The Inhibitory Effect of Soy Protein Isolate on Atherosclerosis in Mice Does Not Require the Presence of LDL Receptors or Alteration of Plasma Lipoproteins

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ABSTRACT The mechanisms by which dietary soy favorably influences lipoprotein metabolism and inhibits atherosclerosis are uncertain. Studies of blood mononuclear cells and cultured hepatocytes have indicated that certain soy peptides (i.e., 7S globulins) stimulate expression of LDL receptors. This pathway represents a hypothetical mechanism by which soy’s hypocholesterolemic and antiatherosclerotic effects may be mediated. However, direct evidence supporting this hypothesis is lacking. To address this, we compared effects of dietary soy protein isolate in two genetically engineered mouse models of atherosclerosis. One mouse [LDL receptor –/- + apolipoprotein (apo) B transgenic] is devoid of LDL receptors and overproduces apolipoprotein B, whereas the other (apoE –/–) has a normal complement of LDL receptors but does not produce apolipoprotein E. Male (n = 10–12/group) and ovariectomized female (n = 10–12/group) mice were studied. There were three treatment groups, which differed principally by the source of the protein component of the diet: 1) casein/lactalbumin (no isoflavones), 2) alcohol-washed soy protein isolate (total isoflavones = 0.04 mg/g), and 3) intact soy protein isolate (total isoflavones = 1.72 mg/g). Atherosclerosis was assessed by quantifying the aortic content of esterified cholesterol. Atherosclerosis was inhibited (relative to the casein/lactalbumin group) by both alcohol-washed (45 and 31%) (P < 0.05) and intact (65 and 41%) (P < 0.05) soy protein isolate in LDL receptor –/- and apoE –/– mice, respectively. There was no sex difference. In a two-way analysis, there were significant effects of type of soy isolate and type of mouse. The antiatherosclerosis effect was enhanced in LDL receptor –/- mice (P < 0.001) and diminished in mice fed alcohol-washed soy protein isolate (P < 0.001). Furthermore, inhibitory effects of soy on atherosclerosis were unrelated to plasma LDL, VLDL or HDL cholesterol concentrations. The results represent direct evidence for the existence of LDL receptor- and plasma lipoprotein-independent pathways by which dietary soy protein isolate inhibits atherosclerosis.

Key Words: • atherosclerosis • mice • soy protein • isoflavones • lipoproteins

Nutrient-Gene Expression

Diet-induced atherosclerosis is reduced in animals fed soy protein-based diets compared with those fed animal protein-based diets (1). Yet, the mechanisms involved remain unknown. Although substantial evidence implicates favorable effects on lipoprotein metabolism (1–4), the components of soy responsible for these effects and the pathways involved remain to be identified. Studies of blood mononuclear cells (5) and cultured hepatocytes (6–8) have indicated that certain peptide components of soy protein (i.e., 7S globulins) stimulate expression of LDL receptors. Although direct evidence is lacking, it is also possible that soy isoflavones may, through their estrogen agonist activity, upregulate LDL receptor expression. This represents a hypothetical pathway by which soy or its isoflavones may exert hypocholesterolemic and antiatherosclerotic effects. This hypothesis is supported by the results of a study in which dietary soy protein was ineffective in lowering total plasma cholesterol or inhibiting diet-induced atherosclerosis in LDL receptor null mice in which a greatly exaggerated hypercholesterolemia was induced by diet (9). However, confirmatory evidence for this finding is lacking.

There is also evidence that an insoluble “high-molecular-weight fraction” of digested soy protein may act as a bile acid sequestrant to enhance bile acid excretion and thereby increase the catabolism of LDL and lower plasma LDL cholesterol (10–12). This represents another pathway by which the consumption of soy or soy products could exert a hypocholesterolemic effect and inhibit atherosclerosis.

