Soy Protein With Isoflavones, but not an Isoflavone-Rich Supplement, Improves Arterial Low-Density Lipoprotein Metabolism and Atherogenesis

Janice D. Wagner, Dawn C. Schwenke, Kathryn A. Greaves, Li Zhang, Mary S. Anthony, Robert M. Blair, Melanie K. Shadoan, J. Koudy Williams

Objective—We sought to determine if arterial LDL metabolism contributes to the decreased atherosclerosis seen with soy and if isolated isoﬂavones would have similar effects.

Methods and Results—Ovariectomized monkeys were fed an atherogenic diet for 20 weeks with a protein source of (1) casein/lactalbumin (CAS, n=20), (2) soy protein isolate (SOY, n=20), or (3) casein/lactalbumin with isolated soy isoﬂavones (ISO, n=17). Plasma lipoprotein concentrations were improved with SOY but not ISO. Arterial LDL metabolism was characterized with one subset (n=12/group) injected with dual-labeled tyramine-cellobiose (TC)-LDL (125I-TC,131I-LDL) 24 hours before necropsy to determine LDL degradation and accumulation, while another subset (n=8/group) was injected with 125I-TC-LDL 1 hour before necropsy to determine LDL permeability and delivery.

Conclusions—Coronary artery LDL degradation was reduced by 50% (P=0.02) with SOY but not with ISO compared with CAS. Neither treatment altered arterial permeability. Reduced LDL degradation with SOY was due to decreased arterial LDL delivery (P=0.02). Carotid artery cholesterol ester was also decreased with SOY, but not with ISO. Plasma isoprostanes or plasma markers of inﬂammation did not differ among treatment groups. Thus, the decreased arterial LDL delivery and subsequent LDL degradation may explain, in part, the atheroprotective effects of soy. (Arterioscler Thromb Vasc Biol. 2003;23:2241-2246.)

Key Words: soy • isoﬂavones • atherosclerosis • lipoproteins • monkey
Of major public health importance is whether pills containing isolated isoflavones can produce the same vascular benefits as soy protein. Studies in people and monkeys have found no beneficial effect of soy isoflavone supplementation on plasma lipids and lipoproteins. However, Nestel et al. did find a significant lipid-independent improvement in systemic arterial compliance with soy isoflavones. Thus, isolated soy isoflavones may have direct effects on the artery despite not altering plasma lipoprotein concentrations.

The purpose of this study was 2-fold. The first aim was to identify potential mechanisms for the cardioprotective effects of soy protein. In previous studies, we found that estrogens have direct effects on the artery, including reducing arterial stiffness. In previous studies, we found that estrogens despite not altering plasma lipoprotein concentrations. Thus, systemic arterial compliance with soy isoflavones. Thus, isolated soy isoflavones would have similar actions on the artery. Second, we wanted to determine if isolated soy isoflavones would have effects similar to the intact soy protein containing a similar amount of isoflavones. In this paper, we extend the findings of our previously published paper, we extend the findings of our previously published study on the effects of these diets on plasma lipids and lipid metabolism. Due to the estrogenic activity of the soy isoflavones, we proposed that soy isoflavones would have similar actions on the artery. Second, we wanted to determine if isolated soy isoflavones would have effects similar to the intact soy protein containing a similar amount of isoflavones. In this paper, we extend the findings of our previously published paper, we extend the findings of our previously published study on the effects of these diets on plasma lipids and lipoproteins to include effects on artery.

Methods

Study Population and Diet Composition

Sixty adult female cynomolgus monkeys (Macaca fascicularis) were imported from the Indonesian Primate Center (Bogor, Indonesia). After quarantine, monkeys were fed a baseline, moderately atherogenic, casein-lactalbumin diet (0.07 mg cholesterol/kJ) for 2 months and were subsequently ovariectomized and randomized to one of three treatment groups (n = 20 per group) stratified by TPC and HDLC concentrations.

