Decreased membrane fluidity and altered susceptibility to peroxidation and lipid composition in overweight and obese female erythrocytes

Roberta Cazzola,[1,*] Mariangela Rondanelli,[†] Samantha Russo-Volpe,[*] Ettore Ferrari,[†] and Benvenuto Cestaro[*]

Department of Preclinical Sciences "LITA Vialba,"[*] University of Milan via G. B. Grassi, 74-20157 Milan, Italy; and Department of Internal Medicine and Medical Therapy,[†] School of Endocrinology and Metabolism and Chair of Geriatrics, "Istituto Santa Margherita," University of Pavia, Piazza Borromeo, 5-27100 Pavia, Italy

Abstract The increased generation of reactive oxygen species that occurs in the condition of obesity may be responsible for oxidative injury to erythrocyte membranes, which could lead to a decrease in tissue oxygenation. Therefore, we have looked into the effects of obesity on both indexes of oxidative damage and physical-chemical properties of erythrocyte membranes in 50 overweight or obese [25 < body mass index (BMI) < 33], normotensive, nondiabetic women and 50 age-matched lean healthy women (BMI < 25). In the obese group compared with the lean group, we found that *a*) the onset of free radical-induced erythrocyte hemolysis and the ratio between reduced and oxidized glutathione were reduced, whereas the rate of free radical-induced damage increased; *b*) the n-3 fatty acid and the phospholipid contents decreased; *c*) the ratio between cholesterol and phospholipids increased; and *d*) the membrane fluidity decreased. These findings suggest an impairment of erythrocyte membrane physical-chemical properties in overweight and obese people as a consequence of oxidative injury that might be part of a pathogenetic mechanism responsible for obesity-related pathologies such as atherosclerosis and hypertension.—Cazzola, R., M. Rondanelli, S. Russo-Volpe, E. Ferrari, and B. Cestaro. **Decreased membrane fluidity and altered susceptibility to peroxidation and lipid composition in overweight and obese female erythrocytes. *J. Lipid Res.* 2004.** 45: **1846–1851.**

Supplementary key words fluorescence anisotropy • oxidative stress • essential fatty acids • membrane cholesterol

Physiological oxidative metabolism and neutrophil activation occurring in the blood give rise to oxygen-reactive substances and other very active radical species that can irreversibly damage the surrounding macromolecules. In particular, these radicals can attack both the amino and thiol groups of proteins and the double bonds of polyunsaturated fatty acids in lipoproteins. Statistically significant correlations have been found between lipoprotein susceptibility to peroxidation, the degree of obesity, and the risk of developing cardiovascular disease (1). Because any increase in the rate of lipoprotein peroxidation not only diminishes their levels of polyunsaturated fatty acids but also consumes and reduces their antioxidant contents (vitamin E, β-carotene, coenzyme Q, etc.), the consequence of these biochemical events in overweight and obese people is also a probable reduction of the "exchange rate" of both polyunsaturated fatty acids and lipophilic antioxidants that are normally transferred in the blood from the donor lipoproteins to the erythrocyte acceptor membranes. A decrease in both the degree of polyunsaturation of lipids and the antioxidant levels of the erythrocyte membrane could thus be expected, together with a decrease of both membrane fluidity and the activity of its membrane-bound enzymes. Because the erythrocyte membrane serves as a variable barrier to oxygen transport, the changes in its composition can induce cellular hypoxia in the tissue bed. Furthermore, because the size, shape, and diffusion capacity of a red blood cell depend on the structure of its membrane, alterations in membrane structure could lead to a decrease in tissue oxygenation (2). Such modifications of oxygen available in cardiovascular cells might be part of a pathogenetic mechanism responsible for obesity and obesity-related pathologies such as atherosclerosis and hypertension. To our knowledge, the physical-chemical properties of the erythrocyte membrane in overweight and obese people have not been thoroughly investigated. With the aim of providing more detailed information on these properties, we performed the following study in which we measured the susceptibility to peroxidation, the lipofuscin levels, the flu-

Manuscript received 12 December 2003 and in revised form 19 May 2004.

