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Growth Stimulatory Activity of Unsaturated Fatty Acids for Normal and Neoplastic Breast Epithelium

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ABSTRACT

Studies with experimental animals first showed that dietary lipids in excess have a very large stimulatory effect on the development of breast tumors either induced by carcinogens or occurring spontaneously. These observations took on added significance when epidemiologists found a strong positive correlation between breast cancer incidence and the level of dietary fat. Although not unequivocably established, the total observations concerning this phenomenon suggest a cause and effect relationship between high dietary lipids and breast cancer development. In an attempt to understand how the lipids might be acting, we have begun to assess the effects of various fatty acids on the growth and function of breast epithelium from both normal and neoplastic tissue. The results to date suggest that the unsaturated fatty acids are needed for mammary cell division and that they may play roles in this process by serving as substrates for prostaglandin synthesis, as membrane structural elements or possibly as activators of C kinase when they are in the form of diglycerides. Whatever the mechanism of growth stimulation, it appears that the fatty acids are rate limiting for growth and that physiologic mechanisms for recruiting fatty acids from proximal fat cells exist within the mammary gland. It thus appears that the fat cell serves as a physiologic buffer and that exceeding this buffer such as by consuming excessive lipids may override this buffering capacity and thus favor the division of normal or neoplastic breast cells.

INTRODUCTION

Although it has been more than 40 years since the demonstration that carcinogen induced or spontaneously developing mammary tumor incidence in experimental animals was modulatable by dietary fat (1), the numerous reports on other tumor model systems that have confirmed this observation have failed to elucidate the basis for this effect. Dietary lipid effects on hormone levels (2) or on carcinogen metabolism or clearance rates (3) have been suggested, but the results from other laboratories are not consistent with either of these possibilities (4,5). The type of lipids which promote the response of mammary cells to carcinogens also has proven variable. Original reports indicated that unsaturated fatty acids were more efficient than saturated fatty acids and suggested that these compounds might act as promoters (6). More recently it has been found that a minimal amount of unsaturated fatty acid (essential fatty acid) is needed and that over and above this minimal amount an increased tumorigenic response is obtainable with additional amounts of either a saturated or an unsaturated fatty acid (7). This latter result is consistent with recent epidemiological surveys suggesting that the strongest link between dietary fat consumption and human mammary cancer incidence relates most strongly to the total amount of fat in the diet rather than to the amount of unsaturated fatty acid in the diet (8). Unfortunately, retrospective estimates of consumption are notoriously unreliable because of subjects' faulty memories and lack of accuracy concerning the actual amount of fat consumed (for example, of the amount of dietary fat available for consumption, how much is discarded in the food preparation process?). In spite of these problems, there is a strong positive correlation between lipid intake and the incidence of mammary cancer in humans and in the efficiency with which carcinogen induction of mammary cancer takes place in experimental animals.

Because of the lack of a consistent demonstration that dietary lipids affect serum mammatrophic hormone levels such as prolactin, estrogens or progestins, we have considered the possibility that lipids may directly affect the growth of the mammary epithelium and thus increase the possibility of neoplastic conversion by increasing the size of the cell population at risk. What has emerged from these studies is the fact that the growth of both normal and neoplastic mammary epithelium is facilitated by unsaturated fatty acids. Experiments both in vivo and in vitro with cultures of mammary epithelium have led us to propose that there is an integration between the mammary epithelium and mammary fat cells that is linked through mammotrophic hormones and an intermediary cell type, the mast cell. The experiments that have led us to this postulate are reviewed in this report.

RESULTS AND DISCUSSION

The Model

First let us introduce the model we have formulated and then describe briefly the experimental evidence which supports the various aspects of the model. Then we wish to speculate on the implications of the model insofar as mammary cancer and dietary lipids are concerned.

