Nutrigenomics, Rumen-Derived Bioactive Fatty Acids, and the Regulation of Milk Fat Synthesis

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Abstract

Mammary synthesis of milk fat continues to be an active research area, with significant advances in the regulation of lipid synthesis by bioactive fatty acids (FAs). The biohydrogenation theory established that diet-induced milk fat depression (MFD) in the dairy cow is caused by an inhibition of mammary synthesis of milk fat by specific FAs produced during ruminal biohydrogenation. The first such FA shown to affect milk fat synthesis was *trans*-10, *cis*-12 conjugated linoleic acid, and its effects have been well characterized, including dose-response relationships. During MFD, lipogenic capacity and transcription of key mammary lipogenic genes are coordinately down-regulated. Results provide strong evidence for sterol response element-binding protein-1 (SREBP1) and Spot 14 as biohydrogenation intermediate responsive lipogenic signaling pathway for ruminants and rodents. The study of MFD and its regulation by specific rumen-derived bioactive FAs represents a successful example of nutrigenomics in present-day animal nutrition research and offers several potential applications in animal agriculture.

Contents

Milk fat depression (MFD): a reduction of up to 50% in milk fat yield with no change in the yield of milk and other milk components. It is often the result of changes in dietary or management inputs that alter rumen fermentation. These alterations result in the formation of specific bioactive fatty acids in the rumen that are absorbed and ultimately lead to a reduction in milk fat synthesis in the mammary gland

MAMMARY FAT SYNTHESIS AND THE LOW-FAT MILK SYNDROME

Mammary epithelial cells have an impressive ability to convert circulating nutrients into milk components. Fat is the major contributor to energy density in whole milk, and it is responsible for many of the physical properties, organoleptic characteristics, and manufacturing qualities of dairy products (70). Although liver and adipose tissue are major lipid synthesizing organs in nonlactating mammals, their quantitative production of lipids pales in comparison to the mammary glands. Thus, Rudolph et al. (122) have described the lactating mammary gland as a "lipid synthesizing machine." For example, modern dairy cows have a daily milk fat output that is energetically greater than their maintenance requirement, and over the typical 305-day lactation, top-producing cows will have a milk fat output nearly double their body weight (11). Likewise, over the typical 20-day lactation, mice may produce an amount of milk fat equivalent to their body weight (122).

Fat is the most variable component of milk in dairy cows, with the amount and composition affected by many factors including genetics, physiological state, and environment (86, 104). Milk fat production, however, is especially responsive to nutrition, providing a practical tool to alter its yield and composition (30, 83). One nutritional situation of practical and biological interest is the low-milk fat syndrome, commonly referred to as milk fat depression (MFD). Diet-induced MFD is classically observed in cows fed highly fermentable diets or diets that contain plant or fish oil supplements (13). First described by French scientist Boussingault in 1845 (141), diet-induced MFD is characterized by a reduction of up to 50% in milk fat yield with no change in the yield of milk and other milk components (13). The basis for diet-induced MFD has perplexed scientists for over a century, and highlights of significant historical milestones unraveling the biology of the low-fat milk syndrome have been reviewed elsewhere (14, 62). The discovery that changes in milk fat yield were negatively correlated with milk fat concentration of *trans*-18:1 fatty acids (FAs) was a key observation (40). Based on this and results from related studies, Bauman & Griinari (13) proposed that diet-induced MFD was due to an inhibition of mammary lipid synthesis by specific FA intermediates produced during ruminal biohydrogenation of dietary polyunsaturated FA (PUFA). Known as the "biohydrogenation theory," investigations over the past decade have verified that diet-induced MFD involves an interrelationship between rumen fermentation processes and mammary synthesis of milk fat.

Nutrigenomics is an umbrella term that refers to the impact of dietary components on physiological processes by altering gene expression, epigenetic effects, proteins, or metabolites (96). Diet-induced MFD represents an exciting

example of nutrigenomics; specifically, an example where bioactive FAs produced as biohydrogenation intermediates during rumen fermentation act to down-regulate the expression of key lipogenic genes involved in milk fat synthesis. The first of these bioactive FAs identified was *trans*-10, *cis*-12 18:2 (conjugated linoleic acid; CLA), and it has been the most extensive investigated. In the following sections we discuss the causative biohydrogenation intermediates that have been found to effect milk fat synthesis, consider dose-response relationships and general metabolic effects, and discuss recent advances and results from molecular approaches to identify the mechanisms by which these bioactive FAs are able to regulate mammary synthesis of fat. Finally, we consider long-term effects and potential applications and implications in animal agriculture.

BIOHYDROGENATION INTERMEDIATES AND DIET-INDUCED MILK FAT DEPRESSION

Diets for lactating dairy cows are low in fat content (~4%–5%), with linoleic acid and linolenic acid being the predominant PUFAs. When dietary lipids enter the rumen, the ester linkages are hydrolyzed (>85%) followed by biohydrogenation of unsaturated FA. Biohydrogenation involves only a few of the species of rumen bacteria, and they carry out these reactions as a protection mechanism against the toxic effects of PUFA and/or to match the FA profile desired for microbial growth. As a consequence of this extensive hydrolysis and biohydrogenation, rumen outflow of FA is mainly saturated free FA. However, some biohydrogenation intermediates, specifically CLA and *trans*-18:1 FA, also escape from the rumen, and these are absorbed and utilized for milk fat synthesis [see reviews by Bauman & Lock (15) and Shingfield et al. (130)].

