Walnut-enriched diet increases the association of LDL from hypercholesterolemic men with human HepG2 cells

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Abstract In a randomized, cross-over feeding trial involving 10 men with polygenic hypercholesterolemia, a control, Mediterranean-type cholesterol-lowering diet, and a diet of similar composition in which walnuts replaced ~35% of energy from unsaturated fat, were given for 6 weeks each. Compared with the control diet, the walnut diet reduced serum total and LDL cholesterol by 4.2% ($P = 0.176$), and 6.0% ($P = 0.087$), respectively. No changes were observed in HDL cholesterol, triglycerides, and apolipoprotein A-I levels or in the relative proportion of protein, triglycerides, phospholipids, and cholesteryl esters in LDL particles. The apolipoprotein B level declined in parallel with LDL cholesterol (6.0% reduction). Whole LDL, particularly the triglyceride fraction, was enriched in polyunsaturated fatty acids from walnuts (linoleic and α-linolenic acids). In comparison with LDL obtained during the control diet, LDL obtained during the walnut diet showed a 50% increase in association rates to the LDL receptor in human hepatoma HepG2 cells. LDL uptake by HepG2 cells was correlated with α-linolenic acid content of the triglyceride plus cholesteryl ester fractions of LDL particles ($r^2 = 0.42$, $P < 0.05$). Changes in the quantity and quality of LDL lipid fatty acids after a walnut-enriched diet facilitate receptor-mediated LDL clearance and may contribute to the cholesterol-lowering effect of walnut consumption.—Muñoz, S., M. Merlos, D. Zambón, C. Rodríguez, J. Sabaté, E. Ros, and J. C. Laguna. **Walnut-enriched diet increases the association of LDL from hypercholesterolemic men with human HepG2 cells.** *J. Lipid Res.* 2001. **42: 2069–2076.**

Supplementary key words fatty acids • LDL receptor • polygenic hypercholesterolemia

There is ample evidence that blood cholesterol levels are reduced by dietary substitution of unsaturated fatty acids from vegetable oils and fats and fish products for saturated fatty acids (SFA) from animal fat (1), and this effect can be predicted by the amounts of fatty acid classes exchanged (1–4). Hence, current guidelines for reducing the risk of cardiovascular disease by dietary and other lifestyle practices emphasize replacement of foods with cholesterol-raising fatty acids by nutrients with a high unsaturated fatty acid content in conjunction with an energy intake suitable for maintaining a normal body weight (5, 6). Among foods with favorable fatty acid profiles, nuts have received particular attention because of the epidemiological association of their frequent intake with protection from coronary heart disease (7–10). Feeding studies that have incorporated different nuts into test diets have shown a consistent cholesterol-lowering effect of regular nut consumption, with an actual response greater than that predicted from the changes in dietary fatty acids (11). Thus the mechanisms underlying plasma cholesterol reduction due to nut consumption remain speculative.

Of the tree nuts, walnuts are unique because they are a rich source of the PUFA 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) (12). Controlled dietary intervention trials also have shown that daily consumption of walnuts has a cholesterol-lowering effect greater than expected from predictive models that include regression coefficients for percentage energy changes in dietary fatty acids (13, 14). In our randomized cross-over trial in hypercholesterolemic men and women (14), the replacement of ~35% of energy from unsaturated fatty acids with walnuts, without changing total fat or SFA intake, reduced serum LDL cholesterol by an average of 0.29 mM [95% confidence interval (CI), −0.41 to −0.15 mM] [−11.2 mg/dl (−16.3 to −6.1 mg/dl)] compared with the control diet, whereas the decrease predicted by the equations based on percentage energy changes in dietary fatty acids ranged from 0.13 to 0.18 mM (5 to 7 mg/dl) (1–4).

We wondered whether LDL compositional changes

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produced by the walnut diet, particularly LDL enrichment with PUFA from walnuts, might increase LDL uptake by liver cells, thus reducing the number of circulating LDL particles and contributing to the observed cholesterol-lowering effect. To test this hypothesis, LDL were isolated for association studies at the end of the control diet and the walnut diet periods in a subset of 10 men successfully completing the trial (14). LDL association with cultured human hepatoma cells (HepG2) was used as a functional test to identify differences in the biological properties of LDL from the two dietary periods. The human cell line HepG2 is recognized as a good surrogate for adult hepatocytes, as it is able to reproduce most hepatocytic metabolic functions (15), in particular the clearance of LDL through functional LDL receptors (16, 17). LDL association with the LDL receptor has been satisfactorily measured by using the fluorescent probe 3,3′-dioctadecylindocarbocyanine (DiI), which is incorporated into the lipoprotein particle in the phospholipid fraction (18, 19). The results suggest that LDL incorporation of PUFA from walnuts increases the rate of association of these particles with the LDL receptor, thus providing a potential explanation for the cholesterol-lowering effect of the walnut diet.

