

# Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance

(flavonoids/antioxidants/genistein/daidzein)

MATTI J. TIKKANEN<sup>\*†</sup>, KRISTIINA WÄHÄLÄ<sup>‡</sup>, SIRPA OJALA<sup>‡</sup>, VEERA VIHMA<sup>\*</sup>, AND HERMAN ADLERCREUTZ<sup>§</sup>

Departments of <sup>\*</sup>Medicine, <sup>‡</sup>Organic Chemistry, and <sup>§</sup>Clinical Chemistry, University of Helsinki, 00290 Helsinki, Finland

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**ABSTRACT** The oxidation of low density lipoproteins (LDLs) is thought to take place in the arterial intima when the particles have become isolated from circulating water-soluble antioxidants. We hypothesized that isoflavonoid antioxidants derived from soy could be incorporated into lipoproteins and possibly could protect them against oxidation, which is regarded as atherogenic. Six healthy volunteers received 3 soy bars [containing the isoflavonoid antioxidants genistein (12 mg) and daidzein (7 mg)] daily for 2 weeks. LDLs were isolated from blood drawn at the end of a 2-week dietary baseline period, after 2 weeks on soy, and after discontinuation of soy. Large increases in plasma isoflavonoid levels occurred during soy feeding, but only minute amounts were stably associated with lipoproteins (less than 1% of plasma isoflavonoids in the LDL fraction). The LDLs were subjected to copper-mediated oxidation *in vitro*. Compared with off soy values, lag phases of LDL oxidation curves were prolonged by a mean of 20 min ( $P < 0.02$ ) during soy intake, indicating a reduced susceptibility to oxidation. The results suggest that intake of soy-derived antioxidants, such as genistein and daidzein, may provide protection against oxidative modification of LDL. As only very small amounts of these substances were detected in purified LDL, modified LDL particles may have been produced *in vivo* by circulating isoflavonoids promoting resistance to oxidation *ex vivo*.

The oxidative modification of low density lipoprotein (LDL) particles is considered to be a prerequisite for the uptake of LDL by macrophages in the artery wall, an initial step in the formation of atheroma. Oxidation probably takes place in microdomains in the arterial intima sequestered from the abundant antioxidants in plasma (1). Lipophilic natural antioxidants are transported in LDL (2–4), and could in theory protect LDL from oxidation when it has left the plasma compartment. Current knowledge is, however, derived from oxidation studies carried out *in vitro*. A common approach is to isolate LDL and add copper ions into the medium, which initiates chain reactions that oxidize LDL lipids extensively. This leads to a rearrangement of fatty acid double bonds producing the characteristic 234 nm absorption of “conjugated dienes” (2). Administration of two lipophilic antioxidants, probucol and  $\alpha$ -tocopherol, has been shown to prolong the lag phase preceding the rapid propagation phase, which reflects conjugated diene production (3, 5), but it is not known whether this indicates protection of LDL *in vivo*.

We thought that the provision of food antioxidants into LDL particles could be a possible strategy for preventing oxidation of this lipoprotein. Dietary soy protein is considered atherogenic (6, 7). We explored the possibility that isofla-

vonoids known to possess significant antioxidative activity in lipid-aqueous systems (8) and contained in soy protein products (9) could reduce the susceptibility of LDL particles to oxidation.<sup>¶</sup>

## MATERIALS AND METHODS

**Design of Soy Feeding Studies.** Six apparently healthy volunteers (3 men and 3 women) participated in the study. They were between 20 and 30 years of age and did not take any medications. Starting 2 weeks before initiation of soy intake they had to eliminate the following items from their diet: all soy products, beans, peas, nuts, sprouts, and seeds and any food that could contain any of these components. No vitamin supplements or vitamin-fortified foods were allowed, and vegetable oil consumption had to be kept at minimum. The study participants were advised to keep their diet as constant as possible during the whole study period.

After the 2 baseline weeks the participants consumed one soy bar (Scientific Hospital Supplies International, Liverpool, England) containing about 12 mg of genistein (4',5,7-trihydroxyisoflavone) and 7 mg of daidzein (4',7-dihydroxyisoflavone) per bar (analyzed by gas chromatography) three times daily for 2 weeks. These soy bars did not contain any added antioxidants or vitamins. The energy content per bar was 123 kJ, and one bar contained 18.3 g of carbohydrate, 2.4 g of fat, and 7.1 g of protein equivalent. Because the bars contained soy protein, the subjects reduced their intake of meat and fish to compensate for this. Following discontinuation of soy intake they continued with the baseline diet for another 2 weeks.

