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Interaction of Transresveratrol with Plasma Lipoproteins

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ABSTRACT. Resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin present in some red wines. Like other phenolic substances, this compound is assumed to protect against atherosclerosis by reducing the peroxidative degradation of low-density lipoproteins (LDL). The *in vitro* efficiency of resveratrol was found to be mainly due to its capacity to chelate copper, although it also scavenges free radicals. In this study, we examined the ability of the compound to associate with lipoproteins *in vitro*. *Trans*-resveratrol added to plasma was distributed between subsequently isolated lipoproteins with a linear dose-response curve. The concentrations as expressed on a protein basis increased with the order of their lipid content: high-density lipoproteins (HDL) < LDL < very low-density lipoproteins (VLDL). This finding reveals the lipophilic character of resveratrol. Other assays showed that resveratrol added to plasma prior to fractionation was, as expressed on a protein basis, more associated with lipoproteins ($d < 1.21$ g/mL) than with lipoprotein-free proteins (5.5 ± 0.7 vs 2.2 ± 0.4 nmol/mg protein). On the other hand, resveratrol inhibited the formation of thiobarbituric acid reactive substances (TBARS) in preparations containing phospholipid unilamellar liposomes oxidized by the water-soluble radical generator 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). A linear dose-response curve was obtained up to 30 μM when the antioxidant was added in the final preparation and up to 200 μM when added before preparing liposomes in order to facilite its incorporation. This suggests that the soluble fraction of resveratrol scavenged free radicals in the aqueous phase before attacking PUFA and within membranes. Taken together, the present data support the hypothesis that resveratrol may be efficient at different sites: in the protein and lipid moieties of LDL and in their aqueous environment. BIOCHEM PHARMACOL **55**;6: 811–816, 1998. © 1998 Elsevier Science Inc.

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Resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin synthesized by a wide range of plant species including *Vitis vinifera*, in response to infection or injury [1, 2]. This compound exerts several therapeutic effects which are partly due to its antioxidant potential. Frankel *et al.* [3] hypothesized that resveratrol could contribute to the beneficial effects of wine drinking because, like other phenolic substances of wine, it inhibited the lipid peroxidation of plasma low-density lipoproteins (LDL). In humans, Fuhrman *et al.* demonstrated that some phenolic substances present in red wine were absorbed and bound to LDL† [4]. However, the role played by resveratrol remains unknown because when present in wines, its concentration is much lower than that of flavonoids and nothing is known about its intestinal absorption. In preliminary studies, we observed that a fraction of *trans*-resveratrol orally administrated to rats was recovered in plasma without apparent alteration. The three-fold higher amounts in the fraction containing LDL and very low-density lipoproteins (VLDL) than in the fraction containing high-density lipoproteins (HDL) suggested that resveratrol had the greatest affinity for lipoproteins having the highest lipid content (unpublished observation).

Indirect assumption of the binding of resveratrol to LDL was obtained by kinetic studies showing that the dose-response curve was linear when the production of conjugated dienes was monitored during LDL peroxidation [5, 6]. Moreover, the finding that the fraction of soluble resveratrol was lower in aqueous solution of LDL than in pure water [5] supports the existence of interactions between resveratrol and LDL. In this study, we carried out *in vitro* studies in order to examine the capacity of *trans*-resveratrol added to plasma to associate with either lipoprotein (LP) class subsequently isolated by ultracentrifugation. The association of resveratrol with LP particles may involve interactions with the protein moiety as well as incorporation into lipid components. This question was examined by testing the ability of the compound to bind to either plasma LP or a defined protein, namely BSA, representative of plasma proteins and to protect phospholipid polyunsatu-

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† *Abbreviations:* AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; ApoB, apolipoprotein B; HDL, high-density lipoproteins; LDL, low-density lipoproteins; LP, lipoprotein; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; VLDL, very low-density lipoproteins.

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rated fatty acids (PUFA) in the absence of proteins. For these assays we used porcine plasma, because it resembles human plasma in the distribution and composition of LP [7] and in the structure of apo B [8].

MATERIALS AND METHODS

Preparation of Lipoproteins

All the assays were carried out with *trans*-resveratrol, which is the commercially available form of resveratrol. This compound and other antioxidants (Trolox and D,L-α-tocopherol) was purchased from Sigma-Aldrich. Fresh blood from standard-fed pigs reared in our Institute was collected in the presence of heparin sodium salt (100 μg/mL). Plasma obtained after low-speed centrifugation was supplemented with preservatives (2 mM benzamidin and 200 mM gentamycin). Lipoprotein fractions were isolated by sequential ultracentrifugation at 145,000 *g* according to Havel *et al.* [9] using saline solutions stored over Chelex-100 (3 g/L). The intervals of density were: VLDL, d < 1.006 g/mL; LDL, d = 1.006–1.063 g/mL; HDL, d = 1.063–1.21 g/mL). In some assays, total lipoproteins were obtained after a 36-hr run. The density of plasma was raised to 1.21 g/mL by adding solid KBr (325 mg/mL). For some assays, plasma with added preservatives was supplemented with resveratrol before fractionation using small volumes of an ethanolic stock solution (10 mM) to reach the final concentrations.