There is also substantial evidence supporting the existence of lipoprotein-independent pathways by which soy inhibits atherosclerosis [reviewed in (13)]. For example, soy isoflavones may directly inhibit atherosclerosis through estrogen receptor-mediated effects on tyrosine kinase activity, macrophage cytokine expression, the migration or proliferation of arterial smooth muscle cells, endothelium-dependent arterial dilation, arterial elasticity and platelet aggregation or activation (13).
Soy isoflavones also have potent antioxidant activity and may therefore inhibit atherosclerosis progression by reducing LDL oxidation (14–18). Direct evidence for the existence of lipoprotein-independent antihernihibitory effects of soy isoflavones is provided by the findings of Yamakoshi et al. (14) and Ni et al. (19). In the Yamakoshi study (14), rabbits were fed atherogenic diets containing protein of nonsoy origin to which isoflavone (primarily genistein and daidzein) concentrates were added. Although plasma lipoprotein patterns were unaffected, atherosclerosis extent was reduced 50 and 90% in the aortic arch and thoracic aorta, respectively, in the rabbits fed the highest isoflavone doses. Ni et al. (19) studied the effects of dietary soy protein isolate on atherosclerosis in apolipoprotein (apo)E−/− mice. In these studies, despite a tendency for an elevation in total plasma cholesterol concentration in mice fed soy protein, aortic atherosclerosis was reduced in mice fed isoflavone-intact soy protein and was unaffected in mice fed isoflavone-deficient soy protein.

The purpose of this study was to assess the respective roles of LDL receptors and soy isoflavones in mediating the antitheroinhibitory effects of soy protein isolate using genetically engineered mouse models of atherosclerosis. We compared the effects of intact and alcohol-washed (i.e., isoflavine-deficient) soy protein isolate on plasma lipoproteins and atherosclerosis in mice devoid of LDL receptors (LDL receptor −/− plus apoB transgenic) and mice with a normal complement of LDL receptors (apoE −/−). The results indicate that soy protein has antitheroinhibitory effects that are enhanced by the presence of its natural isoflavones and that do not require the presence of LDL receptors or effects on plasma lipoproteins.

**MATERIALS AND METHODS**

**Animals and diets.** The mice used in these studies were bred and reared in our animal facility, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. All procedures involving animals were approved by the institutional animal care and use committee. The original breeding pair of LDL receptor −/−, human apoB transgenic mice (20) was provided by Dr. Helen Hobbs, Departments of Internal Medicine and Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX. This mouse is a hybrid cross between the LDL receptor −/− mice (21), which itself is a hybrid of 129sv and C57BL/6 strains, and the human apoB transgenic mouse (22), a hybrid of SJL and C57BL/6B strains. ApoE −/− mice (23), backcrossed > 99% to C57BL6/J, were provided by Dr. Nobuyo Maeda, Department of Pathology and Laboratory Medicine, University of North Carolina-Chapel Hill, Chapel Hill, NC.

At 6 wk of age, male and ovariectomized female mice were assigned randomly to one of three diet groups. There were 10–12 mice of each sex in each diet group. The diets are described in Table 1. At 6 wk of age, male and ovariectomized female mice were assigned randomly to one of three diet groups. There were 10–12 mice of each sex in each diet group. The diets are described in Table 1. The principal difference between the three diets was the source of the protein component as follows: group 1, casein and lactalbumin (control diet devoid of soy protein and isoflavones); group 2, ethanol-extracted soy protein isolate (total isoflavone content = 0.04 mg/g of isolate); and group 3, intact soy protein isolate (total isoflavone content = 1.72 mg/g of isolate). Soy protein–containing diets were supplemented with l-α-methionine (0.3 g/100 g). To equalize total plasma cholesterol concentrations in the two types of mice, the diet fed to the apoE −/− mice was supplemented with crystalline cholesterol (0.14 g/100 g). Intact and ethanol-extracted soy protein isolates were provided by Protein Technologies International, St. Louis, MO.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>Soy (−IF)</th>
<th>Soy (+IF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg dry weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>105.0</td>
<td>105.0</td>
<td>105.0</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Soy (−IF)</td>
<td>202.8</td>
<td>202.8</td>
<td>202.8</td>
</tr>
<tr>
<td>Soy (+IF)</td>
<td>211.8</td>
<td>211.8</td>
<td>211.8</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Dextrin</td>
<td>306.0</td>
<td>306.0</td>
<td>300.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>280.0</td>
<td>280.0</td>
<td>280.0</td>
</tr>
<tr>
<td>Alphacel</td>
<td>100.0</td>
<td>102.6</td>
<td>100.0</td>
</tr>
<tr>
<td>Lard</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>10.0</td>
<td>6.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin mixture2</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Mineral mixture2</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>

1 Soy protein isolates (−IF = isoflavine deficient, +IF = isoflavone intact) were provided by Protein Technologies International, St. Louis, MO. Composition of −IF (g/kg): protein, 878; moisture, 53; fat, 36; ash, 42; +IF: protein, 841; moisture, 49; fat, 6; ash, 43. 2 Vitamin mixture ALN-76A (65,66) and mineral mixture ALN-76 (66) were obtained from Harlan Teklad, Madison, WI.