The model is presented in Figure 1, which depicts a cross

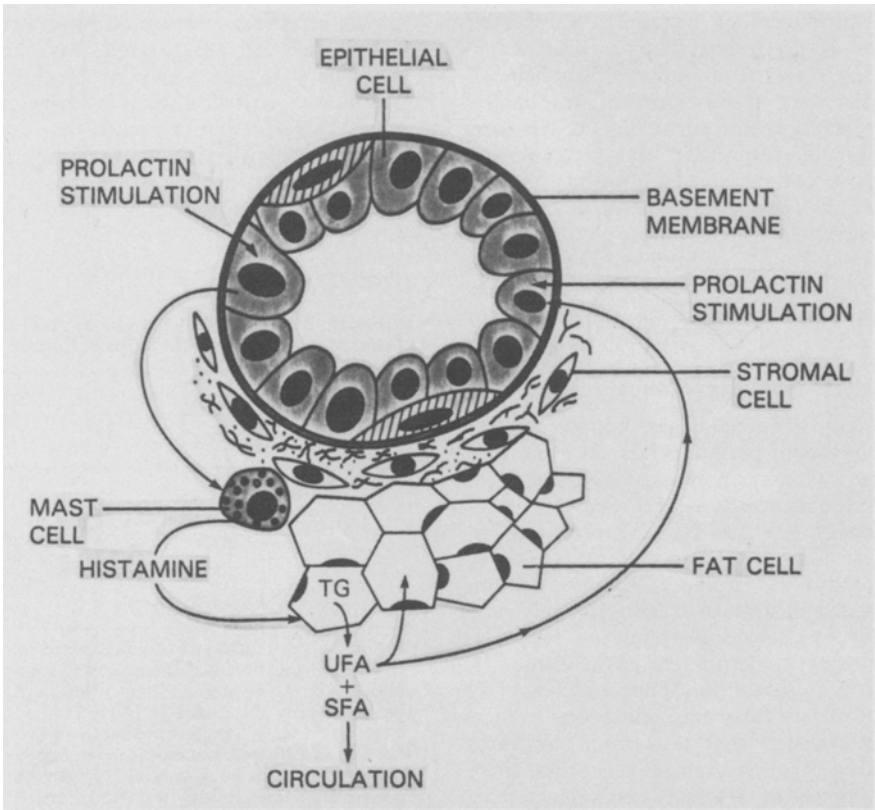

FIG. 1. Model depicting the interrelationships between mammary epithelial cells, mast cells and fat cells in the response to prolactin stimulation. See text for details. TG, triglycerides; UFA; unsaturated fatty acids; SFA, saturated fatty acids.

section of an alveolus of the mammary gland, bounded by a basement membrane which separates the mammary epithelial cells from the surrounding stromal cells, stromal collagen, adipocytes, mast cells, etc. When the glandular epithelium is stimulated to proliferate by a hormone such as prolactin, the epithelium transmits a signal of undefined nature to mast cells in the near vicinity. The mast cells then release histamine which interacts with Hl receptors on the adipocytes leading to lipase activation and the release of free fatty acids.

The adipocytes release both unsaturated and saturated fatty acids and these are differentially handled by the gland. Saturated fatty acids are removed from the gland by the venous effluent. Unsaturated fatty acids are either reassimilated into triglycerides in fat cells or are taken up by the prolactin activated epithelium. These fatty acids then supplant the saturated fatty acids in membrane phospholipids and serve as substrates for prostaglandin syntheses (linoleate and arachidonate). The consequence of one or both of the latter is to enhance mammary cell growth.

EXPERIMENTAL SUPPORT FOR THE MODEL

Prolactin Stimulation of Fatty Acid Release from Mammary Adipocytes

Experiments with intact animals as well as with explant cultures of mammary tissue indicate that prolactin activates lipase in mammary adipocytes. Thus the administration of perphenazine to virgin female rats brings about a 5-10-fold elevation of serum prolactin levels (9). It also causes a rapid and massive proliferation of the mammary epithelium (9), decreases the total amount of mammary fat and increases the relative abundance of unsaturated fatty acids in the gland (10). This latter effect is so large that it must involve the mammary adipocytes which contain about 90-95% of the total mammary lipids as triglycerides.

