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Resveratrol Inhibits Metal Ion-Dependent and Independent Peroxidation of Porcine Low-Density Lipoproteins

Leila Belguendouz, Lucie Fremont, and Alain Linard*
Laboratoire de Nutrition et Sécurité Alimentaire CRJ-INRA 78352 Jouy-en-Josas Cedex, France

ABSTRACT. Resveratrol, a phytoalexin (3, 4′, 5, trihydroxystilbene) present in some red wines, has been reported to inhibit copper-mediated low-density lipoprotein (LDL) oxidation. In this study, we examined the efficiency of this compound in inhibiting metal ion-dependent and independent peroxidation of porcine LDL. At 0.5, 1, or 1.5 μM, *trans*resveratrol prolonged the lag time preceding the onset of conjugated diene formation in a dose-dependent manner, with a slope of the propagation phase 5-fold greater in the presence of Cu SO_4 (5 μM) than in the presence of the free radical generator, AAPH [2, 2′-azobis (2-amidinopropane) dihydrochloride] (1 mM). At 1 μM, *trans*resveratrol prolonged the lag time 3.4- and 1.4-fold in the presence of copper and AAPH, respectively. Isomerisation into *cis*resveratrol significantly lowered the chelating capacity, but did not alter the free radical scavenging capacity. As compared to flavonoids and trolox, *trans*resveratrol showed a much higher ability to prolong the lag time in copper, but not in AAPH-catalyzed oxidation. The kinetics of generation of degradative products in the presence of copper confirmed the strongest protective effects of *trans*resveratrol, because the formation of thiobarbituric acid reactive substances and hydroperoxides was almost completely inhibited at 200 min. By contrast, *trans*resveratrol was less potent than flavonoids (but more than trolox) as a scavenger of free radicals. Our data show that, like flavonoids, resveratrol protects LDL against peroxidative degradation by both chelating and free radical scavenging mechanisms. However, *trans*resveratrol, which is by far the most potent chelator of copper, does not chelate iron. It might contribute to the protective effects of wine polyphenols by removing copper from LDL particles and arterial tissue and, thereby, delaying the consumption of flavonoids and endogenous antioxidants. BIOCHEM PHARMACOL 53;9:1347–1355 © 1997 Elsevier Science Inc.

KEY WORDS. wine polyphenols; flavonoids; resveratrol; lipid peroxidation; low-density lipoprotein; antioxidant

Oxidative modifications of LDL† that alter physicochemical and biological properties of the particles, are thought to play a central role in atherogenesis [1–2]. The changes depend on a common initiating step, the peroxidation of PUFA components, which leads to extensive fragmentation into degradative products, such as conjugated dienes, peroxy radicals, aldehydes, etc. [3]. Hence, the intake of vegetable foods and beverages that contain phenolic antioxidants potentially protects against atherosclerosis. It has been postulated that the high content of polyphenols in red wine (up to 4 g/L) might be responsible for the beneficial effects of moderate drinking on coronary heart disease [4–7]. The main natural polyphenols of wine are flavonoids produced by healthy plants, but some red wines also contain a phytoalexin named resveratrol (3, 4′, 5-trihydroxystilbene) (Fig. 1). This polyphenol is the active component of "kojokon" prepared from roots of Polygonum species and used in Asian traditional medicine to treat several diseases, in particular, lipid disorders [8–9]. In the grapevine, the production of resveratrol as *trans*isomer is a nonspecific response to infection or injury [10]. Because resveratrol synthesis is mainly located at the skin level of grape berries [11], it is present at very much higher levels in red than in white wines [12]. Frankel *et al.* [13] were the first to demonstrate that *trans*resveratrol inhibited LDL oxidation by cupric ion. The effect could be assigned to the chelation of copper because metals act as pro-oxidants by electron transfer, releasing free radicals from PUFA and hydroperoxides. On the other hand, as a polyphenolic compound, resveratrol may inhibit lipid peroxidation by scavenging free radicals. Because some features suggest that both metal ion-dependent and metal ion-independent processes may be relevant to LDL oxidation *in vivo*, we compared the antioxidant properties of the two types of wine polyphenols, resveratrol and flavonoids (quercetin, (+)-catechin and

* Corresponding author. Lucie Fremont, Laboratoire de Nutrition et Sécurité Alimentaire, CRJ-INRA, 78352 Jouy-en-Josas Cedex, France. Tel. 01 34 65 23 03; FAX 01 34 65 23 11.

† *Abbreviations:* AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; LDL, low-density lipoprotein; LOOH, lipid hydroperoxides; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances.

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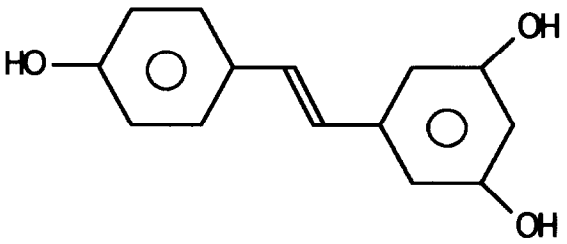

FIG. 1. **Chemical structure of *trans*resveratrol.**

(−)-epicatechin). Trolox, a water-soluble analog of vitamin E, was used as a reference compound. We examined the Cu^{2+}-dependent peroxidation of LDL because copper, which is a strong prooxidant, has the capacity to bind defined sites of the protein moiety of LDL [14]. Therefore, the capacity of any chelator to displace copper by forming redox inactive complexes is related to the structure of the LDL [15]. We used porcine LDL because their composition and structure resemble that of human LDL [16]. The response was compared to that obtained with the water-soluble AAPH that directly induces the peroxidation of PUFA by producing free radicals at a constant rate.

