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The *in vivo* antioxidant activity of soybean isoflavones in human subjects

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Abstract

This paper presents the *in vivo* antioxidant activity of soy isoflavones in human subjects determined by the urinary excretion of secondary lipid peroxidation products. Ten healthy women 18-35 years of age consumed a self-selected diet and avoided legumes, whole grains, and isoflavone containing foods. A powdered soy protein isolate was added daily to their diet that provided 3 levels of isoflavones: control 0.15, low 1.01, and high 2.01 mg/kg body weight. Subjects were randomized to consume all three diets for 13 weeks each, with each subject serving as her own control. Urine samples were analyzed from 24-hr collections at the end of each diet period for lipophilic aldehydes and related carbonyl compounds by HPLC. Results show that six of the individual urinary nonpolar compounds (NPC) levels were significantly lower due to consumption of the high isoflavone diet and one was also significantly lower due to consumption of the low isoflavone diet. The total of the individually measured urinary NPC was significantly lower with consumption of both the low and high isoflavone diets when compared with the control diet.

Keywords: Antioxidants; Human; Isoflavones; Lipid peroxidation; Soy

1. Introduction

Consumption of dietary antioxidants such as vitamins E and C, selenium, and carotenoids has long been associated with a decreased risk of developing heart disease and cancer which are the leading causes of death for Americans [1]. Soy isoflavones and soy protein are also associated with a reduced risk of chronic disease, which may be due to cellular antioxidant

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activity [2]. Although isoflavones are generally extracted along with the protein component of soy, a health claim of protection against heart disease has been awarded to soy protein *per se* without consideration of the isoflavone content [3].

Recent interest in lipid oxidation in relation to heart disease centers mainly around the low-density lipoprotein (LDL) in the blood, which when oxidized is likely to promote atherosclerosis [4]. Individual isoflavones and one of their metabolites have been shown to inhibit LDL oxidation *in vitro* [5–7], and the consumption of a soy food is credited with a reduction in oxidized LDL [8].

Oxidation events are also suspected in the development of cancer [9], with a focus on DNA damage. A decrease in oxidative DNA damage has been credited to two individual isoflavones, genistein and daidzein, in cell cultures [10,11]. Similar observations were noted for soy milk and a soy supplement when ingested by human subjects [12,13]. These studies investigated DNA strand breakage and the addition of hydroxyl groups, however, DNA damage can also be caused by endogenous lipid peroxidation products [14]. Lipid peroxidation occurs as a free radical chain reaction [15] involving the polyunsaturated fatty acids found in many endogenous lipids. This event is associated with the formation of hydroperoxides and free radical intermediates, and the production of secondary oxidation products [16]. Lipid peroxidation can undermine the structural and functional integrity of cell membranes [17] and some of the secondary oxidation products, including certain short chain aldehydes and ketones, can contribute to peroxidative cell damage by reacting with sensitive biomolecules [18], including DNA, LDL, and others. These compounds are sufficiently long lived to damage target molecules distant from the site of their formation [19]. These secondary lipid oxidation products formed *in vivo* are also excreted in the urine, and have recently been measured in nanogram quantities in this laboratory using high performance liquid chromatography (HPLC) [20,21].

The goal of the current investigation was to examine the *in vivo* antioxidant effects of dietary soybean isoflavones in healthy human subjects by measuring the level of urinary secondary lipid oxidation products.

2. Materials and methods

2.1. Materials

2,4-Dinitrophenylhydrazine (DNPH), hexanal, RRR-δ-tocopherol (90%), and DL-α-tocotrienol (90%) were obtained from Sigma (St. Louis, MO); RRR-α-tocopherol (99%), RRR-γ-tocopherol (97%), from Fluka Chemie (Milwaukee, WI), pentan-2-one (97%), hept-2-enal (97%), hepta-2,4-dienal (90%), decanal, and deca-2,4-dienal from Aldrich Chemical Co. (Milwaukee, WI); hydrochloric acid, acetone, methanol, dichloromethane, hexane, and water from EM Science (Gibbstown, NJ); hexane and isopropyl alcohol from Fisher Scientific (Fair Lawn, NJ). All solvents used were HPLC-grade. DNPH derivatives of butanal, butanone, hexanal, octanal, non-2-enal, 4-hydroxyhex-2-enal, 4-hydroxynon-2-enal, 4-hydroxyoct-2-enal, and 4-hydroxydec-2-enal were gifts from H. Esterbauer, Department of Biochemistry, Univerisity of Graz (Graz, Austria).

