Soy Protein Containing Isoflavones Favorably Influences Macrophage Lipoprotein Metabolism but Not the Development of Atherosclerosis in CETP Transgenic Mice

Leiko Asakura[a], Patrícia M. Cazita[a], Lila M. Harada[a], Valéria S. Nunes[a], Jairo A. Berti[b], Alessandro G. Salerno[b], Daniel F.J. Ketelhuth[c], Magnus Gidlund[c], Helena C.F. Oliveira[b], Eder C.R. Quintão[a,*]

[a]Lipids Lab, University of São Paulo Medical School, São Paulo, Brazil, [b]Department of Physiology and Biophysics, Biology Institute at State University of Campinas (UNICAMP), São Paulo, Brazil, and [c]Department of Immunology, Biomedical Sciences Institute at the University of São Paulo, Brazil

ABSTRACT: The possibility that soy protein containing isoflavones influences the development of experimental atherosclerosis has been investigated in ovariectomized mice heterozygous for the human CETP transgene and for the LDL-receptor null allele ($LDLr^{+/-}$ $CETP^{+/-}$). After ovariectomy at 8 wk of age they were fed a fat/cholesterol-rich diet for 19 wk and divided into three experimental groups: dietary unmodified soy protein containing isoflavones (mg/g of diet), either at low-dose (Iso Low, 0.272, $n = 25$), or at high-dose (Iso High, 0.535, $n = 28$); and the atherogenic diet containing an isoflavone-depleted alcohol-washed soy protein as a control group ($n = 28$). Aortic root lipid-stained lesion area (mean $\mu m^2 \times 10^3 \pm$ SD) did not differ among Iso Low (12.3 ± 9.9), Iso High (7.4 ± 6.4), and controls (10.7 ± 12.8). Autoantibody titers against plasma oxidized LDL did not differ among the experimental groups. Using the control mice as the reference value (100%), *in vitro* mouse peritoneal macrophage uptake of labeled acetylated LDL-cholesterol was lower in the Iso High (68%) than in the Iso Low (85%) group. The *in vitro* percent removal by exogenous HDL of labeled unesterified cholesterol from macrophages previously enriched with human [4-^{14}C]-cholesteryl oleate acetylated LDL was enhanced in the Iso High group (50%). In spite of these *in vitro* potentially antiatherogenic actions, soy protein containing isoflavones did not modify the average size of lipid-stained area in the aortic root.

Paper no. L9983 in *Lipids 41,* 655–662 (July 2006).

Although most retrospective epidemiological studies have indicated that hormonal replacement therapy effectively reduces coronary artery disease outcomes in postmenopausal women, recent large intervention trials have failed to demonstrate these beneficial effects (1–3). Due to the controversies surrounding conventional estrogen replacement therapy, soy-based food has been proposed as a dietary alternative to lower the plasma lipid concentration (4), in addition to improving the menopause-related symptoms (5,6) and osteoporosis (7). However, in spite of a suggestion that the intake of phytoestrogens has diminished the incidence of coronary heart disease in Japan (8) a definite proof of this benefit in Western societies is lacking (9–12). In addition, in humans, soy isoflavones have been shown to lower plasma lipids according to two meta-analyses (4,13), but not in two recent studies (14,15). Nonetheless, in several animal species, protection against the development of experimental atherosclerosis has been ascribed to phytoestrogens (16), soy isoflavones (17–20), a specific soy protein (21), and a soy-rich diet (22–24). However, on reviewing these studies, we noticed that in four (17,18,21,22), but not in four others (19,20,23,24), reductions of atherosclerosis and of plasma cholesterol occurred simultaneously. On the other hand, rabbits fed phytoestrogens have shown significantly reduced aortic cholesterol, an effect that the authors deem comparable to that of the estrogen replacement therapy (25). In addition to the fact that dietary soy cannot be strictly compared to isoflavone administration, it is worth noting that with the exception of some experiments involving moderate degrees of hypercholesterolemia (17,18,23), in all other experiments hypercholesterolemia attained by the experimental animals was remarkable or even extremely severe (16,18–22,24). Degrees of hypercholesterolemia attained are not ideal models for the effects of isoflavones in the human population, where plasma cholesterol concentrations are far more modest. Furthermore, these mentioned studies cannot strictly be compared because diets differed according to several components, such as soy, soy isolates, phytoestrogens, and pure isoflavones, making it difficult to interpret them in regard to their usefulness in humans. In spite of these considerations, several potentially beneficial actions of isoflavones concerning protection against premature atherosclerosis have been proposed, such as a reduction of LDL susceptibility to oxidation, improvement in vascular reactivity, and inhibition of pro-inflammatory cytokines, cell adhesion, and platelet aggregation (26,27).

We measured the effects of treatments with low and high doses of soy protein containing isoflavones on the development of aortic root lipid-laden lesions, and, considering the possible antiatherogenic mechanisms involved, we measured the formation of serum antibodies against oxidized LDL and the cholesterol uptake and efflux by mouse peritoneal macrophages *in vitro*. We utilized ovariectomized mice partially deficient in LDL receptor ($LDLr^{+/-}$), expressing a human CETP minigene ($CETP^{+/-}$), as previously reported

*To whom correspondence should be addressed at Av. Dr. Arnaldo, 455, sala 3317, CEP 01246-000, São Paulo, SP, Brazil. E-mail: lipideq@usp.br

(28,29). This model was chosen because the total cholesterol concentration was mild, well within the range of a normal human population, and human CETP was expressed.

EXPERIMENTAL PROCEDURES

Animals. The animal protocols were approved by the University of São Paulo Medical School Ethics Committee (protocol number 191/01). Female $LDLr^{+/-}$ $hCETP^{+/-}$ mice (n = 110) on a C57BL/6 background were obtained from the crossbreeding of female LDLr-knockout (from the Jackson Laboratory, Bar Harbor, ME) with male CETP-transgenic (a kind gift from Dr. H.C.F. Oliveira, Unicamp, Campinas, São Paulo, Brazil). Mice were weaned at 4 wk of age, and thereafter fed an *ad libitum* commercially available diet for 5 wk (Nuvital Nutrientes Ltda., Curitiba, PR, Brazil), housed 5 mice per cage and kept in a 12-h light-dark cycle in a conventional room. At 8 wk of age, they were ovariectomized, and 3 wk afterward their vaginal smears were collected to evaluate ovariectomy effectiveness. Only those animals that presented atrophic vaginal epithelium during a 5-d consecutive follow-up period were included. Animals were separated into three groups: those fed soy protein containing isoflavones at low dose (Iso Low, n = 25), those fed soy protein containing isoflavones at high dose (Iso High, n = 28), and controls fed isoflavone-depleted alcohol-washed soy protein (C, n = 28).

