Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 18 (2007) 46–53
Journal of Nutritional Biochemistry

Soy protein with and without isoflavones fails to substantially increase postprandial antioxidant capacity

Karrie M. Heneman[a], Hebron C. Chang[b], Ronald L. Prior[b], Francene M. Steinberg[a,*]

[a]Department of Nutrition, University of California, Davis, CA 95616-8669, USA
[b]US Department of Agriculture, Agriculture Research Service, Arkansas Children's Nutrition Center, Little Rock, AR 72202, USA

Received 3 November 2005; received in revised form 2 March 2006; accepted 6 March 2006

Abstract

Five methods for the assessment of antioxidant capacity [whole plasma conjugated diene formation, low-density lipoprotein oxidation susceptibility, ferric-reducing ability of plasma, oxygen radical absorbance capacity and perchloric-acid-treated oxygen radical absorbance capacity (PCA-ORAC)] were used in a randomized, double-blind, cross-over study to determine the acute postprandial antioxidant protection imparted by the isoflavone component of soy. On separate days, 16 subjects consumed one of three isocaloric shakes containing 25g of protein in the form of soy, with 107 mg of total aglycone units of isoflavones, soy with trace isoflavones (<4 mg) or total milk protein. Blood was collected at baseline, 4h, 6h, and 8h after consumption. Antioxidant capacity, serum isoflavone levels, fat-soluble antioxidants and plasma vitamin C levels were evaluated. Repeated measures analysis of variance showed no significant differences ($P=.05$) within treatments over time in four of five antioxidant capacity measurements. Significant differences over time between the soy with trace isoflavones and the total milk protein group were observed using the PCA-ORAC assay. It can be concluded that, on an acute basis, a significant increase in serum antioxidant capacity is not detectable following consumption of soy protein.

Keywords: Soy protein; Isoflavones; Antioxidant capacity

1. Introduction

Epidemiological evidence suggests that eastern populations that regularly consume soy have a low relative risk of cardiovascular disease (CVD) and have favorable levels of some chronic disease biomarkers, such as low-density lipoprotein (LDL) and total cholesterol [1–6]. Although many lifestyle factors may contribute to this association, consumption of soy is one dietary component that has a potential to modulate CVD risk. Controversy exists regarding the contribution of soy foods and bioactive components to the reduction of chronic disease risk factors. Randomized controlled trials have produced inconsistent results, which vary from null to moderately robust protective effects. Despite extensive research, the exact mechanisms for the cardioprotective effect of soy have yet to be confirmed. Soy protein components must be present for lipid-lowering effects to be observed, whereas other biologic effects may be related to isoflavones and their metabolites. The mild hypocholesterolemic effect observed with consumption of soy protein is dependent on the degree of hypercholesterolemia in at-risk individuals [7–13]. The shift of LDL particle size to a larger, less atherogenic pattern as a result of soy protein consumption [13] and improvement in endothelial function independent of lipoprotein changes represent two other potential soy-mediated protective mechanisms [9,14].

Soy protein, the soy-derived isoflavones genistein and daidzein, and the metabolite equol are hypothesized to impart antioxidant protection contributing to reduction in oxidative stress and CVD risk. In vitro investigations have demonstrated the hydrogen-donating abilities of isoflavones and metabolites, inhibition of lipid peroxidation and the ability to interact with the oxidants hypochlorous acid and peroxynitrite [15–18]. Cell culture studies suggest that they may act by enhancing the cellular antioxidant network by increasing metallothionein mRNA levels [19], inhibiting peroxynitrite-mediated LDL oxidation by delaying tyrosine nitration [20], activating glutathione peroxidase [21] (thereby increasing levels of cellular reduced glutathione) [22] or inhibiting

* Corresponding author. Tel.: +1 530 752 0160; fax: +1 530 752 8966.
E-mail address: fmsteinberg@ucdavis.edu (F.M. Steinberg).

0955-2863/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2006.03.001

superoxide production and, thus, cell-mediated LDL modification [23].

However, clinical trials investigating the antioxidant hypothesis have failed to observe the protective effects of purified isoflavones [24,25], while studies using soy protein preparations have yielded results both supporting [8,26,27] and disproving [14,28,29] the antioxidant cardioprotective theories of soy and its components. A very recent study reported a small significant increase in plasma antioxidant status, yet without much effect on biomarkers of oxidative stress [30]. Hypotheses for these apparent contradictions include differences in study design; soy preparation [31]; the existence of active components other than isoflavones in the protein itself [32]; variations in the ability of study populations to produce equol, a metabolite with potent antioxidant capabilities [33]; and the possibility that there is no clinical significance to the minor changes observed in some reports. These studies have provided partial explanations for literature discrepancies; however, questions regarding the antioxidant potential of soy and its in vivo components at physiologic doses still remain.

