cholesterol basis, then LDL and HDL of SHE group were found to contain less LPO molecules at the beginning of copper ion-induced oxidation than the control group (p = 0.09 and 0.01 for LDL and HDL, respectively, Table 4).

**Discussion**

Over the last few years, research has focused on soybean constituents such as isoflavones, phytosterols, saponins, water- and fat-soluble vitamins, and minerals [29, 30]. Tanizawa et al. [31] reported that soyasaponins inhibit the in vivo Adriamycin-induced increase of lipid peroxide levels in the myocardium of mice and saponin constituents are also responsible for a reduction of NADPH-induced lipid peroxidation (in vitro) in mice hepatic microsomes. In a small study, Tikkanen et al. [18] also documented that the consumption of soy bars for 2 week resulted in a longer lag phase of copper-induced LDL oxidation than that at baseline. However, there is a contradiction about the in vivo effect of isoflavones and saponins on lipid oxidation [12–14]. It encourages us to investigate the in vivo antioxidant properties of soy hypocotyl, rich in isoflavones and saponins, in whole plasma and isolated lipoprotein fractions.

In this study isoflavones and saponins in soybean hypo-
cotyl occur as glucose-conjugated forms. Once ingested, these glucosides are hydrolyzed to absorbable aglycones [32]. It is commonly assumed that isoflavone aglycones are absorbed quickly compared with glucoside forms. However, the bioavailability of aglycones and glucosides of soy isoflavones is controversial [33, 34]. Soyasaponins may have very low absorbability in the human gut. According to the study of Hu et al. [35], soyasaponins are taken up by gut epithelial cells in a saturable manner in small amounts; in contrast, the uptake of soyasapogenol may depend upon its concentration in the lumen. The absorbed soyasaponin or sapogenol may also contribute to anticancer activity [35].

The most widely used methods for monitoring lipid oxidation are the measurement of lipid peroxides, thiobarbituric acid reactive substance and conjugated diene levels. In our study the addition of SHE to daily diet clearly reduced the plasma and hepatic LPO levels. Moreover, LPO levels in the isolated HDL samples from SHE group were significantly lower than those from the control group. The result indicates reduced lipid peroxidation of the liver, plasma and high density lipoprotein fraction. The chronic SHE administration also slightly decreased the LPO status of LDL, but the differences between the two groups were not significant. The result may be explained with the fact that HDL is the principal vehicle for circulating plasma lipid hydroperoxides and may be more rapidly oxidized than those in LDL in vivo [36]. Lipid peroxides may accumulate more rapidly in HDL than in LDL in vivo because of the antioxidant action of CoQH2 in the LDL [31].

According to the study of Bowry et al. [36], even though HDL particles carry most (≥85%) of the detectable oxidized core lipoprotein lipids, LDL core lipids were oxidized more rapidly after most ubiquinol-10 present in LDL was consumed. Therefore, we have also examined the effect of SHE on the susceptibility of LDL and HDL to oxidation. All samples of SHE group showed similar shifts of the propagation curves to the right as compared to the control group. Our finding that 16 weeks of SHE feeding caused a significant prolongation of lag time of LDL is potentially important, as oxidative modification of LDL is regarded as atherogenic. The elevated concentrations of circulating antioxidant phytochemicals such as isoflavones and saponins during SHE feeding could have protected LDL from oxidation and resulted in a reduction of the initiation rate. Even given a significant reduction of HDL-LPO after SHE administration, there was no significant difference in the formation of conjugated dienes and lag time of HDL oxidation between the two groups. The much lower initiation rate and shorter lag time of HDL oxidation curve may explain the “lack” of a protective effect of SHE against HDL oxidizability in some way.

In this experiment, antioxidant activity of SHE was confirmed. In diabetic rats, the consumption of soybean hypocotyl rich in isoflavones and saponins reduced the liver, plasma and HDL lipid peroxidation and the oxidative modification of LDL. These findings suggest that soybean hypocotyl, the natural combination of isoflavones and saponins, might have a therapeutic value for the prevention and treatment of diabetes-associated atherosclerosis.

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