

Original articles

Effects of resveratrol on oxidative modification of human low density lipoprotein

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Objective To determine the antioxidative effects of resveratrol (RES), a polyphenolic compound in red wine , on the oxidation of human low density lipoprotein (LDL) using two different oxidation systems.

Methods Oxidation of LDL was induced by adding either Cu^{2+} or an azo compound. The extent of LDL modification was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS), the relative electrophoretic mobilities (REM), and the amount of oxidized LDL degradation by macrophages.

Results During Cu^{2+} -induced oxidation , RES reduced TBARS formation in LDL by 70.5% , REM of LDL by 42.3% and the amount of macrophage degradation by 65.7% , respectively. The lag phase of LDL oxidation was also delayed by adding RES both in the copper ion and azo compound-induced oxidation systems.

Conclusion RES can protect LDL against both Cu^{2+} -induced and azo compound-initiated oxidative modification in vitro , which might be due to its free radical scavenging capacity.

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The presence of lipid-laden " foam cells " within the intima of arteries is a characteristic feature of atherosclerotic lesions. Oxidatively modified low density lipoprotein (LDL) is a major atherogenic lipoprotein in vivo , which is taken up by macrophages through the scavenger receptor pathway. LDL contains a number of endogenous antioxidants , including α -tocopherol and β -carotene. It 's only when these antioxidants have been largely consumed that lipid peroxidation takes place in LDL. Previous studies showed that LDL oxidation could be inhibited by adding lipophilic

antioxidants such as α -tocopherol , β -carotene , butylated hydroxytoluene (BHT) or probucol. Probucol , a lipid-lowering drug , might exert a protective effect against the development of spontaneous atherosclerosis in the Watanabe hereditary hyperlipidemic rabbit , by acting as an antioxidant rather than as a lipid-regulating agent.¹ An inverse relationship exists between the intake of natural antioxidants and the incidence of coronary heart disease^{2,3,4} further supporting the importance of LDL oxidation in the development of atherosclerosis. One noted exception to this relationship , however , is the apparent compatibility of a high fat diet with a low incidence of coronary heart disease , often referred to as the " French paradox ". The observed disparity may be linked to the regular consumption of red wine , known to contain several phenolic compounds with antioxidant properties.⁵ One such compound is the polyphenol resveratrol , which was reported to be more active than vitamin E in inhibiting copper ion-catalyzed oxidation of LDL.^{6,7} In this study , the effects of resveratrol (RES) on inhibiting copper ion-induced and azo compound-initiated oxidative modification of LDL were reported.

METHODS

Reagents

RES , 1 , 1 , 3 , 3-tetramethoxypropane , thiobarbituric acid , and ascorbic acid (vitamin C) were purchased from Sigma Chemical Co.. The azo compound (2 , 2 '-azobis

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[2-amidinopropane dihydrochloride], AAPH) was obtained from Polysciences Inc. . DMEM culture media was obtained from Gibco. Other chemicals were analytical grade.

Isolation and labeling of LDL

LDL , with a density range of 1.019 – 1.063 g/ml , was isolated from freshly prepared plasma from two healthy volunteers by sequential ultracentrifugation.⁸ EDTA (0.05%) and gentamycin (80 mg/ml) were added to minimize lipoprotein oxidation and microorganism contamination during preparation. Purified LDL was dialyzed at 4°C for 48 h against PBS (pH7.4) supplemented with 2 mmol/L EDTA. LDL was labeled with Na¹²⁵I by method of McFarlane.⁹ ¹²⁵I -LDL was dialyzed extensively at 4°C over 2 nights again PBS (pH7.4) and its radioactivity was measured.

LDL was sterilized by membrane filtration , and stored in the presence of 2 mmol/L EDTA under N₂ at 4°C and used within 2 weeks. Protein concentration was determined by Lowry 's method.

Isolation and culture of macrophages

Mouse peritoneal macrophages (MPM_S) were isolated and plated in 6-well plates at two million macrophages per well. The culture medium consists of 9 volumes of DMEM and 1 volume of newborn calf serum. The cells were incubated at 37°C under water-saturated air/co₂(19 : 1). After 5 h , the cells were washed with DMEM to remove contaminating cells. Following an overnight incubation , when the extension rate of macrophages exceeded 70% , the cells were ready to be used to measure the degradation of ¹²⁵I -OX-LDL.

