Soy and Alfalfa Phytoestrogen Extracts Become Potent Low-Density Lipoprotein Antioxidants in the Presence of Acerola Cherry Extract

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Postmenopausal women have an increased risk of coronary heart disease. Oxidation of low-density lipoprotein (LDL) has been implicated in atherogenesis, and the presence of modified LDL (LDL^-) in plasma appears to represent LDL oxidation in vivo. Because previous studies have demonstrated a strong antiatherogenic effect of estrogen due to its antioxidant activity and similar antioxidant activity was found for specific isoflavones derived from soy extract, the antioxidant activity of a phytoestrogen extract derived from soy and alfalfa was studied. Copper-mediated LDL oxidation was inhibited in the presence of soy and alfalfa extracts, and this effect was further enhanced in the presence of acerola cherry extract, which is rich in ascorbic acid. Male rabbit aortic endothelial cells pretreated with soy extract were resistant to the toxic effects of high levels of LDL and LDL^-, and a lesser, but significant protection, was also afforded by alfalfa extract. Cell-mediated oxidation of LDL, measured by LDL^- formation, was inhibited in the presence of soy extract but not alfalfa extract. However, in the presence of acerola cherry extract, both soy and alfalfa extracts potently inhibited the formation of LDL^-. These findings show that acerola cherry extract can enhance the antioxidant activity of soy and alfalfa extracts in a variety of LDL oxidation systems. The protective effect of these extracts is attributed to the presence of flavonoids in soy and alfalfa extracts and ascorbic acid in acerola cherry extract, which may act synergistically as antioxidants. It is postulated that this synergistic interaction among phytoestrogens, flavonoids, and ascorbic acid is due to the "peroxidolitic" action of ascorbic acid, which facilitates the copper-dependent decomposition of LDL peroxides to nonradical products; this synergy is complemented by a mechanism in which phytoestrogens stabilize the LDL structure and suppress the propagation of radical chain reactions. The combination of these extracts markedly lowers the concentrations of phytoestrogens required to achieve significant antioxidant activity toward LDL.

Keywords: *Antioxidant; low-density lipoprotein; soy extract; alfalfa extract; acerola extract*

INTRODUCTION

Coronary heart disease is the leading cause of death among U.S. women. Estrogen replacement therapy (ERT) has been shown to markedly reduce the risk of cardiovascular disease (CVD) in postmenopausal women. Nevertheless, the use of ERT remains limited due to concerns about increased risk for breast or endometrial cancers in postmenopausal women, especially with a familial history of these diseases. ERT has also been considered for men but severe complications (Group, 1975) and feminizing effects prevent its use. A new focus has developed to find alternative sources of estrogenic agents that do not possess the unwanted side effects of pharmaceutical grade estrogen. Studies with plant-based estrogenic compounds (i.e., phytoestrogens) have indicated that a number of benefits derived from ERT may be obtained from compounds naturally found in plants with estrogenic activity. Diets that are rich in naturally occurring phytoestrogens, such as the Asian diet, have been epidemiologically linked to reduced incidences of breast and prostate cancer as well as lower incidences of cardiovascular events. (Adlercreutz and Mazur, 1997). This is thought to be the case for phytoestrogens found in soy extracts that are widely used by the public as an alternative estrogen source. The effects of phytoestrogens have been linked to antioxidant activity based on the oxidative resistance of low-density lipoprotein (LDL) obtained from subjects consuming phytoestrogens (Tikkanen et al., 1998).

Phytoestrogens can be divided into three main categories: isoflavones, coumestans, and lignans (Kurzer and Xu, 1997). In this study we concentrated on representative sources of phytoestrogens from a soy extract, which is mainly composed of the isoflavones genistein and daidzein, and from an alfalfa extract, which is composed mainly of coumestrol and apigenin. The previously reported interaction of estrogen (Hwang et al., 2000b) and isoflavones isolated from soy (Hwang et al., 2000c) was also examined utilizing acerola cherry extract, which is rich in ascorbic acid (AA) and flavonoids.

Oxidative modification of LDL is now considered to be an important process that contributes to the development of atherosclerosis (Steinberg, 1997; Berliner and Heineicke, 1996). The prevention of atherosclerosis has therefore been approached through the use of antioxidants that inhibit LDL oxidative modification. Many studies have evaluated the efficacy of α-tocopherol and

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vitamin C, or AA, which represent the central components of the antioxidant defense system (Wakatsuki et al., 1998). Recently, we reported the interaction between 17β-estradiol and AA; we demonstrated that AA can enhance the antioxidant effect of estradiol by preventing LDL oxidation by copper ions or cells (Hwang et al., 2000a,b), which is analogous to the interaction described for vitamin E and AA. A synergistic interaction between genistein, daidzein, and equol with AA has also been demonstrated to prevent LDL oxidation by copper ions (Hwang et al., 2000c).

