Sensitivity of the S14 null mouse to *trans*-10, *cis*-12 conjugated linoleic acid in the regulation of milk fat depression

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ABSTRACT

Trans-10, *cis*-12 conjugated linoleic acid (CLA) is a fatty acid intermediate produced during rumen biohydrogenation of dietary polyunsaturated fatty acids in the cow. *Trans*-10, *cis*-12 CLA inhibits *de novo* milk fat synthesis in the mammary gland through a coordinated downregulation of key lipogenic enzymes and the nuclear protein thyroid hormone responsive spot 14 (S14). A previous study using a mouse model showed that the S14 knockout (KO) mouse was hyperresponsive to CLA. In KO mice, mammary lipogenesis decreased by 82% in response to a 5-day treatment of 20 mg/d *trans*-10, *cis*-12 CLA, whereas in wildtype (WT) controls, rates of lipogenesis decreased by 23%. The objective of the present study was to investigate whether the KO mouse was also hypersensitive to CLA. This was done by testing the lactogenic response of the KO mice to a low dose of CLA. WT and KO C57BL/6J mice were orally administered with either water (control) or 3.5 mg/d of *trans*-10, *cis*-12 CLA for 5 days. In response to CLA treatment, KO dams had a 28.7% reduction in the percentage of *de novo* synthesized fatty acids in milk, whereas in WT dams this reduction (8.9%) was not significant, and there was an interaction of genotype and CLA treatment. Investigations with mammary explants demonstrated that lipogenic capacity decreased in both KO and WT dams, but there was no interaction of genotype and CLA treatment. A compilation of results from the present study and from previous studies suggests that the ED_{50} of CLA of the WT and KO mice are similar, thus indicating that the sensitivity of the two genotypes are similar. Therefore, the S14 KO mouse is hyperresponsive to CLA-induced inhibition of mammary lipogenesis but sensitivity is unaltered.

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INTRODUCTION

Milk fat depression (MFD) is a common economic problem in today's dairy industry and it represents a dietary situation in which the fat content in cow's milk is reduced. MFD is associated with certain diets including ones high in polyunsaturated fat and highly digestible carbohydrate. The biohydrogenation theory established that MFD is caused by an inhibition of milk fat synthesis in the mammary gland by specific bioactive fatty acids produced as intermediates in ruminal biohydrogenation (Bauman and Griinari, 2003). *Trans*-10, *cis*-12 conjugated linoleic acid (CLA) was the first of these biohydrogenation intermediates specifically identified to reduce milk fat synthesis and has been examined in the context of nutrigenomics, the study of the effect of specific bioactive dietary components on gene expression. *Trans*-10, *cis*-12 CLA has been shown to cause a coordinated downregulation in the gene expression of key lipogenic enzymes, thus resulting in reduced milk fat. Specifically, the sterol response element binding protein (SREBP) transcription factor signaling system and the nuclear protein thyroid hormone responsive spot 14 (S14) have been implicated (Harvatine and Bauman, 2006).

Further investigation of the mechanism of CLA-induced MFD has been possible using a lactating mouse model developed in our laboratory. In this mouse model of MFD, oral administration of *trans*-10, *cis*-12 CLA results in a dose-dependent reduction in milk fat, a shift in milk fatty acid profile, and a downregulation of mammary gene expression of lipogenic enzymes much like in the dairy cow. In addition, a functional role of S14 in CLA-induced MFD has been recently demonstrated during CLA treatment of S14 null mice. When treated with CLA at a dose known to induce a near maximal response in wildtype animals, the knockout animals decreased mammary lipogenesis by 82%, which

is significantly greater than the 23% reduction observed in wildtype controls (Harvatine and Bauman, unpublished). Thus, mammary lipogenesis of S14 null mice is hyperresponsive to CLA at this near maximal dose. Lower CLA doses, however, have not been examined, and it is unknown if the S14 null mouse is also hypersensitive to CLA. This information would provide insight into the mechanism of the CLA-induced inhibition of milk fat synthesis. The objective of this thesis is to examine the sensitivity of the S14 knockout mice to CLA by testing the lactogenic response to a low dose of CLA.

REVIEW OF THE LITERATURE

Milk fat

Milk fat accounts for a large proportion of the energy in milk and is also the most variable milk component. Although reduced fat milk is increasingly desired for consumption as fluid milk, other dairy products such as cheese and ice cream require high levels of milk fat. Therefore, from a production standpoint, fat content affects the economic value of milk.

Ruminant milk is estimated to contain over 400 different fatty acids (Bauman and Griinari, 2001). The two sources of fatty acids in milk fat are preformed circulating fatty acids absorbed in the small intestine and mobilized from body reserves, and *de novo* fatty acids synthesized in the mammary gland. In the cow, *de novo* fatty acids constitute about half of the total fatty acids in milk fat. *De novo* fatty acids are short and medium chain fatty acids, from four to fourteen carbons in length plus ~50% of the sixteen carbon fatty acids. In contrast, preformed fatty acids are long chain, including the remaining portion of the sixteen carbon fatty acids and all the fatty acids of eighteen or more carbons. The fat content of milk is affected by genetics and the environment. From a managerial perspective, nutrition is the major environmental factor affecting milk fat and the most practical way of manipulating milk fat levels (Bauman and Griinari, 2003).

Milk fat depression

Milk fat depression (MFD), first described in 1885, is a phenomenon commonly observed in dairy cows and it represents a dietary situation in which the fat content of

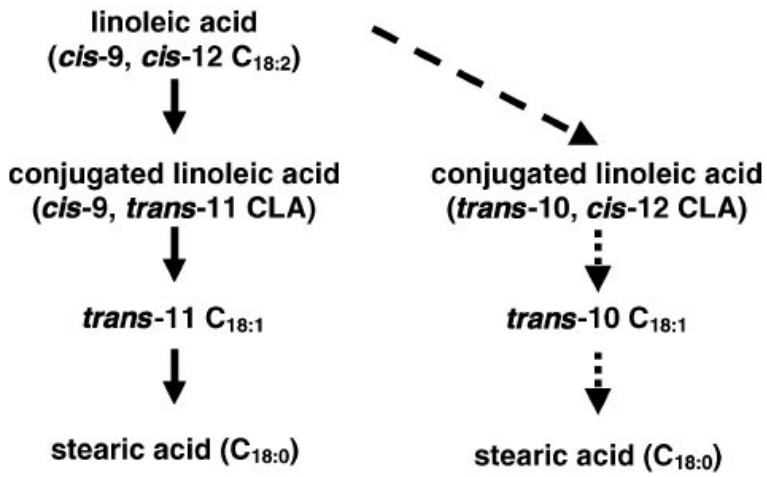

Figure 1. Proposed pathways of ruminal biohydrogenation of linoleic acid. Adapted from Bauman and Griinari (2003).

milk is reduced (Bauman and Griinari, 2003). During MFD cows do not achieve their genetic potential for milk fat synthesis and the value of the milk is decreased, thus creating an economic problem for producers. Only fat is affected in diet-induced MFD; milk yield and protein composition are unchanged. MFD is associated with certain diets including those high in polyunsaturated fat and highly fermentable carbohydrate.