2.2. Subjects

Ten healthy women 18-35 years of age were selected from a previously described study [22]. Exclusion criteria included vegetarian, high fiber, high soy, or low fat diets; regular consumption of vitamin and mineral supplementation greater than the recommended dietary allowances; athleticism; cigarette smoking; antibiotic or hormone use within 6 months; history of chronic disorders; regular use of medication including aspirin; current pregnancy or lactation; irregular menstrual cycles; less than 90% or more than 120% ideal body weight; consumption of more than 2 alcoholic beverages/day; and a history of food allergies.

2.3. Experimental diet

Subjects consumed a self-selected diet *ad libitum* and avoided soy products, flaxseed, whole wheat products, seeds, sprouts, beans, legumes, vitamin/mineral supplements, and alcoholic beverages. One of three powdered soy protein powders (Supro Brand Isolated Soy Protein, Protein Technologies International, St. Louis, MO) was added daily to their diet on the basis of kg BW. The three supplements each contained a similar macronutrient composition (daily average 290 KCal, 53 g protein, 15 g carbohydrate, and 1.9 g fat) but provided one of three levels of isoflavones: 0.15 (Control), 1.01 (Low-Iso), and 2.01 (High-Iso) mg total isoflavones/kg BW/day. The relative proportions of genistein, daidzein, and glycitein averaged 55%, 37%, and 8%, respectively; 97% of the daidzein and genistein and 91% of the glycitein were present as glucoside conjugates in the powdered soy protein isolate.

Subjects were randomized to consume all three soy supplements (Control, Low-Iso, High-Iso), one per diet period. Each of the 3 diet periods continued for ≥90 days with a washout period of about 3 weeks between dietary treatments. Food records were obtained for three consecutive days six times during each diet period [22]. Each subject served as her own control. This protocol was approved by the University of Minnesota Institutional Review Board Human Subjects Committee.

2.4. Urine analysis

Twenty-four hr urine collections were made at the end of each diet period and 20 mL aliquots were obtained and stored at −70° C until analysis as described by Csallany, et al. [20]. Samples were filtered by a stirred Amicon cell equipped with a YC05 Diaflo membrane filter (Amicon Corp. Beverly, MA) under 35 psi of nitrogen pressure to remove compounds with molecular masses larger than 500 dalton.

Urine samples were then reacted with 2,4-dinitrophenylhydrazine (DNPH) overnight at room temperature. The DNPH derivatives were extracted with dichloromethane and separated into nonpolar aldehyde compounds (NPC), polar aldehyde compounds (PC), and osazone (sugar derivative) fractions by thin layer chromatography on silica gel plates (silica gel 60, aluminum backed, 20 × 20 cm, 0.2 mm thickness) purchased from Alltech Associates, Inc. (Deerfield, IL). The NPC and PC groups of lipophilic aldehydes and related carbonyl compounds (LARC) were eluted from the plates with methanol. Separation and quantification of the individual NPC and PC hydrazones was achieved by HPLC using a

mobile phase of methanol/water, 75:25 v/v for NPC and 50:50 v/v for PC. Isocratic elution proceeded for 10 min followed by a linear gradient to 100% methanol for 15 min at a flow rate of 0.8 mL/min. Absorbance of the DNPH derivatives was monitored at 378 nm.

The HPLC system consisted of an Altex 110A solvent metering pump and sample injector (Beckman Instruments, Berkeley, CA), SP8400 UV/Vis detector (Spectra-Physics, Arlington, IL), and HP3380A computing integrator (Hewlett-Packard, San Diego, CA). Separations were performed on an Ultrasphere ODS C18 reverse phase column (25 cm × 4.6 mm i.d., 5 μm particle size) (Altex, Berkeley, CA) with a guard column (2 cm × 2 mm i.d.) (ChromTech, Apple Valley, MN). Disposable syringes used for sample injection were equipped with a 0.2 μm PVDF filter (ChromTech).

Identification of individual urinary NPC and PC DNPH derivatives was accomplished by comparing the retention times of the peaks with the retention times of pure standards. Further identification of compounds was also previously established by co-chromatography with pure standards in three different polarity solvent systems in this laboratory [21].