A question yet to be investigated is whether isoflavones act as acute antioxidants during the postprandial period corresponding to circulating peak plasma levels. It is possible that the postprandial antioxidant effects of soy consumption are subtle and transitory, and therefore may not be readily detectable in longer term studies that examine fasting blood samples.

Several techniques can be used to measure serum or plasma antioxidant capacity [34–36]. Five of the most common methods are whole serum susceptibility to conjugated diene formation (whole serum oxidation), plasma LDL oxidation susceptibility (LDL oxidation), ferric-reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC) and perchloric-acid-treated oxygen radical absorbance capacity (PCA-ORAC) [35]. To our knowledge, a study that compares all five methods has not been conducted; however, FRAP and ORAC assays have been shown to be weakly correlated in some conditions [35]. The objective of the current investigation was to determine whether acute antioxidant protection is imparted by soy protein with and without isoflavones in a healthy, free-living population and to determine whether correlations exist between five different methods of ex vivo antioxidant capacity assessment in this setting. The hypothesis was that consumption of soy protein would promote greater acute antioxidant capacity than control protein, and that soy containing isoflavones would have the greatest effect.

2. Materials and methods

2.1. Participants

Twenty healthy nonsmoking (4 male and 16 female) adults were recruited from the University of California at Davis to participate in this study. The University of California at Davis Institutional Review Board approved the human subjects protocol, and written informed consent was obtained from all participants in compliance with the Helsinki Declaration, as revised in 1983. Participants were excluded if they had a history of chronic disease, consumed a diet significantly high in isoflavones/polyphenols or took multivitamins daily.

2.2. Study design

Participants were assigned to one of three randomization schemes in this double-blind cross-over design experiment such that each subject underwent all three treatments, each separated by a 1-week washout period. The dietary treatments consisted of: (a) soy with isoflavones (Soy^+): isolated soy protein with 110 mg of total aglycone isoflavones; (b) soy with trace isoflavones (Soy^-): ethanol-washed isolated soy protein with <4 mg of total aglycone isoflavones; and (c) total milk protein (TMP): casein with 0 mg of isoflavones as control. The randomization schemes were as follows:

(1)	Soy^+	Soy^-	TMP
(2)	Soy^-	TMP	Soy^+
(3)	TMP	Soy^+	Soy^-

To minimize the possible confounding effects of diet, participants were asked to refrain from consuming soy products for 1 month prior to the study and to consume a diet that was low in flavonoids 24 h before each experimental day. Twenty-four-hour food records were collected to ensure compliance with dietary restrictions. Completed forms were analyzed using NUTRITIONIST V software, version 2 (First Data Bank, San Bruno, CA, USA).

Blood samples were collected in heparin, EDTA and serum tubes at four time points. The first blood draw, or baseline measurement, occurred at approximately 0800 h after a 12-h fast. Subsequently, each subject was asked to consume one of three isocaloric shakes containing 25 g of protein (Solae Co., St. Louis, MO, USA) and flavonoid-free ingredients such as ice cream, rice milk and strawberry syrup (550 kcal, 28.6 g of protein, 12 g of fat and 85.6 g of carbohydrate).

Blood was collected again at 4, 6 and 8 h after shake consumption to capture the peak appearance of isoflavones in the serum [37–39]. After a 4-h blood draw, lunch and water (ad libitum) were provided. This lunch (lean meat and cheese sandwich on white bread; no vegetables or fruits) provided approximately 550 cal and was low in fats, antioxidants and flavonoids. Subjects consumed no other food during the 8-h study period. Immediately after collection, blood samples were spun, aliquoted and stored at −80°C pending further analysis.

2.3. Experimental procedures

2.3.1. Analysis of serum isoflavone content

Analysis of soy isoflavones in serum was carried out according to the methods of Cimino et al. [40]. Total

aglycones of genistein and diadzein were extracted and measured directly on liquid chromatography–mass spectroscopy (LC-MS) following enzymatic deconjugation of conjugated isoflavones. Samples (100 μl) were incubated with a mixture of sulfatase and glucuronidase (Sulfatase H-5; 100 U) for 3 h to obtain the total isoflavone concentration. Serum was extracted twice with 5 ml of hexane to remove the lipids and thrice with 5 ml of diethyl ether, and the organic phase was removed and evaporated to dryness at 50°C under nitrogen. Dried extracts were reconstituted in 0.5 ml of 50% methanol/water containing a known amount of biochanin A and analyzed by LC-MS to determine isoflavone concentrations.

