The Atheroprotective Effect of Dietary Soy Isoflavones in Apolipoprotein E—/— Mice Requires the Presence of Estrogen Receptor-α

Michael R. Adams, Deborah L. Golden, Thomas C. Register, Mary S. Anthony, Jeffrey B. Hodgin, Nobuyo Maeda, J. Koudy Williams

Objective—Although the mechanisms by which dietary soy inhibits atherosclerosis are unclear, one line of evidence implicates an important role for its phytoestrogenic isoflavones. We sought to determine whether soy isoflavones exert atheroprotective effects through estrogen receptor–dependent processes and, if so, which estrogen receptor subtype (ie, α or β) is involved.

Methods and Results—We compared the effects of diets rich in soy protein that were either isoflavone depleted (0.04 mg/g protein isolate) or isoflavone-replete, or Soy(+IF) (1.72 mg/g protein isolate) in apolipoprotein E–deficient (ee) mice that had been crossed with estrogen receptor-α– and -β–deficient mice to produce double-knockout ααee and ββee mice and (estrogen receptor) wild-type controls (AAee and BBee). Both male and ovariectomized female mice were studied (n=10 to 17 per treatment group; total n=201). After 16 weeks, atherosclerosis was assessed by quantifying the aortic content of esterified cholesterol. Atherosclerosis was reduced 20% to 27% (P<0.05) by Soy(+IF) in ββee, BBee, and AAee mice but was unaffected in ααee mice. The inhibitory effect of Soy(+IF) was unrelated to sex, total plasma cholesterol, VLDL, LDL, and HDL cholesterol.

Conclusions—The results indicate a necessary role for estrogen receptor-α–dependent processes in mediating the atheroprotective effects of dietary soy isoflavones. (Arterioscler Thromb Vasc Biol. 2002;22:1050–1056.)

Key Words: atherosclerosis ■ soy ■ mice ■ phytoestrogens ■ estrogen receptors

A recent review article has shown that diet-induced atherosclerosis is reduced in animals that are fed soy protein–based diets compared with those that are fed animal protein–based diets.1 However, the component(s) of soy responsible for this effect and the mechanism(s) involved remain uncertain. Although some evidence implicates favorable effects of certain soy peptides or peptide fractions on plasma lipoproteins,2–7 other evidence implicates nonprotein components, particularly the isoflavones, and lipoprotein-independent processes.8–11 For example, at least three studies have demonstrated plasma lipoprotein-independent inhibitory effects of dietary soy isoflavones on atherosclerosis in experimental animals.9–11 Furthermore, as reviewed by Anthony,8 there is substantial evidence that soy isoflavones influence cellular and pathobiologic processes implicated in atherogenesis, for example, they inhibit tyrosine kinase activity, the production of inflammatory cytokines by macrophages, the migration or proliferation of arterial smooth muscle cells, and platelet aggregation and activation. Soy isoflavones also have potent antioxidant activity and, therefore, may inhibit atherosclerosis initiation or progression by inhibiting lipoprotein oxidation.10,12–15 Many isoflavones have estrogenic activity in classic estrogen target tissues; hence, they have been referred to as phytoestrogens. The major isoflavone components of soy are genistein and daidzein. Although daidzein is a relatively weak estrogen receptor agonist, genistein has been shown to bind estrogen receptor-β with an affinity similar to that of 17β-estradiol.16,17 Furthermore, there is experimental evidence for an estrogen receptor-β–mediated inhibitory effect of genistein on arterial smooth muscle cell proliferation in response to mechanical arterial injury in rats18 and 17β-estradiol on the vascular injury response in mice.19 In addition, recent findings have indicated a necessary role for estrogen receptor-β in the maintenance of normal vascular function.20 Although the role of estrogen receptors in mediating inhibitory effects of isoflavones on atherosclerosis has not been studied previously, Hodgin et al21 have shown that the atheroinhibitory effect of 17β-estradiol in mice depends on the presence of estrogen receptor-α. Taken together, these findings have implicated estrogen receptor–dependent processes and both estrogen receptor-α and -β in mediating the vasculoprotective effects of estrogens.

