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Changes in membrane lipid composition of human erythrocytes after dietary supplementation of ($n-3$) polyunsaturated fatty acids. Maintenance of membrane fluidity

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The effect of dietary ($n-3$) polyunsaturated fatty acids on erythrocyte membrane lipid composition, fluidity, and flexibility was studied in seven healthy subjects. An eight weeks daily supplementation of 3 g of the ($n-3$) fatty acids eicosapentaenoic and docosahexaenoic acid resulted in an increased unsaturation of erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE). This change was accompanied by a slight decrease in PC and PE content ($P < 0.05$) and an increase in sphingomyelin content ($P < 0.01$). The erythrocyte membrane fluidity, measured with electron spin resonance of intact erythrocytes and with fluorescence polarization of erythrocyte ghosts did not change. No change was seen in the viscosity of erythrocyte suspensions of haematocrit = 0.80, measured at various shear rates. The supplementation caused a 42% decrease in plasma triacylglycerol levels. We suggest that the change in the erythrocyte membrane fatty acid composition induced by the dietary supplementation of ($n-3$) fatty acids might be counteracted by a change in the phospholipid class distribution, resulting in overall maintenance of membrane fluidity.

Introduction

Maintenance of membrane fluidity within narrow limits is presumably a prerequisite for proper functioning of a cell. Lipids play a key role in determining the membrane fluidity, and changes in lipid composition have been reported to alter important cellular functions [1–4].

The integrity of membrane lipid fluidity is demonstrated by processes of adaptation to situations where membrane fluidity is temporarily impaired. The adaptation to thermally-induced perturbations of membrane fluidity has been studied extensively in micro-organisms, plants, poikilotherms and hibernating animals [5–8], the adaptation to nutritionally-induced perturbations mainly in tissue cultures [9]. We recently reported compensatory changes in erythrocyte membrane lipid composition in rabbits eating a cholesterol-rich diet [10].

The last decennia diets rich in polyunsaturated fatty acids (mainly linoleic acid C18:2 ($n-6$)) have been recommended for treatment and prevention of hyperlipidaemia and atherosclerosis, and for inhibition of thrombosis. More recently ($n-3$) fatty acids have been recognized being even more potent than linoleic acid [11,12]. Polyunsaturated fat feeding alters the fatty acid com-

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position of cellular membranes [13]; this change can occur rapidly [14,15]. In most studies however no attention has been paid to a possibly subsequent effect on the other chemical modulators of lipid fluidity, viz. the cholesterol/phospholipid ratio and the phospholipid class distribution. In order to restore the original fluidity, a change in membrane fatty acid composition might be counteracted by changes in cholesterol/phospholipid ratio or phospholipid class distribution.

The aim of the present study was to investigate the effect of dietary polyunsaturated fatty acids on the fluidity and its chemical determinants of erythrocyte membranes in man. We used the polyunsaturated fatty acids C20 : 5($n-3$) and C22 : 6($n-3$) because of their efficient incorporation into membranes, even at low doses, and their availability in purified concentrated form [15,16]. Before and after an eight weeks daily supplementation of 3 g of ($n-3$) fatty acids we measured the fatty acid composition of erythrocyte PC and PE, cholesterol/phospholipid ratio and phospholipid class distribution. Membrane flexibility was measured by viscosimetry, and membrane fluidity by electron spin resonance (ESR) using three different fatty acid spin labels, and by fluorescence polarization using diphenylhexatriene.

Subjects and Methods

Subjects

Seven healthy male subjects, aged 23–41 years, volunteered for the trial. Informed consent from each participant and permission for the study from the Medical Ethical Committee of the Free University Hospital Amsterdam were obtained. After a four week run-in period, subjects took 6 capsules, each containing 1 g of a marine lipid concentrate SuperEPA (Pharmacaps Inc., Marlow, Buckinghamshire, U.K.) daily for eight weeks. Each capsule provided 0.5 g of the ($n-3$) fatty acids C20 : 5($n-3$) plus C22 : 6($n-3$) as their methyl esters and 1 I.U. vitamin E. The fatty acid composition of the SuperEPA concentrate is given in Table I. Fasting heparinized blood samples were taken before, at eight weeks, and six weeks after withdrawal of the supplementation. After sampling, blood was placed immediately on ice and centrifuged at 4°C and 1500 × g.