Diets and feeding procedures. Experimental diets were made utilizing the TD 88137 (21% milk saturated fat; 0.2% cholesterol by weight) supplied by Harlan Teklad (Madison, WI). All the experimental diets and the soy protein isolates (Clinical 670 Blend and FXP-H-0140) were a kind gift from Dr. Susan M. Potter (Solae Company, St. Louis, MO). The Clinical 670 Blend contained (mg/g product): genistein, 1.10; daidzein, 0.84; and glicytein, 0.20. The FXP-H-0140 component presented 0.04 mg/g of the product total aglycone. Although the FXP-H-0140 is an isoflavone-free ethanol-extracted product (AWISP), a diet totally devoid of isoflavones is not feasible; hence, insignificant amounts of isoflavones may be detected. The diet made with soy protein containing isoflavones at high dose (Iso High) was prepared using 250 g of Clinical 670 Blend, and the diet made with soy protein containing isoflavones at low dose (Iso Low) was prepared using 125 g of FXP-H-140 and 125 g of Clinical 670 Blend as the source of protein. The diet of the control mice was prepared with 250 g of FXP-H-140. In order to correct for the differences in protein content between FXP-H-140 and Clinical 670 Blend, small amounts (1.1% and 0.57%, respectively) of casein were added to Iso High and Iso Low diets. These diets did not differ regarding the proportions of calories that were represented as protein (19.4%), fat (42.0%), and carbohydrates (38.6%); diets are summarized in Table 1.

During the 19-wk study period, animals were fed the following amounts of isoflavones/100 g diet: 1 mg (C), 27 mg (Iso Low), and 53 mg (Iso High).

Lipid analysis. Plasma total cholesterol (TC) and triacylglycerol (TAG) concentrations were determined by enzymatic assays with commercially available kits (Boehringer Mannheim, Darmstadt, Germany, and Merck KgaA, Buenos Aires, respectively).

TABLE 1
Experimental Diet Composition (g/kg)

	Iso High	Iso Low	Control
Casein	11.49	5.74	0
Soy protein[a]	250	250	250
Free isoflavone	0.535	0.2725	0.01
Sucrose	289	302	305
Corn starch	150	150	150
Anhydrous milk fat	202	202	202
Cholesterol	1.52	1.52	1.52
Cellulose	50	50	50
Minerals	21.7	23.6	25.5
Vitamins	10	10	10
Ethoxyquin	0.04	0.04	0.04
Fat (%)	42.0	41.9	41.8
Carbohydrate (%)	38.6	38.8	38.9

[a]Iso High: Clinical 670 Blend; Iso Low: 50% Clinical 670 Blend plus 50% FXP-H-0140; C: FXP-H-0140.

Histological analysis of atherosclerotic lesions. Mice were anesthetized with ketamine (100 mg/kg, ip, Ketalar, Parke-Davis, São Paulo, Brazil) and xylazine (32 mg/kg, ip, Rompum, Bayer S.A., São Paulo, Brazil) and their hearts perfused *in situ* with phosphate-buffered saline (PBS) followed by 10% PBS-buffered formaldehyde, after which they were excised and fixed in 10% formaldehyde for at least 2 d. The hearts were then embedded sequentially in 5%, 10%, and 25% gelatin. Processing and staining were carried out according to Paigen *et al.* (30). Briefly, the heart was sectioned directly under and parallel to the axis of the atrial leaflets; the upper section was embedded in tissue-freezing medium (TBS, Triangle Biomedical Sciences, Durham, NC) and frozen. Because several other studies revealed a preference for the development of lesions in the aortic root, the segment chosen for analysis extended from beyond the aortic sinus up to the point where the aorta first becomes rounded and was expressed as the sum of the lesions in six 10-µm sections, 80 µm distant from each other in a total aortic length of 480 µm. The slides were stained with oil red O and counterstained with Harris's hematoxylin and with light green. The lipid-stained lesions were quantified as described by Rubin *et al.* (31) using the Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, Silver Spring, MD). The slides were read by an investigator blinded to the experimental groups.

Detection of antibodies to oxidized LDL. Antibodies against holo-oxidized LDL (oxLDL) or antibodies anti-apoB epitope derived from oxLDL (ApoB-D) were measured in mouse plasma by ELISA (28,32). ApoB-D is a 22-amino acid peptide from a region of ApoB-100 not accessible to trypsin. Polystyrene microtiter plates (Costar, Cambridge, MA) were coated with 50 µL of human oxLDL (7.5 µg/mL, 20 mM Cu^{2+}, for 24 h) or 50 µL/well of ApoB-D (1 µg/mL) and kept overnight at 4°C. The plates were blocked with gelatin 1% (Invitrogen Co., Carlsbad, CA, USA) at room temperature for 2 h. Plates were washed twice with PBS (100 µL). Plasma

samples (50 μL, 1:100) were added, and the plates were incubated for 2 h at 4°C, followed by washing with 1% Tween 20 in PBS. A peroxidase-conjugated goat anti-mouse IgG (Pharmigen, San Diego, CA, USA) (50 μL, 1:5.000 in PBS) was added, and after 1 h at room temperature, the plates were washed. Finally, 75 μL of substrate solution (250 mg of tetramethylbenzidine in 50 mL of DMSO, 10 μL of 30% H_2O_2, 12 mL of citrate buffer, pH 5.5) were added, and, after incubation at room temperature for 15 min, the reaction was stopped by adding 25 μL of 2.0 M sulfuric acid. The optical density (OD) was then measured in a microplate reader (Titertek Multiskan MCC/340P, model 2.20, Labsystems, Finland) at 450 nm.