Many theories have attempted to explain the mechanism of MFD, but most have proven inadequate (Bauman and Griinari, 2001). Davis and Brown (1970) observed that changes in milk fat yield correlated with the concentration of *trans* fatty acids in milk. Milk from milk fat-depressed cows contained an increased amount of *trans*-C18:1 fatty acids. Griinari *et al*. (1998) later observed that not all isomers of *trans*-C18:1 fatty acids correlated with low milk concentration. Rather, a specific increase in *trans*-10 C18:1 and its rumen precursor, *trans*-10, *cis*-12 conjugated linoleic acid (CLA), was seen in reduced fat milk. A putative pathway for ruminal biohydrogenation of dietary linoleic acid was proposed (**Figure 1**; Bauman and Griinari 2003). Linoleic acid is normally isomerized to *cis*-9, *trans*-11 CLA, which in turn is saturated to *trans*-11 C18:1 then to stearic acid.

However, under certain dietary conditions, linoleic acid may follow an alternate pathway, in which isomerization to *trans*-10, *cis*-12 CLA occurs first, followed by hydrogenation to *trans*-10 C18:1 then to stearic acid. This alternate pathway has been supported by studies with cultures of rumen bacteria which demonstrated the production of *trans*-10, *cis*-12 CLA from linoleic acid (Wallace *et al*., 2007) and *trans*-10 C18:1 from *trans*-10, *cis*-12 CLA (Kepler *et al*., 1966).

Based on this putative rumen biohydrogenation pathway, the biohydrogenation theory was proposed, which states that MFD is caused by an inhibition of milk fat synthesis in the mammary gland by specific bioactive fatty acids produced as intermediates in ruminal biohydrogenation (Bauman and Griinari, 2003). *Trans*-10, *cis*-12 CLA was the first of these biohydrogenation intermediates specifically demonstrated to inhibit milk fat synthesis. Abomasal infusion of a mixture of CLA isomers including *trans*-10, *cis*-12 CLA resulted in a 55% reduction of milk fat yield (Chouinard *et al*., 1999). Subsequent studies found that the *trans*-10, *cis*-12 CLA was the isomer responsible for the decrease in milk fat (Baumgard *et al*., 2000). The major CLA isomer in milk, *cis*-9, *trans*-11 CLA, was found to have no effect on milk fat yield in this study. Two more CLA isomers have been identified which inhibit milk fat synthesis, *cis*-10, *trans*-12 CLA (Saebo *et al*., 2005) and *trans*-9, *cis*-11 CLA (Perfield *et al*., 2007).

A change in fatty acid composition occurs in both diet-induced and CLA-induced MFD. The yield of fatty acids of all chain lengths is decreased, but the reduction of *de novo* fatty acids is most prominent (Bauman and Griinari, 2003). This results in a decreased proportion of *de novo* fatty acids and an increased proportion of preformed fatty acids in milk fat. MFD is also associated with a decrease in stearoyl CoA desaturase

(SCD) activity. SCD is an enzyme that introduces a *cis*-9 double bond in fatty acids. The desaturase index, the substrate/product ratio often used as a proxy for SCD activity, is increased for fatty acids of several lengths, including $C_{14}/C_{14:1}$, $C_{16}/C_{16:1}$, $C_{18}/C_{18:1}$ (Baumgard *et al*., 2001). High CLA doses lower the desaturase index, but low CLA doses do not alter this index.

Biological effects of *trans*-10, *cis*-12 CLA

Conjugated linoleic acids (CLA) are fatty acids with an 18-carbon chain and a pair of conjugated double bonds, which are two double bonds separated by a one single bond. Over 20 geometric and positional isomers of CLA exist, yet little is known about the biological effects of most of them (Lock and Bauman, 2004). Research on CLA has been done primarily using mixtures of CLA isomers, due to commercial availability and low cost. Studies on individual isomers of CLA has focused on two isomers, *cis*-9, *trans*-11 CLA, which is the major isomer in ruminant products, and *trans*-10, *cis*-12 CLA. *Trans*-10, *cis*-12 CLA is a potent bioactive fatty acid with broad range of biological effects (Bhattacharya *et al.*, 2006). These include decreased body weight gain, decreased body fat accretion, reduced atherogenesis, reduced tumorigenesis, improved glucose tolerance and insulin sensitivity, alteration of inflammatory response mediators, and improved bone health.

Among the first animal studies investigating the effect of CLA on body composition was the one by Park et al. (1997), in which mice fed a diet containing 0.5% CLA had 60% lower body fat and 14% more lean body mass compared to controls. Most animal studies examining the antiobesity effect of CLA have found that CLA reduces

body fat, and the *trans*-10, *cis*-12 isomer has been found to be more potent (Whigham *et al*., 2007). Studies on humans to date have used mostly mixtures of CLA isomers, with a few studies using pure CLA isomers, and have yielded mixed results. Nevertheless, a meta-analysis of 18 human trials has reported that a modest body fat reduction is seen with the median dose of 3.2 g/d used in the studies (Whigham *et al*., 2007).

Trans-10, *cis*-12 CLA has also been shown to reduce in vivo tumorigenesis in animal models and in vitro proliferation in cell culture studies. Rats fed a diet containing 0.5% *trans*-10, *cis*-12 CLA had 33% less premalignant lesions at 6 weeks and 35% less mammary tumors at 24 weeks after treatment with the carcinogen methylnitrosourea (MNU) compared with control rats (Ip *et al*., 2002). In a mouse xenograft study comparing the effects of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA on mammary tumorigenesis, no effect was observed on latency and tumor growth, but both isomers significantly reduced metastasis (Hubbard *et al*., 2003). Both CLA isomers have also been shown to reduce growth and viability of tumor cell lines derived from the mammary gland, prostate, and digestive tract, though the isomers differ in their effectiveness (summarized in Kelley *et al*., 2007). A number of possible mechanisms through which CLA exerts these anticarcinogenic effects have been proposed, and these include apoptosis, increased energy expenditure, increased fat oxidation, decreased adipocyte size, decreased energy intake, and inhibition of enzymes involved in fatty acid metabolism and lipogenesis (Bhattacharya *et al.*, 2006, Li *et al*., 2008). As with the antiobesity effect, human studies on the anticarcinogenic effect of CLA have been inconclusive. The majority of studies found no effect of CLA and there has been no

published human cancer study in vivo that examined the effects of purified CLA isomers (Kelley *et al*., 2007).

In contrast to studies examining the antiobesity and anticarcinogenic effects of CLA, relatively few rodent studies have been conducted on the effect of CLA on lipid synthesis during lactation. In a study by Ringseis *et al*. (2004), rats were fed a 1.47% CLA mixture containing 22 isomers throughout breeding, pregnancy, and lactation. Compared to control rats fed sunflower oil, rats fed CLA had 49% lower fatty acid synthase (FASN) mRNA and 51% lower lipoprotein lipase (LPL) mRNA in the mammary, 80% lower FASN activity in the mammary, 46% lower milk fat content, fewer pups per litter, lower litter weights, and lower triglyceride concentration in liver but not in plasma. Hayashi *et al*. (2007) reported that in rats fed 1.35% of a CLA mixture, milk fat content and pup growth were reduced by 33% and 21%, respectively. CLA decreased short and medium chain fatty acids in milk and activities of lipogenic enzymes such as FASN in mammary and liver. Taken together, the results from these studies suggest that dietary CLA reduces milk fat through reduced *de novo* fatty acid synthesis in the mammary and impaired uptake of preformed fatty acids from the plasma into the mammary gland.

