University of Alberta

The role of diacylglycerol acyltransferase in the accumulation and deposition of triacylglycerol in bovine tissues

by

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Abstract

The production of dairy products with nutraceutic fatty acids such as conjugated linoleic acid (CLA), and cattle with highly marbled beef represent two approaches for dairy and cattle producers to increase the value of their respective products. Bovine diacylglycerol acyltransferase-1 (DGAT1), the enzyme responsible for the final step in the biosynthesis of triacylglycerol, was studied. Intramuscular fat content, DGAT activity, and occurrence of the K232A mutation were measured in Holstein and Charolais bulls, and no significant relationship between DGAT activity and intramuscular fat content was found. With respect to milk fat production, the effect of CLA isomers on the activity and expression of DGAT was investigated in bovine mammary gland epithelial (MAC-T) cells and microsomal fractions of bovine mammary gland tissue. There were no significant effects of CLA isomers or intermediates on the expression of DGAT. Treatment of MAC-T cells with *trans*-10, *cis*-12 18:2 decreased DGAT activity, although the mechanisms are unclear.

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List of abbreviations

A	alanine
ACAT	acyl-CoA: cholesterol acyltransferase
ACC	acetyl-coenzyme A carboxylase
ARAT	acyl-CoA:retinol acyltransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	Celsius
CLA	conjugated linoleic acid
cDNA	complimentary deoxyribonucleic acid
cm	centimetres
CoA	coenzyme A
Ci	Curies
Ct	critical threshold
DAG	*sn*-1,2-diacylglycerol
DEPC	diethylpyrocarbonate
DGAT	diacylglycerol acyltransferase
DGTA	diacylglycerol transacylase
DHA	docosahexaenoic acid
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco's modified Eagle medium
dpm	disintegrations per minute
DTT	dithiothreitol
EC	enzyme commission
EDTA	ethylene diamine tetraacetate
ER	endoplasmic reticulum
FA	fatty acid
FAS	fatty acid synthase
FAME	fatty acid methyl esters

FBS	fœtal bovine serum
g	grams
g	gravity
G3P	*sn*-glycerol-3-phosphate
GC	gas chromatography
GK	glycerol kinase
GPAT	glycerol-3-phosphate acyltransferase
h	hours
HEPES	N-[2-hydroxyethyl]peperazine-N'-[2-ethanesulfonic acid]
i.m.	intramuscular
K	lysine
kg	kilograms
L	litres
LD	*longissimus dorsi*
LPA	lysophosphatidate
LPAAT	lysophosphatidate acyltransferase
LPL	lipoprotein lipase
M	molar
MAG	monoacylglycerol
MES	2-(N-Morpholino)ethanesulfonic acid
MFD	milk fat depression
mg	milligrams
MGAT	monoacylglycerol acyltransferase
min	minutes
mL	millilitres
mM	millimolar
mo	months
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid

N_2 (g)	nitrogen gas
N_2 (l)	liquid nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometers
nmol	nanomoles
PA	phosphatidic acid
PAP	phosphatidate phosphatase
PBS	phosphate buffered saline
PDAT	phospholipid: diacylglycerol acyltransferase
PL	phospholipid
pmol	picomoles
PPARγ	peroxisome proliferator-activated receptor gamma
ppm	parts per million
PUFA	polyunsaturated fatty acids
QTL	quantitative trait locus
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	real-time polymerase chain reaction
s	seconds
SC	subcutaneous
SCD	stearoyl-CoA desaturase
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFA	saturated fatty acid
sn	stereospecific numbering
SNP	single nucleotide polymorphism
SREBP1	sterol response element binding protein 1
ST	*semitendinosus*

TAG	triacylglycerol
TFA	*trans* fatty acids
TG	thyroglobulin
TLC	thin layer chromatography
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
U	units
UV	ultraviolet
v	volume
VLDL	very low density lipoproteins
w	weight
µg	micrograms
µL	microlitres
µM	micromolar

1. Introduction

The challenge of cattle producers is provide predictable high quality products in order to obtain and maintain consumer satisfaction. One strategy is to enhance the content of beneficial fatty acids (FA) in milk fat. Recently, milk enriched in docosahexaenoic acid (DHA all-*cis*-4,7,10,13,16,19 22:6), a nutraceutic FA, was successfully introduced into the market. There is also interest in marketing a milk product enriched in conjugated linoleic acid (CLA) another class of nutraceutic FA. Conjugated linoleic acid is naturally occurring in milk fat, and is readily increased in milk fat by altering the feeding regimen of dairy cattle. Increased CLA content in milk, however, is associated with milk fat depression (MFD), a decrease in overall milk fat output.