Treatment diets were fed for 20 weeks and were designed to be identical in macronutrient and cholesterol composition. Two of the diets contained casein-lactalbumin as a protein source (Teklad) while the third diet contained soy as the protein source (SOY). The chemical composition of the soy protein isolate used in this study was 87% protein, 4.2% moisture, 4.6% fat, and 4.2% ash. Phytosterols were found in the soy protein isolate at a concentration of 13 mg/100 g of protein (dry weight). The soy protein diet and the semipurified extract of soy protein isolate, rich in genistein and daidzein, were supplied by Dupont Protein Technologies. The casein-lactalbumin diets either contained no additives (CAS) or were supplemented with isolated soy isoflavones (ISO). ISO was an alcohol extract containing 68.9% total isoflavones (43.7% genistein, 21.8% daidzein, 3.4% glycitein), 1.8% protein, 0.9% moisture, 0.4% fat, and 0.1% ash. Expressed in aglycone units, the ISO diet contained 6.52 mg of genistein and 3.18 mg of daidzein per 504 kJ, while the ISO diet contained 6.24 mg of genistein and 3.45 mg of daidzein per 504 kJ. Thus, both SOY and ISO diets contained similar amounts of isoflavones, but isoflavones in the ISO diet were present as aglycones while those in the SOY diet were present in glycosylated forms. All diets included cholesterol (0.07 mg/kJ) and contained (as % of energy) 19, 42, and 38% protein, fat, and carbohydrate, respectively. DL-methionine was added to the soy protein to approximately equalize the amounts of sulfur-containing amino acids in the casein and soy diets. Monkeys were fed 504 kJ per kg of body weight per day.

Plasma genistein, daidzein, and equol concentrations were determined by ESA, Inc. by coulometric array detection on a subsample (n = 8 per group) from each group, as reported previously. Mean plasma isoflavone concentrations, measured 2 hours post-feeding at least 9 weeks after starting treatment, were comparable between the ISO and SOY groups (genistein: CAS – not detectable, ISO 86.0 ± 26.0 nmol/L; SOY 110 ± 123.6 nmol/L; daidzein: CAS – not detectable, ISO 80.8 ± 25.9 nmol/L; SOY 92.3 ± 18.5 nmol/L; equol: CAS < 20 nmol/L, ISO 540.2 ± 110.8 nmol/L; SOY 361.7 ± 52.8 nmol/L).

All procedures involving animals were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee. Ovariectomies and catheterizations were performed while monkeys were anesthetized with ketamine hydrochloride (15 mg/kg) and butorphanol (0.05 mg/kg). Three animals died from the ISO group; two from experimental procedures and one from unrelated causes.

Plasma Lipids, Lipoproteins, and Biomarkers of Endothelial Function

Plasma was sampled at baseline and 8, 18, and 20 weeks for lipid determinations. Monkeys were food-deprived for 18 hours and sedated with ketamine (10 mg/kg) before blood sample collection. TPC, HDLC, and TG concentrations were determined using enzymatic methods on a COBAS FARA II analyzer (Roche Diagnostic Systems) as described previously. Analyses for TPC, HDLC, and TGs are in full standardization with the Centers for Disease Control and Prevention National Heart, Lung, and Blood Institute Standardization Program. Aprotinin B-containing lipoprotein cholesterol was calculated as the difference between TPC and HDLC.

Plasma endothelial function biomarkers were analyzed after 18 weeks of treatment. Monocyte chemotactic protein 1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1) were analyzed with commercially available ELISA kits (R&D Systems). Intraassay coefficients of variation were 2.58% and 9.48% for MCP-1 and VCAM-1, respectively. Interassay coefficients of variation were 9.5% and 13.6% for MCP-1 and VCAM-1, respectively.

