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Prevention of LDL α -tocopherol consumption, cholesterol oxidation, and vascular endothelium dysfunction by polyphenolic compounds from red wine

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Abstract

Red wine polyphenolic compounds (RWPCs) have been demonstrated to possess antioxidant properties, and several studies have suggested that they might constitute a relevant dietary factor in the protection from coronary heart disease. The aim of the present study was to determine further the mechanism by which RWPCs can prevent the formation of vasoactive compounds in oxidized LDL. RWPCs were obtained from the Cabernet-Sauvignon grape variety. Human LDL was oxidized in the presence of CuSO_4 (ox-LDL). Vascular reactivity studies were conducted on rabbit aortic rings. RWPCs significantly reduced the formation of 7β -hydroxycholesterol and 7-ketocholesterol and in a lower extent the emergence of lysophosphatidylcholine in ox-LDL. The ability of RWPCs to prevent cholesterol oxide formation was directly dependent on the LDL α -tocopherol content. Once the LDL α -tocopherol has been consumed, RWPCs were no longer effective, indicating that RWPCs act by sparing endogenous α -tocopherol. As a consequence of the preservation of the endogenous α -tocopherol content of LDL, RWPCs could prevent the inhibition of the acetylcholine-mediated endothelium-dependent relaxation of rabbit aorta which was linked to a direct effect on NO release. Independently of a treatment with ox-LDL, RWPC exerted a concentration-dependent and persistent inhibitory effect on the norepinephrine-induced contraction of rabbit aorta. In conclusion, RWPCs can preserve a normal vascular reactivity by acting at different stages of the cascade that leads to lipid oxidation, endothelium dysfunction and vasospasm. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Antioxidant; Lipoprotein; Vasodilation; Vasoconstriction; Endothelium

1. Introduction

A number of studies indicated that oxidized low density lipoproteins (ox-LDL) can impair endothelium-dependent arterial relaxation at an early, still reversible stage of the atherosclerotic process [1,2]. The detailed analysis of lipid derivatives in oxidized LDL revealed that two types of compounds, lysophosphatidylcholine (LPC) and cholesterol oxides, might account for the abnormalities of the vascular tone [3–5]. Cholesterol oxides are stable end-products of the lipid oxidation cascade, and detectable amounts were re-

ported in plasma, arterial wall or food products (for review [6]). In particular, cholesterol oxides were found to be abnormally elevated in hypercholesterolemic plasma [7] and atherosclerotic aortas [8,9], and increased plasma levels of 7β -hydroxycholesterol were recently associated with an increased risk of atherosclerosis in humans [10,11]. In vitro, cholesterol oxides can disturb several aspects of cholesterol homeostasis (including cholesterol biosynthesis, esterification, transfer and efflux) (for review [6]), and they have toxic effects on the three major cell types of the artery wall [12,13]. With regard to endothelium function and vascular tone, cholesterol derivatives oxidized in position 7 (7β -hydroxycholesterol and 7-ketocholesterol) were shown to mimic the inhibitory effect of ox-LDL on the endothelium-dependent arterial relaxation [4]. In fact, recent

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studies in human umbilical vein endothelial cells (HUVEC) revealed that 7β -hydroxycholesterol and 7-ketocholesterol can reduce the histamine-activated release of nitric oxide [14], i.e. the main endothelium-derived relaxing factor.

A number of studies reported the beneficial role of various antioxidants in preventing LDL oxidation and associated vascular damages. The prevention of atherosclerosis by antioxidant therapy was observed in several experimental animal models [15], and some studies revealed an inverse correlation between dietary intake of antioxidant vitamins (vitamin E and vitamin C) and the risk of coronary artery disease in humans (reviewed in [16,17]). In addition to vitamin E, vitamin C and β -carotene, the most common and active antioxidant compounds naturally occurring in food are flavonoids [18–25]. Flavonoids from red wine were shown to prevent the oxidation of isolated LDL [18–25], and the physiological relevance of *in vitro* observations was demonstrated by dietary interventions in healthy human subjects [26–30]. In particular, moderate consumption of red wine results in a significant decrease in the oxidability of LDL as measured either with total plasma or isolated LDL samples [26–30].

Whereas red wine polyphenolic compounds (RWPCs) are known to prevent or delay the formation of conjugated dienes, lipoperoxides, and thiobarbituric acid reactive substances (TBARS) [18,20,24,25,29–31], their effect on the production of cholesterol oxides in LDL, as well as the consequences in terms of vascular reactivity had never been evaluated. In the first part of the present study, the effect of RWPCs on the formation of cholesterol oxides and LPC in isolated human LDL was investigated in conjunction with the consumption of α -tocopherol, the main lipophilic antioxidant in native LDL [32,33]. In a second part of the study, the effect of a RWPC pretreatment of oxidized LDL on their ability to inhibit the endothelium-dependent relaxation was determined on isolated aortic rings. Finally, in the last part of the study, the direct effect of RWPC treatment on the response of isolated aorta to vasoconstrictor and vasorelaxing agents was investigated in the absence of oxidized LDL or lipid derivative supplementations. Part of this work was presented as a poster at the XIIth International Symposium on Atherosclerosis (Stockholm, Sweden, June 2000).

2. Materials and methods

2.1. Drugs

Acetylcholine (ACh), *N*^o-nitro-L-arginine (LNA), indomethacin (INDO), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), (\pm)-arterenol hydrochloride (NE), epicoprostanol, phosphatidyl-

N,N-dimethylethanolamine-dipalmitoyl, α -tocopherol-acetate and ascorbic acid were purchased from Sigma Chemical Co (St Louis, MO). The red wine polyphenolic compounds (RWPCs), obtained from the Cabernet-Sauvignon grape variety, were provided as a dry powder by Dr. M. Moutonnet (Institut National de la Recherche Agronomique, Montpellier, France). As previously reported by Andriambelason and coworkers [34], the procedure for RWPC preparation involved three steps: (i) adsorption of phenolics on a preparative column; (ii) alcoholic desorption; and (iii) gentle evaporation of the alcoholic-eluent. One liter of red wine produced 1.3 g of RWPC dry powder which contained 100 mg/g of total catechins plus proanthocyanidins expressed as catechin (with only 1.0% of each monomeric form of catechin and epicatechin), 64 mg/g of total anthocyanins (including 36% of malvidin-3-glucoside), 5 mg/g of total flavonols and 8.7 mg/g of total phenolic acids (including 19.5% of caftaric acid) [34].