For LC-MS, the extracted samples (100 μl) were injected onto a Discovery RP amide C_{16} column (25 cm×4.6 mm, 5 μm particle size; Supelco, Bellafonte, PA, USA), and isoflavones were eluted with a mobile phase of Solvent A (25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine, pH 4.5) and Solvent B (95% methanol containing 10 mM ammonium acetate and 71 mM triethylamine, pH 5.5) at a flow rate of 1.0 ml/min. Isoflavones were separated using a linear gradient from 45% Solvent B to 65% Solvent B over an 11-min time period. The proportion of Solvent B increased linearly from 65% to 95% over 5 min and was held for another 5 min before returning to initial conditions. The column was equilibrated for 6.5 min prior to any subsequent sample injection. Isoflavones were detected using a PE Sciex API 100 Mass Spectrometer using negative ion monitoring of single ions using a heated nebulizer atmospheric pressure–chemical ionization interface. Isoflavone concentrations were expressed as micromoles per liter in serum after normalization with biochanin A. Intra-assay and interassay coefficients of variation were 5.8% and 10.8%, respectively. The detection limit was 0.8 pmol of each isoflavone injected onto the column.

2.3.2. Serum fat-soluble antioxidant levels

Serum α-tocopherol, retinol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene levels were analyzed using the procedure of Sowell et al. [41]. Fat-soluble compounds were extracted from serum using hexane (Sigma Chemical Co., St. Louis, MO, USA). Aliquots were then dissolved in the mobile phase of 1:1 ethanol and acetonitrile and injected onto a C_{18} reverse-phase high-performance liquid chromatography column with detection at 300, 325 and 450 nm. Peaks were identified and quantified using serum markers of the National Institutes of Standards and Technology.

2.3.3. Plasma ascorbic acid

To determine the levels of ascorbic acid, 0.5 ml of heparinized plasma was added to 2.0 ml of metaphosphoric acid (6.0 g/100 ml) in an eppendorf tube, vortexed and centrifuged at 3000 rpm for 10 min. This initial treatment occurred within 1 h of blood collection. The supernatant was then removed and frozen at −80°C to await further analysis. Within 3 months of collection, samples were analyzed by treatment with a dinitrophenylhydrazine thiourea copper sulfate reagent (Sigma Chemical Co.) and evaluated spectrophotometrically at 520 nm.

2.3.4. Whole plasma oxidation

The assay for whole plasma oxidation was adapted from the method of Schnitzer [42]. Heparinized plasma samples were diluted 200-fold with phosphate-buffered saline (pH 7.4) and placed into a quartz cuvette. After dilution, 10 μl of 50 μM copper sulfate was added to bring the solution to a 1-ml volume with a final copper concentration of 10 μmol/L. Subsequently, the formation of conjugated dienes was monitored at 234 nm with a 12-cell visible spectrophotometer (UV-1601; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) kept at 37°C. Data were collected continuously until conjugated diene formation had reached a plateau and had been graphed as time versus absorbance. Lag time to oxidation was calculated as the intersection between the lag phase and the propagation phase line equations.

2.3.5. LDL oxidation susceptibility

The assay for LDL oxidation was adapted from the method of Esterbauer et al. [43]. Briefly, LDL was isolated from EDTA plasma by sequential density microultracentrifugation according to the method of Brousseau et al. [44] and incubated overnight in dialysis tubing placed in a nitrogen-purged phosphate-buffered solution (pH 7.4) containing Chelex 100 (Bio-Rad, Richmond, CA, USA) to remove EDTA. On the following morning, 75 μg of apo B was quantified by a Lowry protein assay and placed in a quartz cuvette. Phosphate-buffered solution (pH 7.4) and copper sulfate were then added to the LDL to produce a 5-μM copper solution in a final volume of 1 ml. Subsequently, the formation of conjugated dienes was monitored at 234 nm. Lag time to oxidation was determined in the same manner as for the oxidation of whole plasma samples.

2.3.6. FRAP

The FRAP assay was carried out according to the method of Benzie and Strain [45], as modified, to run on an Analette Analyzer (Precisions Systems, Natick, MA, USA). The plasma was mixed with FRAP reagent, and the reaction was monitored at a wavelength of 593 nm.

2.3.7. ORAC and PCA-ORAC

The ORAC and PCA-ORAC methods assays were carried out using the procedure described by Cao et al. [36], which was later modified to be able to use fluorescein as a probe [34]. Perchloric acid was used to extract the nonprotein fractions of heparinized plasma. For preparation of plasma nonprotein fraction, plasma was diluted with 0.5 mol/L PCA (1:1, vol/vol). The samples were then centrifuged at 3000×*g* for 10 min at 4°C, and the supernatants

Table 1
Serum isoflavone levels (ng/100 μl)

Treatment	Genistein		Daidzein	
	Baseline	8 h	Baseline	8 h
Soy^+	0.6 (1.1)	18.9 (34.7)*	0.3 (0.7)	9.8 (16.0)*
Soy^-	0.5 (1.1)	1.0 (2.6)*	0.4 (1.1)	0.3 (0.8)*
TMP	0.4 (0.9)	0.3 (0.6)*	0.2 (0.4)	0.2 (0.3)*

Mean (±S.D.) baseline and 8-h values for serum isoflavone levels. A significant increase in the serum levels of the isoflavones genistein and daidzein was observed in response to Soy^+ treatment, in comparison to both Soy^- and TMP treatments.