MATERIALS AND METHODS

Patients

Ten men with polygenic hypercholesterolemia, who were enrolled in a dietary intervention trial comparing the lipid effects of a diet enriched with walnuts with those of a control Mediterranean diet, were studied (14). The participants were nonsmokers and had no evidence of alcohol abuse or liver, kidney, or endocrine disorders; any lipid-lowering medication was withdrawn 8 weeks before study. None of them had prior coronary heart disease or were taking other medications known to affect plasma lipid levels. Their ages ranged from 48 to 71 years, the body mass index ranged between 23.1 and 30.0 kg/m^2, the systolic blood pressure ranged between 120 and 150 mm Hg, and the diastolic blood pressure ranged between 65 and 90 mm Hg.

Experimental design

The study protocol has been described in detail (14). Essentially, it was a randomized, cross-over trial comparing the effects of two cholesterol-lowering diets on blood lipids, LDL fatty acid composition, and LDL resistance to oxidation. Dietary intervention consisted of two consecutive 6-week diet periods after a 4-week preinclusion period. The diet periods were randomly assigned: one group followed the walnut diet during the first period and then switched to the control diet during the second period, while the other group followed the diets in reverse order. The investigators performing laboratory analyses were blinded with respect to the subjects' diet sequence. The study protocol was approved by the institutional review board of the Hospital Clínic of Barcelona, and all subjects gave informed consent.

Diets

The composition of the two experimental cholesterol-lowering diets has been detailed previously (14). Participants ate on their own, and prepackaged daily allowances of raw, shelled walnuts were provided for daily consumption in amounts varying from 41 to 56 g (the equivalent of 8 to 11 walnuts) during the walnut diet. The control diet was Mediterranean, composed of natural foodstuffs and with olive oil as the main culinary fat. The walnut diet was similar to the control diet, but walnuts replaced approximately 35% of the total energy provided by olive oil and other sources of monounsaturated fatty acids (MUFA). The composition of the two experimental diets was nearly identical with respect to total fat and SFA, protein, carbohydrate, cholesterol, and fiber content, but their unsaturated fat content was dissimilar. The fatty acid composition of the control diet reflected the high MUFA content of olive oil, whereas that of the walnut diet mirrored the constitution of walnut fat, particularly rich in PUFA (**Table 1**). Adherence to the study diets was carefully monitored, as described (14).

Blood sampling and analysis

Blood samples were obtained after an overnight fast twice during the last 2 weeks of each dietary period. Both serum and 0.1% EDTA plasma were collected. The LDL fraction was isolated by sequential ultracentrifugation immediately after separation of plasma. Serum lipid and lipoprotein lipid analyses were performed as described (14). LDL lipid separation and fatty acid analysis were performed by thin-layer chromatography and subsequent gas chromatography of the fatty acid methyl esters (20). Lipoprotein oxidation in the presence of 5 μM $CuSO_4 \cdot 5H_2O$

TABLE 1.   Composition of prescribed and actually observed study diets

Variable	Control Diet		Walnut Diet		P Value between Actual Diets
	Prescribed	Actual[a]	Prescribed	Actual[a]	
Energy, kcal/day	1,600–2,200	1,899 ± 102	1,600–2,200	1,942 ± 178	>0.2
Fat, % energy	30.2	30.9 ± 0.7	32.7	31.8 ± 0.6	0.009
Saturated fatty acids	5.8	6.0 ± 0.5	5.0	5.5 ± 0.6	0.058
Monounsaturated fatty acids	17.6	17.5 ± 0.7	13.2	12.9 ± 0.5	<0.001
Polyunsaturated fatty acids	4.2	3.9 ± 0.4	11.8	11.2 ± 0.3	<0.001
Linoleic (C18:2n-6)	3.3	3.1 ± 0.4	9.6	9.3 ± 0.6	<0.001
α-Linolenic (C18:3n-3)	0.4	0.3 ± 0.1	1.9	1.8 ± 0.1	<0.001
Protein, % energy	18.1	19.1 ± 1.2	16.6	18.8 ± 1.0	>0.2
Carbohydrates, % energy	51.7	48.5 ± 1.4	50.7	48.8 ± 1.6	>0.2
Cholesterol, mg/1,000 kcal	103.9	117.2 ± 22.7	77.8	86.1 ± 14.5	0.002
Soluble fiber, g/1,000 kcal	4.8	3.7 ± 0.4	5.0	3.9 ± 0.4	>0.2
α-Tocopherol, mg/1,000 kcal	5.8	5.2 ± 0.4	5.1	4.4 ± 0.4	<0.001
Vitamin C, mg/1,000 kcal	76.2	87.3 ± 14.9	76.3	80.7 ± 17.6	>0.2

[a] Mean ± SD values estimated from one 3-day food record and six 24-h diet recalls during each diet period.

was determined by measuring conjugated diene kinetics, as described previously (21).