2.5. Tocopherol analysis of soy protein isolates

Nine grams of Control and High-Iso soy isolate powders were placed in separate Soxhlet extraction thimbles and extracted continuously with 100 mL of acetone for 4 hours. The acetone was evaporated by vacuum on a rotary evaporator and the resulting residue was dissolved in 2 mL isopropanol:hexane (1.5:98.5 v/v). Aliquots (20 μL) were injected onto a normal-phase HPLC column (Lichrosorb Si-60 5μ, 4.6 mm × 25 cm, Alltech, Deerfield, IL) using an Altex 110 solvent metering pump and injector. The mobile phase was isopropyl alcohol:hexane (1.5:98.5 v/v) at a flow rate of 1.0 mL/min. Fluoresence was monitored at 295 nm excitation and 330 nm emission for the detection of α-, γ-, δ-tocopherols and α-tocotrienol by a PE 650-10S fluorescent spectrophotometer (Perkin-Elmer, Norwalk, CT) with a SP4270 computing integrator (SpectraPhysics, Santa Clara, CA) [23].

2.6. Statistical analysis

Statistical analyses were performed using the SAS System for Windows, Release 8.02. Paired Student's *t* tests were used for all comparisons. The results are expressed as mean and standard error of the mean (SEM). Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Urinary nonpolar and polar lipophilic aldehydes and related carbonyl compounds

A typical HPLC separation of urinary nonpolar lipophilic aldehydes and related carbonyl compounds (LARC) is illustrated in Fig. 1 . The HPLC elution profile was similar to that produced previously in this laboratory for rat and human urine [20,21]. The nonpolar LARC was measured in isolated urine collections from all subjects during all three diet periods.

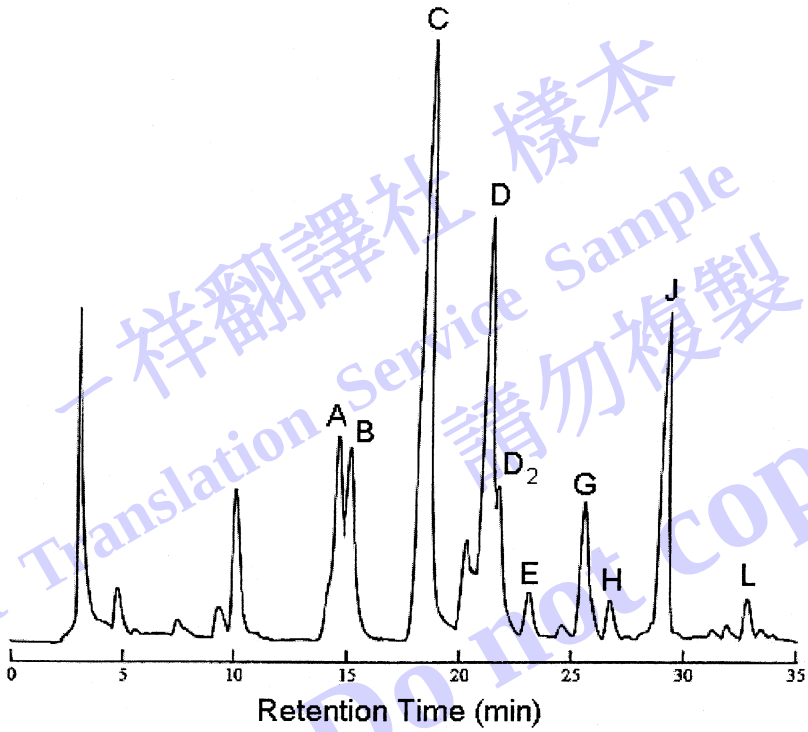

Fig. 1. HPLC separation of the 2,4-dinitrophenylhydrazine (DNPH) derivatives of urinary nonpolar lipophilic aldehydes and related carbonyl compounds from a human subject. A, butanal; B, butanone; D, pentan-2-one; D_2, pentan-3-one; G, hexanal; H, hepta-2,4-dienal; L, non-2-enal. C, E, and J are unidentified. Separation conditions: Ultrasphere ODS column (4.6 mm × 25 cm, 5 μm), isocratic elution with methanol/water (75:25 v/v) for 10 min, followed by a linear gradient to 100% methanol for 15 min; flow rate 0.8 mL/min; detector wavelength, 378 nm; injected volume, 100 μL.

