Effect of dietary fat level on the ability of conjugated linoleic acid (CLA) to inhibit milk fat synthesis in lactating mice

Honors Thesis

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ABSTRACT

Trans-10, *cis*-12 conjugated linoleic acid (CLA) is a fatty acid intermediate produced during rumen biohydrogenation of dietary linoleic acid, and it is a potent inhibitor of milk fat synthesis in the cow. Inhibition of milk fat synthesis by CLA has also been demonstrated in rodent models. The objective of the present study was to determine whether dietary fat level affects the response of the mammary gland to CLA in lactating mice. Wild type C57Bl/6J mice (n = 31) nursing 6-8 pups were fed semi-purified diets containing either 4% fat (LF) or 24% fat (HF) starting 4-6 d after parturition. High oleic acid sunflower oil was substituted for corn starch to increase the fat content of the HF diet. After a 2 d pretreatment period, lactating dams were orally dosed with either water (control) or *trans*-10, *cis*-12 CLA (20 mg/d, divided among three equal doses) for 5 d. CLA treatment decreased growth of the nursing litter similarly for both diets; no effect of dietary fat or interaction with CLA was observed. Dam energy intake was decreased by CLA, but this effect was partially attenuated by increased dietary fat. Milk fat percent was increased 16-17% by the HF diet and decreased 12-13% by CLA. Both CLA and the HF diet reduced the proportion of short and medium chain fatty acids in milk fat. These fatty acids originate from *de novo* synthesis in the mammary gland. Conversely, the milk fat concentration of fatty acids >16 carbons in length was increased 62-63% by the HF diet, and substantial incorporation of dietary oleic acid into milk fat was observed. In conclusion, CLA caused a reduction in milk fat percent and litter growth that was not overcome by increased dietary fat.

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ABBREVIATIONS

6PGDH	6-phosphogluconate dehydrogenase
ACC	Acetyl CoA carboxylase
AGPAT	Acylglycerol phosphate acyltransferase
CoA	Coenzyme A
CLA	Conjugated linoleic acid, often used to specifically denote *trans*-10, *cis*-12 CLA
FABP	Fatty acid binding protein
FASN	Fatty acid synthase
G6PDH	Glucose 6-phosphate dehydrogenase
GPAT	Glycerol phosphate acyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High fat (24% fat diet)
HFCON	High fat control treatment (no CLA)
HFCLA	High fat CLA treatment (20 mg/d CLA)
IP	Intraperitoneal
LF	Low fat (4% fat diet)
LFCON	Low fat control treatment (no CLA)
LFCLA	Low fat CLA treatment (20 mg/d CLA)
LPL	Lipoprotein lipase
LS	Least squares
MFD	Milk fat depression
mRNA	Messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate
NADP-MDH	NADP-malate dehydrogenase
NADPH	Reduced form of NADP
nSREBP	Nuclear fragment of SREBP
RNA	Ribonucleic acid
S14	Thyroid hormone responsive spot 14
SEM	Standard error of the mean
SCD	Stearoyl CoA desaturase
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein

INTRODUCTION

The inhibition of milk fat synthesis by isomers of conjugated linoleic acid (CLA) is a well-studied and physiologically relevant example of nutritional genomics, the modulation of gene expression by specific bioactive dietary components. Originally recognized in dairy cows, these unique fatty acid isomers are produced in the rumen under conditions of altered biohydrogenation and cause a coordinated downregulation in the expression of genes for key lipogenic enzymes (1). The result is a specific reduction in milk fat, commonly known as milk fat depression. Recent research has examined the mechanism by which CLA isomers inhibit milk fat synthesis, and the sterol response element binding protein (SREBP) transcription factor system and the nuclear protein thyroid hormone responsive spot 14 have been implicated (2). To investigate the mechanism further, our lab has validated a lactating mouse model to allow for nutritional genomic investigation (3). Oral administration of *trans*-10, *cis*-12 CLA, the first CLA isomer shown to inhibit milk fat synthesis, caused a dose-dependent decrease in milk fat in the mouse and a shift in fatty acid profile comparable to that observed in the dairy cow (3). Although the reduction in milk fat involves fatty acids of all chain lengths, the greatest decrease is seen for short and medium chain fatty acids that are synthesized in the mammary gland by the process of *de novo* lipogenesis.

The interaction of CLA and diet is an important factor in further understanding the regulation of milk fat synthesis by CLA. Whereas the range of possible dietary interventions is limited in the cow by the necessity of maintaining an appropriate environment for rumen microorganisms, a mouse model provides more freedom to

manipulate diet composition. Of particular interest is the potential role of dietary fat in affecting the response of the mammary gland to CLA. Altering the amount and source of fat in the diet is known to affect the quantity and composition of milk fat in rodents (4-7). This raises the question of whether the changes in milk fat percent and fatty acid profile in response to CLA are modulated by the fat content of the diet, and that is the focus of this thesis.

REVIEW OF LITERATURE

CLA studies in dairy cows: the milk fat depression story

CLA has been studied most extensively in the dairy cow, where it is linked to the phenomenon of milk fat depression (MFD). First described in 1885, MFD is characterized by a reduction in milk fat and a change in fatty acid composition (8). This effect is specific to fat; milk protein and yield are not affected. MFD is observed with certain diets, particularly diets that are low in forage or supplemented with polyunsaturated fatty acids. Although many theories have been proposed to explain the incidence of diet-induced MFD, most have proven inadequate (8). However, the "biohydrogenation theory" proposed by Bauman and Griinari (9) has gained widespread support in recent years. It proposes that low fiber diets cause changes in the rumen environment that alter the pathways of biohydrogenation, leading to the production of unique fatty acid isomers that inhibit milk fat synthesis. This idea grew out of studies from the 1960's that identified an increase in the milk fat content of *trans* 18:1 fatty acid isomers in milk fat-depressed cows (8). These *trans* fatty acids are indicative of

incomplete biohydrogenation by rumen bacteria, and it was suggested that they might directly inhibit milk fat synthesis (9). Later experiments linked MFD with a specific increase in *trans*-10 18:1 rather than *trans* 18:1 isomers in general (10). A putative pathway for the formation of *trans*-10 18:1 was then proposed (**Figure 1**). Dietary linoleic acid is normally isomerized to *cis*-9, *trans*-11 CLA, which is then reduced to *trans*-11 18:1 and finally stearic acid (9). Under conditions of MFD, a minor pathway comes into play in which linoleic acid is instead converted to *trans*-10, *cis*-12 CLA and *trans*-10 18:1 before reaching stearic acid. This alternate pathway has been supported by the isolation of rumen bacteria capable of producing *trans*-10, *cis*-12 CLA (11) or *trans*-10 18:1 (11, 12). Ruminal infusions of *trans*-10, *cis*-12 CLA result in elevated plasma *trans*-10 18:1, providing additional evidence for the precursor/product relationship of these two fatty acids (13).

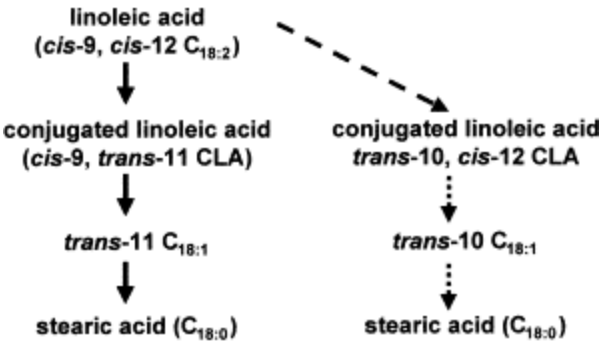

FIGURE 1. Pathways of rumen biohydrogenation of linoleic acid. Adapted from Bauman and Griinari (9).

The first specific inhibitors of milk fat synthesis identified were isomers of CLA. This was discovered in a study investigating the possibility of enhancing the CLA content

of milk to exploit the known positive health effects of CLA (14). A mixture of CLA isomers was abomasally infused, with the unexpected consequence of a 55% reduction in milk fat yield. One of the isomers was *trans*-10, *cis*-12 CLA, which was subsequently demonstrated to cause MFD when infused as a 90% pure preparation (15). Two more biohydrogenation intermediates have since been shown to inhibit milk fat synthesis, *cis*-10, *trans*-12 CLA (16) and *trans*-9, *cis*-11 CLA (17). Others are suspected, but the limited availability of pure isomers has hampered the investigation of bioactive fatty acids.