TABLE I

FATTY ACID COMPOSITION OF THE SUPPLEMENTATION Super EPA

Fatty acid	mol%
14:0	0.7
16:0	4.6
16:1	1.7
18:0	6.6
18:1($n-9$)	13.1
18:2($n-6$)	1.7
18:3($n-3$)	1.0
18:4($n-3$)	2.3
20:1($n-9$)	6.6
20:5($n-3$)	31.0
22:1($n-11$)	4.4
22:4($n-3$)	1.7
22:5($n-3$)	5.8
22:6($n-3$)	18.8

Methods

Membrane lipid composition. Erythrocytes were washed three times with cold isotonic saline, and buffy coat was removed. Cells were frozen at −20°C for at least 2 h and thawed before extraction. Lipids were extracted according to Radin [17] and the following analyses were done: fatty acid composition of PC and PE; the distribution of the phospholipid classes PC, PE, phosphatidylserine and sphingomyelin; cholesterol/phospholipid ratio. The fatty acid composition of phosphatidylserine and sphingomyelin was not analysed, because supplementation of ($n-3$) fatty acids does not change the fatty acid composition of erythrocyte sphingomyelin and that of phosphatidylserine only slightly [15]. Phospholipid unsaturation, expressed as double bond index (dbi, mean of double bonds per molecule fatty acid) was calculated from the total fatty acid composition. Phospholipid classes were separated on silica gel high performance thin-layer plates (Merck, F.R.G.) using chloroform/methanol/25% ammonia (65 : 35 : 5, v/v) as solvent system. The amount of phospholipids was determined as phosphorus after destruction of the phospholipids; fatty acids were determined as their methyl esters and fatty aldehydes (from plasmalogens) as their dimethyl acetals by gas chromatography using a fused silica capillary column coated with Cp Sil 88 (Chrompack, Middelburg, The Netherlands); cholesterol

was determined as trimethylsilyl ether by gas chromatography using a fused silica capillary column coated with Cp Sil 5 (Chrompack) and stigmasterol as internal standard. All lipid analyses have been described in detail previously [15].

Membrane fluidity. Membrane fluidity was assessed with electron spin resonance (ESR) in intact erythrocytes and with fluorescence polarization in erythrocyte ghosts.

For ESR measurements, erythrocytes were washed three times with isotonic phosphate-buffered saline (pH 7.4) at 4°C, and buffy coat was removed. Spin labelling was done by adding a suspension of 150 μl washed packed erythrocytes plus 50 μl phosphate buffered saline to a dried film of stearic acid spin label, prepared by drying an appropriate amount of spin label solution (0.1 mg/ml ethanol) under nitrogen in a small glass tube, and incubating this mixture for at least 15 min at room temperature. Three different stearic acid spin labels (SAL) were used: 5-, 12-, and 16-SAL (EGA Chemie, Steinheim, F.R.G.), which have a stable nitroxide radical ring at the 5th, 12th and 16th carbon position counted from the carboxyl group of the acyl chain, respectively. The concentrations of spin labels were, per 100 μl packed erythrocytes, 10 μg, 12 μg, and 2 μg of 5-SAL, 12-SAL and 16-SAL, respectively. At these concentrations effects of spin-spin interaction, namely extra peak-broadening and concentration-dependent S values, were absent, as was estimated experimentally. ESR spectra were recorded at 37°C on a Varian E3 EPR spectrometer (Varian Instruments, Palo Alto, CA, U.S.A.), equipped with a dewarted insert and a nitrogen flow temperature control system Varian E257. The temperature inside the sample cavity was monitored with a copper-constantan thermocouple and held as constant as possible using a silicone-oil filled quartz NMR tube (accuracy $\pm$ 0.5 K). Instrument settings were: microwave power 5.5 mW; modulation amplitude for the estimation of order parameters 4 G and for the estimation of rotational correlation time 1G; field sweep 100 G; time constant/scan time: 1 s/8 min, 3 s/16 min or 10 s/30 min. Order parameters for 5-, 12-, and 16-SAL and rotational correlation time for 16-SAL were calculated from spectral data, as described by Kamada et al. [18]. Each ESR spectrum was recorded at least twice. High values for order parameters (S) and rotational correlation time (τ_0) correspond to a state of low fluidity.

