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Research Articles

Intake of olive oil can modulate the transbilayer movement of human erythrocyte membrane cholesterol

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Abstract. Transbilayer movement of erythrocyte membrane cholesterol is impaired in patients affected with essential hypertension. This is an inherited disorder, but environmental factors are also involved. Dietary fats might play a role in the prevention and/or treatment of such abnormality in the kinetic pools of membrane cholesterol. We tested this hypothesis by using a diet (in which 30% of the energy came from fat) rich in olive oil or in high-oleic sunflower oil (as natural sources of monounsaturated fatty acids, MUFAs) and determining their influence on the movement of cholesterol into the lipid bilayer of the erythrocyte membrane after a four-week period. We concluded that dietary olive oil is helpful in normalizing the impaired transbilayer movement of membrane cholesterol in erythrocytes of eight normocholesterolaemic and eight hypercholesterolaemic hypertensive patients. However, the effects cannot be attributed exclusively to the content of MUFAs (mainly oleic acid) in the diet, as high-oleic sunflower oil was unable to induce favourable changes.
Key words. Dietary fat; olive oil; high-oleic sunflower oil; cholesterol movement; cholesterol membrane; erythrocyte.

Introduction

In patients with untreated essential hypertension, it has been demonstrated that the distribution [1] and transbilayer movement [2] of erythrocyte membrane cholesterol are impaired. These findings were consistent with the presence of cholesterol-rich domains randomly distributed in the inner monolayer and a lower ability of cholesterol to cross the bilayer in the erythrocyte membrane of hypertensive patients. In addition, recent studies have indicated that both the distribution [3] and transbilayer movement [4] of erythrocyte membrane cholesterol strongly correlated with the activity of erythrocyte Na^+-Li^+ countertransport, a well-known marker of predisposition to essential hypertension [5].
Whereas dietary fatty acids play a role in modifying erythrocyte membrane phospholipid composition and function [6, 7], additional information concerning their effect on kinetic cholesterol pools and structural cholesterol domains would be useful. Interestingly, dietary olive oil (as a natural source of monounsaturated fatty acids, MUFAs) has been found to influence the activity of cation transport systems, including Na^+-Li^+ countertransport, in erythrocytes of postmenopausal hypertensive women [8]. Therefore, it raises the question of whether a MUFA-enriched diet could also have a favourable effect on impaired transbilayer movement of erythrocyte membrane cholesterol. This study was designed to compare the effect of a diet rich in olive oil (OO) with that of refined high-oleic sunflower oil (HOSO) on cholesterol transbilayer movement in the erythrocyte of untreated hypertensive patients, where both diets provided similar concentrations of MUFAs.

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Materials and methods

The study was conducted over two four-week periods, during which each participant followed HOSO or OO diets, with a washout (four-week) period (returning to the baseline diet) between both MUFA diets. Participants were recruited from a religious community in Seville (Spain), because of their regular lifestyle and dietary habits. Before the study, the participants recorded their regular dietary intake on four consecutive weeks (baseline), using 24 hour recall and food frequency questionnaires. The design of the present study was approved by the Institutional Committee on Investigation in Humans (Hospital Universitario Virgen del Rocío, Seville) and all participants gave informed consent.
Sixteen hypertensive (eight with normocholesterolaemia [mean $\pm$ SD age, 55.4 ± 4 years; BMI, 25.7 ± 4 kg/m^2) and eight with hypercholesterolaemia [age, 56.2 ± 3 years; body mass index (BMI), 24.5 ± 2 kg/m^2]) and 12 age-matched control female volunteers (age, 54.2 ± 4 years; BMI, 25.7 ± 3 kg/m^2) were enrolled for this study (table 1). All participants were non-smokers and postmenopausal to avoid interference by sexual hormones

Table 1. Characteristics of healthy subjects (Control), and normocholesterolaemic (HT/NChol) and hypercholesterolaemic (HT/HChol) hypertensive patients enrolled for this study[1].