A number of studies involving the abomasal infusion of *trans*-10, *cis*-12 CLA have yielded similar shifts in the fatty acid composition of milk as those seen during diet-induced MFD. In both cases, the yield of fatty acids of all chain lengths is reduced; however, the decrease in *de novo* synthesized fatty acids is most prominent (8). As a result, these short- and medium-chain fatty acids constitute a smaller proportion of the total fatty acids. High doses of *trans*-10, *cis*-12 CLA (≥7 g/d) inhibit the activity of stearoyl CoA desaturase (SCD) (18), an enzyme in the mammary gland that introduces a *cis*-9 double bond in fatty acids. This also affects the fatty acid composition of milk by increasing the ratios of several SCD substrate/product pairs, including $C_{14}/C_{14:1}$, $C_{16}/C_{16:1}$, and $C_{18}/C_{18:1}$ (18). These ratios are often used as a proxy for SCD activity. Changes in the desaturase index are not consistently observed with lower doses of *trans*-10, *cis*-12 CLA or during diet-induced MFD (19). Thus the inhibition of milk fat synthesis does not necessarily require a reduction in desaturase activity (18).

Mechanism of CLA-induced milk fat depression

Both low fiber diets and *trans*-10, *cis*-12 CLA cause downregulation in the expression of a number of genes encoding key lipogenic enzymes in the mammary gland. These are involved in the uptake and transport of fatty acids from the circulation (lipoprotein lipase [LPL]and fatty acid binding proteins [FABP]), *de novo* synthesis of fatty acids (acetyl CoA carboxylase [ACC] and fatty acid synthase [FASN]), and triglyceride synthesis (glycerol phosphate acyltransferase [GPAT] and acylglycerol phosphate acyltransferase [AGPAT]) (20, 21). Expression of SCD is also reduced, as noted above. The wide range of enzymes affected is consistent with the observed reductions in the yield of fatty acids derived from both *de novo* synthesis and uptake of preformed fatty acids from the blood.

The coordinated down-regulation of genes involved in milk fat synthesis suggested the role of a global regulator of gene expression. The sterol regulatory element binding protein (SREBP) family of transcription factors was identified as a candidate system (20). This was supported by the fact that all of the CLA-responsive genes named above contain sterol regulatory elements (SRE) and are thus potentially responsive to SREBP (20). Proteolytic cleavage of SREBP is required to produce the nuclear fragment (nSREBP) that binds to the SRE of target genes and stimulates transcription (22). Treatment of bovine mammary epithelial cells with *trans*-10, *cis*-12 CLA reduced the level of nSREBP without the altering the amount of the precursor protein, suggesting that CLA inhibits the proteolytic cleavage of SREBP1 (22). In vivo, the abundance of SREBP1 mRNA was decreased during diet-induced MFD and treatment with *trans*-10, *cis*-12 CLA (2). The expression of several proteins involved in the proteolytic activation

of SREBP1 was also reduced in one or both conditions. These observations support the role of the SREBP transcription factor system in the regulation of milk fat synthesis during diet-induced MFD and *trans*-10, *cis*-12 CLA treatment.

Another gene, thyroid hormone responsive spot 14 (S14), has also been implicated in the regulation of gene expression by *trans*-10, *cis*-12 CLA (2). S14 is associated with *de novo* fatty acid synthesis, and its mRNA abundance is well correlated with the lipogenic rates of the tissues in which it is expressed (23). Little is known about its biochemical function, but S14 is a nuclear protein (24) that interacts with transcription factors (25). Its promoter contains a SRE (26), making S14 expression responsive to nSREBP (27). Indeed, mammary gland mRNA abundance of S14 is reduced during diet-induced MFD and treatment with t10, c12 CLA (2). The role of S14 in lipogenesis has been supported by experiments using an antisense oligonucleotide to inactivate S14 mRNA. This decreased the expression of lipogenic enzymes in rat hepatocytes and diminished triglyceride synthesis (28). Additional evidence came from a S14 knockout mouse model in which de novo fatty acid synthesis in the mammary gland was reduced and the milk fat percent was lowered (23). Interestingly, S14 is used as a marker for aggressive breast cancers (29), which are noted for their high rates of lipid synthesis (30). The suppression of S14 expression and lipogenesis by *trans*-10, *cis*-12 CLA provides a link to the observed anticarcinogenic properties of CLA (31).

CLA studies in rodents

Supplementation of CLA as 0.5-2.0% of the diet has been demonstrated to reduce body fat accretion in mice (32, 33), and most rodent studies of CLA examine this anti-

obesity effect. In contrast, the effects of CLA on lactation in rodents occur at much lower doses; as little as 6.5 mg *trans*-10, *cis*-12 CLA per day (equivalent to 0.07% of the diet) has been shown to inhibit milk fat synthesis in mice (3). However, relatively few studies have investigated the effects of CLA in lactating rodents.

The earliest rodent studies of the effects of CLA on milk fat synthesis employed supplements containing a mixture of isomers of CLA (34-36). There are over 20 geometric and positional isomers of CLA (37), and little is known about the biological effects of most of them. Of the eight CLA isomers that have been abomasally infused as pure preparations, three have been demonstrated to inhibit milk fat yield in the dairy cow (1). As mentioned previously, these are *trans*-10, *cis*-12 CLA, *trans*-9, *cis*-11 CLA, and *cis*-10, *trans*-12 CLA. The CLA supplements that have been fed to lactating rodents contain a wide range of CLA isomers, but *trans*-10, *cis*-12 CLA, *cis*-11, *trans*-13 CLA, *cis*-9, *trans*-11 CLA, and *trans*-8, *cis*-10 CLA have predominated in the two studies that reported the composition of the CLA supplement used. These four isomers accounted for 65% of the mix used by Ringseis et al. (35) and 91% of the total CLA fed by Hayashi et al. (36). In these studies, the supplement provided *trans*-10, *cis*-12 CLA as 0.47% (36) or 0.56% of the diet (35).

In both cases, CLA supplementation in lactating rats decreased milk fat and litter growth and shifted the fatty acid composition of the milk (35, 36). These changes were more extensive for Ringseis et al. (35), who reported greater reductions in milk fat (46% vs. 33%) and litter weight or growth (35% vs. 21%) than Hayashi et al. (36). Whereas changes in the fatty acid profile reflected decreased *de novo* lipogenesis in both studies, the decrease in short and medium chain fatty acids reported by Ringseis et al. (35) was

greater (28% vs. 12%); additionally, the incorporation of *trans*-10, *cis*-12 CLA into milk fat was also considerably greater (2.5% vs. 0.6%) despite similar doses of this isomer. These differences may be attributable to variation in the composition of the CLA supplement used and the interval of treatment. Hayashi et al. (36) fed their CLA supplement for the first 16 d of lactation, whereas the rats studied by Ringseis et al. (35) were treated for several weeks prior to breeding, throughout pregnancy, and up to 17 d following parturition. This longer treatment period may have resulted in greater incorporation of CLA isomers into adipose and liver triglycerides during growth. Mobilization of these lipid stores during lactation and uptake by the mammary gland could have contributed to the greater concentration of *trans*-10, *cis*-12 CLA in the milk. However, compared to other species, mobilization of body fat to support lactation typically plays a less substantial role in the adaptation of rodents to the metabolic demands of lactation, as rodents increase their feed intake greatly to supply substrates for milk production (38).

In contrast to these two studies, Chin et al. (34) did not observe an inhibitory effect of CLA supplementation on pup growth. During pregnancy and the first 10 d of lactation, rats were fed a supplement providing 0.25% or 0.5% total CLA as a percentage of the diet. The authors did not provide the isomer composition of the CLA supplement. A 9% increase in average pup weight was observed for the 0.5% CLA group, but there was no significant difference at the 0.25% dose. Mean pup weight was numerically increased for rats fed 0.5% CLA only during lactation, but this difference was not significant. Milk fat percentages were not reported in this paper, but the authors indicated by personal communication that no differences were found. Without

information on the CLA isomers fed by Chin et al. (34), it is difficult to explain the conflicting observations of this study and those by others (35, 36). It is quite possible that the supplements contained different proportions of *trans*-10, *cis*-12 CLA and other isomers that inhibit milk fat synthesis. Also, the highest CLA dose used by Chin et al. (34) was roughly a third of total concentration of CLA isomers fed in the other two studies. Nevertheless, had the supplement contained as little as 10% *trans*-10, *cis*-12 CLA (0.05% of the diet), a decrease in pup growth would be expected based on the response to treatment with pure *trans*-10, *cis*-12 CLA at 0.07% of the diet (3).

