Changes of Membrane Fluidity in Erythrocytes of Obese Children: A Spin Label Study

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ABSTRACT. Red blood cells depend on circulating lipoproteins for the turnover of membrane phospholipids. Thus abnormalities in lipid and lipoprotein metabolism are frequently associated with changes in composition and fluidity of erythrocyte membranes. Using the electron spin resonance technique we studied the physical state of erythrocyte membranes from 15 obese children with a body mass index ranging from 22.8 to 34 kg $\cdot$ m^{-2} and slight alterations of plasma lipids. The spin labels used, 5-doxylstearate and 16-doxylstearate, have shown an increase of the order parameter and of the correlation time, respectively. These observations indicate a decrease of fluidity at the surface and in the hydrophobic core of erythrocyte membranes from obese subjects. The results are statistically significant and they are discussed in relation to clinical aspects of childhood obesity. Studies on erythrocyte membrane molecular organization could represent a sensitive way to find out small metabolic alterations hardly detectable with compositional studies. (*Pediatr Res* 22:141–144, 1987)

Abbreviations

LPL, lipoprotein lipase
LCAT, lecithin-cholesterol acyl transferase
ESR, electron spin resonance
BMI, body mass index
5-NS, 5-doxylstearate
16-NS, 16-doxylstearate
LDL, low-density lipoproteins
HDL, high-density lipoproteins
RBC, red blood cells

Several studies on metabolic and functional alterations in obesity are limited to obese adults; less attention has been devoted to the pediatric obese subjects. In adult obese patients modifications of the activity of the LPL (1) and of the LCAT (2) suggest the hypothesis that obesity could be associated with alterations of the lipoprotein metabolism.

It is known that in certain specific pathological states, inborn errors of metabolism and/or dietary or hormonal disturbances are associated with changes in the surface of lipids of circulating lipoproteins and several lines of evidence correlate these changes with abnormalities in the lipids of erythrocyte membrane (3–7).

Because the molecular organization of the erythrocyte membrane is known in detail, it is possible to correlate changes in membrane composition with modifications in membrane fluid-

ity, moreover it is well known that membrane fluidity affects membrane-bound enzyme activities (8, 9). A reduced level of erythrocyte membrane tocopherol (10) which could explain the erythrocyte sensitivity to peroxidative damage, a lower concentration of insulin receptors (11), and a reduced Na$^+$K$^+$ pump (12) have been shown in erythrocytes of obese children. However until now there is no evidence for their direct or indirect involvement in the pathogenesis of obesity.

The possible correlations between obesity and membrane physical state were studied with the purpose to bring forward a working model in which erythrocyte membrane represents a final system reflecting and amplifying metabolic disorders. To this aim we have studied membrane fluidity using the ESR technique (13–17). Membrane fluidity is strictly related to membrane composition and structure. The ordinary metabolic and hematological laboratory parameters were also determined and the possible relationships between functional and molecular alterations were investigated.

MATERIALS AND METHODS

Patients. Fifteen obese children (11 females and four males; age range 6–14 yr) who had no sign of endocrine or genetic disease and seven normal weight children (four females, three males; similar age range) were studied. All the examined subjects were from the same region of Italy with similar nutritional habits. The BMI (weight in kg divided by square of height in m) taken as measure of obesity (18) ranged from 22.8–34 kg $\cdot$ m^{-2} in the obese subjects and from 14.6–22.2 kg $\cdot$ m^{-2} in the controls. Appropriate informed consent was obtained for this study. Physical characteristics of obese and control children are given in Table 1.

Preparation of erythrocyte membranes. Five ml of blood were drawn from each subject after a 12-h overnight fast and placed in heparinized tubes. Erythrocytes were washed three times with isotonic saline (0.15 M NaCl, 10 mM Tris-HCl pH 7.4) and the buffy coat and plasma were removed each time. Erythrocyte membranes were prepared by hemolizing the washed erythrocytes in 5 mM phosphate buffer pH 8, following the method of Steck and Kant (19). Membrane protein content was estimated by the method of Lowry *et al.* (20) using bovine serum albumin as a standard.

