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Recent advances in the regulation of milk fat synthesis

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In addition to its economic value, milk fat is responsible for many of milk's characteristics and can be markedly affected by diet. Diet-induced milk fat depression (MFD) was first described over a century ago and remains a common problem observed under both intensive and extensive management. The biohydrogenation theory established that MFD is caused by an inhibition of mammary synthesis of milk fat by specific fatty acids (FA) produced as intermediates in ruminal biohydrogenation. During MFD, lipogenic capacity and transcription of key lipid synthesis genes in the mammary gland are down-regulated in a coordinated manner. Our investigations have established that expressions of sterol response element-binding protein 1 (SREBP1) and SREBP-activation proteins are down-regulated during MFD. Importantly, key lipogenic enzymes are transcriptionally regulated via SREBP1. Collectively, these results provide strong evidence for SREBP1 as a central signaling pathway in the regulation of mammary FA synthesis. Spot 14 is also down-regulated during MFD, consistent with a lipogenic role for this novel nuclear protein. In addition, SREBP1c and Spot 14 knock-out mice exhibit reduced milk fat similar to the magnitude and pattern of MFD in the cow. Application of molecular biology approaches has provided the latest chapter in the regulation of milk fat synthesis and is reviewed along with a brief background in nutritional regulation of milk fat synthesis in ruminants.

Keywords: lipid synthesis, mammary, milk fat, milk fat depression

Introduction

Milk fat plays a central role in dairy products and farm efficiency. Fat is a major contributor to the energy density of whole milk and is essential to many of the physical properties, manufacturing qualities and organoleptic characteristics of dairy products. Traditionally, saturated fatty acids (FA) in milk fat have caused concern among human health experts, although more recently milk fat has garnered appreciation as a functional food due to the health-promoting potential of some FA found specifically in ruminant-derived products (Bauman *et al.*, 2006).

From the producers' perspective, milk fat represents a major component of the value of milk, but it is also a significant portion of the energy cost of lactation. Fat is the most variable component of milk and is affected by many factors including genetics, physiological state and environment. However, milk fat is especially responsive to nutrition, providing a practical tool to alter its yield and composition. First described over 150 years ago, diet-induced milk fat depression (MFD), or low-fat milk syndrome, is characterized by a decrease in milk fat yield of up to 50%, with no change in milk yield or in the yield of other milk components (Bauman and Griinari, 2001). MFD is classically observed in ruminants fed highly fermentable diets or in diets that contain plant or fish oil supplements. MFD is also observed in pasture feeding and, although not well studied, appears to be related to increased fermentability and passage rates that occur at some stages of plant growth and possibly slug feeding of grain under some management schemes. Varying levels of MFD are commonly experienced today in both intensively and extensively managed dairy herds, and this represents a level of milk fat production below the genetic potential of the cow. MFD is also a useful variable for evaluating herd management; in many cases onset of diet-induced MFD is an indication of modified ruminal fermentation and in more pronounced cases this can be associated with ruminal acidosis and reduced ruminal efficiency.

Diet-induced MFD involves an inter-relationship between ruminal fermentation and mammary tissue metabolism. This phenomenon has been of research interest over the past century, and has been extensively investigated over the last quarter of the century. The discovery that changes in milk fat yield were negatively correlated with milk fat concentration of *trans* FA provided key insight in understanding MFD (Davis and Brown, 1970). Based on this, Bauman and Griinari (2001) proposed the 'biohydrogenation theory', which states that diet-induced MFD relates to an inhibition of mammary lipid synthesis by specific FA that are intermediates in the

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biohydrogenation of dietary polyunsaturated fatty acid (PUFA), and these are only produced under certain conditions of altered ruminal fermentation. Recent investigations have verified this theory, and over the last decade, research has focused on (1) identifying the causative biohydrogenation intermediates, (2) describing the metabolic phenotype of MFD, (3) delineating dietary risk factors to aid in troubleshooting and mitigating MFD on farms and (4) defining the mechanism(s) by which these bioactive FA isomers are able to regulate mammary synthesis of fat. Broad aspects of the relationship between nutrition and milk fat have been extensively reviewed elsewhere (Chilliard *et al*., 2000; Bauman and Griinari, 2001 and 2003; Lock and Shingfield, 2004; Shingfield and Griinari, 2007). In the following sections, we will provide background and discuss recent advances and the current understanding of MFD based on investigations of diet-induced and conjugated linoleic acid (CLA)-induced MFD, including recent insight provided by molecular approaches.

Background

Ruminant milk fat is estimated to include over 400 individual FA that differ primarily in chain length and number and orientation of unsaturated bonds (Jensen, 2002). Over 95% by mass of the FA are esterified in triglycerides while the remaining are found in phospholipid, cholesterol ester, diglyceride, monoglyceride and free FA fractions. Short- and medium-chain FA (4 to 14 carbons) and a portion of the 16-carbon FA are derived from *de novo* synthesis from acetate and to a lesser extent β-hydroxybutyrate. On a molar basis, about one-half of milk FA are synthesized *de novo* (see review by Bauman and Davis, 1974). Preformed FA account for the remaining 16-carbon and all of the longer-chain FA ($>$16 carbons), and are taken up from the circulating plasma pool. These FA originate from absorption from the digestive tract or mobilization from body reserves. Adipose tissue mobilization accounts for less than 10% of preformed FA in milk fat, except during periods of negative energy balance when their proportion increases substantially (Bauman and Griinari, 2001). Lastly, due to the hydrophobic nature of esterified FA, milk fat is secreted from the mammary epithelial cell as a lipid droplet surrounded by a protein-rich polar lipid coat called the milk fat globule membrane (MFGM; see reviews by Mather and Keenan (1998), Keenan (2001) and Olivier-Bousquet (2002)). The origin of the MFGM and the mechanism of cellular milk fat secretion continue to be areas of intense investigation. Proteomic approaches have identified many of the associated proteins (Reinhardt and Lippolis, 2006; Cavaletto *et al*., 2008), and knock-out mouse models have demonstrated the essential role for some of these proteins in milk fat secretion including butyrophilin (Ogg *et al*., 2004) and xanthine oxidoreductase (McManaman *et al*., 2002).

Historically, nutrition researchers identified an association between diet and milk fat yield, and reports over the last century provide evidence of the interaction between ruminal fermentation and milk fat synthesis (see reviews by Davis and Brown, 1970; Doreau *et al*., 1999; Bauman and Griinari, 2001). Davis and Brown (1970) categorized two types of diets that induce MFD: (1) those that contained high levels of fermentable carbohydrate and low levels of fiber and (2) those that contained high concentrations of unsaturated oils. The extent of MFD with such diets is modified by many factors including associative dietary effects, feed management practices and animal physiological state (see reviews by Sutton, 1989; Grummer, 1991; Palmquist *et al*., 1993; Chilliard *et al*., 2000; Lock *et al*., 2006a). Researchers quickly realized that many diets that caused MFD also resulted in alterations in ruminal environment and fermentation, most notably a decrease in pH and a decrease in the acetate to propionate molar ratio (Bauman and Griinari, 2001).

