Selective Effects of Different Antioxidants on Oxidation of Lipoproteins from Rats

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Abstract. **Lipoprotein oxidation may contribute to development of atherosclerosis, and supplementation with antioxidants may reduce risk for atherosclerotic events. Genistein, a major isoflavone from soy protein, and catechins from green tea have important antioxidant properties. This study compared the effects of various diets containing antioxidant-rich foods or supplements on serum lipids and lipoprotein oxidation of male Sprague-Dawley rats. The control diet used was devoid of vitamin E. Test diets included these ingredients: green tea powder, 20 g/kg; β-carotene, 250 mg/kg; a low isoflavone soy protein isolate; a genistein-rich soy protein isolate; and vitamin E, 4000 mg/kg. Ten-week-old rats were acclimatized for 1 week on a special custom diet without vitamin E. Following randomization and allocation to different diet groups, rats were fed the test diets for 3 weeks. Blood was drawn by cardiac puncture, and the plasma was separated by centrifugation. The VLDL-LDL fraction was isolated by ultracentrifugation. Oxidation kinetics of the VLDL-LDL fraction were determined by measuring the lag phase and formation of conjugated dienes, lipid peroxides, and TBARS.**

The vitamin E diet ($P < 0.001$) and high-genistein diet profoundly decreased all parameters of lipoprotein oxidation. The following alterations were noted with the high-genistein diet compared to the control diet: the lag phase was 49% longer ($P = 0.002$); conjugated diene formation was decreased by 28% ($P = 0.01$); lipid peroxide formation was decreased 31% ($P = 0.0059$); and TBARS production was 35% lower ($P = 0.019$). The low-isoflavone diet increased the lag phase by 43% ($P = 0.0019$) but did not significantly alter other measures of oxidation. Green tea increased only the lag phase by 33% ($P = 0.012$) compared to the control diet. β-Carotene had no significant effect on the oxidation of lipoproteins.

The effect of genistein-rich soy protein isolates on lipoprotein oxidation *in vitro* suggests that either soy isoflavones or other antioxidants derived from soy protein, like vitamin E, may be transported in these lipoproteins. The minimal effects of the isoflavone-poor soy protein isolate suggests that either the small amount of isoflavones present have a potent effect or other components of soy protein are exerting these effects. Further studies are required to examine these results. [P.S.E.B.M. 1998, Vol 218]

Several lines of evidence indicate that the development of atherosclerosis is related to free radical processes, lipid peroxidation, and oxidative modification of low-density lipoproteins (LDL) (1). Oxidation of LDL plays a central role in the pathogenesis of atherosclerosis (1). Oxidized LDL is recognized by the scavenger receptor of macrophages and is able to convert them to lipid-loaded foam cells that are found in the atherosclerotic plaques (2). Recent studies document that administration of antioxidants, such as vitamin E, significantly reduces LDL oxidation in humans (3). Our preliminary work documents that administration of antioxidants to humans leads to significant reduction in LDL oxidation *in vitro* (4). Genistein, a major isoflavone from soy protein, has important antioxidant properties (5). Of additional interest, a recent meta-analysis (6) reviews the persuasive data indicating that soy protein intake by humans leads to significant reduction in serum LDL-cholesterol concentrations. The combination of the

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LDL-lowering effects and antioxidant effects of soy protein and their isoflavones may contribute to the lower rates of coronary heart disease among Asian people who eat generous amounts of soy protein than rates among Western people who eat minuscule amounts of soy protein (6).

Much research has also focused on the potential role of natural antioxidants in protecting LDL. Plant phenols, present in fruits, vegetables, tea, and wine, have received considerable attention because of their potential antioxidant effect. The two active components of the polyphenolic fractions of green tea are catechins and epicatechins (7). A recent study demonstrated that green tea extract as well as its components catechin and epicatechin inhibit LDL oxidation mediated by endothelial cells. They concluded that the polyphenolic components of green tea may have nutritional benefits as physiologically active inhibitors of LDL oxidation (8). The carotenoid, β-carotene, has been shown to function as a radical trapping antioxidant (9) and, *in vitro,* inhibits the oxidation of LDL (10).

