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Resveratrol Has No Effect on Lipoprotein Profile and Does Not Prevent Peroxidation of Serum Lipids in Normal Rats

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Trans-resveratrol, one of the antioxidants found in red wine, has been the subject of controversial reports regarding its protective role against cardiovascular diseases. In this study we synthesized trans-resveratrol and injected it to rats (20 and 40 mg/kg body weight, once a day for 21 days, i.p.) to determine its effect on the serum lipid profile. Synthetic trans-resveratrol was an effective antioxidant *in vitro* against hydroxyl radical (I_{50} = 33 μM). Resveratrol treatment, however, did not have any effect on either the lipid profile or on Cu^{+2}-dependent formation of thiobarbituric-acid-reactive substances (TBARS) from protein-associated lipids. Since the amount of resveratrol used in these experiments was orders of magnitude higher than the amounts found in wine, these results suggest that if resveratrol has any effect against coronary heart diseases, it is not related to its antioxidant role on lipids or to changes in lipoprotein profile.

Keywords: Lipid profile, lipid peroxidation, lipoproteins, resveratrol, wine antioxidants

INTRODUCTION

Reactive oxygen species (ROS) have often been implicated in cardiovascular diseases. For example, the damage caused by reperfusion of ischemic tissues is oxygen-dependent and at least part of it is due to an uncontrolled generation of ROS.[1,2] These species have also been impli-cated in atherosclerosis, as they cause oxidation of lipoproteins which in turn accumulate in macrophages, leading to foam cell formation, ultimately causing vascular stenosis.[3–5] The preventive effect of vitamin E, a powerful antioxidant, on coronary heart disease[6–8] provides support for a role of ROS in the pathogenesis of coronary diseases.

Several groups have found an inverse correlation between red wine intake and prevention of cardiovascular diseases.[9] Since red wine is particularly rich in antioxidants including flavonoids and other phenolic compounds, it has been proposed that its protective effect is due in part to these compounds.[10–12] These results are still under careful scrutiny.[13] For example, De Rijke *et al.* compared the susceptibility to Cu^{+2}-

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dependent oxidation of low-density lipoproteins from normal volunteers and concluded that it was not affected by wine intake.[14] One of the reasons for this finding is that, although wine antioxidants are powerful antioxidants *in vitro*,[11] their halflife in serum appears to be rather short.[15]

Resveratrol is one of several antioxidants found in wine[10,16,17] but its proposed effects are the center of a controversy. Some reports suggest that resveratrol is beneficial as it may be responsible for part of the proposed antioxidant effect of red wine *in vivo*.[12,16] Others indicate that since it is present in very low amounts (6 μM), it could not contribute substantially to the total antioxidant capacity of red wine and its effect *in vivo* should be negligible.[10] Other groups have suggested that wine[18] and even resveratrol alone[17] may be beneficial by increasing plasma HDL, which in turn could protect against cardiovascular heart disease. Yet, a recent report has indicated that resveratrol increases atherosclerosis in cholesterol-supplemented rabbits.[19]

In view of the controversy regarding the role of resveratrol *in vivo*, we decided to treat normal rats with synthetic resveratrol and tested its effect on the lipid profile and the susceptibility of protein lipids to oxidation. Our results show that although resveratrol is a powerful antioxidant *in vitro*, it did not change the lipid profile in plasma and did not affect the susceptibility of lipids associated with serum proteins to copper-mediated oxidation.

MATERIALS AND METHODS

Treatment of Rats with Resveratrol

Trans-resveratrol (the isomer found in wine) was synthesized as described elsewhere.[20] Its molecular weight was determined by mass spectrometry (molecular weight 228 daltons, Mass Spectrometry Center, University of South Alabama). The purity of synthetic resveratrol was determined by high performance liquid chromatography and by comparison of physical properties with literature values.[20]

Twenty rats (CD, female, 300 g) were divided in three groups and injected daily (i.p.), for 21 days with either 0.5 ml of propylene glycol (4 animals, control) or either 20 or 40 mg/kg body weight resveratrol (8 animals in each group) using the same volume of propylene glycol.

At the end of these treatments the animals were anesthetized with pentobarbital (50 mg/kg body weight) and sacrificed immediately after taking two blood samples/animal through heart puncture. One of these two samples contained EDTA as anticoagulant and, after centrifugation, the plasma was shipped to Atherotech (Birmingham, AL) for characterization of the lipid profile of every group. The whole procedure was carried out at 4°C and the lipoprotein analysis was carried out within 24 h of collection. The other sample did not have anticoagulant and was used to separate serum for thiobarbituric acid reactive substances (TBARS) determination.

