Dietary Flavonoids Reduce Lipid Peroxidation in Rats Fed Polyunsaturated or Monounsaturated Fat Diets[1,2]

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ABSTRACT We investigated the influence of dietary flavonoids on α-tocopherol status and LDL peroxidation in rats fed diets enriched in either polyunsaturated fatty acids (PUFA) or monounsaturated fatty acids (MUFA). Diets equalized for α-tocopherol concentrations were or were not supplemented with 8 g/kg diet of flavonoids (quercetin + catechin, 2:1). After 4 wk of feeding, plasma lipid concentrations were lower in rats fed PUFA than in those fed MUFA with a significant correlation between plasma α-tocopherol and cholesterol concentrations, $r = 0.94$, $P < 0.0001$). Dietary lipids influenced the fatty acid composition of VLDL + LDL more than that of HDL or microsomes. The resistance of VLDL + LDL to copper-induced oxidation was higher in rats fed MUFA than in those fed PUFA as assessed by the lower production of conjugated dienes and thiobarbituric acid reactive substances (TBARS) and by the >100% longer lag time for dienes production. ($P < 0.0001$). Dietary flavonoids significantly reduced by 22% the amounts of dienes produced during 12 h of oxidation in rats fed diets rich in PUFA and lengthened lag time 43% in those fed MUFA. Microsomes of rats fed MUFA produced ~50% less TBARS than those of rats fed PUFA ($P < 0.0001$) and they contained more α-tocopherol in rats fed MUFA than in those fed PUFA with higher values ($P < 0.0001$) in both groups supplemented with flavonoids ($P < 0.0001$). Our findings suggest that the intake of dietary flavonoids is beneficial not only when diets are rich in PUFA but also when they are rich in MUFA. It seems likely that these substances contribute to the antioxidant defense and reduce the consumption of α-tocopherol in both lipoproteins and membranes. J. Nutr. 128: 1495–1502, 1998.

KEY WORDS: • *lipid oxidation* • *flavonoids* • *lipoproteins* • *microsomes* • *rat*

The intake of polyunsaturated fatty acids (PUFA)[4] at larger amounts than those required to prevent deficiency has beneficial effects on risk factors associated with coronary heart diseases such as thrombosis and hyperlipemia (reviewed by Goodnight et al. 1982). Nevertheless, high intake of PUFA may be disadvantageous because the oxidative degradation of PUFA can lead to the formation of modified atherogenic LDL (Steinberg et al. 1989). In plasma, circulating LDL are protected from the effects of lipid peroxidation by several antioxidants solubilized in their aqueous environment or associated with particles. In particular, lipophilic substances such as vitamin E (mainly α-tocopherol), β-carotene, lycopene and ubiquinol 10 that are present within particles are major determinants of their resistance to oxidative degradation (Esterbauer et al. 1992). The simultaneous presence of several antioxidants in the body is advantageous because some compounds at low concentrations are efficient for enhancing the activity of α-tocopherol (Thomas et al. 1995). Therefore, the intake of compounds with antioxidative properties is potentially beneficial. In rats, dietary supplementation with caffeic acid, a phenolic antioxidant, has been found to significantly increase the plasma and LDL α-tocopherol concentration (Nardini et al. 1997). In humans, flavonoids ubiquitously distributed in plant foods contribute greatly to the dietary sources of antioxidants. Epidemiologic studies have shown an inverse relationship between dietary intake of flavonoids (Hertog et al. 1993, Keli et al. 1996, Knekt et al. 1996) or vitamin E (Gey et al. 1991, Stampfer et al. 1993) and coronary heart disease. On the other hand, clinical trials have shown that dietary supplementation with vitamin E increased the resistance of LDL to oxidation ex vivo (Abbey et al. 1993b, Dieber-Rotheneder et al. 1991, Wander et al. 1996). Another dietary means of reducing the susceptibility of LDL to lipid peroxidation is the partial substitution of dietary PUFA by monounsaturated fatty acids (MUFA) (Berry et al. 1991, Bonamone et al. 1992, Mata et al. 1992, Mattson and Grundy, 1985, Reaven et al. 1991). Indeed, MUFA are more resistant to oxidative modification than PUFA and, unlike saturated fatty acids, are neutral or have hypolipidemic effects (Grundy 1987). Hence, it can be expected that the requirements of dietary antioxidants for protecting LDL are related to the amounts and type of dietary fats. In this study, we investigated the effects of dietary flavonoids in rats on lipid and oxidation variables in plasma and liver

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[4] Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL-C, HDL cholesterol; LA, linoleic acid; M, diet rich in MUFA; MF, diet M with flavonoids; MUFA, monounsaturated fatty acids; OA, oleic acid; P, diet rich in PUFA; PF, diet P with flavonoids; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; UI, unsaturation index.

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TABLE 1

Nutrient composition of the experimental diets

Component	Amount
	g/kg diet
Casein	220
Cornstarch	405.6
Sucrose	202.8
Fat source	100
Mineral mix[1]	40
Vitamin mix[2]	10
Cellulose	20
DL-Methionine	1.6

[1] Supplied in g/kg of premix: Ca $HPO_4 \cdot 2\ H_2O$, 380; K_2HPO_4, 240; $CaCO_3$, 180; $MgSO_4 \cdot 7H_2O$, 90; NaCl, 69; MgO, 20; $FeSO_4 \cdot 7\ H_2O$, 8.6; Zn $SO_4 \cdot H_2O$, 5; $MnSO_4 \cdot H_2O$, 5; $CuSO_4 \cdot H_2O$, 1; NaF, 0.8; CrK $(SO_4)CrK(SO_4)_2 \cdot 12H_2O$, 0.5; KI, 0.04; $CoCO_3$, 0.02; $Na_2SeO_3 \cdot 5H_2O$, 0.02.

