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Resveratrol Inhibition of Lipid Peroxidation

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To define the molecular mechanism(s) of resveratrol inhibition of lipid peroxidation we have utilized model systems that allow us to study the different reactions involved in this complex process. Resveratrol proved (a) to inhibit more efficiently than either Trolox or ascorbate the Fe^{2+} catalyzed lipid hydroperoxide-dependent peroxidation of sonicated phosphatidylcholine liposomes; (b) to be less effective than Trolox in inhibiting lipid peroxidation initiated by the water soluble AAPH peroxyl radicals; (c) when exogenously added to liposomes, to be more potent than α-tocopherol and Trolox, in the inhibition of peroxidation initiated by the lipid soluble AMVN peroxyl radicals; (d) when incorporated within liposomes, to be a less potent chain-breaking antioxidant than α-tocopherol; (e) to be a weaker antiradical than α-tocopherol in the reduction of the stable radical $DPPH^{\bullet}$. Resveratrol reduced Fe^{3+} but its reduction rate was much slower than that observed in the presence of either ascorbate or Trolox. However, at the concentration inhibiting iron catalyzed lipid peroxidation, resveratrol did not significantly reduce Fe^{3+}, contrary to ascorbate. In their complex, our data indicate that resveratrol inhibits lipid peroxidation mainly by scavenging lipid peroxyl radicals within the membrane, like α-tocopherol. Although it is less effective, its capacity of spontaneously entering the lipid environment confers on it great antioxidant potential.

Keywords: Resveratrol, lipid peroxidation, iron, AAPH, AMVN, phosphatidylcholine liposomes

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl; LOOH, lipid hydroperoxide; Mes, morpholineethanesulphonic acid; PC, phosphatidylcholine

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring phytoalexin found in juice and wine from dark skinned grape cultivars,[1] which has been reported to exert a variety of pharmacological effects. For example, it alters the CD95 signaling-dependent apoptosis in human tumor cells,[2] suppresses cell transformation and induces apoptosis through a p53-dependent pathway,[3] arrests proliferation and induces differentiation of the promyelocytic cell line HL-60,[4] inhibits cyclooxygenase-2 transcription[5] and affects arachidonate metabolism.[6,7] These activities may be important for the anti-cancer,[8]

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anti-inflammatory[9] and anti-platelet[10] properties of resveratrol.

Originally, this molecule attracted interest as the likely active ingredient of wine that might explain the "French paradox", i.e. the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis.[11] Its inhibitory effect on the pathogenesis of atherosclerosis was attributed to the protection, *in vitro*, of human LDL against copper-catalyzed oxidation.[12,13] This protection resulted greater than that exerted by the natural chain-breaking antioxidant α-tocopherol.[12–14] The *in vitro* efficiency of resveratrol was found to be more due to its copper chelation than to free radical scavenging.[15,16] However, recent results obtained *in vivo*, cast doubts on resveratrol effectiveness in preventing serum lipid peroxidation.[17,18] Moreover, a few reports question the proposed molecular mechanism responsible for its action.[12,15]

It thus appears that the experimental data so far obtained give an unclear picture of both the relative antioxidant potency of this compound and its mechanism(s) of action. These informations would be important to ascertain whether the pharmacological effects attributed to resveratrol may be related to its antioxidant activity or to other molecular mechanisms not involving the free radical-dependent pathways of cell proliferation, differentiation and apoptosis.[19–21] To contribute to the comprehension of resveratrol action we studied its effect on model liposomes and in experimental systems, previously well characterized,[22–24] that are suitable to define the target and molecular mechanism(s) of the antioxidant action of this naturally occurring agent with antioxidant capacity.

MATERIALS AND METHODS

Chemicals

Morpholineethanesulphonic acid (Mes), $FeCl_2$, $FeCl_3$, ascorbic acid, Trolox, α-tocopherol, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), resveratrol and all other chemicals, of the highest grade available, were purchased from Sigma-Aldrich (Milano, Italy). Egg phosphatidylcholine was from Lipid Products (Redhill, U.K.) and 1,10-phenanthroline was from Merck (Darmstadt, Germany). The thermolabile azo compounds 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were obtained from Polysciences Inc. (Warrington, PA, USA). All reagents were prepared in Chelex resin-treated distilled water whose pH was brought to 6.5 with HCl.

Liposome Preparations

The standard sonicated egg yolk phosphatidylcholine liposomes (PC) were prepared as previously described.[22–24] The phospholipids (about 18 mg), dried under nitrogen, were added with 6 ml distilled water, vortex mixed for 10 min and stored at 4°C for 1 h. This suspension was subsequently sonicated with a titanium probe sonicator (Labsonic 2000) for 2 min. The vesicle dispersion was then transferred to a small-volume extrusion apparatus (produced by Avestin, Ottawa, Canada) which allowed the extrusion of large unilamellar vesicles through standard 19 mm polycarbonate filters with 0.1 mm pore size. Usually samples were subjected to 37 passes through two filters mounted in tandem in the mini-extruder, as recommended by Hope *et al.*[25] This procedure makes possible the preparation of large unilamellar vesicles (LUVETs) with an average diameter close to the pore size. All extrusion procedures were conducted at 20°C. The multilamellar liposomes containing the azo derivative initiator of lipoperoxidation, AMVN, were prepared in a round bottom tube by adding in the following order: AMVN and, when present, the antioxidant to test followed by PC.[26] After each addition the solvent was carefully removed with a stream of nitrogen in ice. The thin film obtained after evaporation was vortex mixed for 10 min with an aliquot of 5 mM Mes buffer pH 6.5, 0.1 mM EDTA in order to obtain 2 mM AMVN, 8 mM PC and the amount of antioxidant as defined in the