2.2. Isolation of LDL particles

Fresh citrated plasma from five normolipidemic donors was provided by the Centre de Transfusion Sanguine (Hôpital du Bocage, Dijon, France). Low-density lipoproteins (LDL) were isolated by sequential ultracentrifugation. Densities were adjusted by the addition of solid potassium bromide (KBr). The $1.019 < d < 1.063$ g/ml fraction was prepared in a 70-Ti rotor on an L7 ultracentrifuge (Beckman, Palo Alto, CA) with one 15-h, 45,000-rpm run at the lowest density and one 24-h, 45,000-rpm run at the highest density. Isolated LDL were dialyzed overnight against a Tris-buffered saline solution (TBS) (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4).

2.3. Oxidation of LDL

The oxidative modification of LDL was performed as described previously [4]. Briefly, freshly prepared LDL adjusted to 1.2 g protein/l in TBS were incubated for 24 h at 37 °C with a copper sulfate solution (final concentration, 5 μ mol/l). Oxidation was stopped by the addition of EDTA (final concentration, 200 μ mol/l) and BHT (final concentration, 20 μ mol/l). Control, native LDL adjusted to 1.2 g protein/l were supplemented with EDTA (final concentration, 200 μ mol/l) and BHT (final concentration, 20 μ mol/l), and they were maintained at 4°C. Finally, both oxidized and native LDL preparations were dialyzed overnight against Krebs' buffer with the following composition (in mmol/l): NaCl 119, KCl 4.7, KH_2PO_4 1.18, MgSO_4 1.17, CaCl_2 2.5, EDTA 0.027, glucose 11, and NaHCO_3 25. The ability of LDL to affect vascular reactivity was determined within 2 days after their preparation. For biochemical measurements, aliquots of LDL prepara-

tions were stored for a few weeks at $-80\text{ }^{\circ}\text{C}$ under nitrogen.

2.4. Quantitation of oxidation-derived compounds in LDL

Cholesterol oxides were analyzed by capillary gas chromatography as previously described [4,35]. Concentrations of cholesterol and cholesterol oxides were determined from the ratio of the peak area corresponding to one given molecule to the peak area corresponding to the internal standard (epicoprostanol).

LDL phospholipids were assayed by HPLC as previously described [4,36]. The lysophosphatidylcholine (LPC) contents were determined from the ratio of the peak area of the sample to the peak area of the internal standard (phosphatidyl-*N,N*-dimethylethanolamine-dipalmitoyl).

2.5. Quantitation of α -tocopherol in LDL

α -tocopherol was assayed by HPLC as described previously [4,37]. The α -tocopherol contents were determined from the ratio of the peak area of the sample to the peak area of the internal standard (α -tocopherol-acetate).

2.6. Determination of vascular reactivity

2.6.1. Preparation of blood vessels

Vascular reactivity studies were conducted on 3-mm rabbit aortic rings that were mounted in organ baths as previously described [4]. Briefly, the abdominal aorta from 3-kg, New Zealand White rabbits ($n = 18$) of either sex was rapidly removed and transferred into a Krebs' solution bubbled with 95% O_2 and 5% CO_2 . Aortic rings were suspended horizontally between two wire hooks in 20-ml jacketed organ baths containing oxygenated, $37\text{ }^{\circ}\text{C}$ Krebs' solution, and changes in isometric tension were continuously monitored [4]. In some experiments, vascular endothelium was removed by gently rubbing the lumen of the arteries [38]. The destruction of the vascular endothelium was considered to be effective when no more than 10% relaxation was observed after addition of $10\text{ }\mu\text{mol/l}$ of acetylcholine on NE precontracted aortic segments.

These experiments were performed under the framework of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.6.2. Effect of oxidized LDL on vascular reactivity

After a 30-min equilibration period, the aortic rings were precontracted with $0.3\text{ }\mu\text{mol/l}$ NE, a concentration giving 75% of the maximal contraction, and they were subsequently relaxed by cumulative addition of ACh in

the 1 nmol/l to 0.01 mmol/l concentration range. After washout and new equilibration period, aortic rings were incubated for 2 h with either native or oxidized LDL that were pretreated or not with antioxidants prior to oxidation. At the end of the 2-h incubation period, aortic segments were washed, precontracted with NE, and then progressively relaxed with cumulative doses of ACh as described above.

2.6.3. Vascular effects of RWPC

In order to assess the direct relaxing effect of RWPC, NE ($0.3\text{ }\mu\text{mol/l}$) precontracted aortic rings were relaxed by cumulative concentrations of RWPC in the 0.001 to 10 mg/l concentration range. The relaxing effect of RWPC was investigated on rabbit aorta in which endothelium was removed or not by rubbing the lumen of the artery. Arteries with functional endothelium were incubated in the absence or in the presence of *N*^onitro-L-arginine (LNA, $10\text{ }\mu\text{mol/l}$) or indomethacin (INDO, $10\text{ }\mu\text{mol/l}$) for 20 min prior to the NE precontraction.

In another series of experiments, the permanence of the effect of RWPC on vascular reactivity was evaluated by determining the contractility of rabbit aorta to NE. To this end, aortic rings were preincubated for 1 h at $37\text{ }^{\circ}\text{C}$ with or without RWPC (concentrations, 0, 1, 10 and 100 mg/l), and, after washout, concentration–response curves to NE were obtained. In all cases, the vascular effects of RWPC were determined on both intact and deendothelialized arterial rings.

In a last set of experiments, the effects of LNA ($10\text{ }\mu\text{mol/l}$) and INDO ($10\text{ }\mu\text{mol/l}$) on the RWPC-induced inhibition of NE contraction were tested. Rabbit aortas with functional endothelium were preincubated in the absence or in the presence of either LNA ($10\text{ }\mu\text{mol/l}$) or INDO ($10\text{ }\mu\text{mol/l}$) for 20 min. RWPC (10 mg/l) were subsequently added for 1 h or not. After washout, aortic rings were contracted with cumulative concentrations of NE.

2.6.4. Data analysis

The maximal effect (E_{max}) to either ACh, NE or RWPC in rabbit aortic rings were determined from experimental data. EC_{50} values, corresponding to the agonist concentration required to produce a half maximal relaxing effect, were calculated after fitting each curve according to a sigmoid equation of the form:

$$Y = P1 + P2/(1 + e^{P3(\log X - P4)})$$

in which Y is the agonist effect, X is the corresponding agonist concentration; $P1$, the lower plateau response; $P2$, the range between the lower and the maximal plateaus of the concentration–effect curve; $P3$, a negative curvature index indicating the slope independently of the range; and $P4$, $\log \text{EC}_{50}$ [38].

