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Dietary Essential Fatty Acids Are Required for Maintenance and Induction of Prolactin Receptors (40679)

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There is a positive correlation between the incidence of mammary carcinoma and the dietary fat intake by the populations of various countries (1–4). The causal relationship suggested by these demographic observations has been supported by laboratory studies: animals on diets high in unsaturated fats are most likely to develop mammary carcinoma compared to the animals fed diets containing low concentrations of these fatty acids (5–9). Such diets which enhance tumor formation include the essential fatty acids (EFA), linoleic and linolenic acids. These dietary components have previously been shown to alter the fertility of rodents (10) and, therefore, possess the potential to modify certain hormone-related events. These observations plus the fact that some mammary tumors are stimulated to grow by high concentrations of prolactin (11) prompted us to hypothesize that essential fatty acids may alter hormonal effects by modulating the prolactin receptor present on the surface of target cells. Such modifications of the hormone–receptor axis could then alter the response of the cell to normal concentrations of hormones within the body and thereby provide a partial explanation of the role of diet in the development of mammary carcinoma.

Materials and methods. Essential fatty acid-deficient diet (No. TD 77052, Teklad Mills, Madison, Wisc.) was composed (w/w) of 68.5% sucrose, 20% casein, 5% medium chain triglycerides (MCT), 2% nonnutritive fiber, 3.5% mineral mix (Williams-Briggs Mod. No. 170911), and 1% vitamin mix (Teklad Mills No. 40060). MCT consist of resynthesized triglycerides from fatty acids of 8- to 14-carbon chain length hydrolyzed and subsequently purified from coconut oil. The control diet (No. TD 77053, Teklad Mills) was manufactured using the same recipe as above except the MCT was replaced by corn oil[1] and was, therefore, isocaloric with the deficient diet.

C_3H mice (Charles River) were fed EFA-deficient or control diets starting 1–2 weeks after being weaned at age 4 weeks from NIH lab chow-fed dams and were sacrificed by decapitation at various times during the subsequent 32 weeks. Some C_3H mice were also started on the diets in midpregnancy and continued on the same diets until the pups were weaned. These dams and their litters were then maintained on the same dietary regimens until sacrifice at various times thereafter. One group of weanling mice also received subcutaneous injections of 10 μl linoleic acid (Sigma) thrice weekly. Half of these animals were placed on the control diet and the other half on the deficient diet. They were sacrificed after 32 weeks on this regimen.

Ovine prolactin (oPRL, NIH-P-S12, 35 IU/mg) was a gift of the National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases. Iodination of oPRL was performed using a modification of the Thorell and Johansson technique (12). Approximately 1 mCi ^{125}I (Amersham/Searle) was added to 5 μg unlabeled hormone and buffered with 10 μl 0.4 *M* Na-acetate, pH 5.6. One microliter of a lactoperoxidase solution (2.7 mg/ml, Calbiochem grade B enzyme) was added, followed by 1 μl H_2O_2 solution (30% diluted 1:15,000). Thirty seconds later, 100 μl of "transfer solution" (0.02% Na-azide, 1% KI, and 16% sucrose) was added to quench the reaction. The mixture was removed and the iodination vessel was rinsed with 100 μl transfer solution. These solutions were combined and chromatographed on a 1.5 × 90-cm Sephadex G-100 column at 4°, previously equilibrated with 0.025 *M* Tris, pH 7.6. Fractions high on

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[1] Corn oil fatty acid profile, percentage by weight: lauric = 0.02, myristic = 0.13, palmitic = 10.8, palmitoleic = 0.38, heptadecanoic = 0.05, heptadecenoic = 0.03, stearic = 2.18; oleic = 23.0, linoleic = 39.8, linolenic = 0.92 (Raltech Scientific Services, Inc., Madison, Wisconsin 53707).

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the descending limb of the hormone peak were used and had an approximate specific activity of 60 μCi/μg.

Animals were sacrificed by decapitation at the times specified and trunk blood was collected. The excised livers were immediately placed in liquid nitrogen and pulverized. They were then homogenized (Polytron, Brinkmann) for 1 min at 4° in approximately 10 vol of 0.025 *M* Tris buffer, pH 7.6, containing 0.3 *M* sucrose. A preliminary 15,000*g* centrifugation at 4° for 20 min was performed and the supernatant was centrifuged at 100,000*g* for 60 min. These latter cell membrane pellets were stored at −40° for less than 4 days prior to use. Additional livers were obtained from mature female C_3H after receiving either control or deficient diets for 33 weeks and homogenized as above. After a preliminary 120*g* centrifugation at 4° for 20 min to remove nuclei and large particles, the supernatant was used as suspension of receptor for special study in one set of experiments.