Very few studies have examined the effect of individual CLA isomers on lactation. In one study comparing the effects of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA, lactating mice were fed *trans*-10, *cis*-12 CLA (0.91% of diet) or *cis*-9, *trans*-11 CLA (0.96% of diet) from day 4 to 15 of lactation (Loor *et al*., 2003). *Trans*-10, *cis*-12 CLA significantly reduced milk fat concentration, reduced litter weight by 28%, and feed intake by 18% compared with controls, whereas *cis*-9, *trans*-11 CLA showed a smaller

effect. *Trans*-10, *cis*-12 CLA decreased the milk fat percentage of the sum of 12:0, 14:0, and 16:0 by 10.69% compared with control. Lin *et* al. (2004) analyzed some of the cellular changes that occurred in the mammary tissue obtained in the Loor *et* al. (2003) study and found both CLA isomers reduced mammary mRNA levels of acetyl CoA carboxylase (ACC), FASN, and SCD1, and mammary activity of ACC and SCD1. Of the two CLA isomers, *trans*-10, *cis*-12 caused a greater decrease in ACC activity and SCD1 mRNA and activity. Collectively, these results suggest that *trans*-10, *cis*-12 CLA is more potent than the *cis*-9, *trans*-11 isomer in inhibiting *de novo* milk fat synthesis and that the mechanism involves a decrease in gene expression and activity of lipogenic enzymes in mammary tissue.

Mechanism of CLA-induced MFD

Trans-10, *cis*-12 CLA has been shown to cause a coordinated downregulation in the gene expression of key lipogenic enzymes in the mammary gland of the dairy cow during MFD (Baumgard *et al*., 2002, Peterson *et al*., 2003). Affected genes include LPL, fatty acid binding proteins (FABP), ACC, FASN, glycerol phosphate acyltransferase (GPAT), and acylglycerol phosphate acyltransferase (AGPAT). LPL and FABP are involved in the uptake and transport of preformed fatty acids from the circulation. ACC and FASN are both involved in *de novo* fatty acid synthesis. ACC converts acetyl CoA into malonyl CoA, which in turn is incorporated into the newly forming fatty acid by FASN. GPAT and AGPAT are involved in triglyceride synthesis (Harvatine and Bauman, 2006).

The coordinated downregulation of these genes suggests a role for a global regulator of gene expression. The sterol response element binding protein (SREBP) family of transcription factors was proposed as a candidate system (Baumgard *et al*., 2002). A potential role of SREBP as the global moderator of lipid synthesis during MFD is supported by the fact that all of the CLA-responsive genes listed above contain the sterol response element (SRE) in their promoter to which SREBP binds. SREBP1 and SREBP2 are two proteins encoded by separate genes. SREBP1 consists of two subtypes, SREBP1a and SREBP1c, which have differential expression in various organs (Shimomura *et al*., 1997). SREBP1c is the predominant subtype in adipose tissue and is highly expressed in bovine mammary tissue (Harvatine and Bauman, unpublished). SREBP1a is the major subtype in hepatic tissues and most cell lines. Proteolytic cleavage of SREBP1 releases the mature functional nuclear fragment, nSREBP. nSREBP binds to SRE and some E-boxes in sterol-responsive genes to promote transcription (Mater *et al*., 1999).

In bovine mammary epithelial cells treated with *trans*-10, *cis*-12 CLA, nSREBP level was reduced but not the amount of the precursor SREBP1, suggesting that CLA inhibits the proteolytic cleavage of SREBP1 (Peterson *et al*., 2004). In vivo, the mRNA level of SREBP1 was decreased during both diet-induced and CLA-induced MFD (Harvatine and Bauman, 2006). Enzymes involved in processing of SREBP1, including insulin responsive gene (INSIG) 1, INSIG 2, and SREBP cleavage activating protein (SCAP) were also decreased. These observations support that SREBP regulates milk fat synthesis during diet-induced and CLA-induced MFD. An additional protein, thyroid hormone responsive spot 14 (S14), was identified as a candidate regulator of CLA-

mediated inhibition of milk fat synthesis in bovine mammary epithelial cells using microarray analysis (Harvatine and Bauman, 2006).

Thyroid hormone responsive spot 14 (S14)

The thyroid hormone responsive spot 14 is a nuclear protein highly correlated with lipogenesis, but its exact biochemical functions are not known (Harvatine and Bauman, 2006). S14 is highly expressed in tissues which produce lipid for use as a metabolic fuel and in which lipogenesis is regulated by thyroid hormone and dietary constituents (Cunningham *et al*., 1998). These tissues include the liver, brown and white adipose tissues, and lactating mammary tissue. In contrast, S14 mRNA levels in nonlipogenic tissues such as brain, kidney, heart, spleen, testis, pituitary gland, and nonlactating mammary gland are low. Thyroid hormone regulates S14 transcription in lipogenic tissues. For example, in rat liver, the nuclear precursor S14 mRNA accumulated before mature mRNA in response to thyroid hormone (Narayan *et al*., 1984). S14 expression is not controlled by thyroid hormone in nonlipogenic tissues expressing thyroid hormone receptors, suggesting that other tissue-specific factors are necessary for S14 transcription (Jump and Oppenheimer, 1985). Dietary sucrose also increases S14 transcription when thyroid hormone is present. Hypothyroid animals have negligible changes in S14 mRNA levels with sucrose gavage, but response to sucrose was restored when thyroid hormone was coadministered with sucrose, indicating that thyroid hormone and sucrose act synergistically to promote S14 transcription (Mariash *et al*., 1986). On the other hand, polyunsaturated fatty acids (PUFAs) inhibit the expression of S14 (Cunningham *et al*., 1998). Rats fed a diet containing menhaden oil, which is enriched in

long chain polyunsaturated fatty acids, exhibited a rapid and significant reduction in the level of S14 mRNA and S14 transcription in the liver (Jump *et al*., 1993). In cultured hepatocytes, eicosapentaenoic acid significantly reduced S14 mRNA and S14 activity.

At a molecular level, there is evidence that S14 transcription is regulated by SREBP1c. nSREBP1c functionally interacts with the E-box-like sequence in the PUFA response region of the S14 promoter (Mater *et al*., 1999). Overexpression of nSREBP1c, but not other factors known to induce S14 activity, reduced the PUFA-mediated suppression of S14 expression, further supporting the direct interaction of SREBP1c and S14 gene. In breast cancer cells, transfection with a dominant-negative mutant of SREBP1c reduced the induction of S14 expression by progestin to 2.5-fold of basal level, while transfection with constitutively active SREBP1c caused a 330-fold increase in S14 mRNA compared to basal level in response to progestin (Martel *et al*., 2006). Thus, progestin seems to indirectly induce S14 transcription via SREBP1c.