Published, JLR Papers in Press, July 1, 2004.
DOI 10.1194/jlr.M300509-JLR200

[1] To whom correspondence should be addressed.
e-mail: roberta.cazzola@unimi.it

idity, the activity of ATPase and acetylcholinesterase, and the cholesterol and fatty acid compositions of the erythrocyte membrane in two adult female groups: overweight and obese patients and lean healthy subjects. The correlations between all of these indices of erythrocyte antioxidant status, the lipid profile, the activity of ATPase and acetylcholinesterase, membrane fluidity, and the most significant anthropometrics and chemical-clinical parameters were also calculated, and on this basis, their possible contributions to the etiopathogenesis of both obesity and obesity-related cardiovascular pathologies are discussed.

MATERIALS AND METHODS

Subjects

The patients were 50 overweight [25 < body mass index (BMI) < 29] or obese (first level, moderate obesity: 30 < BMI < 33), normotensive, nondiabetic women. Other pathologies were excluded by clinical history and physical examination. Clinical data, alcohol intake, smoking habits, and physical activity were recorded. Thyroid-stimulating hormone, free thyroxine, free triiodothyronine, and free urinary cortisol levels were in the normal range in all patients. No subject was on a restricted diet. Fifty age-matched, lean healthy women (BMI < 25) were studied as a control group. The "Istituto Santa Margherita" Institutional Review Board approved the study protocol, and each subject signed a consent form that stated the purpose of the study and the sampling to be done.

Collection and preparation of blood samples

Blood samples were collected at rest and after a 12 h fasting period. Whole blood (EDTA as an anticoagulant) was used for hematological procedures and packed erythrocyte preparation. In this last case, blood was immediately centrifuged (3,000 *g* for 5 min at 4°C), plasma and buffy coat were removed by careful suction, and the cells were resuspended in a 154 mM NaCl, 50 mM EDTA solution. After mixing by inversion, the samples were centrifuged again at 1,500 *g* for 5 min at 4°C. This washing procedure was repeated twice. Packed erythrocytes were used for pro-oxidant and antioxidant status measurements and membrane isolation.

Hematological assays

White blood cell and red blood cell counts, total cholesterol, HDL cholesterol, triacylglycerol, glucose, protein, hemoglobin, and hematocrit levels were quantified by standard laboratory procedures.

Erythrocyte pro-oxidant and antioxidant status

Free radical-induced hemolysis of erythrocytes was measured on packed erythrocytes resuspended in PBS at 10% hematocrit. Hemolysis was induced by incubating erythrocytes for 6 h at 37°C with 25 mM 2,2′-azobis-(amidinopropane)dihydrochloride (AAPH; Wako Pure Chemical Co.), and the time curve of hemoglobin release was determined by measuring the absorbance at 410 nm as previously described (3). The kinetic profile of each erythrocyte sample, divided into latency, propagation, and steady-state phases, allows the evaluation of two important kinetic parameters: the lag time, expressed in minutes and calculated as the intercept of the linear regression of the propagation phase with that of the latency phase; and the rate of hemolysis in the propagation phase, calculated from the slope of the curve in this phase (**Fig. 1**).

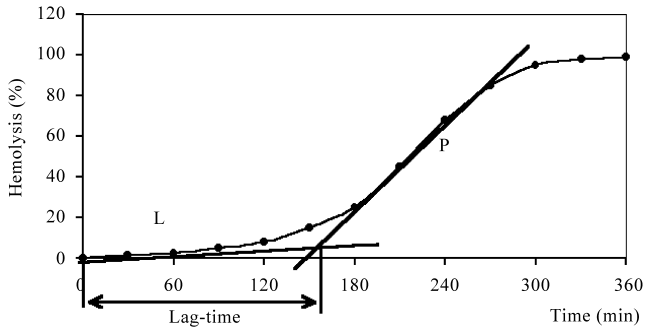

Fig. 1. Free radical-induced hemolysis of erythrocytes. Hemolysis was induced by incubating erythrocytes with 2,2′-azobis-(amidinopropane)dihydrochloride as described in Materials and Methods. The kinetic curve is characterized by an initial latency phase (L), or lag time, followed by a propagation phase (P).

Reduced and oxidized glutathione levels were measured fluorometrically according to Hissin and Hilf (4).

Erythrocyte membrane preparation

The erythrocyte membranes (ghosts) were prepared according to Raccah et al. (5). Membrane proteins were determined according to Lowry et al. (6), and multiple fractions of 0.2 ml were immediately frozen in liquid nitrogen and stored at −80°C until use.