For fluorescence polarization measurements, erythrocytes were washed three times with isotonic phosphate-buffered saline (pH 7.4) at 4°C, and haemoglobin-free ghosts were prepared according to Dodge et al. [19], using hypotonic phosphate buffer (20 mosM, pH 7.4). Ghosts were resuspended in isotonic phosphate-buffered saline and stored at −20°C under nitrogen until analysis. Steady-state fluorescence polarization measurements were done with an Elscint apparatus, model MV-1A (Elscint Ltd., Haifa, Israel) at 25°C according to Van Blitterswijk et al. [20], using the probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Koch Light, Colnbrook, U.K.). Values of the fluorescence polarization P_{DPH} mainly reflect the orientational constraint of the probe and can be converted quantitatively into lipid order parameters S_{DPH}, using a semi-empirical relationship [21,22]. High P_{DPH} and S_{DPH} values correspond to a state of low fluidity.

Erythrocyte deformability. Erythrocytes were washed three times with phosphate buffer (pH 7.4, 300 mosM), to which was added 10 g/l albumin and 5 mmol/l glucose. Suspensions of the washed erythrocytes were prepared in this buffer with a haematocrit of 0.80. Viscosity analysis was done at 37°C and within 6 h after the blood sampling by use of the Contraves Low Shear 30 Rheometer at shear rates ranging from 0.081 − 94.5 s^{-1}.

Plasma lipids. Plasma triacylglycerol was analysed by an enzymatic method (BioMérieux, Marcy-l'Etoille, France), and plasma total cholesterol according to Röschlau et al. [23] using the kit Mono test supplied by Boehringer Mannheim GmbH (F.R.G.).

Statistical analysis. Results are given as mean $\pm$ S.E. Student's t-test for paired observations was used for statistical analysis.

Results

Membrane lipid composition

The fatty acids in erythrocyte PC and PE that showed a significant change after the supplementation are given in Table II and III. In PC of all subjects an increase was seen in C20 : 5(n − 3) and

TABLE II

CONTENT OF RELEVANT FATTY ACIDS (mol%) IN ERYTHROCYTE PHOSPHATIDYLCHOLINE OF HEALTHY SUBJECTS BEFORE, DURING AND AFTER THE ($n-3$) FATTY ACID SUPPLEMENTATION

Values are mean ± S.E., $n = 7$. dbi, double bond index = mean of double bonds per molecule fatty acid. *** $P < 0.001$ and * $P < 0.05$, compared to values before (Student's paired t-test).

	Before	During	After
18:2($n-6$)	24.1 ±0.9	21.1 ±1.1 ***	23.8 ±1.1
20:5($n-3$)	0.4 ±0.1	2.3 ±0.2 ***	0.5 ±0.1
22:6($n-3$)	1.3 ±0.2	2.3 ±0.1 ***	1.6 ±0.1 *
dbi	1.03±0.02	1.12±0.02 ***	1.04±0.01

TABLE III

CONTENT OF RELEVANT FATTY ACIDS (mol%) IN ERYTHROCYTE PHOSPHATIDYLETHANOLAMINE OF HEALTHY SUBJECTS BEFORE, DURING AND AFTER THE ($n-3$) FATTY ACID SUPPLEMENTATION

Values are mean ± S.E., $n = 7$. dbi, double bond index = mean of double bonds per molecule fatty acid. *** $P < 0.001$ and ** $P < 0.01$, compared to values before (Student's paired t-test).