Parameter	Control (n = 12)	HT/NChol (n = 8)	HT/HChol (n = 8)
Plasma lipid levels			
Cholesterol (mmol/l)	4.8 ± 0.6	5.1 ± 0.3	6.5 ± 0.3***
LDL (mmol/l)	3.3 ± 0.4	3.3 ± 0.1	4.7 ± 0.3***
HDL (mmol/l)	1.2 ± 0.2	1.3 ± 0.3	1.0 ± 0.2
Triacylglycerols (mmol/l)	0.9 ± 0.3	0.9 ± 0.3	1.9 ± 0.7**
Blood pressure			
Diastolic (mm Hg)	72.4 ± 5.3	94.9 ± 5.0***	92.0 ± 3.1***
Systolic (mm Hg)	122.3 ± 8.1	161.2 ± 14.4***	163.9 ± 9.6***

Values are expressed as means ± SD.
**Significantly different ($p < 0.01$) from control group.
***Significantly different ($p < 0.001$) from control group.
[1]All the participants had normal glucose tolerance and plasma insulin levels.

and the menstrual cycle. The criterion for hypertension was a diastolic blood pressure (DBP) of ≥90 mm Hg recorded on at least three different occasions after resting for 10 min in the supine position. The criterion for hypercholesterolaemia was a plasma total cholesterol (TC) concentration of ≥6.22 mmol/l and LDL of ≥ 4.14 mmol/l after 12 hours' fast. None of the patients had received any antihypertensive or antihypercholesterolaemic treatment. They underwent a clinical and laboratory examination to rule out any secondary cause of hypertension and none of them had *diabetes mellitus* or *hypothyroidism*. No history of alcohol abuse was presented. The control group maintained a DBP of <90 mm Hg, TC of <5.18 mmol/l, LDL of <3.37 mmol/l, and was in excellent health as defined by laboratory tests.

Diets were based on ordinary food and planned as one-week menus. The only difference between the diets lay in the edible fats, which were in the form of oils (Virgin olive oil: *Olea europaea*; high-oleic sunflower oil: *Helianthus annuus*) for cooking and for salads and occasionally spread on slices of bread. Fatty acid content of two diets (30% fat, 6% saturated fatty acids (SFAs), 21% MUFAs and 3% polyunsaturated fatty acids (PUFAs)) was characterized by a lower amount of SFAs and higher amount of oleic acid (12–13% of total energy) with regard to baseline (30% fat, 11% SFAs, 16% MUFAs and 3% PUFAs). Amounts of protein (22%), carbohydrate (48%), cholesterol (50 mg/MJ), and dietary fiber (2.7 g/MJ) were similar in the diets. Fatty acid content and minor components of the two oils are given in table 2. Three duplicate food portions corresponding to each weekday were collected, homogenized and analysed for their fat content and other nutrients. The energy consumption of participants was ~8.6 MJ. Two investigators were present twice a week in the kitchen during the preparation of the meals and remained blinded, along with the subjects, to changes in erythrocyte membrane cholesterol transbilayer movement and Na^+-Li^+ countertransport activity. The other investigators conducted most laboratory analyses and were blinded to the dietary assignments. Sodium intake (~200 mg/MJ) were similar in the baseline and MUFA-enriched diets.

Kinetics of the transbilayer movement of membrane cholesterol in erythrocytes were determined according to the continuous cholesterol oxidase treatment [2]. Briefly, venous blood (10 ml) was collected in 0.38% citrate. Erythrocytes were then obtained by centrifugation (1750 × g for 5 min at 4 °C) and washed three times in saline buffer (145 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l sodium phosphate; pH 7.4), and three times in assay buffer (310 mmol/l sucrose, 1 mmol/l $MgSO_4$, 5 mmol/l sodium phosphate; pH 7.4) at 4 °C. Cells were suspended in saline buffer to a 3% haematocrit and incubated for 5 min at 4 °C with 185×10^6 Bq [1,2-^{3}H(N)]-cholesterol (from New England Nuclear) (1.88×10^{12} Bq/mmol) in 50 µl ethanol. Radiolabelled cells were then rinsed four times in saline buffer and three times in assay buffer at 4 °C. Washed erythrocytes suspended in assay buffer were treated with cholesterol oxidase from *Brevibacterium* sp. (relative molecular mass approximately 32,500; 6 U/ml) at 37°C. The cells were pre-incubated at 37 °C (for 15 s), then treated

Table 2. Fatty acid and non-fatty acid constituents of high-oleic sunflower oil (HOSO) and olive oil (OO).