Diet-induced MFD is often encountered in modern dairy production, and its occurrence requires two conditions: (*a*) an alteration in the rumen environment and a shift in the bacteria population that is often characterized by a decrease in rumen pH, and (*b*) a dietary source of PUFA (14). As a consequence, there is a shift in the pathways and completeness of rumen biohydrogenation that increases the rumen outflow of biohydrogenation intermediates. Thus, the reduction in milk fat output during diet-induced MFD is highly correlated with increases in the milk fat content of many *trans*-18:1 and CLA isomers (74, 89, 131).

The biohydrogenation theory of MFD proposed that MFD was caused by an inhibition of mammary synthesis of milk fat by specific FAs produced as intermediates in rumen biohydrogenation (13). But which of these biohydrogenation intermediates is the cause of MFD? There are no cell culture systems that maintain the viability of actively secreting mammary epithelial cells, so this has been investigated in dairy cows by postruminal infusions (abomasal or duodenal) of specific FAs as a convenient experimental approach to avoid alterations by rumen bacteria. Initial investigations used mixed isomers of CLA and established proof of concept for the biohydrogenation theory; short-term infusion of CLA mixtures resulted in a dramatic reduction in milk fat secretion, which was reversed when supplementation was terminated (33, 34, 90). Subsequently, Baumgard et al. (19) utilized relatively pure isomers and demonstrated that abomasal infusion of *trans*-10, *cis*-12 CLA resulted in an immediate decrease in milk fat synthesis, whereas *cis*-9, *trans*-11 CLA had no effect. Comparisons between diet-induced MFD and that obtained from *trans*-10, *cis*-12 CLA infusion, however, indicated that there must be additional biohydrogenation intermediates that reduce milk fat synthesis (114, 132). Recently, two additional CLA isomers produced as intermediates in rumen biohydrogenation have been shown to reduce milk fat, *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA; however, each of these isomers has been tested only in a single study conducted at a single dose (110, 125). *Trans*-8, *cis*-10 CLA, *cis*-9, *trans*-11 CLA, *trans*-9, *trans*-11 CLA, *trans*-10, *trans*-12 CLA, and *cis*-11, *trans*-13 CLA are additional

FA: fatty acid

Biohydrogenation: the conversion of unsaturated to saturated fatty acids by rumen bacteria. This extensive conversion also results in the formation of a number of conjugated linoleic acid and *trans* 18:1 fatty acids, some of which are bioactive in the ruminant and other species when taken up by the mammary glands

PUFA: polyunsaturated fatty acid

Nutrigenomics: a broad term that refers to the impact of dietary components on physiological processes by altering gene expression, epigenetic effects, proteins, or metabolites

Conjugated linoleic acids (CLAs): a mixture of positional and geometric isomers of linoleic acid that are present in meat and dairy products derived from ruminants due to their formation as intermediates in ruminal biohydrogenation of PUFA. Individual CLA isomers have specific biological effects

PHVO: partially hydrogenated vegetable oil

intermediates in rumen biohydrogenation that have been examined by postruminal infusions of enrichments or relatively pure preparations; these CLA isomers had no effect on milk fat output, although all were taken up by the mammary gland and incorporated into milk fat (109, 110, 112, 125). On the basis of principal component analysis of studies involving diet-induced MFD, Kadegowda et al. (74) proposed that *trans*-7, *cis*-9 CLA may be another CLA isomer that inhibits milk fat synthesis. This CLA isomer originates predominately from rumen-derived *trans*-7 18:1 via the enzyme Δ9-desaturase in the mammary gland (38, 117). Availability of *trans*-7, *cis*-9 CLA is limited, so effects have not been examined in a postruminal infusion study. This possibility, however, has been indirectly evaluated in studies involving abomasal infusion of sterculic acid, a cyclopropene FA that specifically inhibits Δ9-desaturase (69); although sterculic acid infusion resulted in the expected reduction in the milk fat content of *trans*-7, *cis*-9 CLA and altered the ratio of *trans*-7 18:1 to *trans*-7, *cis*-9 CLA, milk fat output was unaltered (37, 38, 56, 75). Likewise, abomasal infusion of *trans*-9, *trans*-11 CLA (110) or *trans*-10, *trans*-12 CLA (109, 125) and dietary addition of CoEDTA (129, 135) also alter the desaturase index of milk fat, indicating a reduction in Δ9-desaturase activity, but none of these treatments had any affect on milk fat production.

The possible role of *trans*-18:1 isomers in the regulation of milk fat synthesis is also of interest, in part because MFD is observed when large quantities of partially hydrogenated vegetable oils (PHVOs) are abomasally infused [reviewed by Bauman & Griinari (14)]. To establish causative roles for *trans*-C18:1 isomers on milk fat synthesis, purified preparations of individual *trans*-18:1 FA must be tested, and to date no reduction in milk fat secretion has been observed with infusion of these at levels typical for rumen outflow (25 to 45 g/day). *Trans*-18:1 FAs that have been examined to date include *trans*-9, *trans*-10, *trans*-11, and *trans*-12 (56, 88, 120, 134, 140). The bioactivity of *trans*-10 18:1 is of special interest because the reduction in milk fat is highly correlated with rumen outflow and the milk fat content of this FA (66, 74, 89). Lock et al. (88) observed no effect on milk fat output when 43 g/day of relatively pure *trans*-10 18:1 was infused. However, a more recent study by Shingfield et al. (134) infused 247 g/day of a mixture of FA that contained 37.3% *trans*-10 18:1 (supplying ~92 g/day *trans*-10 18:10), and a modest 19% reduction in milk fat output was observed. Reconciling whether the different results with *trans*-10 18:1 are related to the use of physiological levels and/or the presence of other FAs in less pure preparations will require further investigations [see discussion in Harvatine et al. (62) and Shingfield et al. (130)].

