Effects of Vitamin E and Volatile Oils on the Susceptibility of Low-Density Lipoprotein to Oxidative Modification

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ABSTRACT

Antioxidant consumption has been reported to be inversely associated with the incidence of coronary artery disease. To clarify the possible role of vitamin E and volatile oils in the prevention of atherosclerosis, the effects of these compounds on the susceptibility of low-density lipoprotein (LDL) to oxidative modification were investigated. In this study, vitamin E and seven volatile oils "anethol, eugenol, geraniol, limonene, linalool, pulegone and thymol" were added to plasma and incubated at 37° C for 3 h. The LDL fraction was separated by ultracentrifugation and the oxidizability of LDL was estimated by measuring conjugated diene (CD), lipid peroxides and thiobarbituric acid-reactive substances (TBARS) after cupric sulfate solution was added. The data show that vitamin E, thymol and eugenol significantly and dose-dependently prolonged the lag time before initiation of oxidation reaction ($P<0.01$ by ANOVA). Also, vitamin E and thymol suppressed the formation of lipid peroxides and TBARS more markedly than other volatile oils. The ability to prolong lag time, suppression of lipid peroxides and TBARS formation was in the following order: vitamin E > thymol > eugenol > geraniol > linalool > limonene > anethol > pulegone. These data clearly show that LDL exposed to vitamin E and volatile oils *in vitro* reduces oxidizability; therefore have favorable effects in ameliorating atherosclerosis. *Iran. Biomed. J. 7 (2): 79-84, 2003*

INTRODUCTION

Oxidation of low-density lipoprotein (LDL) has been suggested as a causal factor in human atherosclerosis [1]. Oxidatively modified LDL is a potent ligand for scavenger receptors on macrophages that contributes to generation of macrophage-derived foam cells, the hall-mark of early atherosclerotic fatty streak lesions [2]. Many additional mechanisms by which oxidized LDL may contribute to atherosclerosis have been identified [3]. The oxidative modification hypothesis of atherosclerosis is supported by numerous *in vivo* findings, e. g., the presence of epitopes of oxidatively modified LDL in atherosclerotic lesions [4] and elevated titers of circulating auto-antibodies against oxidized LDL in patients with carotid atherosclerosis [5]. Antioxidant compounds provide resistance to this process and have been suggested to lower atherogenicity [6]. Considerable epidemiologic [7, 8], biochemical [9], and clinical [10] evidence has accumulated in support of this hypothesis.

LDL contains different lipophilic antioxidants. The most abundant being α-tocopherol that is the major form of vitamin E. All the other antioxidants (γ-tocopherol, carotenoids, ubiquinol-10) are much less present in LDL. Vitamin E is thought to be the major non-enzymatic antioxidant present in the lipid structures of cells and lipoproteins. It is a donor antioxidant (reductant), which increases the LDL resistance against the oxidative modification. In lipid solutions and dispersions, it inhibits radical formation linearly with time until consumed in the process [1].

Volatile oils are bioactive substances in plants and potentially are present in human plasma in a diet-dependent concentration. Some of them have many hydroxyl and methyl groups in their structure and have been shown to inhibit oxidative modification of LDL when added before initiation of oxidation [12, 13].

However, the mechanisms by which these compounds inhibited LDL oxidation have not been clarified. Because of their amphipathic nature, volatile oils may act within the LDL particle in a manner similar to that of vitamin E.

In this study, the effects of plasma pre-incubation with vitamin E and volatile oils on the susceptibility of LDL to oxidative modification were investigated.

MATERIALS AND METHODS

Chemicals. Vitamin E was obtained from Fluka (Neu-Uim, Germany). Anethol, eugenol, geraniol, limonene, linalool, pulegone and thymol were obtained from Merck (Darmstadt, Germany). Trichloroacetic acid, thiobarbituric acid and 1,1,3.3-tetraethoxypropane were purchased from Sigma (Deisenhofen, Germany).

Addition of vitamin E and volatile oils to plasma in vitro. Fresh blood was collected in the presence of EDTA (1 mg/ml) from a normolipidemic healthy male volunteer after an overnight fast. Plasma was separated by low-speed centrifugation at $1000 \times g$ at 4°C for 15 min. To enrich LDL with vitamin E and volatile oils, each compound was dissolved in 10% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS, pH 7.4) (20 ml 10% DMSO/L plasma) and added to plasma (50, 100 and 200 µmol/L) and then incubated at 37°C for 3 h.