Erythrocyte membranes partially depleted of their cholesterol content were prepared by ghost incubation with β-cyclodextrins according to the method by Ohtani et al. (7).

Erythrocyte membrane peroxidation

Free radical-induced ghost peroxidation was promoted by incubating erythrocyte membranes for different times at 37°C with 25 mM AAPH.

Erythrocyte membrane lipid analysis

Erythrocyte membrane fatty acid composition was determined on lipids extracted in 2:1 chloroform-methanol (Merck, Darmstadt, Germany) containing 0.2% butylated hydroxytoluene (Merck) according to the method of Folch, Lees, and Sloane Stanley (8). The lipid extracts were methylated with methanolic HCl (Merck) at 90°C for 2 h. Fatty acid methyl esters were analyzed using capillary gas chromatography (GC). The GC apparatus (Hewlett Packard 6890 series equipped with autoinjector) was used in split mode (ratio 50:1) with hydrogen as the carrier gas. The analytical column was a fused silica capillary column (BPX70; length 50 m, internal diameter 0.25 mm, film thickness 0.25 μm; SGE Europe Ltd.). The GC autoinjector temperature was 250°C. The GC temperature program was as follows: initial temperature of 150°C for 3 min, increase of 5°C per min to 210°C, and a run time of 60 min. The fatty acid methyl esters were identified according to their retention times in comparison with known standards (Sigma-Aldrich, Dorset, UK) and were quantified by using heptadecanoic acid (Sigma-Aldrich) as the internal standard. The amount of each considered fatty acid was calculated as nanomoles per milligram of protein and expressed as a percentage of the total fatty acid concentration. The degree of unsaturation of erythrocyte membrane (unsaturation index) was calculated as the sum of each unsaturated fatty acid concentration multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.

Vitamin E levels were determined by measuring the ghost α-tocopherol content by HPLC (9).

Total phospholipids and cholesterol of native and peroxidized ghosts were measured according to the method of Alcindor et al. (10).

Lipofuscins, a cross-linked complex of proteins and lipid peroxidation products, were measured fluorometrically on native and peroxidized ghosts as previously described (11).

Measurement of the activity of ATPase and acetylcholinesterase

The Na,K ATPase activity was measured as the ouabain-inhibitable inorganic phosphorus released after incubation of membranes with ATP (5).

The acetylcholinesterase activity was determined according to Vander Jagt et al. (12).

Determination of erythrocyte membrane fluidity

Membrane fluidity was evaluated by measuring the steady-state anisotropy of diphenylhexatriene (Molecular Probes Europe BV, Leiden, The Netherlands) as previously described (13). The diphenylhexatriene probe was excited at a wavelength of 340 nm, and the emission wavelength was set at 420 nm. Samples were then excited with vertically polarized light, and the intensity of emitted light, vertically (I_v) and horizontally (I_h) polarized, was measured. Anisotropy (r_s) was calculated with the equation

$$r_s = I_v - I_h/I_v + 2I_h \qquad (Eq.\ 1)$$

Anthropometric measurements

Body weight, stature, and thickness of skinfold were measured between 9 and 11 AM after an overnight fast. Body weight (kg) was measured on a standing balance, and height (m) was measured using a standing upright scale. BMI was calculated by dividing weight (kg) by height squared (m^2). Skinfold thicknesses (biceps, triceps, suprailiac, subscapular) were measured twice with a Harpender skinfold caliper at 5 min intervals at each site using a standardized technique (14).

Statistical analysis

All measurements were done at least in triplicate, and results are reported as means ± SD. Significant differences between measurements were detected by performing a two-tailed *t*-test. The linear relationships between the covariates were assessed using Pearson's correlations. The level of statistical significance was set at $P \leqslant 0.05$.

RESULTS

As shown in **Table 1**, the differences observed in age and plasma red blood cells, total lymphocytes, and total protein levels between the group of obese and overweight women and the group of control subjects were not significant. Obviously, the BMI and all indices of fat body distribution (biceps, triceps, subscapular, and suprailiac skinfolds) were significantly higher in the group of overweight and obese women.

The levels of hemoglobin and hematocrit were nevertheless significantly lower, whereas the values of glycemia (although in the physiological range) and cholesterolemia were significantly higher in the group of overweight and obese women compared with the control group.