All three studies employing CLA mixtures in lactating rats attest to the relationship of milk fat and pup growth. When a reduction in milk fat was reported, a decrease in pup growth was also seen. This association between milk composition, particularly fat content, and pup growth is consistently observed in conditions other than CLA treatment. For instance, Del Prado et al. (39) altered milk fat concentration by feeding diets with varying fat content and noted corresponding effects on pup growth. Additionally, parameters of pup growth were positively correlated with milk fat across four strains of mice (40). Although other components of milk are responsive to dietary manipulations, the fat content of milk is of particular importance to the nutrition and growth of pups because fat is the primary energy source in milk (38).

Studies of pure isomers of CLA in rodents are even more limited. Loor et al. (41) compared the effects of *trans*-10, *cis*-12 CLA (0.91% of diet) and *cis*-9, *trans*-11 CLA (0.96% of diet) in lactating mice between days 4 and 15 of lactation. Consistent with abomasal infusion studies in dairy cows (15, 42, 43), *cis*-9, *trans*-11 CLA had no effect on milk fat or litter weight whereas *trans*-10, *cis*-12 CLA decreased these parameters by

29% and 25%, respectively. A shift in milk fatty acid profile characteristic of decreased de *novo* lipogenesis was also observed. Additionally, the authors noted that two of the six mice treated with *trans*-10, *cis*-12 CLA drastically reduced their feed intake and stopped lactating. This observation may be related to the dose of *trans*-10, *cis*-12 CLA used, which was nearly twice the amount fed in the studies employing mixtures of CLA isomers.

A study in our lab involving administration of pure *trans*-10, *cis*-12 CLA at varying doses to lactating mice also provided evidence of impairment of lactation due to high doses of CLA (3). Mice were orally dosed with 6.5, 20, or 60 mg/d of *trans*-10, *cis*-12 CLA for a period of 5 d starting at day 6-8 of lactation. Based on the recorded feed intake for each treatment group, these doses were equivalent to 0.07%, 0.23%, and 0.77% of the diet, respectively. The 60 mg dose produced a 49% reduction in pup growth, but no change in milk fat percent was observed. However, mammary lipogenesis, as measured by the rate of incorporation of ^{14}C-labeled glucose into lipid by tissue explants, was decreased by 40%. The considerable reductions in pup growth and lipogenesis without any decrease in milk fat percent were interpreted as evidence for a general inhibition of lactation. A reduction in lactose synthesis would depress milk yield, thus affecting the concentration of other milk components including fat.

Whereas high doses of *trans*-10, *cis*-12 CLA appear to impair lactation, low to moderate doses (0.07-0.23% of diet) cause a specific and dose-dependent reduction in milk fat and consequently pup growth (3). The 6.5 mg dose corresponding to 0.07% of the diet produced numerical decreases in lipogenesis and milk fat. Pup growth and the percent of fat in milk clots collected from the stomachs of the pups were significantly

decreased by 20% and 13%, respectively. The 20 mg dose of *trans*-10, *cis*-12 CLA caused significant reductions in milk fat (20%), clot fat (16%), lipogenesis (30%), and pup growth (19%). All three doses altered the composition of milk fat; the proportion of short and medium chain fatty acids derived from *de novo* lipogenesis was progressively decreased in a dose-dependent manner.

Studies of CLA in rodents have also reported decreases in the mRNA abundance and activity of several key lipogenic enzymes in the mammary gland. Changes include reduced mRNA expression and activity of FASN (3, 35, 36, 44) and ACC (44), two enzymes involved in *de novo* lipogenesis. Also, Hayashi et al. (36) reported decreased activity of glucose 6-phoshphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in mammary tissue. These enzymes are part of the pentose phosphate pathway, which generates reducing equivalents for fatty acid synthesis. However, NADPH is also derived from the malate pathway, and activity of the key enzyme NADP-malate dehydrogenase (NADP-MDH) was unchanged. The authors suggested that NADPH production appeared to not be the rate limiting factor in *de novo* lipogenesis (36). Enzymes involved in other processes related to milk fat production are also downregulated. Decreased abundance of LPL mRNA (35) and decreased mRNA abundance and activity of SCD (44) have been observed in response to treatment with CLA.

Effects of dietary fat on rodent lactation

A number of studies have examined the effects of varying the content and composition of dietary fat on milk fat, mammary lipogenesis, and pup growth. Results

from rodent studies have been somewhat mixed. Several studies have reported that high fat diets increase milk fat percentage (4, 39, 45, 46), while others have found no change (5, 47-50) or even a decrease in milk fat (51). There is also disagreement over whether high fat diets increase pup growth (5, 39, 45) or have no effect (6, 47, 48, 51, 52). Of the studies that measured mammary lipogenesis, most observed a decrease (4-6), whereas one found no change (46).

These differences may be attributable to variations in methodology, percent of dietary fat in the control and high fat diets, treatment period, fat source, species, and/or other factors. For instance, lipogenesis assays were either conducted *in vivo* using tritiated water (4-6) or *in vitro* using mammary explants (46). The latter method measures the lipogenic capacity of mammary tissue in the presence of abundant substrates for fatty acid synthesis, whereas *in vivo* measurements reflect rates of fatty acid synthesis that are occurring as a consequence of the availability of precursors for *de novo* lipogenesis. High fat diets ranged from 15 to 55% fat and were compared to control diets containing 0 to 12% fat. Some investigators substituted additional fat for carbohydrates in the diet; others merely added fat to the control diet. The latter approach changes the proportion of protein and other essential nutrients on a dry matter basis, which can affect the nutritional status of the dam and pups independently of the fat content of the diet. Studies also differed in the period over which the experimental diets were fed; the feeding duration for the experimental diet varied from as little as 3 d to several months. In one case, dams were raised on the high and low fat diets from birth (50). Long term feeding of a high fat diet has been demonstrated to induce obesity and impair mammary development and lactogenesis in mice (53).

Different sources of dietary fat can also contribute to the variation among studies. Common fat choices were corn oil (4, 39, 46, 51) and lard (48, 49, 52). Two studies compared high fat diets of different compositions and observed that the response varied based on the source of dietary fat. In the experiments of Grigor and Warren (5), rats were fed diets containing 20% oil that represented peanut oil, coconut oil, or linseed oil; these high fat diets all reduced the rate of mammary lipogenesis in vivo, although the reduction was greatest for the peanut oil diet. The peanut oil diet was also the only one to produce a significant increase in pup growth. Souza and Williamson (6) found differing responses to diets consisting of 20% tristearin, sunflower oil, triolein, or medium chain fatty acids. Compared to a control diet containing 4% fat, the reduction in mammary lipogenesis, assessed *in vivo* using tritiated water, ranged from 57% for the medium chain fatty acid diet to 92% for the sunflower oil diet. Medium chain fatty acids decreased pup weight by 35%, but no changes were observed for the other diets. Energy intake by the dam also varied by diet; it was significantly increased in the tristearin group and decreased in the medium chain fatty acid group.

Differences in the physiological properties of fatty acids affect their metabolic disposition and their impact on milk fat synthesis. Unlike long chain fatty acids, medium chain fatty acids are not re-esterified and packaged into chylomicra following absorption in the gut (54). As a result, these fatty acids are extensively oxidized for energy in the liver, decreasing their availability for incorporation into milk fat. This is illustrated by the observation of Souza and Williamson (6) that 65% of ^{14}C-labeled octanoate (8:0) supplied in a meal was oxidized to $^{14}CO_2$, and only 0.2% of the absorbed dose was recovered in mammary tissue. In comparison, 38% of ^{14}C-triolein (18:1) added to the

diet was oxidized, and 3.2% ended up in mammary lipids. Dietary polyunsaturated fatty acids such as those found in sunflower oil and peanut oil are known to inhibit lipogenesis by regulating gene transcription (55). These bioactive fatty acids therefore modulate the activity of lipogenic enzymes not just as substrates but also by controlling gene expression. This may account for the more pronounced inhibition of mammary lipogenesis by high fat diets containing polyunsaturated fatty acids from peanut oil or sunflower oil.

The reduction in mammary lipogenesis typically observed due to high fat diets shifts the fatty acid profile of milk fat (e.g. 4). The proportion of short and medium chain fatty acids decreases, reflecting reduced *de novo* synthesis, and long chain fatty acids comprise a greater percent of the total (4). These fatty acids are derived from the uptake of preformed fatty acids from plasma lipoproteins or circulating nonesterified fatty acids. Since rodents adapt to lactation by dramatically increasing feed intake, many of these long chain fatty acids originate from the diet. Consequently, the fatty acid profile of milk fat reflects the fatty acid composition of the diet. This is particularly evident when dietary fat sources are compared (5, 7, 50).