Measurement of Serum Lipid Peroxidation

Rat serum proteins were first separated from other low molecular weight components (*i.e.* ascorbic acid) by gel filtration through Biogel 10DG desalting columns (Biorad, Hercules, CA). The resulting protein fraction was incubated with either 2 or 10 μM $CuSO_4$ at 37°C for 12 hours. The formation of peroxidized lipids was monitored as TBARS as described elsewhere.[21]

Hydroxyl Radical Production

Hydroxyl radical was produced by photolysis of H_2O_2 (100 μM) using a hand operated UV lamp (R-52, Ultraviolet Products, San Gabriel, CA) positioned at 3 cm from the sample. The detection system was reduced cytochrome *c* (20 μM 50 mM potassium phosphate buffer,

pH 7.4) in a quartz cuvette as previously described.[22] The effect of hydroxyl radical on cytochrome *c* was monitored as the decrease in absorbance at 550 nm (ε = 19 $mM^{-1} \cdot cm^{-1}$). Controls were run in the absence of UV light to discount any possible H_2O_2-dependent cytochrome *c* oxidation.

Another control to test the antioxidant effect of resveratrol was performed by determining its inhibitory effect on epinephrine autoxidation. For these studies epinephrine was incubated 50 mM in potassium phosphate at pH 10 and resveratrol was added at increasing concentrations determining the lag time for the reaction. The autoxidation of epinephrine was monitored spectrophotometrically at 480 nm. The results of these experiments only confirmed the antioxidant effect of resveratrol (already shown in Fig. 1 as well as in previous publications) and are only described in the text as "not shown".

RESULTS AND DISCUSSION

Ultraviolet irradiation of hydrogen peroxide produces the hydroxyl radical.[22] The effect of this powerful oxidant on reduced cytochrome *c* was inhibited by synthetic resveratrol in a dose-dependent manner (Fig. 1, I_{50} = 33 μM). These results are in agreement with previous reports describing a powerful antioxidant effect of resveratrol *in vitro*.[10,16,17] As expected, similar results were obtained when testing the effect of resveratrol on other oxidative processes such as epinephrine autoxidation at pH 10 (not shown). In summary, these results indicate that the synthetic resveratrol used in this study was indeed an effective antioxidant.