The parameters indicative of erythrocyte membrane susceptibility to peroxidation (the lag time, indicative of the total amount of antioxidants, and the slope, indicative of the rate of free radical-induced membrane damage), together with the lipofuscin levels (indicative of the malondialdehyde-induced cross-linking between the different glycoproteins) and the ratio between reduced and oxidized glutathione (GSH/GSSG, indicative of the oxidized/reduced status of the erythrocyte cytosol), are reported in **Table 2**. The slope and the lipofuscin levels showed a significant tendency to increase in the group of overweight and obese women. On the contrary, the lag time and the GSH/GSSG ratio were significantly lower in this group of patients.

The lipofuscin levels were directly correlated with the BMI ($P < 0.0001$) and the thicknesses of triceps ($P = 0.007$), subscapular ($P = 0.0003$), and suprailiac ($P = 0.003$) skinfolds, whereas the lag time was inversely correlated with the thicknesses of the biceps ($P = 0.007$), triceps ($P = 0.0001$), subscapular ($P = 0.0001$), and suprailiac ($P = 0.004$) skinfolds.

The total amounts of cholesterol and phospholipids in the native and peroxidized membranes in both obese and control women are reported in **Table 3**. The peroxidation

TABLE 1. Age, hematological, and anthropometric parameters of the study populations

Parameter	BMI ⩽ 25 (N = 50)	25 > BMI > 33 (N = 50)	*P*
Age (years)	34.3 ± 5.4	36.1 ± 5.5	NS
Red blood cells ($\times 10^6/\mu l$)	5.8 ± 1.4	5.5 ± 0.9	NS
Hemoglobin (g/dl)	14.6 ± 1.2	13.4 ± 1.2	<0.0001
Hematocrit (%)	41.7 ± 2.8	39.5 ± 3.1	0.0003
White blood cells ($\times 10^3/\mu l$)	6.1 ± 1.0	6.5 ± 1.2	NS
Proteins (g/dl)	7.4 ± 0.5	7.3 ± 0.4	NS
Glucose (mg/dl)	80.8 ± 5.5	93.3 ± 7.6	<0.0001
Cholesterol (mg/dl)	177 ± 29.4	219 ± 49	<0.0001
HDL cholesterol (mg/dl)	53 ± 13.2	55 ± 11.7	NS
Triacylglycerols (mg/dl)	73 ± 38	86 ± 41	NS
BMI (kg/m^2)	22.6 ± 1.9	29.3 ± 2.8	<0.0001
Skinfolds			
Triceps (mm)	12.9 ± 5.8	28.2 ± 6.0	<0.0001
Biceps (mm)	6.8 ± 25.0	18.9 ± 3.1	<0.0001
Subscapular (mm)	12.0 ± 4.9	29.1 ± 5.4	<0.0001
Suprailiac (mm)	10.2 ± 5.5	29.6 ± 6.9	<0.0001

BMI, body mass index.

TABLE 2. Erythrocyte pro-oxidant and anti-oxidant status

Variable	BMI ⩽ 25 (N = 50)	25 > BMI > 33 (N = 50)	*P*
Lag time (min)	170 ± 19	154 ± 17	<0.0001
Slope (ΔA_{420}/min)	0.75 ± 0.23	0.87 ± 0.24	0.0122
GSH/GSSG	0.86 ± 0.06	0.83 ± 0.05	0.0078
Lipofuscins ($F.U._{360/430}$/mg protein)	690 ± 27	950 ± 32	<0.0001

The reduced-to-oxidized glutathione ratio (GSH/GSSG) and the levels of lipofuscins were determined on native erythrocytes. The lag time and the slope were calculated from the kinetic curves of 2,2′-azo-bis-(amidinopropane)dihydrochloride (AAPH)-induced hemolysis as described in Materials and Methods.

