Human erythrocyte membrane fluidity and insulin binding are independent of dietary *trans* fatty acids

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Substitution of selected saturated fatty acids of the diet of 29 men and 29 women with cis *or* trans *monounsaturated fatty acids did not affect erythrocyte membrane fluidity, insulin binding, and the membrane cholesterol and phospholipid concentrations. Subjects were fed four different controlled diets with a total fatty acid content of 39 to 40 energy percent for four 6-week periods in a Latin square design. The diets were: (1) high oleic acid (16.7 energy percent oleic); (2) moderate* trans *(3.8 energy percent* trans *fatty acids); (3) high* trans *(6.6 energy percent* trans *fatty acids); and saturated (16.2 energy percent lauric + myristic + palmitic acids). There were no significant diet effects on red cell ghost fluidity determined by fluorescence polarization of the hydrocarbon probe 1, 6-diphenyl-1,3,5-hexatriene (DPH) and the polar analog trimethylammonium-DPH (TMA-DPH). There were limited diet effects on fluidity of membranes as determined with DPH-propionic acid (DPH-PA) for the men. Insulin binding was more closely associated with anisotropy of fluorescence of the surface probe, DPH-PA, than with that of the other probes, which is compatible with the localization of the insulin receptor in a domain at the cell membrane surface.* (J. Nutr. Biochem. 5:591–598, 1994.)

Keywords: *trans* fatty acids; insulin binding; membrane fluidity; fluorescent probes; insulin receptor

Introduction

Incorporation of dietary unsaturated fatty acids in erythrocyte membrane phospholipids has been reported[1–7] to result in more fluid cell membranes in laboratory animals and human subjects. These increases in membrane fluidity are related to esterification of *cis* unsaturated fatty acids to the membrane phospholipids.[1–7] Evidence from crystallographic, calorimetric, and optical studies indicates that *trans* unsaturated fatty acyl groups behave much like their saturated counterparts.[8–10] Projection diagrams[10] of the various crystal forms of tristearin, triolein, and trielaidin show a more open structure for triolein. Close packing occurs in the saturated fatty acid moieties of triacylglycerol crystals involving the "Z"-shaped strings of zigzagging polymethylene segments. This geometry is altered by twist angles introduced by the double bonds in *cis* unsaturated fatty acids. The absence of such twist angles in the *trans* compounds results in saturated fatty acids and *trans* unsaturated fatty acids having similar conformations. Thus, tristearin and trielaidin exhibit similar crystal structures.[10] Membranes with *trans* fatty acyl esterification of the phospholipids are therefore expected to exhibit physical properties similar to membranes with phospholipids esterified with saturated fatty acids.

Modulation of membrane fluidity may result in changes in membrane function. Effects of both in vivo and in vitro alteration of membrane fatty acyl unsaturation and fluidity on receptor accessibility and activity have been described and reviewed.[11,12] We have observed[5–7,13,14] dietary fat-induced modification of erythrocyte ghost insulin binding, which could be associated with changes in membrane fluidity. Insulin binding was significantly higher in more fluid erythrocyte membranes obtained from polyunsaturated fatty acid-fed rabbits, miniature swine, and men and women. Barnard et al.[9] reported higher insulin binding to erythrocyte membranes from monkeys fed a diet containing *cis*-octadecenoates than to membranes from monkeys fed a diet high in *trans*-octadecenoates, but 1,6-diphenyl-1,3,5-hexatriene

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(DPH) steady state fluorescence polarization measurements showed no related changes in red blood cell membrane fluidity. Thus, with respect to the insulin receptor, high levels of dietary *trans* fatty acids produced membrane effects in a nonhuman primate similar to those of dietary saturated fats. The effects of dietary *cis* and *trans* fatty acids at levels typical of the US diet on cell membrane fluidity are, however, not known.

The results on insulin binding in the monkey suggest that *trans* fatty acid feeding reduces membrane fluidity. However, this was not corroborated in fluidity measurements by DPH fluorescence polarization. We have suggested[15] that insulin receptor activity may depend on fluidity in a specific membrane microenvironment that is not always detected by steady state polarization measurements with DPH, which readily diffuses to all membrane lipid regions. Several polar DPH derivatives have been used as fluidity probes of specific membrane regions, including the cationic (TMA-DPH)[16,17] 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene and the anionic (DPH-PA)[18] 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid.