A crossover study by Teter et al. (50) demonstrated that isomers of *trans*-18:1 appear in milk fat within 6 h of switching from a diet consisting of predominantly *cis* fatty acids to one high in *trans* isomers. The concentration of *trans* fat in milk reached a stable plateau within 2 d and dropped dramatically within 1 d when *trans* fatty acids were removed from the diet. Teter et al. (50) also compared *cis* and *trans* fat diets at different levels of total dietary fat. The authors did not observe an effect of fat level on milk fat, but the presence of a high concentration of *trans* fatty acids in the diet decreased milk fat

percent for mice fed both low fat and high fat diets. No interaction was found between the amount of fat in the diet and the proportion of *cis* or *trans* fatty acids. The *trans* fat diets contained partially hydrogenated fat from margarine, which has been demonstrated to contain a range of isomers of CLA (56). Some of these CLA isomers may have contributed to the observed effects on milk fat, but the hydrogenation process generates a number of other unusual fatty acids that are potentially bioactive. To the best of our knowledge, the effect of dietary fat level on the ability of pure *trans*-10, *cis*-12 CLA to inhibit milk fat synthesis in lactating rodents has not been examined.

OBJECTIVE

The overall goal of this thesis was to investigate the interrelationship between dietary fat and bioactive fatty acids in the regulation of milk fat synthesis. The specific objective of the present experiment was to examine the effect of dietary fat level on the ability of the *trans*-10, *cis*-12 isomer of CLA to inhibit milk fat synthesis in a lactating mouse model.

MATERIALS AND METHODS

Animals and treatments

C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained in accordance with the National Institutes of Health guidelines for animal care. Experimental procedures were approved by the Cornell University Institutional

Animal Care and Use Committee. Females were bred in groups to wild type males and subsequently housed individually in shoe box cages with continuous access to food and water. Mice were fed a commercial pelleted diet containing 5% fat (Harlan Teklad, Madison, WI) during pregnancy and early lactation. Litters born to dams in their first (n = 17), second (n = 13), or third (n = 1) lactation were adjusted to 6-8 pups by cross-fostering within 2 d of parturition. Dams were randomly assigned to one of four treatments in a 2x2 factorial design. Mice were fed diets either low or high in fat (4% and 24%, respectively) and received oral doses of either water (control) or *trans*-10, *cis*-12 CLA (20 mg/d). Thus the treatments were low fat diet control (LFCON), low fat diet with CLA (LFCLA), high fat diet control (HFCON), and high fat diet with CLA (HFCLA). Experimental diets were provided ad libitum starting between days 4-6 of lactation. CLA treatment began 2 d later and lasted for a period of 5 d.

Pelleted semi-purified diets were prepared by Research Diets (New Brunswick, NJ). The high fat (HF) diet was formulated by substituting Trisun high oleic sunflower oil for cornstarch in the low fat (LF) diet (**Table 1**). Diet fatty acid composition (**Table 2**) was determined by the method of Sukhija and Palmquist (57). Briefly, ground feed samples were heated in 5% methanolic HCl at 70° C for 2 h as part of a one-step method to extract and esterify feed fatty acids. A triglyceride composed of 17:0 was used as an internal standard. Fatty acid methyl esters were quantified by gas chromatography using a fused-silica capillary column (CP-Sil 88; 100 m x 0.25 mm internal diameter; Varian, Inc., Walnut Creek, CA) as described by Perfield et al. (17).

CLA was administered by pipette into the mouth of the dam in three equal doses per day (0900, 1700, and 2400 h). Control mice received a similar volume of water at

each time point. The CLA stock (Natural ASA, Norway) was in free fatty acid form and contained 89.0% *trans*-10, *cis*-12 CLA, 2.0% linoleic acid, and <0.9% of any other individual fatty acid. CLA and other fatty acids were purified from the stock solution by hexane:isopropanol extraction according to Hara and Radin (58).

TABLE 1
Diet composition

	Low fat diet (LF)		High fat diet (HF)	
	g% [a]	kcal%[b]	g%	kcal%
Casein, 80 Mesh	18.96	19.72	23.31	19.72
L-Cystine	0.28	0.30	0.35	0.30
Corn starch	47.39	49.30	16.97	14.35
Maltodextrin 10	9.48	9.86	11.65	9.86
Sucrose	9.48	9.86	11.65	9.86
Cellulose, BW200	4.74	0	5.83	0
Soybean oil	2.37	5.55	2.91	5.55
Trisun high oleic sunflower oil	1.90	4.44	20.68	39.39
Mineral mix S10026	0.95	0	1.17	0
Dicalcium phosphate	1.23	0	1.51	0
Calcium carbonate	0.52	0	0.64	0
Potassium citrate, 1 H_2O	1.56	0	1.92	0
Vitamin mix V10001	0.95	0.99	1.17	0.99
Choline bitartrate	0.19	0	0.23	0
FD&C yellow dye #5	0.002	0	0.003	0
FD&C red dye #40	0.002	0	0	0
FD&C blue dye #1	0	0	0.003	0
Protein	19.2	20.0	23.7	20.0
Carbohydrate	67.3	70.0	41.4	35.0
Fat	4.2	10.0	23.6	45.0
Total kcal/g		3.85		4.73

[a] g/100 g of diet (dry weight)
[b] % energy of diet

TABLE 2
Fatty acid composition of experimental diets

	Low fat diet (LF)	High fat diet (HF)
Fatty acid[a]	g/100 g total fatty acids	
8:0	0.03	0.01
10:0	0.07	0.02
12:0	2.25	1.35
14:0	4.06	0.68
14:1c9	0.01	0.33
16:0	7.12	3.70
16:1c9	0.11	0.08
18:0	3.01	2.52
18:1c9	45.93	78.41
18:2c9,12	30.01	10.49
18:3c9,12,15	2.64	0.64
Others	4.76	1.77

[a] c, cis

Data and sample collection

Dams, litters, and feed were weighed daily between 0900 and 1000 h for temporal analysis of litter growth and dam energy intake. Average litter growth rate and energy intake for the last 3 d of treatment were also used for determination of treatment effects. Following day 5 of CLA treatment, pups were euthanized by CO_2 asphyxiation at 1130 h, and milk clots were collected from their stomachs. Clots were composited by litter, freeze-dried, and stored at -80° C. Later the same day, dams were anesthetized and milked at 1430 h. Oxytocin (7 USP; VEDCO, St. Joseph, MO) was administered IP 5 min prior to IP injection of 0.2 mL of a solution of either tribromoethanol (32 mg/mL; Avertin, Sigma-Aldrich, St. Louis, MO) or ketamine/xylazine (10 mg/mL ketamine

[Ketaset, Fort Dodge Animal Health, Fort Dodge, IA], 1 mg/mL xylazine [Anased, Ben Venue Laboratories, Bedford, OH]). Dams were milked by manually massaging the mammary glands to express droplets of milk, which were collected into a 2 mL siliconized microcentrifuge tube (ISC BioExpress, Kaysville, UT) with the aid of a vacuum source. The milking apparatus and procedure are described more completely by Harvatine et al. (3). Milk samples were immediately diluted 1:1 with isotonic saline and sonicated to improve pipetting ease. Aliquots of diluted milk were stored at -80° C until analysis. After milking, dams were euthanized by cervical dislocation while under anesthesia, and tissues were collected. The liver was weighed, and a #3 mammary gland was fixed in 10% neutral buffered formaldehyde. Mammary tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Cornell Veterinary School Histology Lab (Ithaca, NY).

Sample analysis

Fat content and fatty acid profiles were determined for milk obtained from dams and milk clots collected from pup stomachs. Lipids were extracted in duplicate from approximately 60 mg diluted milk or 20 mg freeze-dried clot using hexane:isopropanol (58). Two internal standards, a triglyceride containing 17:0 and 19:0 fatty acid methyl ester, were added prior to extraction for determination of fat concentration. Milk fatty acids were transmethylated and quantified by gas chromatography as described previously (17). A dual methylation procedure was used for clot lipids; fatty acids were first methylated overnight at 40° C in 1% methanolic sulfuric acid (59) and subsequently transmethylated and quantified as described for milk fat.

Statistical analysis

Endpoint variables were analyzed by the fit model procedure of JMP (Version 7.0; SAS Institute, Cary, NC). The model included the effects of diet (LF vs. HF), CLA (control vs. CLA), and the interaction of diet and CLA. Initial litter weight was added as a covariate in the models for litter weight gain and dam energy intake, and initial dam body weight was used as a covariate in the liver weight model. Effects of diet and CLA were considered significant at $p < 0.05$, and interactions were considered significant at $p < 0.10$. Preplanned contrasts tested the differences between LFCON and LFCLA and between HFCON and HFCLA. Data points with Studentized residuals >2.5 were excluded as outliers.

