# Dietary *trans* fatty acids modulate erythrocyte membrane fatty acyl composition and insulin binding in monkeys

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*The substitution of* trans- *for half of the* cis-*monounsaturated fatty acids in the diet of* Macaca fasicularis *monkeys resulted in alterations in erythrocyte fatty acid composition and insulin receptor properties but not in membrane fluidity. Both* cis *and* trans *diets contained 10% fat and similar fatty acid compositions, except that approximately 50% of the* cis-*octadecenoate (c-18:1) in the* cis *diet was replaced with* trans-*octadecenoate isomers (t-18:1) in the* trans *diet. Compared with the* cis *diet, the* trans *diet resulted in the incorporation of approximately 11% t-18:1, an approximately 50% decrease in c-18:1, an approximately 16% decrease in total saturated fatty acids, and an approximately 20% increase in 18:2(n-6) in erythrocyte membrane lipids. The increase in 18:2(n-6) may reflect on homeostatic mechanisms designed to maintain overall membrane fluidity, as no diet-related changes in fluidity were observed with diphenylhexatriene steady state fluorescence polarization. Values observed for insulin binding and insulin receptor number were higher and binding affinity was lower in monkeys fed the* cis *diet. In the absence of an effect on overall membrane fluidity, altered receptor activity suggests that insulin receptor activity is dynamic, requiring specific fluid membrane subdomains or highly specific fatty acid-protein interactions.*

**Keywords:** *trans* Fatty acids; insulin binding; membrane fluidity

## Introduction

Dietary modifications of membrane lipids can result in alterations in membrane fluidity and associated physiologic processes, such as receptor function.[1-7] The insulin receptor, an integral plasma membrane protein which is in intimate contact with membrane lipids,[8] has been shown to respond to changes in membrane lipid fatty acyl composition. Diet has been reported to affect insulin receptor number, insulin binding, and receptor binding affinity.[2,8-13]

Alterations in insulin receptor properties resulting from diet-induced modifications in membrane fatty acyl composition have been demonstrated in Erlich ascites grown in mice[9] and in erythrocytes of minipigs,[10] rabbits,[2] and women.[11] The results of these studies were consistent with the hypothesis that an increased saturation of plasma membrane phospholipids results in a lower receptor number. Similar conclusions were reached in studies using Friend erythroleukemia cells grown in culture.[13] In most of these studies, insulin receptor affinity was also affected. However, the situation may be more complex as both higher[9,12,13] and lower[2,10] affinities have been associated with increased fatty acyl saturation in membrane phospholipids.

The present study was conducted to examine the

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effects of dietary *trans* fatty acids on erythrocyte membrane fatty acyl composition, fluidity, and insulin binding activity of *Macaca fasicularis* monkeys. Incorporation of *trans*-octadecenoates (t-18:1) has been shown in many tissues,[14] as well as in the erythrocyte membrane.[15] Although t-18:1 fatty acids are unsaturated, their melting points and other physical characteristics are more similar to those of saturated fatty acids.[16] Consequently, it was of interest to determine how erythrocyte membrane t-18:1 would modulate membrane fluidity and insulin binding activity in comparison to dietary *cis* unsaturated fatty acids.

## Materials and methods

### *Animals and diets*

Twelve adult male *M. fasicularis* monkeys fed a Purina 15% Monkey Chow diet (Purina Mills, Richmond, IN, USA) were selected for this study. Monkeys were housed at the National Institutes of Health Primate Research Unit in cages (86 × 59 × 66 cm) in a well-ventilated room (10 to 15 air changes per hour) at a constant temperature of 25°C and with fluorescent illumination 12 hours per day. The primate facility was operated in accordance with the requirements and recommendations of the Guide for the Care and Use of Laboratory Animals.[17]

The monkeys were randomly separated into two groups of six and fed either a diet containing saturated and *cis* unsaturated fatty acids (*cis*-diet) or a diet containing saturated and both *cis* and *trans* unsaturated fatty acids (*trans* diet) for 18 weeks. The diets were open formula natural ingredient diets[18] manufactured by a cold pelleting technique (Zeigler Bros., Inc., Gardners, PA, USA). The formulations of the two diets were identical except for the fats *(Table 1)*. In the *trans* diet, approximately 50% of the monounsaturated fatty acids were t-18:1 and in the *cis* diet, all monounsaturates were *cis* unsaturated fatty acids *(Table 2)*. The *cis* and *trans* diet fats were chosen to yield virtually identical proportions of saturated, monounsaturated, and polyunsaturated fatty acids. Sufficient diet for the entire study was manufactured in a single production batch and stored at 0°C.