The major purpose of this study was to determine if intake of a diet containing isoflavone-rich soy protein affected lipoprotein oxidation in rats. Comparison diets included a low-antioxidant (low–vitamin E diet), a high–vitamin E diet, and an isoflavone-poor soy protein. To further compare the effects of soy isoflavones, other dietary antioxidants, namely β-carotene and green tea containing catechins and other antioxidants, were compared. Since the standard rat chow has different levels of vitamin E, the control casein diet was formulated without vitamin E to eliminate this variable. A secondary objective was to compare the effects of soy proteins, either rich or poor in isoflavones, on serum lipid concentrations. Previously we (11) developed and used a high-cholesterol diet to examine the hypocholesterolemic effects of different dietary fibers for rats; this dietary approach was used here to examine the potential hypocholesterolemic effects of soy protein.

Materials and Methods

Animals. In this experiment, 70 male, 10-week-old Sprague-Dawley rats were housed individually in stainless steel cages. Upon delivery to the VA Medical Center animal research facility, rats were acclimatized for 1 week on a control diet without vitamin E. Before starting the test diets, the rats were weighed, and the five heaviest and the five lightest rats were eliminated from the study. The rats were randomized and then assigned to different diet groups by stratified allocation to have 10 rats in each group. Throughout the study rats were given free access to water.

Diet. Table I presents the nutrient composition of the special custom diet as well as the different test diets. Diets were prepared by ICN (Cleveland, OH); all ingredients, except soy protein, were provided by ICN. The isolated soy proteins and their nutrient analysis were provided by Protein Technologies International (St. Louis, MO). The high-genistein soy protein isolate (HG 670) contained 1.45 mg genistein/g of protein, whereas the low-isoflavone soy protein isolate (termed isoflavone-free, IF 670) contained 0.08

Table I. Composition of Special Custom Diet and Test Diets

Component (g/kg)	Diet I[a]	Diet II[b]	Diet III[c]	Diet IV[d]	Diet V[e]	Diet VI[f]
Sucrose	440	440	440	440	440	440
Corn starch	225	206.2	215.8	221	205	224.75
Casein purified high nitrogen	150	—	—	150	150	150
Cotton seed oil	60	50.6	58.8	60	60	60
DL Methionine	3	3	3	3	3	3
Cholesterol	10	10	10	10	10	10
Cholic acid	2	2	2	2	2	2
Special salt mix[g]	40	40	40	40	40	40
Alphacel non nutritive bulk	60	60	60	60	60	60
VDFM (Without vitamin E)[h]	10	10	10	10	10	10
Soy protein (670 HG)	—	178.2	—	—	—	—
Soy protein (670 IF)	—	—	160.4	—	—	—
Vitamin E powder	—	—	—	4	—	—
Green tea, pulverized (2%)	—	—	—	—	20	—
β Carotene	—	—	—	—	—	0.25

Note. Values represent g of ingredient/kg diet
[a] Diet I: Special custom diet without vitamin E
[b] Diet II: Custom diet with 15% added soy protein (HG 670)
[c] Diet III: Custom diet with 15% added soy protein (IF 670)
[d] Diet IV: Diet containing added vitamin E (1000 units/kg)
[e] Diet V: Diet containing 2% green tea
[f] Diet VI: Diet containing 50 mg/kg β carotene
[g] Special salt mixture contained the following (per kg mix): sodium chloride 139.3 g, monopotassium phosphate 389 g, magnesium sulfate 57.3 g, calcium carbonate 381.4 g, ferrous sulfate 27 g, manganese sulfate 4 g, potassium iodide 0.8 g, zinc sulfate 0.8 g, copper sulfate 0.5 g, cobaltous chloride 25 mg.
[h] Vitamin diet fortification mixture contained the following (per kg mix): retinyl acetate 4 g, Calciferol 260 mg, inositol 11 g, choline chloride 167.2 g, menadione 4.9 g, biotin 40 mg, p-aminobenzoic acid 11 g, ascorbic acid 99 g, niacin 9.8 g, riboflavin 2.2 g, pyridoxine HCl 2.2 g, thiamine HCl 2.2 g, calcium pantothenate 6.6 g, folic acid 200 mg, vitamin B 12 trituration (1.1%) 3 g, sucrose 640 g.

mg genistein/g of protein. The total isoflavone content of the two diets was 2.39 and 0.11 mg/g of protein, respectively.