In this paper, we describe the antioxidant activity of soy (*Glycine max*), alfalfa (*Medicago sativa*), and acerola cherry (*Malpighia glabra*) extracts. Because it is often these extracts, rather than isolated components, that are used as phytoestrogens, a study was undertaken of their antioxidant activities in the form of marketed extracts. We also describe the interaction between soy and alfalfa with acerola cherry extract, based on the oxidation kinetics of LDL measured in vitro and in the presence of cultured rabbit aortic endothelial cells.

MATERIALS AND METHODS

Chemicals and Reagents. EDTA, NaBr, NaCl, and TRIS were obtained from Sigma Chemical Co. (St. Louis, MO). All organic solvents and copper sulfate were of HPLC grade and purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Buffers, media, and cell culture supplies were obtained from GIBCO (New York, NY), and male human serum was from Omega Scientific (Tarzana, CA). Soy, alfalfa, and acerola cherry extracts were obtained from the Rehnborg Center for Nutrition and Wellness, Nutrilite Division of Amway Corp. (Buena Park, CA).

Extract Preparation. Commercial soy and alfalfa extracts were subjected to hydrolysis; 100 g of the extract was incubated with 2 N HCl in 80% methanol for 2 h at 37 °C. This was used to prepare the aglycone forms of the corresponding phytoestrogens in the extracts. Methanol was evaporated to dryness, and the sample was partitioned with methylene chloride that was evaporated to dryness and redissolved in 100 mL of methanol and analyzed by HPLC. Briefly, 20 μL of extracts was analyzed in a C18 column (Waters, Milford, MA). The mobile phase was composed of a 50 mM KH_2PO_4 buffer at pH 3 as solvent A and methanol as solvent B. The following linear gradient was used: 10% solvent B initially, increased to 60% solvent B at 10 min, held at 60% solvent B for 20 min, increased linearly to 75% solvent B within 5 min, held for another 10 min at 75% solvent B, and equilibrated back to 10% solvent B for 10 min. The eluent was monitored at a wavelength of 260 nm for the soy isoflavones and at 340 nm for alfalfa, coumestrol and apigenin. Fluorescence signals were Ex = 249 nm and Em = 419 nm for coumestrol. The amounts of phytochemicals were measured using established calibration curves.

Lipoprotein Separation. Venous blood was obtained from fasting, healthy, adult human volunteers, and plasma was immediately separated by centrifugation at 1500*g* for 10 min at 4 °C. LDL (δ = 1.019−1.063 g/mL) was isolated from freshly separated plasma by preparative ultracentrifugation following a standard technique described in Hodis et al. (1994). The isolated LDL was extensively dialyzed against argon-sparged 0.01 mol/L TRIS buffer, pH 7.2, containing 10 μmol/L EDTA. This EDTA concentration prevents autoxidation of LDL and permits studies in cells without affecting cell growth. Isolated lipoproteins were then sterilized by filtration (0.2 μm Millipore membrane) and stored at 4 °C for up to 4 weeks under nitrogen. Samples were monitored for oxidative modification by means of agarose gel electrophoresis and measurements of LDL^- concentration (see below).

LDL^- Preparation. Separation of LDL^- from native LDL (n-LDL) was accomplished by subjecting the isolated LDL fraction to anion exchange HPLC (Perkin-Elmer series 4 HLPC, Norwalk, CT), as previously described (Hodis et al., 1994). Briefly, LDL samples (500 μg of protein/200 μL) were injected onto an UNO Q-1 anion exchange column (Bio-Rad, Hercules, CA) that was eluted at 1.0 mL/min with helium-sparged and pressurized Tris-HCl (0.01 M), pH 7.2. After 5 min, a linear NaCl gradient from 0 to 0.3 M in 0.01 M Tris-HCl buffer was applied over 5 min and then continued for the remaining 35 min of the analytical run. The eluent was monitored at 280 nm, and the peaks corresponding to the LDL^- fraction were collected using a fraction collector (Frac-100, Pharmacia Biotech, Uppsala, Sweden). The pooled LDL^- was concentrated and desalted using a Millipore 30000 molecular weight cutoff microconcentrator (Millipore Corp., Bedford, MA). Before storage, the LDL^- was diluted in PBS containing 10 μmol/L EDTA to a final concentration of 500 μg/mL protein, sterilized by ultrafiltration (0.2 μM Millipore membrane), and kept at 4 °C under nitrogen for up to 1 week.

