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EFFECT OF ALTERATIONS IN MEMBRANE LIPID UNSATURATION ON THE PROPERTIES OF THE INSULIN RECEPTOR OF EHRLICH ASCITES CELLS

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We have altered the phospholipid composition of the plasma membranes of Ehrlich ascites cells grown in mice and studied the effects on the properties of the insulin receptor of this cell. The insulin receptor of the Ehrlich cell demonstrated all of the binding characteristics of mammalian insulin receptors: specificity for insulin and insulin analogs, saturability, inverse relationship of steady-state binding levels to temperature, and negative cooperativity. Cellular phospholipids enriched in monounsaturated fatty acyl groups were produced by growth in animals that were maintained on a diet rich in coconut oil; cellular phospholipids enriched in polyunsaturated fatty acyl groups were produced in animals fed sunflower oil. Insulin receptors were present in the normal cells at 180000 sites/cell but this fell to 125000 ($p < 0.001$) in cells enriched in monounsaturated fatty acids and rose to 386000 ($p < 0.001$) in cells enriched in polyunsaturated fatty acids. The normal cells had affinity constants ($\bar{K}_e$ and $\bar{K}_f$) of 0.03 and 0.01 nM^{-1}. The cells enriched in monounsaturated fatty acids had an increase in these affinity constants to 0.06 and 0.03 nM^{-1} whereas values of 0.01 and 0.005 nM^{-1} were obtained in the cells enriched in polyunsaturated fatty acids (all comparison $p < 0.001$). Thus, increased unsaturation of plasma membrane phospholipids, produced by dietary manipulations, was associated with an increase in insulin receptor number but a decrease in binding affinity. In contrast, increased saturation of the phospholipids of the plasma membrane was associated with a decrease in receptor number and an increase in affinity. The results can be explained by a model in which the insulin receptor is assumed to be multimeric.

Introduction

The lipid composition of the plasma membrane has been reported to have a marked effect on the properties of hormone receptors including the insulin receptor [1–10]. In previous studies we have demonstrated that increasing the proportion of unsaturated fatty acids in the phospholipid fraction of Friend erythroleukemia cells leads to an increase in insulin receptor concentration but a decrease in receptor affinity. Preliminary data have suggested that such changes are associated with an increase in responsiveness of these cells to insulin [1,11]. We have also demonstrated that insulin receptors, solubilized from avian erythrocytes and reconstituted into liposomes with exogenous phospholipid demonstrate similar alterations [2,3]. After incorporation of the receptors into liposome containing highly unsaturated phospholipid many more receptor sites are available than in similar preparations containing saturated phospholipids. Again, the receptors in the unsaturated preparation demonstrated a lower affinity. Not all cell

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preparations, however, behave identically. Using 3T3-L1 preadipocytes, we [4] and Grunfeld and Baird [5] have demonstrated that increased unsaturation of membrane phospholipids is associated with a decrease in insulin receptor concentration and in insulin's ability to promote 2-deoxyglucose transport.

To further study the relationship of membrane lipid composition to insulin receptor properties and to extend our studies to cells grown in intact animals, we have studied the effect of dietary manipulations on the properties of the insulin receptor of Ehrlich ascites cells grown in mice. Previous studies using these cells have shown that when the cells are grown in animals fed a diet rich in saturated fatty acid they incorporate a substantial amount of saturated fatty acid into their membrane phospholipids [12]. Similarly, when grown in animals fed a diet rich in polyunsaturated fatty acids they incorporate more polyunsaturates into their membrane phospholipids. Alterations of the membrane phospholipids, produced by feeding the animals diets rich in either saturated (coconut) or polyunsaturated (sunflower) oils are associated with major changes in the properties of membrane such as rotational fluidity measured by electron spin resonance [13] or fluorescence polarization and properties of membrane bound enzymes [14] including amino acid transport systems [12].

In the current study we present the properties of the insulin receptor of the Ehrlich ascites cells and demonstrate that the effects of lipid alteration, produced in vivo, upon these properties are very similar to those produced in vitro with the Friend cell [1] and the reconstituted receptor [3] in that increased unsaturation of the plasma membrane was associated with an increase in receptor number but a decrease in receptor affinity.