Procedure. The rats were weighed before starting the test diets. Each rat was fed on a test diet based on the group to which it had been allocated, for a period of 3 weeks. To allow for each dietary group to be fed for a period of 3 weeks, rats were weighed, then sacrificed according to the same staggered schedule as the start of the feeding. Thus, rats from each dietary group were sacrificed daily to ensure that each rat consumed the test diet for 21 days. The rats were anesthetized, the blood drawn by cardiac puncture and then killed by exsanguination. The liver was removed, weighed, and frozen at 20°C until further analysis (11).

Measurements. Rats were weighed at 0 and 3 weeks. Whole blood was collected by cardiac puncture into 16 × 125 mm tubes, and serum was separated by centrifugation. VLDL-LDL was isolated by sequential ultracentrifugation (12). Plasma density was raised to 1.09 g/ml by addition of KBr and transferred to Beckman centrifuge tubes along with saline EDTA (0.1% EDTA). Samples were centrifuged at 55,000 rpm at 4°C for 5 hr and 18 min using a VTi 90 rotor in a Beckman model L8-80 ultracentrifuge. Since the rat has a very small amount of LDL, all further assays were performed on the isolated VLDL-LDL fraction.

Oxidative damage to lipoproteins was measured by several techniques because the time course of formation of different oxidative products differs (13). The kinetics of lipoprotein oxidation were determined by measuring the formation of conjugated dienes spectrophotometrically at 234 nm every 5 min for 3 hr; duplicate samples of 25 μg of protein were incubated with 5 μ*M* Cu at 28°C (13). Lag phase measurements during *in vitro* oxidation served as an indicator of resistance to oxidation. The lag phase was calculated by measuring the tangent of the intercept of the slope of absorbance in the propagation phase with the baseline (13) and was expressed in minutes. Lipid peroxides, a fairly specific measure of lipid peroxidation, were measured at completion of the 3-hr oxidation period by a modification of the method of El-Saadani *et al.* (15). The formation of thiobarbituric reactive substances (TBARS), a nonspecific measure of oxidative damage to lipoproteins, was determined by the method of Kosugi and Kikugawa (14).

Serum cholesterol measurement was based on the catalytic oxidation of cholesterol using the Abbott VP analyzer (16). Serum triglyceride levels were determined by measuring the decrease in absorbance due to the oxidation of NADH to NAD using the Abbott VP Analyzer (17). Liver cholesterol was measured as previously described (11).

Statistical Analysis. Group means were compared by one-way analysis of variance. Means were considered to be significantly different at $P < 0.05$ as determined by the mean *t* test (18).

Results

Body Weight. Initial body weights as well as final body weights were similar for all groups. There were no significant differences in weight gain among groups (Table II). The vitamin E group showed the largest average weight gain (80 g) whereas the green tea group showed the smallest average weight gain (64 g). Weight gain in the other groups was comparable. No significant differences were observed in the liver weights between groups (data not shown, ANOVA $P = 0.3$).

Lipid Responses. (Table II) *Serum cholesterol.* Significant differences in serum cholesterol concentrations were seen ($P = 0.0026$). However, the only group that differed significantly from the control group was the green tea group with serum cholesterol values significantly higher ($P = 0.025$) than control values. The lowest serum cholesterol value of 115 mg/dl was for the low-isoflavone group, but this did not differ significantly from control values.

Serum triglycerides. Significant differences in serum triglyceride concentrations were also seen ($P = 0.0048$). Triglyceride values were significantly lower ($P < 0.001$) for the high-genistein group than for the control group.