The antioxidative potency of *cis*resveratrol was also tested, because it is present in wines at almost equal amounts as *trans*resveratrol, according to the wine variety and wine-making practice [17–18].

MATERIALS AND METHODS

Preparation of LDL

Fresh blood from standard-fed pigs reared in our Institute was collected in the presence of heparin sodium salt (100 mg/L) as anticoagulant and a mixture of 2 mM benzamidin and 184 mM gentamicin as preservatives. After low-speed centrifugation, Chelex-100 (Biorad, Ivry sur Seine, France) was added to plasma (2 g/L) to remove traces of contaminating transition metals. The LDL fraction (d 1.018–1.063 g/mL was isolated by sequential ultracentrifugation according to Havel *et al.* [19] in the presence of aprotinin (100 kallikrein inhibitory units/L) and phenylmethylsulfonylfluoride (1 mM) using saline solutions stored over Chelex-100 (3 g/L). LDL aliquots (nondialyzed) were stored under N_2 at −80°C in the presence of 100 mg/L Chelex-100 and 10% sucrose, by adding a stock solution (50% sucrose, 150 mM NaCl, pH 7.4) according to Rumsey *et al.* [20].

Immediately before oxidation, most of the salt and sucrose were removed by gel filtration on a PD 10 column (Pharmacia, St-Quentin, France) packed with Sephadex G25 and equilibrated with PBS. Then, LDL were filtered through a 0.2 μm Millipore filter (St-Quentin, France) to remove Chelex. The protein content was estimated according to Bradford [21] using the Biorad protein assay with BSA (fraction V) as standard. The LDL stock solution (1.2–1.6 mg protein/mL) was diluted with oxygen-saturated 10 mM PBS, pH 7.4, at the concentration of protein required for the assay.

Antioxidants

*Trans*resveratrol, flavonoids, D, L-α-tocopherol, and Trolox were from Sigma-Aldrich (St-Quentin, France).

The ethanolic stock solutions (9 mM) were stored for less than 1 month at −80°C. Immediately before use, they were diluted in bidistilled water at required concentrations for the assays.

α-Tocopherol Determination

LDL lipids were extracted according to Burton *et al.* [22] by mixing water (0.5 mL), ethanol (1 mL), and LDL (0.5 mL) in a glass tube. After adding n-heptane (1 mL), the mixture was vortex-stirred for 50 sec and the organic phase separated by centrifugation. The extract was dried under N_2 and resolubilized in hexane. The sample was analyzed by HPLC using a Microporasil column (300 × 3.9 mm, 10 μm) supplied by Waters (Milford, MA). The mobile phase was n-hexane/ethyl acetate (100:7.5, v/v) at a flow rate of 1 mL/min. Detection was performed with a Hitachi spectrofluorimeter (Model F-2000, Tokyo, Japan) with excitation at 290 nm and fluorescence emission at 330 nm. Quantification was done with external standards of D, L-α-tocopherol.

Preparation of cis*Resveratrol*

*cis*Resveratrol was obtained by UV irradiation at 254 nm (Camag lamp with the filter removed) of an ethanolic solution (100 μg/mL) of commercial *trans*resveratrol [16]. After 90 min irradiation, *cis*resveratrol was isolated by preparative HPLC. The solution was evaporated, dissolved in methanol, and loaded on a Ultrasphere-ODS column (250 mm × 10 mm, 5 μm) supplied by Beckman, Palo Alto, CA. The mobile phase was water/acetonitrile (50:50, v/v) at a flow rate of 0.8 mL/min. Peaks were detected at 280 nm (*cis*resveratrol) and 307 nm (*trans*resveratrol). The stock solution (9 mM in ethanol) and dilutions of *cis*resveratrol as well as *trans*resveratrol were prepared by weighing the evaporated fraction.

Solubility and Partition of trans*Resveratrol*

An excess of *trans*resveratrol was added to bidistilled water and the mixture was agitated for 15 hr in the dark at ambient temperature (ca. 20°C) and filtered (Millipore, 0.22 μm). The concentration of the solubilized compound was determined by HPLC at 307 nm. The capacity of resveratrol to bind to LDL was tested by adding the compound to porcine LDL (ca. 3 mg protein/mL in 1.5 mL; final concentration of resveratrol, 0.25 mM). After incubation for 1 hr under N_2, the separation of the aqueous phase was obtained by centrifugal ultrafiltration (5000 × *g*) by using an Ultrafree-LC filter (Millipore) that excludes molecules of Mr > 10,000 Da. Resveratrol was extracted by ethyl acetate in both soluble and insoluble fractions.

