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REGULATION OF MILK LIPID SECRETION AND COMPOSITION

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ABSTRACT

Triacylglycerols make up 98% of the lipid content of milk, ranging in different species from 0 to 50% of the total milk volume. The fatty aid composition of the triacylglycerols depends on the species, the dietary fatty acid composition, and the carbohydrate-to-lipid ratio of the diet. The rate of lipid synthesis in the lactating mammary gland depends on the stage of mammary development and is decreased by fasting and starvation in ruminants and rodents but not in species that fast during lactation, such as seals and hibernating bears. Regulatory agents include insulin, prolactin, and non-esterified fatty acids. Dietary *trans* fatty acids may depress milk lipid synthesis under certain conditions. Evidence is presented that fatty acids may play a major regulatory role in acute changes in de novo mammary fatty acid synthesis, acting primarily on the activity of acetyl coenzyme A carboxylase.

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INTRODUCTION

The focus of this article is regulation of the secretion and composition of the major milk lipid, triacylglycerol (TAG). TAG makes up 98% of the lipid in milk and, in humans, contributes 40–50% of the total energy content (64). Total lipid content of milk varies among species: It ranges from a reported 0% in rhinoceros milk, to 4% in humans and ruminants, to as much as 50% in pinnepeds and whales (61). In addition to varying among species, total lipid content of milk also varies within species, depending on such factors as feed (3, 24, 57), e.g. the feeding of grain-rich diets in dairy cows (103), and in humans the stage of lactation (100), and the amount of body lipids (29, 133).

As reviewed in Dils' classic article (30), there is also wide diversity in the fatty acid composition of milk TAG (Table 1). This is caused by a number of factors. Short-chain fatty acids (fewer than eight carbons), especially butyric and hexanoic acids, are found in significant quantities in ruminant milks and probably result from efficient extraction of their precursors, especially β-hydroxybutyrate, from the plasma where high concentrations are due to rumen fermentation (1, 87). The proportion of medium-chain fatty acids, defined here as $C_{8–14}$, is determined both by species and by diet. Elephant milk, for example, is composed almost entirely of medium-chain fatty acids (61), whereas human milk contains 15–35% (64), rodent milks contain 20–50% (40), and seal and guinea pig milks contain 0% (61). Medium-chain fatty acids are synthesized only in mammary alveolar cells using glucose or acetate as precursors. Synthesis is decreased by high-fat diets in cows (38, 103), rats (91, 121), and humans (59). Both saturated and unsaturated long-chain fatty acids ($\geq$16 carbons) enter the mammary alveolar cell from the plasma and are derived from the diet or from lipid stores.[1] Desaturation and elongation of the essential fatty acids, linoleic and linolenic acids, produce the long-chain polyunsaturated fatty acids (LC-PUFA) present in milk (5, 56). Although cultured human mammary epithelial cells contain all the enzymes necessary for LC-PUFA synthesis (39), it is not clear how much of the relatively large quantities of LC-PUFA present in

[1]In ruminants (30) and mice (109), the mammary alveolar cell contains a D^9 desaturase, fatty acyl-coenzyme A desaturase, that converts stearic (18:0) and other unsaturated fatty acids to oleic (18:1) and other monounsaturated fatty acids. In other species, such as rats, rabbits, and—presumably—humans, this form of fatty acyl desaturation takes place in the liver (18), and monounsaturated fatty acids are transported to the mammary gland in the plasma.

Table 1 Major fatty acids of human and bovine milks

Fatty acid (wt%)[a]		Human milk		Bovine milk[d]
		Western diet[b]	Nonwestern (Nigerian) diet[c]	
Saturated fatty acids				
Short chain				
4:0	Butyric acid	—	—	4.5
6:0	Hexanoic acid	0.07	0.01	2.3
Medium chain				
8:0	Octanoic acid	0.17	—	1.3
10:0	Decanoic acid	1.01	0.54	2.7
12:0	Lauric acid	4.94	8.34	3.0
14:0	Myristic acid	5.63	9.57	10.6
Long chain				
16:0	Palmitic acid	20.33	23.35	28.2
18:0	Stearic acid	7.54	10.15	12.6
Monounsaturated fatty acids				
16:1 ω-7c	Palmitoleic acid	3.35	0.91	1.6
18:1 ω-9c	Oleic acid	30.96	18.52	21.4
18:1 ω-9t		2.67	0.86	1.7
Polyunsaturated fatty acids				
18:2 ω-6	Linoleic acid	12.55	11.06	2.9
18:3 ω-3	Linolenic acid	0.69	1.41	0.3
LC-PUFA (ω-6)				
18:3 ω-6	γ-Linolenic acid	0.37	0.12	2.9
20:2 ω-6		0.31	0.26	0.03
20:3 ω-6	Dihomo-γ-linolenic acid	0.36	0.49	0.10
20:4 ω-6	Arachidonic acid	0.47	0.82	0.2
LC-PUFA (ω-3)				
20:5 ω-3	Elcosapentaenoic acid	0.05	0.48	0.08
22:5 ω-3		0.07	0.39	NA
22:6 ω-3	Docosahexaenoic acid	0.23	0.93	0.09

[a]LC-PUFA, Long-chain polyunsaturated fatty acids.

[b]Based on normalized data from 15 investigations (64).

[c]From Reference 72, milks of Nigerian women with diets high in carbohydrate and fiber and low in animal and total fat. Seafood readily available.

[d]From Reference 62: analysis of bovine milk fat. NA, Not available.

human milk (Table 1) is synthesized in the mammary epithelium and how much is derived from the plasma. Because LC-PUFA levels are very low in infant formulas, with lipids often provided by corn oil, there is concern that formula-fed newborns—especially premature infants, who may have inadequate capacities for desaturation of long-chain fatty acids—may not receive sufficient quantities of these compounds (27). Deficiencies of LC-PUFA are thought to lead to imperfect development of the immune system, the brain, and the visual system (27, 75).