Temporal responses of litter weight gain and dam energy intake were analyzed by the mixed procedure of SAS (Version 9.1; SAS Institute, Cary, NC) using a repeated statement. The model included the fixed effects of treatment (LFCON, LFCLA, HFCON, or HFCLA), the interaction of treatment and time, and in the model of energy intake, the covariate initial dam body weight. Repeated measures over time were calculated using the autoregressive covariance structure [AR(1)], and denominator degrees of freedom were estimated using the Kenward Rogers method. Data points with Studentized residuals >3 were excluded from analysis. Preplanned contrasts tested the differences between LFCON and LFCLA and between HFCON and HFCLA at each time point.

RESULTS

Production parameters

CLA treatment decreased litter growth by 19-21% ($p < 0.001$; **Table 3**). No effect of diet or interaction between diet and CLA was observed ($p = 0.18$ and 0.69, respectively). Temporal analysis indicated that the reduction in litter growth due to CLA was evident for all 5 d of treatment for mice on the LF diet and for days 2-4 for mice on the HF diet ($p < 0.05$; **Figure 2A**). Energy intake was increased for dams on the HF diet ($p < 0.01$) and decreased by treatment with CLA ($p < 0.0001$; Table 3). An interaction between diet and CLA was observed ($p < 0.01$); the reduction in voluntary intake due to CLA was greater for mice on the LF diet (27%) than those on the HF diet (14%). Energy intake by dams on the HF diet was nearly double that of dams on the LF diet for the first day that the experimental diets were offered (**Figure 2B**). Energy intakes on the following day were similar. CLA decreased energy intake for the entire 5 d treatment period for mice on the LF diet but only for days 2 and 4 for mice on the HF diet ($p < 0.05$). Administration of CLA to dams resulted in weight loss ($p = 0.04$), but dam live weight was only numerically decreased for dams on the LF diet (Table 3). Dam liver weight was increased 2-4% for mice on the HF diet ($p < 0.01$), but CLA had no effect on liver weights (Table 3).

TABLE 3

Effect of dietary fat level and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on production and milk fat concentration of lactating C57Bl/6J mice

	Treatment[a]				SEM	P		
	LFCON	LFCLA	HFCON	HFCLA		Diet	CLA	Diet*CLA
Litter growth, g/d[b]	2.84	2.30[d]	3.11	2.45[e]	0.08	0.18	<0.001	0.69
Dam energy intake, kcal/d[b]	36.23	26.45[d]	36.24	31.26[e]	0.36	<0.01	<0.0001	<0.01
Dam weight change, g/d[c]	-0.01	-0.15	0.07	0.07	0.04	0.58	0.04	0.63
Liver weight, g	1.88	1.92	1.65	1.72	0.03	<0.01	0.35	0.78
Milk fat, %	19.92	17.44[d]	23.25	20.29[e]	0.38	<0.001	<0.01	0.75
Pup stomach clot fat, %	58.89	49.07[d]	59.16	55.38	0.75	0.04	<0.001	0.05

[a] Treatments were LFCON (4% fat diet, no CLA), LFCLA (4% fat diet, 20 mg/d CLA), HFCON (24% fat diet, no CLA), and HFCLA (24% fat diet, 20 mg/d CLA). Diets were fed to dams for 7 d in midlactation; CLA was orally administered for the last 5 d of the treatment period. Values are LS means; n = 7 for LFCON, and n = 8 for all other treatments.

[b] Average for last 3 d of treatment period

[c] Average for last 3 d of treatment period

[d] Significantly different from LFCON ($p < 0.05$)

[e] Significantly different from HFCON ($p < 0.05$)

Milk and clot fat content and composition

Milk fat percent was increased 16-17% for the HF diet ($p < 0.001$) and decreased 12-13% by CLA ($p < 0.01$; Table 3). No interaction was observed between diet and CLA. The percent of fat in milk clots collected from the stomachs of pups nursing dams on the LF diet was decreased 17% by CLA; a lesser reduction (6%) was observed for pups of dams on the HF diet (interaction: $p = 0.05$; Table 3). Considering both control and CLA-treated animals, the overall effect of the HF diet was an increase in clot fat percent ($p = 0.04$). However, this was primarily due to the less dramatic reduction in clot fat percent due to CLA when the HF diet was fed; clot fat percent was similar for the

LFCON and HFCON treatments. Milk clots from pups nursing HFCLA dams contained a greater percent of fat than those of pups nursing LFCLA dams, but both had lower clot fat percents than the controls.

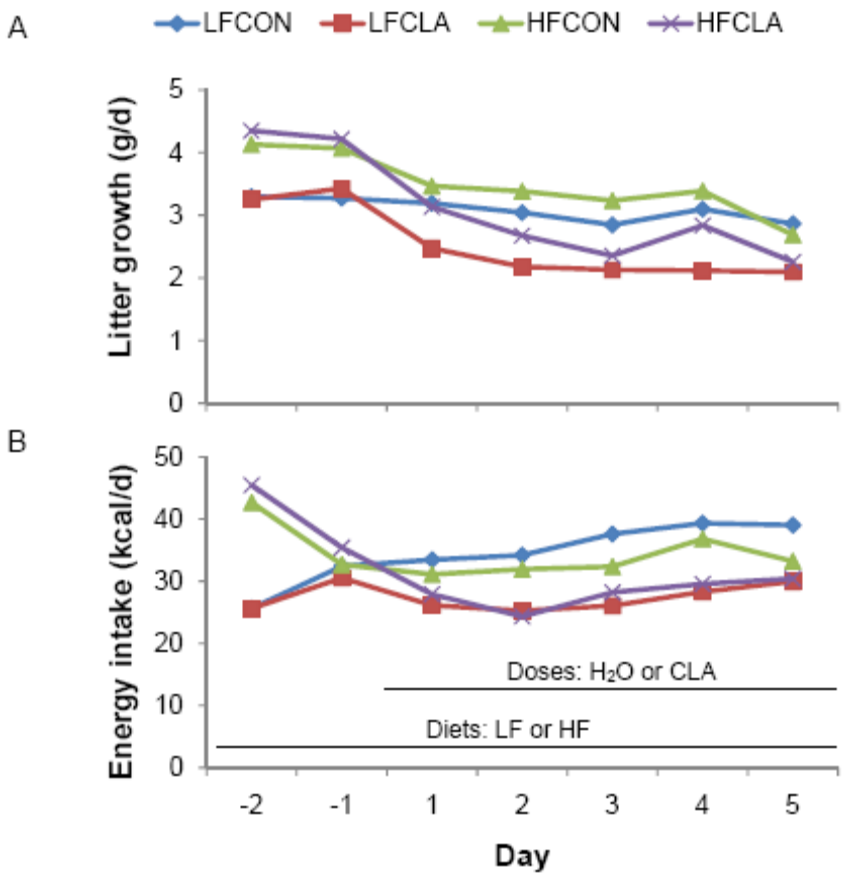

FIGURE 2. Temporal response of litter growth (A) and dam energy intake (B) in lactating C57Bl/6J mice fed low fat (4% fat; LF) or high fat (24% fat; HF) diets and orally dosed with water (control; CON) or *trans*-10, *cis*-12 conjugated linoleic acid (20 mg/d; CLA) in the specified combinations for the intervals indicated. Litter growth rates were significantly different ($p < 0.05$) between LFCON and LFCLA on days 1-5 and between HFCON and HFCLA on days 2-4. Energy intakes were significantly different ($p < 0.05$) between LFCON and LFCLA on days 1-5 and between HFCON and HFCLA on days 2 and 4. Values are LS means; n = 7 for LFCON, and n = 8 for all other treatments.

Increased dietary fat and treatment with CLA both altered the fatty acid profile of milk fat (**Table 4**). The HF diet decreased milk fat concentrations of 8:0, 10:0, 12:0, 14:0, 16:0, and 16:1c9 and increased the concentrations of 18:1c9, 18:2c9,12, 18:3c9,12,15, and 20:0. In particular, the milk fat content of 18:1c9 was increased from 29.29 to 56.46 g/100 g of fatty acids for control mice fed the HF diet. CLA decreased the concentrations of 12:0, 14:0, 16:0, and 18:3c9,12,15 and increased the concentrations of 18:0, 18:1c9, 18:2c9,12, and 20:0. *Trans*-10, *cis*-12 CLA was not detected in the milk of control mice but composed 0.61 and 0.36 g/100 g of fatty acids in the milk of LFCLA and HFCLA mice, respectively. The concentrations of individual fatty acids in clot fat differed somewhat from their concentrations in milk fat, but similar patterns of changes were observed in response to the HF diet and CLA treatment (**Table 5**). In both milk fat and milk clot fat, interactions between diet and CLA were observed for several fatty acids (Tables 4 and 5). Those that were consistently seen were for 10:0, 16:1c9, and 20:0. CLA increased the concentrations of 10:0 and 16:1c9 when the LF diet was fed but not when the HF diet was fed. The concentration of 20:0 was significantly increased by CLA for both diets, but the magnitude of the increase was greater for the LF diet.