[2] Supplied in g/kg of premix triturated in sucrose: retinyl acetate (500,000 IU/g), 1; cholecalciferol (250,000 IU/g), 2.5; *dl*-α-tocopherol (500 IU/g), 10; menadione, 0.1; thiamin · HCl, 1; riboflavin, 1; nicotinic acid, 4.5; calcium pantothenate, 3; pyridoxine · HCl, 1; inositol, 5; biotin, 0.02; folic acid, 0.2; cyanocobalamin, 0.00135; ascorbic acid, 10; *p*-aminobenzoic acid, 5; choline chloride, 75.

microsomes according to the degree of unsaturation of dietary lipids. We used as dietary supplements two major flavonoids of human food sources, i.e., quercetin (flavon-3-ol) and (+)-catechin (flavan-3-ol) found mainly in vegetables (Hollman et al. 1995) and red wine (Frankel et al. 1995), respectively.

MATERIALS AND METHODS

All of the chemicals used were of analytical reagent grade and purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Animals and diets. Male Wistar rats (IFFA CREDO, L'Arbresle, France) bred in our laboratory were maintained at 22 ± 1°C with a 12-h light:dark cycle and had free access to water and food. They were fed diet M rich in MUFA, or diet P, rich in PUFA. Both diets had an (n-6) to (n-3) fatty acid ratio in the range of 5.6 to 6.3, which is adequate for rats (Bourre et al. 1989). The nutrient composition of the experimental diets and the fatty acid composition of the dietary fats are given in **Tables 1** and **2,** respectively. The level of α-tocopherol was equalized in all diets by supplementing the mixture of oils used for diet M with *dl*-α-tocopherol to the level found in the mixture of oils used for diet P (final concentration, ~60 mg/100 g oil mixture). After adjustment with the added vitamin mix, the α-tocopherol concentration was 110 mg/kg diet. Rats were randomly distributed into four groups of 18 animals each, just after weaning (21 d old) in a 2 × 2 factorial design. Two groups of rats were fed the diets without supplementation (diets M and P), whereas the other two received the same diets supplemented with 8 g/kg diet of dietary flavonoids (quercetin + (+)-catechin, 2:1 wt/wt) (diets MF and PF).

The French instructions 88-123 concerning ordinance 87-848 about rules in animal experimentation were followed.

After 4 wk of dietary treatment, rats were food deprived for 15 h before weighing and killing by decapitation between 0800 and 0900 h. Blood was collected on heparin sodium salt (100 mg/L) and a mixture of 2 mmol/L benzamidin and 200 mmol/L gentamycin as preservatives. Plasma samples from each group, obtained after low speed centrifugation, were randomly pooled 3 by 3 into 6 subgroups. After adding 2 g/L Chelex-100 (Biorad, Ivry sur Seine, France) to remove traces of contaminating transition metals and aprotinin (100 kallikrein inhibitory units/L), plasma samples were stored at −80°C until analysis. Liver was rapidly removed, rinsed with ice-cold 150 mmol/L NaCl, blotted and weighed. Individual aliquots were freeze-clamped and stored at −80°C.

Lipoprotein isolation. Lipoprotein classes were isolated by sequential ultracentrifugation at 145,000 × *g* according to Havel et al. (1955) using saline solutions stored over Chelex-100 (3 g/L). Because both VLDL (precursor of LDL) and LDL undergo lipid peroxidation and because rats have relatively small amounts of LDL (Scaccini et al. 1992), the following two fractions were isolated: VLDL + LDL, $d < 1.050$ kg/L and HDL, $d = 1.050–1.21$ kg/L, after a 20- and a 36-h centrifugation, respectively. Just before their analysis, the fractions were filtered through a 0.2-μm Millipore filter (Saint Quentin en Yvelines, France) to remove Chelex.

Preparation of liver membranes. Microsomes were prepared by standard differential centrifugation techniques. All operations were performed at 4°C. Liver tissue was rinsed with ice-cold 6 mmol/L EDTA, 150 mmol/L NaCl, minced with scissors and homogenized in 2 mL/g of buffer (10 mmol/L Tris-HCl, 250 mmol/L sucrose and 1 mmol/L EDTA, pH 7.4) using a Polytron homogenizer (PT 1200 Kinematica, Bioblock, Strasbourg, France) with three pulses at setting 5. The whole homogenate was then filtered through a nylon gauze and centrifuged at 500 × *g* for 5 min. The supernatant was centrifuged at 15,000 × *g* for 15 min followed by centrifugation of the supernatant at 105,000 × *g* for 60 min. The pellet termed "microsomes" was washed to remove EDTA with 5 mmol/L Tris-maleate and 150 mmol/L KCl, pH 7.4. After centrifugation, the pelleted microsomes were suspended in the same buffer and stored at −80°C.

Fatty acid composition. Dietary lipids, plasma lipoproteins and liver microsomes were extracted by the method of Folch et al. (1957) and saponified in 2.7 mol/L KOH at 37°C for 5 h. The fatty acids were esterified by methanol containing 0.36 mol/L HCl and 0.38 mol/L 2,2 dimethoxypropane at 70°C for 35 min and analyzed by gas-liquid chromatography. The Carlo Erba model 4180 (Milan, Italy) chromatograph included an automatic injector on the column, a flame-ionization detector and a CP wax 52 CB (carbowax 20 M) bonded fused silica capillary column (50 m × 0.3 mm i.d.). The assays were carried out with programmed oven temperature increases of 3°C/min from 54 to 200°C. Hydrogen was the carrier gas at a pressure of 80 kPa. Peak areas were measured with an integrator connected to a microcomputer, which expressed the data automatically. Peaks were identified by comparing their equivalent chain lengths with those of authentic fatty acid methyl esters.