Hormone binding activity was determined by measuring the amount of ^{125}I-labeled hormone specifically bound to liver membrane preparations under the following assay conditions: ~60,000 cpm of labeled hormone was incubated with the 100,000*g* liver membrane preparation (0.3–0.6 mg protein) and serial dilutions of unlabeled hormone (0–500 ng) in a final volume of 0.5 ml buffer (0.01 *M* $MgCl_2$ and 0.1% bovine serum albumin in 0.025 *M* Tris at pH 7.6). These preparations were incubated overnight at room temperature and the incubations were terminated by addition of 1 ml chilled buffer at 4°. The tubes were then centrifuged at 2500*g* for 20 min at 4°, and the pellets were rinsed with an additional 1 ml cold buffer and recentrifuged. The supernatant was decanted and the pellet counted in a Packard gamma counter. The counts per minute bound in the presence of 500 ng unlabeled hormone was used as the value of nonspecific binding was subtracted from the total counts in the remaining tubes in that assay group to yield binding values required for Scatchard analysis (13).

Serum samples or suspensions of 100,000*g* membrane pellets were extracted by shaking with 20 vol of chloroform:methanol (3:1) for 30 min at room temperature. The mixture was then centrifuged at 1500*g* for 10 min and the resulting organic phase passed through a glass filter prior to being evaporated to dryness under a nitrogen blanket. The samples were then incubated for 2 hr at 90° in 2 ml 30% KOH/absolute methanol, cooled to room temperature, and acidified to pH 1–2 with concentrated HCl. The free fatty acids were then extracted into 8 vol of hexane and evaporated to dryness with N_2. After solubilizing the samples in 1 ml methanol/phenolphthalein, they were neutralized with 0.1 *N* KOH/methanol, evaporated to dryness, and resolubilized in 1 ml of a uv tag solution composed of 15 mg *α-p*-dibromoacetophenone (Aldrich Chemical Co.) and 1.5 mg 18-Crown-6 reagent (Aldrich Chemical Co.) per 1 ml acetonitrile (Brudick and Jackson Laboratories). The solution was incubated for 30 min at 80° and cleared of particulate matter by centrifugation at 1500*g* for 10 min. Free fatty acids were separated by using high-pressure liquid chromatography (HPLC) techniques on a μBondapak C18 column with the Waters Associates Model 440 absorbance detector and solvent programmer 660 set to curve No. 5 eluting with 1 ml per minute at 40 to 85% acetonitrile over a 3-hr period (14, 15).

Results. The deficient animals gained weight slower and attained only two-thirds of the final weights of their control diet-fed littermates (Fig. 1) even though the average daily food consumption was the same for both groups. These animals exhibited the manifestations of EFA deficiency: lethargy, reddish fur coloring, rough coat, and dermatitis that developed after 22 weeks on the diet. The quantity of fat within both the peritoneal cavity and mammary fat pads was visibly decreased 2 months after starting the deficient diet at weaning and was virtually absent after 6 months.

Livers from both dietary groups were morphologically indistinguishable by light microscopy.

The 100,000*g* membrane pellets, which include plasma membrane and endoplasmic reticulum fractions, were prepared from the livers of these animals and assayed for prolactin binding (7).

Females, which normally have higher levels of liver prolactin receptors than males (16), showed their number of receptors to be

