A Soy Protein Isolate Rich in Genistein and Daidzein and Its Effects on Plasma Isoflavone Concentrations, Platelet Aggregation, Blood Lipids and Fatty Acid Composition of Plasma Phospholipid in Normal Men

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ABSTRACT The effects of consuming a soy protein isolate beverage powder (60 g/d for 28 d) vs. a casein supplement was evaluated in 20 male subjects who were randomly allocated into the two groups. A dramatic rise in plasma isoflavone concentrations was observed after supplementation in the soy protein group, the levels reaching 907 ± 245 nmol/L for genistein (a 110-fold increase) and 498 ± 102 nmol/L for daidzein (a 150-fold increase) as measured by isotope dilution gas chromatography - mass spectrometry. These concentrations are higher than previously reported for the plasma of Japanese subjects consuming a traditional diet (276 nmol/L and 107 nmol/L, respectively). No significant differences in collagen- or 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin F₂α (U46619)-induced platelet aggregation were observed in platelet-rich plasma from the two groups; the increase in plasma isoflavonoids from soy protein supplementation is not sufficient to significantly inhibit platelet aggregation ex vivo. Similarly, plasma total and HDL-cholesterol were not affected by protein supplementation, possibly because the men were normocholesterolemic at entry. Analysis of plasma phospholipid polyunsaturated fatty acid composition showed no differences between soy protein and casein supplementation. Previous investigations reported a significant alteration in fatty acid status in animals fed soy protein relative to those fed casein. The present studies indicate that although soy protein supplementation to a typical Western diet can increase plasma concentrations of isoflavones, this may not necessarily be sufficient to counter heart disease risk factors such as high plasma cholesterol and platelet aggregation. J. Nutr. 126: 2000–2006, 1996.

INDEXING KEY WORDS:
• humans • soy protein • isoflavones • platelet • plasma cholesterol

In the past two decades, investigators have focused on the blood lipid lowering effect of soy protein (Carroll 1991, Carroll and Kurowska 1995). A more recent interest in soy protein stems from the reports on high concentrations of isoflavonoids, particularly genistein and daidzein, which are diphenolic compounds found in soybeans (Dewick 1988). These compounds are thought to provide cancer-protective effects and their potential health benefits have been reviewed (Adlercreutz et al. 1995, Barnes 1995, Setchell and Adlercreutz 1988). It has also been reported that isoflavones can inhibit in vitro platelet aggregation [Gaudette and Holub 1990]. Because the role of tyrosine phosphorylation in platelet responses has gained importance (Thomas and Holub 1992), this inhibition of platelet aggregation was attributed to the inhibition of tyrosine kinases by genistein [Akiyama et al. 1987]. However, it has been reported that genistein and daidzein could also inhibit platelet aggregation by acting as thromboxane A₂ receptor antagonists [McNicol 1992, Nakashima et al. 1991].

To date, no feeding trials >1 d have been published which evaluated the effects of regular daily consumption of a soy protein isolate on plasma concentrations of isoflavones or on platelet aggregation as measured in ex vivo studies. Previous reports of soy protein sup-

1 Supported by the Heart and Stroke Foundation of Ontario and the Ontario Ministry of Agriculture and Food and Rural Affairs.
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plementation include plasma concentrations of isoflavones after one dose [Xu et al. 1994] and urinary excretion after one to several days of supplementation [Cassidy et al. 1994, Hutchins et al. 1995, Kelly et al. 1994, Xu et al. 1994]. Epidemiological studies report isoflavone concentrations in urine and plasma of the Japanese compared with subjects consuming a Western diet [Adlercreutz et al. 1986, 1991 and 1993b]. The present study examined the effect of consuming a soy protein isolate for 28 d on plasma concentrations of genistein and daidzein in healthy men.