Nonpolar compounds separated from the urine include: A, butanal; B, butanone; D, pentan-2-one; D_2, pentan-3-one; G, hexanal; H, hepta-2,4-dienal; L, non-2-enal; and three unidentified compounds (C, E, and J). The comparison of individual NPC (Fig. 2) shows lower values for both levels of isoflavones (Low-Iso and High-Iso) for eight of the ten LARC analyzed. Butanal, pentan-2-one, hexanal, hepta-2,4-dienal, and compound E were significantly lower ($p < 0.05$) and compound J was also significantly lower ($p < 0.01$) for the High-Iso diet, as compared to the Control diet. Compound J was also lower at $p < 0.05$ for the Low-Iso diet, compared to the Control diet.

Fig. 3 shows the total NPC determined by summing the individually measured peak areas for all NPC. Significantly lower values were observed with both levels of dietary isoflavones. The Low-Iso diet achieved $p < 0.01$ level of significance and the High-Iso diet $p < 0.001$, when compared with the Control diet.

Separation of the individual polar LARC was similar to the separation previously reported by this laboratory for animal and human urine [20,21]. Compounds found in the urine of soy isoflavone-fed subjects in the present experiment were identified as 4-hydroxyhex-2-enal, 4-hydroxyoct-2-enal, and 4-hydroxydec-2-enal. As it was expected, 4-Hydroxynon-2-enal

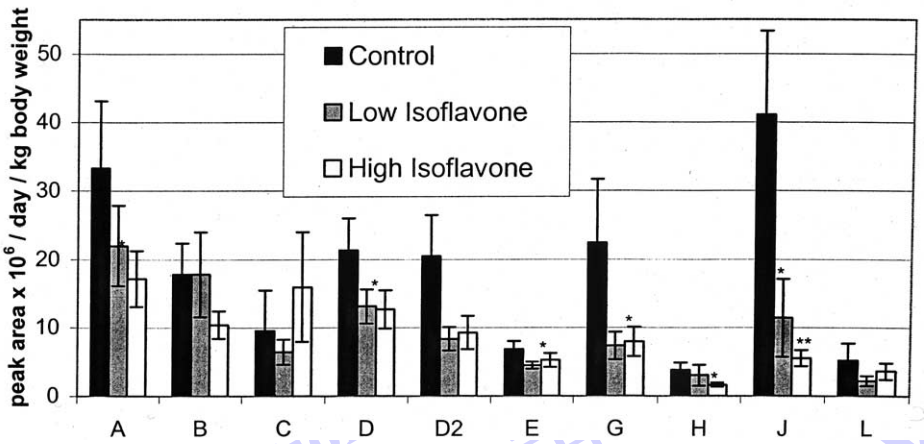

Fig. 2. Comparison of the urinary excretion of individual HPLC-separated nonpolar lipophilic aldehydes and related carbonyl compounds in women consuming control, low, and high isoflavone diets. Values represent the mean ± SEM for all subjects (n = 10) consuming each diet for > 13 weeks. *$p < 0.05$, **$p < 0.01$, when compared with the control diet. A, butanal; B, butanone; D, pentan-2-one; D_2, pentan-3-one; G, hexanal; H, hepta-2,4-dienal; L, non-2-enal. C, E, and J are unidentified.

was not detected in these human urine samples. The remaining polar compounds have not yet been identified. Comparison of the individual polar compounds and the sum of the PC found no significant differences for either the High-Iso or Low-Iso diet when compared to the Control diet. Since no significance was found for urinary PC, chromatographic separation and measurement of the individual and total PC levels is not illustrated in this paper.

Tocopherols were not detected in either the Control or High-Iso soy protein isolate powders. The Low-Iso powder was a 1:1 mixture of the Control and High-Iso isolates, therefore, it was assumed that the Low-Iso powder did not contain any tocopherols either.