Quantitation of α-tocopherol. Lipids were extracted from total plasma and HDL according to Burton et al. (1985) by mixing the sample (0.5 mL), water (0.5 mL) and ethanol (1 mL) in a glass tube. After *n*-hexane (1 mL) was added, the mixture was stirred using a

TABLE 2

Fatty acid composition of dietary fats

Fatty acid	Diet M[1]	Diet P[1]
	g/100 g fatty acids	
16:0	7.4	9.7
18:0	3	4.9
16:1(n-7)	0.3	2.5
18:1(n-9)	73.4	19.6
20:1(n-9)	0.7	0.6
18:2(n-6)	12.9	53.8
20:4(n-6)		0.2
18:3(n-3)	2.3	0.3
18:4(n-3)		0.9
20:5(n-3)		4.4
22:5(n-3)		0.4
22:6(n-3)		2.6
SFA[2]	10.4	14.6
MFA[2]	74.4	22.7
(n-6) PUFA[2]	12.9	54.1
(n-3) PUFA	2.3	8.6
(n-6)/(n-3)	5.6	6.3

[1] Supplied in g/100 g fat source. Diet M (rich in monounsaturated fatty acids): rapeseed oil 13, peanut oil 12, olive oil 65; Diet P (rich in polyunsaturated fatty acids): sunflower oil 73, salmon oil 27.

[2] Abbreviations: SFA, saturated fatty acids; MFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

TABLE 3

Effects of flavonoid supplementation on lipids and α-tocopherol in plasma and liver microsomes of rats fed diets containing monounsaturated or polyunsaturated fatty acids[1,2]

	Diet M	Diet MF	Diet P	Diet PF	SEM	*P*-values[3]		
						Lipid	Flav	L × F
			mmol/L					
Plasma								
Triacylglycerol	1.41[a]	1.47[a]	1.07[b]	0.92[b]	0.05	0.0001	NS	NS
Total cholesterol	1.75[b]	2.05[a]	1.31[c]	1.30[c]	0.05	0.0001	0.02	0.01
HDL cholesterol	1.12[b]	1.32[a]	0.88[c]	0.94[c]	0.05	0.0001	0.02	NS
HDL-C/Total cholesterol	0.64	0.64	0.68	0.72	0.03	NS	NS	NS
			μmol/L					
Total α-tocopherol	16.52[b]	21.39[a]	13.81[c]	13.61[c]	0.65	0.0001	0.002	0.0009
HDL α-tocopherol	8.48[b]	10.22[a]	6.53[c]	6.88[c]	0.42	0.0001	0.02	NS
			nmol/mg protein					
Microsomes								
α-Tocopherol	0.42[b]	0.49[a]	0.27[d]	0.32[c]	0.01	0.0001	0.0001	NS

[1] Diets were rich in either monounsaturated fatty acids (M) or polyunsaturated fatty acids (P), not supplemented (diets M and P) or supplemented (diets MF and PF) with flavonoids (quercetin + catechin, 2:1).

[2] Values are means; $n = 6$ (pooled samples with 3 rats per pool) for plasma; $n = 18$ (individual samples) for microsomes. Within a row, values with different letter superscripts differ ($P < 0.05$).

[3] Two-way ANOVA: Lipid, significant influence of dietary fat source; Flav, significant influence of flavonoid supplementation; L × F, interaction; NS, not significant ($P > 0.05$).

vortex for 50 s, and the organic phase was separated by centrifugation. The aqueous phase was re-extracted, and hexane extract was dried under N_2 and resolubilized in a defined volume of hexane. The sample was analyzed by HPLC using a Microporasil column (300 × 3.9 mm, 10 μm i.d.) supplied by Waters (Milford, MA). The mobile phase was *n*-hexane/ethyl acetate (100:7.5, v/v) at a flow rate of 1 mL/min. Detection was performed with a Hitachi spectrofluorimeter (Model F-2000, Tokyo, Japan) with excitation at 290 nm and fluorescence emission at 330 nm. Quantitation was done with external standards of *dl*-α-tocopherol. The VLDL + LDL α-tocopherol concentration was calculated as the difference between total plasma and HDL concentration. The extraction of α-tocopherol from microsomes was adapted from Buttriss and Diplock (1984). In brief, 1 mL of microsomal suspension (1 mg protein) in 2 mL absolute ethanol containing 10 g/L ascorbic acid was saponified at 70°C for 30 min using 0.3 mL saturated potassium hydroxide. After cooling, 1 mL distilled water was added followed by 1 mL of *n*-hexane for extracting α-tocopherol.

Other assays. Plasma triacylglycerol and cholesterol concentrations were measured using enzymatic colorimetric tests (240052 and 1442341, Boehringer Mannheim, Meylan, France). HDL cholesterol (HDL-C) was determined after precipitation of VLDL + LDL by the dextran sulfate Mg^{2+} procedure of Warnick et al. (1979) adapted for rats (Sjöblom and Eklund 1989). VLDL + LDL cholesterol was estimated as the difference between total cholesterol and HDL-C.