Fasting blood was drawn during the last 2 days of the 2-week baseline period (days -1 and 0), during the last 2 days of the 2-week soy feeding period (days 13 and 14), as well as 12 days after discontinuation of soy intake (day 26).

**Lipid and Lipoprotein Determinations.** Plasma and lipoprotein cholesterol and triglyceride were determined by enzymatic methods (Boehringer Mannheim). The main lipoprotein fractions very low density lipoproteins (VLDLs) (35,000 rpm, 19 hr), LDL (35,000 rpm, 22 hr), and high density lipoproteins (HDLs) (35,000 rpm, 48 hr) were isolated by sequential ultracentrifugation (10) in a Beckman Ti 50.4 rotor in a Beckman L-80 ultracentrifuge. Following ultracentrifugation of HDL, the bottom fraction was taken to represent lipoprotein-deficient serum. Lipoprotein fractions were physically separated by slicing of the ultracentrifuge tubes. When appropriate, EDTA and other small molecular weight substances

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; ID-GC-MS-SIM, isotope dilution-gas chromatography-mass spectrometry in the selected ion monitoring mode.

<sup>†</sup>To whom reprint requests should be addressed. e-mail: matti.j.tikkanen@helsinki.fi.

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were removed from lipoprotein fractions by gel filtration on Sephadex G-25 (Pharmacia) employing  $1.5 \times 15$ -cm columns.

**Determination of Isoflavonoids in Plasma and in Isolated Lipoprotein Fractions.** In the preliminary experiments we first used the original plasma method, which is based on isotope dilution–gas chromatography–mass spectrometry in the selected ion monitoring mode (ID–GC–MS–SIM) by using synthesized deuterated internal standards (11), and analyzed each lipoprotein and the lipoprotein-deficient serum fraction separately. In this method the free + sulfate fraction and the glucuronide fraction are quantitated separately. As very little of the isoflavonoids occurred in the free + sulfate fraction, the values being mostly below the sensitivity limit of the method (11) (results from one subject are shown in Table 1), further studies were carried out with a modified method by combining the two fractions. Initial experiments also included determinations of two isoflavonoid metabolites, equol and *O*-desmethylanagolensin (11), which were detected in very small quantities or were absent from lipoprotein fractions. Further studies therefore focused on the isoflavonoids genistein and daidzein only. We also analyzed the lignans enterodiol and enterolactone (11) to observe whether there were any significant changes in the levels of these compounds. They share a bisphenolic structure with daidzein and genistein, but because they are weak antioxidants (12), the results could be affected. Because lignan concentrations did not change significantly after soy intake, lignan determinations were omitted in later experiments. Because of the causative role of LDL in atherogenesis, we concentrated our further studies on this lipoprotein. Also, the concentrations of  $\alpha$ -tocopherol and ubiquinol-10 in LDL were measured on and off soy to monitor possible alterations by using the method of Lang *et al.* (13) modified by Aejmelaeus (14).

In the simplified version of the original ID–GC–MS–SIM method (11) the sample was diluted with triethylamine sulfate buffer and extracted with 4 ml of 70% methanol by using a Sep-Pak C<sub>18</sub> cartridge (Waters). After evaporation, the sulfates were first hydrolyzed in 2 ml of ethyl acetate/2  $\mu$ l of concentrated HCl overnight at 37°C. Following evaporation of the solvent, glucuronides were hydrolyzed with  $\beta$ -glucuronidase (11). The next steps of the method were exactly as described previously, except that one anion exchange chromatographic step (QAE-Sephadex in the acetate form) was eliminated. The interassay and intraassay coefficients of friction in single assays were 12.5 ( $n = 13$ ) and 10.6% ( $n = 10$ ) for daidzein and 11.4 ( $n = 13$ ) and 6.3% ( $n = 10$ ) for genistein, respectively.