Progress in identifying the biochemical role of S14 has been limited. Using a yeast two-hybrid system, it was demonstrated that S14 forms homodimers, which is a characteristic of many transcription factors (Cunningham *et al*., 1997). In rat hepatocytes, S14 coimmunoprecipitated with a 36-kDa protein, suggesting the existence of a S14-S14-p36 complex. Chou *et al*. (2007) has also suggested that S14 functions as a homodimer and that it interacts physically and functionally with thyroid receptor to regulate gene expression of malic enzyme, which is involved in *de novo* synthesis of long chain fatty acids. Subsequent studies have suggested that S14 also interacts with Zac1, a zinc finger protein which regulates apoptosis and cell cycle arrest, and functions as a p53-dependent transcriptional coactivator of lipogenic enzymes (Chou *et al*. 2008).

In humans, S14 gene is located in the 11q13 amplicon (Cunningham *et al*., 1998). This region has been implicated in body weight regulation in rodents (Taylor and Phillips, 1996) and is also amplified in approximately 20% of breast cancers (Schuuring *et al*., 1992). This amplification confers a poor prognosis, prone to recurrence, and also occurs more frequently in recurrent than primary tumors. Indeed, recent studies have shown that high expression of S14 in primary invasive mammary tumors is associated with recurrence whereas low expression is associated with disease-free survival (Kinlaw *et al*., 2006, Wells *et al*., 2006). Tumor samples (n = 131) were examined and clinical outcome of the patients were monitored for the ensuing 3000 days. S14 was detectable in all tumor samples, regardless of clinical outcome. 32% of tumors with maximal S14 expression recurred. A significant relationship ($P = 0.015$) was observed between level of S14 expression and disease-free survival. Therefore, S14 is a potential tumor marker that predicts aggressive tumor biology and reduced disease-free survival.

In a recent study using breast cancer cells and liposarcoma cells, a mixture of CLA isomers, as well as pure *cis*-9, *trans*-11 CLA and pure *trans*-10, *cis*-12 CLA, caused a decrease in mRNA levels of S14 and FASN and growth inhibition (Donnelly *et al*., 2009). The inhibition was fully (CLA mixture and pure *trans*-10, *cis*-12 CLA) or partially (pure *cis*-9, *trans*-11 CLA) rescued by palmitic acid, suggesting that the anticarcinogenic effect of CLA is mediated via reduced tumor lipogenesis.

Further investigation of the role of S14 has become possible with the creation of a S14 knockout mouse model by Dr. Cary Mariash's laboratory (Zhu *et al*., 2001). In the S14 null mice, the gene is deleted except for the first 21 amino acids. Lack of S14 mRNA in the liver of the knockout mouse validated this model. The S14 null mouse shows a

phenotype in the mammary gland (Zhu *et al*., 2005). Pups nursing on S14 null dams grew significantly less than pups nursing on wildtype dams or heterozygous dams, regardless of the genotype of the pups. Milk clots from S14 null dams had 28% reduced triglyceride levels compared to wildtype dams. No difference in protein levels was observed. Lactating mammary glands of null dams contained 33% less triglycerides than wildtype dams, due to a decrease in medium chain fatty acids. Lipid synthesis rate in the mammary gland was decreased by 62% in null dams compared to wildtype dams. Thus, the reduction in pup growth and milk fat concentration in the S14 null dams was due to a decrease in *de novo* lipid synthesis. However, no reduction in FASN and ACC activity was observed. In fact, ACC activity was increased in null mice. Surprisingly, no change in mRNA levels of lipogenic enzymes was observed. Thus, S14 does not seem to affect gene expression of key lipogenic enzymes as speculated before. More recently, LaFave *et al*. (2006) has reported that malonyl-CoA is higher in the mammary gland of S14 null mice. A possible explanation of this result is that S14 reduces lipogenesis by regulating FASN activity. Thus, the authors proposed that the S14 protein may allosterically regulate the activity of FASN. Adult S14 null mice also exhibit resistance to diet-induced obesity (Anderson *et al*., 2009). S14 null mice had a reduced rate of weight gain as a result of a decrease in fat accumulation. When injected with glucose intrapenitoneally, S14 null mice had a significantly reduced maximal blood glucose level compared to wildtype mice. Similarly to this improved glucose tolerance, the knockout mice exhibited increased insulin sensitivity. Compared to wildtype animals, null animals had a longer duration of repressed blood glucose in response to insulin.

Mouse model of MFD

Our laboratory has developed a lactating mouse model (C57BL/6J) to further investigate the mechanism of *trans*-10, *cis*-12 CLA-induced MFD. In a dose titration experiment with this mouse model, lactating dams were orally dosed with 0 (control), 7, 20, and 60 mg/d of *trans*-10, *cis*-12 CLA for 5 days starting day 6-8 of lactation (Harvatine and Bauman, unpublished). The 60 mg/d dose resulted in a decrease in pup growth, reduction in milk clot fat concentration, an increase in milk protein and a decrease in mammary lipogenic capacity, but did not cause a decrease in milk fat concentration. Thus, the 60 mg/d seemed to be suppressing overall milk synthesis and was deemed as not an appropriate dose to study MFD, in which only milk fat is affected. The 20 mg/d dose induced a maximal decrease in milk fat percent without altering protein concentration, suggesting that the effects were specific to milk fat synthesis. The 20 mg/d and 7 mg/d treatments resulted in a dose-dependent reduction in milk fat, a shift in fatty acid profile, and a downregulation of gene expression of lipogenic enzymes much like in the dairy cow. Thus, 20 mg/d was determined to be the dose that induces a near maximal effect of MFD in the mouse.

To further elucidate the role of S14 in the lactogenic response to *trans*-10, *cis*-12, CLA, I cooperated in a study which compared wildtype and S14 null C57BL/6J mice. Dams of both genotypes were randomized to receive either the control treatment (water) or 20 mg/d of CLA for 5 days starting days 6-8 of lactation. Milk and milk clot fat percent was reduced by 24.6% and 14.2%, respectively, in S14 null mice compared with wildtype mice. CLA treatment lowered total milk fat concentration by 12% in wildtype and 10% in knockout animals, and there was no interaction of genotype and CLA

treatment. In contrast, CLA treatment decreased milk fat concentration of *de novo* synthesized fatty acids (<16 carbons) by 27% in wildtype dams and 72% in S14 null dams. There was a strong interaction of genotype and CLA treatment ($P < 0.01$) for *de novo* fatty acids, as well as for C14 and C16 desaturase indexes ($P < 0.001$). CLA treatment increased milk C14 and C16 desaturase indexes by 640 and 100%, respectively, in S14 null mice, but increases in the wildtype dams were only 37 and 45%, respectively. Mammary tissue lipogenic capacity as measured by ^{14}C glucose incorporation into lipids by tissue explants was similar between control animals of either genotype. CLA decreased lipogenic capacity in both genotypes, and there was a strong interaction of genotype and CLA ($P < 0.001$); CLA decreased mammary lipogenic capacity by 23% in wildtype dams and by 82% in S14 null dams. Overall, results indicated that the lactogenic response of S14 null mice to CLA was much greater compared with wildtype animals at this near maximal dose. These results were unexpected; previous studies have suggested that S14 functions as a transcriptional coactivator of lipogenic enzymes (Chou *et al*., 2008), thus we were expecting that the S14 null dams would not respond to CLA. The hyperresponsiveness of the S14 null mouse to CLA suggests that S14 has an indirect role in lipogenesis, possibly by altering the response of another CLA-responsive factor. Although the hyperresponsiveness of the S14 null mouse at a high CLA dose has been observed, lower CLA doses have not been examined; thus it is unknown if the S14 null mouse is also more sensitive to CLA. The sensitivity of the S14 null mouse relative to the wildtype mouse would provide further insight into the mechanism of the CLA-induced inhibition of milk fat synthesis.