**Plasma lipoproteins and atherosclerosis.** For plasma lipoprotein separations, plasma was injected onto a 30-cm Superose 6 chromatography column that is run at 0.5 mL/min with 9 g/L NaCl containing 0.05% EDTA, pH 7.4, and 0.05% NaN3, as previously described (24). Fractions were collected and pooled according to the elution times for VLDL, LDL, and HDL, and aliquots of isolated fractions were used for enzymatic measurement of cholesterol (25).

Analysis for aortic free and esterified cholesterol content was conducted as described previously (26). The aorta was placed on the platform of a dissecting microscope and the adventitia was carefully and completely dissected away from the intima/media and removed. The intima/media was then detached from the heart and placed in 3 mL of chloroform/methanol (2:1, v/v) containing 5 μg/ml cholesterol as an internal standard, and the lipids were extracted. The lipid extract was separated by filtration and extracts were dried under N2 at 60°C and then dissolved in hexane. Analysis of total and free cholesterol was done with two injections per sample on a DB 17 (0.53 mm i.d. × 15 μm × 1 μm) gas-liquid chromatography column at 250°C and installed in a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph equipped with an HP 7673A automatic injector using column injection and a flame ionization detector. Cholesteryl ester was calculated as the difference between free and total cholesterol, as measured before and after saponification and reextraction of the nonspinnifiable sterol into hexane. The digalactoside tissue protein was then digested and dissolved in 1 mol/L NaOH and total protein was determined (27).

**Plasma isoflavones.** Plasma genistein, daidzein, equol and total isoflavone concentrations for each treatment group were determined from plasma samples obtained by pooling 0.1 mL from each mouse in each treatment group. These analyses were done using combination HPLC/mass spectroscopy (28) in the laboratory of Dr. Stephen Barnes, University of Alabama, Birmingham, Birmingham, AL.

**Data analysis.** To reduce skewness and equalize group variances, all data sets underwent logarithmic or square root transformation before analysis. Three-way, two-way and one-way ANOVA and analysis of covariance were used for detecting effects of treatment on atherosclerosis and plasma lipoproteins. Pairwise comparisons were made using Bonferroni simultaneous confidence intervals. Multiple linear regression was used to assess the relationship between effects of treatment on plasma lipoproteins and effects on atherosclerosis. Analyses were done using BMDP Statistical Software (University of California, Berkeley, CA). Differences were considered significant at \( P < 0.05 \).
RESULTS

Atherosclerosis. These data are summarized in Table 2. Three-way ANOVA revealed significant main effects of type of mouse and diet, but not sex, on atherosclerosis (aortic cholesteryl ester content) and a significant diet/type of mouse interaction term ($P = 0.03$). Atherosclerosis was reduced by both alcohol-washed (45 and 31%; $P = <0.01$) and intact (65 and 41%; $P = <0.01$) soy protein isolate in LDL receptor $−/−$ and apoE $−/−$ mice, respectively. This analysis also revealed that the effect of mouse type was largely explained by the fact that regardless of diet, the extent of atherosclerosis was approximately five times greater in the apoE $−/−$ mice relative to LDL receptor $−/−$ mice. Because of this, and to permit a direct comparison of the effect of diet on atherosclerosis in the two types of mice, a percentage difference, or $Δ$, attributable to diet was calculated by placing aortic cholesteryl ester content of mice in each treatment group in rank order, subtracting the aortic cholesteryl ester content of mice in each of the soy diet groups from that of the corresponding mice in the casein/lactalbumin group, dividing by cholesteryl ester content of the casein/lactalbumin mouse and multiplying by 100. These percentage $Δ$ values were then entered into a two-way (type of mouse $×$ diet) ANOVA. This analysis revealed main effects of both type of mouse and diet. As shown in Figure 1, the atheroinhibitory effect of soy was greater in LDL receptor $−/−$ mice ($P < 0.001$) and was diminished in mice fed alcohol-washed soy protein isolate ($P < 0.001$). Aortic content of free cholesterol did not differ among groups. Consequently, data for total aortic cholesterol paralleled closely those for cholesteryl ester (data not shown).