legends to figures. Appropriate blanks lacking the thermal initiator were also prepared. Lipid peroxidation was triggered by incubating the solutions in a water bath at 41°C. The reaction vessels were kept under air in the dark. In the experiments using water soluble AAPH, the initiator (17 mM) was added to the vortex mixed liposome suspension at the beginning of the measurement, therefore no special care was taken during liposome preparation. The phospholipid content of the liposome suspensions was determined by the method of Marinetti.[27]

Fe^{2+} Determination

Measurement of Fe^{2+} concentration was made by the *o*-phenanthroline method according to Mahler and Elowe.[28] All assays were carried out in 1 ml of 5 mM Mes buffer, pH 6.5. The reactions, initiated by $FeCl_2$ addition, were conducted at room temperature, stopped by the addition of 0.2 ml 25 mM 1,10-phenanthroline and A_{515} was immediately read. The concentrations of the components of the reaction mixtures and the incubation conditions are given in the figure legends. Fe^{3+} reduction was determined by measuring Fe^{2+} generated in 1 ml of 5 mM Mes buffer, pH 6.5 in the presence of 0.2 ml 25 mM 1,10-phenanthroline.

Oxygen Consumption

Lipid peroxidation was measured, indirectly, by monitoring oxygen consumption with a Clark-type electrode. Reaction mixtures containing 5 mM Mes buffer, pH 6.5 and the liposomes were continuously stirred in a 3 ml sealed chamber where the O_2 electrode was immersed via a sealed port. Additions were made via a resealable port on top of the chamber.

Oxidation Index of Liposomes

Lipid peroxidation was measured by determining the oxidation index of the liposomes.[29] The samples containing 100–150 μg phospholipid were extracted with 1 ml butan-1-ol. Phases were separated by centrifugation at 3000 rpm for 10 min and the 200–300 nm ultraviolet spectrum of the upper organic phase was recorded against appropriate blanks containing all reagents but liposomes. The oxidation index of the liposomes was determined by the $A_{234\,nm}/A_{215\,nm}$ ratio.

DPPH• Reduction

Antioxidant solution in methanol (0.75 ml) was added to 1.5 ml of DPPH• solution (20 mg/l in methanol). The blank contained 0.75 ml methanol. The decrease in absorbance of DPPH• was determined at 517 nm after 5 min incubation at room temperature. The percentage of DPPH• reduction was calculated as follows: Reduction percentage $= 1 - A_{compound}/A_{blank}$.[30,31]

RESULTS

Iron Catalyzed Lipid Peroxidation

PC liposomes prepared by sonolysis, when incubated with increasing concentrations of $FeCl_2$ in 5 mM Mes buffer, pH 6.5, oxidize the metal (Figure 1). The pattern of Fe^{2+} oxidation by PC liposomes, after 10 min incubation, was biphasic: as $FeCl_2$ concentration was increased, at first Fe^{2+} oxidation increased, reached a maximum then decreased. The addition to the reaction mixture of resveratrol (50 μM) in ethanol inhibited Fe^{2+} oxidation by PC liposomes whereas an equimolar amount of α-tocopherol was ineffective. Also Trolox and ascorbate strongly reduced Fe^{2+} oxidation. The extent of the inhibition exerted by resveratrol, Trolox and ascorbate on lipid peroxidation, measured both as Fe^{2+} oxidation and increase of the oxidation index of liposomes, was dose-dependent (Figure 2) and resveratrol, with an IC_{50} value of 0.5 μM was the most effective.

As shown in Figure 3, incubation of 150 μM $FeCl_3$ in 5 mM Mes buffer, pH 6.5 with the antioxidants under test resulted in its reduction to Fe^{2+}: in the presence of 100 μM either ascorbate

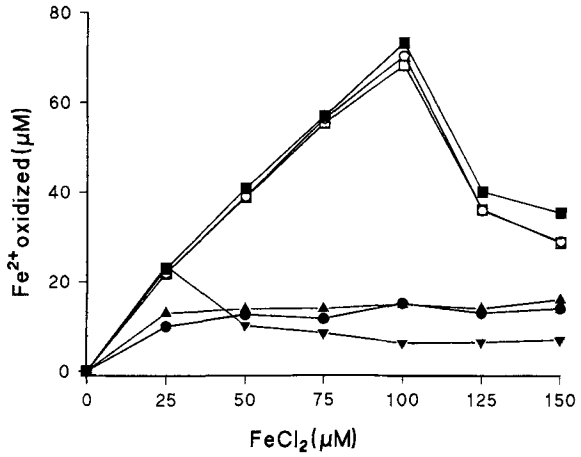

FIGURE 1 Effect of resveratrol on the peroxidation of PC liposomes. Increasing concentrations of $FeCl_2$ were incubated with PC liposomes (150 μg phospholipid), in 5 mM Mes buffer, pH 6.5 in the absence (■) and presence of 50 μM resveratrol (▲), the ethanol vehicle (final concentration in the assay 43 mM) of the drug (□), 50 μM α-tocopherol (○), 50 μM Trolox (●), 50 μM ascorbate (▼). The Fe^{2+} oxidized by the liposomes after 10 min incubation was measured. Values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 5% of the mean. The results are representative of ten experiments with different PC liposome preparations.