Biohydrogenation intermediates

Ruminant diets contain a low percentage of fat, although a significant intake of PUFA is generated from forages and oilseeds and in some cases from fat supplements. Dietary FA are metabolized by ruminal microbes resulting in a large difference between the dietary profile and the profile of FA absorbed from the small intestine (Doreau and Chilliard, 1997; Chilliard *et al*., 2000). Ruminal FA metabolism was summarized in the classic review by Harfoot and Hazlewood (1988) and more recently by others (Palmquist *et al*., 2005; Jenkins *et al*., 2008). Most dietary FA are esterified, and they are almost completely hydrolyzed to free FA in the rumen. Unsaturated free FA are then isomerized (double-bond position or orientation changed) and reduced (saturation of double bond), although the exact mechanisms are not well established (Wallace *et al*., 2007). The resulting saturated FA and some of the biohydrogenation intermediates escape the rumen and are subsequently absorbed.

Davis and Brown (1970) recognized that *trans*-C18:1 FA were increased in milk fat of cows with low-fat milk syndrome. They suggested that these *trans* FA originated from incomplete ruminal biohydrogenation of unsaturated FA and might contribute to the development of MFD. Subsequent studies have demonstrated a clear relationship between *trans* FA and MFD (see reviews by Bauman and Griinari, 2001 and 2003; Shingfield and Griinari, 2007). Briefly, although feeding unsaturated oils induced MFD, feeding completely hydrogenated (saturated FA) oils had minimal effects on milk fat yield. Moreover, abomasal infusion of unsaturated FA did not reduce milk fat yield, but abomasal infusion of partially hydrogenated FA (high *trans* FA) did reduce milk fat yield.

Trans-11 C18:1 and *cis*-9, *trans*-11 CLA are the predominant *trans* FA intermediates produced from the ruminal metabolism of linoleic acid (Figure 1; Harfoot and Hazlewood, 1988); however, ruminal biohydrogenation pathways are dynamic, allowing the production of a wide range of positional and geometric isomers as well as modified FA such as hydroxy and keto derivatives (Palmquist *et al*., 2005; Jenkins *et al*., 2008). These isomers are absorbed and incorporated into milk fat, thereby allowing the milk FA

profile to be used as a proxy of changes occurring in the rumen. The concentration of *trans*-18:1 and CLA isomers in milk fat is very dynamic as summarized in Table 1; however, the high values for many of these FA isomers represent experimental conditions involving less-typical diets (Lock and Bauman, 2004; Shingfield and Griinari, 2007). The predominant metabolic pathways and the microbial capacity for isomerization and biohydrogenation depend on the microbial population and the ruminal environment (Allen, 2000; Palmquist *et al.*, 2005; Jenkins *et al.*, 2008). Dietary factors that affect ruminal fermentation (e.g. high carbohydrate fermentability, high oil, rumensin) modify ruminal FA metabolism through complex associative effects that result in altered ruminal microbial populations, altered pathways of PUFA biohydrogenation (Figure 1), and ruminal outflow of a wide range of biohydrogenation intermediates, and this is reflected in the milk FA composition (Table 1). Although we have focused our discussion on the products of linoleic acid, other PUFA including the long-chain n-3 FA are also biohydrogenated and likely produce unique FA intermediates.

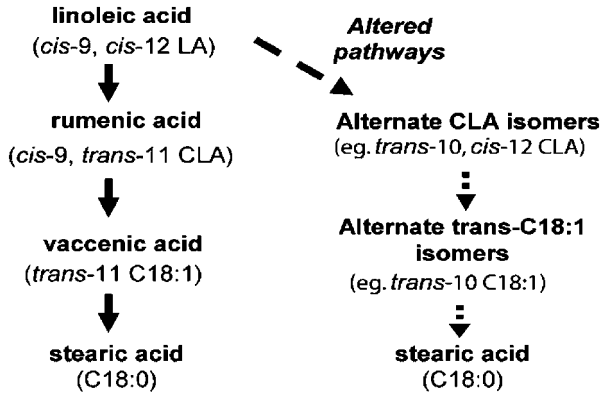

Figure 1 Pathways of ruminal biohydrogenation of linoleic acid (LA) and conjugated linoleic acid (CLA) under normal and altered ruminal fermentation. Adapted from Griinari and Bauman (1999).

Advances in lipid analysis and renewed interest in *trans* FA identified a correlation between MFD and an increase in *trans*-10 C18:1, rather than *trans*-C18:1 FA in general (Griinari *et al.*, 1998). Based on this evidence, Bauman and Griinari (2001) postulated that altered ruminal fermentation experienced in some diets resulted in a shift in biohydrogenation pathways and that either *trans*-10 C18:1 or related metabolites could be the cause of MFD (Bauman and Griinari, 2001). Based on biochemical evidence and a strong relationship between milk *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA, a putative altered biohydrogenation pathway was proposed where linoleic acid was isomerized to *trans*-10, *cis*-12 CLA followed by reduction to *trans*-10 C18:1 and finally a second reduction to C18:0 (Figure 1; Bauman and Griinari, 2001). Increased *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA in duodenal flow and milk demonstrates altered ruminal FA metabolism and are hallmarks of diet-induced MFD. These isomers are normally formed at low rates but ruminal outflows of *trans*-10 C18:1 and to a lesser extent *trans*-10, *cis*-12 CLA are substantially increased during diet-induced MFD (Bauman and Griinari, 2003). Increased ruminal outflow could result from the increased formation or less-complete biohydrogenation of these isomers.

The relationship between specific ruminal FA isomers and milk fat yield is strong and highly repeatable, but it is simply correlative evidence, indicating a possible direct role in

Table 1 *Range of* trans*-C18:1 and conjugated C18:2 concentrations reported for milk fat*[1]

Trans-C18:1		Conjugated C18:2	
Isomer	Total *trans*-18:1 (%)	Isomer	Total conjugated 18:2 (%)
trans-4	0.3 to 2.3	*trans*-6, *trans*-8	nd to 1.4
trans-5	nd to 1.4	*trans*-7, *cis*-9	1.2 to 9.5
trans-6-8	0.5 to 11.3	*trans*-7, *trans*-9	nd to 2.8
trans-9	3.0 to 18.2	*cis*-8, *trans*-10	nd to 1.7
trans-10	3.4 to 29.8	*trans*-8, *cis*-10	nd to 2.3
trans-11	24.5 to 74.9	*trans*-8, *trans*-10	0.2 to 0.7
trans-12	1.9 to 17.9	*cis*-9, *trans*-11	65.6 to 91.2
trans-13+14	<1.0 to 23.1	*trans*-9, *cis*-11	nd to 3.9
trans-15	3.3 to 11.1	*trans*-9, *trans*-11	0.8 to 3.2
trans-16	1.7 to 12.5	*trans*-10, *cis*-12	nd to 1.6
		trans-10, *trans*-12	0.3 to 1.4
		cis-11, *trans*-13	nd to 4.7
		trans-11, *cis*-13	0.1 to 9.3
		trans-11, *trans*-13	0.3 to 6.0
		cis-12, *trans*-14	nd to 1.1
		trans-12, *trans*-14	0.3 to 3.6
		trans-13, *trans*-15	nd to 0.2
		cis–*cis* isomers	0.1 to 4.8

Values less than 0.1% are shown as not detected (nd).
[1]Adapted from summary by Lock and Bauman (2004) and Shingfield *et al.* (2008a).