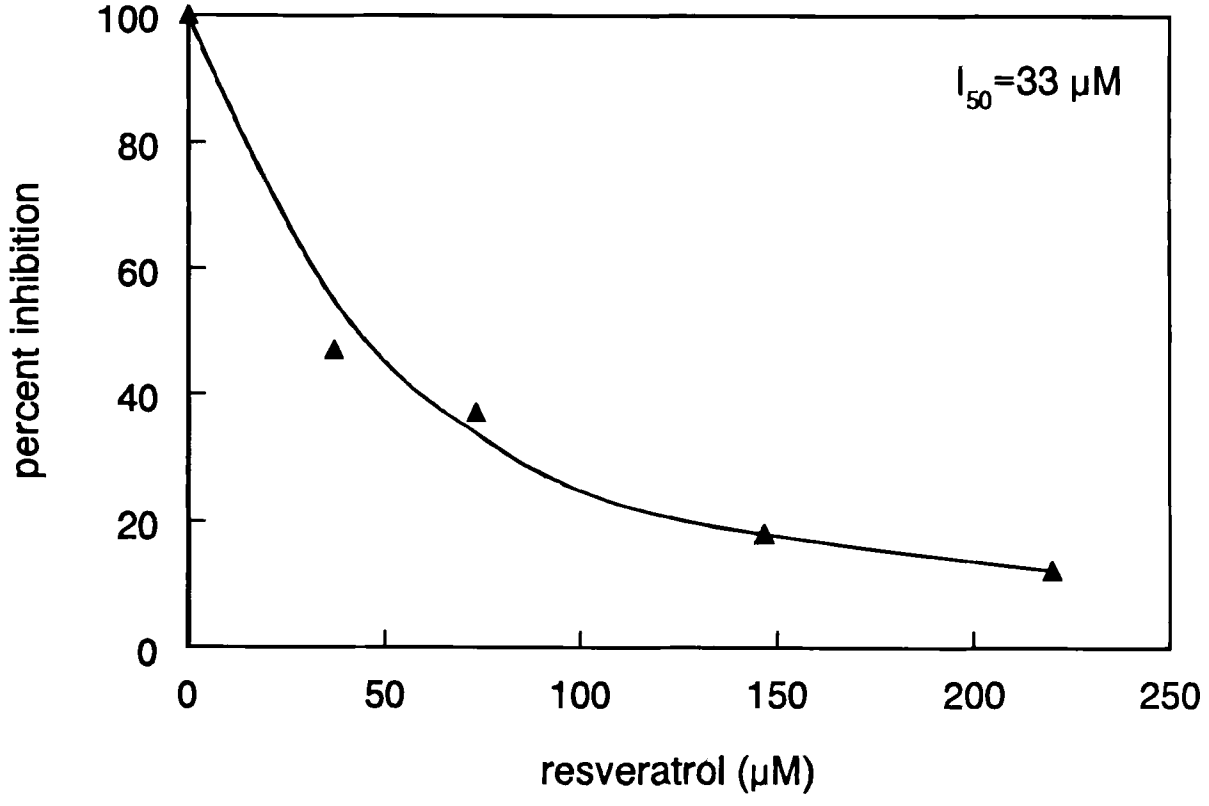

FIGURE 1 Inhibitory effect of resveratrol on the rates of cytochrome *c* oxidation by H_2O_2. For experimental conditions, see Materials and Methods. The points are average of two independent experiments.

Several studies proposed that moderate alcohol consumption increases HDL cholesterol.[9,23] It has been hypothesized that in addition of being an antioxidant resveratrol in the wine may increase plasma HDL activity.[10] The concentration of resveratrol in wine is about 6 μM,[10] approximately 1.4 mg/l. In order to determine the effect of pure resveratrol on the lipid profile of normal control rats, control animals were treated with much higher doses (20 or 40 mg/kg body weight).

The serum lipid profile was not significantly different among all groups (Table I). This treatment did not alter the proportion of cholesterol bound to either HDL, IDL or LDL concentration. Although the total concentration of triacylglycerides appears to decrease with resveratrol concentration, when this trend was analyzed by ANOVA it turned not significant ($p = 0.17$). Similarly, the increasing trend in HDL-cholesterol with resveratrol concentration and the resulting decreasing trend in the LD/HDL ratio showed no significance ($p = 0.62$ and 0.3, respectively).

Resveratrol could also protect against cardiovascular diseases if it became associated with plasma proteins involved in lipid transport (*i.e.*, albumin and lipoproteins), thus preventing lipid peroxidation. To test this possibility, an aliquot of serum from the same rats was first passed through a desalting column to separate low molecular weight antioxidants (*i.e.*, uric acid or vitamin C) from proteins. The resulting mixture of proteins was incubated with submaximal copper concentrations (2 or 10 μM) to induce variable levels of lipid peroxidation (Fig. 2). Resveratrol, like vitamin E[24] delays the initiation of lipid oxidation upon exposure of proteins to copper. A delayed initiation should lead to a smaller extent of TBARS, unless the concentration of oxidant is enough to oxidize all the lipids before the samples are analyzed for TBARS. In these experiments, TBARS formation in controls and samples from resveratrol-treated rats increased with copper concentration (Fig. 2). Thus, the amounts of TBARS produced were not maximal, at least for the lowest copper concentration. However, TBARS accumulation in serum samples from resveratrol-treated rats was not significantly different when compared to controls (Fig. 2). Thus, it may be concluded that there was no delay in the rate of oxidation in samples from resveratrol-treated animals, or in other words, that resveratrol was not associated with the protein fraction.

The susceptibility of protein-associated lipids to copper-dependent lipid peroxidation was not affected by resveratrol treatment, suggesting that although resveratrol may protect lipoproteins when added *in vitro*,[16] it does not become associated with proteins *in vivo* (Fig. 2). This is a major difference with vitamin E which prevents lipoprotein oxidation and therefore prevents foam cells from being formed. Thus, although resveratrol has twice the antioxidant capacity of Trolox[10] (a vitamin E analog) the potential protective effects *in vivo* cannot be compared.

In summary, the results reported herein suggest that resveratrol at doses 3 orders of magnitude higher than those ingested with red wine does not have any significant effect on lipoprotein profile *in vivo* nor does it prevent the oxida-

TABLE I Lipid profile and lipoprotein (a) content in control rats (injected with propylene glycol as a vehicle) and rats after 21 days of treatment with either 20 mg/kg or 40 mg/kg b.w. resveratrol. The values represent averages ± S.D. The groups were compared by ANOVA and the results show no significant differences among treatments.