	HOSO	OO
Fatty acids (%)		
16:0	4.30	11.79
16:1n-7	0.14	0.86
17:0	0.13	0.37
18:0	4.72	2.79
18:1n-9	80.18	79.22
18:2n-6	9.44	3.45
18:3n-3	0.06	0.60
20:0	0.44	0.28
20:1n-9	0.22	0.20
24:0	0.37	0.44
Minor components (mg/kg)		
Hydrocarbons	310	4800
Sterols	3300	1400
Polyphenols	–	2600

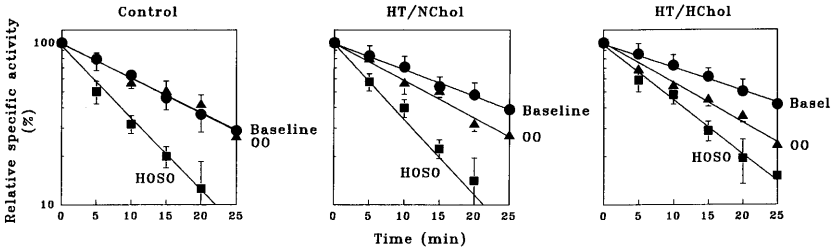

Figure 1. Changes in specific radioactivities of cholestenone with time in erythrocytes of control subjects, and (HT/NChol) normocholesterolaemic and (HT/HChol) hypercholesterolaemic hypertensive patients, during the periods on the high-oleic sunflower oil-rich (HOSO) and olive oil-rich (OO) diets. The data were expressed as the radioactivity of [^{3}H]-labeled sterol/μg of sterol, relative to the initial specific radioactivity of cholestenone. Half-time from these plots means the incubation time to reach 50% decay of the specific radioactivity of cholestenone. Values are expressed as means ± SD.

continuously with enzyme (from 0 to 25 min). To end the reaction, aliquots of the mixture were extracted with chloroform-methanol (2:1, vol/vol). The organic phase was split into two portions for sterol mass analysis and for determination of sterol radioactivity.

Activity of Na^+-Li^+ countertransport was achieved as external Na^+ stimulated Li^+ efflux from Li^+-loaded cells as described [5]. Briefly, erythrocytes were suspended in loading buffer (75 mmol/l Li_2CO_3, 10 mmol/l glucose, 10 mmol/l MOPS-Tris; pH 7.4) to a 10% haematocrit and incubated at 37 °C for 60 min. Cells were then washed four times in magnesium washing solution (115 mmol/l $MgCl_2$, 10 mmol/l MOPS-Tris; pH 7.4). Li^+ efflux was measured by incubating the erythrocytes in sodium-enriched medium (150 mmol/l NaCl, 10 mmol/l glucose, 0.1 mmol/l ouabain, 10 mmol/l MOPS-Tris; pH 7.4) or sodium-free medium (75 mmol/l $MgCl_2$, 85 mmol/l sucrose, 10 mmol/l glucose, 0.1 mmol/l ouabain, 10 mmol/l MOPS-Tris; pH 7.4) at 37 °C for 60 min. At the end of this loading period, cells were centrifuged and the supernatants were used for analysis of lithium by atomic absorption spectrophotometry. Kinetics of Na^+-Li^+ countertransport in the erythrocyte were estimated as the difference between Li^+ efflux into sodium-rich and sodium-free media. The lithium efflux was computed from the linear regression of lithium loss as a function of time.

Haemolysis of cholesterol oxidase-treated erythrocytes was determined by haemoglobin absorbance at 540 nm and 410 nm (Soret's band) and measurement of adenylate kinase and lactate dehydrogenase activities [2, 9].