Although perphenazine stimulation also causes an increase in serum hormones other than prolactin, it is most probable that elevated prolactin causes the changes in mammary lipid composition as shown by the effects of prolactin in explant cultures of mammary tissue (Table I). When the mammary explants are incubated with prolactin, there is a release of free fatty acids into the growth medium where they are trapped on bovine serum albumin and subsequently can be quantitated by gas chromatography (11). The prolactin effect is seen with as little as 50 ng/ml concentration (Table I).

How does prolactin affect the free fatty acid release and what cells are their source? The evidence points to the adipocytes because prolactin addition to cultures of isolated mammary epithelium actually enhances fatty acid uptake into these cells rather than their release of free fatty acids. This is especially true for the unsaturated fatty acids (11).

Prolactin Stimulation of Histamine Production

Cultures of rat mammary epithelium release histamine as shown in Table II. This production of histamine was variable from cell preparation to preparation, and histamine production in response to prolactin was seen only if the cultures contained a few contaminating mast cells. When these contaminants were eliminated by subculturing the mammary epithelium on collagen gels, prolactin no longer stimulated histamine production. These results suggested that prolactin stimulated epithelium activates the mast cells

which are the actual producers of histamine. Consistent with this interpretation is the finding that prolactin receptors are present exclusively on the mammary epithelial cells (12). A mast cell involvement in the hormonal responsiveness of the mammary gland is not surprising. It has long been noted that hormonally responsive breast tumors are very rich in mast cells in comparison to hormonally independent mammary tumors (13). Additionally, it has been shown that histamine levels in the mammary gland rise and fall in concert with the DNA synthetic activity of the epithelium and that antihistamines such as Benadryl (Diphenhydramine) block DNA synthesis in the mammary gland (14).

Histamine and FFA Release from the Mammary Gland

Other investigators have reported that histamine can activate lipases in adipocytes of peripheral tissues (15). This apparently is also the case for mammary adipocytes as shown in Figure 2. At concentrations as low as 10 ng/ml (the amount produced/24 hrs per 10^5 mammary cells), histamine causes a doubling in the amount of free fatty acids released from mammary explants. A mast cell involvement in free fatty acid release is also strongly suggested by the effects of a mast cell degranulator, 48/80 on mammary explant cultures as demonstrated in Table III. This compound, which is a calcium ionophore, increases by 5-fold the total amount of free fatty acid released.

Preliminary results suggest that histamine activates adipocytes by interacting through their Hl receptors, since Benadryl reduces the amount of free fatty acids released by the mammary explants in response to prolactin but Cymetidine (SKF 92334) (a H2 blocker) does not. However, we have yet to demonstrate the presence of high affinity histamine receptors in the gland.

Unsaturated Fatty Acids and Mammary Epithelial Cells

Previously we demonstrated that isolated mammary epithelial cells take up free fatty acids when cultured in the presence of prolactin (11). The unsaturated fatty acid linoleate was depleted from the growth medium at 100 times the efficiency of palmitic acid, while oleic acid depletion was 50 times more efficient than the saturated fatty acid. These results are consistent with a requirement of the epithelium for unsaturated fatty acids for proliferation and an inhibitory effect of saturated fatty acids on this process (10). This is true for both normal and neoplastic mammary cells in culture (10).

The Fate of Assimilated Fatty Acids in Mammary Epithelium

The various means by which unsaturated fatty acids promote mammary cell growth have not been elucidated. However it appears that at least in part they enhance growth by altering membrane composition. This conclusion is based on the compositional change in phospholipids derived from the membranes of growing vs resting mammary epithelium. For example, membranes prepared from isolated mammary epithelial cells of perphenazine treated virgin female rats contained 1.3 to 3.4 times as much linoleic acid per unit phospholipid as did the epithelial membranes of untreated animals. This enrichment of unsaturated fatty acid acyl groups of phospholipids also was observed in membranes of epithelium from pregnant vs non-pregnant animals (11).

