Improvement in the Antioxidant Status of Plasma and Low-Density Lipoprotein in Subjects Receiving a Red Wine Phenolics Mixture

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ABSTRACT: It is commonly accepted that oxidized low-density lipoprotein (ox-LDL) plays an important role in coronary heart disease (CHD) and etiologically related atherogenesis. Consumption of wine may contribute to the low risk of CHD in the Mediterranean population. These findings raise the question of the *in vivo* antioxidant role of wine phenolic compounds after a prolonged supplementation period in healthy human volunteers. We found that subjects, receiving 2 g/d of an alcohol-free red wine-extracted phenolic compound (RWPC) mixture for 14 d (which was equivalent to about 1 L/d of the red wine), exhibited an increase in the plasma antioxidative capacity and in LDL vitamin E by blood sampling under fasting conditions. The fact that the LDL Cu^{2+}-oxidizability was not decreased can be explained by both the lack of phenolic compound affinity for the lipoprotein particle, highlighted by LDL dialysis, and the insufficient increase in LDL vitamin E, as shown by the relationship between vitamin E content and oxidation resistance of LDL evidenced by literature data. These results support that RWPC could play a coantioxidant role, similar to that of vitamin C, possibly accounting for their LDL vitamin E sparing effect and their beneficial role in lowering CHD risks.

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KEY WORDS: Antioxidant status, dietary supplementation, human subjects, low-density lipoprotein oxidizability, plasma antioxidant capacity, red wine phenolic compounds, vitamin E.

Whereas some weaknesses have appeared in the "Lipid Hypothesis" of atherosclerosis (1), there is increasing evidence to support the idea that oxidized low-density lipoprotein (ox-LDL) plays an important role in atherogenesis (2–9). A corollary of the "Oxidative Theory" of atherosclerosis would be that antioxidants prevent atherogenic processes. This has been suggested by an epidemiological approach concerning vitamin E (10), polyphenols (11), or quercetin (12). Decreased oxidizability of LDL in case of LDL incubation in the presence of vitamin E (13) or various phenolics (14–17), but also in case of *in vivo* (dietary) vitamin E supplementation (18–23), is also in favor of this preventing effect. Moreover, recent studies suggest that people who are fed a rather well-defined diet, usually called "Mediterranean diet" (24), statistically show low incidence of coronary heart disease (CHD) (25). It is postulated that French people are protected against CHD risk by the larger amounts of wine (particularly red wine) they drink as compared to Northern European or American people. Conflicting results concerning oxidation resistance of LDL were previously found by supplementation with an integrated red or white wine (26–28) or a red wine with a low alcohol content (29). The aim of the present work was to examine whether the alcohol-free, red wine-extracted phenolic compounds (RWPC) can be effective in preventing oxidative modification of total plasma and/or LDL in healthy human volunteers. To do so, a prolonged period of ingestion was chosen to reasonably ensure that an effect can be observed.

METHODS

Chemicals. The phenolic compounds from a French red wine (Cabernet-Sauvignon grape variety) were prepared and analyzed by I.N.R.A. (Institut National de la Recherche Agronomique, the Narbonne Center, France). Briefly, this preparation involved adsorption of wine phenolic materials on an ADS-4 preparative column (a stationary phase from Applexion, Epone, France), desorption by means of a hydroalcoholic solution (ethanol/water 46:4, vol/vol), and gentle evaporation. The concentrated residue was finally spray-dried to obtain the RWPC powder (see composition in Table 1) given to volunteers in capsule form. One liter of red wine produced 1.3 g of RWPC.

Subjects. Twenty normocholesterolemic men (20–45 yr old) took part in this study. They were nonsmokers and without pharmacological treatment. For each subject, the sequential experiment was as follows: (i) a deprivation period of 10 d (d10 to d1): vitamins E and C, alcohol, wine, fruit juices,

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TABLE 1
Composition of the Red Wine Phenolic Compounds (expressed as mg/g of RWPC)

Catechins + proanthocyanidins[a]		Anthocyanins		Favonols		Phenolic acids	
Total	(±) Catechin	Total	Malvidin-3-glucoside	Total	Quercetin + quercetin-3-glucoside	Total	Caffeoyl-tartaric acid
100	2.0	64	23	5.0	4.2	8.7	1.7

[a]Expressed as (±) catechin; RWPC, red wine-extracted phenolic compound.

coffee, tea, and cola beverages were not allowed to be consumed; (ii) a supplementation period of 14 d (d0 to d13): the dietary restrictions were maintained and the subjects received daily six capsules containing 0.33 g of RWPC (two at each of the three meals). The supplementation was equivalent to the consumption of approximately 1 L of wine daily. The study was approved by the Montpellier University Ethical Committee, and all subjects gave informed consent.