MFD. For example, Kadegowda *et al*. (2008) recently suggested MFD was caused by *trans*-7 C18:1 or *trans*-7, *cis*-9 CLA based on correlations with milk fat percent. Because diet-induced MFD is associated with altered rumen fermentation and biohydrogenation pathways, the rumen outflow and milk fat content of many *trans*-C18:1 and CLA isomers are correlated with MFD (e.g. Loor *et al*., 2005; Kadegowda *et al*., 2008). To demonstrate causative relationships, specific isomers must be tested and since few are commercially available this has often required that they be chemically synthesized and purified by investigators. This approach has been extensively used for CLA isomers and evaluations of bioactivity have included biomedical models. Early experiments with animal models have demonstrated a range of positive biological responses including anti-carcinogenic effects (reviewed by Ip *et al*., 2002; Kelley *et al*., 2007) and reduced adiposity (reviewed by Pariza, 2004), although only a mixed preparation including both *cis*-9, *trans*-11 and *trans*-10, *cis* 12 CLA was available in the original experiments. Similarly, this mixed CLA preparation also reduced milk fat yield in dairy cows (Loor and Herbein, 1998; Chouinard *et al*., 1999a and 1999b). Subsequently, the ability to prepare relatively pure CLA isomers allowed the seminal demonstration by Baumgard *et al*. (2000) that *trans*-10, *cis*-12 CLA markedly reduced milk fat synthesis, whereas *cis*-9, *trans*-11 CLA had no effect (Figure 2). Multiple experiments including dose titrations have shown a clear curvilinear relationship between abomasal infusion of *trans*-10, *cis*-12 CLA and the reduction in milk fat yield. de Veth *et al*. (2004) summarized data from abomasal infusion experiments and showed that 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 (de Veth *et al*., 2004); a recent updated analysis by Shingfield and Griinari (2007) found the same relationship.

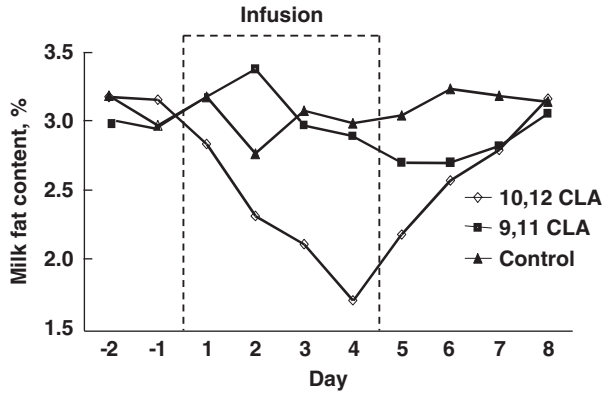

Figure 2 Temporal pattern of milk fat content during abomasal infusion of conjugated linoleic acid (CLA) supplements. Infusions were for 4 days and treatments were control, *cis*-9, *trans*-11 CLA (10 g/day), and *trans*-10, *cis*-12 CLA (10 g/day). Used with permission from Baumgard *et al*. (2000).

Diet-induced MFD results in ruminal outflow of a large number of FA isomers, not just *trans*-10, *cis*-12 CLA. The milk fat concentration of *trans*-10, *cis*-12 CLA is linearly related to the abomasally administered dose, and milk fat *trans*-10, *cis*-12 CLA concentration is curvilinearly related to the decrease in milk fat yield during abomasal infusion (de Veth *et al*., 2004). Therefore, milk fat *trans*-10, *cis*-12 CLA concentration can be used to predict the expected extent of MFD contributable to the *trans*-10, *cis*-12 CLA isomer during diet-induced MFD (Peterson *et al*., 2003). Although there is a relationship between milk fat concentration of *trans*-10, *cis*-12 CLA and milk fat yield during diet-induced MFD, it is clear this CLA isomer only accounts for a modest portion of the MFD observed during most diet-induced MFD conditions (Peterson *et al*., 2003; Griinari and Bauman, 2006; Shingfield *et al*., 2008a). Furthermore, there is often little or no change in *trans*-10, *cis*-12 CLA in the milk fat when MFD is induced by feeding fish oils (reviewed by Shingfield and Griinari, 2007). These observations have provided the impetus to identify additional bioactive FA isomers.

Purified or highly enriched preparations of other CLA isomers have been tested in the dairy cow and results are summarized in Table 2. Thus far, three CLA isomers have been identified as inhibitors of milk fat synthesis, although

Table 2 *Conjugated linoleic acid (CLA) isomers tested for their effect on milk fat yield and milk desaturase index in dairy cows*

CLA isomer tested	Effects on milk fat yield	Effects on milk desaturase index[3]	Reference[4]
trans-8, *cis*-10	NC[1]	NC[1]	A
trans-9, *cis*-11	Inhibition[2]	Decreased[2]	B
cis-9, *trans*-11	NC	NC	C to F
trans-9, *trans*-11	NC	Decreased	B
trans-10, *cis*-12	Inhibition	Decreased	A to E, G to L
cis-10, *trans*-12	Inhibition	Decreased	K
trans-10, *trans*-12	NC	Decreased	K,L
cis-11, *trans*-13	NC	NC	A

[1]NC = no change when abomasally infused at a dose comparable to *trans*-10, *cis*-12 CLA.
[2]Inhibited milk fat yield or decreased desaturase index when abomasally infused at a dose comparable to *trans*-10, *cis*-12 CLA.
[3]Desaturase index is the ratio of product/(substrate + product) for Δ^9-desaturase.
[4]Reference citations are as follows: A = Perfield *et al*. (2004); B = Perfield *et al*. (2007); C = Baumgard *et al*. (2000); D = Baumgard *et al*. (2002a); E = Loor and Herbein (2003); F = Shingfield *et al*. (2007); G = de Veth *et al*. (2004); H = Baumgard *et al*. (2001); I = Peterson *et al*. (2002); J = Harvatine *et al*. (2006); K = Saebo *et al*. (2005a); L = Perfield *et al*. (2006).

Table 3 Trans-*C18:1 fatty acids tested for their effect on milk fat yield in dairy cows*[1]

C18:1 Isomer tested	Dose (g/day)	Isomer in milk fat (%)	Effects on milk fat yield	Reference[4]
trans-9	25	NR[1]	NC[2]	A
	42	3.21	NC	B
trans-10	43	1.11	NC	C
trans-11	12.5	2.86[3]	NC	D
	7.5 to 30	1.63 to 2.72	NC	E
	41	3.20	NC	B
trans-12	12.5	0.99[3]	NC	D
	7 to 29	0.87 to 2.39	NC	E

[1]NR = not reported.
[2]NC = no change in milk fat yield when abomasally infused.
[3]Estimated from graphical presentation.
[4]Reference citations are as follows: A = Rindsig and Schultz (1974); B = Tyburczy *et al.* (2008); C = Lock *et al.* (2007); D = Griinari *et al.* (2000); E = Shingfield *et al.* (2007).

the examination of two of these has involved only a single study conducted at a single dose. Nevertheless, it is clear that small differences in FA structure can result in striking differences in FA action and potency. For example, *trans*-10, *trans*-12 CLA and *trans*-9, *trans*-11 CLA do not reduce milk fat synthesis, but both are potent inhibitors of stearoyl-CoA desaturase (SCD; Δ^9-desaturase) as indicated by changes in the desaturase index; the desaturase index represents the relationship between product and precursor for SCD and is commonly used as a proxy for SCD activity. Likewise, *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA reduce milk fat yield, but the former appears less effective and the latter appears more effective than *trans*-10, *cis*-12 CLA (Table 2; Saebo *et al.*, 2005a; Perfield *et al.*, 2006 and 2007). The biologically active isomers identified to date elicit distinct responses (e.g. 50% decrease in milk fat) with doses less than 10 g/day in the dairy cow. Given the potency of these FA, it is clear that low concentrations of additional unidentified biohydrogenation intermediates could be responsible for a portion of the response observed during diet-induced MFD.