Metal-chelating Capacity of transResveratrol

A solution of *trans*resveratrol (10 μM) in water/acetonitrile (50:50, v/v) was analyzed by HPLC at 307 nm in the absence (reference) and in the presence of increasing amounts of Cu SO_4 (i.e. 0.031-, 0.062-, 0.125-, 0.25-, 0.50-, 0.75-, 1-, 1.5-, 2-fold the concentration of resveratrol).

In other assays, Cu^{2+} was replaced by either Fe^{2+} (Fe SO_4) or Fe^{3+} (Fe Cl_3). The capacity of resveratrol to displace chelated copper was tested by performing the assays in the presence of the 1:1 Cu^{2+}-EDTA complex.

Determination of Peroxidation Products

CONJUGATED DIENES. LDL aliquots (50 μg protein/mL) were incubated at 37°C using a Uvikon 930 spectrophotometer (Kontron, Montigny-le Bretonneux, France) equipped with a six-cell holder. The oxidation was initiated by adding 10 μL of a freshly prepared aqueous solution of either Cu SO_4 or the free radical generator AAPH (Polysciences INC, Warrington, PA). The final concentrations were either 5 μM $CuSO_4$ or 1 mM AAPH. In the case of AAPH-dependent oxidation, EDTA was added to LDL (final concentration 0.1 mM) to chelate adventitious metal ions. In a first set of experiments, the effect of various concentrations of *trans*resveratrol was tested (final concentration 0.5, 1, 1.5 μM). In a second set of experiments, the response to a defined concentration of *trans*resveratrol (1.5 μM) was compared to that of either quercetin, (+)-catechin, (−)-epicatechin, or trolox used at the same concentration. The control cell contained LDL and prooxidants. The very low amounts of ethanol present in antioxidant solutions (<0.03%) did not influence the peroxidation process, because there was no change in the response when assays were performed either in the absence or in the presence of 0.1% ethanol. In assays using AAPH, a blank cuvette containing PBS (without LDL) and AAPH, was used to overcome the background absorbance. The increase in absorbance at 234 nm was recorded every 10 min for up to 7 hr. The output from the spectrophotometer was converted directly into the ASCII file format and used to compute the lag time prior to the onset of oxidation. The maximum diene concentration was determined from the difference between the absorbance at the maximum slope of the absorbance curve and the absorbance at time zero using the extinction coefficient for conjugated dienes at 234 nm ($E = 2.95 \times 10^4\ M^{-1}\ cm^{-1}$). In a third set of experiments, the antioxidant potency of *trans*resveratrol was compared to that of *cis*resveratrol used at the same concentrations (1.5 or 3 μM).

HYDROPEROXIDES. We used an assay based on the oxidative activity of LOOH, which convert iodide to iodine. Iodine reacts further to form the triodide ion, which absorbs at 365 nm. We used the microtiter plate procedure proposed by Wallin and Camejo [23], which includes a modification of the method described by El Saadani *et al.* [24], using a commercially available reagent (CHOD-iodide, Merck, Darmstadt, Germany).

The peroxidation was carried out in polystyrene microtiter plates by adding 10 μL of aqueous solution (of either 0.065 mM Cu SO_4 or 13 mM AAPH) and 20 μL of distilled water (control) or antioxidant (final concentration 0.5, 1, or 1.5 μM) to 100 μL of LDL in PBS (0.22 mg protein/mL). The plates were incubated at 37°C in a slowly shaking bath covered with adhesive polyester film permeable to air. The oxidation was stopped at different time points by cooling on ice bath and addition of 10 μL BHT (final concentration 12.5 μM) and EDTA (final concentration 12.5 μM) in the case of Cu^{2+}-induced oxidation or BHT (final concentration 25 μM) in the case of AAPH-induced oxidation. Then, 190 μL of room-tempered CHOD-iodide reagent was added and plates were further incubated for 60 min at 37°C. The absorbance at 365 nm was measured in a microplate reader (Labsystems, Multiskan MCC/340, Uppsala, Sweden). The concentration of LOOH was calculated from the molar absorption coefficient of $2.46 \times 10^4\ M^{-1}\ cm^{-1}$ for the triiodide ion at 365 nm and a path length in the microtiter plate of 1 cm for the final volume of 330 μL.

TBARS. The TBARS were evaluated according to a modification of the procedure described by Wallin *et al.* [25]. The LDL oxidation was performed in Eppendorf microtest tubes (Polylabo, Strasbourg, France) (1.5 mL) instead of microtiter plates. Samples of LDL containing 25 μg protein were diluted in PBS to the final volume of 500 μL, after adding 10 μL of either 0.25 mM $CuSO_4$ or 50 mM AAPH and 20 μL of antioxidant (final concentration 0.5, 1, or 1.5 μM). The tubes were vortexed and incubated at 37°C for different periods in a slowly shaking bath as described for hydroperoxides. At the end of oxidation, 50 μL of 50% trichloracetic acid and 75 μL of 1.3% thiobarbituric acid in 0.3% NaOH were added. The tubes were incubated at 80°C in a shaking bath for 35 min. After cooling, the tubes were centrifuged for 10 min (1500 × g) at 4°C. A volume of 300 μL supernatant was transferred to microtiter plates and the absorbance was read at 535 nm. The concentration of TBARS was expressed as nmol of malondialdehyde equivalents per mg LDL protein using a freshly diluted 1, 1, 3,3-tetraethoxypropane for the standard curve.