LDL labeling

The LDL were labeled with the fluorescent probe DiI (Molecular Probes, Eugene, OR) by a modification of the method described by Stephan and Yurachek (22). Briefly, a stock solution of DiI was prepared by dissolving 30 mg of DiI in 1 ml of dimethyl sulfoxide, and an appropriate volume was added to the LDL solution, adjusted to 1 mg of LDL protein per ml to yield a final concentration of 150 µg of DiI per mg of LDL protein. The mixture was protected from light and incubated at 37°C for 18 h to provide DiI-labeled LDL (DiI-LDL), which was subsequently layered over with an NaBr solution (density, 1.1 g/ml) and reisolated by ultracentrifugation (100,000 g, 18 h, 20°C), dialyzed (Spectrapor MWCO 3500; Serva, Heidelberg, Germany) against PBS (18 h, 4°C), and sterilized by filtration (Nalgene polyether sulfone filter, 0.22 µm; Nalge Nunc International, Naperville, IL). The final protein concentration was determined by the Bradford method (23). The DiI-LDL was stored at 4°C in the dark and used within 7 days.

DiI and DiI-LDL standard curve

A standard solution of DiI was prepared in isopropanol at a concentration range of 0 to 200 ng/ml and its fluorescence was measured in a Hitachi F2000 fluorometer with excitation and emission wavelengths set at 522 and 564 nm, respectively. Standard solutions of DiI-LDL samples with a concentration range of 100 to 1,000 ng of protein per ml were prepared from a stock solution of 10 µg of LDL protein per ml in isopropanol and their fluorescence was determined as described above. The specific activity of the DiI-LDL samples was calculated as the amount of DiI (micrograms) incorporated into 1 mg of LDL protein.

Cell culture and association assay

Human hepatoma (HepG2) cells were obtained from the European Collection of Cell Culture (Salisbury, Wiltshire, UK). The cells were routinely cultured in DMEM containing 10% fetal bovine serum, penicillin G (100 IU/ml; Sigma, Madrid, Spain), and streptomycin (74.7 IU/ml; Sigma), and maintained at 37°C in equilibration with 5% CO_2-95% air in 10-mm plates. For experiments, HepG2 cells (2.5×10^5 cells/well) were seeded into six-well plates in DMEM supplemented with 10% fetal bovine serum, grown for 72 h, and then switched to DMEM containing 0.5% fatty acid-free bovine serum albumin for an additional 24 h. Afterward, the cells were incubated with 30 µg of DiI-LDL protein per ml for 0, 15, 20, 60, 90, and 120 min at 37°C. At this temperature, both binding and internalization of DiI-LDL particles were measured (15). At the end of each incubation period, the cells were extensively washed with PBS, and 2 ml of isopropanol was added to each well, with gentle shaking of the plates for 15 min. The isopropanol extracts were then transferred to 10 × 25 mm glass tubes and centrifuged at 3,500 g for 10 min, and the fluorescence was determined as described above. DiI content in the extracts was calculated from the DiI standard curve. The cells were dissolved in 2 N NaOH and left overnight at 4°C for protein determination. Taking into account the specific activity for each DiI-LDL sample, the rate of association of the DiI-LDL with the HepG2 cells was obtained from the slope of the adjusted straight line obtained by representing the total amount of DiI-LDL associated with the cells versus time and expressed as micrograms of LDL protein per minute per milligram cellular protein, as described elsewhere (22). The association rate was linear throughout the interval of time studied (**Fig. 1A**). Saturation was obtained at concentrations of DiI-LDL above 80 µg of LDL protein per ml of incubation medium (Fig. 1B). Scatchard