Plasma lipoproteins. Plasma lipoprotein data are summarized in Tables 3 and 4. In three-way analyses, significant main effects and interaction terms were frequently observed for sex, type of mouse, and diet. As with atherosclerosis data, many of the effects related to type of mouse were probably accounted for by differences in lipoprotein metabolism attributable to the difference in the genetic mutations. For this reason, the lipoprotein data were also analyzed in a manner similar to the analysis of the atherosclerosis data. Because there were sex differences in plasma lipoprotein responses, sex was also included as an independent variable. Thus, the percentage $Δ$ values for each variable were entered into a three-way (sex $×$ type of mouse $×$ diet) ANOVA. Effects of treatment on each variable were often opposite in apoE $−/−$ vs. LDL receptor $−/−$ mice and male vs. female mice. Both alcohol-washed and intact soy protein decreased plasma LDL and VLDL cholesterol in ovariectomized female LDL receptor $−/−$ mice but increased them in female apoE $−/−$ mice (Table 4). However, both types of protein isolate generally had minimal effects or decreased LDL and VLDL cholesterol in male mice of both types (Table 4). Both intact and alcohol-washed soy increased plasma HDL cholesterol (Table 4). However, this effect was more pronounced in the LDL receptor $−/−$ mice.

Multiple linear regression was used to determine whether there were associations between plasma lipoproteins and atherosclerosis. In addition to lipoprotein values, sex and diet were entered into these analyses as dummy variables. Due to the differences in lipoprotein patterns and extent of atherosclerosis between the two types of mice, these analyses were done separately for apoE $−/−$ and LDL receptor $−/−$ mice. For apoE $−/−$ mice, the regression equation identified the two types of soy protein diet as the only significant predictors of atherosclerosis extent. For the LDL receptor $−/−$ mice, plasma VLDL cholesterol, HDL cholesterol and intact soy protein diet were identified as significant predictors of atherosclerosis extent. To determine whether effects of diet on atherosclerosis in LDL receptor $−/−$ mice could be accounted for by plasma VLDL and HDL cholesterol concentrations, these two variables were used as covariates in a two-way (sex $×$ diet) ANOVA. Adjusted means for the percentage reduction in extent of atherosclerosis were 24% in mice fed alcohol-washed soy protein isolate and 49% in mice fed intact soy protein isolate ($P < 0.01$) relative to controls. The results of these analyses indicate that the inhibitory effects of diet on atherosclerosis in both types of mouse were independent of effects on plasma lipoproteins.

Plasma isoflavones. Plasma genistein, daidzein, equol and total isoflavone concentrations are summarized in Table 5. With one exception, plasma isoflavones were undetectable in LDL receptor $−/−$ mice in groups fed casein/lactalbumin or alcohol-washed soy protein. Equol represented 70–80% of total plasma isoflavones in mice fed intact soy protein isolate. Total isoflavone concentrations were threefold greater in males than in females.

DISCUSSION

The major findings were the following: 1) aortic cholesterol content is reduced by intact and alcohol-washed (isoflavone-
Table 3

Plasma total cholesterol concentration in two types of mice fed diets with different sources of protein

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Ovariectomized females</th>
<th>Males</th>
<th>Ovariectomized females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein/lactalbumin</td>
<td>25.5 ± 8.5a</td>
<td>28.9 ± 6.3a</td>
<td>26.1 ± 6.5a</td>
<td>16.6 ± 2.6a</td>
</tr>
<tr>
<td>Soy + IF</td>
<td>29.0 ± 6.8a</td>
<td>21.1 ± 5.0b</td>
<td>23.4 ± 4.3a</td>
<td>18.8 ± 3.2a</td>
</tr>
<tr>
<td>Soy - IF</td>
<td>24.7 ± 5.5a</td>
<td>19.1 ± 3.4b</td>
<td>22.6 ± 4.1a</td>
<td>18.9 ± 2.9a</td>
</tr>
</tbody>
</table>

1 Values are means ± sd, n = 10–12. In a three-way analysis, there were main effects of sex (P < 0.001), type of mouse (P < 0.001) and diet (P < 0.04) and a three-way interaction (P < 0.001). Within columns, values labeled with different letters differ (P < 0.05). IF, isolavone.

deficient) soy protein isolate in atherosclerosis-susceptible mice; 2) this effect is enhanced in mice fed intact soy protein relative to those fed alcohol-washed soy protein; 3) the effect was enhanced in LDL receptor −/− + apoB transgenic mice relative to apoE −/− mice; and 4) inhibitory effects of soy protein on atherosclerosis were independent of plasma lipoproteins in both types of mice. These effects were observed in both male and ovariectomized female mice. Female mice were ovariectomized to eliminate the possibility of confounding interactive effects between endogenous estrogen and dietary soy phytoestrogens.