* The significance ($P<.05$) between treatments at different time points was determined by repeated-measures ANOVA with Tukey–Kramer post hoc test.

were removed as plasma nonprotein fraction and diluted for the ORAC assay.

$ORAC_{FL}$ assay was carried out on a FLUOstar Galaxy plate reader, which was equipped with an incubator and two injection pumps. The temperature of the incubator was set to 37°C. Assay procedures were based on the modified $ORAC_{FL}$ method [34] using fluorescein as fluorescent probe. 2,2′ -Azobis(2-amidino-propane) dihydrochloride (AAPH) was used as a peroxyl generator, and Trolox was used as a standard. Forty microliters of sample, blank and Trolox calibration solutions were transferred to 48-well microplates in duplicate based on a set layout. The plate reader was programmed to record the fluorescence of $ORAC_{FL}$ at every cycle. The number of running cycles for the complete $ORAC_{FL}$ analysis was set to 25, and the cycle time was automatically given by the instrument according to the number of wells used in the assay. Peroxyl-radical-induced oxidation started immediately after the addition of AAPH; thus, the third reading cycle can be considered as the starting point of the reaction. The final results were calculated by using the differences of areas under the fluorescent decay curve between the blank and the sample (expressed as μmol Trolox Eq/L).

2.4. Statistical analysis

Serum isoflavone, fat-soluble antioxidant and ascorbic acid levels, and diet components were compared across treatment days using repeated-measures analysis of variance (ANOVA) with a significance level of $P\leq.05$ and using Tukey's honestly significant difference post hoc analysis to determine the differences between treatments over time. The effects of soy protein on measures of serum antioxidant capacity were also determined by ANOVA. Prior to ANOVA, it was determined that there was no interaction between treatment and time. Baseline, 4-h, 6-h and 8-h values were compared for each of the participants across the three test meals. Finally, Pearson's bivariate correlation coefficients were calculated to compare the five serum antioxidant capacity measurements across all three treatments at each time point. These statistical analyses were performed using SPSS 10.0 for Windows.

3. Results

Sixteen of the original 20 subjects completed the intervention. Three women and one man were removed prior to study completion due to inability to comply with the protocol. Initial repeated-measures ANOVA of baseline serum isoflavone levels revealed a statistical outlier, suggesting that this participant was also noncompliant. This subject was therefore removed from the data set, and data from 15 participants were included in the final analysis. No treatment order effect was observed for the data. Dietary analysis (data not shown) revealed no significant differences in the intake of total kilocalories, macronutrients or antioxidant-related micronutrients of the participants between treatment days.

A significant increase ($P\leq.05$) in serum levels of the isoflavones genistein and daidzein was observed in response to Soy^+ treatment (Table 1), reaching an average of 18.9±34.7 and 9.8±16.0 ng/100 μl serum, respectively, at the 8-h time point, as expected based on study design. Baseline serum levels of the antioxidants α-tocopherol, retinol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene,β-carotene and ascorbic acid did not differ significantly between treatment days (Table 2).

The antioxidant capacity of serum after consumption of protein treatments was assessed by five different methods. There were no significant differences between treatments using the whole serum, LDL oxidation, ORAC or FRAP

Table 2
Blood antioxidants

Treatment	α-Tocopherol (nmol/L)	Retinol (nmol/L)	Lutein/zeaxanthin (nmol/L)	β-Cryptoxanthin (nmol/L)
Soy^+	72.00 (11.60)	2.76 (1.01)	0.16 (0.08)	0.05 (0.04)
Soy^-	74.00 (17.00)	2.68 (1.01)	0.14 (0.07)	0.04 (0.02)
TMP	76.80 (13.10)	2.97 (1.40)	0.18 (0.01)	0.05 (0.05)

Treatment	Lycopene (nmol/L)	α-Carotene (nmol/L)	β-Carotene (nmol/L)	Ascorbic acid (mmol/L)
Soy^+	0.47 (0.17)	0.13 (0.07)	0.28 (0.13)	79.49 (17.03)
Soy^-	0.50 (0.30)	0.19 (0.13)	0.34 (0.26)	79.49 (28.39)
TMP	0.50 (0.20)	0.19 (0.13)	0.32 (0.15)	73.81 (28.39)

Mean (±S.D.) baseline values for serum fat-soluble antioxidants and plasma ascorbic acid. Baseline levels of the antioxidants α-tocopherol, retinol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene and vitamin C did not differ significantly between treatment days.
Significance ($P<.05$) was determined by three-factor ANOVA with Tukey–Kramer post hoc test.