The monkeys were weighed, and blood samples were drawn at the beginning of the study and subsequently at 3-week intervals. The monkeys were sedated with ketamine (10 mg/kg body weight) to facilitate blood collection and weighing. Blood was collected (20 ml) via the femoral vein or artery using 10-ml vacutainers containing a 15% solution of $K_2$EDTA. During the 18-week study, food intake was measured by determining the difference between the weight of food offered and the uneaten food collected within 1 hour after feeding.

### *Erythrocyte membrane isolation*

Erythrocytes were separated from plasma and platelets by differential centrifugation, dispersed in isotonic phosphate buffer (310 mOsm, pH 7.4), and washed by repeated centrifugation (20 minutes, 1,000 × *g*). Erythrocyte ghosts were prepared by hypotonic lysis in 20-mOsm phosphate buffer (pH 7.4) according to the procedure of Dodge and Phillips.[19] Red blood cell ghosts were washed repeatedly in the 20-mOsm phosphate buffer to remove hemoglobin and other cytoplasmic contaminants.

**Table 1** Formulation of the experimental diets[a]

| Ingredient | Amount per kilogram diet (g) |
|---|---|
| Casein | 168 |
| Rice gel | 269 |
| Methionine | 3 |
| Glucose | 150 |
| Apple pomace | 100 |
| Beet pulp | 140 |
| Fat[b] | 100 |
| Mineral mix[c] | 50 |
| Vitamin mix[d] | 20 |

[a] Formulations for both the *trans* and the *cis* diets were the same except for the source of the fat

[b] Fat source for the *cis* diet was corn oil, olive oil, and cocoa butter in a ratio of 40.97:49.37:2.50, respectively. The fat source for the *trans* diet was margarine stock

[c] Mineral mix consisted of (g/kg) calcium carbonate, 299.74; cupric sulfate•5$H_2O$, 3.00; potassium phosphate dibasic, 206.23; ferric ammonium citrate, 27.47; magnesium sulfate, 101.91; manganous sulfate, 4.99; calcium phosphate dibasic, 74.93; potassium iodide, 0.06; zinc sulfate, 0.34; sodium chloride, 167.35; and carrier-limecrest, 113.98

[d] Vitamin mix consisted of (g/kg) vitamin A/$D_3$ (650/325k IU/g), 0.30; vitamin A (650 k IU/g), 1.47; vitamin E 50% (500 IU/g), 10.0; hetrazeen 45.5% menadione, 1.10%; vitamin $B_{12}$ (1,000 mg/lb), 3.30; thiamine mononitrate USP (92%), 1.0; riboflavin (50%), 2.0; D-calcium pantothenate USP (92%), 5.00; pyridoxine HCl USP (82%), 1.00; folic acid USP, 0.75; biotin (1%), 2.50; ascorbic acid coated (97.5%), 100.0; choline chloride 60% (52% choline), 41.70; niacinamide USP, 7.50; carrier sucrose, 720.5; ethoxyquin (66.6%), 10.0

### *Fluidity measurements*

Membrane fluidity was assessed by measuring 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization as described by Shinitzky and colleagues.[20,21] Probe incorporation was accomplished by diluting 2 mM DPH in tetrahydrofuran 500-fold into aqueous suspensions of ghosts and incubating with agitation at 35 to 37°C for 2 hours. Steady state fluorescence polarization intensity was measured with an Aminco Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD, USA) equipped with Glan-Thompson prism polarizers. DPH was excited at 366 nm and the fluorescence at 450 nm was detected through a Wratten 2A cutoff filter for wavelengths shorter than 415 nm. Intensities were measured with a photon counter which is suitable for dilute systems. Appropriate dilutions were made to avoid light scattering errors. Anisotropies were obtained from the measured emission intensities polarized parallel and perpendicular to the polarized excitation using standard formulas. An instrumental grating correction factor was included.[22,23]