Methods

Cells. Ehrlich ascites cells were grown in CBA mice fed with either normal lab chow or a semisynthetic chow (16% fat) enriched in saturated fats (coconut oil) or polyunsaturated fats (sunflower oil) as previously reported [12]. The normal chow had about equal amounts of saturated, monounsaturated and polyunsaturated fats, whereas the chow with coconut oil contained 93% saturated fatty acids and the chow with sunflower oil contained 75% polyunsaturates (almost entirely linoleic acid).

Weanling mice were placed on these diets for six weeks, then transplanted with 30 μl of tumor dispersed in 0.3 ml of saline, intraperitoneally. After 13–16 days cells were harvested and washed as previously described [12] and used immediately for insulin binding studies.

Insulin and insulin binding. Porcine monocomponent insulin was the gift of Dr. Ron Chance (Eli Lilly and Co.), as was the porcine proinsulin. Desoctapeptide insulin was generously provided by Dr. J. Walder of the University of Iowa. IGF was purified as previously described [15]. Insulin was iodinated as previously described [1] by a low volume modification of the chloramine T method [16]. Cells were washed and resuspended at $50 \cdot 10^6$/ml in HE buffer (75 mM Hepes, 75 mM NaCl, 2.4 mM KCl, 0.81 mM $MgSO_4$, 0.81 mM sodium acetate, 0.81 mM EDTA, 10^{-3} M bacitracin, 1% bovine serum albumin). Incubations were performed in final volume of 0.5 ml in HE buffer and contained $4 \cdot 10^6$ cells, 20 pM [^{125}I]iodoinsulin and 0–10 μg unlabeled unsulin. Incubations were performed for 2.5 h at 15°C. Duplicate 200-μl aliquots were layered over 200 μl of buffer and sedimented at $8000 \times g$ for 2 min in a Beckman Microfuge. The supernatants were aspirated and the tips containing the pellets removed and counted in a Micromedic Mark IV gamma radiation counter at 70% efficiency.,

Data reduction was performed by a computer program DERC 2 developed at the University of Iowa [17].

Preparation of membranes and lipid analysis. A 20% suspension of cells in buffer (0.25 M sucrose, 0.4 M NaCl, 0.1 M KCl, 5 mM $MgSO_4$, 20 mM Tris, pH 7.2) were ruptured under 900 lb/inch^2 in a nitrogen bomb (Yeda press) as previously described [12]. Plasma membranes were prepared by differential centrifugation followed by isopycnic centrifugation. Analysis of $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase and NADPH-cytochrome *c* oxidase indicated that this preparation was very highly purified [12]. The plasma membranes were extracted with chloroform/methanol (2:1, v/v). Phospholipids were separated from neutral lipids by thin-layer chromatography and the eluted

phospholipids saponified with 0.3 M KOH in ethanol for 45 min at 70°C [12]. The liberated fatty acid were extracted into hexane after acidification, and methylated with 14% BF_3 in methanol. The resultant methyl esters of the fatty acids were separated and quantitated using gas-liquid chromatography and automatic integration [12].