Measurement of aortic cholesterol content was chosen to assess atherosclerosis for several reasons. First, the metabolism of atherogenic lipoproteins by the artery and the resultant accumulation of free and esterified cholesterol is inherent in and believed by most to be a requirement for the initiation and progression of atherosclerosis. Second, the method most frequently used for assessing atherosclerosis in mice, measurement of plaque area at the aortic sinus, is tedious and labor intensive. More importantly, it is not clear whether examination of this single location accurately represents total arterial involvement. Finally, in a separate validation study, we compared aortic surface area involved with atherosclerosis, aortic sinus plaque size and aortic cholesterol content in 23 apoE −/− mice at 4 mo of age (data not shown). Aortic cholesterol correlated with surface area involvement (r = 0.91) and aortic sinus plaque size (r = 0.79; P < 0.001). We concluded that measurement of aortic cholesterol content is a useful quanti-

Table 4

Plasma LDL, VLDL and HDL cholesterol concentrations in two types of mice fed diets with different sources of protein

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Ovariectomized females</th>
<th>Males</th>
<th>Ovariectomized females</th>
<th>Males</th>
<th>Ovariectomized females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein/lactalbumin</td>
<td>19.2 ± 5.6a</td>
<td>23.8 ± 4.8a</td>
<td>5.2 ± 3.4a</td>
<td>4.1 ± 1.7a</td>
<td>1.1 ± 0.4a</td>
<td>1.1 ± 0.3b</td>
</tr>
<tr>
<td>Soy + IF</td>
<td>22.9 ± 4.6a</td>
<td>18.5 ± 3.8b</td>
<td>4.7 ± 2.4a,b</td>
<td>1.9 ± 0.8b</td>
<td>1.4 ± 0.1a</td>
<td>1.5 ± 0.2a</td>
</tr>
<tr>
<td>Soy - IF</td>
<td>19.2 ± 4.4a</td>
<td>16.0 ± 3.2b</td>
<td>3.9 ± 1.5b</td>
<td>1.6 ± 0.5b</td>
<td>1.6 ± 0.4b</td>
<td>1.5 ± 0.4a</td>
</tr>
</tbody>
</table>

Apo E −/− mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Ovariectomized females</th>
<th>Males</th>
<th>Ovariectomized females</th>
<th>Males</th>
<th>Ovariectomized females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein/lactalbumin</td>
<td>7.3 ± 1.4a</td>
<td>5.2 ± 1.2a</td>
<td>18.1 ± 5.4a</td>
<td>10.9 ± 1.7a</td>
<td>0.7 ± 0.1a,b</td>
<td>0.5 ± 0.1a</td>
</tr>
<tr>
<td>Soy + IF</td>
<td>6.0 ± 1.2a</td>
<td>6.1 ± 2.4a</td>
<td>16.4 ± 4.1a</td>
<td>12.1 ± 2.3a</td>
<td>0.9 ± 0.3a</td>
<td>0.6 ± 0.1a</td>
</tr>
<tr>
<td>Soy - IF</td>
<td>6.2 ± 1.6a</td>
<td>5.9 ± 0.8a</td>
<td>15.8 ± 2.9a</td>
<td>12.4 ± 2.5a</td>
<td>0.6 ± 0.2b</td>
<td>0.6 ± 0.1a</td>
</tr>
</tbody>
</table>

1 Values are means ± sd (n = 10–12). Within columns and types of mice, values labeled with different letters differ (P < 0.05). IF, isolavone.
2 In a three-way analysis, there were main effects of type of mouse (P < 0.001) and diet (P < 0.02) but not sex (P < 0.12) and sex/diet (P < 0.05), diet/type of mouse (P < 0.03) and three-way (P < 0.001) interactions.
3 In a three-way analysis, there were main effects of sex (P < 0.001) and type of mouse (P < 0.001) but not diet (P > 0.16) and a sex/type of mouse (P < 0.04) interaction.
4 In a three-way analysis, there were main effects of sex (P < 0.001), type of mouse (P < 0.001) and diet (P < 0.04) and sex/type of mouse (P < 0.02), diet/type of mouse (P < 0.001) and three-way (P < 0.05) interactions.
tative indicator of atherosclerotic involvement of the mouse aorta.