OBJECTIVE

The objective of this thesis was to examine the sensitivity of the S14 knockout mice to CLA by testing the milk fat response to a low dose of CLA.

MATERIALS AND METHODS

Animals and treatments

Experimental procedures for the present study were approved by the Cornell University Institutional Animal Care and Use Committee. Wildtype (WT) and S14 partial knockout (KO) C57BL/6J mice were obtained from Dr. Cary Mariash (University of Minnesota). Dr. Mariash's group has previously reported the phenotype of the S14 KO mice in publications by Zhu *et al*. (2001 and 2005). Experimental mice were the progeny of heterozygous breeding pairs to ensure that the genotype of the parents did not affect the phenotype of the experimental animals. Genotype of the animals was determined by PCR using DNA extracted from tail snips of weanlings (Zhu *et al*., 2001). Wildtype females were bred to knockout males and knockout females were bred to wildtype males to produce heterozygous pups, thus eliminating the possible effect of pup genotype. The dams were subsequently housed in individual cages with free access to food and water. Litter size was adjusted to 6-8 pups on day 0-1 of lactation by cross-fostering to eliminate the effect of litter size on dam lactation. Dams were randomly assigned to one of four treatments in a 2x2 factorial design (genotype and CLA treatment). Dams were fed *ad*

libitum a pelleted rodent diet containing 22% protein and 5% fat (diet #8640, Harlan Teklad, Indianapolis, IN). CLA was in free fatty acid form and contained 89.0% *trans*-10, *cis*-12 CLA, 2.0% linolenic acid, and <0.9% of any other individual fatty acid (Natural ASA, Lysaker, Norway). CLA was diluted 1:2 with oleic acid to provide a reasonable working volume. Mice assigned to the CLA treatment (n = 8 for WT CLA, n = 9 for KO CLA) were orally dosed with 3.5 mg/d of *trans*-10, *cis*-12 CLA and 7 mg/d of oleic acid for 5 days starting on day 6-8 of lactation, and mice in the control groups (n = 7 for WT CON, n = 8 for KO CON) were similarly dosed with the same volume of water. The daily dose was divided in three equal portions and given at 0030, 0930, and 1700 h.

Data and sample collection.

Dams, pups, and feed were weighed daily between 1300 and 1400 h, and the average dam weight gain, litter growth, and dam intake over the last 3 days of treatment were used for analysis. After the fifth day of treatment, dams and pups were euthanized at 1400 h. Pups were gassed with CO_2 before being euthanized by cervical dislocation. Milk clots were collected from the stomach of each pup, composited within each litter, and freeze-dried. Dams were anaesthetized with ketamine-zylazine (1 and 0.1 mg/10 g body weight, respectively) then euthanized by cervical dislocation. The liver was removed from the dams and weighed. One #4 mammary gland from the dams was placed in ice-cold isotonic sucrose buffer and immediately used to determine lipogenic capacity. The other #4 mammary gland was snap-frozen in liquid nitrogen for gene expression analysis.

Sample analysis

Milk clot fatty acid profile

Milk clot samples were analyzed for fat concentration and fatty acid profile according to Harvatine *et al*. (2008). Approximately 20 mg of freeze-dried milk clot was weighed into an extraction tube. 250 μg of both C17:0 (triglyceride) and C19:0 (methyl ester) were added as internal standards. Milk clot lipids were extracted by hexane: isopropynol extraction according to Hara and Radin (1978). The extracted fatty acids were first acid methylated overnight at 40^{o}C in 1% methanolic sulfuric acid and subsequently transmethylated. Methyl esters were quantified by gas chromatography using a fused-silica capillary column (CP-Sil 88; 100 m x 0.25 mm [i.d.] Varian Inc.) and conditions as described by Perfield *et al*. (2006). Milk clot fat concentrations were determined based on dilution of the internal standards.

Mammary lipogenesis assay

Rates of incorporation of ^{14}C glucose into fatty acid were determined in one #4 mammary gland according to Bauman *et al*. (1973) as modified by Harvatine *et al*. (2008). Briefly, mammary tissue explants were prepared using a Stadie-Riggs hand microtome, and approximately 100 mg tissue explants were incubated in triplicate in a vial with 3 mL of a modified Krebs-Ringer bicarbonate buffer at physiological pH. Vials were gassed with a mixture of O_2:CO_2 (95:5), sealed, and incubated for 3 h at 37^{o} C in a shaking water bath. After termination of incubation, tissue was rinsed and saponified, lipids were extracted with petroleum ether, and the radioactive count of extracted lipids was determined (Bauman *et al*., 1970).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from approximately 30 mg of mammary tissue from each dam using the RNeasy Lipid Kit (Qiagen) with on-column DNase treatment (RNase-Free DNase Set; Qiagen) to remove DNA contamination. RNA concentration and integrity were determined by an Agilent 2100 BioAnalyzer (Agilent Technologies). The extracted RNA was reverse transcribed using SuperScript III First Strand Synthesis kit (Invitrogen) with random primers. Quantitative reverse transcriptase-PCR (qRT-PCR) reactions were conducted according to Harvatine and Bauman (2006) using Power SYBR Green (Applied Biosystems) and 400 nM of gene specific forward and reverse primers (Invitrogen; **Appendix, Table 1**). Sample message level was determined relative to a dilution curve of pooled cDNA (ABI Prism, 2001) and analyzed relative to the housekeeping gene $ß_2$-microglobulin (B2M; Vandesompele *et al.*, 2002).

Statistical analysis

Data were analyzed using the fit model procedure of JMP® (Version 7.0, SAS Institute). The model included the fixed effects of genotype (WT and S14 KO) and treatment (CON and CLA), and the interaction of genotype and treatment. Effects of genotype and CLA were considered significant when $P < 0.05$, and interactions were considered significant when $P < 0.1$. The average litter weight gain for the two days immediately prior to CLA treatment was used as a covariant for litter weight gain. The average intake for the two days immediately prior to CLA treatment was used as a covariant for feed intake. The average initial weight of the dams was used as a covariant

for liver weight. Data points with Studentized Residuals greater than 2.75 were considered outliers and excluded from analysis.

RESULTS

Performance parameters

Litter weight gain during the last 3 days of treatment was decreased by both S14 deletion and CLA treatment ($P = 0.05$ and 0.01, respectively; **Table 1**). CLA treatment caused a 28% reduction in litter weight gain in pups of S14 KO dams and no significant decrease in litter weight gain in pups of WT dams, but there was no interaction of genotype and CLA treatment ($P = 0.25$). CLA treatment reduced feed intake and weight gain of dams regardless of genotype ($P = 0.01$ and $P < 0.001$, respectively). Intake was decreased 13% in WT dams with CLA treatment, but there was no significant decrease in KO dams in response to CLA. Neither an effect of genotype nor an interaction of genotype and CLA was observed ($P = 0.40$ and 0.24, respectively). CLA treatment decreased dam weight gain in both genotypes; neither a genotype effect nor an interaction was observed ($P = 0.31$ and 0.46, respectively). Liver weights were unaffected by either genotype or CLA treatment ($P = 0.87$ and 0.68, respectively).