Analysis of Data

Results are expressed as mean ± SD. Differences between the effects of various antioxidants were analyzed by 1-way ANOVA. Comparisons were made using Fisher's least significance difference test. Differences were considered significant at $P < 0.05$.

RESULTS

α-Tocopherol in LDL

The α-tocopherol content of freshly isolated LDL varied little between pigs (N = 8). Indeed, the mean value was 2.02 ± 0.27 nmol/mg LDL protein.

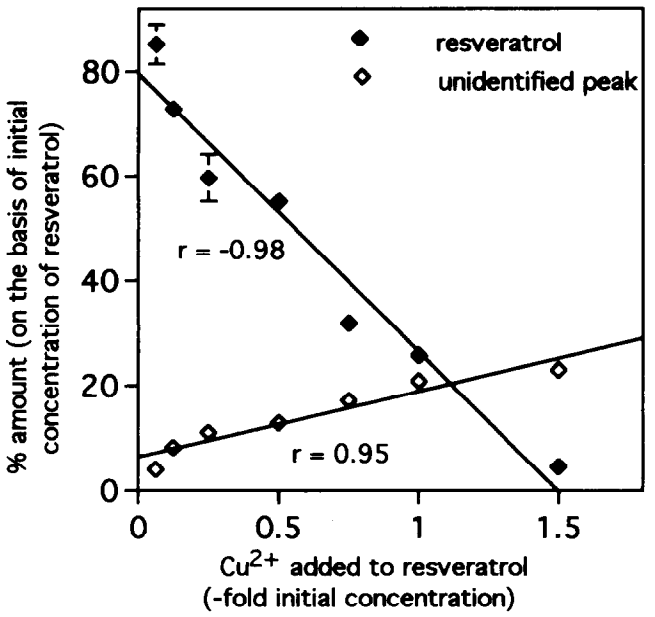

FIG. 2. *trans*Resveratrol disappearance in the presence of copper. *trans*Resveratrol (10μM) was analyzed by HPLC (ultrasphere-ODS column) in water/acetonitrile (50:50, v/v) in the absence and in the presence of increasing concentrations of Cu SO_4. Values for resveratrol (♦) diamonds and for the new peak (◇) diamonds were expressed as percentages of the initial concentration of resveratrol. Values are means (±SD) of 4 separate determinations.

61puUV Spectra

The shape of the UV spectra of native LDL at time zero differed from that of LDL oxidized for 300 min in the presence of either $CuSO_4$ or AAPH. The increase in absorption beyond the maximum value of native LDL (222 nm) revealed the presence of conjugated dienes and degradation products that absorbed in the 220–240 nm region [3]. The addition of various concentrations of resveratrol to LDL samples at time zero did not alter the final spectrum of oxidized LDL (data not shown).

Solubility of trans*Resveratrol and Binding to LDL*

The mean value for solubility in water was 102.9 ± 7.3 nmol/mL (N = 4). The value was lower in the filtrate of an LDL preparation containing resveratrol because the concentration was only 47 ± 4.3 nmol/mL (N = 3). The total recovery of the added amounts was ca. 79% (19% and 60% in soluble and insoluble fractions, respectively).

Metal-Chelating Capacity of trans*Resveratrol*

As shown in Fig. 2, the addition of increasing amounts of Cu^{2+} to 10 μM resveratrol resulted in the proportional reduction of the peak detected at 307 nm. The linear curve (r = −0.98) shows that it had almost completely disappeared at the molar ratio of 1.5:1 (Cu^{2+}/resveratrol). Three minor peaks with a shorter retention time than that of resveratrol appeared. Another peak was also present in proportions linearly correlated with the concentration of Cu^{2+} (r = 0.95). The retention time was close to that of *cis*resveratrol. The chelating capacity of resveratrol was weaker than that of EDTA. Indeed, the addition of EDTA to the resveratrol-Cu^{2+} complex displaced resveratrol, whereas the addition of resveratrol to the EDTA-Cu^{2+} complex had no effect. The replacement of Cu^{2+} by either Fe^{2+} (Fe SO_4) or Fe^{3+} (Fe Cl_3) did not produce chelates. This data was confirmed by UV spectra (Fig. 3). The maximum absorbance of *trans*resveratrol at 307 nm gradually decreased in the presence of increasing amounts of Cu^{2+}, up to the molar ratio of 2:1 (Cu^{2+}/resveratrol). The spectrum did not exhibit the peak absorbance of *cis*resveratrol (250–280 nm). This suggests that the unidentified peak revealed by HPLC might be the chelate or a related compound. We did not observe any new absorption peak. The spectrum of pure *trans*resveratrol remained unchanged in the presence of the Cu^{2+}-EDTA complex as well as in the presence of Fe^{2+}.