The present study of the human red blood cell membrane with these probes was designed to resolve questions pertaining to membrane microheterogeneity, fluidity, and receptor activity as affected by saturated and *cis* and *trans* monounsaturated fatty acids at levels in the US diet.

Methods and materials

Subjects and experimental diets

Male and female subjects 25 to 65 years of age were recruited by advertisement in the area of the Beltsville Agricultural Research Center, Beltsville, MD USA. Subjects were recruited without regard to race or smoking habits. Participants were required to be within 85 to 120% of desirable body mass index (BMI),[19] calculated from height and weight in the life insurance tables,[20] and to be in general good health without history of cancer, heart disease, hypertension, hyperlipidemia, diabetes, peripheral vascular disease, gout, liver or kidney disease, or endocrine disorders. Those taking lipid-lowering drugs were not included in the study. Dietary exclusion criteria included frequent use of dietary supplements, habitual alcohol consumption, and eating patterns not compatible with the study protocol, e.g., vegetarian diets or low fat diets. Exercise was not controlled, but subjects were asked to maintain their normal activity patterns. Sixty-four subjects, 31 men and 33 women, entered the study. Twenty-nine men and 29 women finished all phases of the study. Only results for those subjects who completed all phases of the study are included in the data. All procedures were approved by the Institutional Review Board, Georgetown University School of Medicine, Washington, DC USA.

Four experimental diets were devised to contain approximately 15 energy percent (en%) protein and 39 to 40 en% fatty acids, similar to levels found in self-selected diets of subjects in previous studies.[21–24] The diets are designated OLEIC, highest in *cis* monounsaturates (16.7 en% oleic acid), SAT, highest in saturated fatty acids, MOD TRANS, and HIGH TRANS, the latter two corresponding to different levels of *trans* fatty acids (3.8 en% and 6.6 en% *trans* fatty acids, respectively). Subjects ate each diet for 6 weeks in a Latin square design. The diet with the highest level of saturated fat (SAT) was planned to have 16 en% from the saturated fatty acids lauric, myristic, and palmitic acids and 3 en% from stearic acid. Saturated fatty acids in the other three diets were constant at 10 en% lauric, myristic, and palmitic acids and 3 en% stearic acid. The OLEIC, MOD TRANS, and HIGH TRANS diets were thus reduced 6 en% in lauric, myristic, and palmitic acids. In OLEIC, these acids were replaced with 6 en% oleic acid; in MOD TRANS, with 3 en% *trans* and 3 en% oleic acids; and in HIGH TRANS, with 6 en% *trans* fatty acids. All diets had 6 en% linoleic acid. SAT and OLEIC were as low in *trans* fatty acids, about 0.7 en%, as could be attained with commonly available foods. Dietary *trans* fatty acids primarily as *trans* monoenes were controlled at 3.8 en% and 6.6 en%, concentrations approximating intakes for the US diet.[25] Other fatty acids were controlled at concentrations corresponding to those derived from diet histories from previous studies of subjects whose characteristics were similar to those in the present study.[21–24] In all diets, cholesterol was controlled at about 0.083 mmol/MJ (.135 mg/kcal), providing 400 mg/day at the average energy intake for the study.

Each 6-week study period used a 14-day menu cycle. A nutrient data base for developing study menus and estimating dietary intake was compiled from USDA Handbook No. 8 (revised series 1–17 and 19–21)[26] and from data obtained by analysis of various fats and oils that were used to control the fatty acid composition of the menus. For each diet, composites were prepared and analyzed six times at the 11.72 MJ/day and once at the 7.53 MJ/day level. Fatty acid analyses of the composites were performed by gas chromatography of the fatty acyl methyl esters.[27] Total *trans* fatty acids were determined by Fourier transform infrared spectroscopy.[28] Fatty acid contents of the diets as analyzed are given in *Table 1*. Further details of the diets and related protocols have been published.[25]

Blood sampling and erythrocyte membrane preparation

Morning fasting blood samples were taken at the end of each 6-week experimental diet period. Erythrocytes were collected by centrifugation from one blood sample drawn from each subject into vacuum tubes containing Na_2EDTA. A second blood sample was drawn into a separate tube containing K_2EDTA and an antiprotease to prevent insulin degradation prior to analysis. Plasma was separated and frozen immediately at $-78°$ C for subsequent insulin measurement. Erythrocytes were dispersed in isotonic phosphate buffer (310 mOsm, pH 7.4) and washed by repeated centrifugation (20 min, 1,000*g*) in the same buffer. The washed cells were hypotonically lysed using the method of Dodge et al.[29] in phosphate buffer (20 mOsm, pH 7.4) and washed free of hemoglobin and other cytosolic contaminants.