Milk clot fat content and composition

Milk clots from pups nursing on S14 KO dams had 15.9% less fat than the milk clots from WT dams ($P < 0.001$; **Table 2**). CLA treatment also caused a reduction in

Table 1. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on performance of lactating C57BL/6J wildtype and S14 null mice

	Treatment[1]							
	------WT------		----S14 KO----			*P*[2]		
	CON	CLA	CON	CLA	SEM	S14	CLA	int
Litter weight gain, *g/d*[3]	2.31	2.06	2.19	1.58	0.16	0.05	0.01	0.25
Intake, *g/d*[4]	14.2	12.3	13.2	12.4	0.50	0.40	0.01	0.24
Dam weight gain, *g/d*	2.3	0.5	1.7	0.4	0.38	0.31	<.001	0.46
Dam liver weight, *g*[5]	2.20	2.18	2.21	2.18	0.10	0.87	0.68	0.99

[1]Treatments were arranged in a 2x2 factorial design. Wildtype (WT) and S14 knockout (KO) mice were treated with either water (CON) or 3.5 mg/d *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Litter weight gain, feed intake, and dam weight gain are based on the average of the last 3 days of treatment. Values are LS means; n = 7 for WT CON, n = 9 for KO CLA, and n = 8 for WT CLA and KO CON.
[2]The probability of a main effect of genotype (S14) and *trans*-10, *cis*-12 CLA (CLA) and the interaction of genotype and CLA (int).
[3]The average litter weight gain for the two days immediately prior to CLA treatment was used as a covariant.
[4] The average intake for the two days immediately prior to CLA treatment was used as a covariant.
[5] The average initial weight of the dams was used as a covariant.

milk clot fat percent ($P = 0.02$), but there was no interaction of genotype and CLA treatment ($P = 0.11$). CLA was not detected in the fatty acids of the milk clots of CON dams, but constituted 0.12% and 0.16% of milk clot fatty acids of WT CLA and KO CLA dams, respectively. CLA treatment caused a reduction in the *de novo* synthesized medium chain fatty acids C12:0, 14:0, and 14:1 ($P < 0.001$), and an interaction of genotype and CLA was observed for each of these fatty acids ($P < 0.1$). Examination of the fatty acid data indicates that the KO mice were more responsive to the CLA treatment than the WT mice. The C16:0 component, which includes preformed and *de novo* fatty acids, was also significantly reduced by CLA ($P = 0.009$), although there was no interaction ($P = 0.61$). CLA treatment increased *cis*-C16:1 fatty acids in milk clots from KO dams. Both S14

Table 2. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on milk clot fat concentration and fatty acid profile of C57BL/6J wildtype and S14 null mice

	Treatment[1]							
	-------WT-------		-----S14 KO-----			*P*[2]		
	CON	CLA	CON	CLA	SEM	S14	CLA	int
Milk clot fat, *%*	51.73	46.06	41.67	40.57	1.43	<.001	0.02	0.11
Fatty acid profile, *g/100 g total fatty acids*								
8:0	0.07	0.05	0.09	0.08	0.02	0.38	0.63	0.70
10:0	3.92	3.53	3.84	3.38	0.42	0.77	0.29	0.93
12:0	10.21	9.99	8.52	6.32	0.32	<.001	<.001	0.003
14:0	12.40	11.04	7.81	5.21	0.30	<.001	<.001	0.04
14:1	0.10	0.07	0.09	0.08	0.01	0.50	<.001	0.03
16:0	23.43	21.92	16.93	15.89	0.47	<.001	0.009	0.61
16:1, *cis*	1.23	1.20	1.62	2.14	0.10	<.001	0.01	0.007
18:0	2.32	2.63	1.86	2.16	0.05	<.001	<.001	0.93
18:1, *cis*-9	14.56	17.16	20.62	23.89	0.59	<.001	<.001	0.56
18:1, *cis*-13	1.92	2.25	3.03	3.31	0.08	<.001	<.001	0.70
Linoleic acid	21.42	21.74	22.76	24.97	0.44	<.001	0.006	0.04
Linolenic acid	1.90	1.91	2.14	2.19	0.05	<.001	0.54	0.64
***trans*-10, *cis*-12 CLA**	0.00	0.11	0.00	0.16	0.02	0.15	<.001	0.15
20:2	1.25	1.13	1.65	1.51	0.05	<.001	0.01	0.83
20:3	0.77	0.78	1.18	1.21	0.03	<.001	0.53	0.86
20:4	0.79	1.03	1.17	1.75	0.07	<.001	<.001	0.02
20:5	0.29	0.33	0.34	0.29	0.04	0.95	0.83	0.27
22:4	0.32	0.32	0.58	0.66	0.03	<.001	0.13	0.19
22:5	0.37	0.36	0.54	0.60	0.02	<.001	0.14	0.06
22:6	0.31	0.32	0.36	0.39	0.02	0.003	0.30	0.55
Desaturase index								
14:1/ (14:0 + 14:1)	0.008	0.006	0.011	0.015	.001	<.001	0.15	<.001
16:1/ (16:0 + 16:1)	0.050	0.052	0.090	0.129	.004	<.001	<.001	0.002

[1]Treatments were arranged in a 2x2 factorial design. Wildtype (WT) and S14 knockout (KO) mice were treated with either water (CON) or 3.5 mg/d *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Values are LS means; n = 8 for WT CON, n = 7 for KO CON, and n = 9 for WT CLA and KO CLA.

[2]The probability of a main effect of genotype (S14) and *trans*-10, *cis*-12 CLA (CLA) and the interaction of genotype and CLA (int).

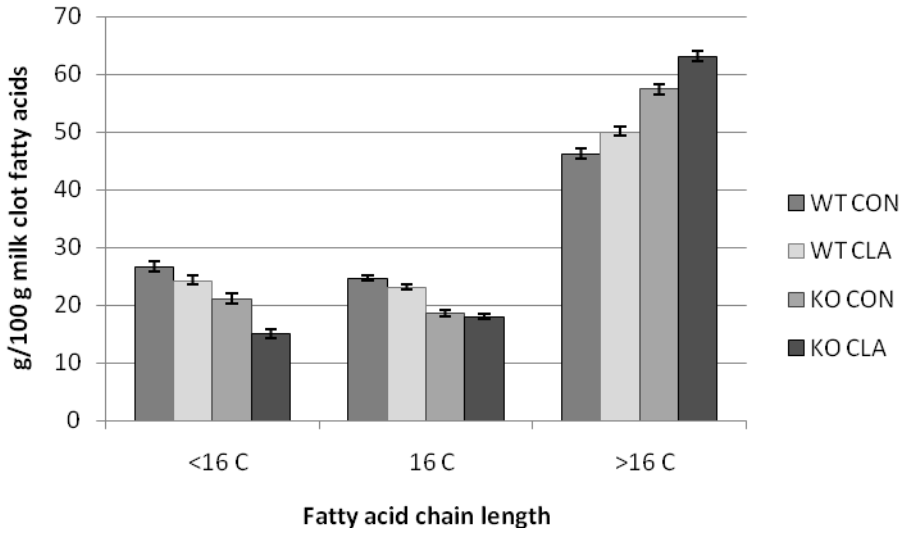

Figure 2. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on milk clot fatty acid profile by carbon length from C57BL/6J wildtype (WT) and S14 knockout (KO) mice
Mice were treated with either water (CON) or 3.5 mg/d *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Values are LS means ± SE, n = 7 – 9.

deletion and CLA caused a reduction in C18:0 ($P < 0.001$ for both), but both caused an increase in *cis*-9 C18:1 and *cis*-13 C18:1 ($P < 0.001$ for all). This may be due to an increase in SCD activity. Both S14 deletion and CLA treatment caused an increase in the percentage of most of the long chain fatty acids, but interaction was not observed for most of these. The increase in the percentage of long chain fatty acids is not so much due to an increase in the amount of these fatty acids, but rather to the decrease in the amount of the short chain and medium chain fatty acids thereby resulting in an increase in the proportion of long chain fatty acids.