Dose-Response Relationships

The dose response between abomasal infusion of *trans*-10, *cis*-12 CLA and the reduction in milk fat yield is a clear curvilinear relationship. de Veth et al. (43) summarized data across experiments involving abomasal infusions and demonstrated that the milk fat response to *trans*-10, *cis*-12 CLA best fit an exponential decay curve with maximal response of ~50% reduction in milk fat at ~7.5 g/day and a one-half maximum response at ~3.5 g/day. Recent summaries have added additional data with lactating dairy cows and verified this curvilinear dose-response relationship among studies that provided *trans*-10, *cis*-12 CLA by postruminal infusion (51, 132) or when *trans*-10, *cis*-12 CLA was fed in a supplement formulated to minimize alterations by rumen bacteria (137). *Trans*-10, *cis*-12 CLA is also incorporated into milk fat, and across the CLA dose range, the transfer efficiency of abomasally infused *trans*-10, *cis*-12 CLA into milk fat averaged 22% (43). The linear relationship in transfer to milk fat is remarkable when one considers that the yield of milk fat is simultaneously decreased as the abomasal dose of *trans*-10, *cis*-12 CLA is increased. As a result of the consistent uptake across this dose range, the relationship between milk fat content of *trans*-10, *cis*-12 CLA and the reduction in milk fat secretion is also curvilinear, and this offers a means to compare effects among species.

The onset of MFD is very rapid, as best illustrated in studies involving abomasal infusion of *trans*-10, *cis*-12 CLA; milk fat percent was progressively decreased following the initiation of infusion with a significant reduction by 10 h (59) and a nadir achieved by 3–4 days (19). Likewise, milk fat synthesis is rescued after termination of *trans*-10, *cis*-12 CLA treatment, with the recovery time course being similar to the progressive pattern of decline (19). As previously discussed, *trans*-10, *cis*-12 CLA–induced MFD is a specific reduction in milk fat yield, with no change in the yields of milk or other milk components. This phenotype is sustainable for long periods, as shown in two studies where *trans*-10, *cis*-12 CLA supplementation continued for 20 weeks (23, 108). The mechanism of CLA is also independent of stage of lactation, as *trans*-10, *cis*-12 CLA reduces milk yield during all phases of the lactation cycle (23, 28, 108), although a larger dose is required in early lactation (97, 101).

CLA-induced MFD has a distinct phenotype, where the only milk component altered is milk fat, but the milk FA composition does differ over the *trans*-10, *cis*-12 CLA dose range. Initial studies showed that the *trans*-10, *cis*-12 CLA-induced reduction in milk fat secretion was due to decreases in FAs of all chain lengths, but effects were most pronounced for those synthesized de novo (33, 34, 90). As investigations focused on *trans*-10, *cis*-12 CLA and expanded to include a range of doses, it was discovered that at lower doses, the reduction in milk fat was distributed more uniformly among FAs synthesized de novo (short- and medium-chain length) and longer-chain FAs taken up from the blood (21, 113). Likewise, an inhibition of Δ9-desaturase that resulted in a marked shift in the FA composition of milk fat was observed only at doses of *trans*-10, *cis*-12 CLA, where milk fat production was reduced by >20%. At lower doses of *trans*-10, *cis*-12 CLA, the ratio of FA representing product/substrate for Δ9-desaturase was unaffected (21, 113).

The maximal reduction in milk fat yield observed in the dose-response summaries with *trans*-10, *cis*-12 CLA is ∼50%, and this is also the maximum observed in diet-induced MFD (14). The basis for this is unknown, but it appears that either *trans*-10, *cis*-12 CLA can only down-regulate its target-signaling molecule(s) by 50% or the target-signaling pathway is only responsible for regulation of about one-half of milk fat yield. Redundant regulation of biological processes is a characteristic of mammalian biology, and such redundancy would be logical for milk fat synthesis because of the importance of milk fat as an energy source for the nursing young. Furthermore, the ability of *trans*-10, *cis*-12 CLA to regulate milk fat synthesis has also been observed in other mammals including mice (73, 91), rats (65, 121), pigs (26, 119), sheep (87, 136), goats (85, 133), and humans (94). Most of these investigations have used dietary supplements containing a mixture of CLA isomers or low-enrichment preparations. Variation in *trans*-10, *cis*-12 CLA dose, interval of administration, and response end point makes it difficult to directly compare results from different lactating mammals to those from cows. Nevertheless, *trans*-10, *cis*-12 CLA supplementation has consistently resulted in a reduction in milk fat content, milk fat yield, and/or growth rate of the nursing neonate. An exception is studies with lactating women, where a reduction in milk fat content was observed in one study (94), and no effect was reported in another (98); interestingly, these two studies were conducted by the same group, and the basis for the difference in milk fat response to *trans*-10, *cis*-12 CLA supplementation is not obvious.

Whole-Animal Metabolism

Early theories to explain diet-induced MFD proposed that milk fat synthesis was limited by substrate availability due to changes in absorbed metabolite profile that occur with diets associated with MFD. Subsequent work, however, determined that milk fat was not limited by deficiency in dietary fat or acetate or by increased propionate, glucose, or insulin (reviewed in 13, 14). Rather, effects were specific for changes in mammary biosynthesis of milk fat. Indeed, as summarized in **Table 1**, results indicate that *trans*-10, *cis*-12

Table 1 Summary of biological responses to *trans*-10, *cis*-12 conjugated linoleic acid in the dairy cow[1]