Results

Properties of the insulin receptor

The insulin receptor of Ehrlich ascites cells demonstrated binding properties similar to those described for all mammalian insulin receptors [18]. When cells were incubated with 20 pM [^{125}I]iodoinsulin, binding was time-dependent (Fig. 1). Steady-state binding levels were reached in about 2 h at 15°C. The total binding was 1.2% per million cells and the nonspecific (i.e. non-saturable) binding was 0.4%. Thus, the specific binding was 0.8% per million cells. Binding was proportional to cell concentration up to at least 15 million cells per ml (data not shown) and was reversible. Addition of excess unlabeled insulin after 2 h of tracer binding led to a rapid release of bound insulin (Fig. 1). There was only a small amount degradation of insulin in this system. Greater than 90% of the tracer was still precipitable by 5% trichloroacetic acid after 4 h of binding at 15° (Fig. 1) and at this time about 80% of the insulin was intact when analyzed by rebinding studies (data not shown). This was due to the inclusion of bacitracin, an inhibitor of insulin degradation [19,20] in the binding mixture. The binding rate was proportional to temperature, but the steady state level of binding was inversely related to temperature. At 4°C specific binding reached 1.2% per million cells, whereas at 15°C this was 0.8%, at 22°C it was 0.6% and at 37°C it was only 0.2% (Fig. 2). The pH optimum for binding was 8.0, and the specific binding was reduced to 50% at pH 7.4 and 8.3 (Fig. 3). The receptor specifically recognized insulin and insulin analogs (Fig. 4). Porcine insulin competed for half of the tracer binding at 20 ng/ml (3.6 nM), porcine proinsulin at about 200 ng/ml (22 nM) and des-octapeptide insulin at 2000 ng/ml (380 nM). These values were in proportion to the biological activity of the insulin preparations measured by glucose

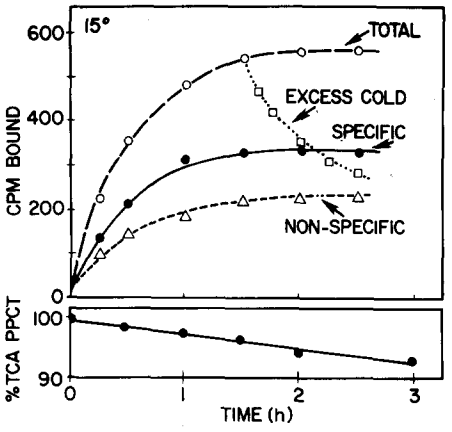

Fig. 1. Insulin binding to Ehrlich ascites cells. In a total volume of 10 ml, $80 \cdot 10^6$ cells were incubated with 20 pM [^{125}I]iodoinsulin ± 1.7 μM unlabeled insulin in HE buffer. At the times indicated duplicate 200-μl aliquots were withdrawn and sedimented in a microfuge as described in Materials and Methods. Specific binding was obtained by subtracting non-specific (non-saturable) binding from total binding. At 90 min, 1.7 μM insulin was added to a portion of the mixture to check reversibility of binding. At each time point, duplicate 100-μl aliquots were withdrawn and mixed with 100 μl of 10% trichloroacetic acid. After incubation at 4°C for 5 min, the mixture was sedimented at 8000 × *g* for 2 min to determine degradation of the insulin tracer.

oxidation by 3T3-L1 cells (data not shown). The receptor was not a growth factor receptor since IGF-6.5 competed poorly, as did multiplication-stimulating activity [18]. Unrelated hormones, such

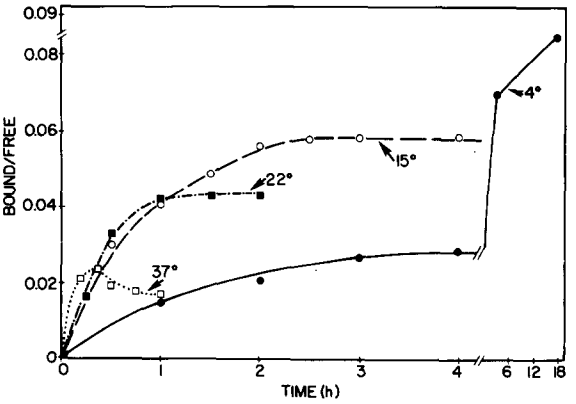

Fig. 2. Effect of temperature on insulin binding. Binding reactions were performed as in Fig. 1 but the incubations were performed at 4, 15, 22 or 37°C. Specific binding was calculated as in Fig. 1 and is presented here.