Table 3
Antioxidant capacity values

Treatment	Baseline	4 h	6 h	8 h
Whole plasma oxidation susceptibility (lag time in min)				
Soy^+	131 (53)	128 (53)	140 (55)	145 (56)
Soy^-	140 (60)	142 (57)	149 (60)	153 (59)
TMP	130 (59)	118 (54)	131 (54)	139 (53)
LDL oxidation susceptibility (lag time in min)				
Soy^+	54 (22)	56 (24)	51 (17)	49 (15)
Soy^-	50 (17)	51 (12)	47 (17)	51 (18)
TMP	51 (17)	57 (27)	55 (17)	49 (12)
FRAP (μmol Trolox Eq/L)				
Soy^+	207 (32)	200 (31)	198 (31)	190 (30)
Soy^-	209 (35)	206 (35)	203 (33)	195 (31)
TMP	206 (37)	192 (33)	190 (33)	184 (33)
ORAC (μmol Trolox Eq/L)				
Soy^+	14,415 (1144)	15,206 (2186)	15,824 (1449)	15,751 (1559)
Soy^-	14,406 (1224)	14,927 (3225)	16,129 (1561)	16,156 (1754)
TMP	14,358 (847)	15,011 (1209)	15,287 (1054)	15,293 (1265)
PCA-ORAC (μmol Trolox Eq/L)				
Soy^+	1116 (136)	1182 (159)	1318 (126)	1250 (163)
Soy^-	1170 (138)	1235 (191)	1352 (114)	1346 (129)*
TMP	1068 (127)	1153 (102)	1263 (118)	1148 (133)*

Mean (±S.D.) change in antioxidant capacity values. There were no significant differences over time among the three protein treatments using whole plasma, LDL oxidation, FRAP or ORAC assay. PCA-ORAC was significantly ($P = .03$) different for Soy^- treatment compared to TMP treatment across the 8-h time period.

* Significance ($P < .05$) was determined by three-factor repeated-measures ANOVA with interaction term and Tukey–Kramer post hoc test.

assay (Table 3). However, serum PCA-ORAC values increased significantly over time in response to Soy^- treatment, in comparison to TMP treatment (Table 3) ($P = .03$).

Correlation analysis was used to compare the five measurements of serum antioxidant capacity. The whole serum/FRAP and ORAC/PCA-ORAC assays were the only pairs of assays to be correlated across all four time points (Table 4).

4. Discussion

Epidemiological and clinical trials suggest that regular consumption of soy is associated with favorable cardio-

Table 4
Correlation between antioxidant capacity values

	Baseline					4 h				
	Whole plasma	LDL oxidation	FRAP	ORAC	PCA-ORAC	Whole plasma	LDL oxidation	FRAP	ORAC	PCA-ORAC
Whole plasma	1.0	0.1	0.3*	−0.4**	0.4**	1.0	−0.1	0.3*	−0.1	0.1
LDL oxidation	0.1	1.0	0.1	0.2	0.2	−0.1	1.0	0.1	−0.1	0.1
FRAP	0.3*	0.1	1.0	0.0	0.1	0.3*	0.1	1.0	0.0	0.2*
ORAC	−0.4**	0.2	0.0	1.0	0.3**	−0.1	−0.1	0.0	1.0	0.3*
PCA-ORAC	0.4**	0.2	0.1	0.3**	1.0	0.1	0.1	0.2*	0.3*	1.0
	6 h					8 h				
	Whole plasma	LDL oxidation	FRAP	ORAC	PCA	Whole plasma	LDL oxidation	FRAP	ORAC	PCA
Whole plasma	1.0	0.4**	0.2*	−0.1	0.3*	1.0	0.4**	0.3*	0.1	0.2
LDL oxidation	0.4**	1.0	0.0	0.3*	0.3*	0.4**	1.0	0.2	0.0	0.1
FRAP	0.2*	0.0	1.0	0.2	0.1	0.3*	0.2	1.0	0.2	0.1
ORAC	−0.1	0.3*	0.2	1.0	0.2*	0.1	0.0	0.2	1.0	0.3*
PCA-ORAC	0.3*	0.3*	0.1	0.2*	1.0	0.2	0.1	0.1	0.3*	1.0

Correlation coefficients for antioxidant values. Pearson's bivariate correlation coefficients were calculated to compare the five oxidation measurements. Of the five methods, the whole plasma/FRAP and ORAC/PCA-ORAC assays were the only pairs of assays to be correlated across all four time points (baseline, 4, 6 and 8 h).

* $P < .05$.

** $P < .01$.

vascular risk factors, but the strength of these associations, their biologic mechanisms and their clinical significance remain somewhat controversial [6,46,47]. One potential mechanism behind cardioprotective effects is that soy imparts antioxidant protection; however, clinical trials investigating this hypothesis have yielded conflicting results [8,14,26–29]. To further explore the antioxidant theory, in the current study, the acute postprandial antioxidant protection imparted after the consumption of 25 g of soy protein, with or without isoflavones, was investigated in a cohort of healthy adults.