TABLE 3. Phospholipid and cholesterol contents of native and peroxidized erythrocyte membranes

Membrane	BMI ⩽ 25 (N = 50)	25 > BMI > 33 (N = 50)	*P*
Native membranes			
Cholesterol (nmol/mg protein)	523 ± 47	623 ± 52	<0.0001
Phospholipids (nmol/mg protein)	1,005 ± 57	842 ± 57	<0.0001
Cholesterol/phospholipids	0.52 ± 0.18	0.74 ± 0.24	<0.0001
Peroxidized membranes[a]			
Cholesterol (μg/mg protein)	461 ± 42	577 ± 52	<0.0001
Phospholipids (nmol/mg protein)	906 ± 54	796 ± 48	<0.0001
Cholesterol/phospholipids	0.51 ± 0.19	0.72 ± 0.22	<0.0001

[a] Ghosts were peroxidized by incubation at 37°C with 25 mM AAPH for 3 h.

promoted a significant decrease in the amounts of phospholipids ($P < 0.0001$) and cholesterol ($P < 0.0001$) in both groups. In the native and peroxidized membranes, the cholesterol-to-phospholipids ratio was significantly higher in the obese than in the control group.

The ratio between cholesterol and phospholipids of the native membrane was directly correlated with the lipofuscin levels ($P < 0.0001$), the BMI ($P < 0.0001$), and the thickness of the triceps ($P = 0.0004$), subscapular ($P = 0.0005$), and suprailiac ($P = 0.0007$) skinfolds and was inversely correlated with the lag time ($P < 0.0001$).

To better clarify the influence of cholesterol on the extent of peroxidation, the obese ghosts were incubated with β-cyclodextrins, a well-known method for reducing the ghost cholesterol content (7). The cholesterol-to-phospholipids ratio was reduced from 0.74 ± 0.24 to 0.49 ± 0.22 by this treatment. The native and cholesterol-depleted ghosts were then submitted to AAPH-induced peroxidation for 30 min. The lipofuscin content after peroxidation was 1,164 ± 98 and 1,004 ± 107 fluorescence units/mg protein in the native and cholesterol-depleted ghosts, respectively ($P > 0.0001$).

TABLE 4. Fatty acid profiles of native erythrocyte membranes

Fatty Acid	BMI ⩽ 25 (N = 50)	25 > BMI > 33 (N = 50)	*P*
	%		
Palmitic	30.13 ± 3.9	29.44 ± 3.1	NS
Palmitoleic	3.0 ± 0.1	2.91 ± 0.1	NS
Stearic	16.54 ± 2.0	16.41 ± 1.4	NS
Oleic	12.19 ± 2.5	13.69 ± 1.2	0.0002
Linoleic	10.68 ± 1.6	9.73 ± 0.9	0.0004
α-Linolenic	0.26 ± 0.2	0.22 ± 0.2	NS
Arachidic	0.43 ± 0.3	0.56 ± 0.2	0.014
Dihomo-γ-linolenic	0.84 ± 0.2	1.11 ± 0.2	<0.0001
Arachidonic	9.94 ± 2.1	11.58 ± 1.1	<0.0001
Eicosapentaenoic	0.64 ± 0.1	0.55 ± 0.15	0.002
Lignoceric	4.32 ± 1	4.24 ± 0.7	NS
Nervonic	5.68 ± 0.7	6.13 ± 0.54	0.0003
Docosapentaenoic	1.92 ± 0.2	0.72 ± 0.2	<0.0001
Docosahexaenoic	3.44 ± 0.5	2.71 ± 0.5	<0.0001
Saturated	51.41 ± 4.5	50.65 ± 4.3	NS
Monounsaturated	20.85 ± 2.4	22.73 ± 2.5	0.0002
PUFA	27.73 ± 3.0	26.61 ± 3.0	NS
PUFA ω-6	21.46 ± 3.28	22.41 ± 3.0	NS
PUFA ω-3	6.27 ± 0.75	4.2 ± 0.6	<0.0001
n-6/ω-3	3.48 ± 0.89	5.5 ± 1.0	<0.0001
U.I.[a]	2.49 ± 0.11	2.34 ± 0.13	<0.0001
Unsaturated/vitamin E	73.7 ± 4.8	75.6 ± 5.1	NS

The fatty acid contents are expressed as molar percentages.

[a] Unsaturation index calculated as the sum of each unsaturated fatty acid concentration multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.