Consequently, we have suggested that a replacement of saturated fatty acyl groups of phospholipids by unsaturated ones promotes mammary cell proliferation. This might occur because of enhanced membrane receptor accessibility or via enhanced membrane transport capabilities. However, a role of the unsaturated fatty acids, arachidonate and linolenate in prostaglandin production which might also stimulate growth also is a distinct possibility. Knazek's work (16) has demonstrated that arachidonic acid addition to liver cell membranes increases the amount of specific high affinity prolactin receptors. Furthermore, he has found that essential fatty acids are required for the induc-

TABLE I

Prolactin Stimulation of Free Fatty Acid Release From Mammary Tissue in Explant Culture

Prolactin Conc. (ng/ml)	% Stimulation of release			
	18:0	16:0	18:1	18:3
0	0	0	0	0
25	1	1	3	5
50	2	11	19	25
100	3	34	49	67
300	3	24	37	46
1000	3	23	36	42

Prolactin stimulation of free fatty acid release from mammary tissue in explant culture. Rat mammary tissue was isolated, sectioned into about 10 mg pieces and cultured in Eagle's medium containing bovine serum and prolactin (NIH S14) as described in Reference 11. Free fatty acids were recovered from the growth medium and quantitated as follows. Labeled carrier fatty acid was added to the medium after removing the tissue. An antioxidant, BHT, also was added and the lipids extracted into chloroform:methanol. The residue after evaporating the solvent was dissolved in a small amount of solvent and chromatographed on silica gel plates with a developing solvent that contained BHT. The free fatty acid areas were located on the plates by autoradiography and these areas recovered. The free fatty acids were converted to their methyl esters and quantitated on Quadrex capillary columns by gas chromatography. More complete details are given in Reference 11.

TABLE II

Prolactin Stimulation of Histamine Release From Isolated Mammary Epithelial Cells

Prolactin conc. (ng/ml)	Mast cells present	Histamine released (ng/ml)
0	Yes	5
10	Yes	22
50	Yes	31
100	Yes	35
200	Yes	30
100	No	4

Prolactin stimulation of histamine release from isolated mammary epithelial cells. Mammary ducts and alveoli were isolated from virgin female Sprague Dawley rats as described by Wicha et al. (10). The isolated epithelium was incubated in Eagle's medium supplemented with bovine serum albumin (100 μg/ml) and prolactin at the indicated concentration. Histamine released into the growth medium was quantitated by the method of Endo (25) after centrifugation to remove the cells. Briefly the method entailed the addition of trace amounts of labeled histamine, precipitation of proteins in the growth medium with cold 5% PCA, neutralization of the sample with KOH and chromatography on cellulose phosphate columns to isolate the histamine from basic amino acids and polyamines. Histamine was then quantitated on a fluorimeter after derivatization with orthothalaldehyde. Incubation was 24 hr and was performed on bacterial plastic dishes on which cell viability is maintained but neither cell growth nor attachment takes place. Mast cell presence in the isolated epithelium was quantitated by staining the cells with acridine orange and examining with a fluorescence microscope. Mast cell contamination (about 1 mast cell per 10,000 epithelial cells) was eliminated by plating the freshly isolated epithelium on rat tail collagen coated dishes on which mast cells but not epithelial cells attach. After 24 hr the mast cell-free epithelium was recovered and plated on bacterial dishes as described.