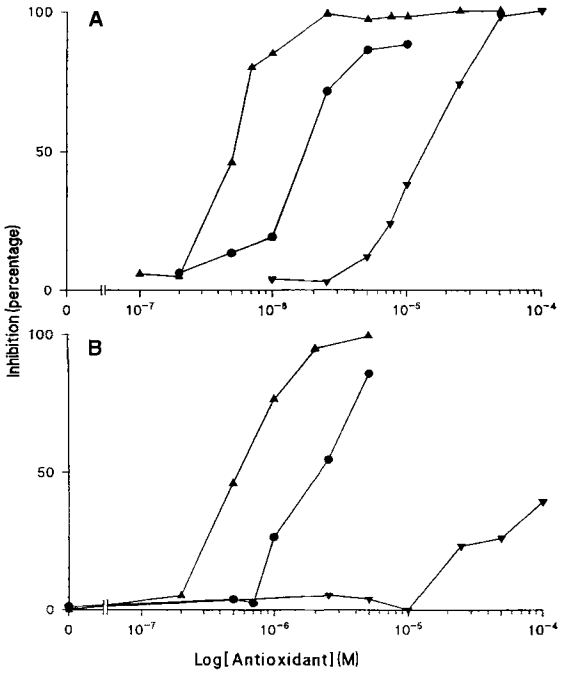

FIGURE 2 Effect of resveratrol concentration on the peroxidation of PC liposomes. PC liposomes (150 μg phospholipid) were incubated in 5 mM Mes buffer, pH 6.5 with 100 μM $FeCl_2$ and increasing concentrations of resveratrol (▲), Trolox (●), ascorbate (▼). Panel A. Peroxidation was evaluated by measuring, after 10 min incubation, Fe^{2+} oxidized by the liposomes. Panel B. Peroxidation was evaluated by measuring the oxidation index of the liposomes. The values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 15% of the mean. The results are representative of four experiments with different PC liposome preparations.

or Trolox a rapid kinetic behaviour was observed whereas α-tocopherol and resveratrol, at the same concentration, reacted more slowly. In a similar manner resveratrol could reduce Cu^{2+} to Cu^{1+} (result not shown).

Sonicated PC liposomes were incubated in 5 mM Mes buffer, pH 6.5, with 125 μM $FeCl_2$ (Table I). Peroxidation was evaluated by change in the oxidation index of liposomes. After a rapid peroxidation during the first 2 min incubation, the rate of the process decreased; a delayed addition of 100 μM $FeCl_3$ after 2 min incubation and a further incubation up to 10 min, exerted a stimulatory effect which was counteracted by the simultaneous addition of each of the antioxidants under test. PC liposomes were also incubated with 100 μM $FeCl_3$ in the absence of $FeCl_2$ (Table I); in this experimental condition no peroxidation occurred and, among the antioxidants tested, only ascorbate could elicit lipid peroxidation whereas the others were uneffective.

Azo Compound Initiated Lipid Peroxidation

The peroxidation of PC liposomes was triggered by the decomposition of the hydrophilic azo compound AAPH. AAPH (17 mM) was exogenously added to PC liposomes (8 mM) in 5 mM Mes buffer, pH 6.5, 0.1 mM EDTA. Peroxidation was monitored by measuring the oxygen uptake associated to this process (Figure 4). PC liposomes were oxidized at a constant rate. In the presence of the water soluble chain-breaking antioxidant Trolox (3 and 10 μM) a clear induction period occurred and after all the antioxidant had been depleted, the reaction proceeded at the same rate observed in its absence. Resveratrol (10 and

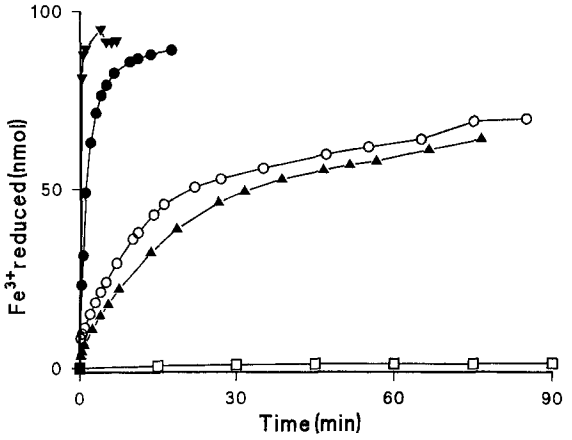

FIGURE 3 Fe^{3+} reduction by resveratrol. $FeCl_3$ (150 μM) was incubated in 1 ml of 5 mM Mes buffer, pH 6.5 containing 5 mM 1,10-phenanthroline, with 100 μM resveratrol (▲), the ethanol vehicle (final concentration in the assay 86 mM) of the drug (□), 100 μM α-tocopherol (○), 100 μM Trolox (●) and 100 μM ascorbate (▼). At the time stated A_{515} was read. Values plotted are means of triplicate assay; SE never exceeded 5% of the mean. The results are representative of three experiments.