Arterial LDL Metabolism

Arterial LDL metabolism was studied after 20 weeks. Blood samples were collected and processed to limit proteolysis and to prevent oxidation. LDLs (1.020 to 1.063 g/mL) were isolated by differential ultracentrifugation and LDL protein was measured. LDLs were either directly labeled with 125I and then covalently coupled to 125I-tyramine cellobiose (TC) or coupled to 125I-TLC only. Before use, LDL preparations were stored in the dark at 4°C under nitrogen to limit oxidative damage. Filter-sterilized LDL preparations (0.45 μm Millipore filter) were injected within 1 week of labeling for experiments described below. In one protocol, LDL degradation and accumulation were studied after injecting 125I-TLC. 125I-LDL into monkeys (SOY, n = 12; CAS, n = 12; ISO, n = 10). LDL particles for these studies were isolated from each individual animal, labeled, and re-injected as described previously. Labeled LDLs were injected via indwelling catheters. Subsequent blood samples were collected into tubes containing EDTA (0.1% final concentration) at 4, 10, 15, 20, 30, 40, and 60 minutes and 2, 4, 6, and 12 hours, and at necropsy 24 hours after injection, in order to calculate the plasma fractional catabolic rate (FCR) of LDL.

In another protocol, LDL permeability and delivery were measured in monkeys injected with 125I-TL-IDL. LDL particles for these studies were isolated from plasma pools. Subsequent blood samples were collected at 4, 10, 15, 20, 30, 40, and 50 minutes and at necropsy 60 minutes after injection to determine the area under the curve for plasma 125I-TL-IDL.

After collecting the final blood sample, the animals were anesthetized deeply with sodium pentobarbital (80 mg/kg body weight, IV). The cardiovascular system was flushed via the left ventricle with 1 L of lactated Ringer’s solution containing 2.7 mmol/L EDTA and 50 μmol/L BHT to prevent oxidative damage. Thoracic and abdominal aorta, carotid and left anterior descending coronary arteries, and carotid bifurcations were removed and immersed for 24 hours in a solution to fix arterial 125I-TC present on both carotid bifurcations were removed and immersed for 24 hours in a
Analysis of LDL Metabolism

The rates of LDL degradation and the calculated concentration of undegraded LDL were determined as described. The $^{125}$I-TC representing LDL degraded by the artery was determined by subtracting the arterial $^{125}$I radioactivity from the total arterial $^{125}$I-TC radioactivity, taking into account the relative activities of these two isotopes in plasma LDL at necropsy. Arterial LDL degradation was first calculated in fractional terms and expressed as a fraction of the plasma LDL pool degraded per hour per gram of tissue (FCR$_{max}$). FCR$_{max}$ was calculated as the product of the whole body FCR and the ratio of LDL degradation products per gram of tissue to that for the whole body. Absolute rates of LDL degradation ($\mu$g LDLC/g tissue/h) were calculated as the product of FCR$_{max}$ and the total plasma LDL cholesterol pool (plasma LDLC cholesterol concentration × plasma volume).

LDL permeability ($\mu$L/g/h) was determined by dividing the tissue radioactivity (cpm/g) by the plasma area under the curve (AUC) (cpm×h/µL) as described. Total LDLC delivery ($\mu$g LDLC/g h) to the artery was estimated by multiplying the LDL permeability by the plasma LDLC concentration.

Radioactivities in all samples were corrected for overlap of the energy spectra of the two isotopes, for background radioactivity, and for isotopic decay. Samples were counted for approximately 60 minutes, giving a $2\sigma$ counting error of $<1.0\%$ for $^{125}$I and $<3.0\%$ for $^{131}$I. Background was counted until a minimum of 10000 counts accumulated, resulting in a $2\sigma$ counting error of $<2\%$.

Arterial Cholesterol and Isoprostane Determinations

Arterial cholesterol content was determined in the carotid artery. Lipid extracts of arterial tissue were prepared, and total and free cholesterol concentrations were determined enzymatically as described previously. Esterified cholesterol was determined as the difference between measured total and free cholesterol.

Arterial isoprostanes were measured in the first subset of animals in the CAS and SOY group as an index of oxidant stress using negative-ion selective ion monitoring, gas chromatography-mass spectroscopy as reported. Briefly, artery sections were extracted using the method of Folch et al., including 0.1 mmol/L DTPA, 80 µmol/L BHT, and 2 mmol/L triphenylphosphine in the extraction to prevent further oxidation. F$_g$ isoprostane concentrations were measured in the organic layer using a Riberma R10–10C quadrupole mass analyzer (Nermag, SA, France) interfaced to a Hewlett Packard model 5890 Series II gas chromatograph as described.