The results from our investigation suggest that consumption of soy protein, with or without isoflavones, results in only a very modest increase in postprandial antioxidant capacity. Statistically significant increases in plasma antioxidant capacity in comparison to the control TMP treatment were observed after participants have consumed Soy^{-} treatment using the PCA-ORAC assay. No other statistically significant changes were observed in antioxidant capacity, as assessed by a variety of methods. Other dietary antioxidants, such as ascorbic acid and fat-soluble vitamins, were not particularly high in this population based on exclusion criteria, dietary records and serum levels. Therefore, this was not felt to be a confounding factor in the results obtained. Additionally, the low antioxidant and flavonoid content of the lunch was not anticipated to substantially affect serum antioxidant concentrations. It is acknowledged, however, that there is no information in the literature regarding the effects of subsequent meals on isoflavone enterohepatic circulation or isoflavone kinetics within the 8-h postprandial period that we examined. Therefore, the possibility that the lunch affected the results cannot be excluded.

These findings are consistent with work completed by other research groups that investigated the effects of chronic soy consumption on antioxidant status in vivo [14,28–30]. When consumed in physiologic amounts by free-living populations, consisting of both hypercholesterolemic and normocholesterolemic individuals, soy protein appears to have modest to no effects on acute and chronic plasma antioxidant capacity. In the current study, the only significant effect was observed with Soy^{-} (rather than with Soy^{+}) preparations, indicating little contribution of isoflavones to the overall antioxidant effect. This is further supported by research failing to detect increased lag time to LDL oxidation after supplementation with isoflavone extracts [48,49]. Furthermore, in vitro work has demonstrated that high concentrations of isoflavones (levels unachievable through diet) must be reached for peroxidation inhibition to occur [15].

It has been suggested that methods for accurately measuring the total antioxidant capacity of plasma after consumption of soy protein or other polyphenol-rich foods have yet to be developed [50] or are not sufficiently sensitive or selective [51]. This may explain why significant antioxidant protection was only observed through the PCA-ORAC assay. Although correlations were observed between the whole plasma/FRAP assays (both use metal to induce oxidation in a relatively unprocessed blood sample, which still contains aqueous components contributing to antioxidant capacity) and the ORAC/PCA-ORAC assays (which only differ in the use of PCA to treat the sample), the lack of correlation between all five measurements of serum antioxidant capacity establishes that each method is distinct. It is not surprising that the susceptibility of isolated LDL to oxidation was not modified by the previous soy-containing meal, since insignificant amounts of isoflavones are carried via these lipoproteins. Indeed, the five methods evaluate antioxidant capacity in differing systems (i.e., aqueous whole plasma vs. isolated lipoproteins vs. small molecules). Additionally, ORAC and PCA-ORAC use AAPH as a peroxyl radical generator, whereas other methods use metal ions (either copper or iron) to induce oxidation. These differences contribute to the inherent difficulties of making statistical and biologic comparisons in the field of antioxidant research. Of the five assays used, the PCA-ORAC method is unique in that it measures the activity of low-molecular-weight antioxidants [52]. In this technique, plasma is pretreated with perchloric acid before analysis so that a protein-free fraction can be obtained. Albumin, which makes a major contribution to plasma antioxidant capacity, is removed from the sample, and subtle changes in antioxidant activity are detected. To our knowledge, this is the first investigation to use the PCA-ORAC assay to measure the antioxidant capacity of human plasma following a soy protein meal.

Alternatively, isoflavonoids may not impact in vivo antioxidant capacity since, in the plasma and urine of humans, over 75% of genistein and diadzein exist in conjugated form as glucuronide or sulfate [40,53]. Genistein and diadzein have only one hydroxyl group on the B-ring and thus have a lower antioxidant capacity compared to other flavones such as quercetin or myricetin, which have two hydroxyl groups on the B-ring [54]. Conjugation with sulfate or glucuronide occurs on these hydroxyl groups; thus, conjugation decreases the antioxidant capacity of the isoflavonoid metabolite. Conjugation can occur on more than one of the hydroxyl groups, and mixed conjugates of sulfate and glucuronide may also occur, all of which would even further lower any measured antioxidant capacity of isoflavonoid metabolites.

Compared to other flavonoids, the quantities of isoflavonoids that are apparently absorbed are much larger, since up to 30% of the amounts consumed appear in the urine [55]. This absorption will not likely be a limitation in altering in vivo antioxidant capacity. The combination of lower antioxidant capacity and extensive conjugation more likely accounts for lack of response following a soy meal.

In conclusion, consumption of soy protein was not associated with a significant increase in acute postprandial antioxidant capacity in four of five assay methods. Any

observed and putative in vivo health effects of soy consumption are likely derived through mechanisms other than direct antioxidant protection.

Acknowledgment

Protein products were provided by Solae Co.