A

B
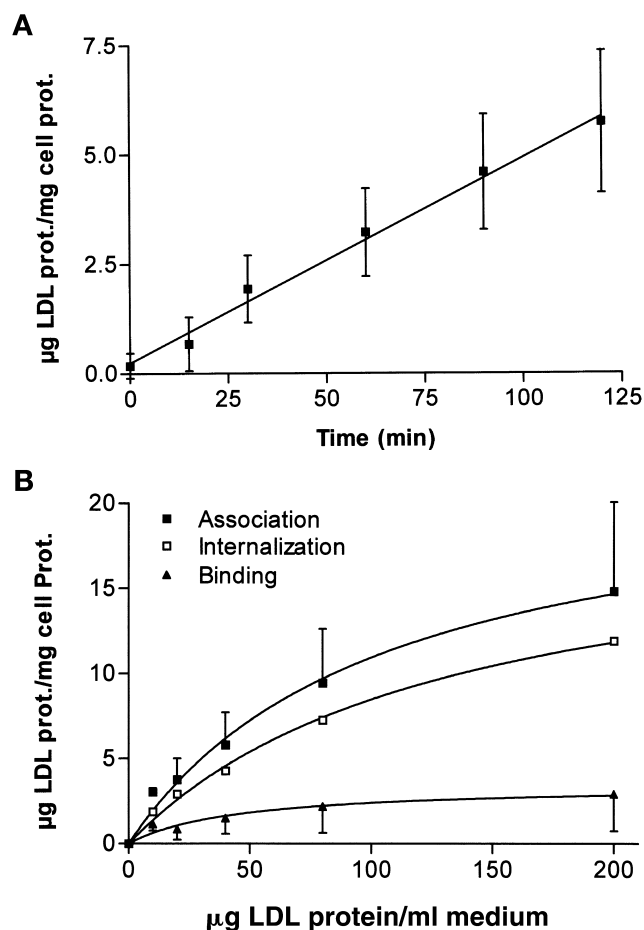
Fig. 1. A: Time dependence of the rate of association of DiI-LDL with HepG2 cells. LDL concentration was fixed at 30 µg of LDL protein per ml of culture medium. Each point is the mean of three different assays; in each assay, two cell preparations for every time point were determined and averaged. B: Dependence of the rate of association (at 37°C) and binding (at 4°C) of DiI-LDL to HepG2 cells with respect to the concentration of LDL protein added to the culture medium. The incubation time was fixed at 2 h. Internalization was calculated as the difference, for each LDL concentration point, of the corresponding mean values for internalization and binding. Each point is the mean of three different assays; in each assay, two cell preparations for every LDL concentration point were determined and averaged.

plots gave a linear representation (bound LDL/free LDL = −0.0096 bound LDL + 0.2132, B_{max} = 22 µg of LDL protein per mg of HepG2 cell protein), pointing to a single high affinity binding site for LDL on the surface of HepG2 cells.

To assess the sensitivity of the technique to changes in LDL receptor activity, cells were incubated in DMEM supplemented with 10% fetal bovine serum in the presence of 1 µM lovastatin for 18 h. Cells were then switched to DMEM containing 0.5% fatty acid-free bovine serum albumin for an additional 24 h, again in the presence of 1 µM lovastatin. Lovastatin produced a 19% increase in LDL association (2 h), using pooled LDL (0.064 ± 0.001 vs. 0.054 ± 0.003 µg of LDL protein per min per mg cell protein for lovastatin-treated and control cells, respectively; $P < 0.01$), which is in accordance with previously published data using this assay (22).

Statistical analysis

For each subject, the results of all measurements made in duplicate at the end of each dietary period were averaged. Descrip-

TABLE 2. Serum lipid and lipoprotein levels at the end of each diet period[a]

Patient	Age	Total Cholesterol		Triglycerides		LDL Cholesterol		HDL Cholesterol		VLDL Cholesterol		ApoB	
		CD	WD	CD	WD	CD	WD	CD	WD	CD	WD	CD	WD
1	52	6.93	5.97	1.67	1.56	4.85	4.12	1.32	1.12	0.71	0.62	169	153
2	56	8.15	7.75	1.94	1.90	5.97	5.50	1.28	1.01	1.05	1.29	209	203
3	71	5.60	5.95	1.52	1.59	3.69	3.98	1.23	1.23	0.84	0.89	140	135
4	63	8.16	7.89	1.86	2.03	6.14	5.84	1.16	1.12	1.28	1.02	218	187
5	48	6.23	5.61	1.31	1.32	4.27	3.85	1.36	1.16	0.59	0.57	148	118
6	72	6.43	6.14	1.48	1.10	4.19	3.98	1.56	1.64	0.61	0.44	150	156
7	64	6.71	5.40	1.05	0.67	4.67	3.48	1.56	1.62	0.48	0.17	150	121
8	56	6.79	6.87	1.16	0.91	4.91	4.69	1.34	1.76	0.58	0.45	156	148
9	55	6.71	6.58	1.50	1.46	4.94	4.89	1.09	1.02	0.63	0.67	168	178
10	54	5.59	6.34	1.27	1.47	4.03	4.47	0.97	1.19	0.56	0.62	148	156
Mean	59.1	6.73	6.45	1.48	1.40	4.77	4.48	1.29	1.29	0.73	0.67	165.3	155.3
SD	8.0	0.88	0.84	0.29	0.42	0.80	0.75	0.19	0.28	0.25	0.32	26.9	27.6
Difference, CD vs. WD (%)		−4.2		−5.1		−6.0		0		−8.0		−6.0	
P value[b]		0.176		>0.2		0.087		>0.2		>0.2		0.075	

[a] CD, control diet; WD, walnut diet. Values are expressed as mM, except for apoB (mg/dl). To convert values for total, LDL, VLDL, and HDL cholesterol to mg/dl, multiply by 38.67. To convert values for triglycerides to mg/dl, multiply by 88.57.