Erythrocyte spin labeling and ESR measurements. The membrane fluidity was studied by means of the ESR technique with two spin labels: 5-NS and 16-NS. These labels which have a nitroxide radical ring at the 5th and 16th carbon position (Fig. 1) probe the physical state of the membrane surface and hydrophobic core, respectively (Fig. 1). The ESR technique has been well established as a valuable tool in the study of both biological and model membranes. The labels purchased from Syva Co (Palo Alto, CA) were dissolved in absolute ethanol at a concentration of 10 mM and kept at −20° C.

Incorporation of the labels into the membranes was accom-

Table 1. *Physical characteristics of obese and control subjects*
(mean ± SD)

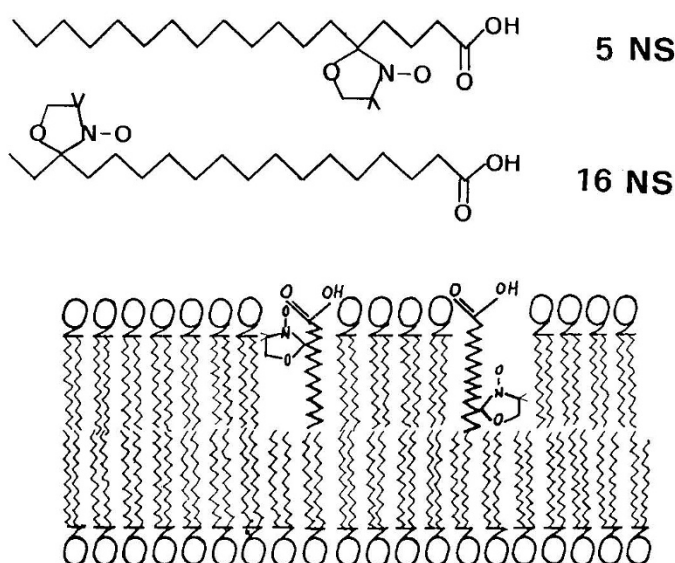| Subjects	Sex	Age (yr)	BMI (kg × m⁻²) (range)
Controls	3 M	12 ± 1	18 ± 3
	4 F		(14.6–22.2)
Obese	4 M	11 ± 3	28.21 ± 3*
	11 F		(22.8–34)

* $p < 0.001$.

5 NS

16 NS

Fig. 1. Chemical formulae and localization in biological membranes of 5-NS and 16-NS.

plished as described elsewhere (13). Ethanol was evaporated under a nitrogen stream and the samples were incubated overnight at 4° C. A probe to phospholipid ratio of 1:100 was used. ESR studies were performed with a Varian E-4 spectrometer (microwave frequency 9.1 Ghz; modulation amplitude 1.6 Gauss; time constant 0.5 s).

The spin labels embedded in biological membranes exhibit their freedom of anisotropic motion in conformity with the position of the nitroxide ring of the alkyl fatty acid chain. The anisotropic motion reflects the molecular motion of phospholipids and depends on order and fluidity. The spectra of 5-NS and of 16-NS embedded in erythrocyte membranes are shown in Figure 2a and b, respectively.

The observed values of the outer ($2T_{\parallel}$) and inner ($2T_{\perp}$) hyperfine splitting (in Gauss) were used to calculate the order parameter (Sn) for 5-NS according to the following equation (16):

$$Sn = 0.568 \frac{T_{\parallel} - T_{\perp}}{a'} \qquad \text{where } a' = (1/3)(T_{\parallel} + 2T_{\perp})$$

where a' is a measure of the polarity, which decreases with decreasing polarity.

For 16 NS the pseudoisotropic motion can be described by the empirical parameter h_0/h_{-1} which is the ratio between the mid-field height (h_0) and the high field height (h_{-1}); however results are also presented using the correlation time (τ_c) calculated according to Kivelson (21).

$$\tau_c = 6.5 \times 10^{-10} \, w_0 \, [(h_0/h_{-1})^{1/2} - 1]$$

where w_0 is the line width.