Fluidity measurements

Fluorescence polarization was measured with DPH, TMA-DPH, and DPH-PA to assess erythrocyte membrane fluidity. Steady state fluorescence polarization measurements were made using the methodologies developed by Shinitzky and Barenholz.[30] Fluorophores (2 mM in dimethylformamide) were diluted (1,000 fold) into the aqueous membrane suspensions, which were then incubated at 35 to 37° C for 2 hours. Steady state fluorescence polarization was measured at 4° C and 37° C with an SLM Model 4800 spectrophotofluorometer (SLM-Aminco, Urbana, IL USA) equipped with Glan-Thompson prism polarizers in the T-optical format. Excitation and emission wavelengths were 366 and 430 nm, respectively. Light scattering errors were minimized by diluting the samples sufficiently to be certain that the anisotropies were concentration-independent.

Insulin binding

Erythrocyte membrane insulin receptor was assayed by measuring insulin binding to ghosts (100 μg protein/tube) incubated in 0.5 mL Tris-HEPES buffer[31] with 0.1 ng ^{125}I-insulin (specific activity 81.4 TBq/nmol) and 0 to 100 μg native porcine insulin/mL at 4° C for 16

to 24 hours. ^{125}I-insulin was purchased from New England Nuclear (Boston, MA USA), and native porcine insulin was a gift from Eli Lilly & Co (Indianapolis, IN USA). After the incubation, 0.2 mL aliquots were layered over 0.2 mL chilled Tris-HEPES buffer and centrifuged (60 s, 7500*g*). The ghost pellet was washed once with 10% sucrose, and radioactivity was determined in a gamma counter (Model A5550, Packard Instrument, Downers Grove, IL USA). Plasma insulin levels were measured by radioimmunoassay.[32]

Chemical analysis

Membrane cholesterol contents were determined enzymatically[33] using cholesterol esterase and cholesterol oxidase. Chemical methods were used for protein[34] and lipid phosphorus[35] analyses. After protein analysis of the aqueous ghost suspensions, lipids were extracted with $CHCl_3/CH_3OH$ by an adaptation[36] of the method of Sperry and Brand,[37] and aliquots were taken for cholesterol and phosphorus analyses.

Statistical analysis

Data were analyzed for variance for main effects and interactions of gender and diet. The statistical analyses only include data for those subjects who completed all four diet phases of the study. Regression analyses were performed[38] to assess relationships between fluorescence anisotropies (as measurements of membrane fluidity) and insulin binding and between fluorescence anisotropies and cholesterol to phospholipid ratios.

Results and discussion

Dietary *trans* fatty acids at the intake levels examined in this study had no effect on erythrocyte membrane fluidity or insulin binding. The fluorescence anisotropy data given in *Tables 2 and 3* display no significant diet-dependent differences for either males or females, either at physiologic temperature or at 4° C, for DPH and TMA-DPH. Data for DPH-PA *(Table 4)* at 37° C display limited diet effects for membranes from the men, but there were no significant diet effects for the women at either temperature. At physiologic temperature, the DPH anisotropy values *(Table 2)* were the lowest of all three probe molecules, reflecting the most fluid regions of the cell membrane, i.e., the center of the hydrocarbon region of the bilayer. Anisotropies for TMA-DPH *(Table 3)* and DPH-PA *(Table 4)* were higher than for DPH, indicating that both are located in more ordered membrane domains, i.e., in proximity to the phospholipid headgroups. All three probes yielded similar anisotropy values at 4° C.