2.7. Other analyses

Proteins were assayed with the use of bicinchoninic acid reagent (Pierce) according to the method of Smith et al. [39]. The assay was performed on a COBAS-FARA centrifugal analyzer (Roche).

2.8. Statistical analysis

Data are expressed as mean \pm S.E. The statistical significance of differences between data means was determined by using either ANOVA, Mann–Whitney *U*-test, or Wilcoxon signed rank test, as appropriate. Coefficients of correlation were calculated by Spearman's rank correlation analysis.

3. Experimental results

3.1. Effect of red wine polyphenolic compounds (RWPCs) on the antioxidant and oxidation-derived lipid compounds in oxidized LDL

In a first attempt to evaluate the ability of RWPCs to inhibit the oxidation process in low density lipoproteins, cholesterol oxides and lysophosphatidylcholine (LPC) were quantitated in oxidized LDL that were pretreated or not with RWPCs. Table 1 shows the amounts of 7α -hydroxycholesterol, 7β -hydroxycholesterol, 7-ketocholesterol and LPC in native and oxidized LDL at the end of a 24-h oxidation in the presence of copper sulfate. As compared to native LDL that were maintained at 4 °C, oxidized LDL showed significantly higher cholesterol oxide and LPC contents (Table 1). RWPCs were able to significantly decrease the formation of oxidation-derived compounds, with a somewhat greater inhibitory effect on the formation of cholesterol oxides (At 20 and 40 mg/l RWPC concentrations: -60.5 ± 16.5 and $-90 \pm 3.5\%$ for 7β -hydroxycholesterol, and -58.3 ± 16.4 and -88.7 ± 3.6 for 7-ketocholesterol, respectively) as com-

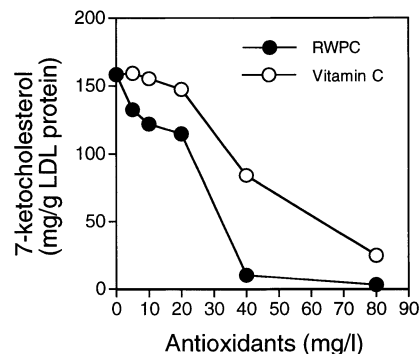


Fig. 1. Concentration-dependent effect of RWPC and vitamin C on the formation of 7-ketocholesterol in oxidized LDL. Briefly, LDL (1.2 g protein/l) were preincubated for 3 h in the presence of either RWPC (closed circles) or vitamin C (open circles), in the concentration range of 0–80 mg/l, prior to oxidation in the presence of CuSO_4 (5 $\mu\text{mol/l}$ for 24 h). 7-Ketocholesterol was assayed by capillary gas chromatography as described in Section 2. Each point corresponds to duplicate determination. Significance of the difference between 'RWPC' and 'vitamin C' curves: $P < 0.05$ by Wilcoxon signed rank test.

pared to LPC (At 20 and 40 mg/l RWPC concentrations: -37.8 ± 25 and $-65.6 \pm 16.1\%$, respectively) (Table 1). As shown in Fig. 1, both RWPCs and vitamin C were able to prevent the formation of cholesterol oxides in a concentration-dependent manner. All along the concentration range studied (5–80 mg/l), RWPCs were consistently more efficient than vitamin C in preventing the formation of 7-ketocholesterol ($P < 0.05$, $n = 5$; Wilcoxon signed rank test). Unlike for vitamin C, 7-ketocholesterol was no longer detectable in the presence of the 40 and 80 mg/l RWPC doses (Fig. 1).

In order to bring some insights into the mechanism by which RWPCs can prevent the formation of cholesterol derivatives in oxidized LDL, the disappearance of endogenous α -tocopherol and the emergence of cholesterol oxides were monitored over a 24-h oxidation period. As shown in Fig. 2, the incubation of control LDL in the presence of copper sulfate caused a rapid depletion of endogenous α -tocopherol that became no

Table 1
Effect of RWPC on the formation of oxidation-derived compounds in LDL particles

	mg/g of LDL protein			
	7α -OH	7β -OH	7-keto	LPC
Native LDL	1.18 ± 0.8	1.34 ± 0.86	1.65 ± 0.72	8.14 ± 2.59
ox-LDL	4.48 ± 1.94	$104.98 \pm 6.9^{***}$	$168.95 \pm 31.15^{***}$	$101.54 \pm 24.88^*$
ox-LDL (RWPC 20 mg/l)	3.87 ± 1.91	$42.54 \pm 18.05^{**}, \ddagger$	$73.64 \pm 26.22^*, \dagger$	72.95 ± 32.18
ox-LDL (RWPC 40 mg/l)	2.67 ± 1.16	$10.74 \pm 3.76, \ddagger$	$20.20 \pm 7.27, \ddagger$	41.38 ± 21.16

LDL particles were either maintained at 4 °C (native LDL) or preincubated for 3 h at 37 °C without addition (ox-LDL) or with RWPC (20 or 40 mg/l) before oxidation. Cholesterol oxides (7α -hydroxycholesterol (7α -OH), 7β -hydroxycholesterol (7β -OH), 7-ketocholesterol (7-keto)) and lysophosphatidylcholine (LPC) were assayed by capillary gas chromatography and HPLC, respectively, as described in Section 2. Values correspond to mean \pm S.E. of five LDL preparations.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. native LDL.

$\dagger P < 0.01$, and $\ddagger P < 0.001$ vs. ox-LDL.

more detectable for incubation times exceeding 2 h (Fig. 2). Concomitantly, whereas neither 7 β -hydroxycholesterol, nor 7-ketocholesterol were detectable within a few hours of incubation, a progressive and substantial rise in cholesterol oxides was observed beyond 4 h of oxidation (Fig. 2). The addition of RWPCs to the incubation medium was able to markedly delay both the disappearance of α -tocopherol and the rise in cholesterol oxides in a concentration-dependent manner. With the highest RWPC dose (40 mg/l), significant α -tocopherol levels were still detected after 24 h of incubation, and no