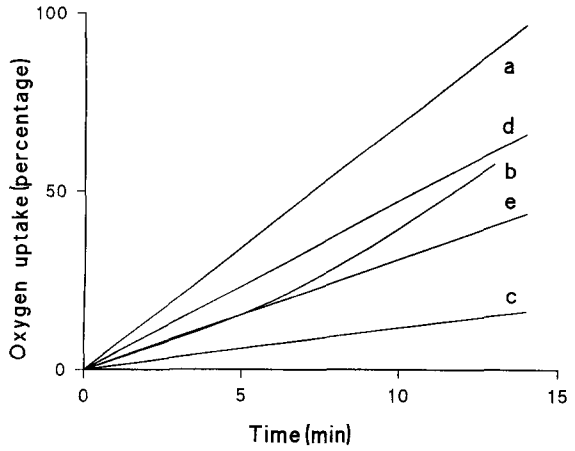

FIGURE 4 Effect of resveratrol on the peroxidation of PC liposomes initiated by AAPH. PC liposomes (15 mM phospholipid) prepared by vortex mixing for 10 min in 5 mM Mes buffer, pH 6.5, 0.1 mM EDTA were incubated at 40°C with 17 mM AAPH in the absence (line a) or presence of either α-tocopherol (3 μM line b, 10 μM line c) or resveratrol (10 μM line d, 20 μM line e). Peroxidation was evaluated by oxygen consumption. The results are representative of four experiments.

TABLE I Antioxidants and $FeCl_3$ influence on the peroxidation of PC liposomes

Assay condition					Oxidation index A_{234}/A_{215}
PC liposomes					0.21 ± 0.03
PC liposomes	+125 μM $FeCl_2$	t_2			0.39 ± 0.02
PC liposomes	+125 μM $FeCl_2$		+100 μM $FeCl_3$		0.76 ± 0.02
PC liposomes	+125 μM $FeCl_2$		+100 μM $FeCl_3$	+1 μM resveratrol	0.24 ± 0.06
PC liposomes	+125 μM $FeCl_2$		+100 μM $FeCl_3$	+1 μM Trolox	0.33 ± 0.03
PC liposomes	+125 μM $FeCl_2$		+100 μM $FeCl_3$	+50 μM ascorbate	0.37 ± 0.02
PC liposomes	+125 μM $FeCl_2$	t_{10}			0.42 ± 0.03
PC liposomes	+100 μM $FeCl_3$				0.22 ± 0.03
PC liposomes	+100 μM $FeCl_3$		+1 μM resveratrol		0.20 ± 0.03
PC liposomes	+100 μM $FeCl_3$		+1 μM Trolox		0.21 ± 0.02
PC liposomes	+100 μM $FeCl_3$		+50 μM ascorbate		0.76 ± 0.02

PC liposomes (140 μg phospholipid) were incubated in 5 mM Mes buffer, pH 6.5 and peroxidation was evaluated by measuring their oxidation index after 10 min incubation (unless stated). The values plotted are means of triplicate assay with the same PC liposome preparation. The results are representative of three experiments with different PC liposome preparations.

20 μM) reduced the peroxidation rate with respect to the control experiment; however an induction period was not observed at the concentrations tested. The peroxidation of PC liposomes was also initiated by the thermal decomposition of the lipid soluble azo initiator AMVN incorporated in PC liposomes (Figure 5). The effect of the compartimentation of the antioxidant was also evaluated. Peroxidation was monitored by measuring change in the oxidation index of liposomes. Control liposomes were oxidized at a constant rate. When 10 μM resveratrol was exogenously added to the reaction mixture it reduced the peroxidation rate with respect to the control experiment; the exogenous addition of 10 μM α-tocopherol was less effective. Exogenously added ascorbate (100 μM) caused a modest decrease in the peroxidation rate (result not shown) whereas