The protein content was determined by the method of Bradford (1976) using the Biorad Protein assay (Biorad, Richmond, CA) with bovine serum albumin (fraction V) as standard.

Measurement of peroxidation. The thiobarbituric acid reactive substances (TBARS) were evaluated according to the procedure described by Wallin et al. (1993), but the oxidation was performed in Eppendorf microtest tubes (Polylabo, Strasbourg, France) instead of microtiter plates. Samples of VLDL + LDL containing 25 μg protein were diluted in 10 mmol/L PBS (pH 7.4) to a final volume of 465 μL after addition of 10 μL of 0.25 mmol/L $CuSO_4$. The tubes were vortexed and incubated at 37°C for different periods in a slowly shaking bath covered with adhesive polyester film permeable to air. At the end of oxidation, 10 μL of 1% butylated hydroxytoluene in ethanol, 50 μL of 3 mol/L trichloracetic acid and 75 μL of 90 mmol/L thiobarbituric acid in 75 mmol/L NaOH were added (final volume 600 μL). The tubes were incubated at 80°C for 40 min. After cooling, the tubes were centrifuged for 10 min (1500 × *g*) at 4°C. A volume of 300 μL supernatant was transferred to microtiter plates and the absorbance was read at 535 nm. The concentration of TBARS was expressed as nanomoles of malondialdehyde equivalents per milligram LDL protein using a freshly diluted l, l, 3,3-tetraethoxypropane for the standard curve. The same protocol was followed to determine the production of TBARS in microsomes, but the method of Laughton et al. (1991) was used to initiate peroxidation. The reaction mixture contained microsomes in PBS (0.5–1 mg protein), 100 μmol/L $FeCl_3$ and 100 μmol/L ascorbic acid at a final volume of 465 μL.

The kinetics of conjugated diene formation in the VLDL + LDL fraction were followed by continuous monitoring of the 234-nm absorption according to Esterbauer et al. (1989). Lipoprotein aliquots (25 mg protein/L) in oxygenated PBS (10 mmol/L, pH 7.4) were incubated at 37°C with 5 μmol/L $CuSO_4$. The increase of absorbance was recorded every 15 min for 24 h in a Uvikon 930 spectrophotometer (Kontron, Montigny le Bretonneux, France). The lag time was defined as the interval (min) between the intercept of the tangent of the slope of the propagation phase and the initial absorbance axis. The maximum diene concentration was determined from the difference between the absorbance at the maximum slope of the absorbance curve and the absorbance at time zero using the extinction coefficient for conjugated dienes at 234 nm [E = 29500 L/(mol · cm)]. The maximal rate of oxidation calculated from the slope of the absorbance curve during the propagation phase was expressed as nanomoles of dienes produced per minute per milligram of LDL protein.

Statistical analysis. Values are presented as means and SEM with significance set at $P < 0.05$. Two-way ANOVA was used to test effects of dietary fat type, supplementation with flavonoids and their interaction on variables. The differences between means were tested using Least Significant Differences (LSD) when the *F*-value was significant. One-way ANOVA was used to test the effects of oxidation time on the production of TBARS in VLDL + LDL in each dietary group. Some variables were submitted to linear regression analysis and