Several well-defined conditions alter either the rate of secretion of milk lipid or the fatty acid composition of milk.

1. INITIATION OF LACTATION AND WEANING After parturition there is a coordinated increase in many of the enzymes of lipid synthesis in the mammary gland, thought to be regulated largely at the level of gene expression (129). Cessation of suckling with milk stasis brings about involution of the mammary epithelium with a coordinate decrease in the same enzymes (139).

2. FASTING AND STARVATION The effects of total food withdrawal on milk lipid synthesis depend on the species and the duration of the fast. In animals with little metabolic reserve in comparison to the rate of milk synthesis, e.g. rats and mice, 24 hr of food deprivation leads to a state of starvation, with profound effects on the rate of synthesis of all milk components, including lipids (138). Other species, e.g. some seals and hibernating bears, fast completely during lactation (101); obviously, in such species food deprivation has no effect on milk secretion.

3. DIETARY COMPOSITION The composition of the diet has profound effects on the composition of milk lipid, often with no effect on the total lipid secretion. Changes in the fatty acid composition of the diet are directly reflected in milk composition. In a classic study, Insull and his colleagues (59) showed clearly that changes in the ratio of fat to carbohydrate in the diet lead to profound changes in the ratio of long-chain to medium-chain fatty acids in the milk, reflecting an alteration in de novo synthesis of fatty acids in the mammary gland.

4. MISCELLANEOUS Decreased body fat as been associated with decreased fat content of human milk (29, 133). Large quantities of dietary *trans* fatty acids inhibit milk fat synthesis in ruminants (35). Diurnal variations have been observed in the fatty acid composition of the milk of several species (24, 90, 132).

Much of our understanding of the biochemistry of milk lipid formation was achieved prior to 1980 (87, 120, 129). The milk fat globule (68, 77), the

composition of human and bovine milks (64, 65), the modes of action of fatty acid synthetase (119) and lipoprotein lipase (31), the regulation of milk lipid content in women (93), and the effect of the starved-refed transition on milk lipid synthesis (138) have all been reviewed recently. Some of this material is summarized here because it provides a basis for integration with new information on the regulation of substrate entry into the lactating mammary cell (98), on milk lipid formation in species that fast during lactation (60, 101), on human milk lipid composition (64), and on the roles of insulin (79) and prolactin (PRL) (34).

TRIACYLGLYCEROL SYNTHESIS AND SECRETION

Before considering the mechanisms involved in regulation of mammary lipid synthesis, it is useful to summarize the biochemistry (Figure 1) and cell biology (Figure 2) involved. Glucose, fatty acids, and glycerol, as well as acetate and β-hydroxybutyrate in ruminants, are the major substrates for fatty acid synthesis, entering the mammary cell from the plasma. Once glucose enters the mammary alveolar cell it has three fates (Figure 1): (*a*) It can be converted to

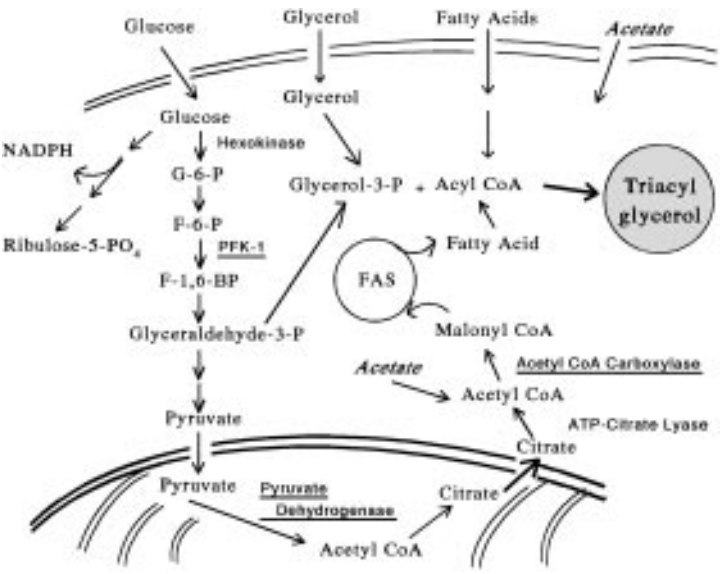

Figure 1 Key steps in glucose utilization and fatty acid synthesis in the mammary alveolar cell. G-6-P, Glucose-6-phosphate; F-6-P, fructose-6-phosphate; PFK-1, phosphofructokinase-1; F-1,6-BP, fructose 1,6-biphosphate; FAS, fatty acid synthase; CoA, Coenzyme A.