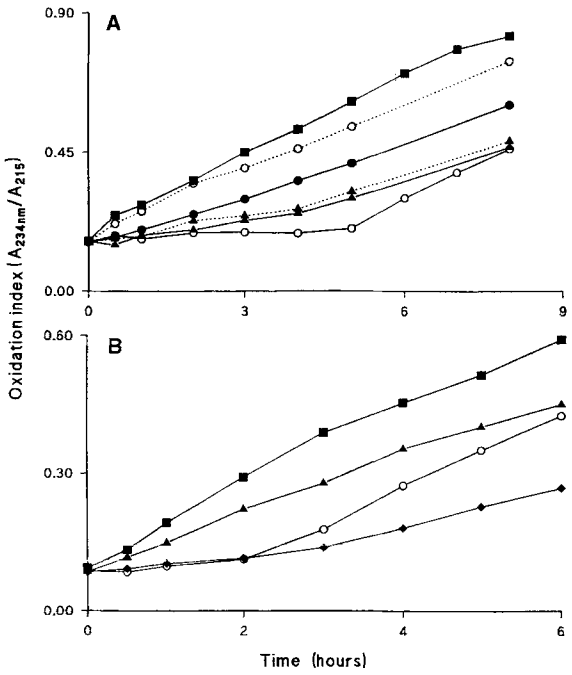

FIGURE 5 Effect of resveratrol on the peroxidation of PC liposomes initiated by AMVN. PC liposomes (8 mM phospholipid) containing 2 mM AMVN were prepared by vortex mixing for 10 min in 5 mM Mes buffer, pH 6.5, 0.1 mM EDTA. Peroxidation was evaluated by change in the oxidation index of liposomes. Panel A. The PC liposome suspension was incubated at 41°C in the absence (■) or presence of 10 μM resveratrol (-▲- incorporated, ..▲.. exogenously added), 10 μM α-tocopherol (-○- incorporated, ..○.. exogenously added), 10 μM Trolox (● exogenously added). Panel B. The PC liposome suspension was incubated at 41°C in the absence (■) or presence of 4 μM resveratrol (▲ incorporated), 4 μM α-tocopherol (○ incorporated), 4 μM resveratrol and 4 μM α-tocopherol (◆ incorporated). At the stated time 50 μl of the suspension were added to 1 ml butan-1-ol. Peroxidation was evaluated by measuring the oxidation index of the liposomes. Values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 15% of the mean. Student's *t*-test for difference of means for paired samples was performed. All treatments gave significantly different values from corresponding controls ($p < 0.01$), except exogenously added α-tocopherol in Figure 5A ($p < 0.05$). The results are representative of four experiments with different PC liposome preparations.

Trolox (10 μM) decreased AMVN initiated lipid peroxidation but it was less effective than resveratrol (Figure 5A). However, when resveratrol and α-tocopherol were incorporated within the liposomes, contrasting results were observed: while resveratrol effect did not significantly differ,

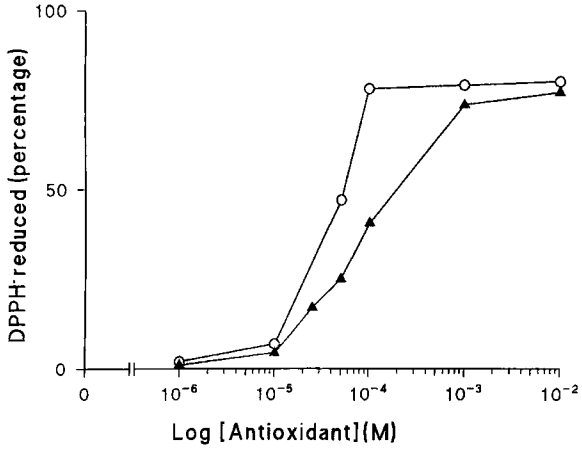

FIGURE 6 Effect of resveratrol concentration on DPPH• reduction. DPPH• (30 μg in methanol) were incubated with increasing concentrations of either resveratrol (▲) or α-tocopherol (○) for 5 min. The percentage of DPPH• reduction was calculated as follows: Reduction percentage = $1 - A_{compound}/A_{blank}$. Values plotted are means of triplicate assay; SE never exceeded 5% of the mean. The results are representative of five experiments.

a strong inhibition of the peroxidation rate and a clear induction period was evident in the presence of α-tocopherol (Figure 5A and B). When resveratrol (4 μM) and an equal amount of α-tocopherol were incorporated together in AMVN containing PC liposomes, the induction time ascribable to α-tocopherol was unchanged (Figure 5B). However a very strong inhibition of lipid peroxidation was observed, apparently resulting from the additive effects of the two antioxidants.

DPPH• Reduction

The stable radical DPPH• in methanolic solution was incubated with increasing concentrations of the antioxidants under test. The reduction of this compound was monitored. As shown in Figure 6, α-tocopherol proved to be a better antioxidant than resveratrol.

DISCUSSION

To assess the molecular mechanisms of resveratrol inhibition of lipid peroxidation we have first

utilized a well characterized experimental model that consists of PC liposomes whose peroxidation is triggered by $FeCl_2$ in 5 mM Mes buffer, pH 6.5.[22–24] As shown in Figures 1 and 2 resveratrol apparently is a better antioxidant than Trolox and α-tocopherol in inhibiting the Fe^{2+} catalyzed lipid peroxidation. These results are consistent with those obtained by Frankel's group[12,13] and by Belguendouz *et al.*[15] when studying the effect of resveratrol on LDL oxidation by copper. The latter authors proposed the copper chelating property of resveratrol as the main cause of its higher capacity to inhibit LDL peroxidation. However, theirs[15] and Frankel's[12] observations that a complete inhibition of LDL oxidation occurred in the presence of 8–10 mol excess of the copper catalyst over resveratrol concentration, question this conclusion. Besides, our data obtained utilizing Fe^{2+}, a metal catalyst which is not chelated by resveratrol,[15] further weaken the hypothesis that the good antioxidant activity of resveratrol is mainly due to its metal chelating activity.