Plasma biological characteristics and chemiluminescence assay for plasma antioxidant capacity (Pl-AOC). Blood samples were drawn on EDTA by venipuncture after an overnight fast at d0 and d14, and plasma was recovered by centrifugation at $1000 \times g$. Apoproteins B and A1 were determined by means of a Turbitimer apparatus with the appropriate method (Behring, Rueil-Malmaison, France). The concentration of LDL was assimilated to that of apo B. Cholesterol, triglycerides, and phospholipids were measured by automated enzymatic methods proposed by Bio-Merieux (Marcy-l'Etoile, France). Pl-AOC was determined by means of a chemiluminescence reaction (30,31) with a mixture of luminol, hydrogen peroxide, and horseradish peroxidase (HRP). Light emission is suppressed in the presence of antioxidative substances that act as radical scavengers and is restored when antioxidants are consumed (32). The time of light suppression—indicative of the antioxidant content of the medium—was measured with a 1251 Luminometer (LKB, Paris, France). Pl-AOC was calculated by determining the time needed to reach 50% of the maximal light emission in the presence of plasma (t_{pl}), and this time was compared to the trolox concentration needed for obtaining the same time. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble tocopherol analog from Aldrich Chemicals (Strasbourg, France), commonly used as an antioxidant standard.

Biological characteristics and oxidizability of LDL. Briefly, LDL (lipoprotein fraction at $1.019 \text{ g/mL} < d < 1.063$ g/mL) was isolated by sequential ultracentrifugation in a TFT 65-13 rotor (Kontron Instruments SA, Montigny Le Bretonneux, France) for 4 h per run at high speed ($300{,}000 \times g$), then dialyzed against deoxygenated phosphate-buffered saline (PBS: 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) that contained 10 μM diethylenetriamine pentaacetic acid (DTPA). LDL vitamin E was assessed by a high-performance liquid chromatography (HPLC) method and electrochemical detection (33). Other biological characteristics were analyzed as for plasma. After lipid extraction of LDL, fatty acids were transesterified and analyzed according to the usual laboratory procedure (34). LDL oxidizability was assessed immediately after its ultracentrifugation and dialysis as indicated above by measuring the conjugated dienes produced during the 5 μM Cu^{2+}-mediated oxidation of 0.1 μM LDL, obtained by adequate dilution (generally 1/20) in oxygenated PBS without DTPA. The incubation was carried out at 37°C under magnetic stirring (35). To investigate the participation of phenolic compounds possibly associated to LDL in the lipoprotein antioxidant protection, 1 μM LDL was preincubated in the presence of various concentrations of RWPC, submitted to Cu^{2+}-mediated oxidation before or after extensive dialysis in 5 μM DTPA-containing PBS, and then diluted 10 times in PBS without DTPA. The final DTPA concentration of the incubation medium used for oxidizability assessment was always within the range of 0.25–0.50 μM, which has previously been verified to be without influence on diene production.

Statistics. Results (mean ± SEM) were statistically analyzed by using a Wilcoxon nonparametric test (StatView™, Alsyd, Meylan, France) .

RESULTS

As shown in Table 2, there was no modification in the plasma biological characteristics, whereas a significant increase in Pl-AOC was found after RWPC supplementation (170.0 ± 10.4 vs. 156.4 ± 10.4 μM trolox; $P = 0.01$).

RWPC supplementation did not affect cholesterol, triglyceride, phospholipid, and fatty acid contents of LDL (Table 3 and Fig. 1A). However, there was a significant increase in LDL vitamin E (the vitamin E/apo B molar ratio was 8.63 ±

TABLE 2
Effect of Red Wine Phenolic Compound Supplementation for Two Weeks on the Plasma of Healthy Subjects[a]

	Biological characteristics		
	d0	d14	*P*
T Chol (mM)	4.04 ± 0.18	4.23 ± 0.28	0.2
TG (mM)	0.67 ± 0.07	0.66 ± 0.06	0.4
PL (mM)	2.17 ± 0.08	2.11 ± 0.07	0.2
Apo B (g/L)	0.78 ± 0.04	0.74 ± 0.03	0.2
Apo A1 (g/L)	1.06 ± 0.04	1.04 ± 0.04	0.2
	Antioxidant capacity		
	d0	d14	*P*
Pl-AOC (μM trolox)	156.4 ± 10.4	170.0 ± 10.4	0.01*

[a]Results are X ± SEM; TChol, total cholesterol; TG, triglycerides; PL, phospholipids; Apo B, apoprotein B; Apo A1, aproprotein A1; Pl-AOC, plasma antioxidant capacity; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; $^*P < 0.05$, $n = 11$.