The constant $k = 6.5 \times 10^{-10}$ s is dependent on the anisotropic hyperfine coupling values and the g-tensor terms when the correlation time τ_c is greater than 10^{-9} s (22).

Although a direct comparison between Sn (for 5-NS) and τ_c (for 16-NS) is not possible, both values are used independently to evaluate membrane fluidity at the surface and in the hydrophobic core, respectively. A decrease in membrane fluidity is associated with greater values of outer hyperfine splitting ($2T_{\parallel}$), order parameter (Sn), peak height ratio (h_0/h_{-1}), and correlation time τ_c.

Plasma lipids. Total cholesterol, triglycerides and total lipids were measured by the methods of Allain *et al.* (23), Bucolo and David (24), and Zoellner and Kirsch (25), respectively. Plasma free fatty acids were measured according to Laurell and Tibbling (26). Lipoprotein analysis was performed using cellulose acetate electrophoresis (27).

Statistics. All results are expressed as means ± SEM if not otherwise indicated. Statistical differences between data from
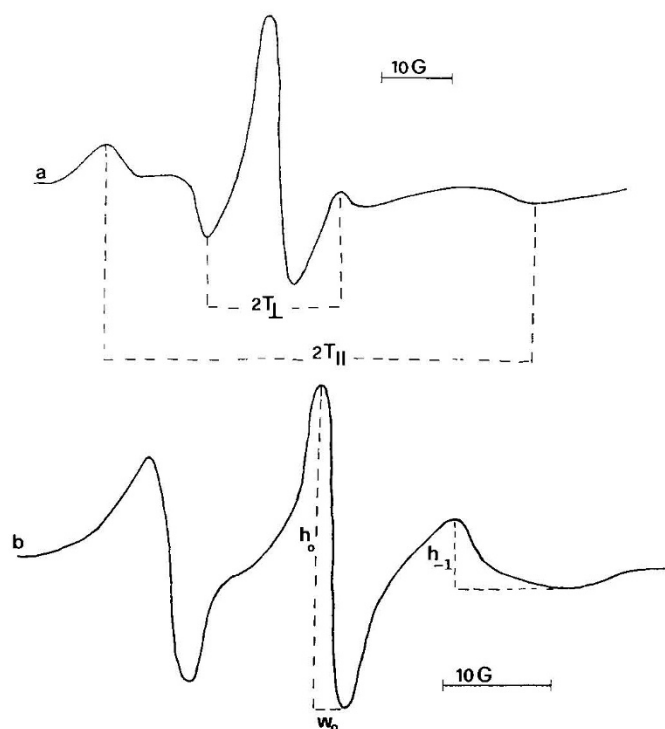

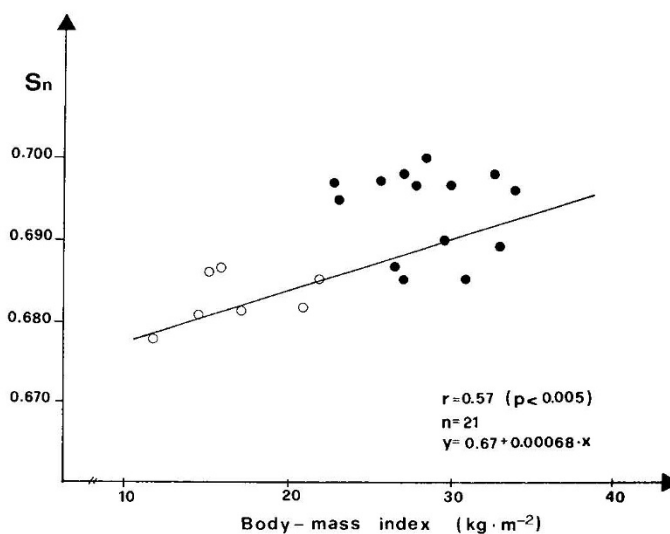Fig. 2. ESR spectra of 5-NS (*a*) and 16-NS (*b*) in erythrocyte membranes.