We further studied the Fe^{2+} catalyzed lipid peroxidation to verify the hypothesis that resveratrol, with its three hydroxyl groups, may interfere with the oxidation state of the metal catalyst. It is known, in fact, that the extent and the kinetics of lipid peroxidation are strictly controlled by Fe^{2+}/Fe^{3+} ratio:[23,24,32,33] they are maximal at the critical Fe^{2+}/Fe^{3+} ratio and decrease when it shifts to either lower or higher values (Figure 1). It is also known that superoxide dismutase, catalase, ascorbate and in general reducing agents, can influence lipid peroxidation by affecting iron reduction/oxidation and the formation of the permissive Fe^{2+}/Fe^{3+} ratio.[33,34] To assess this possibility we checked, first, whether resveratrol can reduce the metal catalyst. The results presented in Figure 3 clearly demonstrates that resveratrol, although less effectively than ascorbate and Trolox, can reduce Fe^{3+}. It is, thus, theoretically possible that resveratrol, like ascorbate, may alter the Fe^{2+}/Fe^{3+} ratio. To test this possibility we performed two sets of experiments (Table I). In the first set, the effect of resveratrol and of the other antioxidants was studied in an experimental condition where a high $FeCl_2$ concentration inhibits lipid peroxidation; this inhibition, as expected, is reverted by $FeCl_3$ addition (Table I). When the experiments were conducted in the presence of the antioxidants, the stimulation of lipid peroxidation by $FeCl_3$ was not observed. This result does not necessarily mean that the compounds, like ascorbate, exert their inhibition by reducing Fe^{3+}. It may be due to their ability to scavenge the lipid radicals generated in the presence of Fe^{3+}. Thus, we performed a second set of experiments where resveratrol and the other antioxidants were tested in an experimental condition where, in the absence of Fe^{2+}, $FeCl_3$ does not elicit lipid peroxidation (Table I). In this experimental condition the presence of a reducing agent, that favours the attainment of the critical Fe^{2+}/Fe^{3+} ratio, should result in a strong stimulation of lipid peroxidation. Of the antioxidant tested only ascorbate, as expected, was stimulatory. These results, in their complex, indicate that resveratrol, unlike ascorbate, does not appear to significantly exert its antioxidative potential by changing the oxidation state of the metal.

We have previously shown that, in the experimental conditions utilized in this study, there is no generation of inorganic oxygen radicals able to initiate lipid peroxidation and, thus, only the lipid hydroperoxide (LOOH)-dependent peroxidation may take place.[23,24,35] This excludes the possibility that the inhibition of lipid peroxidation exerted by resveratrol, and at large by the other antioxidants, occurs by scavenging inorganic oxygen-derived initiators such as $O_2^{\bullet -}$ and $^{\bullet}OH$.[18]

The LOOH-dependent initiation which occurs in our experimental conditions, is triggered by the reductive cleavage of LOOH present in the liposomes, by added $FeCl_2$. The organic alkoxyl radicals generated on the surface and within the liposomes initiate and support the propagation and chain-branching reactions of lipid peroxidation. The effectiveness of resveratrol in inhibiting the metal catalyzed lipid peroxidation, similarly

to Trolox and contrary to the exogenously added α-tocopherol (Figures 1 and 2) suggests that this compound may exert some effect in the aqueous phase by scavenging water soluble/water interacting organic initiators. To assess this possibility we studied resveratrol effect on lipid peroxidation initiated by the water soluble-peroxyl radicals generated by the decomposition of the azo compound AAPH. The results presented in Figure 4 are consistent with those obtained by Belguendouz *et al.*[15] when studying AAPH-mediated LDL oxidation. They indicate that resveratrol is less effective than Trolox in AAPH initiated lipid peroxidation and, thus, demonstrate that resveratrol, contrary to Trolox,[36] is a poor water soluble chain-breaking antioxidant.

We have, then, assessed the possibility that resveratrol may interfere primarily with the lipid phase of peroxidation. To this aim, we studied its effect on the process initiated by the thermal decomposition of the lipid soluble azo initiator AMVN incorporated in PC liposomes. The effect of the compartmentation of the antioxidant was also verified. The results, reported in Figure 5A, point out some interesting aspects. First, resveratrol proved to be more effective in inhibiting the lipid soluble organic radicals than Trolox. This is probably related to the chemical structure of the two compounds: Trolox is likely to scavenge only the organic radicals floating to the surface of the liposome[37] whereas resveratrol may enter the lipid phase and scavenge also the lipid soluble radicals. Second, when exogenously added, resveratrol was more effective than α-tocopherol in inhibiting lipid peroxidation (see also Figure 1); this agrees with the more potent antioxidant properties of wine phenolics compared to α-tocopherol reported by Frenkel's group[12,13] when studying the copper catalyzed oxidation of human LDL. Third, by contrast, when the two antioxidants were incorporated within the liposomes a stronger inhibition of the peroxidation rate and a clear induction period was evident in the presence of α-tocopherol. These results, on one hand, confirm the well known chain-breaking antioxidant potency of this compound which was confirmed by its better antioxidant potency in scavenging the lipid soluble DPPH$^{\bullet}$ radical (Figure 6). On the other hand, the results emphasize the inability of α-tocopherol to spontaneously localize within the liposomes where it exerts its effect and the need to consider the solubility characteristics of the compounds under test when comparative studies are conducted. In their complex these data indicate that resveratrol is likely to exert its inhibitory effect on lipid peroxidation mainly by entering the lipid phase and scavenging lipid soluble radicals. However, its relatively low potency as chain-breaking antioxidant may let believe as modest its contribution to the plasma antioxidant capacity *in vivo* and draw some doubt on its protective effect in cardiovascular diseases. To help in appraising such contribution we studied the effect of equimolar concentrations of resveratrol and α-tocopherol on AMVN initiated lipid peroxidation (Figure 5B). When the two antioxidants were incorporated together in PC liposomes, the induction time ascribable to α-tocopherol was unchanged suggesting that resveratrol can not regenerate the active form of the vitamin. However, a very strong inhibition of lipid peroxidation was observed apparently resulting from the additive effects of the two antioxidants. Resveratrol in rat, is quickly adsorbed reaching a peak plasma concentration (about 80 μM) approximately 60 min after wine ingestion (80 μg/kg);[38] after prolonged administration (daily dosage of natural resveratrol 40 μg/kg for 15 days) plasma concentration reaches a "steady state" of about 30 μM,[38] value comparable to that of α-tocopherol (about 10 μM in rat[39] and 15–25 μM in human[40]). Apparently, thus, a regular consumption of red wine containing 2–6 mg/l resveratrol can provide plasma with a concentration of this antioxidant able to effectively and significantly contribute to the control of oxidative stress.