Data Analysis

Data are presented as the mean±SEM. Statistical analyses were performed using BMDP statistical software (Version 7.0, SPSS, Inc.) and SAS (Version 8.2, SAS Institute). One-way ANOVA and ANCOVA were used to detect differences among treatment groups, and the Duncan multiple range post hoc test was used to determine specific group differences. Repeated measures of ANCOVA were used to analyze treatment effects on plasma lipid concentrations over time. Log transformations were performed if variances differed among groups (carotid cholesterol measures and arterial degradation in carotid, carotid bifurcation, and thoracic aorta). Carotid artery cholesterol was adjusted for baseline plasma apoprotein B lipoprotein cholesterol by ANCOVA because this variable explained a significant portion of the variability. Pearson correlations were used to assess the relation between dependent variables. $P<0.05$ was considered significant.

Results

No differences were found among treatment groups for any of the baseline lipids. Apo B lipoprotein cholesterol was significantly lower with SOY compared with CAS ($P=0.0002$) and ISO ($P<0.0001$) (Figure 1). Similar treatment effects were found for TPC, which was significantly lower with SOY as compared with CAS ($P=0.008$) and ISO ($P=0.0002$) groups (data not shown). HDLC was significantly higher with SOY compared with CAS ($P<0.0001$) and ISO ($P=0.0009$) (Figure 1). Treatment did not influence plasma TGs ($P=0.74$).

SOY resulted in 50% less accumulation of LDL degradation products in coronary arteries ($P=0.02$) (Table 1). This was likely not due to reduced LDL permeability, because treatment did not alter LDL permeability. However, SOY decreased total delivery of LDL to the artery by 60% ($P=0.02$) due to the reduced plasma LDLC, which may

| Table 1. Effect of Soy Protein and Isoflavones on Coronary Artery LDL Metabolism (Mean±SEM) |
|---------------------------------|---------------------------------|-----------------|
| **Diet** | **Degradation Rate** ($\mu$g/g/h) | **Permeability** ($\mu$L/g/h) | **Delivery** ($\mu$L LDLC/g h) |
| CAS | 21.6±3.2 (12) | 0.91±0.15 (8) | 294±47² (8) |
| ISO | 23.0±4.7 (10) | 1.09±0.25 (6) | 306±72² (6) |
| SOY | 10.6±2.0 (12) | 0.91±0.13 (8) | 120±30² (8) |
| P (ANOVA) | 0.02 | NS | 0.02 |

CAS indicates casein-lactalbumin; ISO, casein-lactalbumin plus isoflavones; SOY, soy protein. Numbers in parentheses represent sample size. NS = not significant. Unlike letters represent significant differences ($P<0.05$).
Arterial LDL Degradation

![Figure 2](image_url)

**Figure 2.** Effects of consuming casein-lactalbumin (CAS), casein-lactalbumin plus isoflavones (ISO), and soy protein (SOY) on arterial LDL degradation in coronary arteries (COR, *P* = 0.02), carotid bifurcation (BIF, *P* = 0.17), common carotid artery (CAR, *P* = 0.07), and thoracic (TA, *P* = 0.27) and abdominal (AA, *P* = 0.14) aorta. BIF, CAR, and TA are plotted as retransformed means.

Explain the reduced LDL degradation. The beneficial effect of SOY on arterial LDL degradation was found across other arterial sites; however, these differences were not significant (Figure 2). No significant effects were found with ISO, despite a tendency for increased LDL degradation in some arterial sites.

Consistent with decreased arterial LDL delivery and LDL degradation with SOY, the carotid artery cholesterol content also tended to be less (Table 2). As expected with early atherogenesis, this effect was primarily due to decreased esterified cholesterol, which was significantly less with SOY (*P* = 0.01). Carotid arterial LDL degradation correlated significantly with the esterified cholesterol content (*r* = 0.75, *P* < 0.001).

In order to determine if the decreased atherogenesis with SOY was due to reduced oxidative stress, arterial isoprostane concentrations were measured in CAS and SOY groups. There was no effect of treatment (Table 3). Plasma markers of inflammation (MCP-1 and VCAM) were also unaltered by treatment.