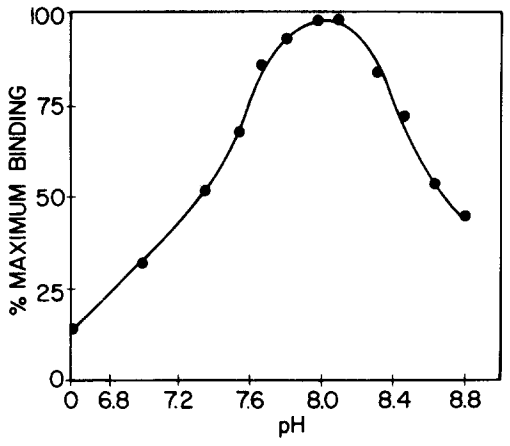

Fig. 3. Effect of pH on insulin binding. Reactions were performed as described in Materials and Methods except that Bistris-proprane ($pK_1 = 9.0$, $pK_2 = 6.8$) was substituted for Hepes and the pH was altered by addition of HCl. At each pH, both a total and nonspecific binding assay were performed, but only the specific binding is shown.

as glucagon and growth hormone, did not compete for binding.

When the competition curve for porcine insulin, shown in Fig. 4, was replotted using Scatchard analysis, a curvilinear plot was obtained (Fig. 5). The receptor concentration, determined from the 'x' intercept of the plot was 9 ng/ml which was equivalent to 180 000 receptors/cell. The curvilinear Scatchard plot was consistent with

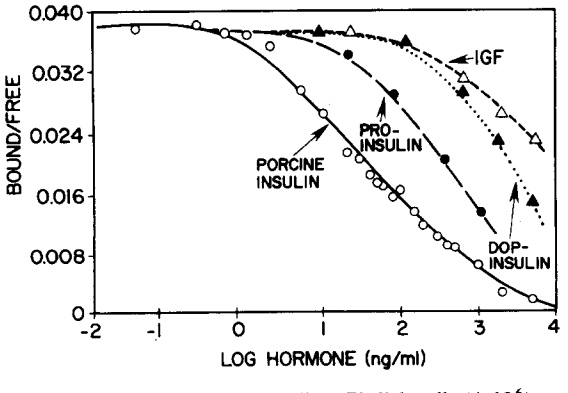

Fig. 4. Specificity of insulin binding. Ehrlich cells ($4 \cdot 10^6$) were incubated in 0.5 ml at 15°C for 2.4 h with 20 pM [^{125}I]iodoinsulin plus varying amount of the indicated analog. Bound and free hormone were separated as described in Materials and Methods.

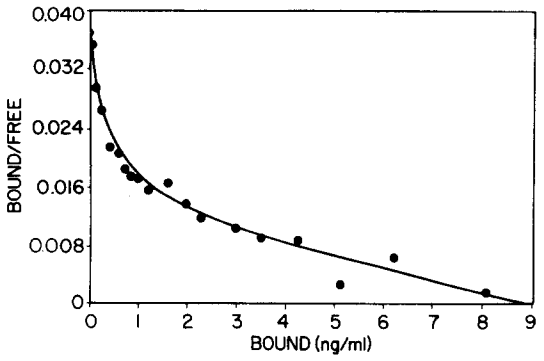

Fig. 5. Scatchard plot of insulin binding. The data of Fig. 4 (for porcine insulin) are recalculated according to the method of Scatchard.

negatively cooperative site-site interactions. Further evidence for negative cooperativity of the insulin receptor of Ehrlich ascites cells was demonstrated by the dilution method of DeMeyts [21]. As shown in Fig. 6 the rate of dissociation of insulin from its receptor was considerably enhanced by the addition of unlabeled insulin. Since the dissociation rate of hormones from independent sites would not be altered by the presence of unlabeled insulin, the data suggest negatively cooperative site-site interactions. Although there is some controversy about the interpretation of these types of experiments, they are characteristic of insulin receptors [18,22,23].