Trans-C18:1 FA have received much less attention due to the technical challenges of making purified preparations. Traditionally, partially hydrogenated vegetable oils (PHVO) have been used to represent an enriched preparation of a range of *trans*-C18:1 FA, and abomasal infusion of 300 to 750 g/day of PHVO caused a reduction in milk fat (Astrup *et al.*, 1976; Selner and Schultz, 1980; Gaynor *et al.*, 1994; Romo *et al.*, 1996). Calcium salts of PHVO (100 to 400 g/day) have also been fed and milk fat yield was modestly reduced (5% to 15%) in one study (Piperova *et al.*, 2004) and unaffected in another (Selberg *et al.*, 2004). The observed milk fat decrease from PHVO has routinely been attributed to *trans*-18:1 FA; however, *trans*-C18:1 represent only about 40% to 60% of the total FA in PHVO, and many other unusual FA isomers, including CLAs, are formed in the production of PHVO (Banni *et al.*, 1994; Jung and Ha, 1999). Thus, when one considers the potency of *trans*-10, *cis*-12 CLA, it is clearly possible that the PHVO-induced reduction in milk fat could be due to small amounts of unique FA other than the *trans*-C18:1 isomers.

To establish causative roles for *trans*-C18:1 isomers on milk fat synthesis, purified preparations of the isomers must be tested and studies that have directly examined the effects of *trans*-C18:1 isomers are summarized in Table 3. In investigating the bioactivity of *trans*-C18:1 FA, the effects of *trans*-10 C18:1 were of special interest because the reduction in milk fat is highly correlated both with rumen outflow and with the milk fat content of *trans*-10 C18:1 (Loor *et al.*, 2005; Hinrichsen *et al.*, 2006; Kadegowda *et al.*, 2008). Duodenal flow of *trans*-10 C18:1 is very dynamic and the maximal reduction in milk fat appears to correspond to a rumen outflow of $\sim$20 to 40 g/day, although much higher outflows can occur, especially in experimental diets that are high in fish oil (Lock *et al.*, 2007; Shingfield and Griinari, 2007). Likewise, a near-maximal reduction in milk fat yield is observed at a milk fat concentration of $\sim$1.5% to 2.5% *trans*-10 18:1, although much higher concentrations do occur with certain experimental diets (Loor *et al.*, 2005; Hinrichsen *et al.*, 2006; Shingfield and Griinari, 2007; Kadegowda *et al.*, 2008). Of specific importance is a recent study that abomasally infused 43 g/day of *trans*-10 C18:1 (Lock *et al.*, 2007). Although *trans*-10 C18:1 was incorporated into milk fat at a concentration predicted to decrease milk fat concentration by 0.4 to 0.5 percentage units (Kadegowda *et al.*, 2008), well within the power of the controlled abomasal infusion experiment, there was no effect on milk fat yield, and milk fat concentration was numerically increased by 0.07 percentage units (Lock *et al.*, 2007). Likewise, no MFD was observed when the milk fat concentration of *trans*-10 C18:1 was increased by feeding high-oleic sunflower oil (Hinrichsen *et al.*, 2006). Abomasal infusions of other *trans*-C18:1 isomers have also reported no effect on milk fat synthesis.

Phenotype of milk fat depression

The characteristics of MFD provide insights into the mechanism(s) by which biohydrogenation intermediates are able to regulate milk fat synthesis. First, MFD is a specific reduction in milk fat yield with no change in the yields of milk or milk protein; this phenotype is sustainable for long periods as shown by studies involving CLA-induced MFD (Perfield *et al.*, 2002; Bernal-Santos *et al.*, 2003; Castañeda-Gutiérrez *et al.*, 2007). In addition, milk fat synthesis is

rescued after termination of CLA treatment, with the time course for recovery being similar to the progressive pattern of decline (Baumgard *et al.*, 2000). Likewise, dietary modifications allow for a return to normal milk fat production following diet-induced MFD. These results demonstrate that the mechanisms of MFD are specific for cellular processes related to lipid synthesis and do not include broader effects such as a generalized impairment of cellular function or induction of apoptosis. Second, the mammary reduction in milk fat synthesis during MFD is rapid. This has been most clearly demonstrated with abomasal infusion of *trans*-10, *cis*-12 CLA in dairy cows where milk fat percent progressively decreased with a significant reduction by 10 h (Harvatine and Bauman, 2007b) and a nadir was achieved by 3 to 4 days (Baumgard *et al.*, 2000). Diet-induced MFD develops over a longer interval ($\sim$ 7 to 18 days) consistent with the adaptations in ruminal fermentation that are required to produce altered biohydrogenation products (Shingfield *et al.*, 2006b); however, MFD was induced more rapidly when cows switched to a low-forage diet and also had ruminal contents exchanged with a cow already adapted to the diet (Satter and Bringe, 1969). Third, the maximal reduction in milk fat yield observed with *trans*-10, *cis*-12 CLA and diet-induced MFD is $\sim$ 50% (Bauman and Griinari, 2003). It appears that either CLA can only down-regulate its target-signaling molecule(s) by 50% or the target-signaling pathway is only responsible for the 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. Finally, yields of FA of all chain lengths are decreased during MFD. However, *de novo*-synthesized FA are decreased to a greater extent, especially in conditions of more pronounced MFD, and this results in a shift in milk FA profile such that the proportion of short- and medium-chain FA are decreased and longer-chain and unsaturated FA increased (Bauman and Griinari, 2001).

Mechanism of milk fat depression

Whole animal metabolism

Investigations with *trans*-10, *cis*-12 CLA infusions offer the opportunity to evaluate the whole animal metabolic phenotype during MFD without the confounding effects associated with diet. CLA-induced MFD had no effects on the plasma concentration of metabolites including glucose, nonesterified fatty acids (NEFA) and β-hydroxy butyrate, or metabolic hormones including insulin, IGF-I and leptin during short- ($<$1 week) and longer-term (up to 20 weeks) treatment (Baumgard *et al.*, 2000 and 2002a; Perfield *et al.*, 2002; Castañeda-Gutiérrez *et al.*, 2005; de Veth *et al.*, 2006). In addition, 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 were unaltered (Baumgard *et al.*, 2002a; de Veth *et al.*, 2006). Hepatic triglyceride concentration was also unaffected during CLA-induced MFD (Bernal-Santos *et al.*, 2003; Castañeda-Gutiérrez *et al.*, 2005). The mechanism of CLA is also independent of the stage of lactation as *trans*-10, *cis-12* CLA reduces milk fat yield during all phases of the lactation cycle (Perfield *et al.*, 2002; Bernal-Santos *et al.*, 2003; Castañeda-Gutiérrez *et al.*, 2005), although a larger dose is required in early lactation (Moore *et al.*, 2004; Odens *et al.*, 2007).