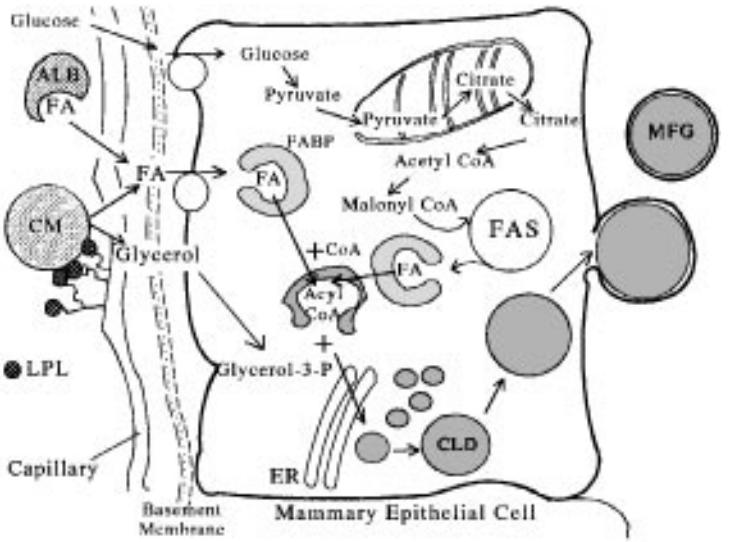

Figure 2 Synthesis and secretion of the milk fat droplet. Fatty acids (FA) enter from albumin (ALB) or from hydrolysis of chylomicron (CM) triacylglycerol by lipoprotein lipase (LPL). Fatty acids may also be synthesized by fatty acid synthase (FAS). The fatty acids are bound to fatty acid–binding proteins (FABP) in the cytoplasm or activated with acetyl-coenzyme A (CoA) and used to synthesize triacylglycerol. Microlipid droplets synthesized in the endoplasmic reticulum (ER) fuse to form cytoplasmic lipid droplets (CLD) that move to and are enveloped by the apical membrane to form the milk fat globule (MFG) that is secreted in a membrane-bound form into milk.

acetyl-coenzyme A (CoA) via pyruvate and citrate synthesis, providing carbon units for fatty acid synthesis via malonyl-CoA. (*b*) It is converted to ribulose-5-phosphate via the pentose phosphate shunt, generating the NADPH necessary to provide reducing units for fatty acid synthesis. [In ruminants, major amounts of NADPH are also generated in the oxidation of isocitrate (129).] (*c*) Finally, the glyceraldehyde-3-phosphate generated in the glycolytic chain can be converted to glycerol-3-phosphate and utilized for TAG formation. Glycerol-3-PO_4 can also be derived from the phosphorylation of glycerol entering from the plasma (135). The major points at which the enzymes of glucose utilization are regulated appear to be the conversion of fructose-6-phosphate to fructose 1,6-biphosphate by phosphofructokinase-1, the metabolism of pyruvate to acetyl-CoA by pyruvate dehydrogenase in the mitochondrion, and the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase in the cytoplasm (89).

The first and rate-limiting (48) step in fatty acid synthesis is conversion of acetyl-CoA to malonyl-CoA, catalyzed by the key enzyme, acetyl-CoA

carboxylase (25). Acetyl-CoA carboxylase is highly regulated at the level of transcription and translation, as well as by phosphorylation (69, 89). After the formation of malonyl-CoA, fatty acid synthase catalyzes a sequence of seven reactions, each of which adds a two-carbon unit derived from malonyl-CoA to a growing fatty acyl chain (119). Each cycle requires two molecules of NADPH. In liver and adipose tissue, when the fatty acid attains a size of 16 carbons, synthesis is terminated by a deacylase, thioesterase I, an integral part of fatty acid synthetase that removes the completed molecule from the enzyme. The cytosol of mammary epithelial cells of nonruminants contains a medium-chain acylthioester hydrolase, thioesterase II, that terminates fatty acid synthesis after the addition of 8–14 carbons, resulting in the de novo synthesis of medium-chain fatty acids.

Long-chain fatty acids are imported from the plasma, where they are either released from TAG in chylomicra or very low density lipoprotein (VLDL) by the enzyme lipoprotein lipase or derived from the pool of non-esterified fatty acids (NEFA) that circulates bound to albumin (Figure 2). The mechanism by which fatty acids traverse the capillary endothelium and interstitial space to reach the alveolar cell is not known. Once at the mammary alveolar cell, fatty acids could cross the plasma membrane by diffusion (67) or via a saturable transport system (116). However, this process has not been studied in the mammary alveolar cell. Transported fatty acids may be activated by combination with CoA and bound to acyl-CoA–binding protein (ACBP) for transfer to TAG (70). They may also be bound immediately after transport into the cell to a fatty acid–binding protein (FABP), one of the more abundant cytosolic proteins in the lactating mammary gland (6, 9). This protein is likely responsible for maintaining a readily available fatty acid pool for TAG synthesis.

Fatty acids, whether formed by de novo synthesis in the mammary cell or imported from the plasma, are activated by combination with CoA and joined with glycerol-3-phosphate by transacylases located in the endoplasmic reticulum to form TAG. The TAG thus formed are incorporated into microlipid droplets that coalesce into larger droplets, the cytoplasmic lipid droplets (127) (Figure 2). These, in turn, progress toward the apical membrane of the cell by mechanisms that are not well understood, become enveloped in plasma membrane, and ultimately pinch off from the cell, bearing a continuous coat of specialized plasma membrane called the milk fat globule membrane. This fascinating process has been well reviewed elsewhere (68, 77).

Hormones and other regulatory agents such as PRL, growth hormone (GH), insulin, and NEFA have been postulated to regulate both the enzyme lipoprotein lipase (51) and the enzymes of glucose metabolism (89). In the next sections, summaries of substrate entry into the mammary alveolar cell, the regulation of lipoprotein lipase, and the control of de novo lipid synthesis set the stage

for discussion of the mechanisms by which changes in the physiological and nutritional environment alter milk fat synthesis.

Transfer of Substrates into the Mammary Alveolar Cell

Glucose transport into the lactating mammary alveolar cell has received considerable attention in a number of laboratories (33, 95, 118). Glucose is transported by facilitated diffusion using the non–insulin-sensitive glucose transporter coded by the GLUT 1 gene (15, 74) or possibly a sodium-sensitive glucose transporter (118). The amount of glucose entering the cell is proportional to the rate of milk synthesis (33, 95). In in vitro studies with isolated acini, glucose transport did not respond directly to insulin (91, 125), and sustained insulin infusion in lactating goats did not increase glucose utilization (124). These studies suggest that, consistent with the presence of GLUT 1, mammary glucose transport is insensitive to insulin. On the other hand, glucose utilization can be shown to be sensitive to insulin under certain experimental conditions, such as starvation in rats (138), perfusion with insulin-free solutions (18a), and an experimental protocol termed the glucose clamp in fasting rats (11, 12) (see below).