Effect of the Concentration of Resveratrol on LDL Oxidation

Figure 4A, B shows the kinetics of oxidation for the formation of conjugated dienes as monitored at 234 nm. The resistance to oxidation of control LDL (without antioxidant) as estimated by the lag time was higher when oxidation was induced by copper (70 min), rather than by AAPH (63 min). The addition of *trans*resveratrol to LDL prolonged the lag time in a dose-dependent manner. Within the concentration range used (0.5, 1, 1.5 μM), the relationship was linear (data not shown), but the slope of the curve was 5-fold greater with copper than with AAPH. The mean values of lag time were 195 ± 20 min, 264 ± 22 min,

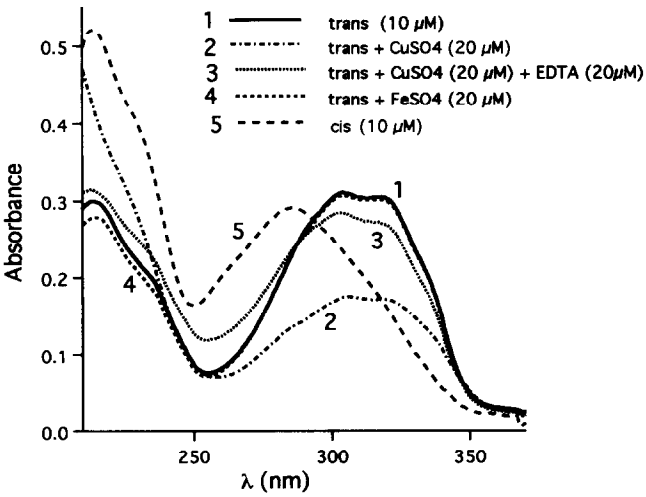

FIG. 3. Absorbance spectra of resveratrol: effect of copper and iron. Spectra were recorded at 30°C against water/acetonitrile (50:50, v/v), which was used to solubilize resveratrol. The curves contained 10 μM resveratrol as *trans*form either alone (1) or in the presence of Cu^{2+}(2), Cu^{2+} + EDTA (3), Fe^{2+} (4), or as the *cis*form (5).

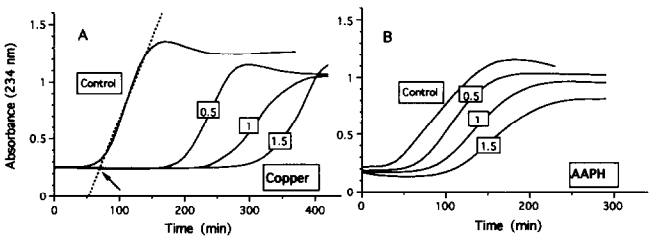

FIG. 4. Effects of *trans*resveratrol concentrations on conjugated diene formation during LDL oxidation. LDL (50 μg protein/mL) were incubated at 37°C for 5 to 7 hr in the presence of 5 μM $CuSO_4$ (A) or 1 mM AAPH (B). The incubation medium did not contain antioxidant (control) or contained 0.5, 1, or 1.5 μM *trans*resveratrol. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min. The results are expressed as absolute absorbance. Values are means of at least 3 determinations. The lag time was defined as the interval (min) between the intercept of the tangent of the slope of the propagation phase and the initial absorbance axis.

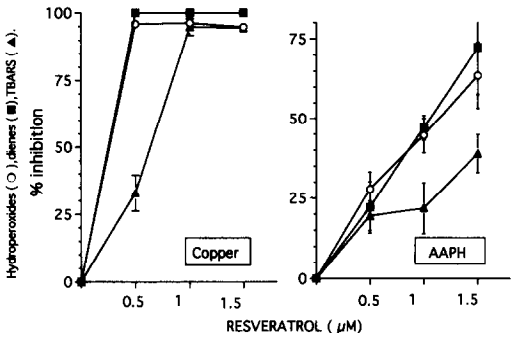

FIG. 5. Concentration-dependent inhibition of LDL peroxidation by *trans*resveratrol. The formation of lipid peroxidation products was determined in LDL (50 μg protein/mL for conjugated dienes and TBARS; 220 μg protein/mL for hydroperoxides) oxidized for 2.5 hr in the presence of 0.5, 1, 1.5 μM *trans*resveratrol. The amounts in control LDL (without resveratrol) were: dienes, 742.4 ± 154.3; TBARS, 81 ± 3.1; LOOH, 938 ± 55 nmol/mg LDL protein with 5 μM Cu^{2+}; dienes, 601.7 ± 118.4; TBARS 52 ± 1.6; LOOH, 2639 ± 24 nmol/mg LDL protein with 1 mM AAPH. Values are means (±SD) of at least 3 determinations.

320 ± 30 min with copper and 72 ± 13 min, 91 ± 7 min, 115 ± 4 min with AAPH, respectively (at least 3 determinations). The maximum production of conjugated dienes was reached after oxidation for 2.5 hr. For this reason, this period of time was selected to compare the amounts of various lipid peroxidation products and the inhibitor effects of antioxidants. In control LDL, the production of conjugated dienes did not significantly differ in the presence of either Cu^{2+} or AAPH (742.4 ± 154.3 vs 601.7 ± 118.4 nmol/mg LDL protein, respectively). The amounts of TBARS produced during the same lapse of time were significantly higher in the presence of Cu^{2+} than in the presence of AAPH (81 ± 3.1 vs 52 ± 1.6 nmol/mg protein, respectively). By contrast, the amounts of LOOH were 2.3-fold lower when Cu^{2+} replaced AAPH (938 ± 55 vs 2639 ± 24 nmol/mg LDL protein, respectively).