Treatment	triacylglycerides (mg/dl)	Cholesterol (mg/dl)				LDL/HDL
		Total	HDL	LDL	IDL	
Control	38.5 ± 9.3	67 ± 9.5	42.5 ± 6.1	16.2 ± 1.3	3.5 ± 0.9	0.39 ± 0.03
20 mg/kg	34.4 ± 6	73.5 ± 20.5	50.6 ± 18.5	16.4 ± 4.5	3.4 ± 0.9	0.35 ± 0.1
40 mg/kg	30.4 ± 4.4	72.4 ± 15.6	51 ± 10.8	15.9 ± 4.3	3.2 ± 0.9	0.31 ± 0.06

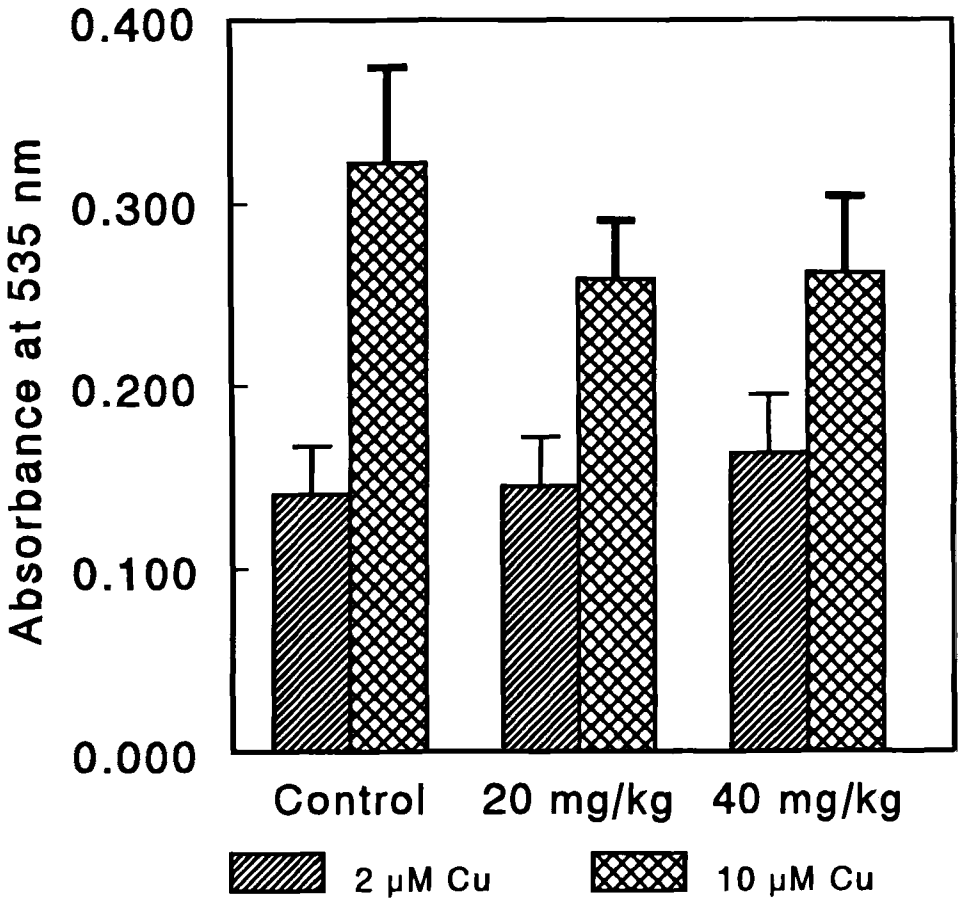

FIGURE 2 TBARS formation during the oxidation of lipids associated with serum proteins from animals treated with either 20 or 40 mg/kg b.w. resveratrol for 21 days. Lipid peroxidation was started by incubating serum samples (12–16 mg protein/ml) with either 2 or 10 μM $CuSO_4$.

tion of plasma lipids *in vivo*. Thus, if resveratrol has any protective effect of against coronary heart disease, it should be attributed to other properties. For example, resveratrol could act like uric acid, as a sacrificial scavenger in circulation[25] or could exert its action through an inhibitory effect on platelet aggregation.[14]

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References

[1] Zweier, J. L., Flaherty, J. T. and Weisfeldt, M. L. (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 1404–1407.

[2] Bolli, R., Jeroudi, M. O., Patel, B. S., DuBose, C. M., Lai, E. K., Roberts, R. and McCay, P. B. (1989). Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 4695–4699.

[3] Noguchi, N., Gotoh, N. and Niki, E. (1993). Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochimica Biophysica Acta: Lipids and Lipid Metabolism*, **1168**, 348–357.

[4] Steinberg, D. (1995). Role of oxidized LDL and antioxidants in atherosclerosis. *Advances in Experimental Medicine and Biology*, **369**, 39–48.

[5] Parthasarathy, S., Steinberg, D. and Witztum, J. L. (1992). The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. *Annual Reviews of Medicine*, **43**, 219–225.