	Before	During	After
18:2($n-6$)	7.3 ±0.5	6.0 ±0.4 ***	6.7 ±0.5 **
20:4($n-6$)	20.2 ±0.4	18.5 ±0.4 **	18.4 ±0.4 **
22:4($n-6$)	5.8 ±0.3	4.8 ±0.2' **	4.5 ±0.2 **
20:5($n-3$)	0.9 ±0.1	3.2 ±0.2 ***	2.4 ±0.1 ***
22:5($n-3$)	3.8 ±0.4	4.8 ±0.1 ***	4.9 ±0.1 ***
22:6($n-3$)	5.0 ±0.4	6.7 ±0.3 ***	6.8 ±0.2 ***
dbi	1.92±0.02	2.08±0.03 **	2.05±0.02 **

C22:6($n-3$) at the expense of linoleic acid C18:2($n-6$). The double bond index increased from 1.03 ± 0.02 to 1.12 ± 0.02 (mean ± S.E., $P < 0.001$). After withdrawal of the supplementation for six weeks the fatty acid composition of PC had returned to basal values. In PE of all subjects an increase was seen in C20:5($n-3$), C22:5($n-3$) and C22:6($n-3$) at the expense of C18:2($n-6$), C20:4($n-6$) and C22:4($n-6$). The double bond index increased from 1.92 ± 0.02 to 2.08 ± 0.03 (mean ± S.E., $P < 0.01$). No change was seen in the dimethyl acetals from PE, indicating that the relative amount of plasmalogens in the PE class had not changed. After withdrawal of the supplementation for six weeks the changes in PE still persisted.

The distribution of the four phospholipid classes PC, PE, PS and sphingomyelin and the

TABLE IV

PHOSPHOLIPID CLASS DISTRIBUTION (mol%) AND CHOLESTEROL/PHOSPHOLIPID MOLE RATIO IN ERYTHROCYTE MEMBRANES OF HEALTHY SUBJECTS BEFORE, DURING AND AFTER THE ($n-3$) FATTY ACID SUPPLEMENTATION

Values are mean ± S.E., $n = 7$. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; C/P, cholesterol/phospholipid ratio. ** $P < 0.01$ and * $P < 0.05$ compared to values before (Student's paired t-test).

	Before	During	After
PC	28.9 ±0.5	28.4 ±0.5 *	29.0 ±0.5
PE	28.9 ±0.3	28.2 ±0.4 *	28.5±0.5
PS	17.6 ±0.5	17.7 ±0.4	17.6 ±0.5
SM	24.7 ±0.4	25.8 ±0.3 **	25.1 ±0.4
C/P	0.82±0.01	0.82±0.01	0.80±0.01

TABLE V

FLUIDITY PARAMETERS OBTAINED WITH ESR MEASUREMENTS (S, τ_0) ON INTACT ERYTHROCYTES AT 37°C AND WITH FLUORESCENCE POLARIZATION MEASUREMENTS (P_{DPH}, S_{DPH}) ON GHOSTS AT 25°C, OF HEALTHY SUBJECTS BEFORE, DURING AND AFTER THE ($n-3$) FATTY ACID SUPPLEMENTATION

Values are mean ± S.E., $n = 7$. ESR, electron spin resonance; S(5-SAL), S(12-SAL and S(16-SAL) are order parameters for stearic acid labelled with a nitroxide radical at the 5th, 12th and 16th carbon position, respectively; τ_0, rotational correlation time for 16-SAL; P_{DPH}, fluorescence polarization of diphenylhexatriene; S_{DPH}, order parameter for diphenylhexatriene.

	Before	During	After
S(5-SAL)	0.663 ± 0.002	0.662 ± 0.002	0.666 ± 0.003
S(12-SAL)	0.509 ± 0.002	0.512 ± 0.002	0.510 ± 0.002
S(16-SAL)	0.344 ± 0.003	0.350 ± 0.002	0.349 ± 0.002
τ_0 (10^{-10} s)	19.7 ± 0.11	19.9 ± 0.06	19.3 ± 0.12
P_{DPH}	0.346 ± 0.002	0.342 ± 0.002	0.344 ± 0.001
S_{DPH}	0.787 ± 0.003	0.780 ± 0.003	0.783 ± 0.002

cholesterol/phospholipid ratio is shown in Table IV. After eight weeks of the supplementation in all subjects a slight decrease was seen in PC and PE ($P < 0.05$) and an increase in sphingomyelin content ($P < 0.01$). No change in the cholesterol/phospholipid ratio was seen.