Fig. 3. Correlation between the order parameter (Sn) of 5-NS and BMI in controls (○) and obese (●) subjects.
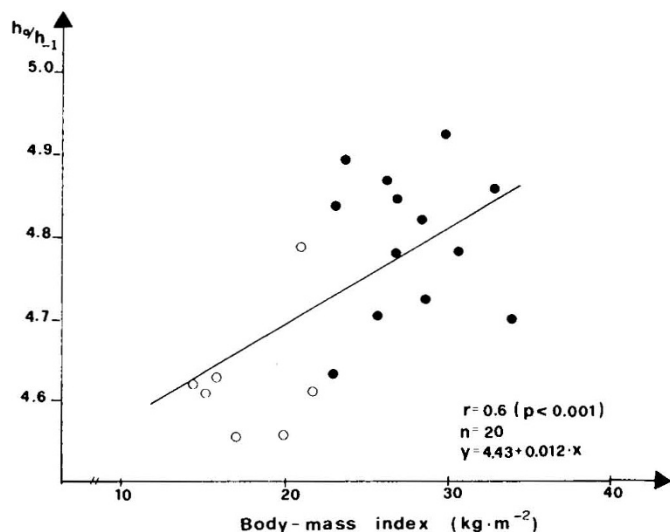
Fig. 4. Correlation between the peak height ratio (h_0/h_{-1}) of 16-NS and BMI in controls (O) and obese (●) subjects.

Table 2. *Plasma lipid analysis in obese children and controls (mean ± SEM)*

	Controls	Obese
Cholesterol (mg dl^{-1})	160 ± 23	159 ± 16
Triglycerides (mg dl^{-1})	66 ± 3	113 ± 8*
Total lipids (mg dl^{-1})	561 ± 26	612 ± 22
Lipoproteins LDL (%)	55 ± 3	63 ± 3*
HDL (%)	44 ± 3	36 ± 1*
LDL/HDL ratio	1.33 ± 0.1	1.83 ± 0.1*
Free fatty acids (mg dl^{-1})	11 ± 0.9	15 ± 1.5

* $p < 0.05$.

obese and nonobese subjects were determined by the Student's t test. Linear regression analysis was used to assess the degree of correlation between BMI and fluidity parameters.

RESULTS

The physical characteristics of the controls and the obese patients studied are summarized in Table 1. The BMI (kg·m^{-2}) was significatively ($p < 0.001$) higher in the obese subjects (28.21 ± 3) with respect to the controls (18 ± 3). No significant age difference was observed between the two groups.

Plasma lipids. Plasma total lipids and free fatty acids (Table 2) were slightly increased in obese patients when compared with normal controls as previously observed (10, 28). There was no significant difference in the plasma total cholesterol values. In the obese patients we observed a significant increase in plasma triglycerides ($p < 0.05$) although the mean value is in the normal range (Table 2). These results are opposite to those of Kitagawa *et al.* (10) and are probably due to the different environment and ethnic origin of the examined population. In our patients it is evident that there is an increase in LDL and a decrease in HDL levels with a consequent significant increase of the LDL/HDL ratio ($p < 0.05$) in relation to the normal value (Table 2).

Membrane fluidity. The ESR spectra of 5-NS in erythrocyte membranes of obese subjects have shown an increase in hyperfine splitting 2TI and in order parameter (Sn) in respect to the controls (Table 3). The increase of the order parameter Sn was highly significant with $p < 0.001$ indicating a decrease of the probe amplitude of motion about the average orientation. A significant increase either of the peak height ratio (h_0/h_{-1}) ($p < 0.05$) or the motion parameter of the probe 16-NS ($p < 0.05$) indicates a decrease of fluidity also in the hydrophobic core of

the membrane (Table 4). It is of interest to stress that the SEM of ESR measurements are extremely small so that the difference in the mean values for the two groups are highly significant.