The presence of *trans*resveratrol in the medium reduced the amounts of peroxidation compounds generated during oxidation of LDL for 2.5 hr (Fig. 5). In the presence of equivalent concentrations of *trans*resveratrol (0.5, 1, 1.5 μM), similar profiles of inhibition were obtained for conjugated dienes, hydroperoxides, and TBARS when using the same prooxidant (either copper or AAPH). With copper, the plateau of maximal inhibition for conjugated dienes (−100%), LOOH (−96%) and TBARS (−95%) were reached with 0.5, 0.5, and 1 μM *trans*resveratrol, respectively. With AAPH, the reduction of both conjugated dienes and LOOH was, as expected, directly related to the concentration of *trans*resveratrol (from 0.5 to 1.5 μM). The decrease ranged from 22 to 72% for dienes and from 28 to 64% for LOOH. The production of TBARS was less affected, because the reduction ranged from 20 to 39%.

Comparative Effects of transResveratrol *and Flavonoids*

The kinetics of generation of conjugated dienes during Cu^{2+}-catalyzed oxidation are presented in Fig. 6A, B. This figure shows that the lag time was more prolonged in the presence of *trans*resveratrol than in the presence of other antioxidants used at the same concentration (1.5 μM). Indeed, *trans*resveratrol prolonged the lag time 4.5-fold, whereas catechin and epicatechin prolonged it 2-fold, tro-

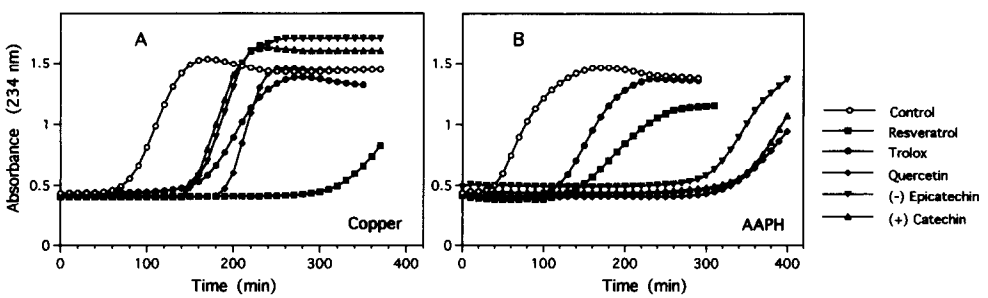

FIG. 6. Time-dependent changes in conjugated diene generation during LDL oxidation. LDL (50 μg protein/mL) were incubated at 37°C for 6.5 hr in the presence of either 5 μM $CuSO_4$ (A) or 1 mM AAPH (B). The incubation medium did not contain antioxidant (control) or contained 1.5 μM of antioxidant. Values are means of absolute absorbance at 234 nm of at least 3 determinations. The bars showing SD have been omitted for clarity.

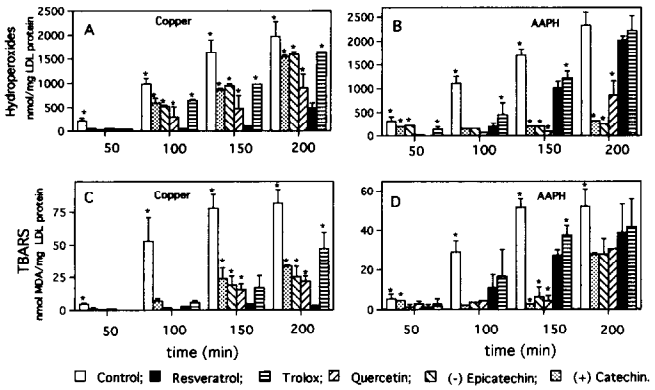

FIG. 7. Time-dependent changes in TBARS and hydroperoxide generation during LDL oxidation. LDL (220 μg protein/mL for hydroperoxides, or 50 μg protein/mL for TBARS) were oxidized for 50, 100, 150, and 200 min in the presence of 5 μM $CuSO_4$(A),(C) or 1 mM AAPH (B), (D). The incubation medium did not contain antioxidant (control) or contained 1.5 μM of antioxidant. Values are means (±SD) of at least 3 determinations of hydroperoxides (A, B) (nmol/mg LDL protein) and TBARS (C, D) (nmol malondialdehyde (MDA)/mg LDL protein). Significance vs oxidation in the presence of *trans*resveratrol is represented by * ($P < 0.05$).

lox 2.2-fold, and quercetin 2.5-fold. In contrast, *trans*resveratrol and trolox were less potent than flavonoids in increasing the resistance to AAPH-induced oxidation. The prolongation of lag time was much lower for *trans*resveratrol and trolox (2-fold) than for epicatechin (4.8-fold) and for catechin or quercetin (5.5-fold).