Stable isotope studies carried out by Hachey and colleagues (50) provided evidence that about 10% of milk TAG is derived from mammary synthesis of fatty acids in well-nourished women on diets that contain about 25% of the calories as fat. About 30% was derived directly from dietary intake (21, 50), presumably via uptake from chylomicra, and the remainder was derived from synthesis or mobilization from other tissues, presumably liver and adipose tissue. In these studies, no attempt was made to differentiate between the fed state, where presumably most lipid substrate would be derived from dietary lipid via chylomicra, and the postabsorptive or fasting state, where lipid substrate would be derived from adipose tissue in the form of NEFA and from liver in the form of VLDL. The availability of both TAG and NEFA differs substantially between the fed and the fasted states. To examine the implications of this fact for plasma lipid utilization for milk lipid synthesis, it is necessary to consider the results of classical studies in lactating goats, where arteriovenous differences of substrates provide reliable estimates of substrate utilization.

In milk from fed goats, virtually all the long-chain fatty acids were derived from plasma TAG in the form of chylomicra and VLDL (1, 98). Isotope studies indicated that plasma NEFA were taken up and released by the lactating mammary gland in approximately equal proportions (135). In the plasma of goats fasted for 29 h, there was a sixfold decrease of plasma TAG, from about 25 mg/100 ml in the fed state to about 4 mg/100 ml, and a fourfold increase in plasma NEFA, from about 0.2 mM in the fed state to 0.7–0.9 mM. Under these conditions, about 40% of the NEFA were extracted from plasma during

passage through the mammary gland and provided 60% of the lipid substrate utilized by the mammary gland, whereas plasma TAG provided only 20% (2). These experiments suggest that the type of plasma lipid utilized for milk lipid synthesis is related to the concentration of the substrate. In support of this idea, in more recent studies the net extraction of TAG, NEFA, cholesterol, and phospholipids from goat plasma was linearly related to the arterial concentration of these substrates (98). In this study (98), there was no net utilization of NEFA at plasma concentrations below 0.2 mM, as was expected, based on the earlier experiments in fed animals (135). These experiments, as well as less extensive studies with rabbits (66) and rats, show that when plasma TAG are low and NEFA are high, as in the fasting animal, plasma NEFA themselves are used directly in large quantity for milk lipid synthesis.

Regulation of Mammary Lipoprotein Lipase

Lipoprotein lipase is a 55-kDa secreted protein whose major activity takes place within the capillary lumen, where it captures TAG-rich lipoproteins, chylomicra and VLDL, and hydrolyzes the TAG (31). Recent evidence suggests that the mammary enzyme is synthesized by the adipocytes rather than the epithelial cells (62). Rat mammary lipoprotein lipase was significantly decreased 24 hr after hypophysectomy or by sealing of the teats and was reversed by injection of PRL (140), leading early investigators to conclude that PRL was a major regulator of the enzyme. In more recent experiments, however, bromocriptine alone had little effect on rat mammary lipoprotein lipase activity and mRNA (7), although bromocriptine combined with anti-GH serum produced a marked reduction in both. This finding is consistent with observations of Da Costa & Williamson (23) that PRL deficiency did not alter triolein uptake into the lactating mammary gland. In addition, injected PRL did not overcome the effects of insulin and PRL deficiency on triolein uptake or lipoprotein lipase activity in the lactating rat mammary gland. These findings suggest that results of the early investigations were actually due to changes in the total secretory activity of the mammary gland, rather than to specific changes in the regulation of lipoprotein lipase (63, 138).

Insulin tends to increase mammary lipoprotein lipase (LPL) activity, at least in rats where the phenomenon has been studied (22). Recent work by Da Costa & Williamson showed that total insulin deficiency decreased uptake of labeled triolein by rat mammary gland in vivo, accompanied by a significant decrease in lipoprotein lipase (23). The effect could be partially reversed by exogenous insulin. In glucose clamp studies in women, insulin increased the LPL activity in milk after a short delay, which suggests that insulin increases mammary LPL in women under circumstances where all other variables are maintained unchanged (97). The physiological significance of these effects is uncertain.

Fatty acids appear to influence lipoprotein lipase activity in at least three ways: by altering its secretion from adipocytes (86), by decreasing lipoprotein lipase attachment to heparin sulfate proteoglycans (113–115), and by end-product inhibition of the enzyme (reviewed in 32). Lipoprotein lipase has four to six specific binding sites for fatty acids, with affinities in the range of 10^{-7} to 10^{-6} M (32). These data support the postulate that fatty acids play a role in the regulation of lipoprotein lipase activity in the mammary gland, possibly acting to decrease enzyme activity during fasting and involution. A regulatory role for fatty acids in maintained lactation may be more problematic, because substrate uptake from TAG has been shown to be related to plasma TAG concentration (98) and lipoprotein lipase does not seem to be rate limiting for this process (28).

In summary, the bulk of the current evidence suggests that the activity of the lipoprotein lipase in the mammary gland responds to changes in the rate of milk synthesis rather than acting as a regulatory point for entry of lipid substrate into the cell. However, a deeper understanding of the mechanisms by which mammary lipoprotein lipase is regulated is necessary before firm conclusions can be drawn.