In Vitro Oxidation of LDL. The kinetics of LDL oxidation were analyzed by adding 10 μM $CuSO_4$ to 200 μg/mL LDL. Formation of conjugated dienes was monitored continuously at 234 nm for up to 16 h using a Beckman DU-650 (Fullerton, CA) spectrophotometer equipped with a six-position automated sample changer. Oxidation kinetics were analyzed on the basis of (1) the oxidation lag time, which was defined as the interval between initiation of oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase; (2) the rate of oxidation during the lag time, defined as the initial oxidation rate before the onset of the propagation phase; and (3) the rate of oxidation during the propagation phase, that is, the *log rate*, which was defined as the maximal rate of oxidation calculated from the slope of the absorbance curve during the propagation phase.

LDL oxidation kinetics were analyzed in the presence and absence of soy, alfalfa, and acerola cherry extracts and the combination of the extracts. The amounts of the ethanol vehicle were the same for all experiments (0.2% v/v).

Cell Culture. Male New Zealand White rabbit aortic endothelial cells (RAEC) were used between passages 19 and 21. Cells were passaged using a 1:2 split ratio, allowed to grow to confluency, and transferred by trypsinization. The doubling time of the cells was ∼38 h in complete medium (DMEM without phenol red containing 15% male serum, 20 μg/mL ECGS, 50 μg/mL gentamycin, and 20 μg/mL heparin). This is referred to as control medium. Five days prior to treatments with LDL preparations and test mixtures, cells were split in complete medium in the presence of 0.5 μM soy extract or 0.1 μM alfalfa extract.

Cytotoxicity Assays. Cells were seeded into 24-well dishes 1 day prior to measurements of cell survival and plating efficiency, which were used as estimates of cytotoxicity following various treatments. The cytotoxicity produced by LDL and LDL^- was determined using nearly confluent cultures grown in the presence of 2% male serum and cell numbers measured after 24 h incubation using a Coulter counter (model ZB, Hialeah, FL). Treatments typically used 1×10^4 cells/cm^2. The parameters related to the measurement of cytotoxicity included plating efficiency (PE) and growth curves based on the surviving fraction (SF) measured at 24 h after treatment with lipoprotein.

Measurement of Cell-Mediated Oxidation. Formation of LDL^- by endothelial cells was used as a measure of cell-mediated LDL oxidation. LDL^- formation was determined after 100 μg of protein/mL of fresh LDL and 1 μM $CuSO_4$ had been added to endothelial cell cultures containing 2×10^5 cells/10 cm^2. Ratios of LDL^- formation relative to total LDL were measured over a 24 h interval for control cells with and without acerola cherry extract (equivalent to 100 μM AA), and cells were pretreated with soy extract corresponding to 0.5 μM equivalents of genistein and daidzein and with alfalfa extract corresponding to 0.1 μM equivalents of coumestrol and apigenin in the presence and absence of acerola cherry extract. At selected time points, aliquots of the media were removed and centrifuged at 800 rpm for 5 min to remove dead cells and cell debris. The supernatant was mixed with 15 mL of

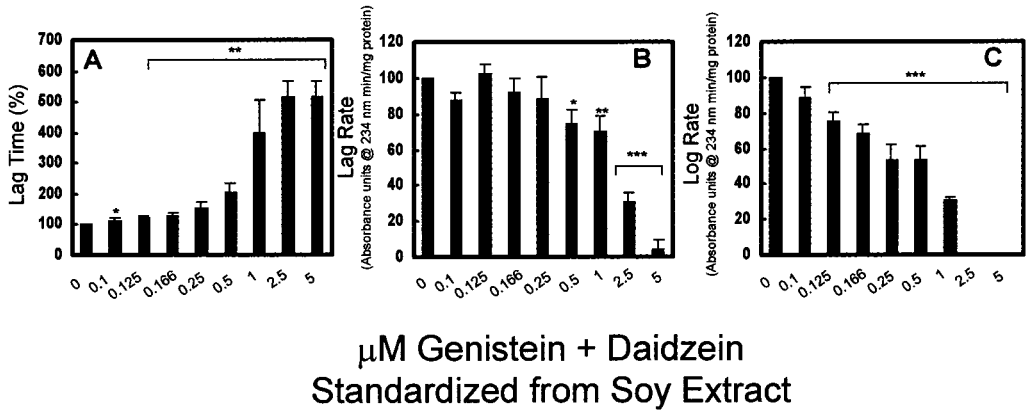

Figure 1. Kinetics of LDL oxidation were measured by the formation of conjugated dienes (OD_{234}). Freshly isolated human LDL (200 μg/mL of protein) was incubated with increasing amounts of soy extract (0−5 μM equivalent genistein and daidzein) in the presence of 10 μM $CuSO_4$ as described under Materials and Methods. The kinetic parameters of lag time (A), lag rate (B), and log rate (C) are shown as a percent of the time/rates measured for control LDL samples incubated with $CuSO_4$. Significant differences compared to control values are indicated by *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$.