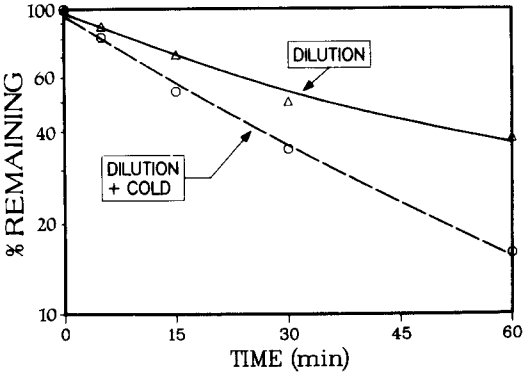

Fig. 6. Kinetic demonstration of negative cooperativity. [^{125}I]Iodoinsulin was associated with the receptor as described in Fig. 1. Aliquots of 100 μl were diluted at 15°C into 10 μl of HE buffer with or without 1 μg/ml insulin. At the indicated times, the tubes were centrifuged at $18000 \times g$ for 3 min at 4°C, the supernatant aspirated and the sediment counted to determine remaining radioactivity.

Effect of diet on membrane lipid composition and insulin binding

Mice were maintained on normal lab chow or on diets highly enriched in saturated fat (coconut oil) or polyunsaturated fat (sunflower oil) and innoculated with Ehrlich ascites cells. The effect of the diet on the fatty acid composition of the plasma membrane of the Ehrlich ascites cells is seen in Table I. Plasma membranes of cells from animals fed sunflower oil were much richer in polyunsaturated fatty acids than those from normal animals, or cells grown in animals fed coconut oil (37.5 vs. 29.4 vs. 18.1). This increase in polyunsaturates was accompanied by a decrease in monounsaturates. There was little change in the saturated fatty acid component. Such increases in polyunsaturates have been shown to be accompanied by increases in membrane fluidity in these and other cells [1,24]. Another crude parameter of membrane fluidity, the mean number of double bonds per fatty acid residue was also increased in the cells grown in animals fed sunflower oil. For these cells it was 1.18 whereas normal cells had a value of 1.07 and cells grown in animals fed coconut oil only 0.91 ($p < 0.001$ for all comparisons).

Insulin binding was evaluated in these three cell types by Scatchard plots. The binding data was analyzed by DERC 2, a computer program using a fourth order polynomial to fit the competition curve. Typical Scatchard plots are shown in Fig. 7. Cells grown in animals fed a normal diet had 180000 sites/cell (Fig. 5), but from animals fed coconut oil had many fewer sites/cell, whereas cells grown in animals fed sunflower oil had many more sites per cell. The insulin binding at very low insulin concentration, however, was greater to the cells grown in animals fed coconut oil. This was due to a higher affinity of the receptor of these cells (Fig. 7, insert). These experiments were repeated 5 times and the results are shown as a histograms in Fig. 8. The mean receptor concentration of the cells grown in control animals was 186000 (±10000) sites/cell. Cells grown in animals fed coconut oil had about 125000 (±5000) sites/cell a value significantly different from control ($p < 0.001$). Cells grown in animals fed sunflower oil had 350000 (±12000) sites per cell, a significant increase over either of the other conditions ($p < 0.001$). Changes in the affinity constants $\bar{K}_e$ and $\bar{K}_f$ were also found. Both the high and low affinity constants of the cells grown in

TABLE I

PHOSPHOLIPID FATTY ACID COMPOSITION OF EHRLICH ASCITES CELL PLASMA MEMBRANE

Sample	Regular	Sun-flower	Coco-nut
Fatty acid composition of phospholipids (%)			
% saturated	46.6 [a]	43.4	44.6
%monounsaturated	22.6	16.9	34.4
%polyunsaturated	29.4	37.5	18.1
Mean number of double bonds	1.07	1.18	0.91
Composition by individual fatty acids (%)			
14:0	3.5 [b]	2.8	4.2
16:0	19.7	19.9	21.2
18:0	14.4	12.4	11.4
22:0	2.7	3.1	2.8
16:1	1.7	1.3	3.5
18:1	16.0	11.2	23.0
24:1	0.4	0.6	2.2
18:2	14.9	22.4	6.0
20:4	6.6	5.8	6.9
22:4	1.9	2.8	0.8
22:5	0.7	3.8	0.6
22:6	3.1	1.2	1.8

[a] A small fraction (1.4–2.9%) remains unidentified in each group.

[b] Minor constituents omitted (<2% in each category).