References

[1] Zhou BF, Stamler J, Dennis B, et al. Nutrient intakes of middle-aged men and women in China, Japan, United Kingdom, and United States in the late 1990s: the INTERMAP study. J Hum Hypertens 2003;17:623–30.
[2] Ho SC, Woo JLF, Leung SSF, Sham ALK, Lam TH, Janus ED. Intake of soy products is associated with better plasma lipid profiles in the Hong Kong Chinese population. J Nutr 2000;130:2590–3.
[3] Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones, and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr 2000;130:2243–50.
[4] Nagata C, Takatsuka N, Kurisu Y, Shimizu H. Decreased serum total cholesterol is associated with high intake of soy products in Japanese men and women. J Nutr 1998;128:209–13.
[5] Setchell KD, Cassidy A. Dietary isoflavones: biological effects and relevance to human health. J Nutr 1999;129:758S–67S.
[6] Zhang X, Shu XO, Gao Y-T, et al. Soy food consumption is associated with lower risk of coronary heart disease in Chinese women. J Nutr 2003;133:2874–8.
[7] Crouse III JR, Morgan T, Terry JG, Ellis J, Vitolins M, Burke GL. A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. Arch Intern Med 1999; 159:2070–6.
[8] Jenkins DJ, Kendall CW, Jackson C-JC, et al. Effects of high- and low-isoflavone soyfoods on blood lipids, oxidized LDL, homocysteine, and blood pressure in hyperlipidemic men and women. Am J Clin Nutr 2002;76:365–72.
[9] Cuevas AM, Irribarra VL, Castillo OA, Yanez MD, Germain AM. Isolated soy protein improves endothelial function in postmenopausal hypercholesterolemic women. Eur J Clin Nutr 2003;57:889–94.
[10] Vigna GB, Pansini F, Bonaccorsi G, et al. Plasma lipoproteins in soy-treated postmenopausal women: a double-blind, placebo-controlled trial. Nutr Metab Cardiovasc Dis 2000;10:315–22.
[11] Baum JA, Teng H, Erdman Jr JW, et al. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. Am J Clin Nutr 1998;68:545–51.
[12] Lichtenstein AH, Jalbert SM, Adlercreutz H, et al. Lipoprotein response to diets high in soy or animal protein with and without isoflavones in moderately hypercholesterolemic subjects. Arterioscler Thromb Vasc Biol 2002;22:1852–8.
[13] Zhan S, Ho SC. Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile. Am J Clin Nutr 2005;81: 397–408.
[14] Steinberg FM, Guthrie NL, Villablanca AC, Kumar K, Murray MJ. Soy protein with isoflavones has favorable effects on endothelial function that are independent of lipid and antioxidant effects in healthy postmenopausal women. Am J Clin Nutr 2003;78:123–30.
[15] Patel RP, Boersma BJ, Crawford JH, et al. Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging. Free Radic Biol Med 2001;31:1570–81.
[16] Kerry N, Abbey M. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation in vitro. Atherosclerosis 1998;140:341–7.
[17] Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 1998;360: 142–8.
[18] Boersma BJ, Patel RP, Kirk M, et al. Chlorination and nitration of soy isoflavones. Arch Biochem Biophys 1999;368:265–75.
[19] Kameoka S, Leavitt P, Chang C, Kuo S-M. Expression of antioxidant proteins in human intestinal Caco-2 cells treated with dietary flavonoids. Cancer Lett 1999;146:161–7.
[20] Lai HH, Yen GC. Inhibitory effect of isoflavones on peroxynitrite-mediated low-density lipoprotein oxidation. Biosci Biotechnol Biochem 2002;66:22–8.
[21] Suzuki K, Koike H, Matsui H, et al. Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. J Cancer 2002;99:846–52.
[22] Guo Q, Rimbach G, Moini H, Weber S, Packer L. ESR and cell culture studies on free radical-scavenging and antioxidant activities of isoflavonoids. Toxicology 2002;179:171–80.
[23] Hwang J, Wang J, Morazzoni P, Hodis HN, Sevanian A. The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification. Free Radic Biol Med 2003;34:1271–82.
[24] Simons LA, von Konigsmark M, Simons J, Celermajer DS. Phytoestrogens do not influence lipoprotein levels or endothelial function in healthy, postmenopausal women. Am J Cardiol 2000; 85:1297–301.
[25] Hsu C-S, Shen WW, Hsueh Y-M, Yeh S-L. Soy isoflavone supplementation in postmenopausal women: effects on plasma lipids, antioxidant enzyme activities and bone density. J Reprod Med 2001; 46:221–6.
[26] Tikkanen MJ, Wahala K, Ojala S, Vihma V, Adlercreutz H. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proc Natl Acad Sci U S A 1998;95:3106–10.
[27] Wiseman H, O'Reilly JD, Adlercreutz H, et al. Isoflavone phytoestrogens consumed in soy decease F_2-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. Am J Clin Nutr 2000;72:395–400.
[28] Swain JH, Alekel DL, Dent SB, Peterson CT, Reddy MB. Iron indexes and total antioxidant status in response to soy protein intake in perimenopausal women. Am J Clin Nutr 2002;76:165–71.
[29] Engelman HM, Alekel DL, Hanson LN, Kanthasamy AG, Reddy MB. Blood lipid and oxidative stress responses to soy protein with isoflavones and phytic acid in postmenopausal women. Am J Clin Nutr 2005;81:590–6.
[30] Vega-Lopez S, Yeum KJ, Lecker JL, et al. Plasma antioxidant capacity in response to diets high in soy or animal protein with or without isoflavones. Am J Clin Nutr 2005;81:43–9.
[31] Gianazza E, Eberini I, Arnoldi A, Wait R, Sitori CR. A proteomic investigation of isolated soy proteins with variable effects in experimental and clinical studies. J Nutr 2003;133:9–14.
[32] Greaves KA, Wilson MD, Rudel LL, Williams JK, Wagner JD. Consumption of soy protein reduces cholesterol absorption compared to casein protein alone or supplemented with an isoflavone extract or conjugated equine estrogen in ovariectomized cynomolgus monkeys. J Nutr 2000;130:820–6.
[33] Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol — a clue to the effectiveness of soy and its isoflavones. J Nutr 2002;132:3577–84.
[34] Prior RL, Hoang H, Gu L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. J Agric Food Chem 2003;51:3273–9.
[35] Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 1998; 44:1309–15.
[36] Cao G, Verdon CP, Wu AHB, Wang H, Prior RL. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clin Chem 1995;41:1738–44.