**Table 2** Diet fatty acid composition[a]

| Fatty acid | *cis* Diet | *trans* Diet |
|---|---|---|
| 16:0 | 10.2 ± 0.10 | 11.1 ± 0.16 |
| 16:1 | 0.3 ± 0.01 | — |
| 18:0 | 3.5 ± 0.06 | 6.1 ± 0.20 |
| 18:1t | — | 25.2 ± 0.23 |
| 18:1c | 54.3 ± 0.16 | 29.3 ± 0.25 |
| 18:2tt | — | 0.3 ± 0.01 |
| 18:2ct | — | 0.2 ± 0.01 |
| 18:2cc | 30.9 ± 0.25 | 27.1 ± 0.62 |
| 18:3ccc | 0.8 ± 0.06 | 0.3 ± 0.03 |

[a] Values are given in weight% as the mean ± SEM of five samples for each diet

### *Insulin studies*

Plasma insulin levels were determined using a radioimmunoassay kit (catalog #0600) from Immunonuclear Corp. (Stillwater, MN, USA). Insulin binding parameters were determined essentially as described by Bhathena et al.[24] and Gambhir et al.,[25] using studies involving the binding of radioactive insulin to right-side-out erythrocyte ghost preparations in the presence and absence of native porcine insulin. The erythrocyte ghost preparations contained 200 to 300 μg protein in 0.5 ml Tris-Hepes buffer,[25] 40 μg/ml of bacitracin, 50 pg of monoiodinated insulin ($^{125}$I-insulin; 81.4 TBq/mmol; New England Nuclear Corporation, Boston, MA, USA), and 0 to 100 μg/ml of native porcine insulin (gift from Eli Lilly & Co., Indianapolis, IN, USA). After incubating the preparations at 4°C for 16 to 18 hours, 0.2-ml aliquots were layered over 0.2 ml of chilled Tris-hepes buffer and centrifuged for 60 seconds at 7,500 × *g* (Microfuge II, Beckman Instruments, Inc., Fullerton, CA, USA) to isolate the ghosts. The ghost pellets were washed once with 10% sucrose and the bound radioactivity was determined in a gamma counter (Model A5550, Packard Instrument, Downers Grove, IL, USA). The percentage of specific insulin bound was determined[25] as the percent specific binding/100 μg ghost protein (insulin binding). Scatchard plots[26] were used to derive insulin receptor numbers and competition-inhibition plots were used to determine receptor binding affinities, as the amount of native insulin (ng/ml) required to displace 50% of the specifically bound radiolabeled insulin.[27]

### *Chemical analysis*

Protein analyses of the ghosts were performed colorimetrically by the procedure of Lowry et al.[28] Cholesterol was determined enzymatically by the method of Allain et al.,[29] and lipid phosphorus was determined after extraction by reducing the phosphomolybdate complex by ascorbic acid, as described by Ames.[30] Lipids were extracted from the erythrocyte ghosts as described by Folch et al.,[31] with the modifications that methylene chloride was substituted for chloroform and butylated hydroxytoluene (BHT) was added (0.01% of expected lipid) to the lipid extracts. Transesterification of the polar lipid fraction was conducted at 80°C for 16 hours in 1.5 N methanolic HCl (1 ml/25 mg lipid) and methylene chloride (2 ml/25 mg lipid). The methyl esters were extracted into hexane and purified by thin layer chromatography using a petroleum ether to diethyl ether (95:5 vol/vol) solvent system. The fatty acid methyl esters (FAME) were analyzed chromatographically using a Hewlett-Packard Model 5830 gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and a glass capillary column (15 m × 0.25 mm ID) coated with SP-2340 (Quadrex, New Haven, CT, USA). Fatty acid methyl ester identification was by comparison of retention times with those of authentic standards (Nu-Chek Prep, Elysian, MN, USA). Quantitation was based on response factors of known mixtures and correction factors for the partially resolved *trans* and *cis* components, determined as previously described.[32] Baseline fatty acid data were obtained by analyzing samples of the Purina 15% Monkey Chow and the erythrocyte membranes isolated from the monkeys prior to initiation of the diet study while they were fed Purina Chow.