TABLE 4

Effect of dietary fat level and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on milk fatty acid profile of lactating C57Bl/6J mice

	Treatment[a]				SEM	P		
	LFCON	LFCLA	HFCON	HFCLA		Feed	CLA	Feed*CLA
Fatty acid, g/100 g[b]								
8:0	0.18	0.28	0.18	0.13	0.02	0.02	0.43	0.04
10:0	4.07	5.80[d]	3.26	2.20	0.26	<0.001	0.53	0.01
12:0	8.99	7.96	5.57	2.90[e]	0.24	<0.0001	<0.001	0.09
14:0	12.91	7.60[d]	6.59	2.65[e]	0.22	<0.0001	<0.0001	0.13
14:1c9	0.42	0.32	0.21	0.27	0.04	0.09	0.83	0.27
16:0	21.29	17.76[d]	11.03	7.97[e]	0.17	<0.0001	<0.0001	0.48
16:1c9	3.07	3.68[d]	0.71	0.68	0.03	<0.0001	0.22	0.08
18:0	1.66	2.11[d]	1.69	2.04[e]	0.05	0.84	<0.001	0.61
18:1c9	29.36	32.33[d]	56.46	65.39[e]	0.47	<0.0001	<0.0001	<0.01
18:2c9,12	8.47	10.11[d]	7.60	8.84[e]	0.00	<0.0001	<0.0001	0.91
18:3c9,12,15	1.84	1.27[d]	1.88	1.56[e]	0.03	0.01	<0.0001	0.03
20:0	0.03	0.10[d]	0.05	0.10[e]	0.62	<0.0001	<0.0001	<0.0001
t10, c12 CLA	0.00	0.61[d]	0.00	0.36[e]	0.03	0.02	<0.0001	0.02
Total by source, g/100 g[c]								
<16 carbons	26.63	22.17[d]	15.88	8.20[e]	0.63	<0.0001	<0.0001	0.21
16 carbons	25.28	21.49[d]	11.74	8.66[e]	0.20	<0.0001	<0.0001	0.39
>16 carbons	41.45	48.25[d]	67.77	78.39[e]	0.56	<0.0001	<0.0001	0.10

[a] Treatments were LFCON (4% fat diet, no CLA), LFCLA (4% fat diet, 20 mg/d CLA), HFCON (24% fat diet, no CLA), and HFCLA (24% fat diet, 20 mg/d CLA). Diets were fed to dams for 7 d in midlactation; CLA was orally administered for the last 5 d of the treatment period. Values are LS means; n = 7 for LFCON, and n = 8 for all other treatments.

[b] c, cis; t, trans

[c] Total by source: fatty acids <16 carbons are derived from mammary *de novo* lipogenesis; fatty acids >16 carbons are taken up preformed from plasma; fatty acids 16 carbons in length originate from both sources.

[d] Significantly different from LFCON ($p < 0.05$)

[e] Significantly different from HFCON ($p < 0.05$)

TABLE 5

Effect of dietary fat level and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on the fatty acid profile of milk clots from the stomachs of C57Bl/6J pups

	Treatment[a]				SEM	P		
	LFCON	LFCLA	HFCON	HFCLA		Feed	CLA	Feed*CLA
Fatty acid, g/100 g[b]								
8:0	0.03	0.01	0.03	0.03	0.00	0.30	0.45	0.53
10:0	3.60	4.72[d]	2.82	2.30	0.10	<0.0001	0.15	<0.001
12:0	11.11	11.16	6.58	3.97[e]	0.14	<0.0001	<0.001	<0.0001
14:0	16.24	11.48[d]	7.96	3.54[e]	0.16	<0.0001	<0.0001	0.59
14:1c9	0.23	0.11[d]	0.06	0.04	0.01	<0.0001	<0.0001	<0.001
16:0	27.45	18.75[d]	12.26	7.66[e]	0.16	<0.0001	<0.0001	<0.0001
16:1c9	2.64	2.82	0.61	0.43[e]	0.03	<0.0001	0.97	0.01
18:0	1.85	1.96[d]	1.62	1.87[e]	0.02	<0.001	<0.0001	0.06
18:1c9	22.17	30.39[d]	55.78	66.69[e]	0.42	<0.0001	<0.0001	0.12
18:2c9,12	7.88	10.43[d]	7.50	8.81[e]	0.08	<0.0001	<0.0001	<0.001
18:3c9,12,15	1.16	1.25[d]	1.71	1.59[e]	0.01	<0.0001	0.66	<0.001
20:0	0.11	0.39[d]	0.18	0.30[e]	0.01	<0.001	<0.0001	<0.0001
t10, c12 CLA	0.00	0.37[d]	0.00	0.28[e]	0.01	0.07	<0.0001	0.107
Total by source, g/100 g[c]								
<16 carbons	31.55	27.55[d]	17.52	10.02[e]	0.35	<0.0001	<0.0001	0.02
16 carbons	30.19	21.59[d]	12.87	8.09[e]	0.17	<0.0001	<0.0001	<0.0001
>16 carbons	33.27	44.62[d]	66.85	79.70[e]	0.45	<0.0001	<0.0001	0.41

[a] Treatments were LFCON (4% fat diet, no CLA), LFCLA (4% fat diet, 20 mg/d CLA), HFCON (24% fat diet, no CLA), and HFCLA (24% fat diet, 20 mg/d CLA). Diets were fed to dams for 7 d in midlactation; CLA was orally administered for the last 5 d of the treatment period. Values are LS means; n = 7 for LFCON, and n = 8 for all other treatments.

[b] c, cis; t, trans

[c] Total by source: fatty acids <16 carbons are derived from mammary *de novo* lipogenesis; fatty acids >16 carbons are taken up preformed from plasma; fatty acids 16 carbons in length originate from both sources.

[d] Significantly different from LFCON ($p < 0.05$)

[e] Significantly different from HFCON ($p < 0.05$)

The HF diet and CLA treatment both decreased the proportion of fatty acids derived from de novo lipogenesis, i.e. those <16 carbons in length ($p < 0.0001$; Tables 4 and 5 and **Figure 3A**). The sum of 16C fatty acids was also reduced in milk fat and milk clot fat by both treatments ($p < 0.0001$). Conversely, the proportion of long chain fatty acids (>16C) was increased by the HF diet and CLA treatment ($p < 0.0001$). Changes in fatty acid distribution due to diet were typically greater in magnitude than those due to CLA treatment. Interactions between diet and CLA were observed for clots; CLA caused greater reductions in <16C and 16C fatty acids when the HF diet was fed than when the LF diet was fed ($p = 0.02$ and $p <0.0001$, respectively).

To take into account the possible dilution of short and medium chain fatty acids in milk fat by an increase in long chain fatty acids, fatty acid distribution was also expressed as the total content of fatty acids by source in whole milk rather than milk fat (**Figure 3B**). This parameter was obtained by multiplying the concentrations of <16C, 16C, and >16C fatty acids in milk fat by the milk fat percent of the dam. This analysis revealed that the HF diet and CLA both decreased the proportion of <16C and 16C fatty acids ($p < 0.0001$ for diet and CLA effects on both <16C and 16C fatty acids), whereas only the HF diet increased the proportion of >16C fatty acids in milk ($p < 0.001$ for diet, $p = 0.78$ for CLA). The HF diet and CLA reduced the content of <16C fatty acids in milk by 30-31% alone and 68% in combination. The proportion of >16C fatty acids in milk was increased 90-92% by the HF diet. No interactions between diet and CLA were observed.