Addition of Resveratrol to Isolated Total Lipoproteins and BSA

Resveratrol was added to LP obtained by flotation at d < 1.21 g/mL in a volume of 2 mL originating from 20 mL plasma (final concentration 0.25 mM). The protein content was 5.4 ± 0.07 mg/mL ($n = 4$). After incubating for 1 hr under N_2 in the dark, the solid phase was separated from the aqueous phase by centrifugal ultrafiltration (5000 *g*) on an Ultrafree LC filter (Millipore) excluding molecules larger than 10 kDa. Resveratrol was extracted from both ultrafiltrate and residue. Likewise, an aqueous solution of BSA (fraction V) at the same protein concentration was supplemented with resveratrol and treated in parallel.

Extraction and Analysis of Resveratrol

Aliquots of plasma or LP fractions (200 to 500 μL) were diluted to 1 mL with bidistilled water. An equal volume of ethyl acetate was added and after 1 min of vortex stirring, the mixture was centrifuged at 1500 *g* for 10 min. The upper layer was removed and the lower layer was twice reextracted with 1 mL ethyl acetate. The extracts were combined and evaporated to dryness under N_2. The residue was solubilized in 200 μL water/acetonitrile (50:50, v/v). The resveratrol content was determined by HPLC using an Ultrasphere-ODS column (250 mm × 10 μm, 5 μm) supplied by Beckman with detection at 307 nm. The mobile phase was water/acetonitrile (50:50, v/v) at a flow rate of 0.8 mL/min. In assays involving ultrafiltration, the residue was extracted five times with 2 mL ethyl acetate and the filter was cut off and introduced into 2 mL methanol. After vortexing the suspension, methanol and ethylacetate extracts were combined, evaporated and treated as described above.

Peroxidation of Liposomes

Preparation of Unilamellar Liposomes. A solution of soybean phosphatidylcholine (Sigma, type IV) in chloroform (80 mg in 4 mL) was prepared in a round-bottom flask. When required, appropriate amounts of either resveratrol or α-tocopherol in ethanol (in the range of 10–500 μM) were added. The solvent was slowly removed by rotating under reduced pressure and 10 mL of 10 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA (pH 7.4) was added. The mixture was vortexed and the resulting dispersion was sonicated using a Braun sonicator (Labsonic 2000) equipped with a needle probe. The power output was 50 W. After *ca.* 5 min of sonication, a clear opalescent suspension of unilamellar liposomes was obtained. Samples were centrifuged for 20 min at 65,000 *g* and 4° to remove titanium fragments and large lipid aggregates. The preparation was either not supplemented or supplemented with an antioxidant at various concentrations (in the range of 10–1000 μM) by using small volumes (10–50 μL) of the ethanolic stock solution of resveratrol. In parallel, resveratrol was replaced by trolox at the same concentrations.

Peroxidation Assays. The oxidation was induced by the water-soluble free radical generator, AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride (Polysciences, Inc.), at the concentration of 10 mM. The production of thiobarbituric acid reactive substances (TBARS) was determined according to Wallin *et al.* [10] by adding 50 μL of 50% trichloracetic acid and 75 μL of 1.3% thiobarbituric acid in 0.3% NaOH as previously described [5]. Tubes were heated at 80° for 35 min and centrifuged after cooling. The absorbance was measured at 535 nm. The concentration of TBARS was expressed as nmol of malondialdehyde equivalents produced in the preparation using a freshly diluted 1,1,3,3-tetra-ethoxypropane for the standard curve.

Other Methods

The protein content of LP fractions was determined according to Bradford [11] using the Biorad Protein Assay (Biorad) with BSA (fraction V) as standard. Total lipids were extracted according to Folch *et al.* [12]. The phospholipid content was estimated from the phosphorus content (P × 25) measured by the method of Bartlett [13].