Liver cholesterol. Values for liver cholesterol concentrations showed a small statistical variation, but none of the individual groups differed significantly from the control group.

Oxidation Studies. (Table III) *Lag phase.* The lag phase differed significantly ($P < 0.001$) between groups. Vitamin E supplementation produced a three-fold increase in the lag phase by increasing values from 56 to 173 min. The high-genistein group had a significant prolongation (83

Table II. Effect of the Different Test Diets on Weight Gain, Serum Cholesterol, Serum Triglyceride and Liver Cholesterol Levels[a]

Measurement	Control	Green tea	Beta-carotene	Low isoflavone	High genistein	Vitamin E	ANOVA P
Weight gain, g	72	64	71	69	71	80	0.62
S.E.M.	0.6	0.4	0.5	0.8	0.5	0.8	
Serum cholesterol, mg/dl	128	151	131	115	121	133	0.0026
S.E.M.	6.9	6.2	5.4	4.7	4.1	7.9	
P vs control		0.025					
Serum triglycerides, mg/dl	218	192	199	210	188	241	0.0048
S.E.M.	9	5.6	12.2	8.7	10.1	13.2	
P vs control					<0.001		
Liver cholesterol, mg/g	46	35.9	38	35.6	38.2	45.4	0.037
S.E.M.	3.3	2.6	2.5	2.7	3	3.2	

[a] Significant differences are shown.

Table III. Effect of Feeding Different Test Diets on the Oxidation of the VLDL-LDL Fraction, Lipid Peroxides and TBARS Formation[a]

Measure	Control	Green tea	Beta-carotene	Low isoflavone	High genistein	Vitamin E	ANOVA P
Lag phase, min	55.7	75.6	74.9	79.6	83.2	173	<0.001
S.E.M.	6.8	6.9	6.8	4.4	5.1	7.2	
P vs Control		0.012		0.0019	0.002	<0.001	
Conjugated dienes, Optical density	0.679	0.611	0.56	0.601	0.489	0.08	<0.001
S.E.M.	0.06	0.06	0.05	0.06	0.04	0.02	
P vs Control					0.01	<0.001	
Lipid peroxides,[b]	982	925	889	846	677	166	<0.001
S.E.M.	118	101	119	95	58	26	
P vs Control					0.0059	<0.001	
TBARS,[c]	66.4	57	51.6	52.2	43.3	8.3	<0.001
S.E.M.	8.3	8.7	4.5	8.4	3.8	3.2	
P vs Control					0.019	<0.001	

[a] Statistically significant differences are indicated.
[b] Values are in nmoles/mg protein
[c] Values are in nmoles MDA/mg protein

min, $P = 0.002$) of lag phase of 49%. The low-isoflavone group had a similar and significant (80 min, $P = 0.002$) prolongation of lag phase, which did not differ statistically from the high-genistein group. Green tea intake also significantly increased (76 min, $P = 0.012$) the lag phase, but beta-carotene did not.

Conjugated diene formation. Figure 1 illustrates the time sequence for conjugated diene formation for the various diets. Lipoproteins from the vitamin E–supplemented group showed minimal oxidation. The high-genistein group showed the next slowest rate of conjugated diene formation. The other diet groups were intermediate between the high-genistein group and the control group.

As Table III summarizes, the vitamin E diet was associated with a reduction in conjugated diene formation (0.08 OD Units) to less than 12% of control values. The high-genistein group also had significantly lower ($P = 0.01$) conjugated diene values (0.49 OD Units) than the control group (0.68 OD Units). The low-isoflavone group had conjugated diene values that did not differ from control values but also did not differ from the high-genistein group. Green tea and beta-carotene intake did not significantly affect conjugated diene values.

Lipid peroxide formation. Vitamin E intake decreased lipid peroxide formation (166 nmoles/mg protein) to less than one-fifth of control values. High-genistein soy protein intake also significantly decreased ($P = 0.006$) lipid peroxides in lipoprotein fractions from control values of 982 nmoles/mg protein to 677 nmoles/mg protein. The other dietary interventions did not significantly affect lipid peroxide values. Values for the high-genistein group did not differ significantly from values for the low-isoflavone group.