The dietary treatments in this study had no significant effect on either the membrane cholesterol or the membrane phospholipid content. The molar cholesterol to phospholipid ratios in *Table 5* show no statistically significant effect of diet. We observed no effects on the membrane cholesterol and phospholipid levels, though the *trans* diets did behave like

Table 1 Fatty acid compositions of the diets (mean ± SD)[a]

	SAT	OLEIC	MOD TRANS	HIGH TRANS
		g/ 100 g dietary fatty acids		
Short-chain[b]	3.3 ± 0.51	1.6 ± 0.28	1.6 ± 0.35	1.3 ± 0.21
Saturated[c]	49.4 ± 0.36	35.4 ± 0.82	34.3 ± 0.50	33.6 ± 0.27
Lauric	9.1 ± 0.16	3.8 ± 0.28	3.7 ± 0.31	2.5 ± 0.06
Myristic	5.7 ± 0.08	3.3 ± 0.13	3.3 ± 0.14	2.8 ± 0.08
Palmitic	25.9 ± 0.31	19.8 ± 0.40	18.2 ± 0.19	19.0 ± 0.11
Stearic	7.7 ± 0.11	7.1 ± 0.13	7.9 ± 0.08	8.2 ± 0.05
Oleic	27.5 ± 0.62	42.4 ± 1.13	35.7 ± 1.31	29.2 ± 1.06
Total *trans*[d]	1.8 ± 0.59	1.9 ± 0.95	9.7 ± 1.21	17.0 ± 0.99
Linoleic	15.3 ± 0.82	15.6 ± 0.93	15.2 ± 0.64	15.8 ± 0.23
Others	2.9 ± 0.55	3.2 ± 0.57	3.4 ± 0.81	3.2 ± 0.33

[a]Average of seven 14-day composites of each diet.
[b]Short chain fatty acids C_6–C_{10}.
[c]Total saturated fatty acids C_{12} to C_{24}.
[d]Total *trans* unsaturated fatty acids determined by Fourier transform infrared spectroscopy.

Table 2 Diphenylhexatriene (DPH) steady state fluorescence anisotropy, r_s

Diet:	SAT	OLEIC	MOD TRANS	HIGH TRANS
temperature				
		males		
37° C	0.2095 ± 0.0010	0.2093 ± 0.0011	0.2093 ± 0.0011	0.2103 ± 0.0010
4° C	0.2955 ± 0.0014	0.2946 ± 0.0012	0.2940 ± 0.0014	0.2953 ± 0.0014
		females		
37° C	0.2105 ± 0.0010	0.2110 ± 0.0012	0.2108 ± 0.0013	0.2106 ± 0.0012
4° C	0.2962 ± 0.0016	0.2979 ± 0.0016	0.2964 ± 0.0013	0.2971 ± 0.0014

Values are means ± SEM.
There were no statistically significant ($P < 0.05$) differences among the anisotropy data at either temperature.

Table 3 Trimethylammoniumdiphenylhexatriene (TMA-DPH) steady state fluorescence anisotropy, r_s

Diet:	SAT	OLEIC	MOD TRANS	HIGH TRANS
temperature				
		males		
37° C	0.2536 ± 0.0010	0.2531 ± 0.0011	0.2508 ± 0.0014	0.2518 ± 0.0009
4° C	0.2989 ± 0.0012	0.3003 ± 0.0013	0.2979 ± 0.0017	0.2992 ± 0.0012
		females		
37° C	0.2534 ± 0.0014	0.2529 ± 0.0010	0.2528 ± 0.0011	0.2527 ± 0.0012
4° C	0.3001 ± 0.0013	0.3000 ± 0.0010	0.2997 ± 0.0012	0.2999 ± 0.0012

Values are means ± SEM.
There were no statistically significant ($P < 0.05$) differences among the anisotropy data at either temperature.

Table 4 Diphenylhexatriene propionic acid (DPH-PA) steady state fluorescence anisotropy, r_s. Values are means ± SEM.[a]

Diet:	SAT	OLEIC	MOD TRANS	HIGH TRANS
temperature				
		males		
37° C	0.2600[ab] ± 0.0007	0.2616[a] ± 0.0008	0.2593[b] ± 0.0006	0.2604[ab] ± 0.0006
4° C	0.3090 ± 0.0007	0.3084 ± 0.0008	0.3075 ± 0.0008	0.3083 ± 0.0008
		females		
37° C	0.2608 ± 0.0009	0.2612 ± 0.0007	0.2601 ± 0.0006	0.2617 ± 0.0007
4° C	0.3083 ± 0.0007	0.3102 ± 0.0009	0.3089 ± 0.0007	0.3095 ± 0.0007

[a]Anisotropy values for the males at 37° C with different superscripts are significantly different. There were no statistically significant ($P < 0.05$) differences among the data for the males at 4° C and for the females at either temperature.