Cell culture studies. Freshly resident mice peritoneal macrophages were harvested in PBS (0.8% NaCl, 0.06% Na_2HPO_4, 0.02% KCl, and 0.04% KH_2PO_4), pH 7.4. Pelleted cells obtained after centrifugation at 500 *g*, 4°C for 3 min were resuspended at a final concentration of 3×10^6 cells/mL in RPMI 1640 medium containing 20% (vol/vol) fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL), (L-glutamine-penicillin-streptomycin, Sigma Chemical, St. Louis, MO, USA). An aliquot (0.5 mL) was transferred into 24-well tissue culture plates and incubated in a humidifier incubator (5% CO_2 atmosphere at 37°C). To remove non-adherent cells after a 2-h incubation period, each plate was washed twice with RPMI 1640 medium without FCS and used for subsequent experiments.

HDL-mediated cellular cholesterol efflux. Adhered mouse peritoneal macrophages were loaded with CE according to the method described by Brown and Goldstein (33). Briefly, macrophages were incubated in RPMI 1640 medium containing 2 mg/mL FA-free BSA in the presence of [^{14}C]cholesteryl oleate-labeled acetylated LDL ([^{14}C]CE-acLDL, 50 μg of protein/mL) for 24 h, at 37°C and washed once with DMEM (Dulbelcco's Minimum Essential Medium) containing antibiotics. [^{14}C]CE-acLDL–loaded macrophages were incubated for 6 h with DMEM containing 2 mg/mL BSA in the presence of human HDL (100 μg protein/mL) as cellular cholesterol acceptor, and the medium was drawn for radioactivity analysis (Ultima Gold) in the LS6000 Beckman Beta Counter. Cells were washed with PBS and dissolved in 0.2 N NaOH for the measurements of cell-associated radioactivity and protein. Efflux rate was defined as the amount of [^{14}C] unesterified cholesterol in the medium expressed as a percentage of total [^{14}C] in the medium plus cells. Blank values were obtained by the incubation of labeled cells in medium containing only 2 mg/mL BSA and no acceptor lipoprotein.

Acetylated LDL cholesteryl ether uptake by cells. Adhered macrophages were incubated in RPMI 1640 medium containing 10% (vol/vol) lipoprotein deficient human serum (3.5 mg protein/mL of medium) in the presence of acetylated LDL (34) labeled with [^{3}H]cholesteryl oleoyl ether ([^{3}H]COE LDL), 50 μg of protein/mL, for 6 h at 37°C in a humidified incubator (5% CO_2 atmosphere). At the end of the incubation, cells were washed with PBS and solubilized in 0.2 N NaOH for the measurement of the cell-associated radioactivity and protein content. Cellular [^{3}H]COE uptake was defined as the amount of ^{3}H in the cells expressed as a percentage of that offered to the cells per mg of cellular protein.

Cholesteryl ester transfer protein activity assay (CETP). Plasma CETP activity was measured by an exogenous method. Briefly, a mixture of human VLDL and LDL (200 μL, 200 mg cholesterol/dL) was incubated with pooled donor human HDL particles (50 μL, 40 mg cholesterol/dL) labeled with [4-^{14}C]-CE and mouse plasma (10 μL), as the source of CETP and 10 μL of Tris buffer, in a final volume of 300 μL. Blanks were prepared with Tris/saline/EDTA buffer (10 mM/140 mM/1 mM), pH 7.4, and control plasma from chow diet–fed C57BL/6 mice that do not express CETP. Incubations were carried out at 37° C for 2 h. The apoB containing lipoproteins were then precipitated with a 1.6% dextran sulfate/1 M $MgCl_2$ solution (1:1) and radioactivity measured in the remaining supernatant in a scintillation solution Ultima Gold (Eastman Kodak Co., Rochester, NY) in the LS6000 Beckman Beta Counter (Beckman Instruments, Palo Alto, CA). The percentage of 4-^{14}C CE transferred from [^{14}C]-CE-HDL to VLDL + LDL was calculated as: [1 – (sample radioactivity/control radioactivity)] × 100.

Hepatic CETP mRNA measurement: extraction of total RNA. The total mouse liver RNA was isolated using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of all RNA samples was evaluated on borate agarose gel electrophoresis after ethidium bromide staining. The reverse transcription was performed with Superscript II pre-amplification system (Invitrogen) from 1 μg of total RNA in a final volume of 20 μL.

Determination of hepatic CETP mRNA level. The hepatic mRNA expression of CETP was determined by RT-PCR using the mouse glyceraldeyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. The sequences of each primer pair (sense and antisense) used in the RT-PCR reaction were the following: human CETP (225 bp, GenBank accession no. NM_000078, 5′-CCA AGG TGA TCC AGA CCG-3′ and 5′-TGG TGT AGC CAT ACT TCA GGG-3′); and GAPDH (394 bp, GenBank accession no. M32599, 5'-CTG CAT CCA CTG GTG CTG-3′ and 5′- AGG GTT TCT TAC TCC TTG GAG G-3′). The RT-PCR reaction was performed in a DNA thermal cycler PTC-200 (MJ Research Inc., Watertown, MA, USA) using the reverse transcription product co-amplified in the presence of sense and antisense primers of GAPDH (7.5 pmol) and CETP genes (15 pmol) in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.3), 200 mM of each dNTP, 5% DMSO, 2 U Taq DNA polymerase (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), in a final volume of 50 μL. The PCR conditions were 94°C for 4 min, and subsequently 28 cycles at 94°C for 1 min, 54°C for 90 s, 72°C for 2 min, and finally at 72°C for 10 min. The PCR products were then submitted to electrophoresis in 2% agarose gel, stained with ethidium bromide, and the band densities were analyzed by the software from Alpha Imager TM 1220 (Alpha Innotec Co., SanLeandro, CA, USA). The relative density of each sample was nor-

malized to the signal of GAPDH and was expressed as the sample/GAPDH ratio.

Lecithin cholesterol acyltransferase assay (LCAT). The LCAT-mediated cholesterol esterification reaction was measured using endogenous substrates (35). Briefly, mice plasma (20 μL) was incubated with [4-^{14}C]-free cholesterol at 37°C for 24 h, and the free cholesterol (4-^{14}C-FC) and cholesterol ester (4-^{14}C-CE) were separated by TLC for radioactivity measurement in the scintillation solution Ultima Gold in a LS6000 Beckman Beta Counter.