TABLE 3
Effect of Red Wine Phenolic Compound Supplementation for Two Weeks upon LDL of Healthy Subjects[a]

	Biological characteristics (mol/mol of apo B)		
	d0	d14	*P*
T Chol	2497.7 ± 153.5	2569.0 ± 134.8	0.2
TG	534.7 ± 37.5	544.2 ± 60.9	0.4
PL	786.1 ± 59.7	856.6 ± 47.3	0.2
Vit E	8.63 ± 0.67	9.98 ± 0.49	0.01*
	Oxidative susceptibility		
	d0	d14	*P*
T lag (min)	65.4 ± 4.1	61.4 ± 2.9	0.08
Oxidation rate[b]	10.5 ± 0.6	10.4 ± 0.4	0.4

[a]Results are X ± SEM; LDL, low-density lipoprotein; for other abbreviations see Table 2.
[b]Oxidation rate is expressed as mol of diene/mol of apo B/min; *$P < 0.05$, $n = 20$.

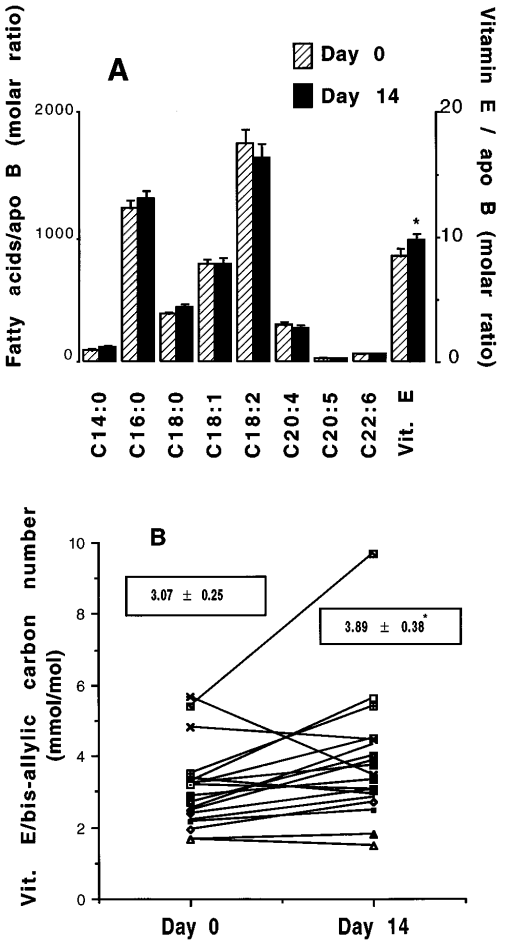

FIG. 1. Effect of red wine phenolic compound supplementation on low-density lipoprotein (LDL) composition. (A) Variation of the main fatty acids and vitamin E; all values were reported to apo B and expressed as a molar ratio. (B) Variation of vitamin E to bis-allylic carbon number molar ratio, resulting from the polyunsaturated fatty acid composition. LDL (lipoprotein fraction at 1.019 g/mL < *d* < 1.063 g/mL) was isolated before (day 0) and after (day 14) red wine-extracted phenolic compound supplementation. Mean ± SEM. *$P < 0.05$.