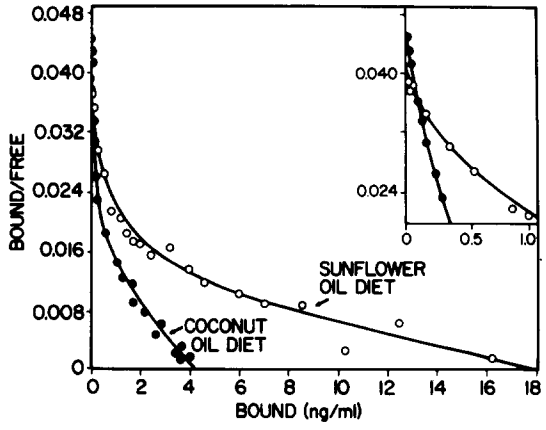

Fig. 7. Effect of membrane lipid saturation on the binding properties insulin receptor. The experiment was identical to that described in Fig. 6, except that cells were harvested from animals maintained on diets rich in either sunflower oil (polyunsaturated) or coconut oil (highly saturated). The insert shows the earliest part of the binding curve in more detail.

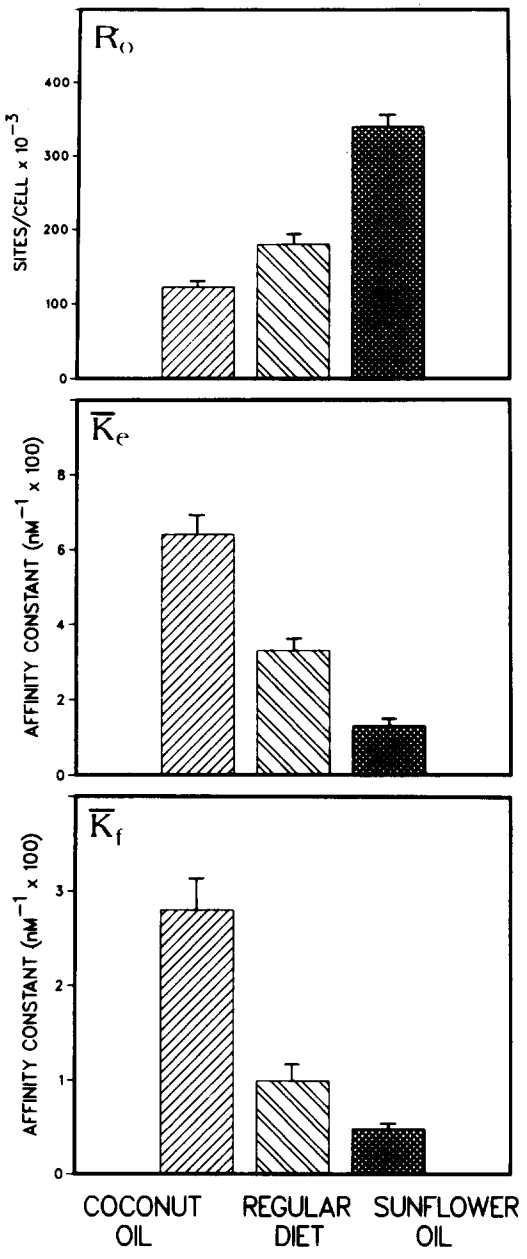

Fig. 8. Effect of membrane lipid saturation on the insulin receptor. Six sets of experiments, identical to Fig. 6, were performed. The mean and standard error are presented for receptor number R_0, and affinity constants $\bar{K}_e$ and $\bar{K}_f$. The confidence limits were $p < 0.001$ for all comparisons in each panel.

animals fed diets rich in polyunsaturated fat were reduced as compared to the values for the cells from animals fed the normal diet ($\bar{K}_e$, $p < 0.001$, $\bar{K}_f$, $p < 0.001$). Conversely the affinity constants of the insulin receptor of cells grown in animals fed a diet rich in coconut oil was higher than that of the receptor of cells grown in animals fed sunflower oil ($\bar{K}_e$, $p < 0.001$; $\bar{K}_f$, $p < 0.001$).