Table 5 Erythrocyte membrane molar cholesterol to phospholipid ratios

Diet:	SAT	OLEIC	MOD TRANS	HIGH TRANS
		males		
	0.80 ± 0.07	0.83 ± 0.11	1.07 ± 0.25	0.94 ± 0.17
		females		
	0.75 ± 0.06	0.73 ± 0.07	0.80 ± 0.08	0.85 ± 0.09

Values are means ± SEM.
There were no statistically significant ($P < 0.05$) differences among the binding data or insulin data for either sex.

saturated fat diets with respect to plasma cholesterolemia and certain aspects of lipoprotein chemistry.[25] Consumption of the *trans* fatty acids resulted in elevations of low density lipoprotein-cholesterol with minor reductions in high density lipoprotein cholesterol. Despite these diet effects on lipoproteins, erythrocyte cholesterol was not affected. The apparent lack of fluidity dependence on the cholesterol to phospho-lipid (C/P) ratios is consistent with the relative constancy of the C/P ratios. Linear regression analyses showed no significant relation between probe anisotropy, as the dependent variable, and the C/P ratio. Cell membrane C/P ratios are generally considered a major determinant of fluidity.[39] We reported a significant dependence of red cell membrane fluidity and insulin binding on the C/P ratio in an earlier study when male subjects were fed fish oil-containing diets.[7] Actually, the data in the present study are compatible with the equation we reported[7] relating DPH fluorescence anisotropy with the C/P ratio in erythrocyte membranes.

There were no statistically significant effects of any of the diets on red blood cell insulin binding or on plasma insulin concentration *(Table 6)*. For each diet, insulin binding values were higher in the females than in the males, but the sex related differences were not significant.

Statistical analyses showed no period-by-diet interactions, indicating that there were no differences in response to diet in the four study periods, thus reducing concern about carryover.

The absence of a diet-dependent effect on fluorescence-polarization-determined fluidity is in agreement with the previous report[14] for erythrocyte membranes from *trans* fatty acid-fed monkeys; however, the study with monkeys did display a *cis-trans* diet effect on insulin binding. The differences between the two studies with respect to receptor function may reflect a species difference or may be related to the higher *trans* level, 25% of the fat, in the monkey-feeding study as opposed to the present study, 9.7% of the fat in MOD TRANS and 17% of the fat in HIGH TRANS. Benga et al.[40] reported that feeding rats semipurified diets elevated in *cis* 18:1, *trans* 18:1, or 18:2 fatty acids had no effect on erythrocyte membrane spectral properties of either stearic acid or methylstearate spin probes, despite differences in membrane fatty acyl composition. They accounted for their findings through either compensatory incorporations of *trans* fatty acids and saturated fatty acids or partitioning of the probes into membrane environments not affected by the fatty acid changes.

The alterations in insulin receptor reported in the earlier study[14] despite the constancy in DPH-determined membrane fluidity suggested that insulin binding and/or function of the insulin receptor are independent of membrane fluidity. There are conflicting reports pertaining to dietary control of mem-

Table 6 Erythrocyte membrane insulin binding and plasma insulin concentration

Diet:	SAT	OLEIC	MOD TRANS	HIGH TRANS
		males		
Binding*	0.403 ± 0.05	0.394 ± 0.05	0.448 ± 0.05	0.401 ± 0.04
insulin†	75.8 ± 8.0	82.7 ± 5.9	80.6 ± 7.7	83.5 ± 10.2
		females		
Binding	0.425 ± 0.05	0.494 ± 0.07	0.460 ± 0.07	0.411 ± 0.06
insulin	97.2 ± 15.4	84.0 ± 12.0	89.8 ± 10.6	73.9 ± 7.0

Values are means ± SEM.
There were no statistically significant ($P < 0.05$) differences among the binding data or insulin data for either sex.
*Percent specific binding / 100 μg erythrocyte ghost protein.
†Plasma insulin as pmol/L.