In conclusion, our data confirm previous evidences for inhibition of lipid peroxidation by resveratrol. However, our results clarify, for the

first time, that the major molecular mechanism responsible of resveratrol inhibition of lipid peroxidation is neither a scavenging of primary inorganic initiators nor a modulation of metal catalysis by chelation or reduction. Resveratrol interacting with the lipophilic environment, appears to exert its effect by scavenging organic initiators, like α-tocopherol does. In spite of its relatively lower potency as chain-breaking antioxidant, its bioavailability[34] and solubility[15,16] may allow this molecule to reach in plasma and tissues such concentrations to significantly contribute to the antioxidant defence *in vivo*. Besides, its phase partition characteristics[16] may spontaneously localize it in membranes and lipoproteins, bypassing the complex machinery that strictly control their composition.[41] In these lipophilic compartments resveratrol may influence cellular physiology affecting free radical-dependent pathways.

Acknowledgements

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References

[1] P. Langcake and R.J. Pryce (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection and injury. *Physiology and Plant Pathology* **9**, 77–86.

[2] M.V. Clement, J.L. Hirpara, S.H. Chawdhury and S. Pervaiz (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* **92**, 996–1002.

[3] C. Huang, W.Y. Ma, A. Goranson and Z. Dong (1999) Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* **20**, 237–242.

[4] F. Della Ragione, V. Cucciolla, A. Borriello, V. Della Pietra, L. Racioppi, G. Soldati, C. Manna, P. Galletti and V. Zappia (1998) Resveratrol arrests the cell division cycle at S/G2 phase transition. *Biochemical and Biophysical Research Communications* **250**, 53–58.

[5] K. Subbaramaiah, W.J. Chung, P. Michaluart, N. Telang, T. Tanabe, H. Inoue, M. Jang, J.M. Pezzuto and A.J. Dannenberg (1998) Resveratrol inhibits cyclooxygenase-2-transcription and activity in phorbol ester-treated human mammary epithelial cells. *Journal of Biological Chemistry* **273**, 21 875–21 882.

[6] Y. Kimura, H. Okuda and M. Kubo (1995) Effects of stilbenes isolated from medicinal plants on arachidonate metabolism and degranulation in human polymorphonuclear leukocytes. *Journal of Ethnopharmacology* **45**, 131–139.

[7] C.R. Pace-Asciak, S. Haln, E.P. Diamandis, G. Soleas and D.M. Goldberg (1995) The red wine phenolics trans-resveratrol and quercitin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clinica Chimica Acta* **235**, 207–219.

[8] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W. Beecher, H.H. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon and J.M. Pezzuto (1997) Cancer chemoprotective activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218–220.

[9] D.S. Jang, B.S. Kang, S.Y. Ryu, I.M. Chang, K.R. Min and Y. Kim (1999) Inhibitory effects of resveratrol analogs on unopsonized zymosan-induced oxygen radical production. *Biochemical Pharmacology* **57**, 705–712.

[10] M.I. Chung, C.M. Teng, K.L. Cheng, F.N. Ko and C.N. Lin (1992) An antiplatelet principle of *Veratrum formosarum*. *Planta Medica* **58**, 274–276.

[11] E.H. Siemann and L.L. Creasy (1992) Concentration of the phytoalexin resveratrol in wine. *American Journal of Enology and Viticulture* **43**, 49–52.

[12] E.N. Frankel, J. Kanner, J.B. German, E. Parks and J.E. Kinsella (1993) Inhibition of oxidation of human-low density lipoprotein by phenolic substances in red wine. *Lancet* **341**, 454–457.

[13] E.N. Frankel, A.L. Waterhouse and J.E. Kinsella (1993) Inhibition of human LDL oxidation by resveratrol. *Lancet* **341**, 1103–1104.

[14] J. Constant (1997) Alcohol, ischemic heart disease, and the French paradox. *Coronary Artery Disease* **8**, 645–649.

[15] L. Belguendouz, L. Fremont and A. Linard (1997) Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochemical Pharmacology* **53**, 1347–1355.

[16] L. Belguendouz, L. Fremont and M.T. Gozzelino (1998) Interaction of transresveratrol with plasma lipoproteins. *Biochemical Pharmacology* **55**, 811–816.