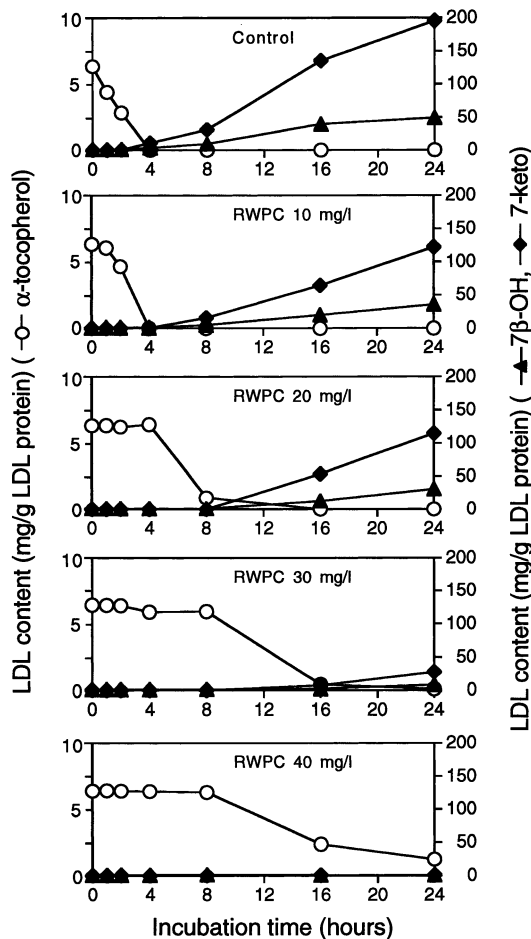


Fig. 2. Concentration-dependent effect of RWPC on the formation of cholesterol oxides (7 β -hydroxycholesterol (closed triangles), 7-ketocholesterol (closed diamonds)) and the disappearance of endogenous α -tocopherol (open circles) in LDL over a 24-h oxidation period. LDL (1.2 g protein/l) were preincubated for 3 h in the presence of RWPC (concentration range, 10–40 mg/l) before oxidation (CuSO₄; 5 μ mol/l for 24 h). Cholesterol oxides and α -tocopherol contents of LDL were determined before and after 0, 1, 2, 4, 8, 16 and 24-h of oxidation. Each point corresponds to duplicate determination. Significance of the difference in vitamin E from homologous 'control' curves: 'RWPC, 10 mg/l', NS; 'RWPC, 20 mg/l', NS; 'RWPC, 30 mg/l', $P < 0.05$; 'RWPC, 40 mg/l', $P < 0.05$. Significance of the difference in 7 β -hydroxycholesterol from homologous 'control' curves: $P < 0.05$ for 10, 20, 30 and 40 mg/l RWPC doses. Significance of the difference in 7-ketocholesterol from homologous 'control' curves: $P < 0.05$ for 10, 20, 30 and 40 mg/l RWPC doses (Wilcoxon signed rank test).

cholesterol derivatives were formed. In contrast, when RWPCs were added at a lower concentration (ranging from 10 to 30 mg/l), α -tocopherol became no more detectable at a given stage along the 24-h incubation period. It is noteworthy that in all cases the total disappearance of endogenous α -tocopherol was a prerequisite to the formation of cholesterol oxides that became detectable when all the α -tocopherol has been lost (Fig. 2).

In order to determine further whether RWPC could exert its effect through the maintenance of the antioxidant potential of LDL, the ability of RWPCs to block the ongoing oxidation process was investigated. To this end, RWPCs (20 mg/l) were added either before ($t = 0$) or after (from 5 to 30 min) the initiation of the 24-h oxidation period. As shown in Fig. 3 (upper panel), α -tocopherol was no more detectable in control LDL after 25 min of incubation in the presence of copper sulfate

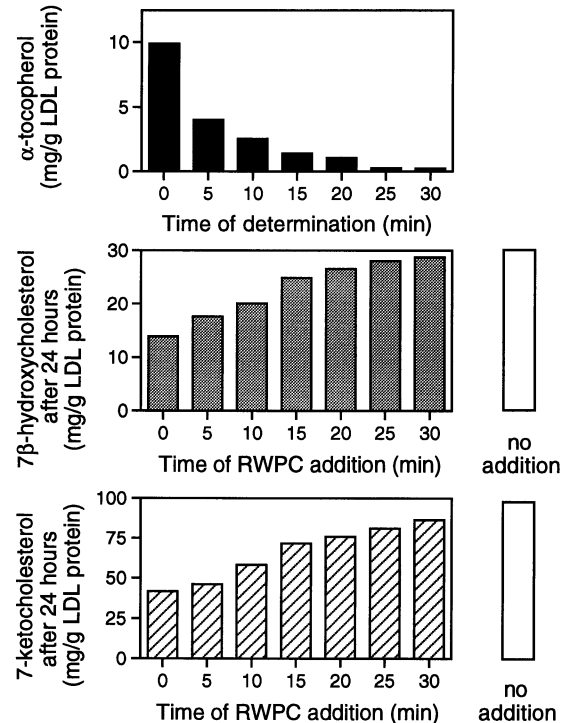


Fig. 3. Determination of the ability of RWPC to block the ongoing oxidation process. The α -tocopherol content of LDL non-supplemented with RWPC (upper panel) was determined over a 30-min period following the initiation of the oxidation process. RWPCs (20 mg/l) were added to LDL (1.2 g protein/l) either before ($t = 0$) or after ($t = 5$ –30 min) the initiation of the 24-h oxidation period in the presence of CuSO₄ (5 μ mol/l for 24 h), and cholesterol derivatives, i.e. 7 β -hydroxycholesterol (middle graph) and 7-ketocholesterol (lower graph), were assayed at the end of the 24-h oxidation period. Open bars 'no addition'; middle and lower graphs) show the amount of cholesterol derivatives that were produced at the end of the 24-h oxidation period in the absence of RWPC supplementation. 7 β -hydroxycholesterol (middle graph) and 7-ketocholesterol (lower graph) correlated significantly with the level of endogenous α -tocopherol (upper graph) at the time of the addition of RWPC: $\rho = -1$, $P < 0.05$ in both cases; Spearman rank correlation analysis.

and in the absence of exogenous antioxidants. RWPCs could be quite effective in blocking the oxidation process, reducing up to 50% of the formation of cholesterol oxides when added at a very early stage of the 24-h incubation period, i.e. within 10 min after the addition of copper sulfate. In contrast, when RWPCs were added too late, i.e. after the α -tocopherol reserve in control LDL has been depleted, they were no longer effective in reducing the amount of cholesterol oxides formed at the end of the 24-h incubation period (Fig. 3). After 24 h of oxidation, the 7β -hydroxycholesterol and 7-ketocholesterol levels in oxidized LDL correlated negatively with the levels of endogenous α -tocopherol at the time of antioxidant introduction in RWPC-treated samples ($\rho = -1$, $P < 0.05$ in both cases; Spearman rank correlation analysis).