Isolation of LDL. LDL (density 1.019-1.063 g/ml) was isolated from the plasma in the 1.019-1.063 g/ml density range by sequential ultracentrifugation [14]. The pooled plasma solvent density was adjusted to 1.019 g/ml with a high- density salt solution (containing NaCl, KBr, and EDTA). After centrifugation at $100,000 \times g$ at 16°C for 1 h, the top layer of supernatant was removed. The density of pooled infranatant was adjusted to 1.063 g/ml and was recentrifuged as before. The LDL, which floats at a relative density of 1.063, was collected and exhaustively dialyzed at 4°C for 12 h in EDTA-free PBS degassed PBS. Control LDL was prepared by the same technique after adding only vehicle (20 min 10% DMSO/L plasma) to the plasma and incubating at 37°C for 3 h. In a preliminary study, we confirmed that DMSO at this concentration did not affect the oxidizability of LDL. The LDL suspension was stored at 4°C under argon in the dark.

Measurement of oxidizability of LDL. The oxidizability of LDL was estimated by measuring three indexes including conjugated dienes (CD), lipid peroxides, and thiobarbituric acid-reactive substances (TBARS).

1) Conjugated dienes. Freshly prepared LDL (50 mg protein/L) was incubated with cupric sulphate solution (final concentration 10 µmol/L) at 37°C in a Hitachi spectrophotometer (Hitachi Sangyo Co., Tokyo) fitted with a peltier heater. The absorbance at 234 nm was automatically recorded at 10-min intervals. Lag time before the initiation of oxidation and the propagation rate was determined according to the methods of Esterbauer *et al.* [15] with some modifications. The output from the spectrophoto-meter was converted directly into the ASCII file format and used to compute the lag time prior to the onset of oxidation. The maximum diene concen-tration was determined from the differences between the absorbance curve and the absorbance at time zero using the extinction coefficient for conjugated dienes at 234 nm (E = 295,000 mol/cm). After incubation for 90 or 180 min with 10 µmol CuSO4/L, the oxidation reaction was stopped by adding EDTA (100 µmol/L final concentration).

2) Lipid peroxides. To measure the content of lipid peroxides in LDL, an assay based on the oxidative activity of lipid peroxide (LOOH), which convert iodide to iodine was used. Iodine forms the triodide ion, which absorbs at 365 nm. For assay, microtiter plate with CHOD-iodide reagents was used according to the manufacturer (Merck, Darmstadt, Germany). The LDL oxidation was carried out in polystyrene microtiter plates. CHOD iodide reagent (190 µL) was added to plates and incubated at 37°C for 60 min. The absorbance at 365 nm was measured in a microplate reader (Labsystems, Muluskan MCC/ 340, Uppsala, Sweden). The concentration of LOOH was calculated from the molar absorption coefficient of 246,000 mol/cm for the triiodide ion and a path length in the microtiter plate of 1 cm for the final volume of 330 µL.

3) TBARS. The concentration of TBARS was also measured using the method described by Buege and Aust [16]. LDL (0.1 ml) was mixed with 1 ml of 0.67% TBA and 0.5 ml 20% trichloroacetic acid and incubated at 100°C for 20 min. After cooling, the reaction mixture was centrifuged at $1,000 \times g$ for 5 min and the absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated using the extinction coefficient of 165,000 mol/cm and expressed as nmol of malondialdehyde (MDA) equivalents per mg LDL protein using a freshly diluted 1,1,3.3-tetra-ethoxypropane for the standard curve.

Statistical analysis. All results are presented as mean ± standard deviations (n = 5). Data between groups were compared by analysis of variance (ANOVA). Fisher's test was used whenever a statistically significant difference between the two groups was shown by ANOVA.

RESULTS

Addition of vitamin E and volatile oils to plasma at 37°C for 3 h resulted in lipoprotein antioxidants enrichment. The efficiency of the enrichment depended on the compound concentration in the medium. Incubation of copper ions with the LDL suspension caused extensive oxidation of the lipoprotein as judged by an accumulation of CD, lipid peroxides and TBARS in the LDL sample. Enriching LDL with antioxidant agents made it more resistant to the copper-induced oxidation in comparison with a native. This effect was demonstrated with all the indices of oxidation used and appeared to be the most pronounced within the first hours of oxidation.