Process	Effect
Milk	
Protein and lactose	Output unchanged
Fat	Output decreased up to 50%
Fatty acid profile	All fatty acids reduced but relatively greater reduction in de novo synthesized fatty acids
Whole-animal metabolism	
Feed intake	Slight reduction (based on meta-analysis of CLA infusion studies)
Ketogenesis	Plasma β-hydroxybutyrate unchanged
Liver lipids	Triglyceride content unchanged
Plasma hormones	
IGF-1	IGF-1 concentration unchanged
Leptin	Leptin concentration unchanged
Glucose homeostasis	
Glucose set-point	Plasma glucose unchanged
Basal insulin	Plasma insulin unchanged
Stimulated	Glucose response to insulin unchanged
Lipolysis	
Basal	Plasma nonesterified fatty acids unchanged
Stimulated	Response to β-adrenergic stimulation unchanged
Tissue-specific metabolism	
Mammary gland	
Lipogenic capacity	Decreased acetate incorporation into fatty acids
Lipid synthesis enzymes	Coordinated decrease in mRNA abundance for enzymes involved in the uptake, synthesis, transport, desaturation, and esterification of fatty acids
Apoptosis	No apparent effect; milk fat rescued after termination of treatment
Cell viability	No apparent effect; continued synthesis of milk protein and lactose
Inflammation	No change in inflammatory cytokine expression
Transcription factors	Decreased expression of SREBP1, SREBP1 activation proteins, and S14
Adipose tissue	
Lipid synthesis enzymes	Increased expression of enzymes involved in fatty acid synthesis, uptake, and desaturation
Leptin	Increased expression of leptin during short-term MFD
Transcription factors	Increased expression of SREBP1 and S14

[1]References for these specific effects are cited in text.

Abbreviations: CLA, conjugated linoleic acid; IGF, insulin growth factor; MFD, milk fat depression; SREBP, sterol regulatory element-binding protein.

CLA-induced MFD had no effects on plasma concentration of metabolites including glucose, NEFA, and βHBA or metabolic hormones including insulin, insulin growth factor (IGF)-I, and leptin during short (<1 week) and longer-term (up to 20 weeks) treatments (18, 19, 28, 42, 108). Likewise, *trans*-10, *cis*-12 CLA-induced MFD did not alter plasma NEFA response to an epinephrine challenge or plasma glucose response to an insulin challenge; thus, homeostatic responses associated with the regulation of lipolysis and glucose uptake are unaltered (18, 42). Interestingly, based on increases in the expression of lipogenic genes, adipose tissue lipid synthesis was increased during short-term treatment with *trans*-10, *cis*-12 CLA-induced MFD (63). Finally, hepatic triglyceride concentration was unaffected

during *trans*-10, *cis*-12 CLA-induced MFD (23, 28). This phenotype contrasts with the lipodystrophy, insulin resistance, and hepatic steatosis reported in growing mice (for review, see 143).

MAMMARY MECHANISMS DURING MILK FAT DEPRESSION

Synthesis of milk fat requires coordination of enzymes involved in metabolite transport, de novo lipogenesis, FA transport, desaturation, and esterification, and the formation, transport, and excretion of the milk lipid droplet. In ruminants, FAs arise from two sources that contribute equally (molar basis)—de novo synthesis within the mammary epithelial cell and uptake of preformed FA from circulation (for review, see 14). The FAs are esterified in the endoplasmic reticulum (ER) and assembled into lipid droplets that move to the apex of the cell. A number of proteins are associated with the milk fat globular membrane (MFGM) that surrounds the lipid droplet, and they are essential for milk fat secretion (16, 29). There is little evidence that *trans*-10, *cis*-12 CLA-induced or diet-induced MFD involves an inhibition of milk fat export; inhibition of milk fat export would be expected to disrupt synthesis and secretion of other milk components because of cytotoxic effects of high cellular lipids.

The fact that *trans*-10, *cis*-12 CLA-induced and diet-induced MFD involve a decrease in milk fat output of both de novo and preformed FAs suggests a coordinated regulation of enzymes of lipid synthesis. We first demonstrated this using mammary tissue from cows with *trans*-10, *cis*-12 CLA-induced MFD (20); a subsequent summary of published results and more recent experiments support the concept of decreased expression of lipid synthesis enzymes for both *trans*-10, *cis*-12 CLA-induced and diet-induced MFD (2, 49, 58, 61, 114, 118). Briefly, mRNA abundance for FA synthase, acetyl-CoA carboxylase, lipoprotein lipase, Δ9-desaturase, fatty acyl-CoA ligase, glycerol-phosphate-acyltransferase, and acyl-glycerol-phosphate-acyltransferase has been reported to be decreased during MFD.

Sterol Response Element-Binding Proteins

ER: endoplasmic reticulum

MFGM: milk fat globular membrane

SREBP1: sterol response element-binding protein-1

Coordinated suppression of mammary lipogenic genes suggests involvement of a central regulator of lipid synthesis. Peterson et al. (115) reported decreased abundance of the active nuclear fragment of sterol response element-binding protein-1 (SREBP1) during *trans*-10, *cis*-12 CLA treatment of a bovine mammary epithelial cell line (MACT). This transcription factor family functions as global regulators of lipid synthesis (128). The full-length inactive SREBP1 protein is complexed with the SREBP chaperone protein (SCAP) and is anchored in the endoplasmic reticulum through association with a third protein, either insulin-induced gene 1 or 2 (INSIG1 or INSIG2). SREBP1 is activated by dissociation of INSIG from the SREBP/SCAP complex, allowing translocation to the Golgi, where it is proteolytically cleaved to "nuclear SREBP1" (nSREBP1), the transcriptionally active fragment. Nuclear SREBP then enters the nucleus and activates transcription of genes involved in lipid metabolism by binding to sterol regulatory response elements (SREs; 52). SREBP1a and SREBP1c are transcribed from a single gene. SREBP1c is the isoform that plays a critical role in the dietary regulation of lipogenic genes, especially those associated with de novo synthesis, and its effects have been extensively characterized in rodents (67, 128).