Regulation of de novo Lipid Synthesis in the Lactating Mammary Cell

Prolactin has long been considered a primary regulator of the synthesis of milk components. The hormone appears to be necessary for initiation of lactation in all species where it has been studied (130) and is necessary for continued milk production in rodents (20) and humans (123) but not in goats and cows (34). Bromocriptine, an inhibitor of PRL secretion, decreases the activity of several enzymes involved in glucose and lipid metabolism in the lactating rat mammary gland (7). Induced lack of both PRL and GH for 48 hr decreased acetyl-CoA carboxylase activity and mRNA by 80% (8), accompanied by cessation of milk production. When GH was maintained under conditions where PRL was inhibited, the rate of milk secretion fell in both goats and rats, but fat synthesis and secretion were maintained for some time (7, 46, 102), suggesting that PRL itself does not directly regulate milk lipid synthesis and that GH can uncouple lipid secretion from the synthesis of other milk components. This observation appeared to be the result of a direct action of GH on the mammary gland, possibly through stimulation of insulin-like growth factor-1 secretion from stromal cells (34). This effect may have physiologic relevance only in ruminants, where—unlike in humans and rats (117)—GH is significantly elevated during lactation (129).

Evidence that both PRL and GH maintain lactation by acting as survival factors for mammary alveolar cells rather than by regulating the activity of specific enzyme pathways has recently been summarized by Flint & Knight (34). If this hypothesis is correct, then the effects of these hormones may be related more

to developmental processes, including both lactogenesis and involution, than to moment-to-moment regulation of the rate of synthesis of milk components.

Insulin is one of the three hormones necessary for the maintenance of the functional mammary epithelium in tissue culture (126), and several enzymes of glucose and lipid metabolism in the lactating mammary gland have repeatedly been shown to be regulated by insulin, including phosphofructokinase-1, pyruvate dehydrogenase, and acetyl-CoA carboxylase (11, 89). A multitude of experiments extending over nearly three decades show that insulin lack or excess in vivo profoundly alters de novo synthesis of fatty acids in the mammary gland. Nonetheless, paradoxes in these data suggest that a reevaluation of the physiologic relevance of insulin effects is necessary.

In support of a role for insulin, observations from both a fasting-refeeding paradigm and glucose clamp experiments with rats suggest an important role for insulin in the regulation of mammary fatty acid synthesis. Williamson and Munday & Hardie showed that insulin both in vivo and in vitro could rescue fatty acid synthesis from the depression occasioned by 24 hr of food deprivation (89, 138). Refeeding for 2.5 hr also rescued mammary fatty acid synthesis in these rats. The effect was blocked by streptozotocin, an inhibitor of insulin release (89), directly implicating insulin in the refeeding effect. Acetyl-CoA carboxylase activity was decreased in the fasted rats as a result of increased phosphorylation of the enzyme; the increased phosphorylation was reversed by insulin treatment, pinpointing phosphorylation of this enzyme as a specific target of insulin action (89). Insulin also reversed the effects of high fat feeding in isolated acini, accompanied by an increase in acetyl-CoA carboxylase activity and a decrease in its phosphorylation (91). It is notable, however, that 6 hr of fasting brought about a marked depression of de novo fatty acid synthesis with no change in the level of phosphorylation or in vitro activity of acetyl-CoA carboxylase (89), suggesting that mechanisms other than changes in acetyl-CoA carboxylase phosphorylation are responsible for rapid changes in fatty acid synthesis.

Further support for a role for insulin has been inferred from an extensive and elegant series of hyperinsulinemic glucose clamp[2] experiments performed with rats by Burnol et al (11–14). Maintenance of plasma insulin at a level four times basal for 1 hr brought about a severalfold increase in both mammary glucose utilization (12) and lipid synthesis (13). The insulin dose–response curve for these effects suggested increased mammary sensitivity to insulin during lactation (14). There was, however, no change in the number of insulin receptors or in the dose-response curve of autophosphorylation or tyrosine kinase activity in

[2]In hyperinsulinemic euglycemic glucose clamp experiments, insulin is infused at a constant rate over a period of time varying from 1 hr (13) to 4 days (79). Plasma glucose is maintained (clamped) at a constant level near normal by infusion of glucose at a rate determined by frequent measurement of plasma glucose.

the mammary gland, suggesting a postreceptor mechanism of action (17). The sites of insulin action in the mammary gland were suggested to be 6-phospho-1-fructokinase (through a demonstrated increase in fructose 2,6-bisphosphate, a potent regulator of 6-phospho-1-fructokinase; 11), acetyl-CoA carboxylase (whose initial activity was doubled; 11), and pyruvate dehydrogenase (17). However, all these experiments were carried out in the postabsorptive state, as determined by careful comparison of portal vein and arterial glucose concentrations. The concentration of plasma NEFA under these conditions was about 0.56 mM (16), a level at which substantial NEFA entry into the mammary gland would be expected (98). Insulin rapidly reduces plasma NEFA to basal levels, raising the possibility that the insulin effects observed might have been mediated by a decrease in NEFA entry into the mammary cells. This possibility was not taken into account by Burnol and coworkers.

In fact, considerable evidence casts doubt on the relevance of changes in insulin to the regulation of mammary fatty acid synthesis under physiologic conditions other than starvation. Plasma insulin levels are usually lower in the lactating than in the nonmated rat (14, 131), human (58, 96), and other species (129). In rats, insulin levels are inversely proportional to the number of pups suckled (43). The low insulin levels appear to be due in part to decreased pancreatic responsiveness, at least in cows (73) and in women (94). Infusion of insulin at rates that maintained plasma insulin levels several times normal, for hours in women (96) or days in cows (45, 79), did not lead to significant changes in lipid secretion into milk, although the proportion of medium-chain fatty acids was slightly increased after four days in the bovine experiment. Finally, de novo fatty acid synthesis in the mammary gland is dramatically decreased in animals fed a high-fat diet (16, 38, 91, 103, 121) under conditions where plasma insulin levels are not significantly different from animals on high carbohydrate regimens. These observations suggest that unless animals are subjected to a relatively severe restriction in food intake, changes in plasma insulin may not play a significant regulatory role in de novo fatty acid synthesis in the lactating mammary gland. In fact, it is most likely that the low insulin levels may shunt nutrients away from other organs to the lactating mammary gland (36, 130). In the face of these observations, it is pertinent to ask to what extent changes in plasma NEFA might be responsible for the apparent effects of insulin on mammary lipogenesis.