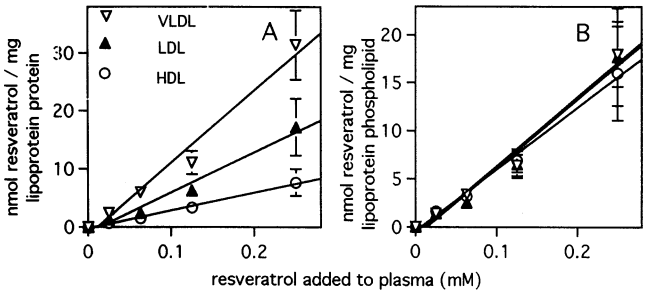

FIG. 1. Distribution of resveratrol added to plasma among subsequently isolated lipoprotein classes. Porcine plasma was supplemented with various concentrations of resveratrol and incubated for 1 hr in the dark. Lipoprotein classes VLDL, LDL and HDL were isolated by sequential ultracentrifugation, and resveratrol was extracted with ethyl acetate. Concentrations in lipoproteins were expressed either on a protein basis (A) or on a phospholipid basis (B). Results are means (±SD) of 5 independent experiments. Differences between classes were significant in (A) and not significant in (B) at $P < 0.05$.

Analysis of Data

Results are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA and Fisher's least significance difference test at the $P < 0.05$ level of significance. The unpaired Student's *t* test was used when only two groups were compared.

RESULTS

Figure 1 shows that resveratrol added to plasma at various concentrations (ranging from 0.025 to 0.25 mM) was recovered in subsequently isolated LP in a dose-dependent manner. The percentage of recovery of amounts added to plasma was: VLDL, 0.20 ± 0.04%; LDL, 0.58 ± 0.18%; and HDL, 2.05 ± 0.61 ($n = 5$). As expressed on a protein basis, the concentrations increased with their lipid content: HDL < LDL < VLDL (Fig. 1A). The recovery was also expressed on a phospholipid basis by using estimated values of the phospholipid content of lipoproteins. Values were obtained by measuring the phospholipid-to-protein ratio in several samples of porcine lipoproteins. The values of the ratio were: VLDL, 1.75 ± 0.06; LDL, 0.97 ± 0.15; and HDL, 0.48 ± 0.03 ($n = 5$). As shown in Fig. 1B, there was no significant difference between lipoprotein classes with the linear increase of recovery as a function of the added dose: the concentration was approximately 18 nmol/mg phospholipid for the highest dose (0.25 mM) added to plasma (*ca.* 14 nmol/μmole phospholipid of mean M_r = 775).

TABLE 2. Binding of resveratrol to lipoproteins (*d* < 1.21 g/mL) and BSA

	Lipoproteins d < 1.21 g/mL	BSA	*P*
Ultrafiltrate			
nmol/mL	13.4 ± 1.6	56.9 ± 10.2	
% recovery	5.3 ± 0.6	21.6 ± 3.9	0.0002
Residue			
nmol/mg protein	40.9 ± 1.7	31.5 ± 2.3	
% recovery	87.6 ± 3.6	69.4 ± 5.0	0.001
% Total recovery	93.0 ± 4.1	91.0 ± 4.7	NS

A volume of 2 mL of either lipoprotein from 20 mL porcine plasma or BSA (5.4 μg protein/mL in each) was incubated for 1 hr in the presence of resveratrol (final concentration 0.25 mM). The preparation was ultrafiltered on filter which excluded molecules > 10 kDa and the resveratrol content was determined in both ultrafiltrate and residue. Values are means (±SD) of four assays. Significance between the percentage of recoveries was assessed by an unpaired Student's *t* test. NS = not significant ($P > 0.05$).

The distribution of resveratrol between total LP (d < 1.21 g/mL) and non-LP plasma proteins (d > 1.21 g/mL) is reported in Table 1. Quantitatively, the major fraction of resveratrol was associated with non-LP proteins. Indeed, after ultracentrifugation of plasma supplemented with resveratrol, the mean recoveries were: 76.4 ± 13.2%, 0.62 ± 0.20% and 3.72 ± 0.47% in infranatant (d > 1.21 g/mL), intermediate fraction (low amounts of a mixture of the denser LP and albumin) and supernatant (LP, d < 1.21 g/mL), respectively. However, as expressed relative to proteins, the LP fraction contained 2.5-fold more resveratrol (5.5 ± 0.7 nmol/mg) than the protein fraction (2.2 ± 0.4 nmol/mg). The total recovery was *ca.* 80%. Because the solubility of resveratrol in aqueous solutions is not negligible, the distribution of resveratrol between fractions of plasma did not exactly reflect the association with either LP particles or proteins. In order to specify the partitioning between the aqueous phase and the residue, a defined volume of either total LP (d < 1.21 g/mL) isolated from plasma or serum albumin (BSA) at the same protein concentration was supplemented with resveratrol and ultrafiltered.