Mammary expression of SREBP1 decreased a similar magnitude as milk fat yield during both diet- and *trans*-10, *cis*-12 CLA-induced MFD (49, 58). Expression of SREBP1 is highly dependent on the concentration of nuclear SREBP1; thus, SREBP1 expression is indicative of SREBP1 signaling (4). Additionally, SREBP1 regulatory proteins (e.g., INSIG1) were also decreased (58). Finally, key lipid synthesis enzymes that are down-regulated during *trans*-10, *cis*-12 CLA-induced and diet-induced MFD contain an SRE in their promoter and are known to be regulated by SREBP1c (58).

SREBP1 appears to play a key role in the regulation of milk fat synthesis. Barber

S14: thyroid hormone–responsive spot 14

et al. (10) reported nearly undetectable nSREBP1 signal in nonlactating tissue and strong nSREBP1 signal in lactating tissue. Transcriptional up-regulation of SREBP1 is also well described in mice (6, 123). In contrast, Bionaz & Loor (25) questioned the importance of SREBP1 in lactation on the basis of a larger magnitude of mRNA expression observed for INSIG1, an SREBP1 regulatory protein, in a trans-lactation study. Care should be taken in interpreting trans-lactation studies, as milk fat yield is affected by different mechanisms in each period (cell differentiation and lactogenesis at the initiation of lactation, galactogenesis during early lactation, and apoptosis during established lactation). Although in vivo inhibition of SREBP1 regulatory proteins during MFD is well established, the critical points and proteins regulating SREBP1 activation have not been described. Specifically, a direct or indirect connection between *trans*-10, *cis*-12 CLA and SREBP1 signaling has not been established, nor has the mechanism been established for inhibition of SREBP1 by other bioactive FAs.

Thyroid Hormone–Responsive Spot 14

Thyroid hormone–responsive spot 14 (S14) was identified as a *trans*-10, *cis*-12 CLA-responsive gene using the bovine Affymetrix GeneChip (58). S14 is highly expressed in lipid-synthesizing tissues, including lactating mammary tissue, and is acutely increased by lipogenic signals such as insulin and high carbohydrate intake (39). In bovine tissues, S14 was highly expressed in liver and adipose tissue and moderately expressed in mammary tissue (58). Previous studies also established that S14 expression was up-regulated in mammary tissue during lactation in humans (144) and mice (124), and this has also been observed for the bovine (25, 58). Using multivariate analysis, a significant relationship between expression of S14 and expression of fatty acid synthase and lipoprotein lipase was observed in bovine mammary tissue ($R^2 = 0.86$ and 0.42, respectively; 58).

Altered expression of S14 has been associated with unique phenotypes involving the regulation of fat synthesis. Abnormalities in the regulation of adipose tissue S14 expression have been reported in obese humans (78, 102). Likewise, gene expression profiling identified differential expression of S14 in livers of chickens selected for growth (35), adipose tissue of chickens selected for adiposity (27), muscle of cattle that differs in marbling (142), and muscle of steers fed a high-starch diet (53). Finally, S14 appears to be a functional component of the high-lipogenic phenotype observed in aggressive breast cancers (77, 144). On the basis of identification of S14 as a *trans*-10, *cis*-12 CLA-responsive gene in the bovine, Donnelly et al. (45) demonstrated down-regulation of S14 as a possible mechanism for the anticancer effects of CLA in a human mammary carcinoma cell line (T47D).

S14 has no assigned biochemical function, but a recent protein crystal structure defined three antiparallel α-helices (36). Originally identified as a protein acutely responsive to thyroid hormone (for review, see 39), S14 is found in both the cytoplasm and nucleus (31, 32, 39), and transport of S14 to the nucleus has recently been reported (32). S14 has been implicated in the transcriptional regulation of lipogenic genes based on antisense and overexpression approaches (32, 80, 93). Taken together, the above findings provide support for a transcriptional coactivator function for nuclear S14.

MIG12 (also called S14 Related) is a paralog to S14 that is expressed in liver and adipose tissue, but not in the mammary gland in mice, and may have redundant function with S14 in nonmammary tissue (147). MIG12, like S14, is found in both the cytoplasm and nucleus (24) and is responsive to changes in metabolic state (63, 139). LaFave et al. (80) proposed a cytoplasmic role for S14 and MIG12 on the basis of a report that MIG12 binds to a microtubular-associated protein (MID1) and functions to cooperatively stabilize microtubules with MID1 (24). In addition, S14 coimmunoprecipitates with FASN, and the

S14 homodimer has a hydrophobic pocket that binds a hydrophobic probe (79). Recent publications have verified redundant functions of S14 and MIG12 in primary hepatocytes (3) and characterized a cytoplasmic function of MIG12 in regulating the inactive dimmer to active polymer transition of acetyl-CoA carboxylase (ACC; 76). Physical interaction of MIG12 and ACC, and MIG12 and S14, has been demonstrated by coimmunoprecipitation (36, 76), which, combined with the previously mentioned association of S14 and FASN, provides support for MIG12 and S14 as linker proteins for lipogenic enzymes. Historically, preformed FA inhibition of ACC has been proposed as a mechanism of the reduced de novo FA in milk on high-fat diets (100). A cytoplasmic function of MIG12 or S14 may explain discordant reports of de novo lipogenesis, lipogenic enzyme mRNA and protein abundance, and ACC activity in high-citrate assays (citrate drives formation of ACC polymer; 147). Mammary expression of MIG12 is not altered during *trans*-10, *cis*-12 CLA or diet-induced MFD in the cow (58), but implications of interactions and overlapping functions provide intriguing mechanisms of S14 in MFD.