Statistical analysis. All comparisons were analyzed by statistical tests using the GraphPad Prism, version 3.0 for Windows (GraphPad Software Inc., San Diego, CA). Differences were considered significant when $P < 0.05$.

RESULTS

The amount of diet consumed did not differ among all groups throughout the study (3.0 ± 0.7 g/d). Iso Low, Iso High, and control groups gained weight from the basal to the final periods (Table 2). At the end of the study, Iso High animals were the heaviest of the three groups.

Plasma lipid and lipoprotein concentrations. Plasma lipid and lipoprotein concentrations were determined 2 wk after ovariectomy (basal values), and again at the end of the study (19th wk). At the basal period, there were no differences among the groups, and at the end of the study, plasma triglycerides levels of Iso Low group were higher than the other groups (Table 3).

The average areas of atherosclerotic lesion did not differ among Iso Low, Iso High, and control groups (Table 4). Because of this lack of difference, we investigated the percent distribution of the lesions according to their size in reference to the control group median size (8.1×10^3 μm^2) so as to define small lesions (below median) and large lesions (above median). We found that the number of mice with small and large lesions were roughly evenly distributed in the Iso Low group, but in the Iso High group, there was a distribution trend favoring the prevalence of smaller lesions: 70% of all mice in this group presented small-type lesions ($P = 0.085$ by κ^2 test).

Antibody titers against oxidized LDL or against an epitope of oxidized apolipoprotein B (ApoB-D) did not differ among the three experimental groups (Table 5).

When compared with the Iso Low and control groups, Iso High mice presented lower mouse peritoneal macrophage up-

TABLE 2
Basal and Final Body Weights[a]

Body weight (g)	Iso Low	Iso High	Control
Basal	18.8 ± 2.9 (25)	20.0 ± 3.2 (27)	19.8 ± 4.1 (27)
Final	27.4 ± 3.9[b] (25)	33.5 ± 7.7[b,c] (28)	29.5 ± 4.8[b] (28)

[a]Mean ± SD. Values in parenthesis represent the number of animals in each group. Statistical comparisons: ANOVA followed by Newman-Keuls multiple comparison test.
[b]Basal vs final, $P < 0.05$.
[c]Final weight of Iso High is greater than the other groups.

TABLE 3
Plasma Lipid and Lipoprotein Concentrations (mmol/L) at Basal and Final Periods[a]

	Iso Low	Iso High	Control
TC			
(Basal)	2.4 ± 0.3 (21)	2.2 ± 0.3 (25)	2.3 ± 0.3 (26)
(Final)	4.0 ± 0.8	4.0 ± 0.8	3.9 ± 0.8
VLDL-C			
(Basal)	0.7 ± 0	0.7 ± 0	0.7 ± 0
(Final)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
LDL-C			
(Basal)	0.8 ± 0	0.7 ± 0	0.8 ± 0
(Final)	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
HDL-C			
(Basal)	0.9 ± 0	0.8 ± 0	0.8 ± 0
(Final)	1.6 ± 0.2	1.6 ± 0.1	1.5 ± 0.2
n-HDL-C			
(Basal)	1.5 ± 0	1.4 ± 0	1.5 ± 0
(Final)	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.2
TAG			
(Basal)	1.1 ± 0.5 (14)	1.3 ± 0.9 (17)	1.3 ± 0.6 (16)
(Final)	1.1 ± 0.8[b] (16)	0.7 ± 0.3 (22)	0.8 ± 0.3 (22)

[a]Values are means ± SD. Values in parentheses represent the number of animals in each group. Lipoprotein fractions were obtained by FPLC from mice plasma pools (n = 3). (Non) n-HDL-C = TC – HDL-C. Statistical comparisons: TC and TAG, ANOVA followed by Newman-Keuls multiple comparison test. VLDL-C, LDL-C, HDL-C, and n-HDL-C, Kruskal-Wallis, followed by Dunns multiple comparison test.
[b]$P < 0.05$, Iso Low differs from Iso High and control.

TABLE 4
Area of Aortic Atherosclerosis Lesion[a]

Iso Low ($n = 24$)	Iso High ($n = 24$)	Control ($n = 22$)
12.3 ± 9.9	7.4 ± 6.4	10.7 ± 12.8

[a]Values are means ± SD, μm$^2 \times 10^3$. Statistical comparison: ANOVA followed by Newman-Keuls multiple comparison test. No differences amongst the experimental groups

TABLE 5
Serum Antibodies Titers Against Oxidized LDL or Oxidized apoB-D Peptide Measured as Optical Densities at 450 nm[a]

	Iso Low (n = 8)	Iso High (n = 10)	Control (n = 13)
ox-LDL	0.76 ± 0.10	0.69 ± 0.16	0.62 ± 0.15
ApoB-D	0.68 ± 0.06	0.67 ± 0.14	0.61 ± 0.14

[a]Statistical comparisons: ANOVA followed by Newman-Keuls multiple comparison test. No differences among the experimental groups.

TABLE 6
Basal and Final Plasma CETP Activity (%) and Hepatic CETP mRNA Expression (%) at the End of the Study

CETP	Iso Low	Iso High	Control
Basal	22.0 ± 7.5 (21)	23.2 ± 7.4 (25)	20.9 ± 5.2 (26)
Final	42.8 ± 8.6[a] (21)	40.8 ± 8.0[a] (25)	43.1 ± 7.6[a] (25)
mRNA	96.3 ± 6.8[b] (19)	95.3 ± 6.3[b] (20)	100 (20)

[a]Paired student's t test: $P < 0.001$, basal vs final in the experimental groups. mRNA values were normalized to GAPDH mRNA content and expressed as relative values.
[b]ANOVA followed by Newman-Keuls multiple comparison test: Iso Low and Iso High lower than control group at $P < 0.05$.

take of [^{3}H]COE acetyl-LDL (68%), and higher ^{14}C-cholesterol efflux rate (50%) from macrophages preloaded with [^{14}C]CE acetyl-LDL (Fig. 1).

Although the hepatic CETP mRNA expression was lower in Iso Low and Iso High than in the control group, plasma CETP activity was not influenced by the isoflavone treatment (Table 6).