0.67 and 9.98 ± 0.49 before and after the supplementation period, respectively, $P = 0.01$; see Fig. 1A), leading to an increment factor of 1.16 ± 0.06 after the supplementation period. Figure 1B shows that the vitamin E/bis-allylic carbon number ratio was significantly higher after supplementation (3.89 ± 0.38 vs. 3.07 ± 0.25 mmol/mol; $P = 0.006$). Table 3 also shows that there was no significant change in the lag time of conjugated diene production (65.4 ± 4.1 and 61.4 ± 2.9 min, $P = 0.08$), indicating that LDL oxidizability was unchanged. Neither was the oxidation rate modified (10.5 ± 0.6 and 10.4 ± 0.4 mol of diene/mol of apo B/min, $P = 0.45$)

We have purposely reported the literature data with respect to the effect of various dietary vitamin E supplementations upon the induced protection of LDL against oxidation (Fig. 2). Such a meta-analysis led to a significant relationship ($r = 0.88$; $P < 0.01$) between the increment of LDL vitamin E and that of the lag time of conjugated diene production (Fig. 3). In the present study, however, such a relationship was not observed (Fig. 3; $r = 0.09$).

RWPC-incubated LDL without subsequent dialysis was used to assess *in-vitro* protection against Cu^{2+}-mediated oxidation.The lag time of conjugated diene production was highly correlated with the RWPC concentration in the incubation medium ($r = 0.98$; $P < 0.001$; Table 4) with a regression coefficient of 12.2%/mg/L. However, subsequent dialysis of LDL led to total suppression of the correlation and loss of any influence of RWPC on the lag time.

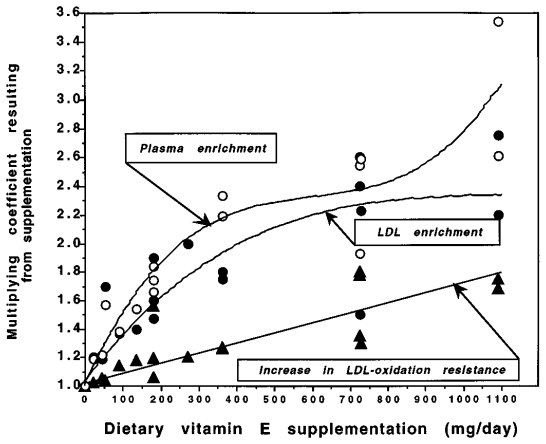

FIG. 2. Effect of dietary vitamin E supplementation on plasma and LDL vitamin E contents and LDL resistance to Cu^{2+}-mediated oxidation (measured as the lag time of conjugated diene production) — literature data. On the vertical axis are plotted the increments (the multiplying coefficient) in the plasma vitamin E, LDL vitamin E, and LDL resistance to oxidation obtained after the period of supplementation. Amounts of vitamin E used for supplementation were recalculated in mg without taking into account the scale of biological activity of the α-tocopherol stereoisomers because of their similar antioxidant properties. The equations were as follows: for plasma vitamin E, $y = 1.0 + 5.9 \times 10^{-3}x - 9.3 \times 10^{-6}x^2 + 5.1 \times 10^{-9}x^3$, $r = 0.97$, $P < 0.01$; for LDL vitamin E, $y = 1.0 + 3.5 \times 10^{-3}x - 3.2 \times 10^{-6}x^2 + 1.0 \times 10^{-9}x^3$, $r = 0.94$, $P < 0.01$; for oxidation resistance of LDL, $y = 1.0 + 7.0 \times 10^{-4}x$, $r = 0.90$, $P < 0.01$. See Figure 1 for abbreviation.

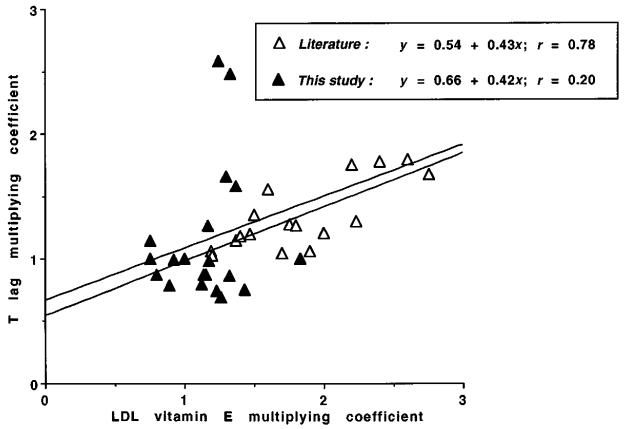

FIG. 3. Effect of the LDL vitamin E enrichment on LDL resistance to Cu^{2+}-mediated oxidation—comparison of data of the present study to those reported in the literature. In this study, LDL (0.1 μM) was incubated with 5 μM Cu^{2+} (as $CuCl_2$) in air-saturated phosphate-buffered saline at 37°C under magnetic stirring. The change in absorbance was monitored at 234 nm (as expression of the conjugated diene production). The lag time increment and LDL–vitamin E enrichment were not significantly correlated ($r = 0.2$, $P > 0.05$). Comparison can be made with the data reported in the literature (see Fig. 2). In this case, the correlation was highly significant ($r = 0.78$, $P < 0.01$). When both of the two types of data were plotted together (correlation line not shown), we obtained a significant correlation ($r = 0.41$, $P = 0.01$). See Figure 1 for abbreviation. For abbreviation see Figure 1.