**Oxidation of LDL *in Vitro*.** A rapid method was used to isolate LDL from plasma (15). All solutions contained EDTA, and tubes were wrapped with foil to protect the samples from light. VLDL was first isolated by ultracentrifugation by using a Beckman Ti 50.4 rotor ( $d = 1.006$  g/ml, 50,000 rpm,  $3\frac{1}{2}$  hr, 15°C) in a Beckman L-80 ultracentrifuge. Following removal of VLDL by tube slicing, the bottom fraction was transferred to

another tube and centrifuged by using the same rotor ( $d = 1.063$  g/ml, 38,000 rpm, 18 hr, 15°C). The LDL-containing top layer was isolated by slicing the centrifuge tubes. Before oxidation experiments, EDTA and other substances not bound to LDL were removed by gel filtration on Sephadex G-25.

The lipid hydroperoxides (“conjugated dienes”) generated during lipid peroxidation of LDL exhibit an absorption maximum around 234 nm and can be directly measured from an aqueous LDL solution (2). In brief, LDL (100  $\mu$ g of protein/ml) in 0.01 M phosphate buffer, pH 7.4/0.16 M NaCl was supplemented with 5  $\mu$ M CuSO<sub>4</sub> as prooxidant (2). Oxidation (formation of conjugated dienes in LDL) was continuously monitored in 1.5-ml quartz cuvettes at 21°C in a Shimadzu UV-1202 spectrophotometer equipped with a Shimadzu cell temperature CPS-controller over a period of 5 hr. The length of the lag phase was defined as the time (minutes) to the intercept of the tangent of the absorbance curve in the propagation phase with baseline. Propagation rate was expressed as the slope of the tangent (change in absorbance/min) (2).

## RESULTS

Before the soy feeding study, preliminary 1-week soy feeding experiments were carried out in 5 volunteers. These experiments showed that very small amounts of soy-derived phytoestrogens were present in free form or as sulfate conjugates in plasma, and therefore most further studies were carried out by using the simplified method (see *Determination of Isoflavonoids in Plasma and in Isolated Lipoprotein Fractions*), which determined free, sulfate, and glucuronide conjugates not separately but in combination after hydrolysis.

**Soy Feeding Study.** No changes were observed in the levels of cholesterol, triglyceride, phospholipid, or protein in LDL (Table 2) or in other lipoprotein fractions or whole plasma in six individuals during administration of soy. After soy feeding, significant amounts of genistein and daidzein were detected in unpurified LDL fractions obtained by tube slicing (Table 2). Twelve days after discontinuation of soy intake, these phytoestrogen levels fell close to baseline levels. The question arose whether water-soluble phytoestrogen metabolites might have contaminated the LDL fractions during separation. Control experiments carried out in other individuals and employing careful purification of LDL by gel filtration revealed that the lipoprotein-bound isoflavonoids constituted a minor part of the total amount contained in the LDL fraction, indicating that the bulk was present unbound in the aqueous phase (or very loosely bound to the lipoprotein). A representative example is shown in Table 1. The proportion of genistein and daidzein in purified LDL was less than 1% of total plasma content of these substances (Table 1). Some of the LDL samples from the original feeding study were sent to another laboratory (Dr. Metsä-Ketelä, University of Tampere) for

Table 1. Isoflavonoid concentrations before and after purification of lipoprotein fractions by gel filtration following intake of one soy bar (12 mg of genistein, 7 mg of daidzein) three times daily for 1 week in a normolipidemic woman

| Lipoprotein fraction | Gel filtration | Glucuronide fraction*      |                           | Free + sulfate fraction†   |                           |
|----------------------|----------------|----------------------------|---------------------------|----------------------------|---------------------------|
|                      |                | Genistein, pmol/mg protein | Daidzein, pmol/mg protein | Genistein, pmol/mg protein | Daidzein, pmol/mg protein |
| VLDL                 | Before         | 53.3                       | 27.1                      | 0.52                       | 0.83                      |
|                      | After          | 1.1 (<0.1%)                | 1.2 (<0.1%)               | 0.70 (0.5%)                | 0.00 (0%)                 |
| LDL                  | Before         | 8.44                       | 1.98                      | 0.31                       | 0.31                      |
|                      | After          | 1.4 (0.1%)                 | 3.8 (0.7%)                | 0.64 (2.1%)                | 0.76 (1.2%)               |
| HDL                  | Before         | 3.92                       | 1.8                       | 0.15                       | 0.29                      |
|                      | After          | 0.28 (<0.1%)               | 0.21 (<0.1%)              | 0.21 (1.2%)                | 0.00 (0%)                 |
| Total plasma         |                | 754 nmol/liter             | 406 nmol/liter            | 23.5 nmol/liter            | 49.2 nmol/liter           |

\*Percent values calculated from total plasma glucuronide level.