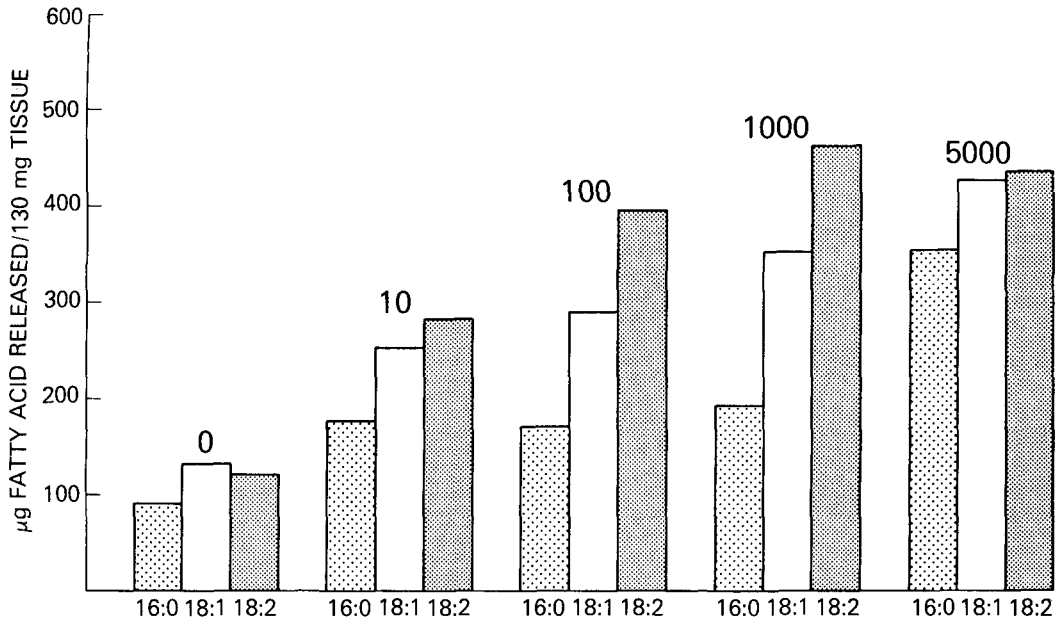

FIG. 2. Histamine stimulated release of free fatty acids from mammary tissue in explant culture. Tissue was cultured and fatty acids released were quantitated as described in Table I. Numbers over the bars on the graph indicate the concentration of histamine present (ng/ml) in the growth medium.

TABLE III

Effect of a Mast Cell Degranulator, 48/80, on Free Fatty Acid Release from Mammary Explants in Culture

Medium supplement	Free fatty acid released (μg/100 mg/24 hr)		
	16:0	18:1	18:3
None	37	49	87
48/80	163	353	276

Effect of a mast cell degranulator, 48/80, on free fatty acid release from mammary explants in culture. Explants were cultivated and the free fatty acids released quantitated as described in Table I.

tion of prolactin receptors in whole animals treated with prolactin (17) and shown that receptor induction is blocked by compounds such as indomethacin which inhibit prostaglandin synthesis (18). The active species in the induction appears to be PGI_2 (19). Other prostaglandins also may play important roles, since we have found that PGE_1 stimulates mammary cell proliferation while PGE_2 and $F_2\alpha$ do not (20). A role of prostaglandins in mammary tumor development also has been suggested by Rao and Abraham (21).

Implications for Dietary Lipid Effects on Mammary Tumorigenesis

All of our findings suggest there is indeed a cause and effect relationship between fat consumption and breast cancer incidence; the link should be more strongly manifest when there is an elevated consumption of unsaturated fatty acids rather than saturated fatty acids. These results need to be considered in relation to the epidemiological studies indicating a higher correlation between total fat consumption and breast cancer incidence than with the consumption of vegetable fat, which is richer in unsaturated fatty acids (8). This latter finding, though subject to many criticisms, is consistent with Carroll's report (7) that essential fatty acids are needed at a certain level for carcinogen-induced tumor development and that over and above this requirement either more saturated or unsaturated fatty acids will enhance tumorigenesis further.

On the face of it the growth stimulating effects of unsaturated fatty acids that we have observed in vivo and in vitro may be a reflection of the basal need of mammary cells, normal or neoplastic, for unsaturated fatty acids. Over and above this need either saturated or unsaturated fatty acids may exert their effects by indirect or direct mechanisms. It is possible to formulate models which could legitimately encompass our results with those of the epidemiological and experimental animal studies. Let us consider the involvement of adipocytes in supplying fatty acids to the mammary epithelium. The uptake and re-release of free fatty acids from adipocytes is a controlled process that normally limits the amount of free fatty acids available to the epithelium. Both the fat cell and the mammary epithelial cell selectively take up unsaturated fatty acids that are available to them. However, saturated fatty acids can compete with unsaturated fatty acids for entry into cells. Our experiments with mammary tumor cells in culture show that linoleic acid uptake into these cells is not dramatically inhibited by saturated fatty acids until the ratio of saturated to unsaturated fatty acids is about 5 to 1. Although similar analyses with mammary adipocytes have not been possible because these cells are extremely difficult to isolate, the relative enrichment of unsaturated vs saturated fatty acids in mammary fat tissue effected by perphenazine treatment in vivo is only about 1.5 to 2 to 1. In other words, a high dietary intake of lipid raises the amount of free fatty acids available to the mammary epithelium because the buffering capacity of fat cells is exceeded. However, the epithelium still can selectively take up the unsaturated fatty acids it requires even in the presence of high amounts of saturated fatty acids. In the case of dietary lipid excess, a mast cell activation for localized increase in free fatty acids would be unnecessary. However, mast cells might be important in this regard with elements other than dietary lipids, such as promoters which enhance cancer development at the progression stage.