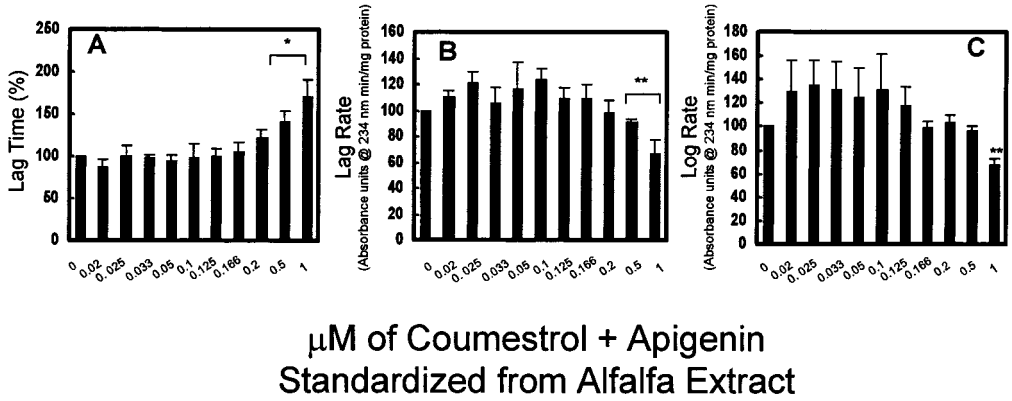

Figure 2. Kinetics of LDL oxidation were measured by the formation of conjugated dienes (OD_{234}). Freshly isolated human LDL (200 μg/mL of protein) was incubated with increasing amounts of alfalfa extract (0−1 μM equivalent coumestrol and apigenin) in the presence of 10 μM $CuSO_4$ as described under Materials and Methods. The kinetic parameters of lag time (A), lag rate (B), and log rate (C) are shown as a percent of the time/rates for control LDL samples incubated with $CuSO_4$. Significant differences compared to control values are indicated by *, $p < 0.05$, and **, $p < 0.005$.

PBS and concentrated using a Millipore 30000 molecular weight cutoff microconcentrator to adjust LDL concentrations and remove low molecular weight components from the medium. These brief reconstituting procedures and adjustments of LDL concentrations did not affect the amounts of LDL^- formation (Sevanian et al., 1996; Hodis et al., 1994). Samples were concentrated to the volume of the initial aliquot removed from the cell culture medium and then analyzed by HLPC for LDL^- content. LDL^- levels were calculated on the basis of the cell number and corrected for LDL^- formation in a cell-free system.

Statistics. All results are expressed as the mean and standard error determined from at least five independent experiments with all measurements performed in duplicate. Determination of statistical significance between various treatment groups utilized the paired two-tailed Student *t* test.

RESULTS

In Vitro Oxidation of LDL Is Inhibited by Soy, Alfalfa, and Acerola Cherry Extracts. Figures 1, 2, and 3 show the effects of soy, alfalfa, and acerola cherry extracts on copper-induced LDL oxidation rates in the presence of phytoestrogens at concentrations ranging from 0.02 to 5 μM (expressed as genistein and daidzein or coumestrol and apigenin equivalents) and from 10.2 to 511 μM acerola cherry extract (expressed in terms of AA concentrations), respectively. The apparent antioxidant effect of soy extract (genistein and daidzein), alfalfa extract (coumestrol and apigenin), and acerola cherry extract (AA) was manifested by a prolongation of the oxidation lag time (Figures 1A, 2A, and 3A). Inhibition of peroxide accumulation during the lag phase (evident as a decreased lag rate) was directly related to the concentrations of soy, alfalfa, and acerola cherry extracts added to the LDL fraction (Figures 1B, 2B, and 3B), with inhibitory effects for soy and alfalfa extracts demonstrating significance ($p < 0.05$) at concentrations >0.5 μM and ≥ 25.6 μM for acerola cherry extract (Figure 3B). Figures 1C, 2C, and 3C show that the oxidation log rates were inhibited ($p < 0.05$) at concentrations ≥ 0.125 μM for soy, ≥ 1.0 μM for alfalfa, and ≥ 102.2 μM for acerola cherry extract.