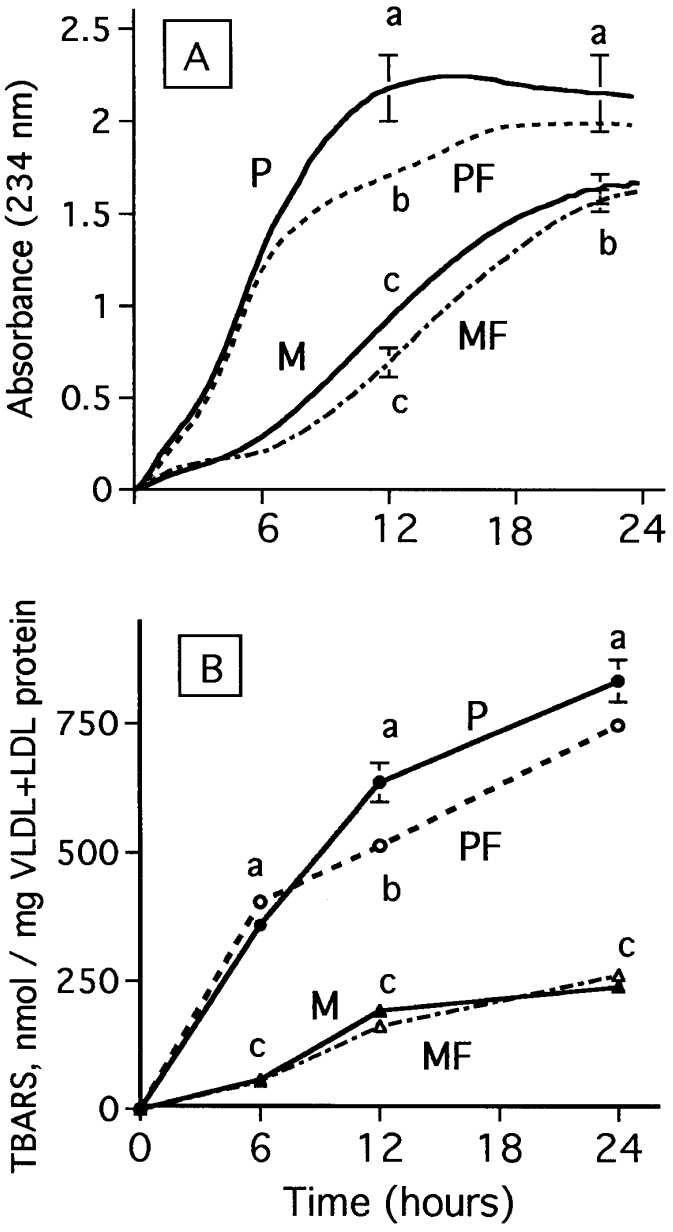

FIGURE 1 Kinetics of copper-catalyzed oxidation of VLDL + LDL in rats fed diets rich in either monounsaturated fatty acids (M) or polyunsaturated fatty acid (P), not supplemented (diets M and P) or supplemented (diets MF and PF) with dietary flavonoids. In *panel A,* the changes in the absorbance at 234 nm were continuously monitored and recorded every 10 min. The initial absorbance was subtracted from all data. Each value of any curve represents the mean of six pooled samples (3 rats per pool), except that at 12 and 22 h, the bars showing SEM have been omitted for clarity. At 12 h, there was a significant difference among groups for the fat source (M vs. P), $P < 0.0001$ and for the effect of flavonoids in rats fed diets rich in polyunsaturated fatty acids (P vs. PF), $P < 0.003$. In *panel B,* the production of thiobarbituric reactive substances (TBARS) was determined after 6, 12 and 24 h of oxidation. Each point represents the mean ± SEM (in some cases, bars are smaller than data points); $n = 6$ (pooled samples with 3 rats per pool). Within any group, the differences between time intervals differed significantly ($P < 0.05$). At any time, there was a significant difference among groups for the fat source (M vs. P), $P < 0.0001$ and at 12 h for the effect of flavonoids in rats fed diets rich in polyunsaturated fatty acids (P vs. PF), $P < 0.003$.

the Pearson correlation coefficient (r) was calculated. The statistical computer program was Super ANOVA (Abacus Concepts, Berkeley, CA).

RESULTS

There were no significant differences among dietary groups for final body weight (216.1 ± 6.3 g) or liver weight (7.2 ± 0.2 g).

The plasma triacylglycerol, total cholesterol, HDL cholesterol (HDL-C) and α-tocopherol levels in food-deprived rats were significantly lower in groups fed PUFA than in those fed MUFA (**Table 3**). However, there was no significant difference in the HDL-C to total cholesterol ratios, indicating that dietary fats affected HDL-C and VLDL + LDL cholesterol similarly. Supplementation with flavonoids did not alter the plasma triacylglycerol levels, whereas it significantly increased both cholesterol and α-tocopherol levels in groups fed MUFA (+17 and +29%, respectively). In total plasma or in any lipoprotein fraction, there was a significant correlation between α-tocopherol and cholesterol concentrations (total plasma, $r = 0.94$, $P < 0.0001$; VLDL + LDL, $r = 0.66$, $P < 0.0004$; HDL, $r = 0.79$, $P < 0.0001$). Plasma α-tocopherol also correlated with plasma triacylglycerol concentration ($r = 0.72$, $P < 0.0001$). The ratio of VLDL + LDL α-tocopherol to VLDL + LDL cholesterol (μmol/mmol) was in the range of 13.7 to 20.5 (16.8 ± 2.4) without significant differences among groups. Assuming that the plasma triacylglycerol concentration is representative of that of VLDL + LDL, the ratio between plasma triacylglycerol and VLDL + LDL cholesterol concentrations is indicative of variations in the average proportion of cholesterol within particles. We found a mean molar ratio of 2.5 ± 0.3, without significant differences among groups. Hence, it can be considered that the plasma α-tocopherol concentrations were linked to the number of VLDL + LDL particles in plasma rather than to the proportion of this compound within particles. Liver microsomes contained more α-tocopherol (>50%) in rats fed MUFA than in those fed PUFA, with higher values (+17–18%) in both groups supplemented with flavonoids (Table 3, $P < 0.0001$).

The kinetics of conjugated diene and TBARS production during copper-catalyzed oxidation of VLDL + LDL are presented in **Figure 1,** and values of the main indices of oxidation are reported in **Table 4.** The oxidation resistance measured as lag time before initiation of oxidation was >100% longer in groups M and MF than in groups P and PF, with significantly higher values (+43%) in group MF than in group M. The lag time did not correlate with the VLDL + LDL α-tocopherol levels as expressed relative to cholesterol concentrations ($r = 0.20$, $P = 0.39$). The rate of diene production was not altered by dietary flavonoids, but the mean value was 150% greater in groups fed PUFA than in those fed MUFA [3.38 vs. 1.36 nmol/(min · mg VLDL + LDL protein) $P = 0.0001$]. The total amounts of dienes produced were greater in groups P and PF than in groups M and MF, without response to dietary flavonoids. The time needed to reach maximal diene production was much shorter in rats fed the nonsupplemented diet P than in rats fed other diets (P, 15 h; PF, 22 h; M, 23 h; and MF, 23 h). At 12 h of incubation when differences between groups M and P were the largest (>100%), supplementation with flavonoids resulted in a lower level of dienes in rats fed PUFA-containing diet. Within each dietary group, the production of TBARS increased significantly over time from time 0 (no TBARS produced) to 24 h of oxidation (Fig. 1; $P < 0.05$ between time intervals). At all times, rats fed diets containing PUFA produced more TBARS than rats fed diets containing

TABLE 4

Effects of flavonoid supplementation on indices of oxidation in plasma lipoproteins and liver microsomes of rats fed diets containing monounsaturated or polyunsaturated fatty acids[1,2]