Mammary metabolism

Synthesis of milk fat requires the coordination of multiple biochemical processes and cellular events in the mammary epithelial cell (Figure 3). Piperova *et al.* (2000) reported decreased mammary enzyme activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) during diet-induced MFD and Baumgard *et al.* (2002b) observed decreased mammary lipogenic capacity during CLA-induced MFD based on ^{14}C acetate incorporation into FA by mammary tissue explants. This demonstrates an inhibition of milk fat synthesis in the mammary gland and is in agreement with the decrease in *de novo*-synthesized FA previously discussed. Mammary lipogenic capacity may be regulated at multiple levels including transcription, translation, protein turnover and enzyme activity. Decreased mammary expression of genes for key enzymes and proteins involved in FA uptake, synthesis, transport and esterification occurred during both diet- and CLA-induced MFD (Piperova *et al.*, 2000; Ahnadi *et al.*, 2002; Baumgard *et al.*, 2002b; Peterson *et al.*, 2003; Harvatine and Bauman, 2006). Combined, these observations define MFD as a decreased mammary capacity for lipid synthesis due to a coordinated transcriptional down-regulation of enzymes and proteins involved in milk fat synthesis.

Sterol response element-binding protein-1

The regulation of lipid synthesis and the role of long-chain PUFA in this regulation have been extensively studied in rodent models and cell culture systems, especially hepatocyte-based systems (see reviews by Duplus and Forest, 2002; Jump *et al.*, 2005; Sampath and Ntambi, 2005). These investigations have provided insight that may be applicable to the mechanism by which biohydrogenation intermediates regulate milk fat synthesis. Although many factors interact to determine tissue rates of lipid synthesis, expression of the genes for key enzymes and proteins in the process is highly regulated by a few well-characterized transcription factors known as 'master regulators' (Duplus and Forest, 2002; Salter and Tarling, 2007). A role for sterol response element-binding protein 1 (SREBP1) in MFD was proposed by Baumgard *et al.* (2002b) based on the function of this transcription factor family as global regulators of lipid metabolism (reviewed by Eberle *et al.*, 2004). SREBP1 is expressed as two isoforms with SREBP1a predominantly involved in the regulation of cholesterol metabolism while SREBP1c predominantly regulates FA synthesis. SREBP1c signaling is inhibited by PUFA in cell culture and rodent models, and this reduction mediates a major portion of the anti-lipogenic response to PUFA (Hannah *et al.*, 2001; Moon *et al.*, 2002). SREBP1 is highly expressed in the mammary

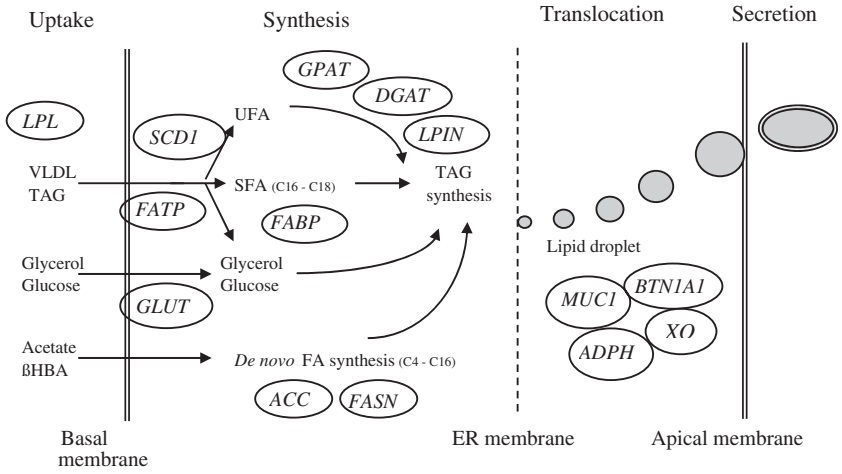

Figure 3 Diagram representing activities and pathways coordinated during the synthesis and secretion of milk fat. Key proteins are shown in ovals: lipoprotein lipase (LPL); stearoyl-CoA desaturase (SCD); fatty acid transport proteins (FATP); glucose transporters (GLUT); glycerol phosphate acyltransferase (GPAT); diacylglycerol acyltransferase (DGAT); lipin (LPIN); fatty acid binding proteins (FABP); acetyl-CoA carboxylase (ACC); fatty acid synthase (FASN); mucin 1 (MUC1); butyrophilin (BTN1A1); xanthine oxidoreductase (XO) and adipophilin (ADPH). FA = fatty acid; UFA = unsaturated fatty acid; SFA = saturated fatty acid; VLDL = very low-density lipoprotein; TAG = triacylglycerol and βHBA = β-hydroxybutyrate. Adapted from McGuire and Bauman (2002).

Table 4 *Summary of SREBP1-regulated genes involved in milk fat synthesis in bovine mammary tissue for which expression is coordinately reduced during diet-induced or* trans*-10,* cis*-12 CLA-induced milk fat depression (MFD)*[1]

Biochemical process/enzymes	Diet-induced MFD (reference[2])	CLA-induced MFD (reference[2])
Synthesis *de novo*		
Acetyl CoA carboxylase	A,B,C	C
Fatty acid synthase	A,B,E	D,E
Preformed fatty acids		
Lipoprotein lipase	A,E	A,E
Fatty acyl CoA ligase	A	
Desaturation		
Stearoyl-CoA desaturase	A,C,E	D
Esterification		
Acylglycerol phosphate acyl transferase	A	D
Glycerol phosphate acyl transferase	A	D

[1]From Harvatine and Bauman (2006).
[2]Reference citations are as follows: A = Peterson *et al.* (2003); B = Piperova *et al.* (2000); C = Ahnadi *et al.* (2002); D = Baumgard *et al.* (2002b); E = Harvatine and Bauman (2006).

gland where it is highly correlated with the expression of FASN and lipoprotein lipase (Harvatine and Bauman, 2006).

The molecular activation of SREBP1 is well described (see reviews by Horton *et al.*, 2002; Eberle *et al.*, 2004; Goldstein *et al.*, 2006). Briefly, the full-length inactive SREBP1c protein is complexed with the SREBP chaperone protein (SCAP) and anchored in the endoplasmic reticulum through association with a third protein, either insulin-induced gene 1 or 2 (INSIG1 or INSIG2). SREBP is activated by the 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. nSREBP1 translocates to the nucleus where it binds to sterol-regulatory elements (SRE) in the promoter/enhancer regions of target genes, recruits co-activators, and stimulates the transcription of genes involved in lipid synthesis. While the sequence of SREBP1 activation and the ability of PUFA to effect this activation are well established, the initial steps in PUFA–SREBP1 interaction are not characterized.