[17] T. Wilson, T.J. Knight, D.C. Beitz, D.S. Lewis and R.L. Engen (1996) Resveratrol promotes atherosclerosis in hypercholesterolemic rabbits. *Life Sciences* **59**, 15–21.

[18] J.F. Turrens, J. Lariccia and M.G. Nair (1997) Resveratrol has no effect on lipoprotein profile and does not prevent peroxidation of serum lipids in normal rats. *Free Radical Research* **27**, 557–562.

[19] K.K. Griendling and M. Ushio-Fukai (1998) Redox control of vascular smooth muscle proliferation. *Journal of Laboratory and Clinical Medicine* **132**, 9–15.

[20] M.B. Hampton and S. Orrenius (1998) Redox regulation of apoptosis cell death in the immune system. *Toxicology Letters* **102–103**, 355–358.

[21] D.R. Green and J.C. Reed (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312.

[22] B. Tadolini, D. Fiorentini, L. Landi and L. Cabrini (1989) Lipid peroxidation. Definition of experimental conditions for selective study of the propagation and termination phases. *Free Radical Research Communications* **5**, 245–252.

[23] B. Tadolini and G. Hakim (1996) The mechanism of iron(III) stimulation of lipid peroxidation. *Free Radical Research* **25**, 221–227.

[24] B. Tadolini, L. Cabrini, C. Menna, G.G. Pinna and G. Hakim (1997) Iron(III) stimulation of lipid hydroperoxide-dependent lipid peroxidation. *Free Radical Research* **27**, 563–576.
[25] M.J. Hope, M.B. Bally, G. Webb and P.R. Cullis (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochimica and Biophysica Acta* **812**, 55–65.
[26] L. Landi, D. Fiorentini, C. Stefanelli, P. Pasquali and G.F. Pedulli (1990) Inhibition of autoxidation of egg yolk phosphatidylcholine in homogeneous solution and in liposomes by oxidized ubiquinone. *Biochimica and Biophysica Acta* **1028**, 223–228.
[27] G.V. Marinetti (1962) Chromatographic separation, identification and analysis of phosphatides. *Journal of Lipid Research* **3**, 1–20.
[28] H.R. Mahler and D.G. Elowe (1954) Studies on metalloflavoproteins. II The role of iron in diphosphopyridine nucleotide cytochrome c reductase. *Journal of Biological Chemistry* **210**, 165–179.
[29] R.A. Klein (1970) The detection of oxidation in liposome preparations. *Biochimica and Biophysica Acta* **210**, 468–489.
[30] M. Joyeux, A. Lobstein, R. Anton and F. Mortier (1995) Comparative antilipoperoxidant, antinecrotic and scavenging properties of terpenes and biflavones from Ginko and some flavonoids. *Planta Medica* **61**, 126–129.
[31] W. Brand-Williams, M.E. Cuvelier and C. Berset (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie* **28**, 25–30.
[32] E.S. Driomina, V.S. Sharov and Y.A. Vladimirov (1993) Fe^{2+}-induced lipid peroxidation kinetics in liposomes: the role of surface Fe^{2+} concentration in switching the reaction from acceleration to decay. *Free Radical Biology and Medicine* **15**, 239–247.
[33] G. Minotti and S.D. Aust (1987) The requirement of iron(III) in the initiation of lipid peroxidation by iron(II) and hydrogen peroxide. *Journal of Biological Chemistry* **262**, 1098–1104.
[34] G. Minotti and S.D. Aust (1992) Redox cycling of iron and lipid peroxidation. *Lipids* **27**, 219–226.
[35] B. Tadolini and F. Franconi (1998) Carvedilol inhibition of lipid peroxidation. A new antioxidative mechanism. *Free Radical Research* **29**, 377–387.
[36] R.T. Dean, J.V. Hunt, A.J. Grant, Y. Yamamoto and E. Niki (1991) Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radical Biology and Medicine* **11**, 161–168.
[37] J.R. Wardlaw, W.H. Sawyer and K.P. Ghiggino (1987) Vertical fluctuations of phospholipid acyl chains in bilayers. *FEBS Letters* **223**, 20–24.
[38] A.A.E. Bertelli, L. Giovannini, R. Stradi, A. Bertelli and J.P. Tillement (1996) Plasma, urine and tissue levels of *trans*- and *cis*-resveratrol (3,4′,5-trihydroxystilbene) after short-term or prolonged administration of red wine to rats. *International Journal of Tissue Reactivity* **18**, 67–71.
[39] K. Engelhart, A.M. Jentzsch, P. Furst and H.K. Biesalski (1998) Short-term parenteral application of alpha-tocopherol leads to increased concentration in plasma and tissues of the rat. *Free Radical Research* **29**, 421–426.
[40] P.M. Farrell (1980) Deficiency states, pharmacological effects and nutrient requirements. In *"Vitamin E. A Comprehensive Treatise"* (Machlin, L.J. Ed.) M. Dekker Inc., New York and Basel, pp. 520–620.
[41] D.J. Rader and H.B. Brewer Jr. (1993) Abetalipoproteinemia. New insights into lipoprotein assembly and vitamin E metabolism from a rare genetic disease. *Journal of American Medical Association* **270**, 865–869.