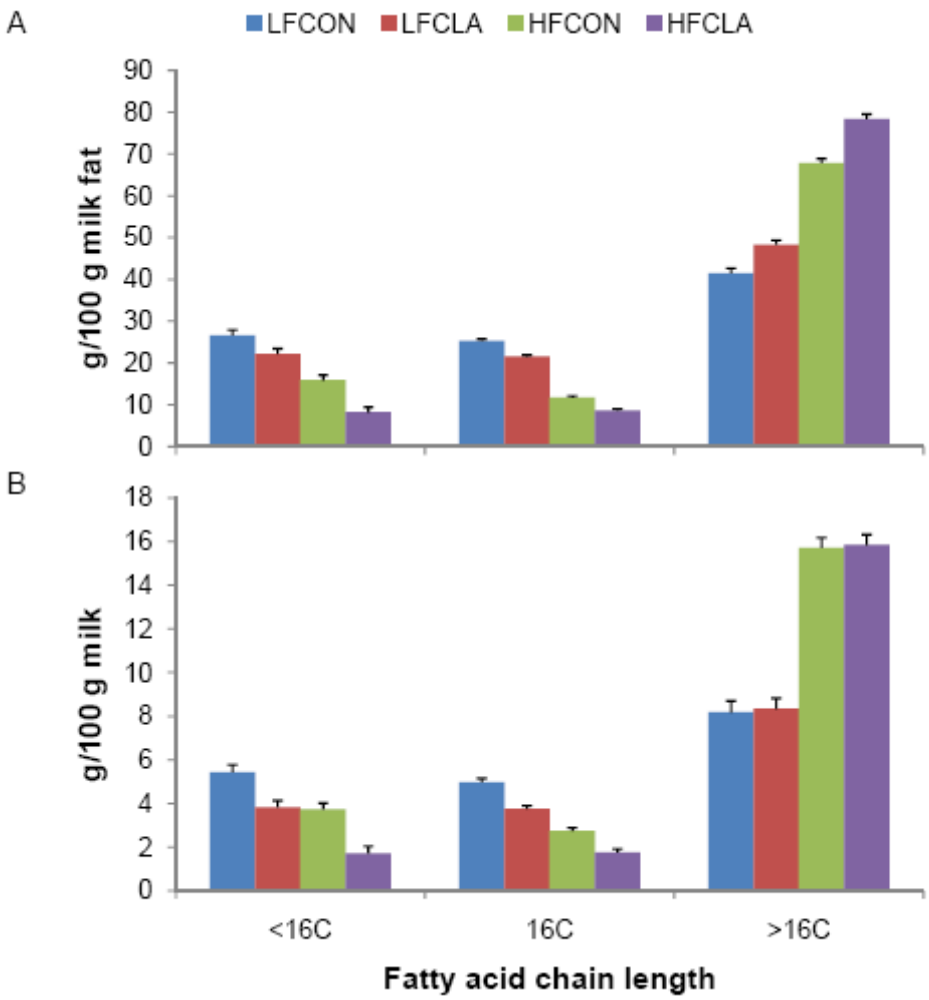

FIGURE 3. Effect of dietary fat level and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on concentrations of fatty acids of varying chain length in milk fat (A) and whole milk (B) of lactating C57Bl/6J mice. Treatments were LFCON (4% fat diet, no CLA), LFCLA (4% fat diet, 20 mg/d CLA), HFCON (24% fat diet, no CLA), and HFCLA (24% fat diet, 20 mg/d CLA). Diets were fed to dams for 7 d in midlactation; CLA was orally administered for the last 5 d of the treatment period. Fatty acids <16 carbons are derived from mammary *de novo* lipogenesis, and fatty acids >16 carbons are taken up preformed from plasma. Fatty acids 16 carbons in length originate from both sources. Values are LS means + SEM; n = 7 for LFCON, and n = 8 for all other treatments.

Comparable analysis of the fatty acid distribution in whole clots revealed that both the HF diet and CLA significantly altered the clot content of <16C, 16C, and >16C fatty acids ($p < 0.001$; data not shown). However, CLA increased the proportion of >16C fatty acids by only 11-12%, compared to a 101-102% increase due to the HF diet. The concentration of >16C fatty acids in clots was decreased 37% by the HF diet alone, 46% by CLA alone, and 70% by the HF diet and CLA in combination. An interaction between diet and CLA was observed such that CLA caused a lesser reduction in 16C fatty acids in combination with the HF diet than alone ($p < 0.0001$).

Histology

Mammary tissue from a subset of dams (n = 4) from each treatment group was examined, and no discernable differences in tissue architecture, prevalence of cell populations, or cell morphology were observed among treatments (data not shown).

DISCUSSION

Consistent with previous studies investigating the effects of CLA isomers on rodent lactation (3, 35, 36, 41), treatment with *trans*-10, *cis*-12 CLA decreased milk fat percent in lactating mice (Table 3). However, the magnitude of this reduction was less than expected based on a previous dose-response experiment completed in our lab (3). In the present study, oral administration of 20 mg/d of CLA caused a 12% decrease in milk fat percent and the incorporation of CLA into milk fat at a concentration of 0.61 g/100 g of fatty acids for mice on the LF diet (Tables 3 and 4). In contrast, in our earlier study,

the same dose previously produced a 20% decrease in milk fat percent and the incorporation of CLA into milk fat at 1.35 g/100 g (3). The response in the present experiment was more similar to that when mice were treated with a lower dose of 6.5 mg/d of CLA, which decreased milk fat by 11% and resulted in 0.47 g/100 g CLA in milk fat (3). The length, timing, and method of CLA treatment were the same in both studies. In the dose-response experiment (3), mice were fed a chow diet containing 5% fat, compared to a semi-purified diet containing 4% fat in the current study (Table 1). Although the nutrient composition of the diets was similar, the absorption of fatty acids may be influenced by whether they are supplied in conjunction with a semi-purified diet or a chow diet. An additional difference is that in the present study, the CLA stock was further purified before administration by extraction using hexane:isopropanol. This process may have removed salts or other compounds that aid the absorption of fatty acids in the gut. This could have reduced the absorption of CLA in the intestine such that the effective dose was lower in the present experiment. This is supported by the fact that the magnitude of the reduction in milk fat percent due to CLA treatment was consistent with the degree of incorporation of CLA into milk fat based results obtained when a lower dose of CLA (6.5 mg/d) was administered (3).

A reduction in short and medium chain fatty acids was primarily responsible for the decrease in milk fat due to treatment with CLA (Table 4 and Figure 3). These fatty acids are derived from *de novo* lipogenesis in the mammary gland, which involves the enzymes acetyl CoA carboxylase (ACC) and fatty acid synthase (FASN). ACC catalyzes the first and rate-limiting step in fatty acid synthesis, the conversion of acetyl CoA into malonyl CoA, and FASN is a multi-enzyme complex that catalyzes a sequence of seven

reactions in which two-carbon units from malonyl CoA are added to the growing fatty acyl chain (38). The mammary activity and expression of ACC and FASN are reduced by CLA treatment in rodents (3, 35, 36, 44) and cows (2, 20). As noted previously, the expression of genes encoding these key lipogenic enzymes and others is regulated by the transcription factor SREBP1c, and CLA is thought to inhibit the proteolytic cleavage of SREBP1c that is required for it to function as a transcriptional activator (22). Thus, CLA treatment appears to decrease *de novo* lipogenesis by inhibiting the activation of SREBP1c, which results in a reduction in the transcription of lipogenic genes such as ACC and FASN.

A decrease in the synthesis of short and medium chain fatty acids would result in an increase in the proportion of long chain fatty acids that are incorporated into milk fat. This was indeed observed in response to CLA treatment (Figure 3A), but after accounting for the decrease in milk fat percent, the total content of >16C fatty acids in whole milk was not affected by CLA (Figure 3B). This is in contrast to observations in the cow that the reduction in milk fat yield due to CLA treatment involves fatty acids of all chain lengths (8). Conclusions based on milk fat yield are not strictly comparable to those based only on milk composition, but in the cow, treatment with CLA does not affect milk yield (8). Thus, the contribution of different fatty acids to milk fat yield should reflect their concentrations in whole milk. To the best of our knowledge, the effect of CLA on milk production in mice has not been determined due to the greater difficulty of measuring milk yield in rodents. If milk yield is indeed unaffected by CLA as it is in the cow, the lack of a reduction in >16C fatty acids in milk may represent a lesser effect of

CLA on the incorporation of preformed long chain fatty acids into milk fat in the mouse than in the cow.

An important enzyme in this process is lipoprotein lipase (LPL), which cleaves fatty acids from circulating lipoproteins to allow their uptake into cells and subsequent re-esterification for secretion in milk fat. Transcription of the gene encoding LPL is stimulated by SREBP1c, and LPL expression is downregulated in cows treated with CLA (2, 22). Measurements of gene expression in the rodent mammary gland during treatment with CLA found no difference (3) or a decrease (35) in the abundance of LPL mRNA. Replication of these measurements as well as determination of LPL activity and the expression of fatty acid binding proteins (FABP) would help elucidate whether the uptake of preformed fatty acids by the mammary gland is less responsive to CLA in rodents than in cows.