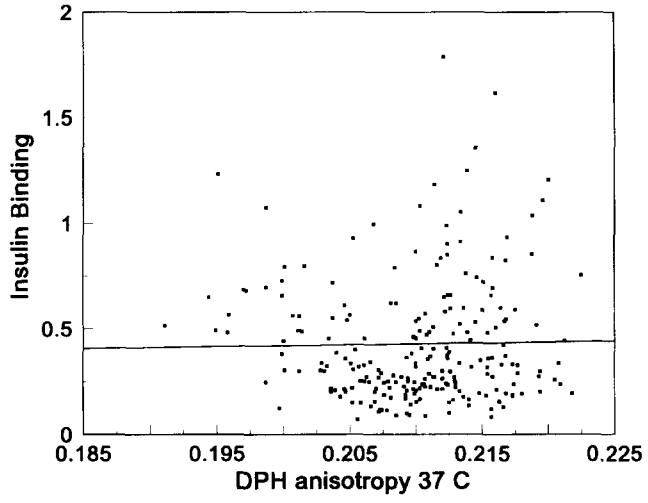

Figure 1 Insulin binding (IB, the percent specific binding per 100 μg erythrocyte ghost protein) and membrane fluidity as DPH fluorescence anisotropy, r_{DPH}, at 37° C. Regression analysis yielded the statistically nonsignificant ($P < 0.78$) equation: $IB = 0.243 + 0.883r_{DPH}$.

brane fluidity resulting in modulation of receptor activity.[6,13,41–46] We have studied diet effects on fluidity and the insulin receptor in several species and have observed situations where these phenomena were independent of one another or of the diet treatment.[5,15] We have suggested that the apparent independence of insulin binding from membrane fluidity may be due to the differences in the measurement temperatures or may result from membrane heterogeneities. Both of these possibilities are addressed in the present work. Membrane fluidity was assessed at both 37° C and 4° C with three different fluorescent probes, while insulin binding was measured at 4° C. Analyses were performed to determine if there is a significant relationship between insulin binding and any of the fluorescence anisotropies, whether they are measured at 4° C or at 37° C. There was no significant relationship between insulin binding and DPH anisotropy at 37° C ($P < 0.78$) or 4° C ($P < 0.12$), in agreement with results of the monkey-feeding study.[14] Linear regression analysis of the data shown in *Figure 1* yielded the nonsignificant equation: $IB = 0.243 + 0.883$ (r_{DPH}); ($r^2 = 0.003$ and $P < 0.78$) between insulin binding and the steady state fluorescence anisotropy for DPH at 37° C. Significant associations exist between insulin binding and the fluorescence anisotropies for the polar probes at either temperature. The most significant relationship was between insulin binding and DPH-PA anisotropy at 4° C. There the data followed the equation: $IB = 5.723 - 17.148$ ($r_{DPH\text{-}PA}$) with $r^2 = 0.06$ and $P < 0.0002$. The relationship between insulin binding and the steady state DPH-PA anisotropy at 4° C is shown graphically in *Figure 2*. These polar probes describe specific membrane regions, i.e., in proximity to the phospholipid headgroups, hence our results support the concept that insulin binding to its receptor would be modulated by membrane fluidity in a specific microenvironment.[15]

Microdomain organization of cell membranes is recognized with nonrandom distribution of membrane components in the bilayer.[47–49] Nonhomogeneous lateral distribution of membrane lipids or membrane proteins has been shown for such systems. Fluidity in these domains has been studied with the various DPH derivatives[16,18,50,51] using steady state and lifetime methods. Kitagawa et al.[51] have described association of TMA-DPH with the inner membrane leaflet and DPH-PA with the outer membrane leaflet in platelets. Membrane lipid domains are associated with the distribution of the phospholipids. Transbilayer asymmetric distribution of phospholipids

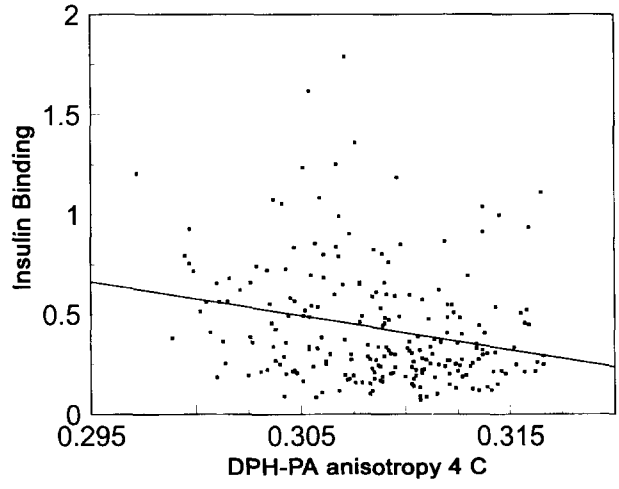

Figure 2 Insulin binding (IB, the percent specific binding per 100 μg erythrocyte ghost protein) and membrane fluidity as DPH-PA fluorescence anisotropy, $r_{DPH\text{-}PA}$, at 4° C. Regression analysis yielded the statistically significant ($P < 0.0002$) equation: $IB = 5.723 - 17.723r_{DPH\text{-}PA}$.