Peterson *et al.* (2004) were the first to investigate SREBP1 signaling in the bovine and reported decreased abundance of nSREBP1 during *trans*-10, *cis*-12 CLA inhibition of FA synthesis in MAC-T mammary epithelial cell cultures. Subsequently, Harvatine and Bauman (2006) demonstrated decreased expression of SREBP1 and the proteins involved in the translocation and activation of SREBPs in mammary tissue from cows during CLA- and diet-induced MFD. SREBP1 stimulates its own transcription, so SREBP1 expression provides an index of nSREBP1 abundance (Amemiya-Kudo *et al.*, 2000).

Key enzymes involved in lipid synthesis that are down-regulated during CLA- and diet-induced MFD contain an sterol response element (SRE) response element in their promoter, and are known to be regulated by SREBP1 (Table 4; Harvatine and Bauman, 2006). In mice, SREBP1 is up-regulated at the

initiation of lactation, and disruption of the SREBP1c gene results in a 41% decrease in milk fat concentration when fed a low-fat diet (Rudolph *et al.*, 2005). Interestingly, this inhibition approximates the maximum reduction in milk fat synthesis observed during bovine MFD as discussed earlier. Overall, decreased expression of SREBP1, SREBP1 activation proteins and SREBP1-regulated genes for key enzymes involved in lipid synthesis provides strong evidence for SREBP1 as a central signaling pathway in the regulation of fatty acid synthesis in bovine mammary epithelial cells.

Thyroid hormone responsive spot 14

Mammalian regulation typically includes redundant systems for signal amplification and for regulation of biochemical processes. We identified thyroid hormone responsive spot 14 (S14) as a *trans*-10, *cis*-12 CLA responsive gene in the microarray analysis of bovine mammary cultures (Harvatine and Bauman, 2006). The S14 gene encodes a nuclear protein that is closely associated with the regulation of lipid synthesis in lipogenic tissues, including the bovine mammary gland (Cunningham *et al.*, 1998; Harvatine and Bauman, 2006). Furthermore, we established that the expression of S14 in the bovine mammary gland is down-regulated in both CLA- and diet-induced MFD (Harvatine and Bauman, 2006). Although its exact biochemical function is not known, S14 is found in the nucleus and is a putative transcriptional coactivator (Cunningham *et al.*, 1998; Chou *et al.*, 2007 and 2008) that is highly responsive to pro-lipogenic signals including SREBP1 activation (Martel *et al.*, 2006).

The expression of S14 is positively associated with conditions of excessive lipid synthesis, including human obesity, chicken lines selected for increased growth and adiposity, muscle of cattle selected for marbling and high lipogenic cancers (summarized in Harvatine and Bauman, 2006). Studies of the function of S14 have provided interesting insight, and also many inconsistencies (see review by LaFave *et al.*, 2006). Knock-down of S14 in hepatocyte culture resulted in decreased expression of lipogenic enzymes (reviewed by Cunningham *et al.*, 1998), but the S14 knock-out mouse had increased hepatic lipogenesis (Zhu *et al.*, 2001). Of special interest in considering possible relevance to MFD, S14 knock-out mice had a 62% reduction in mammary lipogenesis and a 26% reduction in milk clot triglyceride concentration, which was predominantly due to decreased *de novo* fatty acid synthesis; however, activities of mammary lipogenic enzymes were unaltered (Zhu *et al.*, 2005).

Nuclear receptor family proteins

Genes from the nuclear hormone receptor (NR) family are also central regulators of metabolism (Francis *et al.*, 2003). The peroxisome proliferator-activated receptors (PPARs), in particular, have been speculated to play a role in MFD (Baumgard *et al.*, 2002b; Bernard *et al.*, 2006 and 2008). Cellular free FA are natural ligands for the PPARs and CLA is a potent agonist of PPARα and PPARγ. However, PPARα and PPARγ are activated equally well by *trans*-10, *cis*-12 CLA, which induces MFD, and by *cis*-9, *trans*-11 CLA, which does not (Moya-Camarena *et al.*, 1999; Yu *et al.*, 2002).

Any suggested roles for nuclear receptors must take into consideration their tissue-specific expression pattern. Using a panel of bovine tissues, we have surveyed the expression of possible PUFA-responsive nuclear receptors as a means to provide an initial assessment of their potential role in bovine mammary lipid synthesis. For example, expression of HNF4α, a member of the nuclear receptor family, is 15 500-fold higher in bovine liver than in lactating mammary tissue, and the expression in mammary tissue does not differ between lactating and nonlactating tissue (Harvatine and Bauman, 2007a). In the case of the PPARs, PPARα was predominantly expressed in tissues with high rates of FA oxidation (e.g. liver, muscle, heart), PPARγ was predominantly expressed in adipose tissue, and PPPARβ/δ expression was not different between lactating and non-lactating mammary tissue. Furthermore, mammary tissue expression was not modified by CLA- or diet-induced MFD for any of the PPAR genes (Harvatine and Bauman, 2007a).

Nuclear receptor activity and function is modified by ligand-binding, post-translational modifications and by association with various co-repressors and co-activators (Tan *et al.*, 2005; Feige *et al.*, 2006). Ligand binding of PPARα and PPARβ/δ increases FA oxidation, and ligand binding of PPARγ increases FA transport and lipogenesis; none of these changes are consistent with the phenotype of MFD. Kennedy *et al.* (2008) recently reported that *trans*-10, *cis*-12 CLA antagonized ligand activation of PPARγ in adipocyte cell culture, presumably via extra cellular signal-regulated kinases (ERK), specifically ERK-stimulated phosphorylation of PPARγ. Ligand-dependent and -independent repressor mechanisms are well described for the PPARs, but they function primarily to reduce inflammatory and immune responses (Ricote and Glass, 2007); indeed a hematopoietic and endothelial cell-specific PPARγ knock-out resulted in increased levels of inflammatory molecules in milk (Wan *et al.*, 2007). Lastly, CLA treatment reduced body fat in PPARα knock-out mice, demonstrating a PPARα-independent mechanism for CLA in growing mice (Peters *et al.*, 2001). Overall, these results and patterns do not offer support for HNF4α or the PPARs in the regulation of milk fat synthesis in the bovine mammary gland.

Protein kinase B/Akt

Numerous growth factors and hormones, including insulin and IGF1, activate phosphoinositide 3-kinase (PI3K), which subsequently results in phosphorylation and activation of protein kinase B/Akt (Yang *et al.*, 2004). In the mouse, expression of Akt1 is up-regulated at the initiation of lactation. Deletion of the Akt1 gene in mice decreased milk yield, presumably due to a failure to stimulate glucose uptake and metabolism in the mammary gland during the initiation of lactation, but had no effect on milk fat concentration (Boxer *et al.*, 2006). However, mammary-specific over-expression of a constituently active Akt1 more than doubled the milk fat concentration (Schwertfeger *et al.*,

2003). Activation of Akt1 increases lipid synthesis through modification of multiple levels of SREBP1 regulation (Porstmann *et al*., 2005). First, Akt1 promotes processing of SREBP1, increasing nSREBP1 synthesis (Porstmann *et al*., 2005; Du *et al*., 2006). Secondly, nSREBP1 is targeted to proteosomal degradation by GSK3β-dependent phosphorylation, and Akt1 may increase nSREBP1 abundance by inactivating GSK3β (Rudolph *et al*., 2007; Jump *et al*., 2008). Botolin *et al*. (2006) reported that n-3 PUFA decreased insulin-stimulated activation of Akt1 and increased proteasome degradation of nSREBP1, although a constituently active Akt1 did not overcome this effect. In addition, long-chain PUFA induced GSK3β phosphorylation (Jump *et al*., 2008). Overall, these investigations raise the possibility that Akt1 could mediate effects of bioactive FA on SREBP1 signaling, but its regulation has not yet been investigated in MFD.