[b] Paired t-test, two tailed.

tive values are expressed as means ± SD. Statistical analyses included two-tailed paired t-tests for the comparison of changes in outcome variables in response to dietary treatment. Differences with $P < 0.05$ were considered significant. Diet period and carryover effects for the two-period cross-over design were analyzed according to Fleiss (24). Correlation analysis between variables was performed by linear regression, using standard software (GraphPad Prism, San Diego, CA).

RESULTS

The nutrient content of the self-reported diets was well matched to that of the planned diets (Table 1). As expected, there were significant differences in unsaturated fatty acid intake between the two diets, reflecting the different fatty acid composition of the main fatty foods exchanged (olive oil and walnuts). Other nutrients had differences that were small but statistically significant. This can be explained by the use of calorie-adjusted nutrient values and the participant's close adherence to the prescribed diets, resulting in small standard deviations. Body weight was stable throughout the two dietary periods: 74.4 ± 7.0 kg after the control diet and 74.5 ± 7.4 kg after the walnut diet ($P > 0.2$). Serum lipid, lipoprotein cholesterol, and apolipoprotein B values while the subjects were on the control diet or the walnut diet are shown in **Table 2**.

Total cholesterol, LDL cholesterol, and apolipoprotein B levels decreased in parallel in all but two participants (patients 3 and 10 in Table 2) during the walnut diet, whereas no changes in HDL cholesterol and VLDL cholesterol levels were observed. There was no evidence of a carryover effect. The resistance of LDL to oxidation, measured as the lag time of conjugated diene production to an in vitro 5 μM Cu^{2+} challenge, was similar during the control diet and the walnut diet (39.7 ± 6.2 vs. 36.7 ± 5.0 min, respectively, $P > 0.2$; **Table 3**). Essentially, lipoprotein changes and LDL oxidizability reported here do not differ from those observed in the main study (14).

The relative proportions of the different constituents of LDL particles did not differ significantly with the two diets, although there was a trend to a decrease in phospholipid content following the walnut diet (data not shown). Notably, the cholesterol:protein ratio in LDL was essentially the same with the two diets (2.20 ± 0.24 vs. 2.25 ± 0.54 for the control and walnut diet, respectively). The analysis of fatty acids from LDL lipids during the two periods confirmed that participants had closely adhered to the prescribed diets. As expected from the high PUFA content of walnuts, the walnut diet resulted in an enrichment in linoleic acid and α-linolenic acid, with reciprocal changes in oleic acid in the whole particle (**Table 4** and **Table 5**) and in each of the three lipid

TABLE 3. LDL oxidation-related analytes (n = 6)

	CD	WD	% Change WD vs. CD	P
A_0 (absorbance units)	0.375 ± 0.030	0.333 ± 0.031	−10.3 ± 12.9	0.106
C_{max} (nmol CDS/mg LDL protein)	731.9 ± 63.3	776.2 ± 38.3	6.5 ± 7.5	0.099
V_{max} (nmol CDS/min per mg LDL protein)	29.7 ± 3.4	40.7 ± 6.3	38.5 ± 25.4	0.012
Lag time (min)	39.7 ± 6.2	36.7 ± 5.0	−6.5 ± 14.2	0.247

CD, control diet; WD, walnut diet; CDS, conjugated dienes.

TABLE 4. Quantitative fatty acid composition in whole LDL[a]

Patient	Palmitic (16:0) CD	WD	Stearic (18:0) CD	WD	Oleic (18:1n-9) CD	WD	Linoleic (18:2n-6) CD	WD	γ-Linolenic (18:3n-3) CD	WD	α-Linolenic (18:3n-3) CD	WD	Arachidonic (20:4n-6) CD	WD	Eicosapentaenoic (20:5n-3) CD	WD	Docosahexaenoic (22:6n-3) CD	WD
1	273.5	176.8	90.6	54.4	294.3	156.5	430.0	347.1	77.6	109.9	7.27	9.05	136.5	68.2	23.9	11.1	46.3	16.0
2	235.4	172.7	63.7	83.7	203.9	118.4	393.2	350.4	71.1	75.2	5.81	9.32	103.2	87.3	8.9	7.8	28.3	28.9
3	209.6	183.6	59.8	78.3	254.7	178.7	383.6	437.5	100.7	88.5	5.06	9.14	69.8	58.5	9.6	6.5	20.8	22.2
4	265.1	166.0	69.2	42.7	293.8	193.0	329.2	365.5	69.1	41.9	4.70	11.19	85.1	64.4	32.0	12.4	30.6	16.6
5	298.6	244.3	73.3	103.9	336.4	186.8	422.1	491.9	116.4	81.2	3.96	6.40	92.0	93.1	14.2	12.3	20.8	34.9
6	157.0	210.8	54.1	65.9	173.8	168.7	252.1	440.0	72.9	73.3	2.56	5.57	65.5	80.3	12.4	10.9	20.9	19.8
7	261.7	149.3	65.8	52.9	236.9	101.9	470.3	295.4	98.5	86.2	5.13	4.78	123.5	66.1	21.5	7.8	17.4	16.3
8	156.9	239.9	54.8	77.1	149.0	201.0	343.6	559.3	63.9	149.4	1.63	10.97	66.4	58.7	16.7	22.7	27.0	21.3
9	158.5	240.8	50.8	83.5	147.3	204.3	240.0	509.6	68.2	110.2	2.63	9.19	67.5	89.3	18.8	11.3	26.9	26.6
10	204.2	246.5	62.5	96.1	188.9	172.5	372.9	413.9	124.4	104.9	4.14	9.11	75.8	109.2	11.9	15.1	20.3	31.4
Mean	222.1	203.1	64.5	73.8	227.9	168.2	363.7	421.0	86.3	92.1	4.29	8.47	88.5	77.5	17.0	11.8	25.9	23.4
SD	52.8	37.5	11.5	19.6	65.9	34.1	74.4	83.0	21.9	28.7	1.69	2.17	25.3	17.0	7.2	4.6	8.4	6.7
Difference, CD vs. WD (%)		−9.0		11.4		−26.2		15.8		10.7		97.5		−12.0		−31.0		−10.0
P value[b]		>0.2		>0.2		0.020		>0.2		>0.2		<0.001		>0.2		0.072		>0.2