Lecithin cholesterol acyl transferase (LCAT), hepatic (HL), and peripheral lipoprotein lipase (LPL) activities did not differ among all the groups (data not shown).

DISCUSSION

Considering that in addition to plasma cholesterol and lipoprotein concentrations, several other factors play roles in the pathogenesis of atherosclerosis, in this work we investigated the effects of soy protein containing isoflavones on the cholesterol uptake and efflux by mouse peritoneal macrophages, levels of antibodies against oxidized LDL, and average size distribution of the lipid-laden area in the aortic root.

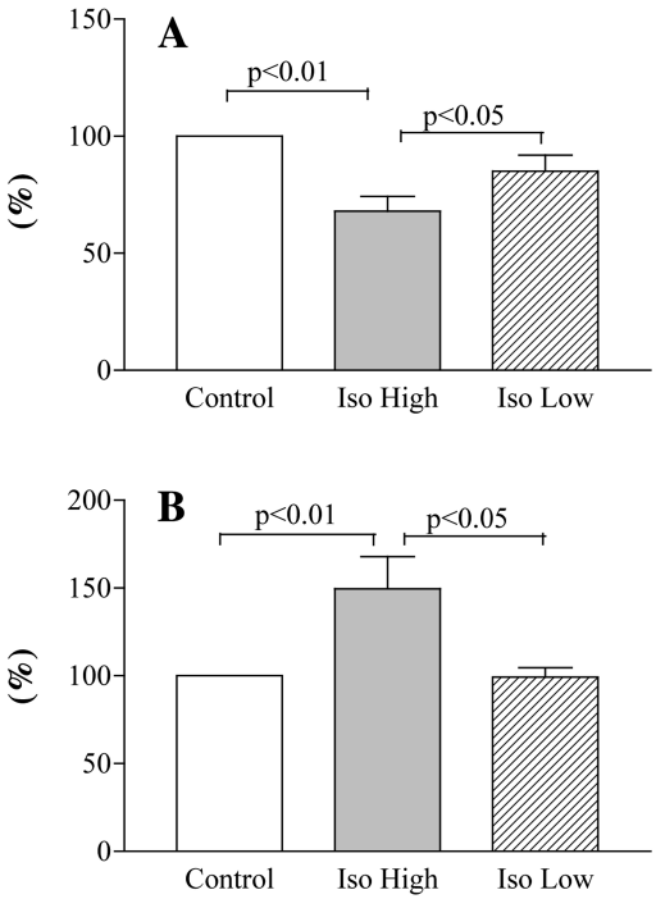

FIG. 1. Mouse peritoneal macrophage uptake of [^{3}H]COE acetyl-LDL (A) and HDL-mediated efflux of cellular unesterified ^{14}C-cholesterol (^{14}C present in the medium) in macrophages that had previously been loaded with [^{14}C]CE acetyl-LDL (B). Data are relative to the control group. Eight replicates of cell pools of from six to eight mice in each experimental condition are shown. Statistical comparison: ANOVA followed by Newman-Keuls multiple comparison test displayed in the figures.

Macrophages drawn from the Iso High group took up significantly less cholesterol and delivered into the culture medium significantly more of the internalized cholesterol than the Iso Low and control groups (Fig. 1). However, these results did not significantly modify the average size of lipid-stained area in the aortic root of isoflavone-treated mice (Fig. 2).

It is known that antibodies against ox-LDL are positively associated with the ox-LDL content in the atherosclerotic lesions in LDL receptor knockout mice (36), as well as with the severity of coronary arterial disease in humans (37). Nonetheless, the results remain controversial in humans; Heikkinen *et al.* (38) did not find differences in ox-LDL antibodies titers in postmenopausal women after one year on hormonal replacement, whereas Uint *et al.* (39) described increased anti-ox-LDL titers after 90 d on this treatment. In this regard, Wen *et al.* (40) and Santanam *et al.* (41) showed that, except for plasma concentrations over 7343 pmol/L, estrogens at physiological concentrations (499 to 4,993 pg/mL) do not protect LDL particles against oxidation. On the other hand, Damasceno *et al.* (32) found that rabbits fed soy protein containing isoflavones have lower autoantibodies against ox-LDL compared with casein-fed rabbits. However, in our study, antibodies against ox-LDL and against apoB-D did not differ among the experimental groups. Regarding the antioxidant action of isoflavones on LDL, although Samman *et al.* (42) did not find differences in the oxidation susceptibility of human LDL after isoflavones or placebo treatment, Meng *et al.* (27) described an increased lag time during the oxidation process, indicating resistance to oxidation likely due to the incorporation of esterified isoflavones into the LDL particles.

Measuring CETP activity in plasma was necessary in our study because plasma CETP inhibition has potentially antiatherogenic effects in humans and in experimental animals (43), but CETP activity was not influenced by the added isoflavones.

In general, estrogen treatment protects against aortic fat accumulation in rabbits, monkeys, and mice, as reviewed by Hodgin and Maeda (44). Nonetheless, in the majority of these investigations, including in our own, in transgenic mice expressing the human CETP (28,29), this protection seems to be related to the lowering of plasma total cholesterol in the non-HDL-C fraction, namely, in apoB-containing lipoproteins.

The present investigation showed that soy protein containing isoflavones resulted in (1) a trend for the formation of smaller lipid-laden aortic lesions, (2) impairment of the macrophage cholesterol loading, and (3) stimulation of HDL-mediated cellular cholesterol efflux, conferring a potentially antiatherogenic effect. Nonetheless, a cautionary note regarding our study concerns the fact that soy protein containing isoflavones and other alcohol isolated soy protein lower but do not eliminate the ability of soy protein to reduce atherosclerosis and thus as suggested by others may have influenced our results because alcohol-extracted soy protein was utilized in our investigation (45).