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We have previously shown that the consumption of a diet rich in soy protein has athero-inhibitory effects in atherosclerosis-susceptible mice and that these effects are diminished when the natural soy isoflavones are removed from the soy by ethanol extraction. Furthermore, these effects are independent of the presence or absence of LDL receptors and effects on plasma lipoproteins. The purpose of the study described here was to determine whether estrogen receptor-dependent processes mediate the athero-inhibitory effects of soy isoflavones and the relative roles of estrogen receptor-α and -β in mediating any such effects. We describe for the first time a critical role for estrogen receptor-mediated processes and, specifically, for estrogen receptor-α.

Methods

Mice and Diets

The mice used in these studies were bred and reared in our animal facilities, which are 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 of Wake Forest University School of Medicine.

Apolipoprotein E-deficient (ApoE−/− ee) mice22 and mice heterozygous for an insertional mutation of estrogen receptor-α (Aα)23 or -β (Bβ),24 each backcrossed greater than 99% to C57BL/6J, were intercrossed to yield males and females heterozygous for both mutations (AαEe or BβEe).25 These double heterozygotes were further mated with ee mice to generate mice heterozygous for estrogen receptor-α or -β and homozygous for the apoE mutation (Aαee or Bβee). Intercrossing these mice produced the subjects for this study, that is, mice lacking estrogen receptor-α or -β (Aαee or Bβee) and litter mate controls having intact estrogen receptor-α or -β (AαEe or BβEe).

At 6 weeks of age, male and ovariectomized female mice of each genotype were assigned randomly to two diet groups. There were 10 to 17 mice of each sex and genotype in each diet group (total n=201).

The diets are described in Table 1. The principal difference between the diets was the source of the protein component: either ethanol-extracted soy protein isolate, abbreviated as Soy(-IF) (total isoflavone content=0.04 mg/g of isolate), or intact soy protein isolate, abbreviated as Soy(+IF) (total isoflavone content=1.72 mg/g of isolate). Soy protein isolates were provided by Protein Technologies International (St. Louis, Mo).

After 16 weeks, mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) and 1 mL of blood was collected by cardiac puncture. Mice were then killed with pentobarbital (200 mg/kg). The heart and aorta were promptly removed and placed in 10% neutral buffered formalin for subsequent processing. Plasma was promptly separated at 5000 g and 4°C and stored at −20°C.

Atherosclerosis and Plasma Lipoproteins

Plasma lipoproteins were separated by high-performance liquid chromatography,26 and aliquots of isolated lipoprotein fractions were used for enzymatic determination of cholesterol.26

Analysis for aortic free and esterified cholesterol content was conducted as described previously.27 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 placed in 3 mL of chloroform/methanol (2:1, v/v) containing 5α-cholestanate 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 free and total cholesterol was performed with two injections per sample on a DB 17 (0.53 mm i.d. × 15 m × 1 μm) gas-liquid chromatograph, column at 250°C and installed in a Hewlett Packard (Palo Alto, Calif) 5890 gas chromatograph equipped with an HP 7673A automatic injector using on-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 nonsaponifiable sterol into hexane. The dilapectated tissue protein was then digested and dissolved in 1 mol/L NaOH and total protein was determined.28

Data Analysis

To reduce skewness and equalize group variances, all data sets underwent logarithmic transformation before analysis. Three-way, two-way, and one-way ANOVA and ANCOVA were used for detecting effects of diet, sex, and genotype on atherosclerosis and plasma lipoproteins. Pairwise comparisons were made using Duncan’s New Multiple Range Test or two-tailed t test. Multiple linear regression was used to assess the relationship between effects of treatment on plasma lipoproteins and effects on atherosclerosis and to select covariates for analysis of covariance. Analyses were performed using BMDP Statistical Software (University of California, Berkeley, Calif).