Insight from Rodent Models

Rodent models have been extensively used for investigation of dietary regulation of milk synthesis as well as the investigation of the mechanisms of *trans*-10, *cis*-12 CLA and other bioactive FAs in adipose tissue. This provides a rich literature for hypothesis development, but care must be taken in extrapolation because of metabolic and experimental differences with bovine literature. The lactating dairy cow is not amenable to transgenic approaches, and maintaining the biosynthesis and secretion of milk components is difficult to achieve in mammary cell culture. To further examine mechanistic aspects of mammary de novo FA synthesis and utilize functional genomic procedures, an in vivo rodent model of *trans*-10, *cis*-12 CLA and dietary regulation of milk fat synthesis during established lactation would be valuable.

Lactation is a highly conserved function across mammals, and Lemay et al. (82) recently reported a high genetic conservation of genes related to lactation, including greater than 98% amino acid sequence homology in milk fat globular membrane proteins. Lactational response to *trans*-10, *cis*-12 CLA is also highly conserved among mammals, and as discussed above, *trans*-10, *cis*-12 CLA inhibition of milk fat synthesis has been reported in several species in addition to the extensive work in cows, sheep, and goats. Early work with lactating rodents reported a diet-induced reduction in milk fat synthesis with diets containing PHVO (7, 138); PHVO typically contains a high concentration of *trans* FA including various CLA isomers (8). Purified CLA preparations have been used in rodent lactation experiments (65, 73, 91, 121), although the CLA dose has generally been much higher than that required for maximal MFD in the cow (17). CLA doses in rodent studies have routinely been 0.3% to 1% of the diet, compared to *trans*-10, *cis*-12 CLA at ~0.05% of the diet, to obtain maximum inhibition in dairy cows; however, inhibition of de novo fatty acid synthesis in lactating mice does occur at lower doses of CLA (60), and this has implications relating to the mechanism(s) (64). It is important to note that excessively high doses of *trans*-10, *cis*-12 CLA in the cow (*trans*-10, *cis*-12 CLA at ~0.25% of the diet) have been shown to cause an apparent apoptosis of mammary epithelial cells, as evidenced by a generalized reduction in the yield of milk and all milk components and a dramatic increase in milk somatic cell count (22).

The mechanism of *trans*-10, *cis*-12 CLA has been extensively examined in rodent models of obesity. Previous experiments using high doses of *trans*-10, *cis*-12 CLA (0.5% to 1% of diet) have reported dramatic loss of adipose tissue (lipodystrophy), inflammation, insulin resistance, and hepatic steatosis. However, recent investigations have demonstrated that a low dose of *trans*-10, *cis*-12 CLA was effective in reducing adipose tissue lipogenesis in mice without off-target induction of inflammation, insulin resistance, and hepatic steatosis (47, 106, 107). Thus, it is increasingly apparent

that low and high doses of *trans*-10, *cis*-12 CLA induce drastically different phenotypes presumably through different mechanisms. Future work with low doses of *trans*-10, *cis*-12 CLA in rodent models is needed to provide additional insight into the physiological mechanisms for the regulation of milk fat synthesis by biohydrogenation intermediates.

Mouse milk fat has a lower proportion and a different pattern of de novo synthesized FA (C10 to C16) than dairy cow milk fat (C4 to C16; 14, 100). However, this proportion is adequate to examine the regulation of their synthesis, which responds to dietary changes similar to dairy cows (for reviews, see 9, 145). In growing rodents, FA synthesis occurs at high rates in both liver and adipose tissue, and this differs from ruminants, where little or no lipogenesis occurs in the liver (12). Similar to the bovine, adipose tissue lipogenesis is drastically decreased during lactation in rodents (<2% that of mammary), but this is less clear for liver (see summary by 9, 145). This raises the question of whether hepatic de novo synthesis might contribute to milk FA in rodents. Most studies with rodents have indicated that in vivo rates of hepatic lipogenesis do not differ between pregnancy and lactation and do not respond to physiological manipulations in a manner parallel to mammary lipogenic rates (for reviews, see 1, 145). Overall, although there are specie differences, the previous discussion and recent validations provide confidence in the use of the lactating mouse model to investigate regulation of de novo lipid synthesis as it relates to the lactating dairy cow.

The SREBP1c null mouse has a 41% decrease in milk fat concentration (123). Interestingly, the extent of inhibition in this model approximates the maximum reduction (~50%) in milk fat synthesis observed during bovine MFD. We have conducted a preliminary study to test the ability of *trans*-10, *cis*-12 CLA to induce MFD in a mouse with mammary-specific disruption of the SCAP gene, thus eliminating SREBP1 activation (123). At the *trans*-10, *cis*-12 CLA dose used (20 mg/d, ~0.23% of diet), there was a 25% decrease in de novo synthesized FA in the wild-type mice but no change in the SCAP-null mice (48).

A S14-null mouse was created to investigate the function of this gene, but it has provided few clear answers (80, 148). Surprisingly, the S14-null mouse has increased hepatic lipogenesis (148). Anderson et al. (5) also reported that S14-null mice were protected from diet-induced obesity. Zhu et al. (147) reported that the S14-null mouse fed a moderate-fat breeder diet had a 62% reduction in mammary lipid synthesis but no reduction in lipogenic enzyme message or activity. Interestingly, mammary ACC activity was increased over 70% in the S14-null mouse using the traditional high-citrate assay (147), and mammary tissue malonyl-CoA concentration increased in S14-null dams, supporting increased ACC activity in vivo (80). S14 has a functional role in milk fat synthesis in the mouse, thereby providing an important implication for the *trans*-10, *cis*-12 CLA-induced decrease in S14 expression during MFD for mice and cows, although the exact mechanism is not clear.