[6] Stampfer, M. J. and Rimm, E. B. (1995). Epidemiologic evidence for vitamin E in prevention of cardiovascular

disease. *The American Journal of Clinical Nutrition*, **62 Suppl.**, 1365S–1369S.
[7] Kushi, L. H., Folsom, A. R., Prineas, R. J., Mink, P. J., Wu, Y. and Bostick, R. M. (1996). Dietary antioxidant vitamins and death from c oronary heart disease in postmenopausal women. *New England Journal of Medicine*, **334**, 1156–1162.
[8] Stephens, N. G., Parsons, A., Schofield, P. M., Kelly, F., Cheeseman, K., Mitchinson, M. J. and Brown, M. J. (1996). Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *The Lancet*, **347**, 781–786.
[9] Kushi, L. H., Lenart, E. B. and Willett, W. C. (1995). Health implications of Mediterranean diets in light of contemporary knowledge. 2. Meat, wine, fats and oils. *The American Journal of Clinical Nutrition*, **61**, 1416S– 1427S.
[10] Rice-Evans, C. A. and Miller, N. J. (1996). Antioxidant activities of flavonoids as bioactive components of food. *Biochemical Society Transactions*, **24**, 790–795.
[11] Miller, N. J. and Rice-Evans, C. A. (1995). Antioxidant activity of resveratrol in red wine. *Clinical Chemistry*, **41**,1789.
[12] Whitehead, T. P., Robinson, D., Allaway, S., Syms, J. and Hale, A. (1995). Effect of red wine ingestion on the anti-oxidant capacity of serum. *Clinical Chemistry*, **41**, 32–35.
[13] Keli, S. O., Hertog, M. G. L., Feskens, E. J. M. and Kromhout, D. (1996). Dietary flavonoids, antioxidant vitamins, and incidence of stroke—The zutphen study. *Archives of Internal Medicine*, **156**, 637–642.
[14] Pace-Asciak, C. R., Rounova, O., Hahn, S. E., Diamandis, E. P. and Goldberg, D. M. (1996). Wines and grape juices as modulators of platelet aggregation in healthy human subjects. *Clinica Chimica Acta*, **246**, 163–182.
[15] Maxwell, S. and Thorpe, G. (1996). Tea flavonoids have little short term impact on serum antioxidant activity. *British Medical Journal*, **313**, 229.
[16] Frankel, E. N., Waterhouse, A. L. and Kinsella, J. E. (1993). Inhibition of human LDL oxidation by resveratrol. *The Lancet*, **341**, 1103–1104.
[17] Celotti, E., Ferrarini, R., Zironi, R. and Conte, L. S. (1996). Resveratrol content of some wines obtained from dried Valpolicella grapes: Recioto and Amarone. *Journal of Chromatography A*, **730**, 47–52.
[18] Van Tol, A., Groener, J. E., Scheek, L. M., Van Gent, T., Veenstra, J., Van de Pol, H., Hendriks, H. F. and Schaafsma, G. (1995). Induction of net mass lipid transfer reactions in plasma by wine consumption with dinner. *European Journal of Clinical Investigation*, **25**, 390–395.
[19] Wilson, T., Knight, T. J., Beitz, D. C., Lewis, D. S. and Engen, R. I. (1996). Resveratrol promotes atherosclerosis in hypercholesterolemic rabbits. *Life Sciences*, **59**, PL15–PL21.
[20] Moreno-Manas, M. and Pleixats, R. (1985). Dehydroacetic acid chemistry: a new synthesis of resveratrol, a phytoalexine of *Vitis vinifera*. *Anales de Quimica*, **81**, 157–161.
[21] Ohkawa, H. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**, 351–358.
[22] Turrens, J. F. and McCord, J. M. (1988). How relevant is the reoxidation of ferrocytochrome *c* by hydrogen peroxide when determining superoxide anio n production? *FEBS Letters*, **227**, 43–46.
[23] Cohn, B. A., Brand, R. J. and Hulley, S. B. (1989). Correlates of high density lipoprotein cholesterol in women studied by the method of co-twin control. *American Journal of Epidemiology*, **129**, 988–999.
[24] Kleinveld, H. A., Naber, A. H. J., Stalenhoef, A. F. H. and Demacker, P. N. M. (1993). Oxidation resistance, oxidation rate, and extent of oxidation of human low-density lipoprotein depend of the ratio oleic acid content to linoleic acid content: studies in vitamin E deficient subjects. *Free Radicals in Biology and Medicine*, **15**, 273–280.
[25] Vásquez-Vivar, J., Santos, A. M., Junqueira, V. B. C. and Augusto, O. (1996). Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals. *Biochemical Journal*, **314**, 869–876.