### *Diet analysis*

Diet samples were analyzed for crude protein, ether extract, nitrogen free extract (NFE), crude fiber, and moisture by proximate analysis in accordance with Association of Official Agricultural Chemists procedures[33] (Lancaster Laboratories, Inc, Lancaster, PA, USA). Diet samples were collected regularly during the study to monitor fatty acid stability.

### *Statistical analysis*

Fatty acid methyl ester data were analyzed by two-way analysis of variance (ANOVA). Significant differences determined by ANOVA were analyzed using Duncan's multiple comparison test. Data for insulin binding, insulin receptor affinity, and insulin receptor number were analyzed by the Student's *t* test. 1,6-Diphenyl-1,3,5-hexatriene anisotropy, $r_s$, data were analyzed by univariate repeated measures ANOVA. All statistical analyses were performed using computer methodology of the Statistical Analysis System.[34]

## Results

The fatty acid profile for erythrocyte membrane lipid isolated from monkeys fed the Purina Chow was similar to that reported by others for *Macaca radiata*.[35] However, in this study, 0.5 mol% of t-18:1 was observed in the membrane lipid, even though *trans* fatty acids were not detected in the lipids extracted from the Purina Chow.

Proximate analysis of the experimental diets indicated that the nutrient compositions of the *cis* and *trans* diets were similar and that both diets provided the nutrients required by *M. fasicularis* monkeys. As expected, the major difference between the diets was that about half of the octadecenoate in the *trans* diet

**Table 3** Erythrocyte membrane fatty acid composition for monkeys fed the *cis* and *trans* diets for 18 weeks[a]

| Fatty acid | *cis*-Fed | *trans*-Fed |
|---|---|---|
| 16:0 | 24.8 ± 0.30[c] | 20.8 ± 0.26[d] |
| 18:0 | 14.1 ± 0.12[c] | 12.8 ± 0.15[d] |
| 18:1t | 0.2 ± 0.05[d] | 10.9 ± 0.24[c] |
| 18:1c | 14.1 ± 0.25[c] | 8.6 ± 0.12[d] |
| 18:2 (n-6) | 15.6 ± 0.36[d] | 19.1 ± 0.44[c] |
| 20:2 (n-6) | 0.6 ± 0.05 | 0.6 ± 0.03 |
| 22:0 | 1.3 ± 0.08 | 1.5 ± 0.09 |
| 20:3 (n-6) | 1.7 ± 0.10 | 1.2 ± 0.10 |
| 20:4 (n-6) | 17.2 ± 0.43 | 16.3 ± 0.25 |
| 24:0 | 2.1 ± 0.12[c] | 1.0 ± 0.10[d] |
| 24:1 (n-9) | 2.2 ± 0.08[c] | 0.9 ± 0.07[d] |
| 22:4 (n-6) | 0.8 ± 0.06 | 0.7 ± 0.05 |
| 22:6 (n-3) | 3.0 ± 0.14 | 3.1 ± 0.23 |
| Others[b] | 2.3 ± 0.17 | 2.5 ± 0.09 |

[a] Values are given in mol% as mean ± SEM for six monkeys on each diet. Values for the same fatty acid with different superscript letters are significantly different ($P < 0.05$)
[b] "Others" includes 16:1t, 17:0, 20:0, 22:5 (n-6), and 20:1 (n-9)

**Table 4** Anisotropy and insulin binding parameters for monkey erythrocyte membranes at week 18 of diet study[a]

| Diet | Anisotropy | Insulin binding[b] | Receptor number[c] | Binding affinity[d] |
|---|---|---|---|---|
| *cis* | 0.20 ± .002 | 1.0 ± .19 | 0.07 ±.003[e] | 56.0 ±6.21[e] |
| *trans* | 0.20 ± .004 | 0.8 ± .14 | 0.03 ±.004[f] | 6.0 ±1.13[f] |

[a] Values are mean ± SEM for six monkeys on each diet. Values in a column with different superscript letters are significantly different ($P < 0.05$)
[b] Percent specific binding/100 μg erythrocyte ghost protein
[c] Number of receptors was determined for each monkey from the intercept on the abscissa of a Scatchard plot
[d] Affinity of the receptors is measured from the competition-inhibition plot as the amount (ng) of native insulin required to displace 50% of the bound tracer

consisted of *trans* isomers, whereas all of it was *cis*-octadecenoate in the *cis* diet (*Table 2*). Food intake and body weight data indicated that both diets were palatable. All monkeys showed a statistically significant ($P < 0.05$) increase in the mean body weight, from 5.5 ± 0.25 kg to 6.00 ± 0.30 kg through the 18-week study.