Other Mechanisms

Investigations with model systems have shown a great diversity of FA-sensor proteins (for reviews, see 46, 71, 126). Other candidate regulators have been investigated on the basis of biomedical literature and microarray data. First, HNF4α is not expected to be an important regulator of mammary lipogenesis, as expression is 15,500-fold higher in bovine liver, a nonlipogenic tissue, than in lactating mammary, and expression does not differ between lactating and nonlactating mammary tissue (62). In addition, expression of the liver X receptors (LXRs) does not change during MFD in dairy cows (62), although McFadden & Corl (94b) report increased lipogenesis in mammary epithelial cells treated with an LXR agonist. Peroxisome proliferator-activated receptor (PPAR)γ was increased in lactating compared to nonlactating mammary tissue (25), but

these results may be related to differentiation and the initiation of milk synthesis rather than the regulation of milk fat synthesis during established lactation. Furthermore, the literature does not support a direct mechanism for CLA through PPARγ; PPARα and PPARγ are activated equally well by *trans*-10, *cis*-12 CLA, which induces MFD, and *cis*-9, *trans*-11 CLA, which does not (99, 146). Additionally, PPARγ agonist increased expression of lipid synthesis enzymes in a bovine mammary epithelial cell line (72), which is the opposite response to that observed for lipogenic genes during diet-induced MFD.

Evidence strongly supports a role of SREBP1 and S14 in MFD, although the mechanisms whereby *trans*-10, *cis*-12 CLA inhibits these signaling factors have not been established. Cross-talk is common in regulatory systems including the regulation of lipid synthesis; we find the most convincing arguments and data for factors that connect with the regulation of SREBP1c and S14, although efforts to investigate other candidates should continue. Recently, ER stress has gained prominence as a possible causative factor of insulin resistance and metabolic disease (for reviews, see 54, 68), and mammary deletion of protein kinase-like endoplasmic reticulum kinase (PERK) in the mouse reduced milk fat synthesis, which was proposed to occur through inhibition of SREBP1. We have observed increased expression of other ER stress factors, tribbles homolog-3 (TRB3) and activating transcription factor 4 (ATF4), in the mammary gland during some instances of MFD without induction of other classical markers of ER stress, including X-box binding protein 1 mRNA splicing and induction of protein chaperones [e.g., heat shock protein 5 (60)]. Recent publications with growing mice also report increased expression of TRB3 in adipose tissue of *trans*-10, *cis*-12 CLA-treated mice (81) and activation of some components of ER stress, but no change in chaperone proteins during *trans*-10, *cis*-12 CLA treatment of mouse mammary tumor cells (103).

APPLICATIONS IN ANIMAL AGRICULTURE

The ability of *trans*-10, *cis*-12 CLA and other unique rumen-derived biohydrogenation intermediates to profoundly affect milk fat synthesis in dairy cattle and other commercially relevant lactating farm animals raises two important issues. First, what are the nutritional and management factors that lead to an increase in rumen production of these unique bioactive biohydrogenation intermediates that results in reduced milk fat synthesis, and second, are there situations where a controlled reduction in milk fat synthesis is beneficial with respect to sparing of nutrients for other metabolic processes? These aspects are briefly discussed below. The reader is directed to other reviews for additional information on these issues (55, 84).

Relationship to Diet-Induced Milk Fat Depression in Dairy Cattle

The relationship between dietary factors and milk fat synthesis has been long recognized in ruminant nutrition. Prior to development of the biohydrogenation theory, ration-balancing strategies to reduce MFD were simply based on correlative evidence and prior experience. The biohydrogenation theory has provided a foundation for mechanistic and principal-based approaches to optimizing milk fat production. Available evidence supports biohydrogenation intermediates as the only cause of diet-induced milk fat depression observed in research experiments and field cases. While nutritional physiologists have investigated the phenotype and mechanism of biohydrogenation intermediates over the past dozen years, ruminant nutritionists have investigated ruminal biohydrogenation pathways and dietary factors that modify them, and nutrition consultants have applied this knowledge to ration balancing. An understanding of the causative components has significantly reduced expense and waste in attempting to alleviate MFD and has provided troubleshooting tools for dairy producers.

Reduced milk fat, however, is still commonly observed today because of the complex interaction of dietary and environmental factors that dictate ruminal biohydrogenation.

The changes in ruminal microbial processes during development of MFD are centered on both an altered rumen environment and an alteration in the rumen pathways of PUFA biohydrogenation (105). In general, no single dietary factor is responsible for MFD, and it is often the interactions between various dietary components that increase the rumen outflow of biohydrogenation intermediates associated with MFD. Dietary components can increase the risk of MFD by increasing substrate supply (18-carbon unsaturated FA), altering rumen biohydrogenation pathways, and altering rates of biohydrogenation. Further research is required to better understand the ruminal conditions that promote the formation of biohydrogenation intermediates (e.g., *trans*-10, *cis*-12 CLA) that may trigger MFD. An improved understanding of these events will provide the critical framework with which to better troubleshoot MFD on commercial dairy farms.

Potential Use of *trans*-10, *cis*-12 CLA as a Management Tool

Dietary supplementation with *trans*-10, *cis*-12 CLA to reduce milk fat yield has potential use as a management tool in milk production. Milk fat is the major "cost" of milk synthesis, accounting for over one-half of the energy needed for milk synthesis; consequently, a reduction in milk fat output will result in a sparing of nutrients that can be used for other purposes. This could have application in commercial situations; for example, in markets where milk production is regulated by a quota system based on milk fat and in situations where cows cannot consume sufficient nutrients to meet their requirements. The ability to selectively reduce milk fat yield could reduce energy demands during times when nutrient intake is inadequate, such as the onset of lactation and the early lactation period, and under adverse environmental conditions such as heat stress or weather-related feed shortages (see 55).