Table 4 shows the percentage levels of the different fatty acids (saturated, monounsaturated, and polyunsaturated) together with the PUFA/vitamin E ratio in the native erythrocyte membrane. The most significant findings emerging from a detailed analysis of these data are as follows:

a) In the patient group, there was a significant decrease in the percentage of ω-3 fatty acids (eicosapentaenoic, docosapentaenoic, and docosahexaenoic fatty acids); on the contrary, the ω-6 fatty acids arachidonic and dihomo-γ-linolenic acids appeared to be significantly higher. Furthermore, in the obese group, the percentages of arachidic (saturated), oleic, and nervonic (monounsaturated) acids were significantly higher.

b) The sum of all ω-3 polyunsaturated fatty acids appeared to be significantly lower, but this decrease is mostly attributable to the decrease of the ω-3 species with the highest degree of unsaturation, namely, eicosapentaenoic and docosapentaenoic acids (with five double bonds per molecule) and docosahexaenoic acid (with six double bonds per molecule). The significant reduction of the membrane unsaturation index is mostly attributable to these changes.

c) The ratio between the ω-6 and ω-3 fatty acids [in particular, between the most polyunsaturated ω-6 (arachidonic acid) and ω-3 (docosahexaenoic acid and eicosapentaenoic acid) species], was dramatically higher in the patient group.

d) The ratio between the total polyunsaturated fatty acids and vitamin E was higher in the patient group, but not significantly.

The r_s index of fluorescence anisotropy, which is inversely related to membrane fluidity, and the activity of Na,K ATPase and acetylcholinesterase are reported in **Table 5**. The r_s values were significantly higher, the ATPase showed a nonsignificant tendency to decrease, and the acetylcholinesterase decreased significantly in the patient group.

The r_s values were directly correlated with the native lipofuscin levels ($P = 0.005$), the BMI ($P = 0.001$), and the thickness of the triceps ($P = 0.0034$), subscapular ($P = 0.003$), and suprailiac ($P = 0.005$) skinfolds.

TABLE 5. Fluorescence anisotropy and activities of Na,K ATPase and acetylcholinesterase of native erythrocyte membranes

Parameter	BMI ≤ 25 (N = 50)	25 > BMI > 33 (N = 50)	*P*
Anisotropy	0.2216 ± 0.0029	0.2256 ± 0.0026	<0.0001
Na,K ATPase (nmol P/mg protein/h)	251 ± 32	239 ± 29	0.0523
Acetylcholinesterase (nmol hydrolysate/mg protein/min)	1.23 ± 0.12	1.18 ± 0.13	0.0484

DISCUSSION

The experimental results reported above demonstrate a significantly higher susceptibility to peroxidation, together with a higher ratio between cholesterol and phospholipids and a significant increase in the cholesterol content of the erythrocyte membranes of overweight and obese patients in comparison with the normal weight control group. The higher levels of cholesterol may be one of the possible explanations for the higher pro-oxidant status in the erythrocyte membrane: cholesterol has been shown, in part, to undergo oxidation, giving rise to a variety of epoxides and alcohols (15). In hypercholesterolemia, the cholesterol contents of erythrocytes, platelets, leukocytes, and endothelial cells have been shown to increase, and this increase has been reported to cause the enhanced production of oxygen free radicals (16, 17). Erythrocytes, in particular, because of their intrinsic potential for free radical generation (attributable to their very high oxygen content), might be a very suitable environment for cholesterol to exert its pro-oxidant reaction (18). When the membranes of the obese women were in part depleted of their cholesterol content, by means of a preincubation with the β-cyclodextrins, their susceptibility to peroxidation was significantly reduced.

Another possible explanation for the higher pro-oxidant status of the erythrocyte membrane in overweight and obese patients is the increased susceptibility of lipoproteins to peroxidation previously reported in these patients (19, 20): this lipoprotein pro-oxidant state promotes an increase of both lipid hydroperoxides and radicals (that are intensely formed in the course of peroxidative processes) and a noticeable decrease of lipophilic antioxidants, such as vitamin E, β-carotene, coenzyme Q, etc.

Because lipid radicals and hydroperoxides, together with lipophilic antioxidants, are normally transferred from donor lipoproteins to the acceptor erythrocyte membrane, what we can expect in overweight and obese women is that a higher amount of lipid radicals and a lower amount of antioxidants could be exchanged between plasma lipoproteins and erythrocyte membranes, thus contributing to an increase in erythrocyte susceptibility to peroxidation.