						P-values[3]		
	Diet M	Diet MF	Diet P	Diet PF	SEM	Lipid	Flav	L × F
VLDL + LDL								
Lag time for dienes, *min*	214[b]	306[a]	91[c]	105[c]	16	0.0001	0.006	0.03
			nmol/mg protein					
Dienes (12 h)	630[c]	469[c]	1477[a]	1156[b]	68	0.0001	0.003	NS
Total dienes	1123[b]	1087[b]	1519[a]	1346[a]	64	0.0001	NS	NS
TBARS (12 h)[4]	193[c]	163[c]	636[a]	514[b]	22	0.0001	0.003	NS
TBARS (24 h)	240[b]	262[b]	834[a]	747[a]	28	0.0001	NS	NS
Microsomes								
TBARS (1 h)	33.3[c]	30.0[c]	62.5[b]	65.7[a]	1.1	0.0001	NS	0.006

[1] Diets were rich in either monounsaturated fatty acids (M) or polyunsaturated fatty acids (P), not supplemented (diets M and P) or supplemented (diets MF and PF) with flavonoids (quercetin + catechin, 2:1).

[2] Values are means; $n = 6$ (pooled samples with 3 rats per pool) for plasma; $n = 18$ (individual samples) for microsomes. Within a row, values with different letter superscripts differ ($P < 0.05$).

[3] Two-way ANOVA: Lipid, significant influence of dietary fat source; Flav, significant influence of flavonoid supplementation L × F, interaction; NS, not significant ($P > 0.05$).

[4] Thiobarbituric acid reactive substances (TBARS) were measured after inducing lipid oxidation with $CuSO_4$ (VLDL + LDL) or with $FeCl_3$-ascorbic acid (microsomes).

MUFA, with a significant lowering effect of flavonoids at 12 h in rats fed PUFA. The two indices used for testing the oxidizability of lipoproteins were in good agreement, as assessed by the strong correlation between diene and TBARS production at 12 h ($r = 0.95$, $P < 0.0001$). Similarly, liver microsomes were affected by the type of dietary lipids. Indeed, the amounts of TBARS produced during 1 h of oxidation were ~50% lower in groups fed MUFA than in those fed PUFA, and group PF produced more TBARS than group P.

As expected, VLDL + LDL fatty acid distribution was more influenced by dietary fatty acids than HDL (**Table 5**). The proportion of oleic acid (OA) was >150% higher in groups fed MUFA than in those fed PUFA, with values 300% higher in VLDL + LDL than in HDL. The VLDL + LDL fraction contained at least 160% more OA than linoleic acid (LA) in groups fed MUFA, whereas the opposite relative proportions were found in groups fed PUFA. Despite the differences between dietary groups in LA intake, the main derivative, arachidonic acid (AA), was only slightly lower in VLDL + LDL and even 25% greater in HDL of rats fed MUFA compared with rats fed PUFA. The differences among groups in responses to dietary (n-3) PUFA were reflected in VLDL + LDL by the levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which were much higher in groups fed PUFA than in those fed MUFA. However, in HDL, the response to dietary long-chain (n-3) PUFA was observed for EPA but not for DHA. Finally, in VLDL + LDL, the unsaturation index (UI) was 63–73% lower in rats fed MUFA than in those fed PUFA, whereas in HDL, UI was higher in rats fed diets M and MF than in those fed diets P and PF. Whatever the class of lipoproteins, the intake of flavonoids did not substantially affect the distribution of fatty acids. However, in HDL of rats fed MUFA, supplementation had a reducing effect on the proportion of EPA and DHA (−50 and −13%, respectively, $P < 0.05$).

Liver microsomes were affected mainly by dietary lipids as shown by the differences among groups in their OA and LA contents (**Table 6**). Like HDL, microsomes contained more AA in groups fed MUFA than in those fed PUFA. The proportion of LA correlated with the production of TBARS during iron-catalyzed oxidation ($r = 0.94$, $P < 0.0001$). The supplementation with flavonoids had little effect, but liver microsomes incorporated less (n-3) PUFA in group MF than in group M.

DISCUSSION

As expected, replacing dietary MUFA by PUFA reduced the plasma lipid concentrations. In rats, the response is due mainly to the intake of long-chain (n-3) PUFA. Indeed, these fatty acids reduce plasma triacylglycerols in humans (Harris et al. 1983) as well as in rats (Wong et al. 1984) and both LDL and HDL cholesterol in rats (Balasubramaniam et al. 1985). In general, the plasma vitamin E concentration strongly correlates with that of total lipids (Bjornson et al. 1976). Therefore, the relationship between α-tocopherol and either triacylglycerol or cholesterol levels could be expected. It is unknown whether dietary flavonoids primarily affected the concentration of α-tocopherol rather than that of cholesterol in rats fed MUFA.