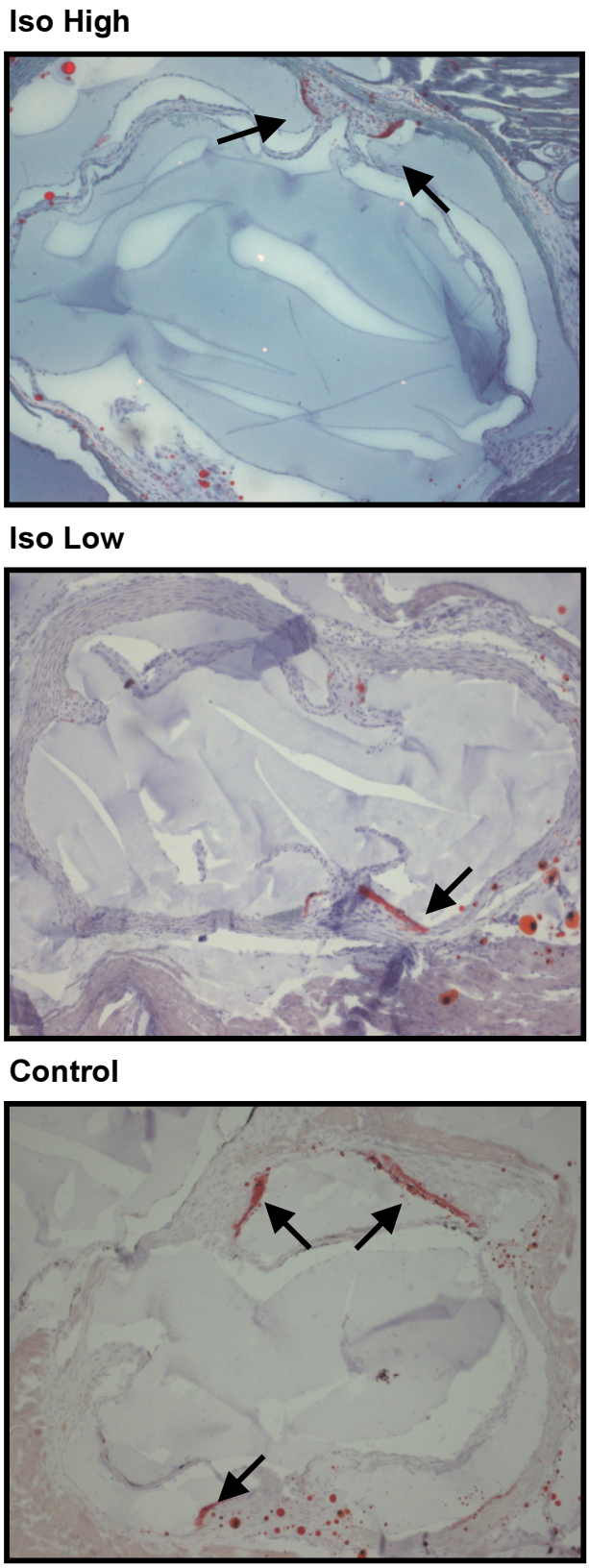

FIG. 2. Histological typical lesions stained with Oil red O and counterstained with Harris hematoxylin and with light green are indicated by arrows. Size increased 10 times.

Our study on the effects of soy protein containing isoflavones on atherosclerosis differs from other investigations in that we chose an experimental model where severe hypercholesterolemia was avoided. We then observed that soy protein containing isoflavones did not reduce the plasma cholesterol concentration or the degree of experimental atherosclerosis, a finding that agrees with another experimental study (23). On the other hand, in a few animal experiments where the degree of hypercholesterolemia was mild, soy isoflavone reduction or prevention of the development of the experimental atherosclerosis seemed consequent to the reduction of plasma cholesterol (17,18). However, in few studies where severe hypercholesterolemia was induced soy isoflavones protected against atherosclerosis without modifying the concentration of plasma cholesterol (19,20). Nonetheless, our study sheds light on the beneficial effects of soy protein containing isoflavones on the macrophage cholesterol metabolism that could have been more efficacious to protect against experimental atherosclerosis in the presence of severe hypercholesterolemia.

These effects need to be taken into account for the human use of this supplemental nutrient, considering that in humans the effects of isoflavones on plasma lipid concentration are controversial and that no trials have been published on the effects of isoflavones on cardiovascular disease (46,47).

ACKNOWLEDGMENTS

This study was supported by a grant (01/00994-9) from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We are grateful to Solae Company for their contribution of the soy products and the diets and to Mr. Pedro A.F. Neto for caring for the animals.

REFERENCES

1. Grady, D., Herrington, D., Bittner, V., Blumenthal, R., Davidson, M., Hlatky, M., Hsia, J., Hulley, S., Herd, A., Khan, S., Newby, L.K., Waters, D., Vittinghoff, E., and Wenger, N. (2002) HERS Research Group. Cardiovascular Disease Outcomes During 6.8 Years of Hormone Therapy: Heart and Estrogen/Progestin Replacement Study Follow-up (HERS II), *JAMA 288*, 49–57.
2. Hulley, S., Furberg, C., Barrett-Connor, E., Cauley, J., Grady, D., Haskell, W., Knopp, R., Lowery, M., Satterfield, S., Schrott, H., Vittinghoff, E., and Hunninghake, D. (2002) HERS Research Group. Noncardiovascular Disease Outcomes During 6.8 Years of Hormone Therapy: Heart and Estrogen/Progestin Replacement Study Follow-up (HERS II), *JAMA 288*, 58–66.
3. Writing Group for the Women's Health Initiative Investigators. (2002) Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results from the Women's Health Initiative Randomized Controlled Trial, *JAMA 288*, 321–333.
4. Zhuo, X.G., Melby, M.K., and Watanabe, S. (2004) Soy Isoflavone Intake Lowers Serum LDL Cholesterol: A Meta-analysis of Eight Randomized Controlled Trials in Humans, *J. Nutr. 134*, 2395–2400.
5. Albertazzi, P., Pansini, F., Bonaccorsi, G., Zanotti, L., Forini, E., and De Aloysio, D. (1998) The Effect of Dietary Soy Supplementation on Hot Flushes, *Obstet. Gynecol. 91*, 96–11.
6. Crisafulli, A., Marini, H., Bitto, A., Altavilla, D., Squadrito, G., Romeo, A., Adamo, E.B., Marini, R., D'Anna, R., Corrado, F.,