Membrane fluidity

Fluidity parameters as measured by ESR and fluorescence polarization are given in Table V. The order parameters (S) of the three stearic acid spin labels 5-, 12-, and 16-SAL, and the rotational correlation time τ_0 (16-SAL) did not change by the supplementation. A slight but insignificant decrease in P_{DPH} and S_{DPH} was seen.

TABLE VI

VISCOSITY OF SUSPENSIONS OF ERYTHROCYTES (Ht = 0.80) FROM HEALTHY SUBJECTS BEFORE, DURING AND AFTER THE ($n-3$) FATTY ACID SUPPLEMENTATION, MEASURED AT VARIOUS SHEAR RATES

Values are mean ± S.E., $n = 7$.

Shear rate (s^{-1})	Viscosity (mPascal·s)		
	Before	During	After
0.081	192 ± 4.3	190. ± 2.2	192 ± 6.0
0.945	50.5 ± 0.8	50.0 ± 0.4	50.9 ± 1.2
8.11	23.9 ± 0.2	23.6 ± 0.2	24.1 ± 0.3
20.4	18.5 ± 0.2	18.2 ± 0.2	18.6 ± 0.2
51.2	15.1 ± 0.2	14.9 ± 0.2	15.2 ± 0.2
94.5	13.5 ± 0.2	13.3 ± 0.1	13.5 ± 0.2

Erythrocyte deformability

The viscosity of erythrocyte suspensions of haematocrit (Ht) = 0.80, measured at various shear rates, is given in Table VI. Viscosity values tended to decrease after the eight weeks of the supplementation, however, no significance was reached.

Plasma lipids

The supplementation had no effect on plasma total cholesterol levels: 5.2 ± 0.5 and 5.2 ± 0.4 mmol/l (mean ± S.E., before and after the supplementation, respectively). Plasma triacylglycerol concentrations decreased significantly from 1.2 ± 0.2 to 0.7 ± 0.1 mmol/l (mean ± S.E., $P < 0.02$). Six weeks after withdrawal of the supplementation the plasma triacylglycerol level had returned to basal value: 1.2 ± 0.2 (mean ± S.E.).

Discussion

The effect of the ($n-3$) fatty acid supplementation on the fatty acid composition of erythrocyte PC and PE is similar to the effect of cod-liver oil as described previously [15]. The rates of appearance and disappearance of the ($n-3$) fatty acids in PC and PE are different and the slow disappearance of ($n-3$) polyunsaturated fatty acids from PE is demonstrated by the still increased levels of these acids in PE six weeks after withdrawal of the supplementation (Table III). PE, largely located in the inner layer of the membrane, is thought to be renewed by acylation of lysophospholipid [24], which is apparently a slow

process. PC, located mainly in the outer layer, is renewed by exchange of PC between plasma lipoproteins and erythrocyte membrane [24,25]. The rates of changes in erythrocyte PC fatty acid composition therefore depend on the rate of changes in plasma lipoprotein PC fatty acid composition, which is relatively fast [14,15].

The supplementation caused an increase in the unsaturation of PC and PE, due to substitution of $(n-3)$ polyunsaturated fatty acids for less unsaturated ones. Although it is widely assumed that changes in degree of unsaturation affect membrane fluidity, we did not find a change in fluidity parameters. There are several possibilities to explain this lack in change. Firstly, the changes in phospholipid class distribution, especially the increase in sphingomyelin (Table IV) might have contributed to counteracting the effect of increased unsaturation. This is in line with the concept of self-adaptive modification of erythrocyte membrane lipids as described by Yawata et al. [26]. They measured normal membrane fluidity in erythrocytes from a patient with phosphatidylcholine–lecithin : cholesterol acyltransferase, despite increased membrane cholesterol/phospholipid ratio due to the abnormal lipoprotein composition. They suggested that the increased membrane phospholipid unsaturation and the altered phospholipid class distribution counteracts the fluidity decreasing effect of the abnormal cholesterol/phospholipid ratio. Secondly, it is possible that the change in fatty acid composition and double bond index per se does not lead to a measurable change in fluidity. Physical properties of phospholipids in a membrane are determined not only by the length and degree of unsaturation of the acyl chains, but also by their positions in the molecule. Switching of fatty acids between the *sn*-1 and *sn*-2 positions, or combining two saturated or two unsaturated fatty acids within one molecule have important effects on fluidity parameters [27,28]. Although the $(n-3)$ fatty acid supplementation did not alter the amount of saturated fatty acids relative to the amount of polyunsaturated ones (the incorporation of the $(n-3)$ polyunsaturated fatty acids was at the expense of other polyunsaturated acids such as 18 : 2, 20 : 4 and 22 : 4), a switch of fatty acids between position *sn*-1 and *sn*-2 can not be excluded. To know the positions of the fatty acids, analysis of molecular species is needed.