Of course, it is also possible that saturated fatty acids inherently and directly promote mammary tumorigenesis by acting on the mammary epithelium. We know that saturated fatty acids inhibit the growth of both normal and neoplastic cells, but this inhibition is a little more dramatic in the case of normal cells (10). This difference may be significant, however, because the presence of normal mammary epithelial cells has been shown to suppress the conversion of preneoplastic to neoplastic mammary cells following transplantation into mammary fat pads (22). A differential inhibition of normal cells thus could favor tumor development. Clearly we are a long way from understanding how dietary lipids might influence breast cancer susceptibility but hopefully our observations and hypotheses will shed some light on the issue, or at least suggest new experimental approaches. Other exciting new ideas already are being brought forth on this subject. For example, Castenaga (23) recently has reported that tumor promoters bind to and activate a calcium and lipid dependent kinase, C kinase. The significance of this observation may be great because C kinase is markedly activated by unsaturated diacylglycerols (24) and thus these compounds may be natural promoters that act as transmembrane signals generated when growth factors interact with membrane receptors and activate phospholipase A_2.

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Lipid Peroxide Catalyzed Chemical Carcinogenesis

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ABSTRACT

Peroxides, including lipid peroxides, with heme catalysts cause the binding of C^{14}-acetylaminofluorene to DNA if microsomes are present. This binding was 96% inhibited by paraoxon, a deacetylase inhibitor. It is concluded that peroxide-peroxidase systems rapidly oxidize acetylated arylamines to proximate carcinogens following deacetylation by microsomal deacetylases. The DNA binding observed was greater than that observed with the liver microsomal mixed function oxidase catalyzed activation to N-OH-acetylaminofluorene, which binds to DNA following deacetylation by microsomal deacetylase. Lipid peroxidation or prostaglandin synthesis should therefore enhance carcinogenesis induced by arylamides.

INTRODUCTION

Multiple mechanisms for metabolic activation of chemical carcinogens exist. The activation by different target tissues also may reflect the different activating systems present. It is thought the initial activation usually involves a mixed function oxidase activity of the endoplasmic reticulum, and that this activation involves a two-electron oxidation of the polycyclic aromatic hydrocarbon to an epoxide or of an arylamine to an N-hydroxyarylamine. However, recently an initial one-electron oxidation to free radicals catalyzed by peroxidases or prostaglandin synthetase has been suggested as a first step for the activation of chemical carcinogens (1-4). Most tissues contain all three systems. However, the uterus, thyroid, salivary gland, Zymbal gland or Harderian glands are target tissues, with little cytochrome P450 and highly active peroxidases (5-8). The kidney medulla and bladder are target tissues with active prostaglandin synthetase (2). The liver hepatocyte, on the other hand, has very high levels of the mixed function oxidase activity of cytochrome P450 and little peroxidase or prostaglandin synthetase activity. The liver Kupffer cells contain a peroxidase (9). Even the apparent two-electron oxidation mechanism of mixed function oxidase function may still involve free radicals (10). It is well established that carcinogenesis induced by irradiation or ultraviolet light is free radical mediated.

Dietary fatty acid hydroperoxides can be toxic to the gastrointestinal tract and can be carcinogenic (11). Enhanced in vivo lipid peroxidation is associated with carcinogenesis induced by chlorinated hydrocarbons (12), hydrazines (13) and metals (14). Furthermore, enhanced in vivo lipid peroxidation following choline deficiency is associated with carcinogenesis (Farber, E., personal communication).