Discussion

Our results indicate that the Ehrlich ascites cell possesses an insulin receptor with binding properties characteristic of the receptor of all mammalian cells. Mammalian insulin receptors have a series of properties that makes them unique [18] and the receptor of the Ehrlich cell demonstrated all of these properties: The Ehrlich cell binds insulin and compounds with insulin-like activity (porcine insulin, proinsulin, desocta-peptide insulin and IGF 6.5) in proportion to their biological activity. It has a sharp pH optimum for binding insulin at pH 8.0 and demonstrates the peculiar temperature dependence that is characteristic of insulin receptors. The insulin receptor of the Ehrlichs cell also demonstrated a ligand-induced acceleration of dissociation, consistent with negative cooperativity [21].

In this study we were able to modify the fatty acid composition of the plasmalemmal phospholipids. This resulted in alteration of some of the properties of the insulin receptor. Previous studies using these cells have shown a clear association between the polyunsaturated fatty acid content in the plasma membrane and fluidity as measured by electron spin resonance of nitroxide-labeled fatty acids [13,24]. The increased unsaturation of the membrane of Ehrlich ascites cells is associated with an increase in receptor number. This is similar to the results that we have obtained with Friend erythroleukemia cells in culture [1] and with erythrocyte receptors reconstituted into artificial membranes [3], but different from the results with cultured pre-adipocytes [4,5]. The magnitude of the change is substantial, about a 2-fold change in receptor number with either increasing saturation or unsaturation of the membrane phospholipids. For a typical insulin response, with spare receptors, this would be expected to cause a 2-fold change in insulin sensitivity [20]. There were, however, changes in affinity as well, so that the overall binding of tracer concentrations of insulin was similar. Thus, at low

physiological insulin levels (10–50 μU/ml) binding to all three varieties of cells was similar, but at higher physiological concentrations (100–150 μU/ml) and at concentrations that occur in portal blood (1000 μU/ml) binding to the cells enriched in unsaturated fatty acids is much higher than to control cells or those enriched in monounsaturated fatty acids.

The reason for a decreased number of receptor of increased affinity, found in cells containing less polyunsaturated fatty acid is uncertain. Similar results were also found by Livingston and coworkers [25] after treating cells with lectins. Wheat germ agglutinin, a reagent expected to crosslink receptors, caused a marked increase is affinity of the insulin receptor of fat and liver membranes, accompanied by a decrease in receptor number. The magnitude of this effect was considerably smaller in placental membranes. Insulin binding has been shown to be increased in these systems by treatments which disrupt phospholipid structure. Thus, treatment with phospholipase, mild detergents such as digitonin or extraction with organic solvents led to an increase in receptor concentration [26]. These results cannot be explained by previous models of the insulin receptor [25]. However, the results presented in this manuscript, along with those found in Friend erythroleukemia cells [1] and insulin receptor reconstitution studies [3], suggest a model for the quarterary structure of the receptor. In this model, the receptor may exist two states, a monomeric and a polymeric state. The monomeric state, which may be the unit described by Jacobs et al. [27], Pilch and Czech [28], Yip et al. [29] and Harrison and Itin [30], would have low affinity, but every site would be available for insulin binding. The multimeric state, composed of many 'monomers' would have a very high affinity for insulin, but in aggregating would 'hide' many of the binding sites. The monomeric, small form is favored under conditions of high membrane lipid unsaturation and increased fluidity and perhaps by such maneuvers as phospholipase *c* or mild detergent treatment [26] that disrupt membrane lipid structure, whereas the multimeric form would be favored by conditions of high membrane lipid saturation and low membrane fluidity and by receptor aggregating agents such as wheat germ agglutinin [25].

Our previous studies with cultured cells have indicated that the number of insulin receptors per cell can be increased by increasing the unsaturation and presumably, the fluidity of the plasma membrane and that such changes may be associated with an increase in the responsiveness of the cells to insulin [1]. This study extends our previous observations to a cell grown in an intact animal. Since the effect can be produced in vivo, it is possible that this type of dietary manipulations can be used to increase sensitivity to insulin in hormone-resistant states of animals and man.

Acknowledgements

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