Analyses throughout the study established that the fatty acid compositions of erythrocyte membrane lipids were stabilized by the 15th week on the experimental diets, at which time the fatty acid compositions were nearly identical to those shown in *Table 3* for membranes isolated at week 18 of the study. As can be seen, the *trans* diet resulted in the incorporation of 10.9 mol% of t-18:1 and lower ($P < 0.05$) proportions of 16:0, 18:0, c-18:1, 24:0, and 24:1 compared with the *cis* diet. In contrast, compared with the *cis* diet, higher proportions of 18:2(n-6) were observed in the erythrocyte membrane lipids of monkeys fed the *trans* diet ($P < 0.005$).

No difference in the ratio of phospholipid to cholesterol was observed in erythrocyte membranes isolated from the two groups of animals. Also, the plasma insulin levels of monkeys fed the *cis* diet (22.5 ng/ml ± 7.25) were not different from those fed the *trans* diet (22.9 ng/ml ± 5.38).

Membrane fluidity, insulin binding, receptor number, and binding affinity data for erythrocyte membranes isolated from monkeys fed the two experimental diets are given in *Table 4*. There was no statistically significant difference in erythrocyte membrane fluidity or in the insulin binding value. However, compared with the values derived from animals fed the *cis* diet, the value for insulin receptor number, which is derived from data in *Figure 1*, was lower ($P < 0.05$), and binding affinity, derived from data in *Figure 2*, was greater ($P < 0.05$) in membranes isolated from animals fed the *trans* diet.

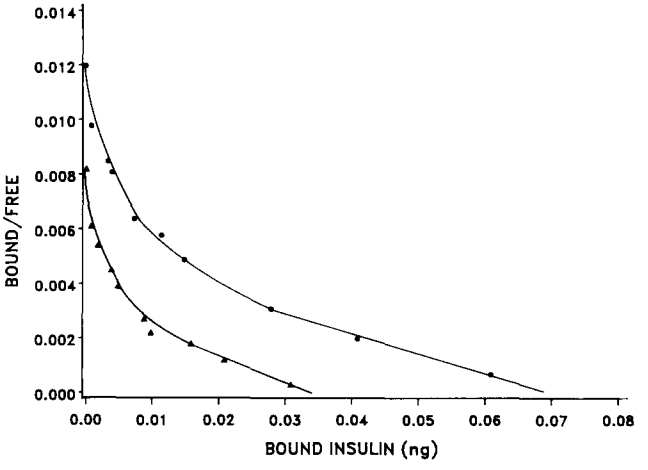


**Figure 1** Scatchard analysis of insulin binding data for erythrocyte ghosts from monkeys fed the *cis* (●●) or *trans* (▲▲) diet. Each point is the average for data from six monkeys

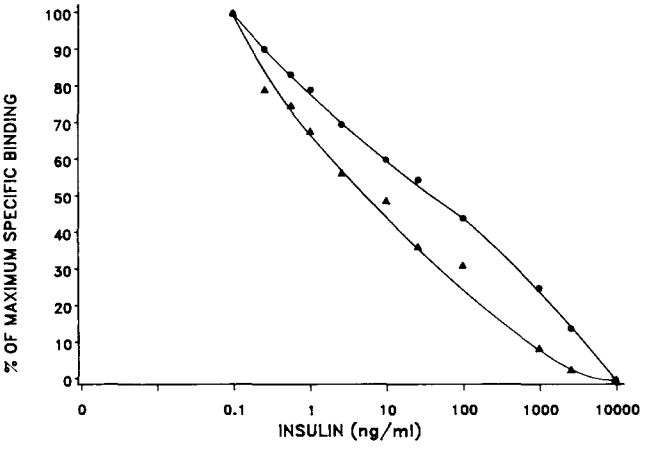


**Figure 2** Competition-inhibition plot of insulin binding to erythrocyte ghosts from monkeys fed the *cis* (●●) or *trans* (▲▲) diet. Each point is the average for data from six monkeys