Results

Plasma Lipoproteins

Total plasma cholesterol data are summarized in Table 2. Similar patterns were observed for LDL, VLDL, and HDL.
TABLE 2. Total Plasma Cholesterol Concentrations at 16 Weeks (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>ααee</th>
<th>AAee</th>
<th>ββee</th>
<th>BBee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Soy (-IF)</td>
<td>1166±246</td>
<td>971±261</td>
<td>965±346</td>
<td>819±139</td>
</tr>
<tr>
<td>Soy (+IF)</td>
<td>1110±299</td>
<td>921±280</td>
<td>938±298</td>
<td>781±236</td>
</tr>
</tbody>
</table>

Values are mean±SD.
Three-way analysis revealed main effects of sex (P<0.01) and genotype (P<0.01) but not diet (P=0.37).

TABLE 3. Aortic Cholesterol Ester Concentration (ng/mg Protein)

<table>
<thead>
<tr>
<th></th>
<th>ααee</th>
<th>AAee</th>
<th>ββee</th>
<th>BBee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Soy (-IF)</td>
<td>53.1±22.9</td>
<td>66.0±21.7</td>
<td>70.4±24.2</td>
<td>85.2±32.8</td>
</tr>
<tr>
<td>Soy (+IF)</td>
<td>63.2±55.7</td>
<td>66.2±33.5</td>
<td>52.8±22.6</td>
<td>65.2±32.5</td>
</tr>
</tbody>
</table>

Values are mean±SD.
Three-way analysis revealed main effects of genotype (P<0.0001) and diet (P<0.03) but not sex (P=0.09).

Atherosclerosis data are summarized in Table 3, stratified by sex, genotype, and diet. Three-way ANOVA revealed main effects of diet (P<0.03) and genotype (P<0.0001), but not sex (P=0.09), on atherosclerosis extent (aortic cholesteryl ester content). Therefore, the data are summarized in Figure 1 stratified by diet and genotype but not sex. Although treatment had no effect on lipoproteins, there were small (nonsignificant) differences among groups in some variables. For this reason, total plasma cholesterol, LDL cholesterol, VLDL cholesterol, and HDL cholesterol were used in a multiple regression equation to determine which were significant predictors of atherosclerosis. For ααee and AAee mice, no lipoprotein variable was a significant predictor. For ββee and BBee, VLDL and HDL cholesterol were selected as significant predictors and together accounted for 9% of the variance in atherosclerosis extent. To determine whether effects of diet on atherosclerosis were accounted for by variation in plasma lipoproteins and to reduce lipoprotein-associated variability within treatment groups, ANCOVA, with significant lipoprotein predictors as covariates, was used to assess effects of diet and calculate mean atherosclerosis extent. These results are depicted in Figure 1. Atherosclerosis extent was reduced 20% to 27% by consumption of Soy(+IF) in ββee, BBee, and AAee mice (P<0.05) but was unchanged (P>0.20) in ααee mice. As the result of higher total plasma cholesterol, LDL, and VLDL cholesterol concentrations, atherosclerosis extent was approximately 40% greater in ααee and AAee mice than in ββee and BBee mice (Figure 1).

Therefore, to facilitate a direct comparison of the genotypes, the percent reductions in atherosclerosis extent attributable to consumption of the Soy(+IF) diet were calculated and are summarized in Figure 2. As shown, the reductions in ββee, BBee, and AAee mice were similar and ranged from 20% to 27% (P<0.05) whereas there was a nonsignificant 2% increase in ααee mice (P>0.20).

Discussion

The component(s) of soy responsible for its atheroprotective effects and the mechanism(s) involved are uncertain. We and others have previously described evidence for a role of both the protein and isoflavone components. The findings described here represent the first evidence directly implicating estrogen receptor–dependent processes and, more specifically, processes mediated by estrogen receptor-α. The spe-
A similar pattern of effects of isoflavones on atherosclerosis was observed in male and ovariectomized female mice. Although female mice were ovariectomized to remove the possibility of interactive effects between endogenous estrogen and dietary soy phytoestrogens, males were not orchietomized. Also, although male mice had slightly less evidence of atherosclerosis than ovariectomized female mice, this difference did not reach statistical significance ($P=0.09$). Nonetheless, it seems reasonable to speculate that the small amounts of endogenous estrogen produced by peripheral aromatization of endogenous androgens in male mice may account for this tendency.