Almost similar responses were obtained for the production of either TBARS or hydroperoxides (Fig. 7A–D). Within the interval of 50 to 200 min during Cu^{2+}-catalyzed oxidation, the values obtained with either antioxidant significantly differed from control values. The stronger inhibiting effect of *trans*resveratrol was assessed by the absence, or presence at very low levels, of degradative products during the course of the reaction. Both flavonoids, catechin and epicatechin, were as efficient as trolox, whereas quercetin was significantly more efficient in lowering the production of LOOH at 100 min and later. The 3 flavonoids and trolox were equivalent in reducing the production of TBARS up to 150 min, but trolox was significantly less efficient at 200 min.

Data from AAPH-catalyzed oxidation show that *trans*resveratrol was slightly more potent than trolox. For this antioxidant, the difference was significant at 150 min for TBARS and at 50, 100, 150 min for LOOH. However, resveratrol was less potent than flavonoids as free radical scavenger. Indeed, *trans*resveratrol significantly reduced the production of LOOH by 82% at 100 min and by 43% at 150 min. Likewise, the production of TBARS was significantly reduced by 62, 47, and 25% at 100, 150 and 200 min of oxidation, respectively. By contrast, flavonoids almost completely inhibited the formation of LOOH and TBARS up to 150 min. At the following time (200 min), catechin, epicatechin and, to a lesser extent, quercetin were still active against LOOH whereas all flavonoids were equivalent to either *trans*resveratrol or trolox in reducing the production of TBARS.

*Effect of cis*Resveratrol

The antioxidant effect of *cis*resveratrol was estimated from kinetics of conjugated diene formation during LDL oxidation (50 μg/mL). Figure 8 shows that *cis*resveratrol protected porcine LDL to a lesser extent than *trans*resveratrol when oxidation was induced by Cu^{2+}. In the presence of 1.5 and 3 μM of *cis*isomer instead of the *trans*isomer, the lag time was shortened by 59% and 36%, respectively. By contrast, there was no change in lag time during AAPH-catalyzed oxidation.

DISCUSSION

Few studies have reported on the resistance of porcine LDL to peroxidative degradation. Knipping et al. [26] used LDL at the concentration of 0.25 mg/mL, corresponding to ca. 50 μg LDL protein/mL, as in this study (for dienes and TBARS), but the authors used less Cu^{2+} (1.66 μM) for oxidation. After a 3-hr incubation, they found a lesser amount of conjugated dienes (ca. 450 nmol/mg LDL protein) but almost equal amounts of TBARS (65 nmol) and as much hydroperoxide (1000 nmol) as in our experiments. Likewise, Miura et al. [27] obtained ca. 70 and 800 nmol/mg

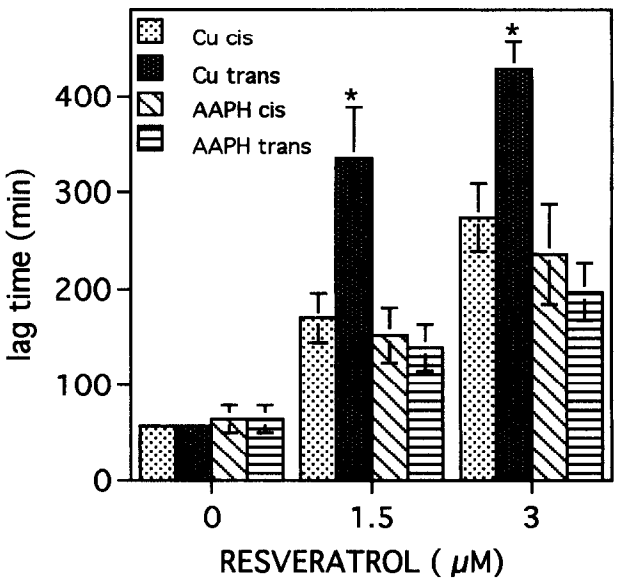

FIG. 8. Effect of *cis*resveratrol and *trans*resveratrol on the onset of conjugated diene formation during LDL oxidation. LDL (50 mg protein/mL) were incubated at 37°C for 7 hr in the presence of either 1.5 μM or 3 μM resveratrol as either *cis* or *trans*form. The lag time was calculated as described in the text. Results are means (±SD) of at least 3 determinations. Significance between *cis* and *trans*isomers in the presence of the same pro-oxidant is represented by * ($P < 0.05$).

LDL protein of TBARS and peroxides, respectively. For dienes, the increase in absorbance of 1.2 units corresponded to approximately 350 nmol/mg LDL protein. They used 5 μM Cu^{2+} to catalyze the oxidation of 100 or 200 μg LDL protein/mL. The production of higher amounts of conjugated dienes in our experiments, as compared to the above-mentioned authors, might be related to the lower LDL protein-to-Cu^{2+} ratio, which could increase the capacity of PUFA to be peroxidized. Using concentrations proposed by Kleinveld *et al.* [28], namely 50 μg LDL protein/mL and 5 μM Cu^{2+}, we found 50% higher amounts of conjugated dienes than these authors for human LDL. This is possibly linked to the antioxidant content of LDL, lower in pigs than in humans [26].

In regard to α-tocopherol, we found values ca. 10-fold higher than those reported by Knipping *et al.* [26]. Indeed, the latter found 0.35–1.41 nmol/mg LDL_1, which corresponds to 0.07–0.28 nmol/mg LDL protein. In humans, the concentration is much higher because the range of values reaches 6–12 mol/LDL particle [29]. This represents 12–24 nmol/mg LDL protein.