The results show that in the obese subjects there is an increase of the order in the surface and more restricted mobility in the hydrophobic core indicating a decrease of membrane fluidity. Relative to the controls every obese subject studied showed similar modifications of the ESR parameters for both the spin probes used (Table 5) suggesting that the erythrocyte membrane could be a suitable model in the study of obesity in childhood. The increments, expressed as percent increase calculated for each obese compared with the control means shown in Tables 3 and 4, ranged between 0.4 to 2.6 for Sn and between 0.5 and 6.9 for h_0/h_{-1}. The relevance of the changes in ESR parameters Sn and h_0/h_{-1} to the pathophysiology of the obese state is supported by the positive correlation with the BMI (Figs. 3 and 4).

DISCUSSION

In the erythrocyte membranes of obese patients, we observed a decrease of fluidity by means of the spin label method. The alterations in the physical state of RBC membrane were evident even in the absence of remarkable changes in plasma lipids and lipoproteins. The positive correlation between the BMI and the erythrocyte membrane physical state, suggests that the decrease in membrane fluidity could be considered as a parameter which

Table 3. *Outer hyperfine splitting ($2T_{11}$) and order parameter (Sn) for 5-NS in erythrocyte membranes from obese and controls (mean ± SEM)*

	n	$2T_{11}$	Sn
Controls	7	56.86 ± 0.09	0.682 ± 0.001
Obese	15	57.18 ± 0.10	0.692 ± 0.001*

* $p < 0.001$.

Table 4. *Peak height ratio (h_0/h_{-1}) and motion parameter (τ_c) of 16-NS in erythrocyte membranes from obese and controls (mean ± SEM)*

	n	h_0/h_{-1}	τ_c(s × 10^{-10})
Controls	7	4.64 ± 0.07	18.22 ± 0.08
Obese	15	4.77 ± 0.03*	19.07 ± 0.26*

* $p < 0.05$.

Table 5. *BMI and values of nitroxide spectral parameters of obese patients**

BMI	Sn	h_0/h_{-1}
22.8	2.2	0.5
23	2	5.2
23.5	0.5	6.3
25.6	2.2	1.9
26.5	0.4	5.6
26.6	0.7	5.2
27.1	2.4	3.7
27.8	2.2	4.5
28.5	2.6	2.4
29.6	1.2	6.9
30	2.2	3.9
30.2	0.4	3.7
32.6	2.6	4.5
32.9	2.4	5.2
34	2	1.9

* Values of the parameters Sn for 5NS and h_0/h_{-1} for 16-NS are expressed as percent increase calculated for each obese compared with control means shown in Tables 3 and 4.

characterizes the modifications present in the RBC in obesity.

Mammalian RBC lack the ability to synthesize lipids *de novo* (29) so that the cholesterol content and phospholipid composition which influence membrane fluidity depend on the exchange with the plasma lipoprotein surface. This mechanism serves adaptive and homeostatic roles under normal conditions and alterations in lipoprotein structure could lead to abnormalities in the red cell lipid composition as shown in type IV hypertriglyceridemia and in LCAT deficiency (5). It has been shown that abnormal behavior of RBC is detectable even when only 0.5–1% of the original membrane phospholipids have been substituted with desaturated species (30). Furthermore, *in vivo* the survival of cells with this type of modified composition is reduced and some phospholipids substitutions even cause instantaneous removal of erythrocytes from the circulation. These observations suggest that the erythrocyte membrane responds to small variations in plasma lipids with changes in its own composition and physical state.

Various membrane functions such as permeability (31), activities of bound enzymes (32), and hormone receptor functions (33) are modulated by membrane fluidity as a consequence of the role of lipid in controlling optimal conformation of membrane proteins (34). The relationship between pathological manifestations of obesity and alterations in membrane fluidity and hence in membrane functions clearly deserves further investigation.

The results presented herein demonstrate that studies of the erythrocyte membrane by the ESR probe technique detect small modifications in the molecular organization of the membrane which nevertheless have major consequences in terms of its rigidity and fluidity.

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