A summary of the fatty acid profile according to carbon length makes more obvious the effect of genotype and CLA on *de novo* vs preformed fatty acids (**Figure 2**). At this low CLA dose, KO dams had a 28.7% reduction in the percentage of *de novo* fatty acids less than 16 carbons in length, whereas in WT dams this reduction (8.9%) was not

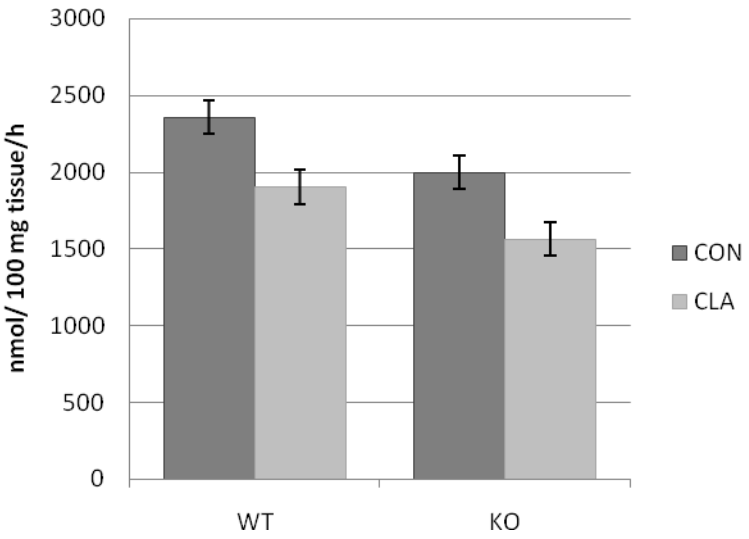

Figure 3. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on rate of lipogenesis of mammary tissue explants from C57BL/6J wildtype (WT) and S14 knockout (KO) mice. Lipogeneiss was based on incorporation on ^{14}C glucose into fatty acids. Mice were treated with either water (CON) or 3.5 mg/d *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Values are LS means ± SE, n = 7 – 9.

significant. There was a significant interaction of genotype and CLA for the percentage of <C16 fatty acids ($P = 0.04$). The proportion of preformed (>C16) fatty acids increased with both S14 deletion and CLA treatment ($P < 0.001$, Table 2), but there was no interaction ($P = 0.32$).

SCD activity as determined by the desaturase indexes was significantly higher in KO dams relative to WT dams ($P < 0.001$). For both C14 and C16 desaturase indexes, there was a significant interaction of genotype and CLA treatment ($P < 0.001$ and $P = 0.002$, respectively).

Mammary lipogenic capacity

Mammary lipogenic capacity as determined by incorporation of ^{14}C glucose into fatty acids in tissue explants was decreased by both S14 deletion and CLA treatment ($P =$

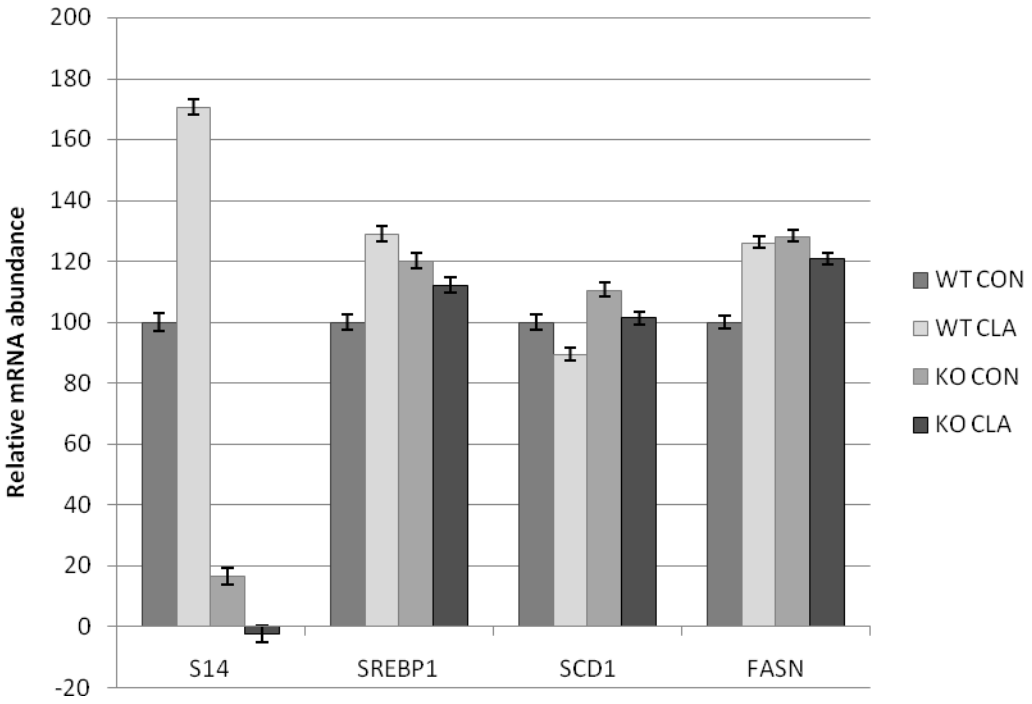

Figure 4. Effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on mammary expression of select lipogenic genes of C57BL/6J wildtype (WT) and S14 knockout (KO) mice..
Mice were treated with either water (CON) or 3.5 mg/d *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Values are LS means ± SE, n = 7-9. The expression level of wildtype controls (WT CON) was normalized to 100 for each gene.

0.003 and 0.0003, respectively; **Figure 3**). However, no interaction of genotype and CLA treatment was observed ($P = 0.93$).

Mammary gene expression

Mammary expression of S14 was at background for KO dams (**Figure 4**) and much lower than WT dams ($P < 0.0001$). Surprisingly, S14 mRNA level significantly increased in response to CLA in WT mice. No statistical difference was observed for any of the other genes.