Although there was a consistent pattern of effects of isoflavones on atherosclerosis within genotype, there were two unexpected genotype-related differences in atherosclerosis susceptibility. First, atherosclerosis was less extensive in $\beta\beta$ee and $BB$ee mice relative to $aa$ee and $AA$ee mice. This can probably be accounted for by the fact that the congenic $aa$ and $\beta\beta$ mice were created at different times from different founder populations. Also, several successive generations of each double knockout heterozygote ($AA$ee and $BB$ee) mouse have been required to produce double knockout subjects for study. For these reasons, there are small genetic differences between the congenics, some of which may influence susceptibility to atherosclerosis. Second, it appears (Figure 1) that $aa$ee mice have less evidence of atherosclerosis than $AA$ee mice fed the Soy(-IF)-containing diet. If the only difference between the two was the presence of estrogen receptor-$\alpha$, one would predict that extent of atherosclerosis would be similar in the two mice when fed an estrogen- or isoflavone-deficient diet. The most likely explanation for this relates to the fact that the difference does not reach statistical significance ($P<0.10$). Less likely, and much more speculative, is the possibility that in disrupting the $\alpha$-receptor gene, adjacent DNA that codes for atherogenic factors has also been disrupted.

Because there was no evidence for a role of plasma lipoproteins in mediating the antiatherosclerotic effects of Soy(-IF) observed in our study, it remains possible that these effects may be accounted for by direct arterial effects of the estrogenic isoflavones. Genistein, daidzein, and their respective glycosides are the major isoflavones found in soybeans and soy protein isolates. The consumption of soy or soy protein isolate results in the elevation of plasma concentrations of these isoflavones, primarily in the form of glucuronides. Ingested isoflavones are subject to extensive metabolism. In one third to two thirds of human beings, and most if not all nonhuman animals, ingested daidzein is transformed to equol by bacterial microflora of the intestinal tract and appears in the circulation as a glucuronide.

Although daidzein binds estrogen receptors weakly, genistein$^{16,17}$ and equol$^{31}$ bind estrogen receptor-$\beta$ with an affinity similar to that of 17$\beta$estradiol. In competitive binding assays, equol has been shown to have 10- to 100-fold greater binding affinity for estrogen receptors than its precursor, daidzein$^{32,33}$ and a greater affinity for estrogen receptor-$\beta$ than estrogen receptor-$\alpha$. However, its uterotropic activity in mice is similar to that of genistein.$^{34}$ In addition, because of the fact that their binding to sex hormone–binding globulin is relatively low,$^{35}$ plasma concentrations of free (bioavailable) genistein and equol are relatively high, thus enhancing their estrogenic potency. Further contributing to its relative bioactivity, equol is known to remain in the circulation longer than genistein or daidzein.$^{36,37}$ The microfloral transformation of daidzein to equol is particularly active in mice and rats, in which equol is the major circulating isoflavone.$^{11,38}$ Taken together, these findings indicate a possible role for equol in mediating atheroprotective effects of soy and suggest possible clinical advantages to be gained by identifying means to maximize the intestinal conversion of daidzein to equol in human beings.

The finding of a necessary role for estrogen receptor-$\alpha$ and not estrogen receptor-$\beta$ in isoflavone-induced atheroprotection is interesting in light of the data indicating weaker binding affinities of genistein and equol for estrogen receptor-$\alpha$ compared with estrogen receptor-$\beta$.$^{16,17,31}$ The most likely explanation for this seeming inconsistency is that the agonistic bioactivity of a ligand is not necessarily correlated to its binding affinity. This is perhaps best demonstrated by the effects of estrogen receptor ligands, such as tamoxifen,
which bind estrogen receptors with high affinity yet actually repress gene expression. Furthermore, in vitro studies have demonstrated that genistein and equol have greater transactivation activity with estrogen receptor-α than with estrogen receptor-β. In addition, it is likely that the relative distribution of these receptors in tissues and cell types involved in atherosclerosis is more important than relative binding affinities of the ligands. For example, preferential expression of estrogen receptor-α relative to estrogen receptor-β in vascular smooth muscle or intimal macrophages may render differences in binding affinity irrelevant. Studies involving induced tissue-specific expression of receptor types would be needed to address this question. Nonetheless, our findings support the existence of substantial isoflavone-dependent transcriptional regulation that is mediated through estrogen receptor-α.