REGULATION OF MILK LIPID COMPOSITION AND SECRETION

Having discussed the mechanisms of milk synthesis and the possible points at which regulatory agents act, we now examine the particular mechanisms that

mediate the effects of initiation as well as cessation of lactation, of starvation, of dietary and body lipid content, and of high-concentrate diets in cows. A central thesis of this discussion is that treatments that decrease milk synthesis in general decrease the activity of lipoprotein lipase and de novo fatty acid synthesis in parallel (129), whereas changes in the supply of plasma fatty acids are balanced by reciprocal changes in de novo fatty acid synthesis. We propose that fatty acids themselves play a major regulatory role in de novo fatty acid synthesis in the mammary gland.

Initiation of Lactation and Weaning

The activity of most of the enzymes involved in milk fat synthesis increases rapidly after parturition. These changes have been studied in many species (51, 63, 76, 78, 81, 87, 137). Some of the more complete data on lipogenic enzymes were published by Mellenberger & Bauman (81), who characterized nine enzymes involved in the synthesis of fatty acids from day 15 of pregnancy to day 22 of lactation in the rabbit. The activities of the key enzymes, acetyl-CoA carboxylase and fatty acid synthase, paralleled fatty acid synthesis, which increased about 10-fold between days 15 and 24 of pregnancy and another 5-fold during lactation (Figure 3). The activities of several other enzymes—ATP citrate lyase, acetyl-CoA synthetase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase—increased with a similar pattern. Because

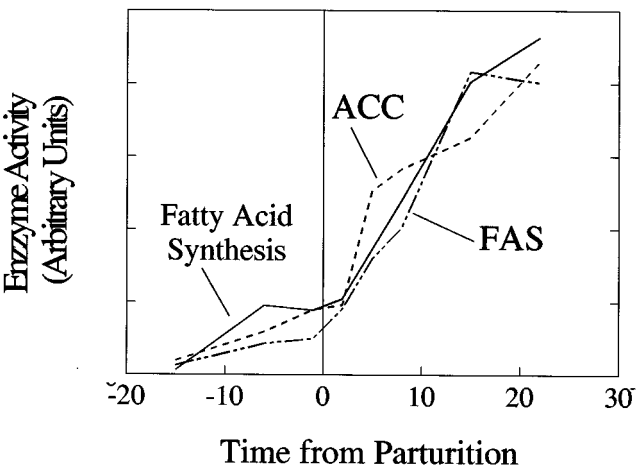

Figure 3 Activity of acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) in mammary homogenates as compared to the rate of glucose and acetate incorporation into fatty acids in mammary slices from pregnant and lactating rabbits. Redrawn from Reference 81.

most of the regulation of fatty acid synthase is thought to occur at the level of gene expression (19, 37), it is expected that increases in the activities of these enzymes reflect increases in mRNA.

Molecular regulation of acetyl-CoA carboxylase has been studied in rat mammary glands where a fourfold increase in mRNA was found between day 1 prior to parturition and day 1 following parturition (69), coinciding with a 10-fold increase in the citrate- and phosphatase-stimulated activity of the enzyme (80). The mRNA was transcribed from promoter II, its so-called housekeeping promoter (69). This promoter contains an elaborate array of *cis*-acting elements, including response elements for insulin, glucose, glucocorticoids, and tumor necrosis factor, as well as five Sp1 sites that serve as powerful transcription enhancers (25). Unlike the mechanism of the increase in activities of the lipogenic enzymes, the increase in lipoprotein lipase activity observed during lactogenesis (51, 78) was mainly post-transcriptional (63), consistent with the metabolic regulation of this enzyme in other organs (31).

Atwood et al interpreted the concentrations of metabolites in sow milk to mean that lipogenic enzyme activities increase only after day 2 postpartum and that suckling is necessary (4). The concept that milk removal from the gland may be necessary to stimulate the coordinate increase in expression of lipogenic enzymes at lactogenesis is supported by the data of Martyn & Hansen from rats (76). Involution of the mammary epithelium after weaning is accompanied by a decrease in the activity and, presumably, the mRNA for most of the enzymes involved in lipid synthesis (66). A feedback inhibitor of lactation found in milk (104) prevents development of fatty acid synthase in an in vitro mammary cell system (136), which suggests that an autocrine inhibitor of milk secretion may mediate these effects. As an alternative hypothesis, Williamson et al (139) suggested that accumulation of medium-chain TAG in the alveoli leads to inhibition of fatty acid synthesis during milk stasis.

Because proliferation and apoptosis are increasingly recognized as important regulators of the functional capacity of the mammary gland for milk synthesis, the relation between these processes and development of the enzymes involved in synthesis of milk lipid is an important area for study. Transcriptional regulation of fatty acid synthase (108, 134) has been partially elucidated in liver and adipose tissue, so the stage is set for new progress in understanding the coordinate changes in metabolic enzymes during lactogenesis and weaning.

Effects of Fasting on the Enzymes of Mammary Lipid Synthesis

One of the earliest and most revealing studies on the effects of fasting on mammary lipid synthesis was carried out by Annison and his colleagues with lactating dairy goats (2). These investigators found that milk yield fell 60% after

24 hr of fasting, whereas lipid secretion fell only about 20%. At the same time there was a significant fall in endogenous mammary lipogenesis, as signaled by a marked fall in incorporation of acetate into milk fatty acids, and a progressive decline in the proportions of fatty acids with chain lengths of C_4 to C_{14}. The authors felt that these declines were related to decreased glucose availability. However, because there are alternate sources of substrate for the production of NADPH for lipid synthesis in ruminants (87), other mechanisms might have been involved. Later studies, largely with rats, also implicate insulin, PRL, and acetoacetic acid (91, 138).