Another strategy for adding value to beef is to increase the degree of marbling fat in skeletal muscle to improve the palatability of beef. As marbling is a heritable trait, breeders have attempted to select for cattle predisposed for high marbling. The main obstacle, however, is predicting whether young cattle will have high marbling before they are slaughtered. Various attempts have been made to develop molecular and biochemical markers for marbling.

When looking at strategies to increase CLA content in milk or marbling in beef, it is important to consider triacylglycerol (TAG) biosynthetic enzymes, in particular diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), as it catalyzes the final step in TAG biosynthesis and is considered an important regulatory enzyme. DGAT is present in all tissues involved in TAG biosynthesis. Interest in mammalian DGAT was raised when mice in which the *Dgat1* gene had been disrupted were generated (Smith *et al.*, 2000).

The *Dgat1*$^{-/-}$ mice had decreased body fat and females were incapable of lactation. Fat biosynthesis was not completely eradicated in the *Dgat1*$^{-/-}$ mice, leading to the discovery of a second *Dgat* gene, designated *Dgat2* (Lardizabal *et al.*, 2001) (Cases *et al.*, 2001). Both *Dgat1* and *Dgat2* have different properties and different roles in the regulation of TAG biosynthesis.

Mutations in the bovine *DGAT1* gene have resulted in changes in both milk and intramuscular (i.m.) fat content (Winter *et al.*, 2002) (Grisart *et al.*, 2004). Because of this fact, the first component of this thesis focuses on the effects of CLA on the activity and expression of DGAT, possibly explaining CLA induced MFD. The effect of various CLA isomers on the expression and activity of DGAT in bovine mammary gland cells and bovine mammary gland tissue was investigated. It is possible that cattle which have a higher degree of i.m. fat also have a higher degree of DGAT activity. The second component of this thesis involved investigating DGAT as a potential biochemical marker for i.m. fat deposition. Muscle tissue was obtained from two different breeds of cattle each differing in i.m. fat content, and correlations between i.m. fat content in the samples and microsomal DGAT activity were investigated.

2. Literature review

2.1. Triacylglycerol biosynthesis in ruminants

2.1.1. Fatty acid modification and biosynthesis

The FA substrates for ruminant TAG biosynthesis come preformed in the diet, arise from the breakdown and metabolism of fat stores, or are synthesized *de novo*. The main FA in ruminant diets are α-linolenic acid (α-18:3, *cis*-9, *cis*-12, *cis*-15 18:3) from forage crops, and linoleic acid (*cis*-9, *cis*-12 18:2) from oilseeds. Forages contain only a small amount of fat and concentrates are often supplemented with lipid for economical reasons. Ruminants have four stomach compartments; the reticulum, the rumen, the omasum, and the abomasum. The reticulum acts to prohibit the further movement of indigestible material through the digestive tract. In the rumen, dietary TAGs are rapidly hydrolyzed to FA by microbial lipases. Following hydrolysis, unsaturated FA are biohydrogenated by select species of ruminal bacteria (Doreau and Chilliard, 1997). As a result, the majority of FA reaching the small intestine are saturated, although some unsaturated FA bypass the rumen. Certain tissues, including the small intestine, express stearoyl-CoA desaturase (SCD, EC 1.14.19.1), which catalyzes the conversion of stearoyl-CoA (18:0-CoA) to oleoyl-CoA (*cis*-9 18:1-CoA).

Because of the combination of microflora in the rumen, there exists a unique class of linoleic acid containing conjugated double bonds (i.e., bonds which are not separated by a methylene group) known collectively as conjugated linoleic acid

(CLA,Figure 1). The principal isomer of CLA in ruminant fat is *cis*-9, *trans*-11 18:2, which is commonly referred to as rumenic acid (Kramer *et al.*, 1998). *Cis*-9, *trans*-11 18:2 results from the biohydrogenation of *cis*-9, *cis*-12 18:2 by a linoleic acid isomerase (EC 5.2.1.5) from the ruminal bacteria *Butyrivibrio fibrosolvens* (Kepler and Tove, 1967). It is then directly absorbed by the small intestine, or further metabolized into *trans*-11 18:1 by a CLA reductase (Hughes *et al.*, 1982) and 18:0. Oleic acid is not converted exclusively to 18:0 by ruminal microbes, but also a number of *trans* isomers including *trans*-11 18:1 (Mosley *et al.*, 2002), that are then converted back into *cis*-9, *trans*-11 18:2 by SCD in the mammary gland. Formation of *cis*-9, *trans*-11 18:2 via the mammary pathway is quantitatively more important than direct absorption in the lactating cow (Griinari *et al.*, 2000). α-Linolenic acid can also be biohydrogenated to produce *trans*-11 18:1 and 18:0, but it does not appear that CLA is an intermediate in this process (Destaillats *et al.*, 2005).