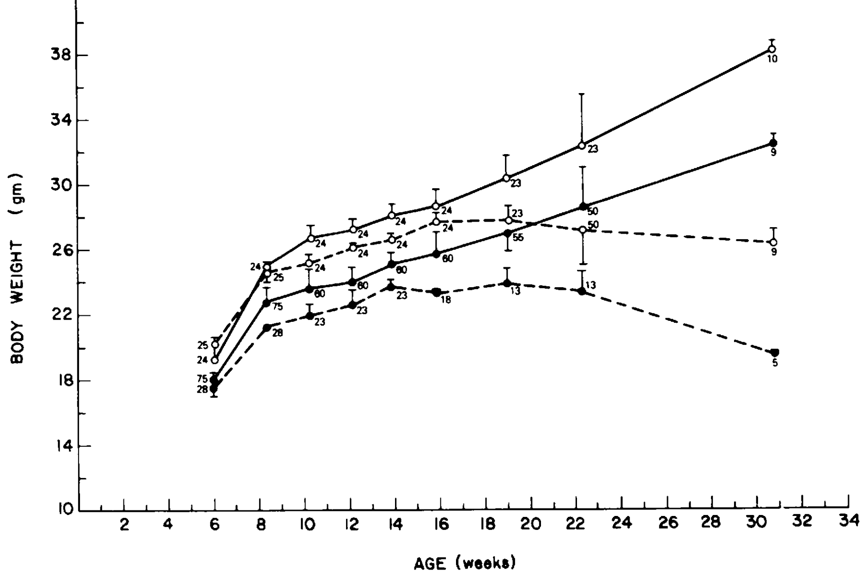

FIG. 1. Weight of C_3H male (○) and female (●) mice as a function of age and type of diet. Animals were weaned from mothers that had been fed standard NIH lab chow through pregnancy and nursing. The pups were continued on the standard diet until being placed on either the control (——) or deficient (- - -) diets 1–2 weeks after weaning. Vertical bars represent 1 SEM. Numbers represent the quantity of mice used to obtain each data point.

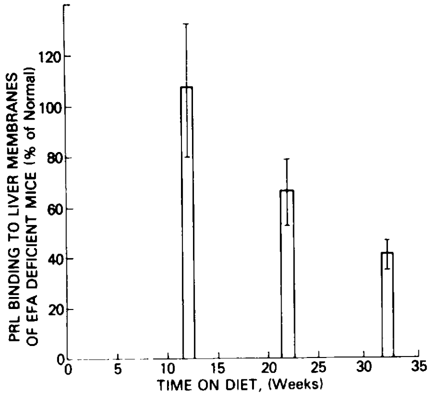

FIG. 2. Ovine PRL binding to 100,000g liver membrane pellets obtained from female C_3H mice fed essential fatty acid-deficient diets for the periods of time depicted on the abscissa. The ordinate represents the ratio of specific oPRL binding per milligram of protein observed in EFA-deficient diet-fed animals to that of control diet-fed animals. Bars represent standard errors. Scatchard analyses of these binding data demonstrate that the gradual decrease in binding represents a decrease in receptor number rather than a change in $K_a \approx 10 \times 10^9\ M^{-1}$.

65 and 41% of controls after 22 and 32 weeks of being fed the deficient diet (Fig. 2). Scatchard analyses (13) revealed the apparent affinity constant, K_a, to be $10.5 \pm 0.6 \times 10^9$ M^{-1}, unchanged from the value of $9.8 \pm 0.6 \times 10^9\ M^{-1}$ for the control membranes. The changes observed, therefore, represent a decrease in the number of binding sites. Supplementation of these deficient animals by thrice weekly subcutaneous injections of 10 μl of linoleic acid (Sigma Chemical Co.) raised the number of receptors to 75% of controls after 32 weeks of dietary treatment. A similar pattern was observed at 16 weeks of age if the diet was started while *in utero*, ~1 week prepartum: deficient animals possess 59 ± 17% of the number of control prolactin receptors. Binding studies were also performed using crude tissue homogenate to determine if dietary-induced alterations in the sedimentation characteristics of the subcellular fractions were responsible for these observations. Pregnant dams maintained on their diets for 32 weeks after parturition had only 52 ± 12 and 46 ± 12% of the binding capacity of controls in the 100,000g membrane pellets and 120g membrane supernatants, respectively. These studies showed that the EFA deficiency-induced decrease in oPRL binding was not due to an altered subcellular centrifugation profile but rather

to an actual change in the binding properties of the cell membranes.