It was of further interest in the present study to evaluate the potential impact of soy protein vs. casein on the polyunsaturated fatty acid status in humans [the fatty acid composition of plasma phospholipid] based on evidence from animal studies which have shown a consistent effect on fatty acid desaturation when soy protein is fed rather than casein (Sugano et al. 1988, Terasawa et al. 1994). It was reported that there is a decreased activity of Δ5-desaturase [mainly in n-6 metabolism] and an increased conversion of eicosapentaenoic acid [EPA, 20:5n-3] to docosapentaenoic acid [DPA, 22:5n-3] (Terasawa et al. 1994). Although the importance of these alterations is not known, it has been suggested that they affect eicosanoid metabolism in rats (Sugano et al. 1988). Recently it was indicated that these effects may be beneficial for humans, namely, vegetarians, who consume soy regularly (Hubbard et al. 1994). However, no controlled study addressing these effects on humans has previously been reported.

The present results indicate that there was a marked increase in plasma isoflavone concentrations (after 28 d of consuming a soy protein supplement) and a reversibility of these concentrations to basal levels during an equivalent washout period. Despite the high plasma concentrations of genistein and daidzein attained, there were no significant differences in platelet aggregation, total cholesterol and HDL-cholesterol and plasma phospholipid fatty acid compositions were unaffected.

### SUBJECTS AND METHODS

**Subjects and experimental design.** The subjects were 20 healthy male volunteers selected from the staff and students of the University of Guelph who reported on a screening questionnaire eating <1 soy meal/wk. Approval for the study was granted by the Human Ethics Committee of the University of Guelph and written informed consent was obtained from each subject. The 20 subjects were randomly assigned to two protein supplementation groups, experimental and control. The

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4 Abbreviations used: DPA, docosapentaenoic acid, EPA, eicosapentaenoic acid; PPP, platelet-poor plasma; PRP, platelet-rich plasma; U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α.
ean, Canada) as an anticoagulant. Whole blood was centrifuged at 200 \( \times \) g for 17 min to obtain platelet-rich plasma (PRP) which was removed (~40 mL); the remaining blood was centrifuged at 1250 \( \times \) g for 15 min to obtain platelet-poor plasma (PPP) (Tremoli et al. 1995). The PRP was used in aggregation studies, and the PPP was used for the measurement of plasma total and HDL-cholesterol levels, plasma phytoestrogen levels, and plasma total phospholipid fatty acid composition. The PPP was stored at \(-20^\circ\)C until all samples were collected and thawed just before analyses.

**Phytoestrogen measurement.** Plasma genistein, daidzein, enterolactone and enterodiol were measured by isotope dilution gas chromatography-mass spectrometry (GC/MS) in the selected ion monitoring mode by Dr. H. Adlercreutz (University of Helsinki, Finland) as described previously (Adlercreutz et al. 1993a and 1994) including detailed presentation of the reliability of the method. Minor modifications were made so that smaller volumes of plasma samples could be used.

**Platelet aggregation.** Platelet aggregation was performed within 2 h of blood collection. Platelets were counted in PRP using a Coulter Counter model ZM (Coulter Electronics, Burlington, Canada) and adjusted to a final concentration of 2.5 \( \times \) 10\(^5\) platelets/L using autologous PPP. Aliquots (0.5 mL) of adjusted PRP were preincubated for 1 min in siliconized cuvettes with stirring at 900 rpm at 37\(^\circ\)C in a dual-channel 800B aggregometer (Payton Instruments, Ion Trace, Scarborough, Canada) before addition of the aggregating agent. Collagen (Hormone-Chemie, Munchen, Germany) was added at three levels with the final concentrations of 10, 3, 1.5 mg/L and the thromboxane analogue 9,11-dideoxy-11\(\alpha\),9\(\alpha\)-epoxymethanoprostaglandin F\(_{2\alpha}\) (U46619) (Upjohn, Kalamazoo, MI) was added at two levels with the final concentrations of 600 nmol/L and 1 \(\mu\)mol/L. The platelets were allowed to aggregate for 2 min following the addition of agonist and then the level of aggregation was measured (Born 1962).

**Cholesterol measurement.** Total cholesterol was measured enzymatically with a diagnostic test (Sigma Diagnostics Procedure No. 352, St. Louis, MO). HDL-cholesterol was isolated by using a dextran sulfate and magnesium ion solution to precipitate the VLDL and LDL from the PPP sample. The HDL fraction was then assayed by an enzymatic assay (Sigma Diagnostics Procedure No. 352-3). Analysis of all time points for each subject was performed in a single assay. The results were adjusted for the dilution by the anticoagulant.