[a] Values are expressed as micrograms of fatty acid per milligram of LDL protein.

[b] Paired t-test, two tailed.

fractions (triglycerides, phospholipids, and cholesteryl esters) present in LDL (**Table 6**). Therefore, the relative proportion of PUFA increased whereas that of MUFA was reduced after the walnut diet, thus increasing the unsaturation index of the whole particle measured as PUFA/SFA + MUFA. The incorporation of fatty acids from walnuts into the different lipid fractions of LDL was not homogeneous. The triglyceride fraction was more enriched in linoleic acid and, especially, α-linolenic acid than the cholesteryl ester and phospholipid fractions. Interestingly, the walnut diet produced a significant decrease (14%, $P < 0.01$) in the unsaturation index of LDL surface components (phospholipids) in relation to the unsaturation index of LDL core lipids (triglycerides and cholesteryl esters) (Table 6).

Cellular assays demonstrated a significant ($P < 0.05$) 50% increase in the binding to HepG2 cell receptors of LDL obtained during the walnut diet compared with LDL harvested from the same patients during the control diet (**Fig. 2**). Considering only the eight individual patients who showed a reduction in LDL cholesterol during the walnut diet, the increase in the association rate was even higher, reaching 72% (0.023 ± 0.009 vs. 0.037 ± 0.014 μg of LDL protein per min per mg cell protein for the control diet and the walnut diet, respectively).

Correlation analyses among the different variables showed that part of the decrease in LDL cholesterol levels with the walnut diet could be attributed to the increase in LDL binding to receptors ($r^2 = 0.31$; $P = 0.097$). Moreover, LDL binding was correlated to α-linolenic acid en-

TABLE 5. Quantitative fatty acid composition in whole LDL[a]

Patient	SFA CD	WD	MUFA CD	WD	PUFA CD	WD	n-6 CD	WD	n-3 CD	WD
1	373.4	235.1	327.8	174.7	759.0	587.4	670.6	542.0	83.9	39.1
2	304.0	261.6	225.2	129.7	643.7	582.2	591.2	528.9	50.6	50.7
3	273.2	266.5	274.8	192.1	621.2	647.1	583.3	604.6	37.7	40.4
4	342.4	215.6	323.9	211.8	595.7	532.9	517.5	488.2	72.8	42.3
5	381.2	355.4	373.8	204.1	721.9	744.6	676.6	684.7	43.5	58.9
6	216.7	282.8	190.5	193.9	459.6	655.0	413.7	613.5	39.9	41.5
7	333.7	205.0	262.0	112.7	770.8	498.7	720.0	465.4	48.3	31.7
8	213.2	320.1	158.8	216.5	540.1	849.5	490.2	791.9	49.3	57.6
9	215.1	331.2	165.8	222.0	445.6	797.1	390.5	738.3	53.6	56.2
10	271.5	348.2	207.4	191.1	633.2	718.2	593.0	655.2	39.1	60.9
Mean	292.4	282.2	251.0	184.9	619.1	661.3	564.7	611.3	51.9	47.9
SD	64.6	54.6	73.8	36.5	113.7	115.0	110.9	107.6	15.2	10.2
Difference, CD vs. WD (%)		−3.5		−26.3		6.8		8.3		−7.6
P value[b]		>0.2		0.037		>0.2		>0.2		>0.2

SFA, saturated fatty acids (14:0 + 16:0 + 18:0 + 20:0); MUFA, monounsaturated fatty acids (16:1 + 18:1 + 20:1); PUFA, polyunsaturated fatty acids (18:2n-6 + 18:3n-6 + 18:3n-3 + 20:3n-6 + 20:3n-9 + 20:4n-6 + 20:5n-3 + 22:4n-6 + 22:5n-3 + 22:6n-3).