Primary and secondary mechanisms

The regulation of milk fat synthesis has predominantly involved animals that were established in MFD. This approach cannot differentiate between causative mechanisms and responses that may have occurred as secondary adaptations to the reduction in milk fat synthesis. Recently we conducted an *in vivo* investigation that involved sequential mammary biopsies during *trans*-10, *cis*-12 CLA infusion; we observed that SREBP1 and S14 were early-phase responders during MFD and down-regulation of their expression was extensive by 30 h after the initiation of CLA treatment (Harvatine and Bauman, 2007b).

The initial cellular steps by which PUFA or CLA modifies SREBP1 signaling have not been elucidated, although this is currently an area of intense investigation. In the case of CLA, an active metabolite derived from *trans*-10, *cis*-12 CLA is a possibility given the well-established pathways for the metabolism of long-chain PUFA to produce eicosanoids. The initial enzyme in metabolism of *trans*-10, *cis*-12 CLA is Δ^6 desaturase that forms *cis*-6, *trans*-10, *cis*-12 C18:3. We have examined this conjugated diene 18:3 isomer at a concentration comparable to that found effective for the *trans*-10, *cis*-12 CLA reduction of milk fat synthesis; while it was taken up and incorporated into milk fat, *cis*-6, *trans*-10, *cis*-12 C18:3 had no effect on milk fat yield (Saebo *et al*., 2005b). Thus, this metabolite and by inference related down-stream metabolites do not appear to have a direct effect or be involved in the mechanism for the regulation of milk fat synthesis by *trans*-10, *cis*-12 CLA.

Milk fat fluidity and milk fat synthesis

Milk fat fluidity is primarily determined by the FA chain length and by the number and orientation of FA double bonds. Fluidity is an important consideration in secretion of milk fat from the mammary epithelial cell (Timmen and Patton, 1988) and it is affected by the profile of the FA that is available for use in the synthesis of milk fat triglycerides. In diet-induced MFD the profile of FA is markedly altered and this is a characteristic of the biohydrogenation theory. Collectively, these changes would reduce the fluidity of milk fat and they include the following: an increase in *trans*-C18:1 FA originating from rumen biohydrogenation processes; a decrease in short- and medium-chain FA due to the inhibition of mammary *de novo* FA synthesis; and a shift in oleic acid (decrease) and stearic acid (increase) due to the inhibition of SCD.

SCD plays an important role in the supply of unsaturated FA for milk fat synthesis. SCD is dynamically regulated and this has functional consequences in metabolic regulation as highlighted by the work of Ntambi and coworkers with the SCD1 null mouse that is protected from obesity and insulin resistance (Ntambi and Miyazaki, 2004; Miyazaki *et al*., 2007). Alterations in milk fat desaturase index are not always observed in MFD, but it is altered in many situations of diet-induced MFD and with higher doses of *trans*-10, *cis*-12 CLA (>25% decrease in milk fat; summarized by Perfield *et al*., 2006). Furthermore, the alteration in the desaturase index is acute during CLA-induced MFD occurring within 6 h after treatment is initiated (Harvatine *et al*., 2006). The rapid changes in the desaturase index occur prior to any reduction in milk fat synthesis and this relates to the very short half-life of SCD (2 to 4 h; Oshino and Sato, 1972; Toyama *et al*., 2007) relative to key enzymes involved in *de novo* synthesis (48 to 76 h; Craig *et al*., 1972; Volpe and Vagelos, 1973; Volpe and Marasa, 1975).

Based on correlative evidence, a decreased activity of SCD and the resulting decrease in oleic acid have been proposed to cause diet-induced MFD (Loor and Herbein, 2003; Loor *et al*., 2005). Recently, Shingfield and Griinari (2007) proposed this as an extension of the biohydrogenation theory of MFD, suggesting that the specific inhibition of SCD over extended periods would induce MFD through changes in the mammary supply of stearic and oleic acids. They based this proposal primarily on Bickerstaffe and Johnson (1972), who reported an immediate change in the desaturase index and a progressive decrease in milk fat percentage when sterculic acid, an inhibitor of SCD, was infused. The report by Bickerstaffe and Johnson (1972) represents observations of a single goat with no replication or controls; however, our calculations from their graphic representation indicate that milk fat percent was decreased ~20% by day 6 and ~33% by day 16 of sterculic acid infusion. A number of well-controlled studies with lactating cows have altered the desaturase index through inhibition of SCD, and none have observed any effects on milk fat yield even though the milk fat contents of oleic and stearic acids were markedly altered. These have all been shorter term, 4 to 9 days, and have included abomasal infusions of sterculic oil (Griinari *et al*., 2000; Corl *et al*., 2001; Kay *et al*., 2004), *trans*-9, *trans*-11 CLA (Perfield *et al*., 2007) or *trans*-10, *trans*-12 CLA (Saebo *et al*., 2005a; Perfield *et al*., 2006a), and dietary administration of the rumen marker CoEDTA (Shingfield *et al*., 2006a and 2008b). In addition, the anti-lipogenic effects of CLA in the growing mouse were found to be independent of SCD1 using the SCD1 null mouse (Kang *et al*., 2004). Thus, while changes in SCD will impact milk fluidity, the mammary gland must have a remarkable ability to maintain milk fat

secretion over a substantial range in FA profile. Clearly, decreased activity of the SCD is not a prerequisite for MFD, and there is no direct evidence supporting the inhibition of SCD as a specific, independent causative factor in diet-induced MFD.

Consideration of historical theories to explain milk fat depression

The investigation of diet-induced MFD has a rich history that has included many theories to explain reduced milk fat synthesis. Most of these theories postulated that limitations in substrate supply for milk fat synthesis caused MFD, generally based on changes in absorbed metabolites as a consequence of alterations in ruminal fermentation. Over several decades, researchers have tested theories based on substrate limitations and found little to no evidence in their support; these theories will be briefly discussed here but have been comprehensively reviewed elsewhere (Bauman and Griinari, 2001; Griinari and Bauman, 2006; Shingfield and Griinari, 2007).