A further consequence of these metabolic pro-oxidant events is the significant decrease that we found both in erythrocyte membrane unsaturation index (mostly attributable to a reduction of long-chain ω-3 fatty acids, which are very sensitive to the peroxidative processes) and in erythrocyte levels of reduced glutathione (which is more intensely consumed in the course of the peroxidation process). Moreover, the peroxidation of polyunsaturated fatty acids, particularly the peroxidation of the highly polyunsaturated ω-3 fatty acids, has certainly contributed to increase the production of aldehydes (such as malondialdehyde), which are known to promote those cross-linking bonds between membrane proteins and phospholipids. The cross-linked polymeric complexes are the probable cause of the higher level of lipofuscins that we found in the patient group.

Thus, there is a lot of evidence that links the higher erythrocyte membrane susceptibility to peroxidation in overweight and obese women with the statistically significant higher values of the fluorescence anisotropy (indicative of a more "rigid" status of the membrane) that we measured in this group:

a) The lower content in the membrane of the polyunsaturated ω-3 fatty acids, which helps to maintain the membrane in a more "fluid" state.

b) The lower values of all of the parameters indicative of the erythrocyte antioxidant defenses (namely, the reduced values of lag time, indicative of the reduced levels of lipophilic antioxidant in the membrane, and the decrease of reduced glutathione, indicative of the decreased status of hydrophilic antioxidant in the cytosol) that normally contribute to the prevention of peroxidation of more polyunsaturated fatty acids.

c) The formation of lipofuscins, which strongly contribute to decreasing both the "degree of freedom" and the mobility of both membrane proteins and phospholipids "immobilized" in these polymeric complexes.

d) The increase in the ratio between cholesterol and phospholipid, which is well known as a contributor to maintaining the membrane in a more rigid state.

Moreover, it is important to note that the level of nervonic acid (a fatty acid normally present only in sphingomyelins) was significantly higher in the obese group. This evidence further contributes to explaining the higher rigidity found in the patient group: it is well known, in fact, that sphingomyelins are a rigidifying agent of the membrane (21).

This decrease in erythrocyte membrane fluidity in overweight and obese women could thus contribute to reducing the rate of blood flow (in particular in the microcirculation) and the oxygen diffusion through the erythrocyte membrane and its exchange with tissues. It is well known that higher cholesterol content in the erythrocyte membrane and the consequent decrease of its fluidity negatively influence both the oxygen release and the deformability of the red blood cells (2, 22, 23). The reduced availability of oxygen could thus contribute to both reducing the aerobic metabolism of glucose and fatty acid and consequently reducing the "thermogenesis" in muscle, brown adipose tissue, etc., and increasing the hypoxic state both in heart and endothelial cells (thus contributing to increasing the incidence of all of the cardiovascular pathologies that are often associated with obesity).

Finally, this decrease in membrane fluidity promoted by the higher pro-oxidant status in erythrocytes could be the expression of a more generalized phenomenon involving other tissues as well, in particular, muscle tissue: a significant direct correlation between membrane fluidity and insulin resistance has already been observed both in non-insulin-dependent diabetes mellitus (24) and in obese patients (21). In the light of current experimental data, it could be of interest to investigate whether or not a suitable dietary integration of ω-3 fatty acids and antioxidants could promote significant beneficial effects for the improvement of the fluidity of erythrocyte membranes in particular and all other tissues in general, and if this improvement could be of some utility in the prevention of both obesity and all of the other cardiovascular diseases correlated with it.