Data were evaluated by using a two-tailed paired *t*-test. The significance of the differences between the groups was assessed by analysis of variance (ANOVA) with Tukey's post-hoc comparison of the means. Correlations were determined by linear regression analysis using Pearson's correlation coefficient. The analyses were done with the GraphPAD InStat (GraphPAD Software, San Diego, CA, USA) and CoStat (CoHort Software, Berkeley, CA, USA) statistical packages.

Results

All participants responded in a similar manner to the two diets and completed the study according to schedule. Compliance with the diets was estimated to be ~90% from the evaluation of daily food questionnaires and by analysis of the fatty acid composition of the plasma cholesterol ester fraction in each patient [10]. There was a significant increase of oleic acid during MUFA-enriched diets, suggesting good adherence to the diets. Body weight was maintained after both MUFA dietary periods.

When intact erythrocytes are pulsed with [^{3}H]-cholesterol at 4 °C the subsequent incubation of labelled cells at 37 °C initiates the equilibration of inner and outer leaflet cholesterol through transbilayer movement [11]. The treatment of [^{3}H]-cholesterol-labelled erythrocytes with cholesterol oxidase, which attacks a cholesterol at the 3β-hydroxyl position to form Δ^4-cholestenone [12], causes a decrease in the specific radioactivity of cholestenone with time at 37 °C and represents mixing of the pool of unlabelled cholesterol (2). By this method, plots are constructed where 50% decay (half-time) reflects transbilayer movement of [^{3}H]-cholesterol [2, 11]. Therefore, an increase in the half-time for the decrease in specific radioactivity of cholestenone means a decrease in the rate of cholesterol transbilayer movement. During this enzyme treatment, leakage of haemoglobin was not apparent and no more than 5% haemolysis was found either by means of adenylate kinase or lactate dehydrogenase activities at the end of the longest incubation time. Under conditions of cell integrity, it is assumed that the reactive cholesterol represents a sampling of cholesterol in the outer monolayer of the plasma membrane [11].

The specific radioactivity of cholestenone decreased with time of incubation at 37 °C in [^{3}H]-cholesterol-labelled erythrocytes of controls and hypertensive women after a continuous cholesterol oxidase treatment, with different kinetics at baseline and after HOSO or OO diets (fig. 1). Thus, the half-time (mean ± SD) for the decrease in specific radioactivity of

cholestenone was significantly higher in hypertensive patients than in controls (table 3), ranging from 32% in the hypertensive normocholesterolaemic (HT/NChol) group to 46% in the hypertensive hypercholesterolaemic (HT/HChol) group.

This reduction in the movement of cholesterol across the erythrocyte membrane was suddenly impaired after the HOSO diet, as there was a 60% decrease in the half-time for the process with no significant differences between groups. Interestingly, the OO diet resulted in a tendency to normalize the kinetics of transbilayer movement of membrane cholesterol either for the HT/NChol or HT/HChol groups and, more importantly, the values of the half-time were similar to the value of the control group at baseline.

Discussion

This study was undertaken to determine the effect of MUFA-enriched diets on the transbilayer movement of erythrocyte membrane cholesterol in normotensive normolipidaemic healthy subjects and patients with untreated essential hypertension, either with or without concomitant hypercholesterolaemia. MUFA derived mainly from HOSO and OO. The diets were based on ordinary food and contained similar amounts of total fats, proteins, carbohydrates, cholesterol, and other nutrients. However, significant differences were noted in the minor components of two oils, including polyphenols that were present in OO but not in HOSO.