†Percent values calculated from total plasma free + sulfate level.

Table 2. The effect of 2 weeks of soy feeding on the content (mean  $\pm$  SD) of isoflavonoid phytoestrogens in LDL fractions isolated from six individuals

|  | Baseline       |                | Soy feeding                   |                               | Off soy        |
|--|----------------|----------------|-------------------------------|-------------------------------|----------------|
| Day  | -1             | 0              | 13                            | 14                            | 26             |
| Genistein, pmol/mg of LDL protein*                           | 2.3 $\pm$ 2.9  | 1.6 $\pm$ 1.8  | 172.4 $\pm$ 68.1 <sup>†</sup> | 175.0 $\pm$ 80.1 <sup>†</sup> | 2.4 $\pm$ 1.1  |
| Daidzein, pmol/mg of LDL protein*                            | 1.1 $\pm$ 1.8  | 3.5 $\pm$ 4.0  | 70.7 $\pm$ 63.3 <sup>‡</sup>  | 72.1 $\pm$ 45.2 <sup>†</sup>  | 2.9 $\pm$ 2.9  |
| Cholesterol, mmol/liter                                      | 3.4 $\pm$ 0.8  | 3.3 $\pm$ 0.7  | 3.4 $\pm$ 0.7                 | 3.4 $\pm$ 0.9                 | 3.3 $\pm$ 0.9  |
| Triglyceride, mmol/liter                                     | 0.4 $\pm$ 0.1  | 0.3 $\pm$ 0.1  | 0.4 $\pm$ 0.1                 | 0.4 $\pm$ 0.1                 | 0.4 $\pm$ 0.1  |
| $\alpha$ -Tocopherol, pmol/nmol of phospholipid <sup>§</sup> | 10.5 $\pm$ 2.2 | 10.3 $\pm$ 2.1 | 10.3 $\pm$ 2.1                | 9.8 $\pm$ 2.2                 | 10.4 $\pm$ 2.0 |
| Ubiquinol-10, fmol/nmol of phospholipid <sup>§</sup>         | 511 $\pm$ 146  | 514 $\pm$ 209  | 470 $\pm$ 193                 | 466 $\pm$ 184                 | 465 $\pm$ 203  |

\*Fractions not purified by gel filtration on Sephadex G-25. Pooled samples were later gel filtered and reanalyzed showing genistein and daidzein levels not significantly different from baseline.

<sup>†</sup> $P < 0.001$  vs. baseline (paired  $t$  test).

<sup>‡</sup> $P < 0.05$  vs. baseline (paired  $t$  test).

<sup>§</sup>Determination carried out in five of six individuals.

vitamin measurements. The remaining parts of these samples were recovered 1 year later, pooled, and reanalyzed following purification by gel filtration on Sephadex G-25. The results indicated that minute amounts of phytoestrogen (1–2 pmol/mg of LDL protein) were detectable, which could not be proven to differ significantly from baseline.

**Copper-Induced Oxidation of LDL *in Vitro*.** The isolated LDLs before, during, and after supplementation with soy were subjected to copper ( $\text{Cu}^{2+}$ )-induced oxidation. The results indicated a significant mean prolongation of the lag phase by more than 20 min after 2 weeks of soy feeding (Fig. 1; Table 3). There was a nonsignificant decrease in the propagation rate on day 13, and the maximum absorbance was slightly but significantly reduced. Whereas all oxidation experiments were carried out with fresh blood samples, part of the LDL samples were stored at  $-70^\circ\text{C}$ . The oxidation reactions were later repeated by carrying out simultaneous experiments on samples obtained at baseline, on soy and off soy in five of six individuals. The repeat measurements gave essentially similar results (mean lag times were: day -1, 129 min; day 0, 141 min; day 13, 171 min; day 14, 171 min; day 26, 134 min). Isoflavonoid concentrations in LDL before purification by gel filtration correlated positively with lag times [genistein:  $r = 0.723$ ;  $P < 0.001$ ; daidzein:  $r = 0.508$ ;  $P = 0.004$  ( $n = 30$ )].