Commercial application of *trans*-10, *cis*-12 CLA as a management tool requires a CLA formulation that must have two characteristics: It must offer protection of *trans*-10, *cis*-12 CLA from alterations by rumen bacteria, and it must subsequently become available for absorption in the small intestine. To date, the majority of research on rumen-protected *trans*-10, *cis*-12 CLA has used supplements consisting of calcium salts of free FA. A consistent reduction in the level of milk fat has also been observed in studies using calcium salts of *trans*-10, *cis*-12 CLA over treatment periods ranging from 3 to 20 weeks involving primiparious and multiparious cows at different stages of lactation and under different dietary and management practices (23, 50, 97, 108, 116, 127). The preparation of dietary supplements containing *trans*-10, *cis*-12 CLA using other methods of rumen-protection has been investigated less extensively compared to calcium salts of *trans*-10, *cis*-12 CLA, but supplements have included formulations where the protection was by treatment with formaldehyde, the formation of amide bonds, and lipid encapsulation (44, 111).

In some feeding and management systems, the reduction in milk fat yield has allowed for a repartitioning of nutrients to support increased milk and milk protein yield (e.g., 23, 87, 92, 95, 101). Producers may also find it advantageous to induce MFD during periods of limited feedstuff availability, such as inadequate rainfall in pasture-based systems or for a short period while breeding. Changes in body weight or body composition are difficult to adequately quantify in ruminants, but increased rumen-empty body weight gain (57) and increased expression of lipid synthesis enzymes and lipogenic signaling in adipose tissue (63) have been reported during MFD. Inducing MFD during breeding periods may also be a useful management practice to improve short-term energy balance and subsequently reproductive efficiency, although caution is important in application of classical MFD diets. Consequently, it appears that during

times of inadequate nutrient intake, inducing a reduction in milk fat increases available energy that can be repartitioned toward the synthesis of milk or milk protein, and there are currently commercial CLA supplements available in some countries that are marketed for this specific purpose. Finally, a recent multistudy survival analysis demonstrated that dietary supplements of *trans*-10, *cis*-12 CLA improved time to conception and pregnancy rates, although effects appeared to be independent of effects on energy status (41). Additional aspects of the potential application of dietary supplements of *trans*-10, *cis*-12 CLA as a management tool are discussed by Griinari & Bauman (55).

FUTURE DIRECTIONS

The study of MFD may arguably be the most complete and successful example of nutrigenomics in present-day animal science research and offers the potential for many valuable applications. Knowledge of the basis for MFD allows the development of feeding strategies and provides the opportunity to troubleshoot commercial problems in low milk fat production. Investigations of MFD have also highlighted key regulatory mechanisms in mammary lipid synthesis, and this provides a platform for the development of methods to alter milk fat yield and improve the FA profile of milk fat.

MFD is dependent on complex interactions of dietary, animal, and environmental factors. Our understanding of the complex rumen dynamics that result in the production of *trans*-10, *cis*-12 CLA and related bioactive FAs has lagged behind the significant advances made in the impact of these FA on mammary synthesis of milk fat. Identifying these interactions will require mechanistic experimental approaches, and results will aid in development of nutritional models capable of predicting ruminal biohydrogenation intermediate synthesis and milk fat yield. An important component of these efforts will be the identification of additional rumen-derived bioactive fatty acids that can regulate tissue rates of FA synthesis. Furthermore, little attention has been given to the investigation of environmental factors such as environmental temperature, housing design, and feeding behavior.

Investigation of the cellular mechanisms in the regulation of milk fat synthesis during established lactation will continue to be a fruitful direction for future research. The presence of the bioactive CLA isomers in milk fat is a clear indication of their uptake by mammary epithelial cells, but their direct or indirect interaction with candidate signaling mechanisms is unknown. Results to date clearly show that the knowledge of the regulation of milk fat synthesis by specific rumen-derived bioactive FAs applies across species, and thus this exciting example of nutrigenomics has broader implications and applications. Recent advances demonstrate the importance of both the cow and mouse as models to investigate the role of bioactive fatty acids in the regulation of milk fat synthesis during lactation and the value of their continued use. Future advances using these animal models will have important implications for our general understanding of mammary biology as well as invaluable applications in the dairy industry.

SUMMARY POINTS

1. Diet-induced milk fat depression is a reduction in milk fat caused by specific bioactive fatty acids produced during ruminal biohydrogenation under some dietary conditions.
2. Multiple conjugated linoleic acid isomers have been observed to reduce milk fat synthesis in the cow, but most mechanistic research has focused on *trans*-10, *cis*-12 conjugated linoleic acid.

3. Whole-animal metabolism, including glucose and insulin signaling, are not modified during diet-induced milk fat depression.
4. During milk fat depression, mammary lipid synthesis capacity is decreased due to a coordinated down-regulation of lipid synthesis enzymes.
5. SREBP1 and S14 are down-regulated in mammary tissue during milk fat depression, but their direct interaction with bioactive fatty acids of ruminal biohydrogenation has not been delineated.
6. Results demonstrate the value of both the dairy cow and mouse as models to investigate the role of bioactive fatty acids in the regulation of milk fat synthesis during lactation.
7. The mechanistic understanding of the regulation of milk fat synthesis gained from investigations of diet-induced milk fat depression has had a substantial impact on dairy management and nutrition strategies.
8. The study of milk fat synthesis and its regulation by unique bioactive fatty acids is one of the most complete and successful examples of nutrigenomics in present-day animal science research.

DISCLOSURE STATEMENT

D.E.B. is coinventor on CLA-use patents that are assigned to Cornell Research Foundation, Inc. K.J.H. and A.L.L. are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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