Bartolone, S., Frisina, N., and Squadrito, F. (2004) Effects of Genistein on Hot Flushes in Early Postmenopausal Women: A Randomized, Double-Blind EPT- and Placebo-Controlled Study, *Menopause 11*, 400–404.
7. Horiuchi, T., Onouchi, T., Takahashi, M., Ito, H., and Orimo, H. (2000) Effect of Soy Protein on Bone Metabolism in Postmenopausal Japanese Women, *Osteoporos. Int. 11*, 721–724.
8. Cassidy, A., and Griffin, B. (1999) Phyto-oestrogens: A Potential Role in the Prevention of CHD? *Proc. Nutr. Soc. 58*, 193–199.
9. Vitolins, M.Z., Anthony, M., and Burke, G.L. (2001) Soy Protein Isoflavones, Lipids and Arterial Disease, *Curr. Opin. Lipidol. 12*, 437–437.
10. Sacks, F.M., Lichtenstein, A., Van Horn, L., Harris, W., Kris-Etherton, P., and Winston, M. American Heart Association Nutrition Committee. (2006) Soy Protein, Isoflavones, and Cardiovascular Health: An American Heart Association Science Advisory for Professionals from the Nutrition Committee, *Circulation 113*, 1034–1044.
11. Van der Schouw, Y.T., Kreijkamp-Kaspers, S., Peeters, P.H., Keinan-Boker, L., Rimm, E.B., and Grobee, D.E. (2005) Prospective Study on Usual Dietary Phytoestrogen Intake and Cardiovascular Disease Risk in Western Women, *Circulation 111*, 466–471.
12. Clarkson, T.B. (2002) Soy, Soy Phytoestrogens and Cardiovascular Disease, *J. Nutr. 132*, 66S–569S.
13. Anderson, J.W., Johnsotone, B.M., and Cook-Newell, M.E. (1995) Meta-analysis of the Effects of Soy Protein Intake on Serum Lipids, *N. Engl. J. Med. 333*, 276–282.
14. Engelman, H.M., Alekel, D.L., Hanson, L.N., Kanthasamy, A.G., and Reddy, M.B. (2005) Blood Lipid and Oxidative Stress Responses to Soy Protein with Isoflavones and Phytic Acid in Postmenopausal Women, *Am. J. Clin. Nutr. 81*, 590–596.
15. Kreijkamp-Kaspers, S., Kok, L., Grobbee, D.E., de Haan, E.H., Aleman, A., Lampe, J.W., and Van der Schouw, Y.T. (2004) Effect of Soy Protein Containing Isoflavones on Cognitive Function, Bone Mineral Density, and Plasma Lipids in Postmenopausal Women: A Randomized Controlled Trial, *JAMA 292*, 65–74.
16. Clarkson, T.B., Anthony, M.S., and Morgan, T.M. (2001) Inhibition of Postmenopausal Atherosclerosis Progression: A Comparison of the Effects of Conjugated Equine Estrogens and Soy Phytoestrogens, *J. Clin. Endocrinol. Metab. 86*, 41–47.
17. Lucas, E.A., Lightfoot, S.A., Hammond, L.J., Devareddy, L., Khalil, D.A., Daggy, B.P., Soung do, Y., and Arjmandi, B.H. (2003) Soy Isoflavones Prevent Ovariectomy-Induced Atherosclerotic Lesions in Golden Syrian Hamster Model of Postmenopausal Hyperlipidemia, *Menopause 10*, 314–321.
18. Kirk, E.A., Sutherland, P., Wang, S.A., Chait, A., and LeBoueuf, R.C. (1998) Dietary Isoflavones Reduce Plasma Cholesterol and Atherosclerosis in C57BL/6 Mice but Not LDL Receptor-Deficient Mice, *J. Nutr. 128*, 954–959.
19. Adams, M.R., Golden, D.L., Register, T.C., Anthony, M.S., Hodgin, J.B., Maeda, N., and Williams, J.K. (2002) The Atheroprotective Effect of Dietary Soy Isoflavones in Apolipoprotein E–/– Mice Requires the Presence of Estrogen Receptor-Alpha, *Arterioscler. Thromb. Vasc. Biol. 22*, 1859–1864.
20. Yamakoshi, J., Piskula, M.K., Izumi, T., Tobe, K., Saito, M., Kataoka, S., Obata, A., and Kikuchi, M. (2000) Isoflavone Aglycone-Rich Extract Without Soy Protein Attenuates Atherosclerosis Development in Cholesterol-Fed Rabbits, *J. Nutr. 130*,1887–1893.
21. Adams, M.R., Golden, D.L., Franke, A.A., Potter, S.M., Smith, H.S., and Anthony, M.S. (2004) Dietary Soy Beta-Conglycinin (7S Globulin) Inhibits Atherosclerosis in Mice, *J. Nutr. 134*, 511–516.
22. Mortensen, A., Pilegaard, K., Frandsen, H., and Breinholt, V. (2004) Effect of a Soy Supplement on Spontaneous Atherosclerosis in Low Density Lipoprotein Receptor Knockout (LDLR–/–) Mice, *Asia Pac. J. Clin. Nutr. 13*(Suppl.), S102 (abs).
23. Wagner, J.D., Schwenke, D.C., Greaves, K.A., Zhang, L., Anthony, M.S., Blair, R.M., Shadoan, M.K., and Willianms, J.K. (2003) Soy Protein with Isoflavones, but Not Isoflavone-Rich Supplement, Improves Arterial Low-Density Lipoprotein Metabolism and Atherogenesis, *Arterioscler. Thromb. Vasc. Biol. 23*, 2241–2246.
24. Adams, M.R., Golden, D.L., Anthony, M.S., Register, T.C., and Williams J.K. (2002) The Inhibitory Effect of Soy Protein Isolate on Atherosclerosis in Mice Does Not Require the Presence of LDL Receptors or Alteration of Plasma Lipoproteins, *J. Nutr. 132*, 43–49.
25. Alexandersen, P., Haarbo, J., Breinholt, V., and Christiansen, C. (2001) Dietary Phytoestrogens and Estrogen Inhibit Experimental Atherosclerosis, *Climacteric. 4*, 151–159.
26. Cassidy, A., De Pascual, T.S., and Rimbach, G. (2003) Molecular Mechanisms by Dietary Isoflavones Potentially Prevent Atherosclerosis, *Expert Rev. Mol. Med. 5*, 1–15.
27. Meng, Q.H., Lewis, P., Wahala, K., Adlercreutz, H., and Tikkanen, M. (1992) Incorporation of Esterified Soybean Isoflavones with Antioxidant Activity into Low Density Lipoprotein, *Biochim. Biophys. Acta 1438*, 369–376.
28. Cazita, P.M., Berti, J.A., Aoki, C., Gidlund, M., Harada, L.M., Nunes, V.S., Quintão, E.C., and Oliveira, H.C. (2003) Cholesteryl Ester Transfer Protein Expression Attenuates Atherosclerosis in Ovariectomized Mice, *J. Lipid Res. 44*, 33–40.
29. Casquero, A.C., Berti, J.A., Salerno, A.G., Bighetti, E.J., Cazita, P.M., Ketelhuth, D.F., Gidlund, M., and Oliveira, H.C. (2006) Atherosclerosis Is Enhanced by Testosterone Deficiency and Attenuated by CETP Expression in Transgenic Mice, *J. Lipid Res. 47*, 1526–1534.
30. Paigen, B., Morrow, A., Holmes, P.A., Mitchell, D., and Willians, R.A. (1987) Quantitative Assessment of Atherosclerotic Lesions Mice, *Atherosclerosis 68*, 231–240.
31. Rubin, E.M., Krauss, R.M., Spangler, E.A., Verstuyft, J.G., and Glift, S.M. (1991) Inhibition of Early Atherogenesis in Transgenic Mice by Human Apolipoprotein AI, *Nature 353*, 265–267.
32. Damasceno, N.R., Goto, H., Rodrigues, F.M., Dias, C.T., Okawabata, F.S., Abdalla, D.S., and Gidlund, M. (2000) Soy Protein Isolate Reduces the Oxidizability of LDL and the Generation of Oxidized LDL Auto Antibodies in Rabbits with Diet-Induced Atherosclerosis, *J. Nutr. 130*, 2641–2647.
33. Brown, M.S., and Goldstein, J.L. (1983) Lipoprotein Metabolism in the Macrophage: Implications for Cholesterol Deposition in Atherosclerosis, *Annu. Rev. Biochem. 52*, 223–261.
34. Basu, S.K., Goldstein, J.L., Anderson, G.W., and Brown, M.S. (1976) Degradation of Cationized Low Density Lipoprotein and Regulation of Cholesterol Metabolism in Homozygous Familial Hypercholesterolemia Fibroblasts, *Proc. Natl. Acad. Sci. USA 73*, 3178–3182.
35. Dobiasova, M., Stribrna, J., Pritchard, P.H., and Frohlich, J.J. (1992) Cholesterol Esterification Rate in Plasma Depleted of Very Low and Low Density Lipoproteins Is Controlled by the Proportion of HDL2 and HDL3 Subclasses: Study in Hypertensive and Normal Middle-Aged and Septuagenarian Men, *J. Lipid Res. 33*, 1411–1418.
36. Tsimikas, S., Palinski, W., and Witztum, J.L. (2001) Circulating Autoantibodies to Oxidized LDL Correlate with Arterial Accumulation and Depletion of Oxidized LDL in LDL Receptor–Deficient Mice, *Arterioscler. Thromb. Vasc. Biol. 21*, 95–100.
37. Ehara, S., Ueda, M., Naruko, T., Haze, K., Itoh, A., Otsuka, M., Komatsu, R., Matsuo, T., Itabe, H., Takano, T., Tsukamoto, Y., Yoshiyama, M., Takeuchi, K., Yoshikawa, J., and Becker, A.E. (2001) Elevated Levels of Oxidized Low Density Lipoprotein