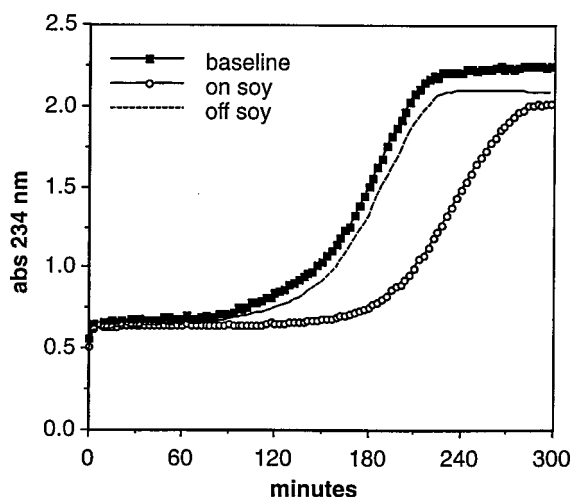


FIG. 1. Examples of continuous monitoring of  $\text{Cu}^{2+}$ -mediated LDL oxidation by the diene absorption. LDL ( $100 \mu\text{g}$  of protein/ml) in PBS (pH 7.4) was supplemented with  $5 \mu\text{M}$   $\text{CuSO}_4$ , and the change of diene absorption at 234 nm was recorded in 1.5-ml quartz cuvettes at  $21^\circ\text{C}$  in a Shimadzu UV-1202 spectrophotometer. The oxidation curves were obtained from one soy feeding study participant at baseline, after 2 weeks on soy (3 soy bars/day), and 12 days after discontinuation of soy intake. Note the shift of the curve to the right during soy intake, followed by return to the original position after discontinuation of soy intake.

## DISCUSSION

Dietary isoflavonoid phytoestrogens (16) are a group of bisphenolic compounds with weak estrogenic activity (12, 17). They (mainly genistein and daidzein) occur particularly in soybeans in the form of glycosides (genistin and daidzin) or in the free form in fermented soy products. They have been shown to influence sex hormone metabolism, protein synthesis, growth factor activity, and malignant cell proliferation (17). Genistein, and to a smaller extent, daidzein, have been shown to exert anticarcinogenic effects (17, 18). However, they are also the source of antioxidant activity in soybeans and soy products (9).

Our finding that 2 weeks of soy feeding caused a significant prolongation of lag time in six individuals is potentially important, as oxidative modification of LDL is regarded as atherogenic. However, the underlying mechanism is difficult to explain. The surface layer of the large molecular weight LDL consists of phospholipid, free cholesterol, and apolipoprotein B surrounding the core, which contains esterified cholesterol and small amounts of triglyceride. Copper-induced oxidation probably starts from the polyunsaturated fatty acids in the surface phospholipid layer and then propagates to the core (1). In theory, the prolongation of the lag phase during soy intake could be caused by LDL-bound antioxidants, such as genistein and daidzein, acting as peroxyl radical scavengers. Glucuronide or sulfate conjugates could probably associate with the LDL surface layer, the lipophilic ends of the molecules sticking into the lipid core, with the hydrophilic glucuronide or sulfate parts remaining on the outside. No data on the isoflavonoid content of lipoproteins have been published previously. We detected only very small amounts of these substances in LDL from which small molecular weight contaminants had been removed by gel filtration. These levels corresponded to isoflavonoid:LDL molar ratios ranging between 1:400 and 1:600. Compared with  $\alpha$ -tocopherol, a natural antioxidant carried in lipoproteins with a mean molar ratio of about 6:1 (2), the relative amounts of soybean phytoestrogens appear too small to explain their antioxidant effects by direct interception of free radicals. Also, it is highly unlikely that metal-chelating capabilities described for some flavonoid substances (19) could operate significantly at such small concentrations.

Although it is difficult to envisage how extremely small concentrations of isoflavonoids present in LDL particles could explain the prolongation of lag time observed in our study, several alternative mechanisms are possible. First, elevated concentrations of circulating isoflavonoid phytoestrogens during soy feeding could have protected the LDL *in vivo* resulting in particles with diminished concentrations of preformed lipid hydroperoxides. Preformed hydroperoxides enhance the initiation rate of oxidation (20), which, in turn, influences the length of the lag time. Accordingly, the prolonged lag times during soy intake could reflect lower initiation rates because