3.2. Effect of RWPC pretreatment of oxidized LDL on their ability to inhibit the endothelium-dependent relaxation

Human plasma LDL, pretreated or not with RWPCs, were oxidized with copper sulfate and their ability to inhibit the endothelium-dependent relaxation was determined on isolated rabbit aortic segments (see Section 2). As shown in Fig. 4, and in agreement with previous studies [4], oxidized LDL (protein, 1 g/l) inhibited the acetylcholine-mediated endothelium-dependent arterial relaxation. This was accompanied by significant decreases in the maximal arterial relaxation (E_{\max}) as well as in the pD_2 values that correspond to the negative

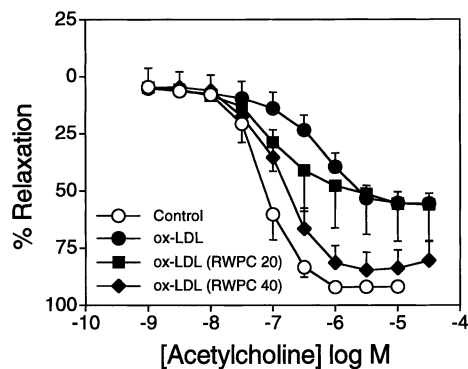


Fig. 4. Effect of the pretreatment of oxidized LDL with RWPC on their ability to inhibit the endothelium-dependent relaxation of rabbit aorta. The graph shows the concentration–response curves to ACh of rabbit aortic rings that were incubated for 2 h in the absence (open circles) or in the presence (closed symbols) of oxidized LDL (final concentration 1 g protein/l). Before oxidation, LDL were preincubated in the absence of any addition (closed circles) or in the presence of RWPC (final concentrations: 20 mg/l (closed squares) or 40 mg/l (closed diamonds)). At the end of the 2-h incubation period, aortic rings were washed and subsequently precontracted with 0.3 μ mol/l NE before addition of ACh. Each point represent the mean \pm S.E. of five distinct experiments. Statistical analysis was performed on pD_2 and E_{\max} values that were determined for each curve (see Table 2).

Table 2

Comparative effects of oxidized LDL and oxidized LDL pretreated with RWPC on maximal relaxation (E_{\max}) and sensitivity (pD_2) of rabbit aorta to ACh

	pD_2 ($-\log EC_{50}$)	E_{\max} (%)
Control	7.13 ± 0.14	94.2 ± 1.9
ox-LDL	$6.14 \pm 0.26^{**}$	$58.2 \pm 9.5^*$
ox-LDL (RWPC 20 mg/l)	$6.40 \pm 0.31^*$	$56.0 \pm 16.1^{**}$
ox-LDL (RWPC 40 mg/l)	$6.88 \pm 0.16^{***}$	85.0 ± 7.7

Aortic segments were incubated for 2 h with LDL pretreated or not with RWPC (20 or 40 mg/l) prior to oxidation. Control segments without LDL addition were incubated in parallel in Krebs' buffer. At the end of the incubation period a concentration–response curve to ACh was obtained on NE-precontracted aortas. The maximal relaxation (E_{\max}) and pD_2 values are mean \pm S.E. of five distinct experiments. * $P < 0.05$, ** $P < 0.01$ vs. 'control' group and *** $P < 0.01$ vs. 'ox-LDL' group.

logarithm of the ACh concentration required to obtain a half-maximal relaxing effect (EC_{50}) (Table 2). The preincubation of LDL with RWPCs significantly improved the arterial relaxation, with a significant rise in E_{\max} and pD_2 values (Fig. 4). With the highest RWPC concentration studied (40 mg/l), endothelium-dependent relaxation tended to be normalized and E_{\max} and pD_2 values determined with RWPC-pretreated, oxidized LDL did not differ anymore from those measured with control arterial segments (Fig. 4, Table 2).

3.3. Effect of the treatment of arterial segments with RWPCs on vascular reactivity

In a last part of the study, the direct effect of RWPCs on vascular reactivity was investigated on abdominal aortic segments that were mounted in organ baths.

Fig. 5 shows the progressive relaxation of intact precontracted aortic rings that was induced by cumulative additions of RWPCs. The direct relaxing effect of RWPCs was completely abolished by removing the endothelium, and the RWPC-mediated relaxation of intact aortic rings could be blocked by a pretreatment with LNA, but not by a pretreatment with Indomethacin (Fig. 5).

Finally, we searched for the ability of RWPCs to modify the contraction of aortic rings. To this end, rabbit aortic segments were preincubated with various concentrations of RWPCs (1, 10 and 100 mg/l), and, after a washing step, they were contracted with cumulative doses of NE. As shown in Fig. 6, RWPCs could exert a persistent effect at the arterial wall by reducing the sensitivity of the artery to the contractile effect of NE. The pD_2 values for NE measured for RWPC-treated rings were significantly lower than corresponding values determined with control, untreated segments (pD_2 : 7.43 ± 0.11 in control segments vs. 6.19 ± 0.23 and

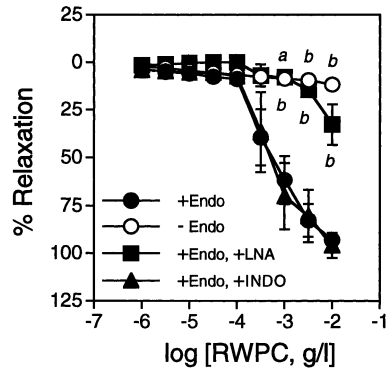


Fig. 5. Effect of N^o -nitro-L-arginine and indomethacin on the concentration-dependent relaxation of rabbit aorta to RWPC. Cumulative concentrations of RWPC (final concentration range, 0.001–10 mg/l) were added on NE-precontracted rabbit aorta in which endothelium was removed (open circles) or not (closed symbols) by rubbing the lumen of the artery. Arteries with functional endothelium were incubated in the presence of 10 μ mol/l of N^o -nitro-L-arginine (closed squares) or indomethacin (closed triangles) for 20 min prior to NE precontraction. Each point represents the mean \pm S.E. of three to six distinct experiments. Significantly different from '+ Endo' arteries: ^a $P < 0.05$; ^b $P < 0.01$.

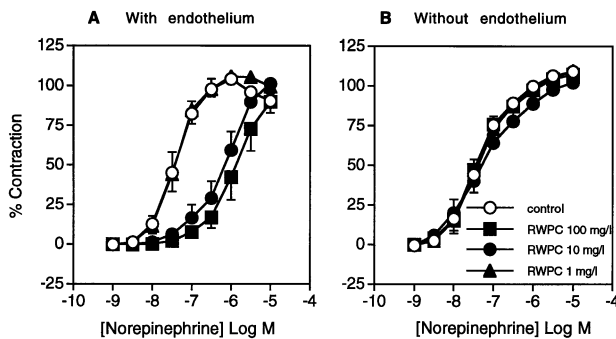


Fig. 6. Concentration-dependent effect of RWPC on the contraction of rabbit aorta to NE. Rabbit aortas with (panel A) or without (panel B) functional endothelium were preincubated for 1 h in the absence (open circle) or in the presence of RWPC (1 mg/l, closed triangles; 10 mg/l, closed circles; 100 mg/l, closed squares). After washout, concentration–response curves to NE were performed. Each point represents the mean \pm S.E. of four distinct experiments. Statistical analysis was performed on pD_2 and E_{max} values that were determined for each curve (see main text of Section 3).