The first documented theory appeared over a century ago and proposed that MFD was caused by a limitation in supply of FA due to low dietary fat (Van Soest, 1994); however, subsequent work showed that limiting dietary fat reduces milk yield but has very little effect on milk fat percent (Virtanen, 1966; Storry *et al.*, 1967; Banks *et al.*, 1976). Another theory based on substrate supply proposed that a deficiency of acetate caused MFD (Davis and Brown, 1970; Doreau and Chilliard, 1997). A reduction in the ruminal molar ratio of acetate to propionate is highly correlated with MFD in diets that are high in fermentable carbohydrate. However, the reduced ratio of acetate to propionate is predominantly due to an increased ruminal production of propionate (Bauman and Griinari, 2001 and 2003), and ruminal infusion of acetate to cows that were MFD had only a marginal impact on milk fat yield (Davis and Brown, 1970).

Lastly, the glucogenic-insulin theory of MFD proposed that increased ruminal propionate and increased hepatic gluconeogenesis stimulated insulin secretion, resulting in an inhibition of adipose tissue lipolysis and increased uptake of lipogenic precursors by extra-mammary tissues that are insulin sensitive (McClymont and Vallance, 1962; Annison *et al.*, 1974). Direct testing of the theory by infusion of propionate found variable responses from no effect to a maximal milk fat reduction of 16% (summarized by Davis and Brown 1970; Bauman and Griinari, 2001). The effect of insulin was also directly tested using hyperinsulinemic–euglycemic clamps and multiple experiments with well-fed cows showed that a four-fold increase in plasma insulin had a minimal effect (average 5% reduction) on milk fat yield (McGuire *et al.*, 1995; Griinari *et al.*, 1997; Mackle *et al.*, 1999). In contrast, hyperinsulinemic–euglycemic clamp of cows in early lactation resulted in a 35% reduction in milk fat yield (Corl *et al.*, 2006). The difference in magnitude of response for cows in established and early lactation can be explained by the effect of energy

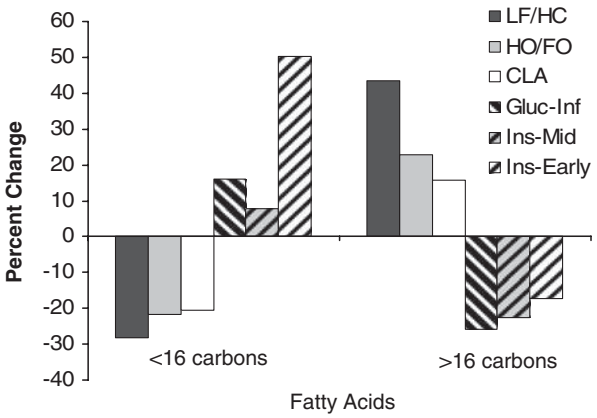

Figure 4 Change in the concentration of *de novo* synthesized (<16 carbon) and preformed (>16 carbon) FA in milk fat that occur with diet-induced and CLA-induced milk fat depression (MFD) and treatments testing glucogenic-insulin regulation of MFD. Examples of classical MFD include a low-forage/high-concentrate diet (LF/HC) that reduced milk fat yield ~ 60% (Storry *et al.*, 1967), a high-oil plus fish oil diet (HO/FO) that reduced milk fat yield 38% (Harvatine and Bauman, 2006) and abomasal infusion of 14 g/day of *trans*-10, *cis*-12 CLA that reduced milk fat yield 50% (Baumgard *et al.*, 2001). Treatments testing the glucogenic-insulin theory include duodenal infusion of 1500 g/day of glucose (Gluc-Inf) that reduced milk fat yield 16% (Hurtaud *et al.*, 1998), hyperinsulinemic–euglycemic clamp during mid lactation (Ins-Mid) that reduced milk fat yield 7% (Griinari *et al.*, 1997), and hyperinsulinemic–euglycemic clamp during early lactation (Ins-Early) that reduced milk fat yield 27% (Corl *et al.*, 2006). Figure development was based on Bauman and Griinari (2001).

balance on the contribution of preformed FA to milk fat; only 4% to 8% of milk FA originate from body fat reserves when cows are in a positive energy balance (Palmquist and Mattos, 1978; Pullen *et al.*, 1989), but the contribution is much greater during negative energy balance because of mobilization of body fat reserves. Most experimental and commercial instances of CLA- and diet-induced MFD occur in cows in positive energy balance and result in a much greater reduction in MFD than observed in testing of the glucogenic-insulin theory. Secondly, the reduction in milk fat observed during testing of the glucogenic-insluin theory represents a different mechanism than classical MFD; the pattern of change in milk composition for several situations where milk fat is reduced is presented in Figure 4. In the case of hyperinsulinemic–euglycemic clamp experiments, the reduction in milk fat was predominantly long-chain preformed FA, consistent with insulin's well-established anti-lipolytic effects. This contrasts with classical diet-induced and CLA-induced MFD that is characterized by a more pronounced decrease in *de novo*-synthesized FA. Overall, the historical theories of MFD were developed from concepts based on observational data, but they failed to be supported by direct experimentation and mechanistic investigations.

Insights gained from milk fat depression

Research in the regulation of milk fat synthesis has focused on investigations of MFD rather than on situations or models where milk fat synthesis is enhanced. Nevertheless,

MFD represents a biologically significant and physiologically relevant example where a metabolite(s) produced in digestive processes is regulating metabolism, and the basis for this regulation can be explained at the molecular level. Many cows do not achieve their genetic potential for milk fat synthesis because of subtle diet-induced MFD. The study of MFD may arguably be the most complete and successful example of nutri-genomics in present-day animal science research and provides many valuable applications. For example, 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 highlighted key regulatory mechanisms in mammary lipid synthesis and this provides a platform for the development of methods to enhance milk fat yield and improve the fatty acid profile of milk fat. For example, SREBP1 and the SREBP1 regulatory proteins are being used as candidate genes for identification of single-nucleotide polymorphisms that may explain genetic differences in milk fat yield (Medrano and Rincon, 2007) and FA composition of bovine fat (Hoashi *et al.*, 2007).

Under certain marketing systems and management schemes, it may be advantageous to reduce milk fat yield (Griinari and Bauman, 2003), and 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. Bernal-Santos *et al.*, 2003; Mackle *et al.*, 2003; Lock *et al.*, 2006b; Odens *et al.*, 2007). 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 (BW) or body composition are difficult to adequately quantify in ruminants, but increased rumen-empty BW gain has been reported during diet-induced MFD (Harvatine and Allen, 2006). In agreement with increased energy balance, we have also observed increased expression of enzymes and protein involved in lipid synthesis and lipogenic signaling in adipose tissue during short-term CLA-induced MFD (Harvatine *et al.*, 2007). 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.

Conclusion

MFD results from an interaction between ruminal fermentation processes and mammary tissue metabolism. Investigation of milk fat synthesis over the past 100 years has resulted in numerous theories based on observational differences in dietary associations, alterations in ruminal fermentation, and adaptations in animal metabolism. To date, the biohydrogenation theory is the only proposed mechanism that has provided causative evidence and withstood rigorous examination. The mechanism by which biohydrogenation intermediates reduce milk fat synthesis has and will continue to provide insight into the regulation of milk fat synthesis. MFD continues to be a real-world condition that reduces the efficiency and productivity of dairy cows, but understanding its fundamental basis will allow for effective management and intervention strategies. Furthermore, advances in understanding the regulation of fat synthesis will undoubtedly have broader implications and applications.

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