Munday & Williamson utilized the fasted, lactating rat extensively to dissect the effects of hormones on endogenous synthesis of fatty acids in the lactating mammary gland (reviewed in 91, 138). As described above, insulin was thought to be a major player in maintaining or restoring mammary lipogenesis, because streptozotocin, a permanent inhibitor of insulin secretion, decreases de novo fatty acid synthesis more than 85% and prevents its restoration on refeeding (91). However, starvation severely inhibits milk secretion in rats (42), as it does in goats (33), so it is not clear whether this experimental paradigm allows examination of specific regulation of mammary lipid synthesis or represents the overall effects of decreased milk synthesis.

Starvation does not alter milk secretion in all species. Although there is a paucity of studies of women, those few data available suggest that short-term (20 hr) (96) or long-term (7 days) (54) fasting during lactation has little effect on milk output. More dramatically, the many species (bears, true seals, and baleen whales) that fast entirely during lactation (60, 101) are adapted to mobilize large stores of body lipids to milk synthesis and, in general, secrete milks that are high in fat and low in carbohydrate, an adaptation that conserves glucogenic substrates in the mother. What is lacking are studies of fasting in species with moderate stores of body fat; such species could serve as more suitable models for human lactation.

Effects of Dietary Composition on the Composition of Milk Triacylglycerol

The total concentration of milk TAG, although highly variable between species, is relatively constant in a given species or individual consuming an adequate number of calories. The composition of the lipid constitutents of milk, on the other hand, is highly variable and depends on the lipid composition of the diet and the relative proportions of lipid and carbohydrate in the diet.

Effects of changes in the fatty acid composition of the diet are well demonstrated by the increase in the proportion of polyunsaturated fatty acids in the milk of American women, from about 8% in 1959, when animal fats were the primary source of cooking lipids, to about 16% in 1977, when corn oil had largely

supplanted animal fats in cooking (47, 71, 82). In a cross-cultural example, Nigerian women, who consume large amounts of fish, have a much larger proportion of the ω-3 series of LC-PUFA than do European women (Table 1) (72). Alterations in milk fatty acid composition occur rapidly following a change in dietary lipid composition, with adjustments in dietary lipid occurring well within 24 hr of the change in diet (21).

Changes in the proportion of lipid to carbohydrate in the diet have another type of effect on milk lipid composition. In women on western diets, who consume 25–40% of their calories as lipids, mammary synthesis of medium-chain fatty acids comprises only about 10–12% of the milk fatty acids (Table 1). On low-fat, high-carbohydrate diets such as those consumed by the Nigerian women, the proportion of medium-chain fatty acids increases to 18% (Table 1). In some populations and individuals, the proportion of medium-chain fatty acids may be as high as 30% (49, 92), or even 45% (59), in response to a decrease in dietary lipid. Similar effects have been observed in cows (38, 103) and rats (91, 121). In general, increases in medium-chain fatty acids are accompanied by a relatively small or by no change in the total fat content of the milk (40, 44), implying a balance between lipid synthesis in the mammary gland and import of plasma-derived fatty acids. These changes are not accompanied by measurable changes in insulin, PRL, or plasma glucose, and the activities of the enzymes lipoprotein lipase (28) and fatty acid synthase (41) appear to be only marginally affected. We propose below that inhibition of acetyl-CoA carboxylase by imported NEFA is a major regulator of this balance.

Miscellaneous

EFFECTS OF BODY LIPID CONTENT ON MILK LIPID SYNTHESIS IN HUMANS The one maternal factor that may alter milk lipid content in women is the proportion of body fat. In several studies, milk lipid or caloric density was inversely related to maternal body fat (10, 100, 105–107), particularly in poorly nourished women. The mechanism of this effect is unknown, but we speculate that it relates to decreased capacity for mobilization of stored lipid in the fasting state.

HIGH CONCENTRATE FEEDING REGIMENS AND *TRANS* FATTY ACIDS When dairy cows are fed diets containing high concentrations of fermentable starch, a significant depression in total milk fat content occurs, often accompanied by a decrease in the percentage of de novo synthesized fatty acids (103). It has long been postulated that such diets increase plasma insulin, causing increased partitioning of nutrients to adipose tissue and away from milk. However, in recent experiments utilizing the glucose clamp technology to maintain high plasma levels of insulin for four days in dairy cows (45, 79), there was no alteration in milk fat synthesis. Further, in another study, Gaynor and colleagues (35)

showed that there was no change in the glucose and insulin levels in the serum of those cows showing a substantial decrease in milk fat in response to a high-concentrate diet. Both these studies provide strong evidence against a role for insulin in the depression of milk fat synthesis by high-concentrate diets.

Davis & Brown (26) suggested nearly 30 years ago that *trans* fatty acids might be responsible for the low–milk-fat syndrome. Griinari et al (45) suggest that certain types of grain diets result in incomplete hydrogenation of $C_{18:2}$ and increased absorption of *trans*-$C_{18:1}$. Although no direct evidence concerning this mechanism is currently available, Gaynor et al (35) suggest that *trans*-$C_{18:1}$ may reduce the activities of stearyl-CoA desaturase, acetyl-CoA carboxylase, and acyl transferases in mammary alveolar cells, causing a depression of both de novo fatty acid synthesis and incorportation of fatty acids into TAG.

DIURNAL VARIATION The fatty acid composition of the milk of several species has been shown to undergo diurnal variation (24, 90, 132). The most likely source of this variation is a change in the entry of preformed fatty acids into the mammary cell during the switch from the fed to the fasting state. Such a change is likely to be most prominent when high-carbohydrate, low-fat diets are fed, and the high-insulin, relatively low-TAG content of the plasma in the fed state leads to enhanced de novo fatty acid synthesis.