The existence of direct, lipoprotein-independent atheroprotective effects of soy isoflavones are further supported by our previous findings and those of Ni et al. We previously showed that the consumption of soy protein isolate by mice has potent atheroinhibitory effects that were diminished by the removal of its natural isoflavones and did not depend on LDL receptors or effects on plasma lipoproteins, which is in agreement with these results are those of Ni et al. These authors also studied apoE−/− mice and showed that, despite having no effect on plasma lipoproteins, the consumption of a diet rich in isoflavone-replete soy protein isolate resulted in an inhibition of aortic atherosclerosis relative to mice fed a casein-based diet. Yamakoshi et al. fed rabbits atherogenic diets supplemented with concentrates of soy isoflavones (94% genistein and daidzein) but containing no soy protein and observed marked reductions in aortic atherosclerosis relative to rabbits fed an isoflavone-free diet. Again, there were no effects of treatment on plasma lipoproteins.

Further supporting the existence of direct atheroprotective effects of soy isoflavones, both phytoestrogens and mammalian estrogens have been shown to have effects on pathobiologic processes implicated in the initiation and progression of atherosclerosis. For example, as reviewed by Anthony, soy isoflavones have been shown to inhibit tyrosine kinase activity, macrophage cytokine expression, the migration and proliferation of arterial smooth muscle cells, and platelet activation or aggregation.

Soy isoflavones also have potent antioxidant activity. Mammalian estrogens inhibit the oxidation of LDL, inhibit the arterial uptake and metabolism of plasma LDL, inhibit the expression of molecules involved in monocyte chemotraction and adhesion, and inhibit the expression of cytokines and inflammatory mediators (eg, interleukin-6, E-selectin, and intercellular adhesion molecule-1). Moreover, there is substantial evidence for a functional interaction between estrogen receptor-α and the proinflammatory transcription factor nuclear factor-κB, in the induction of numerous inflammatory genes, for example, vascular cell adhesion molecule-1, tumor necrosis factor-α, and RANTES both in vitro and in vivo. Taken together, these findings indicate that both phytoestrogens and mammalian estrogens are capable of interfering with the arterial LDL uptake-oxidation-inflammation pathway implicated in the initiation and progression of atherosclerosis.

The relative role of estrogen receptor–dependent processes and relative contributions of estrogen receptor-α and -β in mediating such effects remain to be completely clarified. Although there is evidence for favorable estrogen receptor-β-mediated effects of genistein and 17β-estradiol on the arterial response to injury and 17β-estradiol on endothelium-dependent arterial responses, it has also been shown that the inhibitory effect of 17β-estradiol on atherosclerosis depends on the presence of estrogen receptor-α. Our findings further support the existence of a critical role of estrogen receptor-α in mediating atheroprotective effects of mammalian or phytoestrogens. Furthermore, in a recent study, human monocyte-derived macrophages were found to express estrogen receptor-α abundantly, whereas estrogen receptor-β could not be detected. When considered with the evidence indicating a functional (repressive) interaction between estrogen receptor and nuclear factor-κB, these findings suggest that estrogen receptor-α and -β have roles that may depend on the particular pathobiologic process (eg, smooth muscle response to injury versus inflammation/atherosclerosis) or cell type (smooth muscle versus monocyte/macrophage versus endothelial cell) being studied. Thus, the ultimate effect on atherosclerosis may depend on the relative balance of receptor subtypes expressed in these multiple cell types and the consequent influences on the multiple pathobiologic processes involved in atherogenesis.

Regardless of mechanism, we conclude that consumption of the natural soy isoflavones contained in soy protein isolate inhibits atherosclerosis progression in mice by processes that depend on the presence of estrogen receptor-α.

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