Inhibition of LDL Oxidation Is Enhanced by Acerola Cherry Extract. LDL was incubated with acerola cherry extract containing 25 and 51 μM equivalents of AA in the presence and absence of 0.1 μM genistein/daidzein equivalents of soy extract or 0.2 μM coumestrol/apigenin equivalents of alfalfa extract as described above. Inhibition of copper-mediated LDL oxidation by soy extract was significantly enhanced in the presence of 25 μM acerola cherry extract ($p < 0.05$), and an even greater inhibition was found ($p < 0.005$) with higher concentrations of acerola cherry extract (51 μM) as shown in Figure 4A. Acerola cherry extract also enhanced the inhibition of LDL oxidation by alfalfa

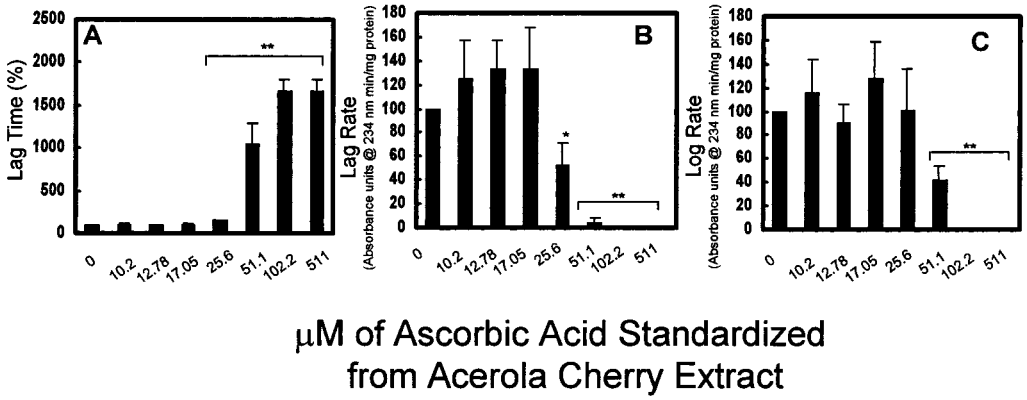

Figure 3. Kinetics of LDL oxidation were measured by the formation of conjugated dienes (OD_{234}). Freshly isolated human LDL (200 μg/mL of protein) was incubated with increasing amounts of acerola extract (0−511 μM equivalent ascorbic acid) in the presence of 10 μM $CuSO_4$ as described under Materials and Methods. The kinetic parameters of lag time (A), lag rate (B), and log rate (C) are shown as a percent of the time/rates for control LDL samples incubated with $CuSO_4$. Significant differences compared to control values are indicated by *, $p < 0.005$, and **, $p < 0.0005$.

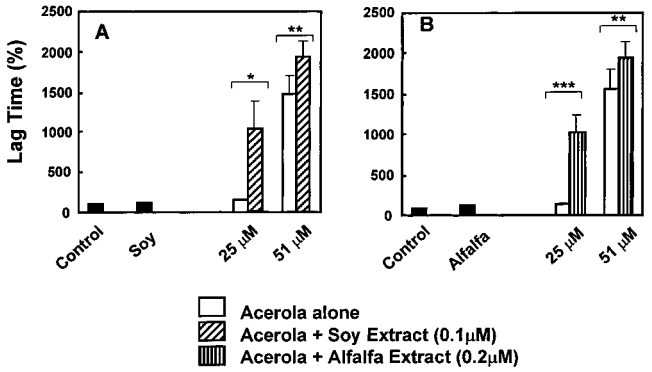

Figure 4. Oxidation of freshly isolated LDL (200 μg/mL of protein) by 10 μM $CuSO_4$ in the presence of 0.1 μM genistein/daidzein (G/D) equivalent from soy extract, 25 and 51 μM AA equivalent from acerola cherry extract (□), and 0.1 μM G/D equivalent from soy extract in the presence of 25 and 51 μM AA equivalent from acerola cherry extract (■) as described under Materials and Methods (A). Oxidation of freshly isolated LDL (200 μg/mL of protein) by 10 μM $CuSO_4$ in the presence of 0.2 μM of coumestrol/apigenin (C/A) equivalent from alfalfa extract, 25 and 51 μM AA equivalent from acerola cherry extract (□), and 0.2 μM C/A equivalent from alfalfa extract in the presence of 25 and 51 μM AA equivalent from acerola cherry extract (■) as described under Materials and Methods (B). Significant differences between 25 and 51 μM AA equivalent from acerola cherry extract in the presence of soy and alfalfa extracts are indicated by *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$.