**Plasma total phospholipid analysis.** The lipids were first extracted from PPP samples by a modified method of Bligh and Dyer (1959). Five milliliters chloroform/methanol (2:1, v/v) and 0.9 mL water were added to 50 \(\mu\)L of plasma samples, vortexed and refrigerated overnight. The mixture was centrifuged at 1200 \(\times\) g for 5 min and the chloroform phase, containing the total lipid fraction, was removed. The phospholipid fraction was isolated from the extracted lipids by thin layer chromatography in a neutral lipid solvent system (heptane/isopropanol ether/acidic acid, 60:40:2, v/v/v) (Mercer and Holub 1979) using Silica Gel 60 HR plates (C. Merck, Darmstadt, Germany). Fatty acid methyl esters were formed by the addition of 2 mL of 6% \(\text{H}_2\text{SO}_4\) in methanol and stored at \(80^\circ\)C for 3 h. To extract the methyl esters, 2 mL petroleum ether and 1 mL water were added, vortexed and centrifuged at 1200 \(\times\) g for 5 min and the petroleum ether phase removed. Fatty acid methyl ester derivatives formed from the isolated plasma phospholipid fraction were analyzed by gas-liquid chromatography to determine the fatty acid content as described previously (Holub and Skeaff 1987).

**Statistical analysis.** All data are reported as means \(\pm\) SEM. Data that were not normally distributed were transformed (log or square) before analyses to achieve normality. When observations were missing, least-squared means were calculated so that means could be compared. Split-plot design including time and treatment as factors was used in all analyses except for comparison between dietary intakes when a t test was performed (Kirk 1968). Statistical analyses were done using the SAS system (SAS Institute, Cary, NC).

## RESULTS

Plasma genistein and daidzein concentrations were not different between the groups at d 0 (Table 2). A dramatic increase was observed in the experimental group at the end of the soy supplementation period (d 28) but no change was seen in the control group. After the washout period (d 56), the levels of the isoflavones in the soy-supplemented group had returned to basal levels and were not different from the control group. The plasma enterodiol and enterolactone concentrations were not different between groups at any time point during the study and were not affected by the protein supplementation in either group (Table 2). The isoflavonoid metabolites were not measured in this study.

The level of platelet aggregation was not significantly different between groups at entry. The level of aggregation postsupplementation (d 28) was not significantly different from d 0 or 56 for either treatment group and was not significantly different between the groups (Fig. 1) at any level of collagen (10, 3, 1.5 mg/L) or U46619 (1 \(\mu\)mol/L) tested.

The plasma total and HDL-cholesterol concentrations at entry were 4.40 \(\pm\) 0.31 mmol/L and 1.14 \(\pm\) 0.05 mmol/L for the soy protein isolate group and 4.35 \(\pm\) 0.21 mmol/L and 0.96 \(\pm\) 0.06 mmol/L for the casein group, respectively. These concentrations were not significantly altered after the 28 d of soy protein or casein feeding.

Both treatment groups had similar plasma total phospholipid fatty acid compositions at entry (Table
TABLE 2

Plasma phytoestrogen concentrations in men before and after soy protein isolate or casein supplementation1

<table>
<thead>
<tr>
<th></th>
<th>Soy-supplemented</th>
<th>Casein-supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td></td>
<td>nmol/L</td>
<td>nmol/L</td>
</tr>
<tr>
<td>Daidzein</td>
<td>3.43 ± 1.6a</td>
<td>498.02 ± 101.7b</td>
</tr>
<tr>
<td>Genistein</td>
<td>8.20 ± 2.7a</td>
<td>906.79 ± 245.0b</td>
</tr>
<tr>
<td>Enterolactone</td>
<td>13.9 ± 3.6</td>
<td>15.3 ± 6.0</td>
</tr>
<tr>
<td>Enterodiol</td>
<td>1.1 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 10. Data were log transformed prior to analyses. Values in a row with different superscripts are significantly different (P < 0.05).