We measured membrane lipid fluidity with fluorescence polarization and ESR. For the fluorescence polarization measurements the fluorophore diphenylhexatriene (DPH) was used, which monitors the dynamic behaviour of the hydrofobic regions of the membrane. If frozen ghosts are used, both leaflets of the lipid bilayer are labelled, and the obtained P_{DPH} and S_{DPH} values are indicative of the packing of apolar moieties in the whole membrane. The P_{DPH} and S_{DPH} values tended to decrease during the $(n-3)$ fatty acid supplementation; however the difference between the means was within the limit of accuracy of the individual P_{DPH} measurements.

For the ESR measurements we labelled intact erythrocytes with three different fatty acid spin labels, thus getting information on the fluidity gradient of the outer layer of the membrane. The fluidity of the core of the membrane can be probed using 16-SAL which has the spin label near the methyl end of the fatty acid. We expected the incorporation of the $(n-3)$ fatty acids into the membrane to affect the fluidity of the core more than the surface. However, we did not find an effect of the supplementation on the order parameter (S) nor on the rotational correlation time (τ_0) of 16-SAL. A change in S or τ_0 would have indicated a change in the range of motion (S) or in the rate of motion (τ_0). The concomitant relative increase in membrane sphingomyelin, which possesses very long saturated fatty acyl chains (24 : 0) reaching far into the core of the bilayer, might be responsible for this lack in effect.

The S values which we measured with 5-SAL were similar to those reported by Kamada et al. [18]. However, for 16-SAL and, to a lesser extent 12-SAL, they measured lower S values than we did. This can possibly be ascribed to the concentration of spin probe in the membrane. We have used experimentally determined low probe concentrations to avoid spin-spin interactions [29,30] and found that especially the concentration of 16-SAL must be kept very low. When we used the higher concentrations (as Kamada et al. did) we also found lower S values.

The value of the viscosity of erythrocyte suspensions of high haematocrits can be used as a

measure of cell deformability. Measured at low shear rates, the viscosity values reflect mainly membrane flexibility [31–33]. A change in the fatty acid composition of erythrocyte membrane phospholipids might be associated with a change in erythrocyte deformability [16]. The ($n-3$) fatty acid supplementation did not cause a change in erythrocyte membrane flexibility, which might be consistent with the lack in change in fluidity.

The plasma lipid lowering effect of fish oil/($n-3$) fatty acids is known for several years [11]. Especially plasma triacylglycerol levels are affected. The supplementation of 3 g of ($n-3$) fatty acids caused a decrease in plasma triacylglycerol concentration of 42%: from 1.2 to 0.7 mmol/l. This decrease is of the same order as that found in healthy subjects taking very large amounts of ($n-3$) fatty acids: 24 g of ($n-3$) fatty acids daily [34], or fish oil up to 30% of the daily energy need [35]. Apparently, supplementation above the moderate amounts used by us does not give further decrease of plasma triacylglycerol levels.

In summary, we demonstrate that altering the fatty acid composition of erythrocytes by dietary supplementation of ($n-3$) polyunsaturated fatty acids in a way that brings about a significant change in double bond index does not alter membrane fluidity as monitored by the different techniques electron spin resonance and fluorescence polarization.

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