Other investigators (18) have shown that rats eating diets containing 20% (w/w) corn oil have higher serum levels of PRL than rats eating diets containing 5% (w/w) corn oil. It has also been shown that PRL will induce its own receptor when injected ip into male mice (19). For these reasons, there was concern that the EFA deficiency was in some way lowering the serum levels of PRL and thereby suppressing the number of PRL receptors. This question was answered when 20 C_3H males receiving either the deficient or control diets for 22 weeks were injected with 50 μg bPRL or the saline carrier ip every 4 hr, for a total of 11 injections. The animals were sacrificed 8 hr after the last injection. The dietary controls responded with a four-fold increase in the number of prolactin receptors, whereas no increase was observed in deficient animals (Fig. 3). No suppression of PRL binding was noted in the saline-injected deficient mice when compared to saline-injected control diet mice. This indicated that even if prolactin levels had been suppressed by the EFA deficiency, the binding capacity could not be returned to normal even though the serum PRL had been returned to a level that would induce binding activity in the animal. This indicates that the primary change in the receptor is due to a modification of the membrane itself rather than a change in the concentration of hormone responsible for receptor binding activity. Assays by HPLC of lipid extracted from the hepatic microsomal fractions of the deficient animals showed linoleic and palmitoleic/arachidonic acids to be ~12% of control values. HPLC analyses of sera from animals fed the deficient diet for 12 weeks showed linoleic and arachidonic/palmitoleic acids to be 4 and 25% of the control values, respectively. These changes resulted in an increase in the fluorescence depolarization which reflects an increase in the viscosity of the 100,000*g* membrane pellets obtained from the livers of animals fed the EFA-deficient diets for 22 weeks when compared to the control diet-fed animals (20, 21).

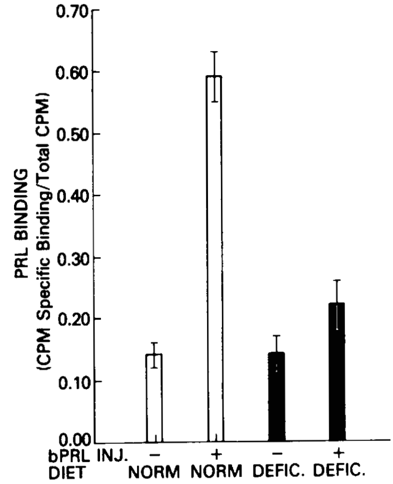

FIG. 3. Effect of an EFA-deficient diet on the ability to induce prolactin receptors. Ordinate is specific binding of oPRL (cpm) to 100,000*g* liver membrane pellet per milligram of protein divided by the total cpm added to each incubation tube (52,800 cpm). Abscissa depicts the *in vivo* treatment prior to sacrifice. Male C_3H mice were maintained on either EFA-deficient or control diets for 22 weeks and then injected ip with either 50 μg bPRL or Tris–saline carrier every 4 hr × 11 and sacrificed 8 hr after the last injection. Animals fed normal diets responded with fourfold increase in PRL receptor number when injected with bPRL while the EFA-deficient animals did not demonstrate a significant change in receptor numbers. Vertical bars represent standard error.

Discussion. While the dietary-induced suppression of PRL binding was marked in the female, males showed no such percentage decrease. The reason for this is unclear but may be due to the fact that females are more susceptible to PRL-receptor modification because of either the inherently larger numbers of receptor sites in the basal states (17) or the susceptibility of the controlling hormonal factors to exogenous manipulation.

The PRL receptors present in very low levels in males may, however, be less susceptible than females to suppression by exogenous means; further suppression of what could be considered rudimentary levels may become increasingly more difficult as the number of sites decreases. The deficiency in dietary linoleic acid may exert these effects directly on the target tissue or may act indirectly via an intermediate tissue.

Receptors for certain hormones and antigens are considered to float within the lipid bilayer of the cell surface (22–25). Binding to receptors may, therefore, be modified not only by altering the structure of the binding site itself, but also by changing the character of the matrix in which it exists. The latter may then affect the receptor configuration and/or its position relative to the extracellular, intramembranous, or intracellular environments (26). While the activity of several cell surface receptors has been modified *in vitro* by altering the lipids of either cell membranes or artificial vesicles (27–32), the data herein reported lend *in vivo* relevance to such observations. The essential fatty acids appear to be necessary not only for the maintenance of the hepatic prolactin receptor but also for its induction *in vivo*. The fact that induction did not occur when the deficient animals were treated with bPRL indicates that diet-induced suppression of endogenous prolactin (33) was not responsible but rather that another effect on the receptor–lipid complex was achieved. This may be a result of changes in the structural composition of the bilayer or the effect of decreasing the availability of these essential fatty acids for use as precursors of prostaglandins.

These data indicate that the response of tissues to lactogenic hormones can be modified by specific types of fat within the diet.

Summary. C_3H mice maintained on diets lacking the essential fatty acids exhibited a progressive decrease with time in the number of prolactin receptors detectable in their hepatic microsomal membranes. These deficient animals were also insensitive to induction of receptor by treatment with exogenous prolactin. These data show that the quality of dietary lipids modifies the prolactin receptor *in vivo*.

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