Although atherosclerosis was reduced in both types of mice, the magnitude of the effect was greater in the LDL receptor −/− + apob transgenic mice. The reason for this remains unclear, although it probably relates to the differences in metabolic responses to the genetic defects. Deficiency of apoE results in delayed clearance of lipoproteins and the elevation of plasma concentrations of chylomicrons and VLDL, which become the atherogenic stimuli in these mice. In contrast, deficiency of LDL receptors results in the delayed removal of intermediate density lipoproteins and LDL and these become the atherogenic stimuli in these mice. Plasma LDL concentrations are further elevated by the presence of the apoB transgene in these mice.

The atheroinhibitory effect of soy has been known for decades (1). A probable role of soy isoflavones in mediating this effect has become apparent much more recently. Sugano and colleagues (10–12) first showed that an alcohol-extracted soy fraction was less effective than the unextracted fraction in lowering plasma cholesterol concentration in rats. Anthony et al. (2,29) studied the effects of alcohol-extracted and intact soy fraction was less effective than the unextracted fraction in lowering plasma cholesterol concentration in rats. Anthony et al. (2,29) studied the effects of alcohol-extracted and intact soy protein isolates on plasma lipoproteins and atherosclerosis. (2,29) studied the effects of alcohol-extracted and intact soy protein isolates on plasma lipoproteins and atherosclerosis. Furthermore, in these mice, there was no statistical association between effects of soy consumption on plasma lipoproteins and effects on atherosclerosis. Furthermore, in apoE −/− mice, atherosclerosis was inhibited in spite of increases in plasma LDL and VLDL cholesterol. The lack of a relationship between atherosclerosis inhibition, the presence or absence of LDL receptor expression and changes in plasma lipoproteins would also seem to eliminate a role for an antiatherosclerotic effect of soy peptides mediated by increased LDL receptor expression or enhanced bile acid excretion. The possibility remains that soy peptides affect atherosclerosis by lipoprotein-independent means.

Kirk et al. (9) also studied the effects of isoflavones contained in soy protein isolate on atherosclerosis in mice. In that study, no difference in atherosclerosis was found between LDL receptor −/− mice fed diets containing alcohol-washed protein relative to those consuming intact soy protein. A group fed protein from nonsoy sources was not included. This result seems to conflict with our finding that the atheroinhibitory effect of soy was enhanced in LDL receptor −/− mice fed intact soy protein isolate compared with those fed alcohol-washed soy protein. However, this conflict is probably accounted for by differences in the experimental diet between the studies. In the Kirk study (9), the diet contained 1.0% cholesterol. This resulted in average plasma cholesterol concentrations of 4000 mg/dL (105 mmol/L), a concentration that is ∼20-fold higher than those found in humans with borderline hypercholesterolemia and fourfold higher than concentrations seen in our study. We postulate that soy isoflavones, or other interventions, may be ineffective in the face of such an extreme atherogenic stimulus, regardless of the presence or absence of LDL receptors.

It seems possible that the antiatherosclerotic effects of soy protein isolate observed in our study may be accounted for by direct atheroinhibitory effects of phytoestrogenic soy isoflavones. Genistein, daidzein and their respective glycosides are the major isoflavones found in soybeans and soy protein isolate. Dietary consumption results in elevation of plasma concentrations of these isoflavones, primarily in the form of glucuronides (33). In approximately one third to two thirds of

### Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Equol Males</th>
<th>Equol Ovariectomized Females</th>
<th>Genistein Males</th>
<th>Genistein Ovariectomized Females</th>
<th>Daidzein Males</th>
<th>Daidzein Ovariectomized Females</th>
<th>Total isoflavone Males</th>
<th>Total isoflavone Ovariectomized Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein/lactalbumin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Soy + IF</td>
<td>1914 ± 61</td>
<td>761 ± 188</td>
<td>447 ± 15</td>
<td>149 ± 32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Soy − IF</td>
<td>13 ± 4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 Values are means ± so of duplicate samples. ND, not detected. IF, isoflavone.
humans (33), ingested daidzein is transformed to equol by bacterial microflora in the intestinal tract and appears in the circulation as a glucuronide. Although daidzein binds estrogen receptors weakly, genistein binds estrogen receptor β with an affinity similar to that of 17β estradiol (34,35). In competitive binding assays, equol has been shown to have 10- to 100-fold greater binding affinity for estrogen receptors than its precursor, daidzein (36,37). Its uterotrophic activity in mice is similar to that of genistein (38). In addition, due to the fact that their binding to sex hormone binding globulin and albumin is relatively low (39), plasma concentrations of free (bioavailable) genistein and equol are relatively high, thus further enhancing estrogenic potency. Further contributing to its relative bioactivity, equol is known to remain in the circulation longer than genistein or daidzein (40,41). The microfloral biotransformation of daidzein to equol is particularly active in mice and rats, in which equol is the major circulating isoform (33). In our study, plasma equol concentrations were approximately two to three times the combined concentrations of genistein and daidzein. When considered with the evidence regarding equol’s relative estrogenic bioactivity, our finding that the antiatherosclerotic potency of soy protein isolate is maximal in mice fed intact soy protein suggests that there may be clinical advantages to be gained by identifying means to enhance the intestinal conversion of daidzein to equol.

Although there is substantial evidence for the existence of antiatherogenic effects of soy isoflavones, the possibility remains that other substances removed by alcohol washing may also be involved. Although there has been no systematic study of the subject, the known alcohol-soluble components of soy protein include saponins, phytosterols, and globulins. We have been unable to detect the presence of phytosterols in intact or alcohol-washed soy. This is probably the result of the processing of soy protein isolate before alcohol extraction. Although we have not measured the saponin content of the isolates used here, it has been reported that soy protein isolate contains <1% saponins (42). Furthermore, there is substantial evidence that soy saponins have no effect on lipoprotein metabolism (43–46) or atherosclerosis (14). Finally, although there is no evidence to support the possibility, it remains possible that the biologically active soy globulins, particularly the 7S globulin (8), may be denatured or removed by alcohol washing, thus negating or reducing its hypolipoproteinemic or antiatherosclerotic properties. Thus, the evidence continues to favor the probability that isoflavones are the hypolipoproteinemic and antiatherosclerotic substances removed by alcohol washing.

Mammalian estrogens have direct effects on the artery (47). Estrogens inhibit the arterial uptake and metabolism of plasma LDL (48,49), inhibit expression of molecules involved in monocyte chemotraction (50,51) and adhesion (52), inhibit expression of cytokines and inflammatory mediators (e.g., interleukin-6) (53,54), E-selectin (55,56) and intercellular adhesion molecule-1 (55,56) and inhibit the oxidation of LDL (57,58). These findings indicate that estrogen may act directly to interfere with the arterial LDL uptake—oxidation—inflammation pathway implicated in atherosclerosis initiation and progression. The evidence regarding possible lipoprotein-independent pathways by which soy isoflavones may inhibit atherosclerosis was reviewed recently by Anthony (2). In addition to those pathways described for mammalian estrogens, these include inhibitory effects on the proliferative response to arterial injury (59), improvement in endothelium-dependent vasomotor function (60), inhibitory effects on expression and/or activity of growth factors (61), inhibition of tyrosine kinase activity (61), inhibition of platelet aggregation or activation (62–64) and potent antioxidant activity (14–18). Direct evidence for lipoprotein-independent atheroinhibitory effects of soy isoflavones is provided by the findings of Yamakoshi et al. (14) and Ni et al. (19). In the Yamakoshi study (14), rabbits fed atherogenic diets supplemented with concentrates of soy isoflavones (94% genistein and daidzein), but containing no soy protein, had marked reductions in aortic atherosclerosis relative to rabbits fed diets not supplemented with isoflavones. Ni et al. (19) studied the effects of dietary soy protein isolate on atherosclerosis in apoE−/− mice. In these studies, despite a tendency toward increased total plasma cholesterol in mice fed diets containing isoflavone-intact soy protein, aortic atherosclerosis was reduced in these mice. Furthermore, there was no effect of isoflavone-deficient soy on lipoproteins or atherosclerosis.

Regardless of the mechanisms, we make the following conclusions: 1) consumption of soy protein isolate results in substantial inhibition of the progression of atherosclerosis in mice with genetically engineered susceptibility; 2) this effect is diminished in mice fed isoflavone-deficient soy protein; and 3) it is independent of plasma lipoprotein concentrations and the presence or absence of LDL receptors. These findings indicate the probable importance of direct inhibitory effects of soy isoflavones and their metabolites on the initiation and progression of atherosclerosis.

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LITERATURE CITED