TABLE 1. Recovery of resveratrol in fractions of porcine plasma

Resveratrol in plasma	Supernatant LP d < 1.21 g/mL	Intermediate fraction	Infranatant
Recovery (μmol)	0.19 ± 0.02	0.03 ± 0.01	3.82 ± 0.66
Recovery (%)	3.72 ± 0.47	0.62 ± 0.20	76.4 ± 13.2
Protein in fraction from plasma (mg/mL)	1.7 ± 0.15	1.1 ± 0.41	86.9 ± 4.9
Protein (nmol/mg)	5.5 ± 0.7	1.6 ± 0.3	2.2 ± 0.4

Resveratrol was added to 20 mL of porcine plasma (final concentration 0.25 mM). After ultracentrifugation (145,000 *g*) at the density of 1.21 g/mL for 36 hr, three fractions were obtained: supernatant, containing total lipoproteins of d < 1.21 g/mL (2 mL); protein-poor intermediate fraction (7 mL); infranatant containing the majority of plasma proteins (13 mL). Values are means (±SD) of 12 assays.

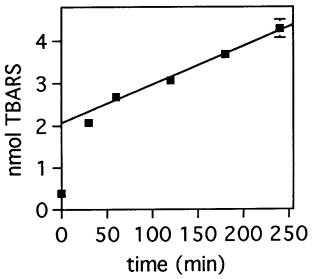

FIG. 2. Time-course of TBARS production in phospholipid liposomes. Soybean phosphatidylcholine unilamellar liposomes were prepared by sonication of multilamellar vesicles in Tris-HCl buffer. The oxidation induced by 10 mM AAPH was performed at 37°, and the production of TBARS was determined at different times up to 250 min. Values expressed as nmol malondialdehyde produced in the medium are means (± SD) of four assays (in triplicate) with different preparations of liposomes.

As shown in Table 2, the major fraction of resveratrol remained in the residue. There were no significant differences between preparations for the total recovery of resveratrol (91–93%), but large differences were found between recoveries in aqueous phase and residue. The values of partition coefficient were 0.06 and 0.3 for LP and BSA, respectively. The finding that resveratrol had a greater capacity to bind to lipoproteins than to proteins confirms data reported in Table 1.

Figure 2 shows that the TBARS content of unilamellar liposomes increased during incubation. Phospholipid PUFA were practically not peroxidized during the preparation of liposomes, as assessed by the very low amounts of TBARS found at time zero (0.3 nmol). The time 120 min, which is situated in the linear portion of the curve, was chosen to test the activity of antioxidants. In assays using liposomes supplemented with resveratrol, the dose-response curve for the decrease in TBARS production was linear up to 30 μM ($r = -0.99$) (Fig. 3A). Higher concentrations had little effect on the response, which did not change between 200 and 1000 μM (not shown). At the same concentrations, trolox was far less active. The dose-response curve was also linear up to 30 μM.

The addition of resveratrol to phospholipids prior to sonication favored the incorporation of the compound within membrane structures. Figure 3B shows that the dose-response curve was linear up to 200 μM ($r = -0.99$). The response obtained with α-tocopherol resembled that obtained with resveratrol, but the slope began to decrease at a lower concentration.

DISCUSSION

Our findings show that in lipoproteins, resveratrol is predominantly associated with their lipid moiety. Likewise, the distribution of α-tocopherol closely correlates with the total lipid content of LP [14]. In this study, we did not specify the distribution of resveratrol between lipid components. By calculating the concentration relative to lipoprotein phospholipids, we found that values linearly increased with the dose added to plasma without differences between lipoprotein classes. This observation supports the view that a fraction of resveratrol was able to associate with surface phospholipids in a defined ratio independently of the proportions of other components. On the other hand, our data related to the distribution of resveratrol between plasma LP and non-LP proteins support the hypothesis that the compound can associate with proteins. The existence of intermolecular bonds between polyphenols and proteins was demonstrated for quercetin, a flavonol which has a high affinity for albumin [15]. Interactions may be hydrophobic and involve phenolic groups to form hydrogen bonds.

We specified the partition between solubilized and associated forms by ultrafiltrating preparations of either LP ($d < 1.21$ g/mL) or BSA supplemented with the compound. As previously observed [5], less resveratrol was solubilized in either LP or protein solutions than in pure water. This likely resulted from interactions with the bulk solution. The higher recovery of resveratrol in the LP than in the protein residue likely reflects the association of resveratrol with both lipid and protein moieties of LP. This may also depend on differences between binding capacities of proteins.