TBARS. Vitamin E intake decreased TBAR formation to one-eighth (8 nmoles MDA/mg protein) of control values. The high-genistein group had TBARS values (43 nmoles MDA/mg protein) that were one-third lower than control values (66 nmoles MDA/mg protein, $P = 0.02$). The other diets were associated with slightly, but not significantly, lower TBARS values than the control group.

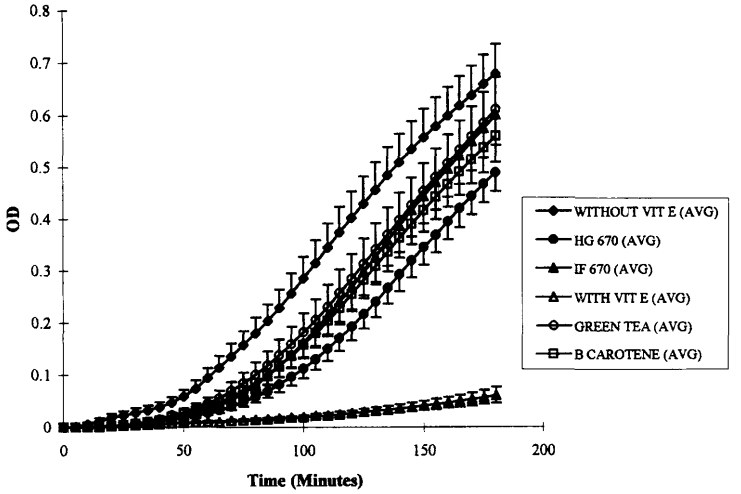

Figure 1. Mean oxidation curves (with SEM bars) for LDL samples from rats fed on the six test diets. Oxidation kinetics of the VLDL-LDL fraction (25 μg protein) were determined by measuring conjugated dienes using 5 μ*M* Cu at 28°C for 3 hr, in duplicate samples with measurements every 5 min. Abbreviations are: VIT E, Vitamin E; HG 670, high-genistein soy protein isolate; and IF 670, low-isoflavone soy protein isolate. Values differed from control (without vitamin E): VIT E ($P < 0.001$) and HG 670 ($P < 0.01$).

Values for the high-genistein group did not differ significantly from those for the low-isoflavone soy protein isolate group.

Discussion

In this study we compared the effects of different dietary antioxidant sources on oxidation kinetics of rat lipoproteins. To minimize dietary antioxidant intake, we administered a semisynthetic diet without vitamin E supplementation for a one-week run-in period and for the three-week diet study. When large amounts of vitamin E (4000 mg/kg diet) were included in the diet as a positive control, lipoprotein oxidation was substantially lower than control values. The high-genistein soy protein isolate diet also significantly decreased all measured aspects of lipoprotein oxidation. The low-isoflavone and green tea diets were associated with increases in the lag phase but not with significant alterations in other measures of lipoprotein oxidation.

This rat model was developed to examine the hypocholesterolemic effects of different dietary fibers (11) and includes 1% cholesterol and 0.2% cholic acid. This large dietary cholesterol and bile acid intake may have obscured effects of soy protein on serum cholesterol values. The low-isoflavone soy protein isolate diet was accompanied by 10% lower serum cholesterol values than the control diet, but these differences were not statistically significant. The high-genistein soy protein isolate diet was associated with significantly lower triglyceride values than control values. The significantly higher serum cholesterol values on the green tea diet may relate to an increase in HDL-cholesterol concentrations, which were not measured in this study.

Pharmacological amounts of vitamin E (4000 mg/kg diet) and beta-carotene (250 mg/kg diet) were used in this experiment. The effects of vitamin E on lipoprotein oxidation were dramatic whereas those of beta-carotene were negligible. The amounts of soy protein, 15% of diet, were in the physiologic range for rat diets (11) and provided less than 15% of energy intake. The levels of genistein (217.5 mg/kg diet) and total isoflavones (358.5 mg/kg diet) provided, however, would be much higher than consumed by most populations. The green tea intake (20 g/1000 kg diet) probably provided polyphenols in the range used by frequent tea drinkers (19).