REFERENCES

1. Dandona, P., P. Mohanty, H. Ghanim, A. Aljada, R. Browne, W. Hamouda, A. Prabhala, A. Afzal, and R. Garg. 2001. The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. *J. Clin. Endocrinol. Metab.* **86:** 355–362.
2. Buchwald, H., H. J. Menchaca, V. N. Michalek, T. D. Rohde, D. B. Hunninghake, and T. J. O'Dea. 2000. Plasma cholesterol: an influencing factor in red blood cell oxygen release and cellular oxygen availability. *J. Am. Coll. Surg.* **191:** 490–497.
3. Zou, C. G., N. S. Agar, and G. L. Jones. 2001. Oxidative insult to human red blood cells induced by free radical initiator AAPH and its inhibition by commercial antioxidant mixture. *Life Sci.* **69:** 75–86.
4. Hissin, P. J., and R. Hilf. 1976. A fluorimetric method for the determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74:** 214–226.
5. Raccah, D., F. Dadoun, T. Coste, and P. Vague. 1996. Decreased Na/K ATPase ouabain binding sites in red blood cells of patients with insulin-dependent diabetes and healthy north African control subjects: relationship with insult and diabetic neuropathy. *Horm. Metab. Res.* **28:** 128–132.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
7. Othani, Y., T. Irie, K. Ubkama, K. Fukunaga, and J. Pitha. 1989. Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. *Eur. J. Biochem.* **186:** 17–22.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
9. Viani, P., R. Cazzola, G. Cervato, P. Gatti, and B. Cestaro. 1996. Pyrene lipids as markers of peroxidative processes in different regions of low and high density lipoproteins. *Biochim. Biophys. Acta.* **1315:** 78–86.
10. Alcindor, L. G., M. Kadra, H. Antebi, M. J. Guerrito, M. C. Piot, and G. Heraud. 1989. A rapid assay for cholesterol and choline phospholipids in erythrocyte membranes. In Perspective Biology. G. Siest, editor. J. Libbey Eurotext, Montrouge, France. 181–184.
11. Dillard, C. J., and A. L. Tappel. 1984. Fluorescent damage products of lipid peroxidation. *Methods Enzymol.* **105:** 337–341.
12. Vander Jagt, D. L., C. Intress, J. E. Heidrich, J. E. Mrema, K. H. Rieckmann, and H. G. Heidrich. 1982. Marker enzymes of Plasmodium falciparum and human erythrocytes as indicator of parasite purity. *J. Parasitol.* **68:** 1068–1071.
13. Cazzola, R., S. Russo-Volpe, G. Cervato, and B. Cestaro. 2003. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur. J. Clin. Invest.* **33:** 924–930.
14. Frisancho, A. R. 1984. New standards of weight and body composition by frame size and height for assessment of nutritional status of adults and the elderly. *Am. J. Clin. Nutr.* **40:** 808–819.
15. Adachi, J., M. Asano, T. Naito, Y. Ueno, and Y. Tatsuno. 1998. Chemiluminescent determination of cholesterol hydroperoxides in human erythrocyte membrane. *Lipids.* **33:** 1235–1240.
16. Hodis, H. N., D. W. Crawford, and A. Sevanian. 1991. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis.* **89:** 117–126.
17. Peng, S. K., B. Hu, and R. J. Morin. 1991. Angiotoxicity and atherogenicity of cholesterol oxides. *J. Clin. Lab. Anal.* **5:** 144–152.
18. Hatherill, J. R., G. O. Till, and P. A. Ward. 1991. Mechanisms of oxidant-induced changes in erythrocytes. *Agents Actions.* **32:** 351–358.
19. Cominacini, L., U. Garbin, A. M. Pastorino, A. Fratta Pasini, M. Campagnola, A. De Santis, A. Davoli, and V. Lo Cascio. 1994. Increased susceptibility of LDL to in vitro oxidation in patients with insulin-dependent and non-insulin-dependent diabetes mellitus. *Diabetes Res.* **26:** 173–184.
20. Olusi, S. O. 2002. Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans. *Int. J. Obes. Relat. Metab. Disord.* **26:** 1159–1164.
21. Candilors, H., N. Zeghari, O. Ziegler, M. Donner, and P. Drouin. 1996. Hyperinsulinemia is related to erythrocyte membrane fluidity changes in obese nondiabetic women. *J. Clin. Endocrinol. Metab.* **81:** 2912–2918.
22. Dumas, D., S. Muller, F. Gouin, F. Baros, M. L. Viriot, and J. F. Stoltz. 1997. Membrane fluidity and oxygen diffusion in cholesterol-enriched erythrocyte membrane. *Arch. Biochem. Biophys.* **341:** 34–39.
23. Rifkind, J. M., and O. O. Abugo. 1994. Alterations in erythrocyte deformability under hypoxia: implication for impaired oxygen transport. In Oxygen Transport to Tissue XVI. M. C. Hogan, editor. Plenum Press, New York. 345–351.
24. Tong, P., T. Thomas, T. Berrish, D. Humphriss, L. Barriocanal, M. Stewart, M. Walker, R. Wilkinson, and K. G. Alberti. 1995. Cell membrane dynamics and insulin resistance in non-insulin-dependent diabetes mellitus. *Lancet.* **345:** 357–358.