We also investigated the capacity of resveratrol to act independently of protein binding. The capacity of resveratrol to inhibit the peroxidation of liposomal and microso-

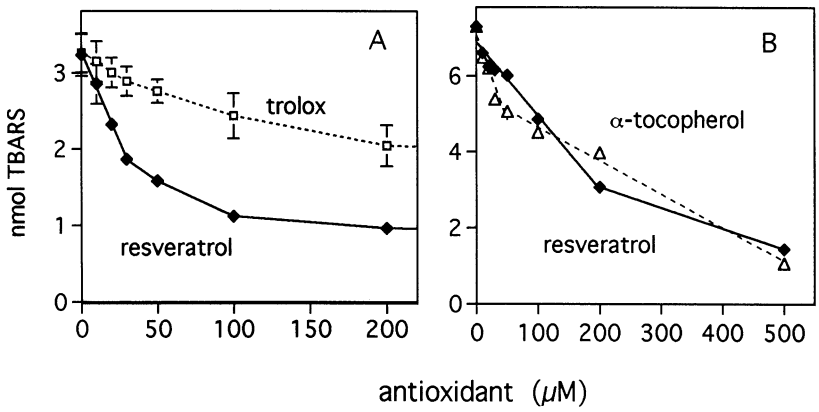

FIG. 3. Inhibition of the oxidation of phospholipid liposomes by various concentrations of resveratrol, trolox or α-tocopherol. The assay was carried out as described in Fig. 2. In (A), resveratrol (black diamonds) or trolox (white squares) was added to the final preparation of liposomes. In (B), resveratrol (black diamonds) or α-tocopherol (white triangles) was added to phospholipids prior to the formation of liposomes. The production of TBARS was determined after 2 hr oxidation at 37°.

mal membranes has been previously reported [16]. The dose-response curve obtained when the compound was added to unilamellar liposomes may be compared to that obtained with water-soluble antioxidants. Actually, in the presence of the water-soluble initiator AAPH, uric acid and ascorbic acid scavenge free radicals in the aqueous phase before they attack PUFA [17]. The lower efficiency of resveratrol at concentrations higher than 30 μM may be due to the decrease in the amount of resveratrol solubilized in the aqueous phase. The dose-response curve was linear up to a concentration seven-fold higher when resveratrol was added prior to the formation of liposomes rather than in the final preparation. This finding suggests that a fraction of the compound was well incorporated within membranes. Hence, it could scavenge free radicals at two levels: in the soluble phase (before they attack liposomes) and inside membranes (when they reach PUFA).

Taken together, the present data suggest that, at least *in vitro*, resveratrol is distributed between lipid, protein and aqueous phases. If *in vivo*, resveratrol is present in LDL particles and in their environment, it can potentially scavenge free radicals and chelate copper.

We previously found that resveratrol was a potent chelator of free copper ions [5]. Considering that the formation of LDL-Cu complexes plays a major role in the copper-catalyzed peroxidation of LDL [18], it would be of interest to investigate the capacity of resveratrol to remove copper bound to apoB. Such an effect has been reported for EDTA, which prevents the lipid peroxidation of human LDL-Cu complexes [18].

In humans, it has not been established to what extent the compound is absorbed by the intestine and whether circulating forms are identical to ingested forms (*cis-* and *trans-*isomers). Depending on the grape origin and wine-making practices, the concentration in wine ranges between 0.5 and 15 mg/L as *cis-* and *trans-*isomers [19–23]. We have previously established that both forms equally scavenge free radicals, whereas the *trans* form is a better chelator of copper than the *cis* form [5]. The present study was limited to the effects of the *trans* isomer. Therefore, the questions as to whether isomerisation occurs *in vivo* and how the two forms are distributed and metabolized and whether they are equally efficient merit investigation.

A recent study carried out in rats [24] showed that after oral administration of red wine containing 6.5 mg/L resveratrol (*cis-* and *trans-*isomers), both forms quickly entered the bloodstream and were incorporated into different organs.

Compared to flavonoids, only small amounts of resveratrol are ingested by wine drinkers. However, as an amphiphilic compound, resveratrol may contribute to their antioxidant potential and thereby reduce the consumption of endogenous antioxidants. This assumption is supported by the finding that several lipophilic and hydrophilic natural compounds are efficient in maintaining the body's stock of α-tocopherol at low concentrations [25]. Although plant extracts containing resveratrol have long been used in Asian folk medicine, the biological properties of the compound have been investigated for only a few years. It has been found that besides its antioxidant effects, resveratrol is capable of inhibiting platelet aggregation and eicosanoid synthesis [26]. It also possesses anticancer properties [27]. Accordingly, resveratrol present in red wine may be beneficial for health.

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