Our results show that the half-time for the decrease in specific radioactivity of cholestenone was increased in the erythrocyte of hypertensive women, which means a decrease in the rate of cholesterol transbilayer movement [2, 11]. This modification of kinetic behaviour of structural cholesterol domains in the bilayer of erythrocyte membrane was further impaired after the HOSO dietary period, the extent of which was found to be the same for the control, HT/NChol and HT/HChol groups. However, the altered half-times for hypertensive groups decreased towards normal after the OO diet, reaching similar values to those found for the control group at baseline. This effect of the OO diet agreed with the reduction in the abnormally increased activity of erythrocyte Na^+-Li^+ countertransport [13], the major cation transport parameter associated with the variations of erythrocyte membrane and serum lipids of hypertensive patients [8, 14, 15]. Indeed, our data have also revealed a significant correlation between cholesterol transbilayer movement and Na^+-Li^+ countertransport after different diets, in agreement with a previous study [4]. There was higher activity of Na^+-Li^+ countertransport at lower half-time for the decrease in specific radioactivity of cholestenone, suggesting that an increase in the transbilayer movement of membrane cholesterol might play a major role in such erythrocyte sodium transport system.

Kinetic cholesterol pools (rapidly exchangeable, very slowly exchangeable, and nonexchangeable) and structural cholesterol domains (including lateral domains in the same monolayer) are closely linked in the human erythrocyte membrane [16–18]. However, the mechanism(s) associated with the modulation of membrane cholesterol distribution and the significance of this asymmetry for non-receptor mediated cholesterol transport and for membrane function (especially membrane-bound proteins) are still unknown. Interestingly, when cells (mouse LM fibroblast and rat aortic smooth muscle cell lines) are cultured with unsaturated fatty acids in the medium, the transbilayer cholesterol gradient may be reversed [19, 20] and the plasma membrane cholesterol efflux may be increased [21]. These data support the notion that transbilayer movement and distribution of membrane cholesterol can be regulated by fat-enriched diets, the physiological consequences of which are of major interest to the prevention of metabolic diseases. Our diets provided a similar content of oleic acid (18:1n-9), but HOSO had nearly three-fold higher linoleic acid (18:2n-6) whereas OO had ten-fold higher linolenic acid (18:3n-3). In addition, intake of OO is associated with a reduced incidence of coronary heart disease [22], particularly when hypertension is coexistent [23]. It is therefore tentative to speculate that the effect of the OO-enriched diet on reducing the systolic and diastolic blood pressures in normotensive and hypertensive women [8, 24] could be mediated by its effect on kinetic cholesterol pools in the membrane of vascular cells.

Certainly, we can claim that a MUFA-enriched diet, derived mainly from OO, may be considered as helpful in restoring the impaired membrane cholesterol transbilayer movement in erythrocytes of patients with untreated essential hypertension. HOSO was unable to

Table 3. Half-times for the decrease in specific radioactivity of cholestenone in erythrocyte membrane during the periods on the high-oleic sunflower oil-rich (HOSO) and olive oil-rich (OO) diets[1].

Participant group	Baseline	HOSO	OO
Control (n = 12)	13.9 ± 2.6	5.9 ± 2.6^{a}	14.0 ± 2.9^{b}
HT/NChol (n = 8)	18.3 ± 3.9*	6.9 ± 3.2^{a}	$13.2 \pm 1.6^{a,b}$
HT/HChol (n = 8)	20.3 ± 6.9*	8.4 ± 3.9^{a}	$11.6 \pm 1.8^{a,b}$

Values are expressed as means (in minutes) $\pm$ SD.
*Significantly different ($p < 0.01$) from control group.
[a]Significantly different ($p < 0.001$) from baseline diet.
[b]Significantly different ($p < 0.001$) from HOSO diet.
HT/NChol, hypertensive and normocholesterolaemic patients.
HT/HChol, hypertensive and hypercholesterolaemic patients.
[1]According to the cholesterol oxidase method [1, 11], the content of cholesterol in the outer monolayer of erythrocyte membrane was around 15% (of total cholesterol) for control group, 8% for HT/NChol and 7% for HT/HChol at baseline; 25% for control group, 22% for HT/NChol and 23% for HT/HChol after HOSO diet; and 14% for control group, 13% for HT/NChol and 11% for HT/HChol after OO diet.

induce the same changes as OO and therefore we cannot rule out the possibility that other minor components rather than oleic acid found in OO [25, 26] might contribute to the observed differences.

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