### Discussion

This study extends our previous report on the effects of soy and isolated isoflavones on plasma lipids and lipoproteins to include effects on arterial LDL metabolism and atherogenesis. First, the beneficial effects of soy on plasma lipids and lipoproteins translate to beneficial effects in the artery. Arterial LDL degradation and carotid artery cholesterol ester content are reduced due to a decrease in arterial LDL delivery.

#### Table 2. Effect of Soy Protein and Isoflavones on Carotid Artery Cholesterol Content (Mean±SEM)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total Cholesterol (mg/g)</th>
<th>Esterified Cholesterol (mg/g)</th>
<th>Free Cholesterol (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td>1.8±0.3 (20)</td>
<td>0.58±0.21* (20)</td>
<td>1.3±0.14 (20)</td>
</tr>
<tr>
<td>ISO</td>
<td>1.9±0.2 (17)</td>
<td>0.56±0.17* (17)</td>
<td>1.3±0.9 (17)</td>
</tr>
<tr>
<td>SOY</td>
<td>1.3±0.1 (20)</td>
<td>0.18±0.03* (20)</td>
<td>1.1±0.1 (20)</td>
</tr>
</tbody>
</table>

*P* (ANOVA)

|       | 0.08 | 0.01 | NS |

CAS indicates casein-lactalbumin; ISO, casein-lactalbumin plus isoflavones; SOY, soy protein. Numbers in parentheses represent sample size. NS = not significant. Unlike letters represent significant differences (*P* < 0.05).

#### Table 3. Effect of Soy Protein and Isoflavones on Arterial Isoprostanes and Plasma Inflammatory Markers (Mean±SEM)

<table>
<thead>
<tr>
<th>Isoprostanes</th>
<th>MCP-1</th>
<th>VCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>4.93±0.67 (12)</td>
<td>271±16 (20)</td>
</tr>
<tr>
<td>ISO</td>
<td>258±9 (17)</td>
<td>232±16 (17)</td>
</tr>
<tr>
<td>SOY</td>
<td>4.55±0.77 (11)</td>
<td>246±16 (20)</td>
</tr>
</tbody>
</table>

*P* (ANOVA)

CAS indicates casein-lactalbumin; ISO, casein-lactalbumin plus isoflavones; SOY, soy protein; MCP-1, monocyte chemotactic protein 1; VCAM, vascular cell adhesion molecule. Numbers in parentheses represent sample size. NS = not significant.

### Previous Results

Previous results from our group have suggested that soy protein with isoflavones had beneficial effects on atherosclerosis. Similarly, Kirk et al reported an inhibition of atherosclerosis in LDL receptor-intact mice fed soy with isoflavones compared with those with alcohol-washed soy (which removes isoflavones and other soluble components). Further, Crouse et al have demonstrated a dose-dependent improvement in plasma lipids with increasing concentrations of isoflavones in soy protein, strongly suggesting a role for isoflavones. Other studies have found that an alcohol-extractable component of soy protein is largely responsible for the beneficial effects on plasma lipids and lipoproteins. Studies in rats and hamsters have shown decreased LDL concentrations when a casein-based diet was supplemented with an alcohol extract of soy protein. The extract had been treated with acetone to precipitate sugars, saponins, and proteins and thus was mostly phytoestrogens (78.9%).

In contrast, studies in rabbits and people using isolated soy isoflavones have not found beneficial effects on plasma lipoproteins. The study in rabbits found an isoflavone aglycone-rich extract decreased aortic atherosclerosis despite no changes in serum lipid profiles. There was a decrease in oxidative stress in both plasma (cholesterol ester hydroperoxides) and aorta (malondialdehyde), suggesting that antioxidant effects of isoflavones may have mediated the decreased atherosclerosis. Reasons for the differences between these results and ours are not clear. It is likely that different doses or preparations of isoflavones may have different effects. Also, differences in absorption or metabolism of the isoflavones between species may result in different responses.