DISCUSSION

Consistent with our previous studies, CLA treatment reduced litter weight gain, feed intake, and dam weight gain. Since milk fat synthesis was reduced due to CLA, dams required less energy for mammary lipogenesis and this is consistent with the reduction in feed intake. The reduction of fat in the milk clots in response to CLA most likely caused the decrease in litter weight gain that would occur as a result of reduced energy intake. S14 deletion caused a decrease in litter weight gain but not in feed intake or dam weight gain. As with previous studies, KO dams had reduced mammary lipogenic capacity, resulting in decreased percentage of *de novo* fatty acids out of total milk clot fat. Probably due to the low dose used in the current experiment, an interaction of genotype and CLA was observed for percent *de novo* fatty acids and for many individual fatty acids, but not for the performance data or for mammary lipogenic capacity. Consistent with our previous study, KO dams showed a greater increase in SCD activity than WT dams as determined by desaturase indexes.

Mammary tissue expression of lipogenic genes did not differ between WT and KO mice, except for S14. This is consistent with the results of our previous study with KO mice (Harvatine, Tanino, and Bauman, unpublished) and with the results from Zhu *et al*. (2005). Similarly, CLA appears to be inhibiting mammary lipogenesis by mechanisms other than the regulation of lipogenic gene expression. Surprisingly, CLA caused an increase in S14 mRNA level in the WT, rather than the expected coordinated downregulation of lipogenic gene expression. This contradicts with our previous result with the 20 mg/d dose of CLA, in which CLA treatment decreased mammary S14 mRNA level in WT mice. The reason for the increased S14 transcription is not apparent.

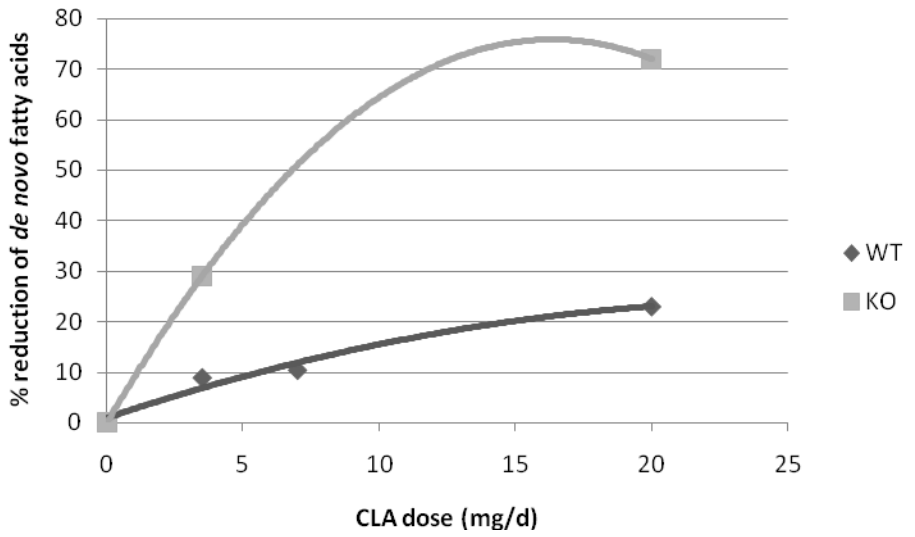

Figure 5. Dose-dependent effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) in the percent reduction of mammary *de novo* fatty acid synthesis in wildtype (WT) and S14 knockout (KO) C57BL/6J mice.
Data points are from the current experiment and from previous experiments in our laboratory (Harvatine, Tanino, and Bauman, unpublished). Data points were fit with a best-fit polynomial equation. R^2= 0.999 for WT and R^2= 0.973 for KO.

The results suggest overall that the S14 null mouse has a greater reduction in lipogenesis in response to the low dose of CLA than WT, although the mechanism remains far from clear. When combined with data from previous experiments in our lab investigating WT and KO mice, it seems that the S14 null mice show dose-responsiveness with similar sensitivity, rather than hypersensitivity (**Figure 5**). From the limited number of data points currently available, the ED_{50} (effective dose at half-maximal response) for the WT mouse is estimated to be around 6 mg/d. The ED_{50} for the KO mouse seems to be around 5 mg/d. The similar ED_{50} of the two curves suggest that the lactogenic response to CLA of the WT and KO mice are similar in sensitivity. The results from this study also suggest that lack of S14 does not affect the dose-responsiveness of other factors involved in CLA-induced inhibition of milk fat synthesis.

Much remains unknown about the role of S14 in the lactogenic response to CLA. Previous studies have suggested that S14 functions as a transcriptional coactivator of lipogenic enzymes (Chou *et al*., 2008). However, the S14 null mouse did not show a downregulation of these genes in mammary tissue in previous studies (Zhu *et al*., 2005, Harvatine, Tanino, and Bauman, unpublished) and in the present study. The hyperresponsiveness of the S14 null mouse to CLA suggests that the S14 protein may play an indirect role in lipogenesis, possibly by altering the response of another factor involved in milk fat synthesis. For instance, S14 may repress an inhibitor of lipogenic gene transcription. This would be consistent with the localization of S14 in the nucleus and its putative lipogenic effect. However, it is not consistent with the lack of change in gene expression in the knockout mice.

Alternatively, S14 may be regulating protein activity. Support for this comes from our interactions, in which we recently learned that a group at University of Texas Southwestern Medical Center has found that the S14 related protein (Mig 12) appears to play a role in catalyzing conversion of ACC to the polymeric form and in the interaction between ACC and FAS. Given the significant sequence homology between Mig 12 and S14, a similar role may be played by S14. To investigate this potential function of S14, the amount of polymeric ACC should be analyzed in the mammary tissue samples of the current experiment. This can be done either by a native Western blot which distinguishes the polymeric and protomeric forms of ACC by molecular weight, or by the use of avidin, which inactivates protomeric ACC but does not affect polymeric ACC activity (Ashcraft *et al*., 1980). If CLA treatment is found to decrease the amount of polymeric ACC in WT but not in S14 null mice, then S14 likely has a role in ACC polymerization. This possible

function of S14 would explain the lower milk fat and lack of change in mammary gene expression in S14 null mice. Nevertheless, it would not explain the hyperresponsiveness of the S14 null mice to CLA. Much of the lactogenic response of the S14 null mouse to CLA, therefore, would still remain in question. Further investigation of the role of S14 in CLA-induced MFD will provide insight into possible solutions to MFD in the dairy cow, as well as the other biological roles of CLA.

APPENDIX

Table 1. Murine primers used in quantitative RT-PCR analysis

Gene/Primers	Sequence	Length	Accession No.
B2M[1]		166	NM_009735
51.F	CATGGCTCGCTCGGTGACC		
216.R	AATGTGAGGCGGGTGGAACTG		
FASN[2]		158	NM_007988
891.F	AGAGATCCCGAGACGCTTCT		
1048.R	GCCTGGTAGGCATTCTGTAGT		
S14		117	NM_009381
283.F	TGAGAACGACGCTGCTGAAAC		
399.R	AGGTGGGTAAGGATGTGATGGAG		
SCD1		102	NM_009127
113.F	TGGGAAAGTGAGGCGAGCAACTG		
214.R	AGGGAGGTGCAGTGATGGTGGTG		
SREBP1c[3]			
96.F	GGAGCCATGGATTGCACATT		
198.R	GCTTCCAGAGAGGAGGCC		

[1]Primer sequences from RTprimerDB (Pattyn *et al.*, 2006)
[2]Primer sequences from Primer bank (30911099a3; Wang *et al.*, 2003)
[3]Primer sequences from Hegarty *et al.* (2005)

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