The differences among groups in the resistance of VLDL + LDL to oxidative degradation was related mainly to the intake of PUFA and flavonoids. However, it has to be considered that diets rich in MUFA included a high proportion of olive oil, which contains substantial amounts of polyphenolic antioxidants (Carmena et al. 1996, Scaccini et al. 1992, Visioli et al. 1995, Wiseman et al. 1996). Lag time before the onset of lipid peroxidation is determined mainly by the antioxidant content of LDL (Esterbauer et al. 1989). As in humans receiving their habitual diet (Esterbauer et al. 1992, Kleinveld et al. 1992), we found no correlation of lag time with the LDL-α-tocopherol concentrations. In contrast, a positive correlation was found in studies using diets supplemented with relatively high amounts of α-tocopherol. In this case, LDL particles were enriched in α-tocopherol (Dieber-Rotheneder et al. 1991). Assuming that VLDL + LDL from the four dietary groups in

TABLE 5

Effects of flavonoid supplementation on the fatty acid composition of plasma lipoproteins in rats fed diets containing monounsaturated or polyunsaturated fatty acids[1,2]

	VLDL + LDL					*P*-value[3]		
Fatty acid	Diet M	Diet MF	Diet P	Diet PF	SEM	Lipid	Flav	L × F
	mol/100 mol							
16:0	22.44[a]	21.64[a]	11.05[c]	15.48[b]	1.34	0.0001	NS	0.06
18:0	5.28[a]	5.34[a]	2.65[b]	4.31[a]	0.40	0.0002	0.04	NS
18:1(n-9)	40.72[b]	44.15[a]	15.86[c]	13.54[d]	0.61	0.0001	NS	0.0001
18:1(n-7)	2.79[a]	2.97[a]	2.17[b]	2.08[b]	0.09	0.0001	NS	NS
18:2(n-6)	15.34[c]	13.34[c]	41.73[a]	39.13[b]	0.84	0.0001	0.01	NS
20:4(n-6)	8.47[c]	9.03[bc]	10.01[a]	9.79[ab]	0.32	0.002	NS	NS
20:5(n-3)	1.26[b]	0.66[b]	6.91[a]	6.53[a]	0.29	0.0001	NS	NS
22:6(n-3)	3.71[b]	2.88[b]	9.63[a]	9.14[a]	0.29	0.0001	0.03	NS
(n-6)/(n-3)	4.79[b]	6.33[a]	3.13[c]	3.12[c]	0.13	0.0001	0.0001	0.0001
(20:4)/(18:2)	0.55[b]	0.68[a]	0.24[c]	0.25[c]	0.02	0.0001	0.0008	0.005
UI[4]	4.98[c]	4.82[c]	18.70[a]	12.87[b]	1.81	0.0001	NS	NS
	HDL					*P*-value		
Fatty acid	Diet M	Diet MF	Diet P	Diet PF	SEM	Lipid	Flav	L × F
16:0	8.57[c]	10.87[b]	16.78[a]	15.71[a]	0.54	0.0001	NS	0.007
18:0	5.30[c]	7.47[b]	11.10[a]	11.27[a]	0.32	0.0001	0.002	0.008
18:1 (n-9)	10.61[a]	10.21[a]	3.37[b]	3.14[b]	0.27	0.0001	NS	NS
18:1(n-7)	2.09[a]	2.09[a]	1.27[b]	1.24[b]	0.04	0.0001	NS	NS
18:2(n-6)	11.57[b]	9.99[b]	18.95[a]	19.24[a]	0.52	0.0001	NS	NS
20:4(n-6)	55.23[a]	53.88[a]	40.92[b]	41.90[b]	0.60	0.0001	NS	NS
20:5(n-3)	0.80[b]	0.43[c]	2.17[a]	2.14[a]	0.08	0.0001	0.02	0.05
22:6(n-3)	5.83[a]	5.06[b]	5.44[ab]	5.40[ab]	0.15	NS	0.02	0.03
(n-6)/(n-3)	10.08[b]	11.63[a]	7.87[c]	8.12[c]	0.20	0.0001	0.0006	0.01
(20:4)/(18:2)	4.77[b]	5.39[a]	2.18[c]	2.19[c]	0.12	0.0001	0.02	0.03
UI[4]	21.85[a]	15.59[b]	8.98[c]	9.42[c]	0.95	0.0001	0.009	0.003

[1] Diets were rich in either monounsaturated fatty acids (M) or polyunsaturated fatty acids (P), not supplemented (diets M and P) or supplemented (diets MF and PF) with flavonoids (quercetin + catechin, 2:1).

[2] Values are means; $n = 6$ (pooled samples with 3 rats per pool). Within a defined plasma fraction (either VLDL + LDL or HDL) of a row, values with different letter superscripts differ ($P < 0.05$).

[3] Two-way ANOVA: Lipid, significant influence of dietary fat source; Flav, significant influence of flavonoid supplementation; L × F, interaction; NS, not significant ($P > 0.05$).

[4] UI, unsaturation index = sum ($a \times b$)/S where a is the relative molar percentage of each unsaturated fatty acid, b is the number of double bonds for that particular fatty acid and S is the molar percentage of total saturated fatty acids.

our study had the same average proportion of α-tocopherol, the differences among lag times depended mainly on the presence of other antioxidants, which were either associated with particles or solubilized in their aqueous environment. In this experiment, the VLDL + LDL fraction was not dialyzed after isolation. Hence, some hydrophilic endogenous antioxidants and a part of the ingested flavonoids could be solubilized in the preparation. This assumption is supported by the longer lag time in group MF than in group M. Apparently, dietary flavonoids had no effect on lag time in rats fed PUFA but a lengthening effect cannot be excluded. Indeed, lag time could not be accurately measured because the oxidation of PUFA began shortly after starting the assay.