Cu²⁺ -induced oxidation of LDL

After extensive dialysis against EDTA-free PBS (pH7.4) at 4°C , LDL was incubated (500 μg protein/ml) at 37°C for 18 h with CuCl₂ (final concentration , 40 μmol/L) and resveratrol (final concentrations 0 , 25 , 50 , 100 and 200 μmol/L , dissolved in 1% DMSO , v/v). Aliquots were taken at the indicated times to measure TBARS and REM. Oxidation was stopped by adding 2.7 mmol/L EDTA. In order to further observe the kinetics of LDL oxidation , LDL (500 μg/ml) was incubated with 40 μmol/L CuCl₂ and 50 μmol/L resveratrol at 37°C . At the indicated times , the reaction was stopped with 2.7 mmol/L EDTA and the amount of TBARS was determined.¹⁰

Azo compound-initiated oxidation of LDL

LDL (500 μg/ml) dialyzed extensively against EDTA-free PBS (pH7.4) at 4°C was incubated with the azo compound (final concentration , 10 mmol/L) , with or without addition of 50 μmol/L resveratrol. The oxidation was stopped with 10 mmol/L vitamin C at the indicated time

points and the amount of TBARS was determined.¹¹

Extent of modification of LDL

The amount of TBARS was determined as described.¹² The results are expressed as formation of nmol equivalents of malondialdehyde (MDA) , using freshly diluted 1 , 1 , 3 , 3-tetramethoxypropane as the standard.

REM determination

The net negative charge of LDL was determined by agarose gel electrophoresis. REM was calculated as the mobility of oxidized LDL relative to that of native LDL (NLDL).¹²

Measurement of LDL degradation by MPM_S

¹²⁵I -LDL (100 μg protein/ml) was incubated at 37°C for 18 h in PBS containing 40 μmol/L freshly dissolved CuCl₂ with or without RES. It was then diluted to 10 μg LDL protein/ml with serum-containing medium and incubated for 5 h in wells that contained macrophages or were cell-free. The degradation by macrophages was measured by the method of Goldstein.¹³

Statistical analysis

The results are presented as mean ± SD. The student *t*-test was used to analyze the difference between groups. The level of significance was set at *P* < 0.05.

RESULTS

Cu²⁺ -induced oxidation of LDL

The amount of TBARS in LDL increased about 6-fold during 18 h of Cu²⁺ -induced oxidation (from 6.27 ± 0.57 nmol MDA/mg LDL to 37.18 ± 1.09 nmol MDA/mg LDL , n = 6 , *P* < 0.01). The addition of 25 μmol/L RES in the reaction mixture did not significantly decrease the MDA formation. At higher concentrations (50 , 100 or 200 μmol/L) , RES significantly reduced the amount of TBARS in LDL by 70.5% , 78.7% , and 82.1% , respectively , as compared to no added RES (n = 6 , *P* < 0.01) (Fig. 1A). Similarly , 18 h of oxidation resulted in an increase of REM from 1.0 to 2.6 (n = 6 , *P* < 0.01) , which was significantly attenuated by the presence of resveratrol. When 50 , 100 or 200 μmol/L RES was added , the REM of LDL was significantly reduced by 42.3% , 50% or 53.8% , respectively (n = 6 , *P* < 0.01) (Fig. 1B). The REM of LDL after 18 h of oxidation with RES was equivalent to that after 8 h of oxidation without RES (Fig. 2). No additional MDA formation was observed in LDL when more than 100 μmol/L RES was used.

The uptake and degradation of LDL by MPM_S increased markedly after 18 h of oxidation (Table). About ten-fold more oxidized LDL than native LDL was degraded by MPM_S (10.84 ± 1.07 μg/mg cell protein versus 1.15 ± 0.14

$\mu\text{g}/\text{mg}$ cell protein ; $n = 3$). LDL oxidized in the presence of RES (final concentration of 50 , 100 $\mu\text{mol}/\text{L}$) showed reduced degradation by MPM_s compared with LDL oxidized for 18 h in the absence of the drug (65.7% , 79.1% , respectively ; $n = 3$; $P < 0.01$).