TABLE 4
Effect of Coincubation of LDL and RWPC upon the Lag Time of Conjugated Diene Production Resulting from Cu^{2+}-Mediated Oxidation of LDL: Without or After Extensive Dialysis. Assessment of LDL Oxidizability

RWPC concentration (mg/L)	Lag time (%)[a] (means ± SEM)	
	Without dialysis[b]	After dialysis
0	100	100
1	105.2 ± 12.0	120.4 ± 41.8
2.5	118.3 ± 32.9	94.1 ± 22.5
5	153.2 ± 32.7	92.4 ± 13.8
10	207.6 ± 44.8	94.0 ± 9.7
15	223.9 ± 36.5	106.2 ± 12.0
20	325.9 ± 31.4	103.7 ± 1.4

[a]Values are reported to the basis of 100 in the absence of RWPC.
[b]The lag time was significantly correlated with RWPC-incubation concentration: ($y = 95.4 + 10.6x$; $r = 0.98$). See Tables 1 and 3 for abbreviations.

DISCUSSION

It can clearly be concluded from the present results (Table 1) that the Pl-AOC was significantly increased by a daily ingestion for 2 wk of an alcohol-free RWPC dry extract. The daily amount ingested was equivalent to consumption of 1 L of wine. Similar results have already been obtained in postprandial conditions and after ingestion of wine (32,36–38).

The present results, however, are of particular interest because of the fasting conditions under which they have been obtained. They provide evidence for an effect of the phenolic compounds beyond the postprandial period and draw our attention to the retention of their antioxidant effect in plasma. This may be in favor of an efficient role in the hemovascular compartment, specifically in the *in vivo* protection of LDL against oxidation.

However, this increased antioxidant capacity of plasma appears not to be due to an increase in oxidation resistance of LDL itself, as shown by the unchanged lag time of LDL oxidation, in spite of an increase in the vitamin E content of LDL and a decrease in the bis-allylic carbon number (the highly oxidizable carbons of the fatty acid chains). The lack of effect of the increased vitamin E content of LDL appears not to be surprising if we compare the present results to those reported in the literature (18–23 and Fig. 2). Figure 2 shows the effect of vitamin E supplementation on the vitamin E enrichment of LDL and the parallel increase in the oxidation resistance of LDL. It clearly appears that a 16% vitamin E enrichment corresponds to a consumption of about 30–40 mg/day of vitamin E and leads to a nonsignificant increase in the LDL resistance. This is also shown in Figure 3 by plotting the lag times vs. the vitamin E enrichment factor obtained in the present study or from literature data.

In-vitro incubation of LDL in the presence of RWPC was performed to show that subsequent dialysis of the lipoprotein particles leads to the release of phenolics, illustrating that these compounds are loosely associated with LDL, probably at the surface of the LDL particles as can be supported from their hydrophilic properties. This location supports a role of phenolics similar to that already described for vitamin C (39,40) and a protecting (a sparing) role of phenolics toward vitamin E. Such a role could explain the significant LDL increase in vitamin E due to RWPC dietary supplementation we presently observed.

Although RWPC and/or the active components derived from RWPC appear(s) to be largely unknown, *in vivo* the increase in the antioxidant capacity of plasma during the fasting period may provide indirect evidence that antioxidant materials supplied by ingestion of RWPC dry extract are absorbed at the level of the intestinal barrier and enter the plasma in the absence of alcohol. It remains plausible that RWPC intake modifies the redox status of the hemovascular compartment in a way that leads (according to the oxidative theory of atherosclerosis) to a decrease in the risk of cardiovascular disease .

In any case, the *ex vivo* part of this study represents the first step of a more exhaustive approach, which will consist of examining, in humans, the appearance/disappearance of phenolic compounds and antioxidative capacity in plasma after administration of different varieties of wine (currently explored in our laboratory).

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