Table 1 shows the effects of vitamin E and seven volatile oils on the susceptibility of LDL to the copper-induced oxidation. Vitamin E, thymol and eugenol significantly increased the lag time before the onset of CD formation ($P<0.01$ by ANOVA). Prolongation of the lag time by these components was dose-dependent. Continuos registration of LDL absorbance at 234 nm showed that vitamin E (200 μmol/L) had the strongest effects and prolonged the lag time three times more than that of the control. Also, among volatile oils, thymol and eugenol had the most effects, and prolonged the lag time two times more than that of the control. The lag time was significantly increased in the LDL separated from pre-treated plasma with 50 μmol/L vitamin E, thymol, and eugenol, or with 100 μmol/L vitamin E, thymol, eugenol, linalool, geraniol and anethol, or with 200 μmol/L vitamin E, thymol, eugenol, anethol, geraniol, linalool, limonene and pulegone ($P<0.01$ and $P<0.05$ by Fisher's test). The capacity to prolong the lag time was in the following order: vitamin E > thymol > eugenol > anethol > geraniol > linalool > limonene > pulegone.

Suppression of TBARS and lipid peroxides formation were observed in the LDL samples to which these agents had been added. In these experiments, vitamin E exerted strongest effect, and among the volatile oils, thymol suppressed the formation of TBARS and lipid peroxides more markedly than others. The effects were proportional to the doses (Table 2).

TBARS and lipid peroxide formation in the medium were significantly reduced in the LDL separated from plasma that had been incubated with 50 μmol/L vitamin E, thymol,, and eugenol, or with 100 μmol/L vitamin E, thymol, eugenol, geraniol and linalool, or with 200 μmol/L vitamin E, thymol, eugenol, geraniol, linalool and limonene ($P<0.05$ and $P<0.0001$). The suppression ability of TBARS and lipid peroxides formation was in the following order: vitamin E > thymol > eugenol > geraniol > linalool > limonene > anethol > pulegone (Table 3).

Table 1. The effect of vitamin E and volatile oils addition (50, 100 and 200 μmol/L) on lag time of plasma on susceptibility of LDL to copper-induced oxidation[*].

	Lag time (min)			
	None (control)	50 μmol/L	100 μmol/L	200 μmol/L
Vitamin E	58.1 ± 3.0	69.9 ± 4.9[a]	91.2 ± 5.8[a]	176.0 ± 9.8[b]
Anethol	58.1 ± 3.0	61.3 ± 3.5	76.1 ± 10.8[a]	100.1 ± 12.5[a]
Eugenol	58.1 ± 3.0	66.7 ± 4.5[a]	90.0 ± 8.1[a]	120.3 ± 11.45[a]
Geraniol	58.1 ± 3.0	60.9 ± 4.9	77.7 ± 9.0[a]	98.2 ± 10.1[a]
Limonene	58.1 ± 3.0	58.5 ± 9.2	62.9 ± 11.3	70.8 ± 8.0[a]
Linalool	58.1 ± 3.0	61.2 ± 7.3	78.3 ± 7.9[a]	80.1 ± 3.9[a]
Pulegone	58.1 ± 3.0	57.1 ± 7.7	59.9 ± 9.8	66.9 ± 6.9[a]
Thymol	58.1 ± 3.0	69.1 ± 6.6[a]	90.0 ± 8.8[a]	131.9 ± 14.0[b]

[*]Mean ± SD of three separate experiments. Lag times for vitamin E, anethol, eugenol, geraniol, limonene, linalool, pulegone and thymol differ significantly among the groups. $P<0.01$(ANOVA); [a, b]Significantly differ from control (Fisher's test):

[a]*P*<0.01 and [b]*P*<0.05.

Table 2. Lipid peroxides measurment after vitamin E and volatile oils addition (50, 100 and 200 µmol/L) to plasma on susceptibility of LDL to Cu-induced oxidation[*].

	Lipid peroxides (nmol/mg protein)			
	None (control)	50 µmol/L	100 µmol/L	200 µmol/L
Vitamin E	214.0 ± 2.0	196.00 ± 5.50[a]	79.33 ± 7.15[b]	25.90 ± 8.20[b]
Anethol	214.0 ± 2.0	212.50 ± 5.00	209.44 ± 14.40	202.35 ± 4.16
Eugenol	214.0 ± 2.0	209.90 ± 11.55	139.50 ± 8.40[b]	87.75 ± 15.36[b]
Geraniol	214.0 ± 2.0	207.96 ± 4.35	159.73 ± 8.50[a]	92.24 ± 10.24[b]
Limonene	214.0 ± 2.0	212.00 ± 2.30	205.62 ± 12.33	191.69 ± 7.14[a]
Linalool	214.0 ± 2.0	208.87 ± 13.20	179.47 ± 10.92[a]	111.24 ± 16.45[b]
Pulegone	214.0 ± 2.0	213.34 ± 2.21	212.48 ± 8.13	204.79 ± 6.34
Thymol	214.0 ± 2.0	197.52 ± 4.80[a]	129.44 ± 14.54[b]	59.83 ± 10.62[b]

[*]Mean ± SD; n = 4. One representative experiment of three is shown; the other two experiments yielded similar results. Lipid peroxides differ significantly between groups, *P*<0.01(ANOVA). [a,b]Significantly different from control: [a] *P*<0.05 and [b]*P*<0.0001.