The partial hydrosolubility of resveratrol raises the question of partition between phases. *In vivo*, wine drinking supplies low amounts of resveratrol (0.5 to 10 mg/L). Hence, it is likely that, even if the compound is well absorbed by the intestine, the fraction that enters the plasma is probably below the limit of solubility. Accordingly, the compound can be diluted throughout the plasma unless it binds to proteins and lipoproteins. Vinson et al. [30] reported that resveratrol had a lipoprotein-bound antioxidant activity equivalent to that of α-tocopherol. This is consistent with the present finding showing that, when LDL was incubated with resveratrol, the concentration in the filtrate was more than 2-fold lower than expected from the value of solubility with a partition coefficient, aqueous phase/residue of 0.32. This observation demonstrates that resveratrol has the capacity to bind to LDL. On the other hand, in the peroxidation assays where resveratrol was used at much lower concentrations (to the limit of solubility in water), the linear dose-response curve indicated that the compound was bound to lipoproteins.

Our data demonstrate that *trans*resveratrol was very efficient in protecting porcine LDL against PUFA peroxidation, with a higher capacity to inhibit copper- rather than AAPH-mediated LDL peroxidation. The chelating properties of *trans*resveratrol were much stronger than those of tested flavonoids and trolox. This finding differs from that of Frankel *et al.* [13], who observed that, at the concentration of 10 μM, *trans*resveratrol was less efficient than quercetin and epicatechin in inhibiting the formation of hexanal during copper-catalyzed oxidation of human LDL. The discrepancy between our results and those of the authors cited above might be due to differences in the experimental design and tests used. On the other hand, *trans*resveratrol was found to be very efficient in protecting rat liver microsomes against lipid peroxidation. The concentration required to inhibit the production of TBARS by 50% was 3-fold lower with *trans*resveratrol than with quercetin [31].

The efficiency of flavonoids in protecting human LDL against oxidative modifications is known [32, 33]. Recently, Miura *et al.* [27] investigated the effects of tea polyphenols on porcine LDL. They observed that the flavonoids tested in the present study, (−)-epicatechin and (+)-catechin, increased the lag time preceding the formation of conjugated dienes. With respect to control values, the prolongation was more than 100 min. Both compounds reduced the formation of TBARS and peroxides. In other assays [34], quercetin was found to be the most active among the flavonoids tested, because the lag time was prolonged up to 235 min in the presence of 0.5 μM antioxidant. The values were somewhat higher than those found in our assays. This may be related to the fact that fewer LDL were incubated in the presence of the same concentration (5 μM) of $CuSO_4$. Although both chelating and antiradical properties of flavonoids are involved in the inhibition of lipid peroxidation, the antioxidative potential of flavonoids predominantly relies on their radical-scavenging capacity. Indeed, flavonoids can easily donate hydrogen because they possess several hydroxyl groups in propitious positions [35].

It is worth noting that the free radical scavenging activity of *trans*resveratrol, less pronounced than that of flavonoids, was almost similar to that of trolox, which only possesses one hydroxyl group. Resveratrol is a stilbene with three hydroxyl groups; their number and position remained unchanged when *trans*resveratrol was isomerized into *cis*resveratrol. Accordingly, the radical scavenging effect of *cis*-resveratrol should be similar to that of *trans*resveratrol. This assumption was ascertained by the equal duration of lag time when oxidation was induced by the free radical generator AAPH. By contrast, the antioxidant potency of *cis*-resveratrol was approximately half that of *trans*resveratrol when Cu^{2+} replaced AAPH in initiating LDL peroxidation. This finding shows that the *trans*structure is of prime importance for the chelating properties of resveratrol. Moreover, the spacial position of hydroxyl groups is likely more propitious to the chelation of copper in the *trans*isomer than in the *cis*isomer.

It is recognized that several antioxidants are involved in the resistance of LDL to oxidative stress. Thus, the antioxidant effect of α-tocopherol is enhanced by, or even dependent on, the presence of coantioxidants [36]. In LDL, ubiquinol-10 has a very powerful sparing effect on the consumption of α-tocopherol, despite a 10-fold lower concentration [37]. In particular, ubiquinol-10 as well as the hydrosoluble vitamin C has the capacity to regenerate α-tocopherol from α-tocopheroxyl [38].

Likewise, it is conceivable that resveratrol interacts with other compounds, thereby improving the efficiency of the antioxidant system. In particular, the *trans*isomer, which possesses a higher chelating capacity than flavonoids, might spare these substances by accelerating the removal of copper present in LDL particles and in environmental arterial

tissue. The protective effects of resveratrol against cardiovascular diseases may also be due to its capacity to inhibit eicosanoid synthesis and platelet aggregation [39], and to its vasorelaxing activity on the aorta [40]. Moreover, the inability of resveratrol to form chelates with iron may be advantageous. Thus, it does not interfere with iron absorption as do other polyphenols and tannins [41]. The findings of other authors and the results of the present studies exhibit several interesting properties of resveratrol that justify further investigation as to their biological properties and their pathways of absorption and metabolism.

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