Table. Resveratrol inhibits oxidation and biological modification of LDL catalyzed by Cu^{2+}

LDL sample	REM	Macrophage degradation($\mu\text{g}\cdot 5\text{h}^{-1}\cdot \text{mg}^{-1}$)
Native , unincubated	1.00	1.15 \pm 0.14
Incubated 18h with Cu^{2+}	2.33	10.84 \pm 1.07 ^{###}
+ DMSO (1% v/v)	2.17	9.92 \pm 0.42*
+ RES 10 $\mu\text{mol}/\text{L}$	2.00	9.34 \pm 1.02*
+ RES 50 $\mu\text{mol}/\text{L}$	1.50	3.72 \pm 0.92***
+ RES 100 $\mu\text{mol}/\text{L}$	1.33	2.27 \pm 0.58***

^{###} $P < 0.01$ versus NLDL ; * $P > 0.05$ versus Cu^{2+} ; *** $P < 0.01$ versus Cu^{2+} .

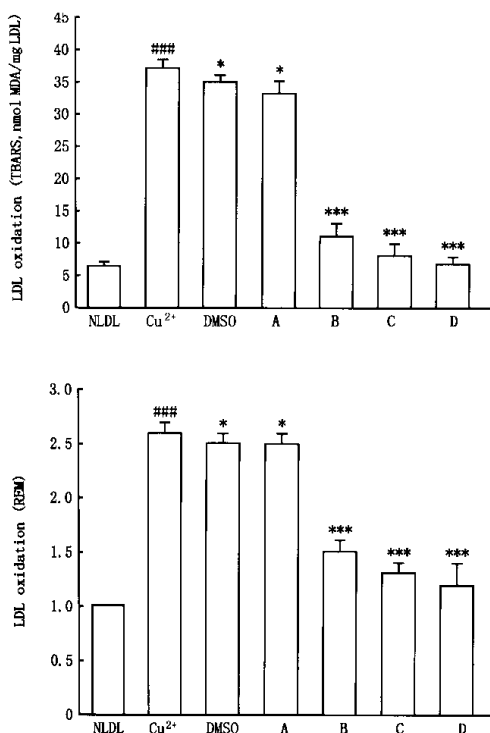


Fig. 1. Bar graphs showing amounts of TBARS (Top) and REM (Bottom) in LDL during Cu^{2+} -induced oxidation. LDL (500 $\mu\text{g}/\text{ml}$) incubated at 37 $^{\circ}\text{C}$ with 40 $\mu\text{mol}/\text{L}$ CuCl_2 in PBS in the absence or presence of 25 – 200 $\mu\text{mol}/\text{L}$ RES for 18 hours. Results expressed in mean \pm SD from 6 separate experiments. TBARS : thiobarbituric acid reactive substances ; REM : relative electrophoretic mobilities. A : RES 25 $\mu\text{mol}/\text{L}$; B : RES 50 $\mu\text{mol}/\text{L}$; C : RES 100 $\mu\text{mol}/\text{L}$; D : RES 200 $\mu\text{mol}/\text{L}$. ^{###} $P < 0.01$ versus NLDL ; * $P > 0.05$ versus Cu^{2+} ; *** $P < 0.01$ versus Cu^{2+} .

The kinetics of MDA formation in LDL during Cu^{2+} -induced oxidation was also investigated. Figure 3A shows the effects of 50 $\mu\text{mol}/\text{L}$ RES on the amount of TBARS generated during LDL oxidation , confirming that the polyphenolic compound caused a significant retardation of LDL oxidation.

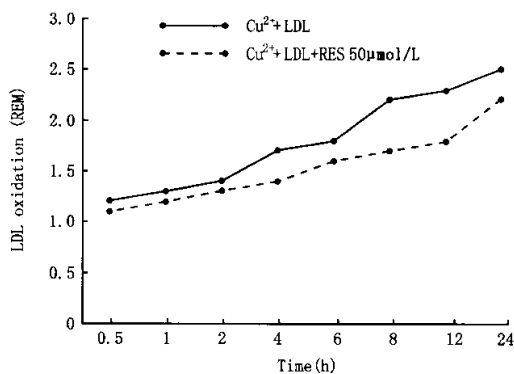


Fig.2. Effect of 50 $\mu\text{mol}/\text{L}$ RES on the relative electrophoretic mobility (REM) of Cu^{2+} -induced oxidation of LDL.

Azo compound-initiated oxidation of LDL

To further validate the antioxidant effect of resveratrol , LDL was subjected to metal ion-independent oxidation initiated by the water-soluble azo compound AAPH. Addition of 50 $\mu\text{mol}/\text{L}$ RES resulted in a significant prolongation of the lag phase associated with oxidation of LDL by AAPH , from 1 h to approximately 12 h (Fig. 3B).