5.88 ± 0.20 in counterparts treated with 10 and 100 mg/l of RWPCs, respectively; $n = 4$; $P < 0.01$ in both cases) (Fig. 6). In contrast to Indomethacin that did not improve the sensitivity to NE of arterial rings treated with 10 mg/l RWPCs (pD_2 after Indo: 6.02 ± 0.07 in RWPC-treated rings vs. 7.30 ± 0.06 in untreated rings; $n = 3$; $P < 0.05$), LNA was able to normalize the contractile response to NE, and similar sensitivities to NE were measured with arterial rings that were pre-treated or not with 10 mg/l RWPCs (pD_2 after LNA: 7.37 ± 0.12 in RWPC-treated rings vs. 7.49 ± 0.06 in control rings; ns) (Fig. 7).

4. Discussion

The present study demonstrated that red wine polyphenolic compounds (RWPCs) can preserve a normal vascular reactivity by acting at different stages of the cascade that leads to lipoprotein oxidation, endothelium dysfunction and vasospasm. RWPCs can delay the consumption of α -tocopherol during oxidation of low-density lipoproteins (LDL), reducing the concomitant formation of oxidation-derived lipid species, including 7 β -hydroxycholesterol and 7-ketocholesterol. As a consequence, the acetylcholine-mediated relaxation of precontracted aortic segments was normalized by the pretreatment of oxidized LDL with RWPCs. Finally, in addition to the direct relaxing effect of RWPCs on precontracted arterial segments [34], the present study brought new evidence in favour of a persistent inhibitory effect of RWPCs on the vascular response to constrictor agents. In a first part of the study, the antioxidant potency of RWPCs, as well as their effect in terms of generation of oxidation lipid derivatives were characterized. The present work brought the first direct evidence in favor of the ability of RWPCs to inhibit the formation of cholesterol oxides (mainly 7 β -hydroxycholesterol and 7-ketocholesterol) and to a lesser extent the emergence of LPC in oxidized LDL. In this respect, RWPCs appeared to be more effective than vitamin C, another hydrosoluble antioxidative compound. The protective effect of RWPCs appeared to be a function of the α -tocopherol content of LDL, and it closely related to the decay of α -tocopherol during the first, initiating step of the lipoprotein oxidation process. Although an effective blockade of the production of conjugated dienes and TBARS by a wine phenolic extract was previously reported to persist despite the exhaustion of vitamin E [25], other water soluble antioxidants (including ascorbic acid, caffeic acid, and an anthocyanoside extract from *Vaccinium myrtillus*) were proven to prevent LDL oxidation mainly through the retardation of vitamin E consumption [24,40,41]. In fact, a number of biochemical studies evidenced that lipid peroxidation in LDL is observed only after the antioxidant defenses, mostly α -tocopherol, have been lost [42,43]. The ability of water soluble antioxidants to prevent the degradation of the LDL α -tocopherol pool might relate either to their capacity to be oxidized themselves in preference to α -tocopherol or to regenerate the α -tocopheroxyl radical [44].

It is now recognized that oxidized LDL largely contribute to the vascular endothelium dysfunction that occurs at an early stage of the atherosclerosis process [1,2]. The deleterious effect of oxidized LDL has been shown to result mainly from the accumulation of oxidized lipid derivatives, i.e. lysophosphatidylcholine [3] and cholesterol oxides [4,5], in the lipoprotein structure. In particular, cholesterol derivatives oxidized

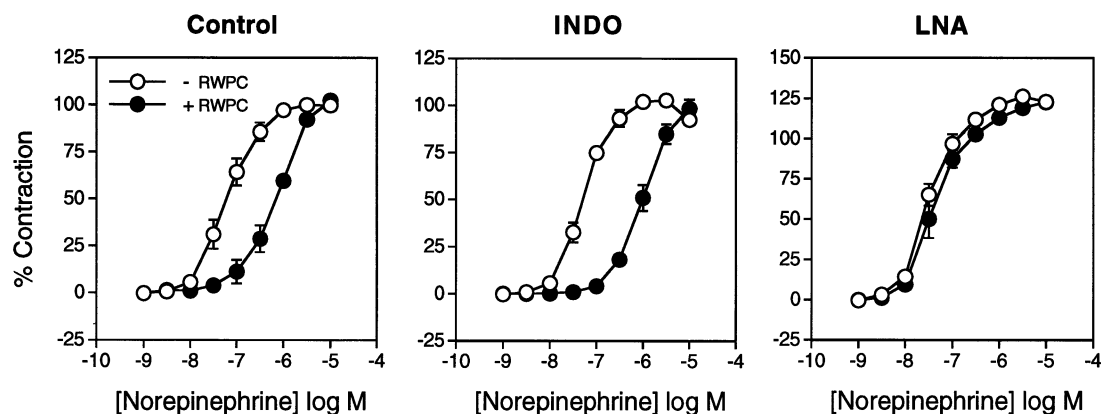


Fig. 7. Effect of indomethacin and N^G -nitro-L-arginine on the inhibition of NE contraction induced by RWPC in rabbit aorta. Rabbit aortas with functional endothelium were preincubated in the absence (control) or in the presence of either indomethacin (INDO, 10 $\mu\text{mol/l}$) or N^G -nitro-L-arginine (LNA, 10 $\mu\text{mol/l}$) for 20 min. RWPC (10 mg/l) were subsequently added (closed circles) or not (open circles) for 1 h incubation period. After washout, aortic rings were contracted with cumulative concentrations of NE. Each point represents the mean \pm S.E. of four distinct experiments. Statistical analysis was performed on pD_2 and E_{max} values that were determined for each curve (see main text of Section 3).

in position 7, i.e. 7β -hydroxycholesterol and 7-ketocholesterol, were shown to mimic the ability of oxidized LDL to inhibit the endothelium-dependent relaxation of rabbit aorta [4] as the result of the impairment of the release of nitric oxide from endothelial cells [14]. The results of the present study demonstrate for the first time that RWPCs can prevent the deleterious effect of oxLDL on the endothelium-dependent arterial relaxation. Since the inhibitory effect of oxidized LDL on the arterial relaxation was previously shown to depend mainly on their cholesterol oxide content [4], the RWPC-mediated blockade of the formation of cholesterol derivatives may well account, at least in part, for the observed beneficial effect. In this experimental context, and as described above, it must be borne in mind that RWPCs clearly acted in concert with α -tocopherol that constituted an essential coantioxidant in preventing the formation of vasoactive cholesterol oxides.