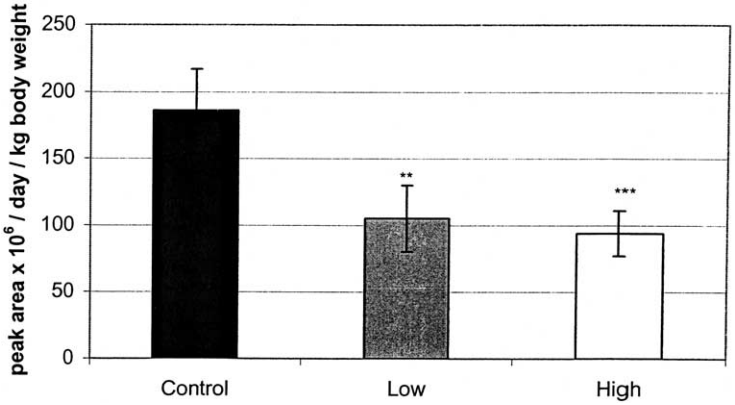

Fig. 3. Comparison of the sum of individually measured urinary nonpolar lipophilic aldehydes and related carbonyl compounds from women consuming control, low, and high isoflavone diets. Values represent the mean ± SEM for all subjects (n = 10) consuming each diet for > 13 weeks. **$p < 0.01$, ***$p < 0.001$, when compared with the control diet.

4. Discussion

Cumulative oxidative damage to cellular lipids and DNA is though to be a significant contributor to the development of cancer and heart disease. Recent interest in lipid oxidation is centered mainly around LDL, which when oxidized is likely to promote atherosclerosis [4]. Lipid peroxidation of polyunsaturated fatty acids is associated with the formation of hydroperoxides, free radical intermediates, and secondary oxidation products such as aldehydes and certain carbonyl compounds [16]. The low molecular weight aldehydes are sufficiently long lived to damage target molecules distant from the site of their formation [24]. Lipid peroxidation *in vivo* can undermine the structural and functional integrity of cell membranes, and its products can impair cell function by reacting with various macromolecules including proteins and nucleic acids [17]. A variety of aldehydes and carbonyl compounds are produced as degradation products of lipid peroxidation *in vivo* and excreted in the urine. These urinary products have been measured in this laboratory in nanogram quantities and found to be one of the most sensitive ways to measure lipid peroxidation and antioxidant protection *in vivo* [20,21,25].

Dietary sources of antioxidants are critical in protection against oxidative damage. Compounds in soybean with antioxidant properties include flavonoids, (isoflavones, genistein and daidzein), tri-terpenoids, carotenoids, tocopherols, and saponins [26]. While the amount and contribution of individual antioxidants in soybeans are not known, there is strong evidence that additive and synergistic interactions occur among antioxidants which significantly strengthen the protective effects [27]. Soybeans and soy foods are some the most important dietary sources of isoflavones. Genistein and daidzein, the two major soy isoflavones, either individually or together with their metabolized products, have been shown to exert some antioxidant action in a variety of experimental conditions *in vitro* or *ex vivo* [5–7,10,12,13].

In the present experiment the *in vivo* antioxidant effect of soybean isoflavones was investigated in human female subjects by measuring the extent of lipid peroxidation and urinary aldehyde excretion after consuming diets with three levels of soy isoflavones. Results show that both low and high levels of isoflavone consumption (1.0 and 2.0 mg total isoflavones/kg BW/day, respectively) exhibited antioxidant activity as compared to the control (0.15 mg isoflavones/kg BW/day), as measured by the significant decrease in urinary aldehydic secondary lipid peroxidation products. Due to higher consumption of soy isoflavones, there was a lesser amount of some of the urinary secondary lipid oxidation products as compared to lower isoflavone consumption (Fig. 2). Both low and high isoflavone consumption also decreased the total amount of excreted aldehydes, as compared to the control, with the higher level of consumption resulting in a more significant difference than the low level of isoflavone consumption (Fig. 3). The antioxidant effects can be attributed solely to the isoflavones since the three soy powders contained no tocopherols.

The present results show the *in vivo* antioxidant activity of dietary soy isoflavones in healthy female subjects as evidenced by the significantly lower excretion of urinary secondary lipid oxidation products, a measure of lipid peroxidation *in vivo*. These results also show that these lipid oxidation products, the urinary aldehydes and related carbonyl compounds, are very sensitive noninvasive biomarkers that can be used to measure *in vivo* lipid peroxidation and antioxidant action in human subjects as well as animal models.

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