Show a Positive Relationship with the Severity of Acute Coronary Syndromes, *Circulation 103*, 1955–1960.
38. Heikkinen, A.M., Niskanen, L., Yla-Herttuala, S., Luoma, J., Tuppurainen, M.T., Komulainen, M., and Saarikoski, S. (1998) Postmenopausal Hormone Replacement Therapy and Autoantibodies Against Oxidized LDL, *Maturitas 29*, 155–161.
39. Uint, L., Gebara, O.C.E., Pinto, L.B., Wajngarten, M., Boschov, P., da Luz, P.L., and Gidlund, M. (2003) Hormone Replacement Therapy Increases Levels of Antibodies Against Heat Shock Protein 65 and Certain Species of Oxidized Low Density Lipoprotein, *Braz. J. Med. Biol. Res. 36*, 491–494.
40. Wen, Y., Doyle, M.C.T., Cooke, T., and Feeley, J. (2000) Effect of Menopause on Low-Density Lipoprotein Oxidation: Is Estrogen an Important Determinant? *Maturitas 34*, 233–238.
41. Santanam, N., Sher-Brewer, R., McClatchey, R., Castellano, P.Z., Murphy, A.A., Voelkel, S., and Parthasarathy, S. (1998) Estradiol as an Antioxidant: Incompatible with Its Physiological Concentrations and Function, *J. Lipid Res. 39*, 2111–2118.
42. Samman, S., Wall, P.M.L., Chan, G.S.M., Smith, S.J., and Petocz, P. (1999) The Effect of Supplementation with Isoflavones on Plasma Lipids and Oxidisability of Low Density Lipoprotein in Premenopausal Women, *Atherosclerosis 147*, 277–283.
43. Barter, P.J., and Kastelein, J.J. (2006) Targeting Cholesteryl Ester Transfer Protein for the Prevention and Management of Cardiovascular Disease, *J. Am. Coll. Cardiol. 47*(3), 492–499.
44. Hodgin, J.B., and Maeda, N. (2002) Estrogen and Mouse Models of Atherosclerosis, *Endocrinology 143*, 4495–4501.
45. Clair, R.S., and Anthony, M. (2005) Soy, Isoflavones and Atherosclerosis. *Handb. Exp. Pharmacol. 170*, 301–323.
46. van der Schouw, Y.T., de Kleijn, M.J., Peeters, P.H., and Grobbee, D.E. (2000) Phyto-oestrogens and Cardiovascular Disease Risk, *Nutr. Metab. Cardiovasc. Dis. 10*, 154–167.
47. Cassidy, A., and Hooper, L. (2006) Phytoestrogens and Cardiovascular Disease, *J. Br. Menopause Soc. 12*, 49–56.

[Received April 7, 2006; accepted August 1, 2006]