[a] Values are expressed as micrograms of fatty acid per milligram LDL protein.

[b] Paired t-test, two tailed.

TABLE 6. Changes in percent molar fatty acid composition and unsaturation index of triglycerides, phospholipids, and cholesteryl esters in LDL particles[a]

Fatty Acid	Lipid Fraction	Control Diet	Walnut Diet	Percent Change	P^b
Oleic acid, C18:1n-9	TG	35.3 (4.1)	28.8 (3.8)	−18	<0.001
	PL	11.7 (1.6)	9.4 (1.0)	−19	<0.001
	CE	19.1 (2.2)	14.5 (2.2)	−24	<0.001
Linoleic acid, C18:2n-6	TG	15.5 (4.4)	23.4 (2.9)	51	<0.001
	PL	19.6 (2.0)	24.5 (1.7)	25	<0.001
	CE	43.9 (3.7)	50.8 (2.7)	16	<0.001
α-Linolenic acid, C18:3n-3	TG	0.69 (0.26)	1.93 (0.76)	181	<0.001
	PL	0.20 (0.11)	0.27 (0.09)	34	>0.2
	CE	0.40 (0.13)	0.79 (0.24)	96	<0.001
Unsaturation index	TG	0.45 (0.10)	0.68 (0.14)	49	<0.001
	PL	0.71 (0.06)	0.84 (0.08)	19	<0.001
	CE	1.74 (0.26)	2.31 (0.27)	33	<0.001
	Whole LDL	1.18 (0.20)	1.43 (011)	21	<0.001
Unsaturation index surface: unsaturation index core		0.33 (0.04)	0.28 (0.05)	−14	<0.01

[a] Values are means (SD) of 10 patients. Unsaturation index obtained from the ratio PUFA/MUFA + SFA; unsaturation index surface:unsaturation index core ratio obtained from the ratio unsaturation index phospholipids: unsaturation index triglycerides + cholesteryl esters.

[b] Paired t test, two tailed.

richment in LDL core lipids (triglycerides plus cholesteryl esters) ($r^2 = 0.41$; $P < 0.05$).

DISCUSSION

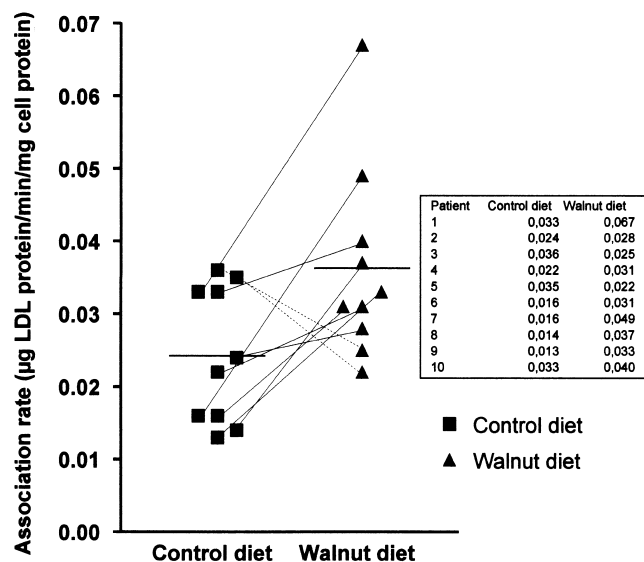In a subgroup of 10 hypercholesterolemic men participating in a larger dietary intervention trial, the inclusion

Fig. 2. Rate of association of DiI-labeled LDL obtained during the control and walnut diet periods to cultured HepG2 cells (n = 10). Experimental conditions are described in Materials and Methods. Horizontal lines correspond to the mean association rate for each dietary period: control, 0.024 ± 0.009 µg of LDL protein per min per mg cell protein; walnut, 0.036 ± 0.013 µg of LDL protein per min per mg cell protein ($P < 0.05$). Individual values for the rate of association of DiI-labeled LDL (expressed as micrograms of LDL protein per minute per milligram of cell protein) are shown in the inset.

of walnuts (about 50 g/day) in a Mediterranean-type hypocholesterolemic diet produced an additional decrease in serum cholesterol and LDL cholesterol. Although in this subset of patients the decrease in LDL cholesterol with the walnut diet in comparison with the control diet did not reach statistical significance (−6%, $P = 0.09$), the effect was similar in magnitude to that observed in the main study involving 49 patients (−5.9%; $P < 0.001$). This is attributable to the low statistical power of the sample in the present study. The decrease in plasma lipids was associated with a proportional decrease in serum apolipoprotein B, suggesting that the observed effect was due to a reduction of apolipoprotein B-rich circulating particles.