DISCUSSION

In this study, the copper-catalyzed oxidation of LDL, isolated from plasma pre-incubated with vitamin E and volatile oils, was significantly inhibited in a dose-dependent manner as assessed by the lag time before the initiation of oxidation and the formation of TBARS and lipid peroxides. To add these lipophilic agents to LDL *in vitro*, EDTA-containing plasma was incubated at 37°C for 3 h with each of these agents (50, 100 and 200 µmol/L). Then, LDL was separated and dialyzed against PBS before the induction of oxidation. The compounds that were on the surface of or within LDL particles were considered to be responsible for inhibiting LDL oxidation.

The data support the suggestion that vitamin E plays an important role in the prevention of LDL oxidation mediated by copper ions *in vitro*. In other studies, the oxidation of LDL induced by metal ions was inhibited in the presence of antioxidant vitamins such as vitamin E [17-19].

Table 3. TBARS measurement after the addition of vitamin E and volatile oils (50, 100 and 200 µmol/L) to plasma on susceptibility of LDL to Cu-induced oxidation[*].

	TBARS (nmol MDA/mg protein)			
	None (control)	50 µmol/L	100 µmol/L	200 µmol/L
Vitamin E	15.6 0± 1.60	9.42 ± 0.57[a]	6.62 ± 0.93[b]	3.23 ± 0.29[b]
Anethol	15.60 ± 1.60	15.28 ± 3.20	14.83 ± 2.96	14.03 ± 3.96
Eugenol	15.60 ± 1.60	12.85 ± 1.13	8.76 ± 0.91[a]	6.37 ± 0.79[b]
Geraniol	15.60 ± 1.60	12.29 ± 1.52	9.12 ± 1.01[a]	6.86 ± 1.18[b]
Limonene	15.60 ± 1.60	13.93 ± 1.06	11.52 ± 1.14	8.25 ± 2.02[a]
Linalool	15.60 ± 1.60	13.10 ± 0.94	10.22 ± 0.83[a]	7.14 ± 1.67[b]
Pulegone	15.60 ± 1.60	14.94 ± 1.86	14.88 ± 3.71	14.49 ± 4.10

Thymol	15.60 ± 1.60	9.51 ± 0.88^{a}	8.39 ± 0.47^{a}	4.89 ± 1.68^{b}

*Mean $\pm$ SD; n=4. One representative experiment of three is shown; the other two experiments yielded similar results. TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde. TBARS differ significantly between groups, $P<0.01$ (ANOVA). a,b Significantly differ from control: a $P<0.05$ ad b $P<0.0001$.

Vitamin E is known to be a highly efficient lipid-soluble antioxidant protecting the unsaturated lipids in a variety of biological and model systems including cell membranes, LDL and liposomes [20]. Vitamin E and lipophilic antioxidants that associated with LDL act as chain-breaking antioxidants [21] that suppress lipid peroxidation by reducing chain-carrying lipid peroxyl radicals (LOO) to LOOH or by forming covalent adducts with these radicals [22, 23]. In comparison with other lipophilic antioxidants, vitamin E is the most efficient in the lipid phase. It contains shielding methyl groups adjacent to the phenolic hydroxyl group and it is optimally positioned in the LDL particle. In addition, vitamin E suppresses the oxidation of LDL during an initial lag time until it is depleted, and then oxidation accelerates, taking place at the same rate as if it had not been present [24].

The efficiency of volatile oils and phenols in protection of LDL against oxidative modification is likewise unknown. Miura *et al.* [25] investigated the effects of phenolic compounds on porcine LDL. They observed that some of phenols increased the lag time preceding the formation of CD, and reduced the formation of TBARS and lipid peroxides. Vinson *et al.* [26] reported that herbal lipophilic products had a lipoprotein-bound antioxidant activity equivalent to that of vitamin E.

These data show that all volatile oils used, significantly decreased the susceptibility of LDL to oxidative modification. These compounds may be more easily incorporated into LDL. Incubation of volatile oils to plasma over a long period of time may enrich the LDL particles sufficiently to make them less susceptible to oxidative reaction.

Also, the results showed that among these volatile oils, thymol and eugenol had strongest inhibitory activity against oxidation. These are two compounds that have phenol group in their structures, which are considered to be necessary for antioxidant activity. This may be one reason for their effects in ameliorating atherosclerosis.

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