Table 3. The effect of 2 weeks of soy feeding on the copper-induced oxidation of LDL isolated from six individuals (mean  $\pm$  SD)

| Day                                       | Baseline      |               | Soy feeding    |               | Off soy       |
|---|---------------|---------------|----------------|---------------|---------------|
|   | -1            | 0             | 13             | 14            | 26            |
| Lag phase, min                            | 147 $\pm$ 9   | 150 $\pm$ 10  | 169 $\pm$ 17*  | 173 $\pm$ 19† | 143 $\pm$ 15  |
| Propagation rate, $\Delta$ absorbance/min | 2.5 $\pm$ 0.3 | 2.4 $\pm$ 0.3 | 2.1 $\pm$ 0.3  | 2.1 $\pm$ 0.3 | 2.1 $\pm$ 0.4 |
| Maximum increase, absorbance              | 1.7 $\pm$ 0.1 | 1.5 $\pm$ 0.1 | 1.4 $\pm$ 0.1‡ | 1.4 $\pm$ 0.2 | 1.4 $\pm$ 0.3 |

\* $P < 0.005$  vs. baseline (paired  $t$  test).† $P < 0.02$  vs. baseline (paired  $t$  test).‡ $P < 0.05$  vs. baseline (paired  $t$  test).

of reduced hydroperoxide formation *in vivo*. The positive correlation observed between genistein and daidzein concentrations in the unpurified LDL fractions and lag times gives some support to this idea.

A second possibility relates to the fact that the binding of  $\text{Cu}^{2+}$  to apolipoprotein B of LDL initiates lipid peroxidation (20, 21). Conceivably, isoflavonoid phytoestrogens known to bind tightly to soybean protein (9) could become attached to binding sites on apolipoprotein B normally occupied by  $\text{Cu}^{2+}$ , inhibiting lipid peroxide formation. A stable modification of LDL could result in oxidation resistance even after LDL has been isolated from circulating isoflavonoids and other antioxidants. This type of mechanism has been suggested for dehydroascorbic acid, a water-soluble antioxidant, which, when coincubated with LDL, renders it resistant to oxidation (22). It is not clear whether our extraction procedure would be able to liberate the isoflavonoids from such binding sites to allow reliable measurement.

Another possibility that cannot be excluded is that the LDL could have contained other antioxidative isoflavonoid metabolites not detectable with our methodology. Initial experiments suggested that the known metabolites equol and *O*-desmethylangolensin were present in much smaller quantities than genistein and daidzein, if at all. Some reports have suggested that endogenous human steroids may be converted to lipoidal derivatives and stored in fat tissue (23). There is no evidence that isoflavonoid phytoestrogens are converted to such lipoidal metabolites in the human organism. However, should such lipophilic derivatives have been present in our samples, they would probably have escaped detection by our phytoestrogen assay.

Finally, it is possible that soy intake could have altered LDL particle size although lipid and protein composition was not altered. LDL size alterations have been described during oral estrogen replacement therapy (24), and this could influence oxidizability, which is reportedly dependent on particle size (25).

Although measurement of oxidation resistance is susceptible to methodological errors, the validity of our results is supported by several findings. First, all individuals showed similar shifts of the propagation curves to the right during soy intake and opposite shifts off soy. Second, repeat oxidation experiments on samples stored at  $-70^\circ\text{C}$  with simultaneous measurement of baseline, on soy, and off soy LDL samples supported the findings obtained with fresh samples.

Although our results demonstrate a possible role for genistein and daidzein in promoting resistance of LDL against oxidation, caution is needed in interpreting the results. There is no convincing evidence that copper-induced oxidizability is of relevance with regard to oxidation resistance *in vivo*. Nevertheless, our results indicate that intake of isoflavonoid food antioxidants can in some way alter LDL particles so that they exhibit resistance to oxidation *ex vivo*. From this follows the theoretical possibility of producing stably modified LDL resistant to oxidation (also after sequestration in the artery wall) by the intake of common food-derived substances. Soy protein in the diet has also been shown to lower cholesterol both in animals and man, although no significant changes occurred in human subjects with initially normal cholesterol

levels (below 5 mmol/liter) (26), in line with our findings. The underlying mechanism is unknown, but because relatively large amounts of the weakly estrogenic isoflavonoids are bound to soy proteins, the possibility exists that they stimulate hepatic LDL receptor activity in analogy with the effect of estrogen administration. Recent evidence obtained in studies with rhesus monkeys demonstrated that in the female (but not in the male) monkey the hypocholesterolemic effect was because of the presence of isoflavonoids (27). The low rates of coronary heart disease in populations consuming soy add to the interest in the possibility that we are dealing with a dual cholesterol-lowering and antioxidative effect.

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