A CENTRAL REGULATORY ROLE FOR FATTY ACIDS

The considerations discussed above lead us to propose that fatty acids are the major regulators of de novo fatty acid synthesis in the lactating mammary gland, acting primarily on acetyl-CoA carboxylase. This hypothesis would explain why de novo fatty acid synthesis in the lactating mammary gland is decreased in both the high-fat fed animal, where lipoprotein lipase provides fatty acids for TAG synthesis, and in the fasting animal, where adipose tissue lipolysis provides large quantities of NEFA to the mammary gland. Only in the fed animal on a high-carbohydrate diet, e.g. the chow-fed rat, would the plasma source of preformed fatty acids be low, because little dietary TAG would be available and lipolysis of depot lipids would be suppressed by insulin.

The concept that NEFA play a central role in the regulation of lipid synthesis was first proposed by Miller & Levy more than 30 years ago (85). The idea has resurfaced at intervals since then (83), most recently in a review by Williamson et al (139). In a very early study with cows, Moore & Steele (88) observed that increased mammary uptake of fatty acids from the plasma decreased fatty acid synthesis from acetate and β-hydroxybutyric acid. Rao & Abraham observed that fatty acids (decanoate and palmitate) inhibited the in vitro incorporation of [^{14}C]glucose into fatty acids in mammary tissue slices from lactating rats

(110). More recently, Hansen & Knudsen (52, 53) and Robinson & Williamson (112) found that oleic acid inhibited fatty acid synthesis in isolated mammary acini from both cows and rats in vitro, and Heesom et al (55) found that C_8 and C_{10} inhibited conversion of glucose into lipids in the same preparation. These observations suggest that fatty acids play a role in the regulation of fatty acid synthesis in the mammary gland. The possibility that this effect is mediated by acetyl-CoA carboxylase is suggested by the observations of Miller and colleagues, who found that the medium-chain fatty acids in rat milk were potent inhibitors of mammary acetyl-CoA carboxylase in the mammary gland (84). Long-, but not medium-, chain fatty acyl CoA were also inhibitory (99), with palmitoyl CoA having an inhibitory constant of 5.5 nM. Similar effects were observed by Vernon in rat and sheep adipose tissue (128).

In the 1970s, the idea that fatty acids could play a regulatory role was discounted, because it was thought that concentrations of fatty acids high enough to inhibit lipid synthesis would have detergent actions that would be deleterious to both cell membranes and enzymes (87). However, the discovery of high concentrations of FABP (9, 122) and acyl-CoA binding protein (70, 111) that keep the free concentrations of NEFA and their CoA derivatives strictly limited, along with the apparent high affinity of palmitoyl CoA, at least remove this problem. More recent focus on insulin has diverted attention away from fatty acids. However, the data cited above suggest that the precise role of fatty acids in the regulation of milk lipid synthesis requires elucidation.

PERSPECTIVES

Over the past 30 years, the biochemical pathways important in substrate uptake and utilization for TAG synthesis in the mammary alveolar cell and elsewhere in the body have been elucidated, many of the enzymes have been purified, and the structures of the proteins and their cDNAs—if not their genes—have been resolved. The cellular localization of most of the components of the system is understood, and a general picture is beginning to appear of the anatomical pathways by which substrates are shuttled from the blood stream across the plasma membrane of the alveolar cell through the cytoplasm, the mitochondria, and the endoplasmic reticulum to emerge as a cytoplasmic lipid droplet (Figure 2). The mechanisms by which these droplets coalesce and become enveloped with apical membrane to be secreted as the mature milk fat globule (68, 77), although not reviewed here, are a fascinating chapter in the cell biology of the mammary alveolar cell. With all of these elements in place, we are poised to come to an understanding of the regulatory mechanisms that integrate lipid synthesis with both the developmental biology of the mammary gland and the nutritional environment of the mother.

Methodologies and model systems are available to allow approaches to the following questions.

1. How is the concentration of LC-PUFA in the milk regulated? To what extent are these fatty acids made in mammary alveolar cells and to what extent are they derived from the plasma?

2. How are fatty acids delivered from the capillary across the interstitial space to the basal membrane of the mammary alveolar cell? Are there specific transport systems in the mammary cell membrane that facilitate fatty acid transfer into the cytoplasm? If so, do they have any specificity, and are they regulated?

3. How is the increase in the concentration of the enzymes of glucose metabolism and lipid synthesis developmentally coordinated at lactogenesis and involution? What is the molecular basis for the relationship between regulation of these pathways and regulation of milk protein and carbohydrate synthesis?

4. Are fatty acids the major regulators of de novo fatty acid synthesis in the mammary alveolar cell? If so, is acetyl-CoA carboxylase their major site of action? Is there specificity in the interaction of fatty acids with acetyl-CoA carboxylase and other enzymes in the pathway? Do *trans* fatty acids inhibit acetyl-CoA carboxylase and other enzymes of lipid synthesis, and if so, do they have a higher affinity for these enzymes than their corresponding *cis* isomers?

Our knowledge base is now sufficient to seek answers to most of these questions at the cellular and molecular level. This process will not be made easier by the nearly complete lack of in vitro model systems that secrete milk fat or that undergo the coordinated series of events that bring about the onset of milk secretion at lactogenesis. This means that most of these questions will have to be approached using in vivo systems or freshly isolated tissues. Fortunately, elegant experimental paradigms like the glucose clamp technology are available that allow components of the in vivo system to be studied under well-controlled circumstances. Such paradigms have the advantage that clear-cut results can often be obtained under conditions that are clearly relevant to the physiology of the animal.

Although this review has focused on regulation of mammary lipid synthesis, the milk-specific components of the TAG, such as LC-PUFA and medium-chain fatty acids, are likely to have functional consequences for the infant as well. Elucidating the nature of these consequences should be an equally important part of future research.

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