A reduction of serum and LDL cholesterol has been consistently found in studies using diets supplemented with nuts (11). In the present study we observed that the decrease in LDL cholesterol levels after the walnut diet was accompanied by an increased association of LDL with the receptors present in human hepatoma cells (HepG2). The increase in LDL association explained approximately 30% of the LDL cholesterol decrease, suggesting the presence of other factors accounting for the hypocholesterolemic effects of the walnut diet. Nevertheless, to our knowledge this is the first time that the relative contribution of plasma LDL association with hepatic LDL receptors has been quantified and correlated with changes in the composition of LDL fatty acids.

It is well known that the quality of ingested fats modifies the composition and physicochemical properties of cellular membranes, leading to changes in the microenvironment of proteins, such as receptors or enzymes, penetrating the asymmetric lipid bilayer (25). The ingestion of SFA increases blood cholesterol by reducing the activity of hepatic LDL receptors (26, 27). Conversely, the hypocholesterolemic activity of n-6 PUFA such as linoleic acid has been attributed to enhanced LDL uptake and degrada-

tion due to facilitated LDL receptor activity (28, 29), and to increased activity of the hepatic scavenger receptor BI or HDL receptor (30). Increased cellular degradation of LDL, however, may be due not only to increased affinity of the receptor for plasma LDL because PUFA in surrounding membrane phospholipids facilitate expression of receptor-binding sites, but also to changes in the affinity of circulating LDL particles for the receptor. Thus, it has been reported that LDL uptake by HepG2 cells is increased in triglyceride-rich LDL particles (31). Conversely, triglyceride depletion of LDL by fish oil treatment in hypertriglyceridemic patients results in lower binding to fibroblasts (32). The lipid composition data in our study and the relative constancy of lipid-to-protein ratios during the two dietary periods indicate that the increased binding to HepG2 cells of LDL formed during the walnut diet probably does not result from an increase in particle size. However, important differences were apparent in the fatty acid composition of the esterified lipid components (Tables 4 and 5). The overall results from compositional analyses of LDL obtained during the two dietary periods suggest that enrichment of the particles with the fatty acids predominating in each diet was an important determinant of their affinity for LDL receptors in HepG2 cells. The short incubation time (2 h) used in the association experiments is insufficient to modify substantially the fatty acid composition of membrane phospholipids present in HepG2 cells (33). Thus, under our experimental conditions, changes in the affinity of LDL particles for the LDL receptor may be ascribed to changes in LDL composition rather than to modifications of the receptor microenvironment.

Fatty acids incorporated into LDL triglycerides were previously identified as the most sensitive marker of participant's adherence to the prescribed diets (14). The enrichment in PUFA, particularly α-linolenic acid, was localized mainly in the triglyceride-rich core of the LDL particle, whereas the phospholipid-rich surface was much less affected. Interestingly, there was a significant correlation ($r^2 = 0.42$, $P < 0.05$) between the increase in α-linolenic acid in the core (triglycerides plus cholesteryl esters) of LDL isolated from patients during the walnut diet, related to the LDL obtained from the same patients during the control diet, and the increase in the rate of association of the corresponding LDL to HepG2 cells. Thus, LDL-core enrichment in this quantitatively minor fatty acid, by changing the shape of the lipoprotein, may be in part responsible for increased LDL clearance and reduction of circulating LDL particles during the walnut diet. Of note, the conformation of apolipoprotein B in LDL can be altered depending on the curvature radius of the particle (34, 35), thus affecting the exposure of epitopes responsible for recognition by the LDL receptor. High dietary intake of α-linolenic acid has been associated with reduced risk of coronary artery disease in both epidemiological studies (36–38) and a landmark clinical trial, the Lyon Diet Heart Study (39, 40). Reduced coronary risk could be attributed to improvement of the serum lipid profile, but the recognized antiarrhythmic and antithrombotic properties of α-linolenic acid may also contribute to this beneficial effect (41).

Epidemiological and clinical studies suggest that frequent nut consumption may be protective against coronary artery disease (7–10), a beneficial effect that may be attributed, at least in part, to improvement of the serum lipid profile. In clinical trials, the cholesterol-lowering effect of regular nut consumption is greater than that predicted from changes in common dietary fatty acids in the tested diets (11). In the present study we describe the changes in lipoprotein composition as well as the heterogeneous incorporation of the PUFA provided by walnuts into the different lipid fractions present in LDL following a controlled hypocholesterolemic diet enriched with walnuts. Moreover, we put forward the importance of changes in LDL fatty acid composition in the clearance process by the LDL receptor, suggesting an additional mechanism that may contribute to the reduction of LDL cholesterol levels. Nevertheless, it should be mentioned that other events, including changes in plasma membrane fatty acid composition, impairment of LDL synthesis, and hypolipidemic effects derived from nonlipid constituents present in nuts, may also occur and contribute to the overall cholesterol-lowering effect of regular walnut consumption.

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