## Discussion

Membrane phospholipid fatty acyl composition has been reported to modulate a number of cell functions, including receptor-mediated processes.[1,2,11-13,36] Such fatty acyl alterations of membrane function may or may not be accompanied by measurable changes in membrane fluidity.[37] In the present study, the substitution of *trans* for *cis* fatty acids in the diet effectively altered membrane composition and insulin receptor parameters but not overall membrane fluidity.

Most *trans* fatty acids have physical properties similar to saturated fatty acids.[14] Since substitution of saturated fatty acids for *cis*-unsaturated fatty acids has been shown to result in decreased membrane fluidity,[38,39] we expected similar changes in membrane fluidity due to the presence of *trans* fatty acids. The lack of an effect in the present study may be rationalized in terms of compensatory mechanisms whereby membrane fluidity is maintained. In this study, the anticipated loss in fluidity following membrane incorporation of *trans* fatty acids may have been offset by the decrease in saturated fatty acids and the increase in linoleic acid in the erythrocytes of monkeys fed the *trans* diet.

Despite the lack of an effect on bulk membrane fluidity, alterations in insulin receptor parameters were observed in the monkeys fed the *trans* diet. It is unlikely that the differences in receptor values observed were due to down-regulation of the insulin receptor as no diet-related differences were observed in plasma insulin levels. Thus, the receptor differences must have resulted from alterations in membrane fatty acid composition. Since *trans* fatty acids do have physical properties similar to saturated fatty acids, it is not surprising that these insulin receptor data are consistent with reports of decreased insulin receptor number and greater receptor affinity when membrane saturation is increased.[9,12,13]

The alterations in receptor properties observed in this study, despite the constancy of membrane fluidity, suggest that these properties are independent of membrane fluidity. The literature contains conflicting reports concerning dietary control of membrane fluidity with resultant modulation of receptor activity.[2,9,10,12,36,40-42] We have studied the effect of differences in dietary fat on erythrocyte membrane properties in several species and observed situations in which both insulin binding and fluidity were affected[11] as well as those in which neither was appreciably affected.[43] Nevertheless, it is important to recognize the limitations in such studies of membrane fluidity assessed by steady state polarization measurements, using DPH as the fluorescent probe. This probe readily diffuses to all membrane lipid regions[44,45]; hence, the observed anisotropies describe an average or bulk membrane fluidity, which represents the contributions of many membrane subdomains, but these may or may not correspond to the fluidity in specific membrane microenvironments. As insulin receptor properties are probably dependent on the fluidity in a specific membrane subdomain, the apparent lack of an association between erythrocyte membrane fluidity and receptor properties in the present study may be viewed as an inability of steady state polarization measurements to always represent the actual fluidity of the appropriate membrane microenvironment.

Differences in membrane lipid domain fluidities have also been invoked by others involved in insulin receptor research[42] and may help explain the findings of Benga et al.,[46] who altered rat red blood cell ghost fatty acyl composition but observed no effects on the spectral parameters of spin-labeled probes. Fiorini et al.[47] provided evidence for erythrocyte membrane heterogeneity using multifrequency phase fluorometry. They measured the fluorescence lifetimes of DPH in red blood cell ghosts and in extracted membrane lipids and observed a broader Lorentzian distribution function for DPH fluorescence lifetime in the membrane samples than in liposomes prepared from the extracted lipids. The broadening of the distribution function for the major lifetime component of 11 ns was associated with structural heterogeneity in the red blood cell membrane.

In conclusion, the results of the present study show that dietary *trans* fatty acids incorporated into monkey erythrocyte membranes affect insulin receptors without affecting the overall membrane fluidity. The results are consistent with the concept that insulin receptors require specific membrane subdomains to provide appropriately fluid regions or that specific fatty acid protein interactions are required for insulin receptor activity. Whatever the specific mechanism, it appears that *trans* and saturated fatty acids function similarly with respect to the insulin receptor.

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