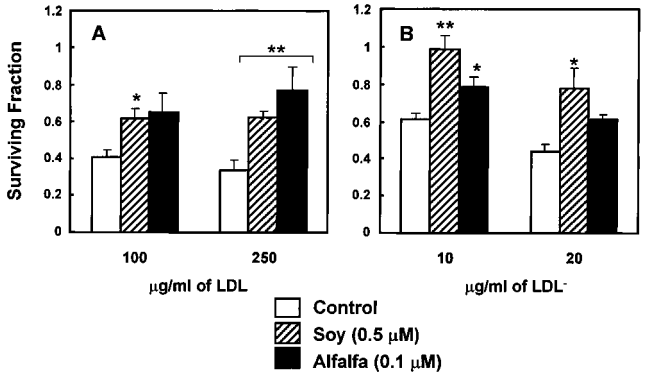

Figure 5. Cytotoxicity of 100 and 250 μg/mL LDL (A) and 10 and 20 μg/mL LDL^- (B) as measured by the SF in RAECC or RAEC preincubated with soy and alfalfa extract for 5 days as described under Materials and Methods. Significant differences compared to control cells are indicated by *, $p < 0.05$; and **, $p < 0.005$.

extract ($p < 0.0005$ at 25 μM and $p < 0.005$ at 51 μM) as shown in Figure 4B.

Soy and Alfalfa Extract Treated Cells Are More Resistant to LDL and LDL^-. Male rabbit endothelial cells preincubated with soy extract (0.5 μM genistein and daidzein equivalents) and alfalfa extract (0.1 μM coumestrol and apigenin equivalents) for 5 days were more resistant to the cytotoxic effects of high concentrations of LDL or LDL^-, as shown in Figure 5A,B. Cells pretreated with soy and alfalfa extracts were resistant to the cytotoxic effect of 250 μg/mL LDL, which caused a significant reduction in the SF of control cells. Treatments with 10 and 20 μg/mL of LDL^- also significantly reduced the SF in control cells; however, cells pretreated with soy extract were resistant up to 20 μg/mL of LDL^- ($p < 0.05$), whereas alfalfa extract treated cells were resistant to 10 μg/mL of LDL^- ($p < 0.05$).

LDL^- Formation Is Decreased in Cells Pretreated with Soy, Alfalfa, and Acerola Cherry Extracts. Pretreatments with soy, alfalfa, and acerola cherry extracts (corresponding to 0.5 μM genistein and daidzein, 0.1 μM coumestrol and apigenin, and 102 μM AA) markedly reduced the extent to which 100 μg/mL of LDL was converted to LDL^- after 24 h as determined by HPLC analysis of total LDL recovered from the medium. Figure 6 shows that control cells converted LDL to LDL^- to a greater extent as compared to cells pretreated with soy ($p < 0.005$) and acerola cherry extracts ($p < 0.0005$). Preincubation of soy and acerola cherry extract in combination further inhibited the formation of LDL^- as compared to soy or acerola cherry extract alone. Although pretreatment with alfalfa extract alone did not inhibit LDL^- formation, a significant reduction was found with the combination of alfalfa and acerola cherry extracts ($p < 0.0005$).

DISCUSSION

Genistein and daidzein are major components of soy extract and represent the isoflavones that are ascribed phytoestrogenic activity. The levels of these isoflavones range from 0.55 to 0.68 μM/L in human plasma after consumption of soy products (Barnes et al., 1996). Morton et al. (1994) reported that plasma levels of genistein and daidzein reach levels of 0.55 and 1.23 μM/