The lipoprotein profile of the rat differs from that of the human in that the rat has high levels of HDL and low levels of LDL (20). In this study we isolated the VLDL-LDL fraction for oxidation kinetic studies. In the human, the oxidation kinetics of VLDL are fairly similar to those of LDL (Anderson JW, unpublished data), and the oxidation kinetics of the VLDL-LDL fraction from the rat were fairly similar to that observed for human LDL in our laboratory. With extensive experience with LDL, HDL, and VLDL oxidation kinetics for the human, we tailored these study conditions to the rat lipoprotein fraction under study (21). However, these observations are not translatable to the LDL of humans.

Our observations suggest that genistein or some potent antioxidant present in the isolated soy protein may be absorbed, transported to the liver, and incorporated into the VLDL fraction. This soy protein antioxidant is then preserved in the VLDL-LDL fraction and acts to inhibit oxidation. The amount of soy isoflavone (358.5 mg/kg diet) provided was less than 9% of the vitamin E (4000 mg/kg diet) provided. Nevertheless, the high-genistein soy protein isolate diet was accompanied by significant inhibition of lipoprotein oxidation. Genistein, accounting for approximately 59% of the isoflavones present in the mature soybean (6), appears to be the best candidate for the potent antioxidant effects associated with soy protein. Genistein has *in vitro* antioxidant properties (5) and is a potent enzyme inhibitor (22). Genistein also has a host of other hypothetical roles including inhibition of angiogenesis (23), protection of bone from osteoporosis (24, 25), and decreasing risk for breast cancer (26–29) and prostate cancer (25). The role of genistein and other soy isoflavones in protecting lipoproteins from oxidation requires much further study.

Measurement of the lag phase during the *in vitro* oxidation of LDL was introduced and popularized by Esterbauer and colleagues (13, 30) and is a very sensitive indicator of the susceptibility of LDL to *in vitro* oxidation. Our observations that green tea intake significantly prolongs the lag time suggests that the polyphenolic compounds such as certain catechins (7) may also be transported in lipoproteins and inhibit their oxidation. Human (19) as well as animal (31) data suggest that tea consumption may selectively decrease LDL and increase HDL concentrations. Although we did not estimate LDL-cholesterol and HDL-cholesterol concentrations in this study, the significant increase in serum cholesterol concentrations may relate to an increase in HDL-cholesterol. Further research is required to delineate the effects of tea intake on serum lipoprotein concentrations and susceptibility of LDL to oxidation. These factors may combine to contribute to a reduction in risk for atherosclerotic disease (19).

Conclusion

Our studies incorporating high-antioxidant ingredients into the diets of rats indicate that dietary antioxidants have a major role in regulation of lipoprotein oxidation. Antioxidant-rich diets were compared to a vitamin E–deficient diet as the control. Vitamin E, at pharmacological concentrations of 4000 mg/kg diet, profoundly inhibited the oxidation of the VLDL-LDL fraction. The high-genistein soy protein isolate diet also significantly decreased lipoprotein oxidation as evidenced by an increased lag phase during lipoprotein oxidation and lower production of conjugated dienes, lipid peroxides, and TBARS. The low-isoflavone soy protein isolate diet was associated with a significant prolongation of the lag phase for lipoprotein oxidation. Incorporating green tea, at 2% of diet, also increased the lag phase significantly.

These studies suggest that genistein or other potent an-

tioxidants from soy protein may be incorporated into lipoproteins, as is vitamin E, and have the ability to inhibit lipoprotein oxidation *in vitro,* as studied here, and probably also in the subendothelial tissue where the atherosclerotic process begins. In addition to the important effect of soy protein intake on lipoprotein concentrations (6), soy protein also appears to convey protective antioxidant properties to circulating lipoproteins.

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