The decrease in milk fat percent due to CLA treatment was accompanied by reductions in the growth rate of the nursing litter and the energy intake of the dam (Table 3 and Figure 2). These responses are probably a direct result of the decreased energy content of the milk. Fat is the major energy source in milk, and its synthesis presents a significant energetic cost to the lactating dam (1). Assuming no change in milk yield, any treatment that decreases milk fat percent would thus be expected to decrease pup growth and the energy requirement of the dam. This would in turn decrease energy intake, as this parameter is highly correlated with energy demand in lactating rodents. For instance, decreasing the energetic cost of lactation by artificially reducing litter size leads to a decrease in energy intake (60), and increasing maintenance energy requirements by keeping mice in a cold environment results in greater energy intake (61).

Similarly, pup growth is positively correlated with milk fat percent across mouse strains (40) and when milk fat percent is altered by varying the fat content of the diet (39).

As increased intake of dietary fat often results in greater milk fat percent, we sought to determine whether feeding a high fat diet would overcome the reduction in milk fat induced by CLA treatment. The fat content of the experimental diet was increased by substituting high oleic sunflower oil for cornstarch to keep the density of other nutrients such as protein constant when expressed on an energy basis (Table 1). Soybean oil was included in both experimental diets to provide essential fatty acids. High oleic acid sunflower oil was chosen as the primary fat source to provide fatty acids that could be readily incorporated into milk fat but that lacked significant bioactive properties. Oils rich in medium chain fatty acids were avoided because these fatty acids are extensively oxidized in the liver, reducing their availability to the mammary gland (6, 54). An oil low in polyunsaturated fatty acids was desired because some of these fatty acids are reported to regulate gene transcription (55). The sunflower oil selected contained approximately 85% oleic acid; oleic acid thus comprised 46% and 78% of the fatty acids by weight in the LF and HF diets, respectively (Table 2). With the exception of oleic acid and 14:1c9, all fatty acids measured were found in greater proportion in the LF diet than in the HF diet.

The HF diet resulted in an increase in milk fat percent (Table 3), and the composition of milk fat reflected that of dietary fat (Tables 2 and 4). The observed increase in oleic acid in milk fat was particularly striking; it rose from 29 to 56 g/100 g of fatty acids in the control mice when the HF diet was fed (Table 4). This increase was predominantly due to incorporation of dietary oleic acid into milk fat, as long chain fatty

acids are not synthesized in the mammary gland. In addition to an increase in the proportion of fatty acids taken up preformed from plasma, the fatty acid profile of milk fat also reflected a reduction in the proportion of fatty acids derived from *de novo* lipogenesis (Table 4, Figure 3A). Accounting for the increase in milk fat percent revealed that this was not merely due to dilution of short and medium chain fatty acids by an increase in long chain fatty acids (Figure 3B). Thus an inhibition of *de novo* lipogenesis was likely, as has been demonstrated by decreased rates of mammary lipogenesis in rodents fed high fat diets (4-6). Inhibition of ACC by non-esterified fatty acids has been proposed as a mechanism for the reduction in lipogenesis due to increased dietary fat (38).

Although the HF diet increased milk fat percent, litter growth rate was only numerically increased (Table 3). Several studies have reported the similar results (6, 47, 48, 51, 52), whereas others have observed significant increases in pup growth (5, 39, 45). As discussed previously, differences in methodology may have contributed to this inconsistency. An increase in energy intake was seen for dams on the HF diet (Table 3). This was may have been related the greater palatability of the diet, as mice were observed to overeat when first offered the HF diet (Figure 2B). Energy intake on the second day was closer to that of mice on the LF diet, but it remained elevated for the duration of the treatment period. Del Prado et al. (39) compared milk fat synthesis and litter growth for lactating rats fed low or high fat diets ad libitum or pair fed the high fat diet on an equal energy basis to rats consuming the low fat diet. They found that elevated fat content in the diet increased milk fat percent and litter growth irrespective of energy consumption.

Therefore, the observed increase in milk fat content for dams on the HF diet in the present experiment was likely not solely a consequence of increased energy intake.

Although increased dietary fat intake caused an increase in milk fat percent, it was unable to overcome the CLA-induced inhibition of milk fat synthesis (Table 3). Milk fat content was decreased equally by CLA for mice fed either diet. Whereas the HF diet increased the incorporation of dietary long chain fatty acids into milk fat, it inhibited the synthesis of short and medium chain fatty acids to an equal or greater extent than did CLA treatment (Figure 3B). Transfer of dietary fatty acids to milk fat was, therefore, not enough to compensate for the reduction in lipogenesis, so no interaction was found between diet and CLA (Table 3). Interactions were observed for other parameters, such as energy intake (Table 3). The greater palatability of the HF diet presumably caused the CLA-treated dams to consume energy in excess of their requirements. Consistent with this hypothesis, the body weight of HFCLA dams changed by +0.07 g/d during the treatment period compared to a change of -0.15 g/d for the LFCLA dams (Table 3). However, variation in recorded weights was high due to movements of the dams during weighing, so neither treatment significantly differed from its respective control. Interactions between feed and CLA were also noted for the concentrations of multiple fatty acids in milk fat and clot fat, but in only three cases (10:0, 16:1c9, and 20:0) was the same effect observed for the same fatty acid in both milk and clot fat (Tables 4 and 5). A reason for these interactions is not apparent.

In general, effects of diet and CLA on the fatty acid profile of milk clots reflected changes in milk fatty acid composition, but differences in the concentrations of individual fatty acids were observed (Tables 4 and 5). These are thought be related to circadian

patterns of feed consumption and lipogenesis as well as differences in the timing of milk and clot collection with respect to the photoperiod and the final CLA dose. Feed intake by lactating rodents is greatest at night, and this is associated with a peak in mammary lipogenesis due to the greater availability of substrates for fatty acid synthesis (39). Dams in the present experiment were milked in the afternoon, which coincides with a period of lower feed intake and lipogenesis. In contrast, milk clots from the stomachs of pups provide a composite of milk samples over time; labeled milk can be recovered from clots up to 20 h after its ingestion by rat pups (62). Therefore, the fatty acid profile of clots exhibits less diurnal variation. Indeed, the proportions of >16C and 16C fatty acids in clot fat were greater than those in milk fat, since clots presumably reflected the fatty acid composition of milk synthesized during the period of greater lipogenesis during the preceding night rather than just during the day (Tables 4 and 5). The concentration of CLA in clot fat was less than that measured in milk fat (Tables 4 and 5). This was also related to the timing of milking and clot collection. Clots were recovered 2-3 h after the final dose of CLA was administered, leaving little time for ingested CLA to be absorbed by the dam, incorporated into milk fat, and consumed by the pups. Thus the milk most recently ingested was produced toward the end of the interval between CLA doses and likely contained less CLA. On the other hand, dams were milked 5-6 h after the last CLA dose, so the composition of milk fat reflected greater availability of absorbed CLA for incorporation into milk fat.

Biological effects of CLA isomers have been observed in many tissues and cell types, and a number of mechanisms have been investigated. The reductions in body fat accretion and carcinogenesis attributed to isomers of CLA have been linked to increased

apoptosis of adipocytes (63) and neoplastic mammary epithelial cells (64). High concentrations of *trans*-10, *cis*-12 CLA have been shown to induce apoptosis in cultured bovine mammary epithelial cells, and this has been proposed as a mechanism for the effects of CLA on lactation (65). Increased cytokine expression and macrophage infiltration have also been observed in adipose tissue of rodents treated with CLA (66). In the present study, examination of mammary tissue from mice treated with CLA yielded no signs of inflammation or increased apoptosis. No gross differences were discernable among treatments, thus, these mechanisms were unlikely to have contributed to alterations in milk composition.

In conclusion, treatment of lactating mice with *trans*-10, *cis*-12 CLA decreased milk fat percent and growth of the nursing litter. This was not overcome by increased intake of dietary fat, although milk fat percent was greater for mice fed a high fat diet than those fed a low fat diet. Both CLA treatment and consumption of a high fat diet resulted in changes in the fatty acid profile of milk fat that suggested depressed *de novo* lipogenesis in the mammary gland. The high fat diet, but not CLA, increased the incorporation of preformed fatty acids derived from plasma into milk fat. These long chain fatty acids originated primarily from the diet, and substantial transfer of dietary oleic acid to milk fat was observed for mice fed a high fat diet rich in oleic acid. The composition of milk clots collected from the stomachs of nursing pups reflected that of milk, but some differences were noted. Histological observations of mammary tissue did not support CLA-induced inflammation or apoptosis of mammary epithelial cells as potential mechanisms for the observed decreases in lipogenesis and milk fat percent in response to CLA treatment. Alternatively, a mechanism was discussed involving reduced

activation of the transcription factor SREBP1c, resulting in decreased expression of genes encoding key lipogenic enzymes in the mammary gland.

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