in the human erythrocyte membrane has long been recognized,[52–54] with phosphatidyl choline and sphingomyelin as the predominant headgroups in the outer leaflet of the membrane bilayer and phosphatidyl ethanolamine and phosphatidyl serine as the dominant headgroups of the cytofacial leaflet phospholipids. Hence the negatively charged DPH-PA, which binds to positively charged headgroups, e.g., phosphatidyl choline, describes the lipid environment of the outer leaflet, and the positively charged quaternary ammonium TMA-DPH, which associates with the negatively charged phosphatidyl serine or the neutral phosphatidyl ethanolamine, describes the lipid environment of the cytofacial leaflet. Our finding that insulin binding is significantly related ($P < 0.0002$) to DPH-PA-determined fluidity is compatible with the accepted concept that the insulin receptor is on the outer cell surface.[55] The insulin receptor is described[56] as a multisubunit protein with the α unit located on the outer surface of the cell. It is the α subunit that binds insulin. Hence it is reasonable that insulin binding would more likely be associated with the DPH-PA-determined fluidity than that determined with the other probes.

Anisotropy data for the polar probes *(Tables 3 and 4)* consistently show $r_{DPH\text{-}PA} > r_{TMA\text{-}DPH}$, indicating that the exofacial leaflet lipids of the erythrocyte membrane were less fluid than the cytofacial lipids. Kitagawa et al.,[51] concluded from similar data that the outer leaflet was less fluid than the inner leaflet in bovine platelet plasma membranes. Electron spin resonance (ESR) studies by Tanaka and Ohnishi[57] and Seigneuret et al.[58] and a dye binding study by Williamson et al.[59] showed that the inner half of the erythrocyte membrane is more fluid than the outer half; however, there are conflicting reports.[60,61] Fluidity differences between the membrane leaflets result from nonhomogeneous distributions of cholesterol or unsaturated fatty acids. Schroeder et al. in their review[62] state that cholesterol is enriched in the inner leaflet of most mammalian membranes. Regulation of the asymmetric transbilayer distribution of cholesterol in membranes is highly sensitive to incorporation of polyunsaturated fatty acids.[63–65] Schroeder et al.[62] stated that cholesterol is distributed asymmetrically across the red blood cell membrane, with 75% in the inner cytofacial leaflet and the remainder in the exofacial leaflet, with most integral membrane proteins localized in the cholesterol poor outer leaflet. Hence the size, dynamics, and lipid distribution within these domains may be important modulators of membrane protein functions and receptor-effector coupled systems. Brasaemle et al.[66] measured the rate of cholesterol transfer across the human red blood cell membrane and stated that their data were consistent with asymmetric cholesterol distribution, with the majority in the inner leaflet. Their data indicate that 10 to 14% of the cholesterol is in the exofacial leaflet and 87% is in the cytofacial leaflet. Schachter et al.[67] provided evidence that perturbations of the fluidity of a given membrane hemileaflet influence the membrane proteins and their associated functions. Our data do not show reduced fluidity in the inner leaflet of the erythrocyte membrane; however, our finding that insulin binding and probably activity of the insulin receptor are related to fluidity as determined with DPH-PA, which usually describes the outer leaflet environment, extends these conclusions[62,67] pertaining to membrane lipid asymmetry and the role of the exofacial membrane leaflet in modulating the activity of the insulin receptor.

In summary, we have shown that consumption of foods containing *trans* fatty acids at levels comparable to the US diet (3.8 en%) and somewhat higher (6.6 en%) has no effect on red blood cell membrane fluidity, insulin binding, and cholesterol and phospholipid levels. Results with several fluorescent probe molecules were consistent with the notion that the insulin receptor is located at the outer membrane leaflet and its function may be related to fluidity in that domain.

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