In a last part of the present study, RWPCs were shown to be able to exert a direct beneficial effect on vascular reactivity, independently of their ability to prevent the formation of vasoactive lipids in oxidized LDL. In agreement with previous studies that were conducted with the same extract on rat aorta [34], RWPCs could relax precontracted rabbit aortic rings in a concentration-dependent manner. Since the direct relaxing effect of RWPCs was completely suppressed by endothelium removal or competitive inhibitors of NO synthesis ([34,45], present study), it is clearly mediated through the NO pathway. Although the precise mechanism of action of RWPCs on vascular endothelium remains to be clearly established, previous studies suggested that the relaxing effect might relate to enhanced synthesis of NO rather than to diminished NO breakdown [34,46]. Thus, the relaxing response of isolated rat aortic rings induced by SIN-1, i.e. an

exogenous NO and O_2^- donor, was not significantly modified by RWPCs [34]. In fact, recent studies indicated that RWPCs promote the endothelium-dependent relaxation of aortic rings through an extracellular Ca^{2+} -dependent mechanism that leads to the activation of NO synthase [46]. In the present studies, we could demonstrate that RWPCs are also able to modify the contraction of aortic rings to norepinephrine (NE). Again, the alteration by a RWPC pretreatment of the contraction response was linked to a direct effect on NO release since the decreased sensitivity to NE of RWPC-treated arterial segments was completely abolished in the presence of LNA, a competitive NO synthase inhibitor. While the latter observations on rabbit aorta are in good agreement with previous ones on rat aorta [45], they further demonstrated that RWPCs can exert a long-lasting effect, and the significant inhibition of the arterial contraction in the present study was still observed following removal of RWPCs from the experimental medium. Based on present observations with isolated LDL, RWPCs may also spare cellular α -tocopherol through a coantioxidant cooperativity mechanism. In support of the possible contribution of endothelium α -tocopherol to the vascular effects of RWPCs, differences in vascular α -tocopherol content result in strikingly different susceptibilities to the oxLDL-mediated impairment of the endothelium-dependent arterial relaxation [47–49].

The physiological relevance of ex vivo observations on isolated arterial rings is in the scope of the present studies. The total polyphenol content of LDL in human plasma significantly increased after red wine consumption [29,30], reaching levels similar to those of the present ex vivo studies. Moreover, the reduced progression of atherosclerosis in apoE-deficient mice or in cholesterol-fed rabbits following consumption of either red wine or its major polyphenolic compounds,

proanthocyanidins, or ginger extract is associated with a reduced susceptibility of LDL to oxidation [50–52]. Although human studies did not confirm unambiguously a beneficial effect of vitamin E supplementation on NO bioactivity and vascular function, the most recent advances pointed to the key role of water soluble, phase transfer antioxidants in acting in concert with α -tocopherol [44]. In this respect, the present study indicates that RWPCs might well constitute efficient candidates in regenerating α -tocopherol both at the lipoprotein and endothelium levels. Since the total antioxidant capacity of red wine is likely to result from the combined activity of a wide range of compounds [24,31,53], the strongest beneficial effect of RWPCs, as compared to vitamin C, might actually reflect synergistic actions of different polyphenol classes.

In conclusion, the present study demonstrated that the preservation of the LDL α -tocopherol content by RWPCs can block the formation of vasoactive cholesterol oxides in LDL, as well as the subsequent alterations of the arterial relaxation and contractility. Since in the present study RWPCs did not exert any beneficial effects once α -tocopherol has been consumed, the combination of water soluble RWPCs and hydrophobic α -tocopherol, in preference to α -tocopherol supplementation alone, might constitute a promising approach in the prevention of endothelium dysfunction and atherosclerosis in vivo.

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References

- [1] Cox DA, Cohen ML. Effects of oxidized low-density lipoprotein on vascular contraction and relaxation: clinical and pharmacological implications in atherosclerosis. *Pharmacol Rev* 1996;48:3–19.
- [2] Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. *J Vasc Res* 1996;33:181–94.
- [3] Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990;344:160–2.
- [4] Deckert V, Perségol L, Viens L, et al. Inhibitors of arterial relaxation among components of human oxidized low-density lipoproteins. Cholesterol derivatives oxidized in position 7 are potent inhibitors of endothelium-dependent relaxation. *Circulation* 1997;95:723–31.
- [5] Mougnot N, Lesnik P, Ramirez-Gil JF, Nataf P, Diczfalussy U, Chapman JM, Lechat P. Effect of the oxidation state of LDL on the modulation of arterial vasomotor response in vitro. *Atherosclerosis* 1997;133:183–92.
- [6] Brown AJ, Jessup W. Oxysterols and atherosclerosis. *Atherosclerosis* 1999;142:1–28.
- [7] Hodis HN, Crawford DW, Sevanian A. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis* 1991;89:117–26.
- [8] Carpenter KLH, Taylor SE, Ballantine JA, Fussell B, Halliwell B, Mitchinson MJ. Lipids and oxidised lipids in human atheroma and normal aorta. *Biochim Biophys Acta* 1993;1167:121–30.
- [9] Breuer O, Dzeletovic S, Lund E, Diczfalussy U. The oxysterols cholest-5-ene-3 β ,4 α -diol, cholest-5-ene-3 β ,4 β -diol and cholestane-3- β ,5 α ,6 α -triol are formed during in vitro oxidation of low density lipoprotein, and are present in human atherosclerotic plaques. *Biochim Biophys Acta* 1996;1302:145–52.
- [10] Salonen JT, Nyyssönen K, Salonen R, Porkkala-Sarataho E, Tuomainen T-P, Diczfalussy U, Björkhem I. Lipoprotein oxidation and progression of carotid atherosclerosis. *Circulation* 1997;95:840–5.
- [11] Zieden B, Kaminskas A, Kristenson M, Kucinskiene Z, Vessby B, Olsson AG, Diczfalussy U. Increased plasma 7 β -hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. *Arterioscler Thromb Vasc Biol* 1999;19:967–71.
- [12] Lizard G, Deckert V, Dubrez L, Moisan M, Gambert P, Lagrost L. Induction of apoptosis in endothelial cells treated with cholesterol oxides. *Am J Pathol* 1996;148:1625–38.
- [13] Lizard G, Monier S, Cordelet C, et al. Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7 β -hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. *Arterioscler Thromb Vasc Biol* 1999;19:1190–200.
- [14] Deckert V, Brunet A, Lantoine F, et al. Inhibition by cholesterol oxides of NO release from human vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 1998;18:1054–60.
- [15] Steinberg D. Oxidative modification of LDL and atherogenesis. *Circulation* 1997;95:1062–71.
- [16] Mügge A. The role of reactive oxygen species in atherosclerosis. *Z Kardiol* 1998;87:851–64.
- [17] Tribble DL. Antioxidant consumption and risk of coronary heart disease: emphasis on vitamin C, vitamin E and β -carotene. *Circulation* 1999;99:591–5.
- [18] Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 1993;341:454–5.
- [19] Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. *Lancet* 1993;341:1103–4.
- [20] Kanner J, Frankel E, Granit R, German B, Kinsella JE. Natural antioxidants in grapes wines. *J Agric Food Chem* 1994;42:64–9.
- [21] Lanningham-Foster L, Chen C, Chance DS, Loo G. Grape extract inhibits lipid peroxidation of human low density lipoprotein. *Biol Pharm Bull* 1995;18:1347–51.
- [22] Cook NC, Samman S. Flavonoids—chemistry, metabolism, cardioprotective effects and dietary sources. *J Nutr Biochem* 1996;7:66–76.
- [23] Paganga G, Al-Hashim H, Khodr H, et al. Mechanisms of antioxidant activities of quercetin and catechin. *Redox Rep* 1996;2:359–64.
- [24] Abu-Amsha R, Croft KD, Puddey IB, Proudfoot JM, Beilin LJ. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation in vitro: Identification and mechanism of action of some cinnamic acid derivatives from red wine. *Clin Sci* 1996;91:449–58.
- [25] Hurtado I, Caldu P, Gonzalo A, Ramon JM, Minguez S, Fiol C. Antioxidative capacity of wine on human LDL oxidation in vitro:

- effect of skin contact in winemaking of white wine. *J Agric Food Chem* 1997;45:1283–9.
- [26] Maxwell S, Cruickshank A, Thorpe G. Red wine and antioxidant activity in serum. *Lancet* 1994;344:193–4.
- [27] Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T, Itakura H. Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* 1994;344:1152.
- [28] Whitehead TP, Robinson D, Allaway S, Syms J, Hale A. Effect of red wine ingestion on the antioxidant capacity of serum. *Clin Chem* 1995;41:32–5.
- [29] Fuhrman B, Lavy A, Aviram M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 1995;61:549–54.
- [30] Nigdikar SV, Williams NR, Griffin BA, Howard AN. Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo. *Am J Clin Nutr* 1998;68:258–65.
- [31] Ghiselli A, Nardini M, Baldi A, Scaccini C. Antioxidant activity of different phenolic fractions separated from an Italian red wine. *J Agric Food Chem* 1998;46:361–7.
- [32] Behrens WA, Madere R. Distribution of alpha-tocopherol in human plasma lipoproteins. *Am J Clin Nutr* 1985;35:691–6.
- [33] Ogihara T, Miki M, Kitagawa M, Mino M. Distribution of tocopherol among human plasma lipoproteins. *Clin Chim Acta* 1989;174:299–306.
- [34] Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet J-C, Andriantsitohaina R. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br J Pharmacol* 1997;120:1053–8.
- [35] Park SW, Addis PB. Capillary column gas-liquid chromatographic resolution of oxidized cholesterol derivatives. *Anal Biochem* 1985;149:275–83.
- [36] Hax WMA. High-performance liquid chromatographic separation and photometric detection of phospholipids. *J Chromatogr* 1977;142:735–41.
- [37] Miller KW, Yang CS. An isocratic high performance liquid chromatography method for the simultaneous analysis of plasma retinol, α -tocopherol and various carotenoids. *Anal Biochem* 1985;145:21–6.
- [38] Deckert V, Pruneau D, Elghozi J-L. Mediation by 5-HT_{1D} receptors of 5-hydroxytryptamine-induced contractions of rabbit middle and posterior cerebral arteries. *Br J Pharmacol* 1994;112:939–45.
- [39] Smith PK, Krohn R, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76–85.
- [40] Viana M, Barbas C, Bonet B, Bonet MV, Castro M, Fraile MV, Herrera E. In vitro effects of a flavonoid-rich extract on LDL oxidation. *Atherosclerosis* 1996;123:83–91.
- [41] Otero P, Viana M, Herrera E, Bonet B. Antioxidant and prooxidant effects of ascorbic acid, dehydroascorbic and flavonoids on LDL submitted to different degrees of oxidation. *Free Radic Res* 1997;27:619–26.
- [42] Esterbauer H, Dieber-Rotheneder M, Waeg G, Striegl G, Jürgens G. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem Res Toxicol* 1991;3:77–92.
- [43] Jessup W, Rankin SM, De Whalley CV, Hoult JRS, Leake DS. α -Tocopherol consumption during low density lipoprotein oxidation. *Biochem J* 1990;265:399–405.
- [44] Upston JM, Terentis AC, Stocker R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. *FASEB J* 1999;13:977–94.
- [45] Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol* 1993;265:H774–8.
- [46] Andriambeloson E, Stoclet J-C, Andriantsitohaina R. Mechanism of endothelial nitric oxide-dependent vasorelaxation induced by wine polyphenols in rat thoracic aorta. *J Cardiol Pharmacol* 1999;33:248–54.
- [47] Keaney JF, Jr, Guo Y, Cunningham D, Shwaery GT, Xu A, Vita JA. Vascular incorporation of α -tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. *J Clin Invest* 1996;98:386–94.
- [48] Keaney JF, Jr, Simon DI, Freedman JE. Vitamin E and vascular homeostasis: implications for atherosclerosis. *FASEB J* 1999;13:965–75.
- [49] Desrumaux C, Deckert V, Athias A, et al. Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering α -tocopherol to endothelial cells. *FASEB J* 1999;13:83–892.
- [50] Hayek T, Fuhrman B, Vaya J, et al. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* 1997;17:2744–52.
- [51] Yamakoshi J, Kataoka S, Koga T, Ariga T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* 1999;142:139–49.
- [52] Fuhrman B, Rosenblat M, Hayek T, Coleman R, Aviram M. Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice. *J Nutr* 2000;130:1124–31.
- [53] Fremon L, Belguendouz L, Delpal S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sci* 1999;64:2511–21.