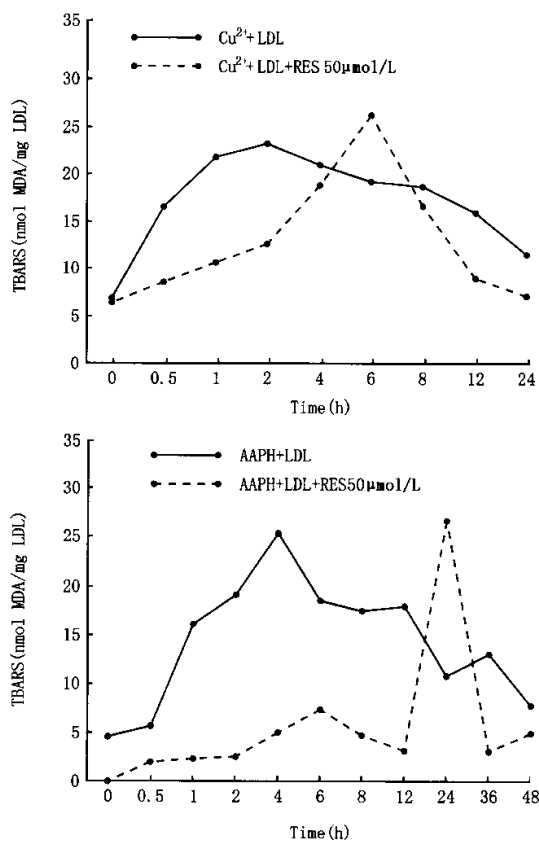


Fig.3. Line graphs showing the formation of MDA in LDL during Cu^{2+} -induced oxidation (Top) and AAPH-initiated oxidation (Bottom). LDL (500 $\mu\text{g}/\text{ml}$) incubated at 37 $^{\circ}\text{C}$ with 40 $\mu\text{mol}/\text{L}$ CuCl_2 or 10 mmol/L AAPH in PBS in the absence or presence of RES (50 $\mu\text{mol}/\text{L}$) for the indicated periods of time. Each point represents the mean of two experiments. TBARS : thiobarbituric acid reactive substances.

DISCUSSION

Many clinical and experimental studies have strongly supported the notion that elevated plasma concentration of LDL, especially OX-LDL is associated with accelerated atherogenesis.¹⁴ LDL oxidation could be induced in vitro by Cu²⁺-induced or azo compound-initiated oxidative modification. In this study, we determined the extent of LDL oxidation and evaluated the effects of RES on LDL oxidative modification by these two methods.

RES (3,4,5-trihydroxystilbene) was first found as a phytoalexin in plants,¹⁵ where it is synthesized in response to ultraviolet light irradiation, fungal infection and exposure to ozone. RES is distributed in 72 kinds of plants including human foods such as grapes, peanuts, mulberries and berries. It is lipophilic, having a molecular weight of 228.2. And its structure suggests that it may have antioxidative properties. Early in the 1980's, a Japanese¹⁶ reported the effects of stilbene components of the roots of *Polygonum cuspidatum* on lipid metabolism. He found that RES inhibited the deposition of triglyceride and cholesterol in the liver of rats fed with a corn oil-cholesterol-cholic acid mixture, and reduced triglyceride synthesis in the liver. In 1993, Frankel⁶ reported that RES inhibited LDL oxidation induced by copper ion. Results of these experiments demonstrated that RES effectively inhibited LDL modification induced by copper ion and azo compound, in a time- and concentration-dependent manner, showing a reduction in the amount of MDA formed, suppression of the change in relative electrophoretic mobility of OX-LDL and decrease in the LDL degradation by macrophages. When 100 μmol/L resveratrol was incubated with LDL, the oxidation was almost completely inhibited.

In order to clarify the antioxidative mechanism of resveratrol, both AAPH oxidation and copper ion oxidation were evaluated. AAPH is a water-soluble azo compound and which is thermally decomposed to produce peroxy radicals, which in turn acts on LDL to result in its peroxidation. The addition of 50 μmol/L RES significantly inhibited LDL oxidation and prolonged the lag phase from 1 h to 12 h, without significantly affecting the maximum output of TBARS. This suggests that RES, like α-tocopherol, might act as a free-radical trap to halt the progression of LDL oxidation. Thus, as a strong antioxidant and mild lipids-regulated agent, RES would prevent to a certain extent from the development of atherosclerosis.

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