3). No significant differences between the two groups at d 28 (postsupplementation) were observed in any of the fatty acids examined. Also included in Table 3 are relevant ratios of fatty acids which provide the desaturation indices of certain pathways. There was no significant effect of type of protein supplementation on the corresponding ratios [(22:5 + 22:6)/20:5, (20:3 + 20:4)/18:2] which were calculated.

DISCUSSION

The present investigation examined the effects of a soy protein supplement (rich in genistein and daidzein) on certain variables compared with a casein supplement which served as a control. A unique feature of this study is that, to our knowledge, this is the first report of plasma isoflavones in a controlled intervention trial in which soy protein was fed over an extended interval. Soy protein supplementation at 60 g/d dramatically increased plasma concentrations of genistein and daidzein whereas lignan concentrations were not altered. Lignans are generally found in grains such as flax and rye but are not present in purified soy protein. These results agree with other related studies which have shown an increase in urinary isoflavonoid excretion after a soy challenge (Cassidy et al. 1994, Hutchins et al. 1995, Kelly et al. 1994, Xu et al. 1994) and with reports of plasma concentrations in subjects consuming a traditional Japanese diet (Adlercreutz et al. 1991 and 1993b). It is noteworthy that the genistein and daidzein concentrations observed in this controlled study [e.g., mean values of 907 nmol/L and 498 nmol/L, respectively] are higher than those reported previously in the plasma of Japanese men (Adlercreutz et al. 1993b), 276 nmol/L and 107 nmol/L, respectively. Japanese men may have genistein concentrations in plasma exceeding 2000 nmol/L [Adlercreutz, unpublished]. This study also reports the reversibility of these high concentrations to basal levels during a washout period of length equal to the period of supplementation [d 28]. The high concentrations observed in the soy-supplemented group confirm the compliance of these volunteers because genistein and daidzein are limited in nature to the *Leguminosae* family with the greatest amounts found in the soybean (Dewick 1988). Conversely, the low levels reported in the casein-supplemented group and basally in the soy-supplemented group confirm that the volunteers were not consuming a considerable amount of soy products in their diet. Further, although the increase of the isoflavones in the plasma of the soy protein group was great, it was quite variable among subjects. This could be due to the timing of the soy protein consumption [Xu et al. 1994] or the composition of the gut flora [Axelson et al. 1984]. The metabolism of isoflavones in the gut is variable among individuals and remains to be elucidated; it is currently the focus of many investigations.
Despite the studies which have shown that genistein and daidzein could inhibit platelet aggregation in vitro [Gaudette and Holub 1990, McNicol 1992, Nakashima et al. 1991], the concentration of these compounds in human plasma was not high enough to cause a significant inhibition of platelet aggregation in this ex vivo aggregation study. The isoflavones used to inhibit aggregation in the in vitro studies are in the free form only and are generally at concentrations higher than those which can be reached in the plasma through soy protein consumption [Gaudette and Holub 1990, McNicol 1992, Murphy et al. 1993, Nakashima et al. 1991]. Conversely, the isoflavones in human plasma exist mainly in the inactive glucuronide conjugated form, and only a small amount (~10%) exists in the active free and sulphate conjugated forms [Adlercreutz et al. 1993a, 1993b and 1994]. In the present study there was a slight tendency toward inhibition of platelet aggregation at two levels of collagen (10 and 3 mg/L) and U46619 (1 μmol/L) that did not reach statistical significance. Future studies are indicated to determine if a larger group of subjects or a higher plasma concentration of isoflavones is required to reach a statistically significant inhibition of platelet aggregation in ex vivo studies.

The lack of effect on total cholesterol concentrations in the soy protein group is in agreement with others who found that soy had little effect in normocholesterolemic individuals [Carroll 1991]. As reviewed by Carroll [1991] and Carroll and Kurowska [1995], hypercholesterolemic subjects generally exhibit a decrease in total and LDL-cholesterol after soy protein consumption relative to normocholesterolemic subjects. It is possible that we would have seen a lowering of the total cholesterol levels had the volunteers been hyperlipidemic at entry. Only a few studies have reported an HDL-cholesterol raising effect due to the consumption of soy protein; most studies have shown little or no effect on HDL-cholesterol levels [Carroll 1991]. The present results indicate a similar lack of effect of soy protein on HDL-cholesterol levels in normocholesterolemic volunteers. Only recently have isoflavones been examined separately to determine if these compounds are responsible for the lipid lowering effects of soy. The administration of purified isoflavones to animals has shown variable results on blood lipids [Anthony et al. 1995, Balmir et al. 1995, Kurowska et al. 1994]. One study conducted on hypercholesterolemic humans failed to show an effect of purified isoflavones on blood lipid levels [Colquhoun et al. 1994].