The cardioprotective effects of soy protein containing isoflavones have been postulated to be due to the estrogenic effects of the isoflavones. These compounds are structurally similar to estradiol, but while estradiol binds similarly to both estrogen receptor (ER) α and ERβ, the isoflavones bind with relative greater affinity to ERα compared with ERβ. The relative expression of ERα and ERβ differs greatly between individual tissues, but both subtypes are found in cardiovascular tissues and may mediate some of the cardiovascular protective effects. Genistein, for example, results in changes to the artery. Second, adding isolated soy isoflavones to the casein/lactalbumin diet did not appear to reduce atherogenesis.
in arterial ER subtypes after arterial injury and decreases neointimal formation.29

Other soy protein components, including the 7S globulin (β-conglycinin) and both a low- and high-molecular-weight fraction of soy, have been proposed to mediate the effects on lipoprotein metabolism.6 Soy research is currently hampered by the fact that alcohol-washing the protein to separate protein components from isoflavones appears to disrupt the structure of the individual components. Also, despite similar plasma levels of the isoflavones with SOY and ISO, we cannot be assured of comparable metabolic fates. For example, the aglycones (present in ISO) are absorbed faster but the bioavailability is less than when ingested in glycosylated forms (present in SOY).30,31 Also the metabolism of daidzein to equol is often higher after ingestion of glycosides due to the longer intestinal transit time. In this study, the blood sample taken 2 hours after ingestion was likely prior to the complete metabolism of daidzein, thus resulting in slightly lower levels with SOY compared with ISO. As equol is a more estrogenic isoflavone with greater antioxidant activity, this metabolism could be important.32 Detailed metabolic studies will be needed to further assess this area.

We have found that with conventional estrogens a large portion of the beneficial effects appears to be mediated by direct effects on the artery. Further, when parenteral replacement is used, there are few changes in plasma lipoproteins, yet arterial LDL metabolism16 and atherosclerosis1 are inhibited 70% and 50%, respectively. Thus, we had proposed that even though we found no effect of isolated isoflavones on plasma lipoproteins, beneficial effects on atherogenesis might still occur due to direct antiatherogenic effects on the artery.

In addition to the rabbit study by Yamakoshi et al,26 others have found isoflavones to have direct effects on the artery. Nestel et al12 found improved arterial compliance with isolated isoflavones, despite no effect on plasma lipids. But in this same study isoflavones did not alter forearm flow-mediated dilation or antioxidant activity. In premenopausal monkeys, intravenous genistein changed the constrictor response to acetylcholine to dilation within 20 minutes of administration.33 A similar finding in women was reported.34 Genistein, at doses from 10 to 300 nmol/min (resulting in physiological levels), was infused intravenously and forearm blood flow determined. A dose-dependent increase in forearm blood flow was seen in both men and premenopausal women. Estradiol produced equivalent increases in blood flow, but daidzein was ineffective. The increases with estradiol and genistein were both blocked by a nitric oxide (NO) synthase inhibitor, suggesting dilation results from activation of the l-arginine/NO pathway.34

This study was specifically of short duration to allow investigation of early events in atherogenesis. For example, if there were differences in atherosclerosis extent, one would expect to see differences in oxidative stress, inflammatory markers, and smooth muscle cell migration. It is likely that if the study period were longer we would have seen decreased atherosclerosis with soy as well as decreased oxidative stress, as published previously.35 Our observation that the treatments did not alter plasma markers of chemotaxis (MCP-1) and cell adhesion (VCAM-1) as well as reports that estrogens decrease plasma levels of these markers in both monkeys and women16,37 may suggest some mechanistic differences between isoflavones and estradiol.

In conclusion, the early events associated with the cardioprotective effect of soy consumption are explained in part by decreases in arterial LDL delivery as reported in male monkeys.19 This does not occur with intake of isolated isoflavones. As the plasma isoflavone concentrations in this study were similar between the SOY and ISO groups, it appears that both were absorbed similarly. Thus, the nature of the active components is unclear. Further studies to differentiate the effects of soy components compared with the intact soy protein containing isoflavones are ongoing.

Acknowledgments

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