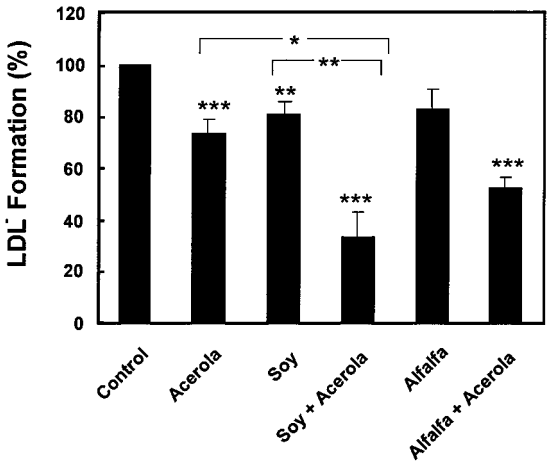

Figure 6. LDL⁻ formation mediated by cells after addition of 100 μg/mL LDL protein. Cells were incubated under standard conditions (control) and preincubated with acerola, soy, soy and acerola, alfalfa, and alfalfa and acerola extracts for 5 days as described under Materials and Methods. All treatment conditions are expressed as a percent of control cells, which were set at 100%. Differences compared to control values are indicated by **, $p < 0.005$; and ***, $p < 0.0005$. Significant differences between acerola and soy plus acerola are indicated by *, $p < 0.05$; and between soy and soy plus acerola are indicated by **, $p < 0.005$.

L, respectively, in postmenopausal women consuming soybean flour. Because the quantities of these components vary in extract preparations, the amounts used in this study were presented on the basis of the content of genistein and daidzein in the soy extract and of coumestrol and apigenin in the alfalfa extract. These extracts were diluted in ethanol to give appropriate stock solutions that were added to LDL or cultured cells to give final extract concentrations containing the indicated equivalents of these phytoestrogens. The same approach was used for acerola cherry extract, which was diluted with water to give final stock concentrations based on AA content. Thus, the concentration ranges of extracts studied were 0.1−5 μM equivalents of genistein and daidzein for soy extract, 0.02−1.0 μM equivalents of coumestrol and apigenin for alfalfa extract, and 10.2−511 μM AA for acerola cherry extract. These concentrations are within the ranges found in human plasma after consumption of soy products as noted above. There is very little information about blood levels of key components such as coumestrol and apigenin after consumption of alfalfa extracts, with limited data from human and animal studies (Kurzer and Xu, 1997; Janssen et al., 1998). Supplementation of humans with AA results in plasma AA levels that correspond to the concentration of AA equivalents in the acerola cherry extract (Asenjo, 1959). Acerola cherry is considered to be one of the richest sources of vitamin C (Clein, 1957) and was shown in human trials to be a better source of bioavailable vitamin C than USP vitamin C (Tang, 1995). On the basis of these properties and its use as a natural source of vitamin C, acerola cherry extract was used in this study.

The antioxidant activity of soy phytoestrogens has received increasing attention, and although studies have been performed with isolated phytoestrogens, this is the first report to our knowledge that describes antioxidant activity of naturally occurring phytoestrogens. Because both soy and alfalfa extracts have been considered and utilized as sources for phytoestrogens, applications in terms of positive estrogenic health effects, particularly prevention of CVD, warrant investigation. To this end, evidence of antioxidant activity toward LDL and prevention of oxidized LDL-induced cytotoxicity suggests that these phytoestrogens may have antiatherogenic activity. Alfalfa extract has not been investigated in terms of LDL antioxidant activity to our knowledge. However, evidence exists for potential antiatherosclerotic activity because alfalfa extract has been shown to inhibit the absorption of dietary cholesterol (Molgaard et al., 1987) and to decrease serum cholesterol in animals (Malinow et al., 1980). Nevertheless, there is no documentation about serum levels of the principal phytoestrogens found in alfalfa extract after ingestion. The levels used in this study were selected so as to be similar and comparable to the concentrations of soy phytoestrogens. In the latter case, there is ample evidence of measurable serum levels after ingestion of the soy products as noted above.

The biochemical basis for the effects of the isoflavones present in these extracts remains speculative. One mechanism for their action as antioxidants is based on properties analogous to those of 17β-estradiol in terms of inhibiting LDL oxidation (Neugarten et al., 1995; Tang et al., 1997; Maziere et al., 1991). Binding to LDL apo B and preservation of protein structure are thought to be involved in the antioxidant effect (Parasassi et al., 2000). A stabilization of particle structure during lipid peroxidation was found in terms of the general polarization of a fluorescent probe (Laurdan) that coincided with inhibition of lipid peroxidation. It is thought that changes in LDL structure (particularly for apo B) are highly sensitive to the formation of lipid peroxidation products (Mowri et al., 1984); interactions between estrogen or phytoestrogens and apo B−lipid domains stabilize LDL structure, which prevents the progression of lipid peroxidation.