The production of conjugated dienes, as well as that of TBARS depended mainly on the relative content of PUFA and MUFA in VLDL + LDL. Similar results were obtained in studies of rabbits (Parthasarathy et al. 1990) and humans (Abbey et al. 1993a, Reaven et al. 1991 and 1993). The fatty acid composition of VLDL + LDL also affected the rate of oxidation as seen in LDL from nonhuman primates (Thomas et al. 1994). Thus, even if the response to dietary flavonoids differed between dietary groups (longer lag time in MUFA-fed rats and lesser extent of oxidation in PUFA-fed rats), these substances could be effective with any type of dietary fat to protect VLDL + LDL against peroxidative degradation. Similarly, in humans fed their habitual diet, Fuhrman et al. (1995) observed that after daily consumption of red wine rich in polyphenols (vs. white wine poor in polyphenols), LDL were enriched in these substances and their propensity to undergo peroxidation induced by copper was reduced. They found a longer lag time, and the production of conjugated dienes, as well as that of TBARS, was lower.

We observed that liver microsomes also responded to dietary treatments. Murphy and Mavis (1981) showed that the susceptibility of microsomes to lipid peroxidation depended on their α-tocopherol content. The positive correlation between the α-tocopherol concentration and that of fatty acids containing three or more double bonds suggested to these authors that α-tocopherol had a greater affinity for these fatty acids than for the others present in microsomes. Our findings are not consistent with this hypothesis because α-tocopherol correlated with linoleic acid, which was more affected by dietary treatments than the more unsaturated fatty acids. The production of TBARS in microsomes depended mainly on their PUFA content, without reduction in supplemented groups. This does not mean that flavonoids were ineffective

TABLE 6

Effects of flavonoid supplementation on the fatty acid composition of liver microsomes in rats fed diets containing monounsaturated or polyunsaturated fatty acids[1,2]

						P-value[3]		
Fatty acid	Diet M	Diet MF	Diet P	Diet PF	SEM	Lipid	Flav	L × F
			mol/100 mol					
16:0	17.88[c]	18.50[b]	20.13[a]	17.68[c]	0.16	0.0001	0.0001	0.0001
18:0	16.87[b]	19.44[a]	17.63[b]	14.76[c]	0.38	0.0001	NS	0.0001
18:1(n-9)	11.07[b]	11.83[a]	3.59[c]	3.31[c]	0.12	0.0001	0.04	0.0001
18:1(n-7)	2.71[b]	2.73[a]	1.77[b]	1.82[b]	0.04	0.0001	NS	NS
18:2(n-6)	10.76[c]	8.74[d]	18.25[b]	20.35[a]	0.24	0.0001	NS	0.0001
20:4(n-6)	30.79[a]	30.58[a]	25.65[c]	28.08[b]	0.32	0.0001	0.001	0.0001
20:5(n-3)	0.59[c]	0.34[d]	1.95[b]	2.25[a]	0.06	0.0001	NS	0.0001
22:5(n-3)	0.62[b]	0.49[c]	1.03[a]	1.09[a]	0.02	0.0001	NS	0.0001
22:6(n-3)	8.70[c]	7.33[d]	10.02[b]	10.66[a]	0.16	0.0001	0.03	0.0001
(n-6)/(n-3)	4.22[b]	4.83[a]	3.39[c]	3.48[c]	0.08	0.0001	0.0001	0.001
(20:4)/(18:2)	2.86[b]	3.50[a]	1.41[c]	1.38[c]	0.04	0.0001	0.0001	0.0001
UI[4]	6.27[b]	5.34[d]	5.82[c]	7.44[a]	0.13	0.0001	0.01	0.0001

[1] Diets were rich in either monounsaturated fatty acids (M) or polyunsaturated fatty acids (P), not supplemented (diets M and P) or supplemented (diets MF and PF) with flavonoids (quercetin + catechin, 2:1).

[2] Values are means; $n = 18$ individual samples. Within a row, values with different letter superscripts differ ($P < 0.05$).

[3] Two-way ANOVA: Lipid, significant influence of dietary fat source; Flav, significant influence of flavonoid supplementation; L × F, interaction; NS, not significant ($P > 0.05$).

[4] UI, unsaturation index = sum ($a \times b$)/S where a is the relative molar percentage of each unsaturated fatty acid, b is the number of double bonds for that particular fatty acid and S is the molar percentage of total saturated fatty acids.

in protecting membranes. Indeed, the α-tocopherol contents of microsomes were higher in supplemented groups than in the corresponding nonsupplemented groups, suggesting that flavonoids spared α-tocopherol associated with microsomes. Moreover, an effect on the enzymatic antioxidant system of membranes cannot be excluded. It is possible that flavonoids, which have the capacity to bind to proteins (Manach et al. 1995), are associated with microsomes as well as with VLDL + LDL. The difference between microsomes and VLDL + LDL for oxidizability could be linked to their physical state. Indeed, membranes were obtained in the form of pellets, whereas lipoproteins were solubilized in a volume of plasma. Hence, soluble flavonoids that are not associated with proteins could be present in the preparation of lipoproteins but not in that of microsomes.

In this study, diets had comparable (n-6) to (n-3) fatty acid ratios and (n-3) PUFA were administered as fish oils. In the analyzed compartments, the AA to LA ratio was >100% higher in groups fed MUFA than in those fed PUFA. This was likely due to the inhibition of Δ 6-desaturation of LA by dietary DHA (Brenner and Peluffo 1967). As a result, lipoproteins as well as microsomes of groups fed MUFA had a high AA content, which almost reached or exceeded that of groups fed PUFA. The data obtained in rats fed two types of dietary fats commonly consumed by humans provide useful information by showing that dietary flavonoids decreased the susceptibility of VLDL + LDL to copper-induced peroxidation. In conclusion, our findings suggest that supplementation with dietary flavonoids is not only beneficial when diets are rich in PUFA, but also when they are rich in MUFA. These substances might protect circulating and membrane lipids by sparing vitamin E and endogenous antioxidants.

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