There are fairly consistent reports concerning the impact of soy protein, relative to casein, on fatty acid desaturation in rodent studies [Sugano et al. 1988, Terasawa et al. 1994]. It has been suggested that a similar effect may occur in vegetarians who consume soy protein and that this may be beneficial to these individuals through an impact on eicosanoid metabolism [Hubbard et al. 1994]. Because the effect in humans had not been addressed previously, the present study included analyses of the plasma total phospholipid (fatty acid) composition in soy protein isolate– vs. casein-supplemented subjects. We could not reproduce any of the effects on fatty acid desaturation observed in rats in the soy protein–supplemented humans relative to those who consumed a casein supplement. Despite some minor alterations in the fatty acid profile that occurred after protein supplementation, none of these changes were similar to those reported in rats. The type of protein

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Soy-supplemented</th>
<th>Casein-supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>16:0</td>
<td>30.6 ± 0.7ab</td>
<td>30.0 ± 0.9ab</td>
</tr>
<tr>
<td>18:0</td>
<td>13.8 ± 0.3</td>
<td>14.1 ± 0.4</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>12.9 ± 0.5</td>
<td>13.6 ± 0.3</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>19.1 ± 0.7</td>
<td>18.8 ± 0.8</td>
</tr>
<tr>
<td>20:3(n-6)</td>
<td>2.3 ± 0.2a</td>
<td>2.6 ± 0.2b</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>8.5 ± 0.3ab</td>
<td>8.0 ± 0.3a</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>0.60 ± 0.08a</td>
<td>0.80 ± 0.11b</td>
</tr>
<tr>
<td>22:4(n-6)</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>22:5(n-3)</td>
<td>0.55 ± 0.06a</td>
<td>0.66 ± 0.04ab</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>(n-6)/(n-3) ratio</td>
<td>3.2 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>20:3(n-6) + 20:4(n-6)/18:2(n-6)</td>
<td>0.6 ± 0.03</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>22:5(n-3) + 22:6(n-3)/20:5(n-3)</td>
<td>5.6 ± 0.7a</td>
<td>4.5 ± 0.6b</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 10. Values in a row with different superscripts are significantly different, P < 0.05.
supplemented had little effect on the fatty acid profile of the subjects. Therefore, it appears that soy supplementation is not exerting an effect on fatty acid desaturation in humans and will not likely affect eicosanoid metabolism as was previously suggested for rats [Sugano et al. 1988]. This does not rule out a beneficial effect in vegetarians because the present study included only omnivorous volunteers consuming a protein supplement.

In summary, there was a dramatic increase in plasma concentrations of isoflavones after a period of regular soy supplementation, and an accompanying decrease in these concentrations after a washout period. These concentrations, while surpassing plasma levels reported in Japanese subjects consuming traditional soy foods, were not sufficient to cause a significant reduction of platelet aggregation. In addition, the soy supplementation had no effect on the total or HDL-cholesterol concentrations of the normocholesterolemic subjects or their polyunsaturated fatty acid status [n-6 and n-3 families] as measured in the plasma phospholipid fraction. It is possible that any effects of the soy supplementation were masked by the high fat, low fiber Western diet that the subjects typically consumed. Future studies might consider substitution of soy rather than supplementation or might include soy with a low fat, high fiber diet to determine any interactive effects.

ACKNOWLEDGMENTS

We would like to thank Margaret Berry, Indu Mani, Julie Conquer, Marco Turini and Diana Philbrick for their help in all aspects of this investigation. We would also like to thank E. C. Henly and Lisa Unlu at Protein Technologies International [St. Louis, MO] for supplying the protein supplements which made this investigation possible.

LITERATURE CITED