A principal finding of this study is that soy and alfalfa extracts and acerola cherry extract, which is rich in AA, interact synergistically in preventing LDL oxidation in vitro and in a cell culture model for LDL-induced cytotoxicity. The interaction of phytoestrogens and AA during the inhibition of copper-dependent initiation and propagation of LDL oxidation is of considerable significance, because it points out an interaction in the blood that could potentiate the effects of these agents as antioxidants. It also highlights the importance of antioxidant interactions in providing overall antioxidant defense, whereby synergism may become an important mechanism in overall antioxidant defense. The synergism evident in the interaction of phytoestrogens and AA may be due to a combination of distinct processes. The "peroxidolytic" action of AA would facilitate the decomposition of peroxides in plasma to nonradical products and limit the formation of lipid radicals that would require quenching by other antioxidants in lipoproteins. This process would thereby sustain the antioxidant activity of LDL, and other lipoprotein antioxidants, including the phytoestrogens.

Retsky et al. (1999) described the antioxidant effect of AA as a prevention of peroxide-dependent LDL oxidation involving a reductive decomposition of preformed hydroperoxides. Elimination of these peroxides would cancel a key event in LDL oxidation, namely, peroxide-mediated oxidation. AA is also reported to oxidize histidine residues on LDL apo B to 2-oxohistidine, which prevents copper binding to the particles, so

a catalytic initiation of lipid peroxidation by copper bound to LDL would be inhibited. A combination of "peroxidolytic" and "anticomplexing" actions of AA may, thereby, account for the strong suppression of LDL oxidation, and only after AA is expended can peroxides begin to accumulate. The antioxidant behavior of phytoestrogens would then be conditional on the limited formation of peroxides during the lag phase interval.

The antioxidant effect of the phytoestrogen extracts was also found under cell culture conditions in which a significant inhibition of cell-mediated LDL oxidation was found for soy extract alone and for soy and alfalfa extracts when combined with acerola extract. The protective mechanism is not clear in this case but may relate to the cytoprotective action of these compounds. All compounds were able to protect endothelial cells from LDL-mediated toxicity when high concentrations were applied to cultures. Although beyond the scope of this project, the formation of reactive oxygen species (ROS) accompanies cell injury induced by high LDL−cholesterol exposure (Heinle and Liebich, 1980), and this may be a mechanism by which these phytoestrogens are protective. How the injury is manifested in relation to ROS production is unclear, but the relationship to ROS formation is evident through the level of LDL oxidative modification, measured as LDL^- formation. The inhibition of LDL^- formation by cells treated with the phytoestrogens and/or phytoestrogens and acerola extract is in agreement with reports showing that antioxidants prevent modification of LDL to more cytotoxic species, such as oxidized LDL (Belcher et al., 1993; El-Swefy et al., 2000). There are several other possibilities for the protective effects, among which the inhibition of LDL−cholesterol retention despite high levels of exposure and binding and uptake may be one example, taken from the effects described for estrogens (Sulistiyani and St. Clair, 1997). Also, ascorbate and estrogens are reported to enhance nitric oxide (NO) formation and preserve EDRF activity in the face of an oxidative challenge (May, 1999; Kim et al., 1999). Because NO is a potent cellular and LDL antioxidant (Hogg and Kalyanaraman, 1999), the protection found in this study may be due to a preservation or enhancement in NO levels and is being further investigated.

The antioxidant behavior of the major phytoestrogenic compounds from soy extract was recently shown by the inhibition of LDL oxidation analogously to 17β-estradiol (Shwaery et al., 1997; Huber et al., 1990; Tang et al., 1996). The mechanism of action has been proposed to involve a preservation of apo B structure during LDL oxidation. This is evidenced by the general polarization (GP) of the fluorescent probe, laurdan, that precedes the propagation of LDL lipid peroxidation (Brunelli et al., 2000). We postulate that changes in LDL structure, particularly for apo B, are highly sensitive to lipid peroxidation, resulting in marked disruption of order that predisposes lipids to further peroxidation (Mowri et al., 1984). Estrogen or phytoestrogens interact with apo B−lipid domains and prevent the structural changes that facilitate the progression of lipid peroxidation (Brunelli et al., 2000). This protective effect is analogous to the resistance of highly organized lipid bilayers to peroxidation (Sevanian et al., 1990). Thus, an AA-based plant extract and phytoestrogens may protect LDL by separate and independent mechanisms rather than by a similar free radical scavenging process and thereby increase the efficacy of the other LDL antioxidants. Nevertheless, it is not possible to ascribe the effects of the extracts studied solely to the isoflavones or ascorbate because other components may be contributing to the antioxidant action. However, because a similar level and type of activity, in the same test systems, was found using purified isoflavones from soy extract, it is likely that at least part of the antioxidant actions observed are due to their interactions with LDL.

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