[37] King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. Am J Clin Nutr 1998;67:867–72.
[38] Setchell KDR. Absorption and metabolism of soy isoflavones — from food to dietary supplements and adults to infants. J Nutr 2000;130: 654S–655S.
[39] Setchell KD, Brown NM, Desai P, et al. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr 2001;131:1362S–75S.
[40] Cimino CO, Shelnutt SR, Ronis MJJ, Badger TM. An LC/MS method to determine concentrations of isoflavones and their sulfate and glucuronide conjugates in urine. Clin Chim Acta 1999;287:69–82.
[41] Sowell AL, Huff DL, Yeager PR, Caudill SP, Gunter EW. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, *trans*-β-carotene and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin Chem 1994;40:411–6.
[42] Schnitzer E. Lipid oxidation in unfractionated serum and plasma. Chem Phys Lipids 1998;92:151–70.
[43] Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radic Res Commun 1989;6:67–75.
[44] Brousseau T, Clavey V, Bard J, Fruchart J. Sequential ultracentrifugation micromethod for separation of serum lipoproteins and assays of lipids, apolipoproteins, and lipoprotein particles. Clin Chem 1993;39: 960–964.
[45] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70–6.
[46] Weggemans RM, Trautwein EA. Relation between soy-associated isoflavones and LDL and HDL cholesterol concentrations in humans: a meta-analysis. Eur J Clin Nutr 2003;57:940–6.
[47] de Kleijn MJ, van der Schouw YT, Wilson PWF, Grobbee DE, Jacques PF. Dietary intake of phytoestrogens is associated with a favorable metabolic cardiovascular risk profile in postmenopausal US women: the Framingham Study. J Nutr 2001;132: 276–82.
[48] Samman S, Wall PML, Chan GSM, Smith SJ, Petocz P. The effect of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in premenopausal women. Atherosclerosis 1999;147:277–83.
[49] Hodgson JM, Puddey IB, Croft KD, Mori TA, Rivera J, Beilin LJ. Isoflavonoids do not inhibit in vivo lipid peroxidation in subjects with high-normal blood pressure. Atherosclerosis 1999; 145:167–72.
[50] Kris-Etherton PM, West SG. Soy protein with or without isoflavones: in search of a cardioprotective mechanism of action. Am J Clin Nutr 2005;81:5–6.
[51] Collins AR. Assays for oxidative stress and antioxidant status: applications to research into the biological effectiveness of polyphenols. Am J Clin Nutr 2005;81:261S–7S.
[52] Cao G, Shukitt-Hale B, Bickford PC, Joseph JA, McEwen J, Prior RL. Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. J Appl Physiol 1999;86: 1817–22.
[53] Zhang Y, Hendrich S, Murphy PA. Glucuronides are the main isoflavone metabolites in women. J Nutr 2003;133:399–404.
[54] Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure–activity relationships. Free Radic Biol Med 1997;22:749–60.
[55] Shelnutt SR, Cimino CO, Wiggins PA, Ronis MJ, Badger TM. Pharmacokinetics of the glucuronide and sulfate conjugates of genistein and daidzein in men and women after consumption of a soy beverage. Am J Clin Nutr 2002;76:588–94.