Trans-7, *cis*-9 18:2 was also identified as a major component of CLA in milk fat (Yurawecz *et al.*, 1998). This isomer is thought to result exclusively from endogenous synthesis by tissues such as the mammary gland, as it is undetectable in ruminal fluids (Corl *et al.*, 2002). Because inhibitors of SCD affect the milk fat content of *trans*-7, *cis*-9 18:2, it is possible that this isomer results from the desaturation of *trans*-7 18:1 present in the rumen by SCD (Corl *et al.*, 2002).

Linoleic acid is also converted into *trans*-10, *cis*-12 18:2 by certain ruminal bacteria, including a *Propionibacter* species (Pariza *et al.*, 2001). A *cis*-9, *trans*-10 isomerase present in the bacteria catalyzes the conversion of *cis*-9, *cis*-12 18:2 to *trans*-10, *cis*-12 18:2. Although not a typical intermediate of linoleic acid hydrogenation, certain diets (i.e., high-concentrate diets) decrease the pH of the rumen, altering

bacterial populations and increasing production of this isomer (Piperova *et al.*, 2000). Cow's milk also contains *trans*-10 18:1, which is thought to occur via the biohydrogenation of *trans*-10, *cis*-12 18:2 (Griinari *et al.*, 1998). Mammals, however, do not have a delta-12 desaturase, so *trans*-10 18:1 is not converted back into *trans*-10, *cis*-12 18:2. Therefore, the occurrence of the *trans*-10, *cis*-12 18:2 isomer in tissues is the sole result of absorption from the rumen.

In the omasum, excess water is removed from the digesta before it reaches the small intestine. The small intestine consists of the duodenum, the ileum and the jejunum. The FA reaching the duodenum are adsorbed to feed particles and bacteria. Bile salts and lysophospholipids facilitate the solubilization of FA into micelles, which allows for the absorption of lipids into the jejunum. In the liver, FA are re-esterified, packaged into chylomicrons and very low-density lipoproteins (VLDL), and transported by the lymph to lipogenic tissues depending on the metabolic type of the animal. For example, certain cattle known as accretion types store nutrients as muscle and fat, whereas secretion type cattle secrete nutrients as milk fat (Bellmann *et al.*, 2004). Both chylomicrons and VLDL are rapidly metabolized by lipoprotein lipase (LPL EC 3.1.1.34), generating FA and *sn*-2-monoacylglycerol.

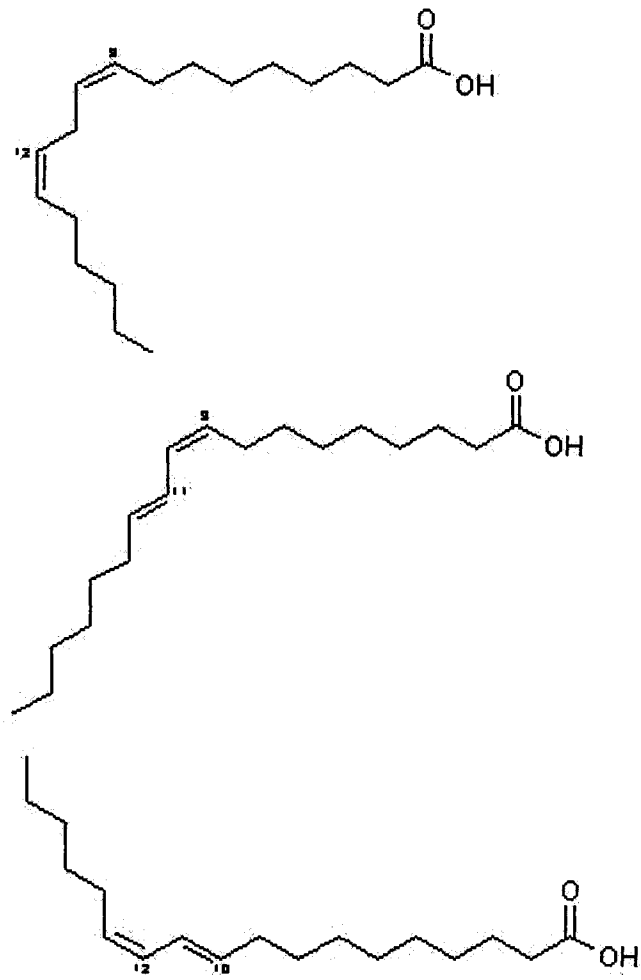

Figure 1. Schematic depicting examples of linoleic acid and conjugated linoleic acid.

Top- *cis*-9, *cis*-12 18:2; Centre- *cis*-9, *trans*-11 18:2; Bottom- *trans*-10, *cis*-12 18:2

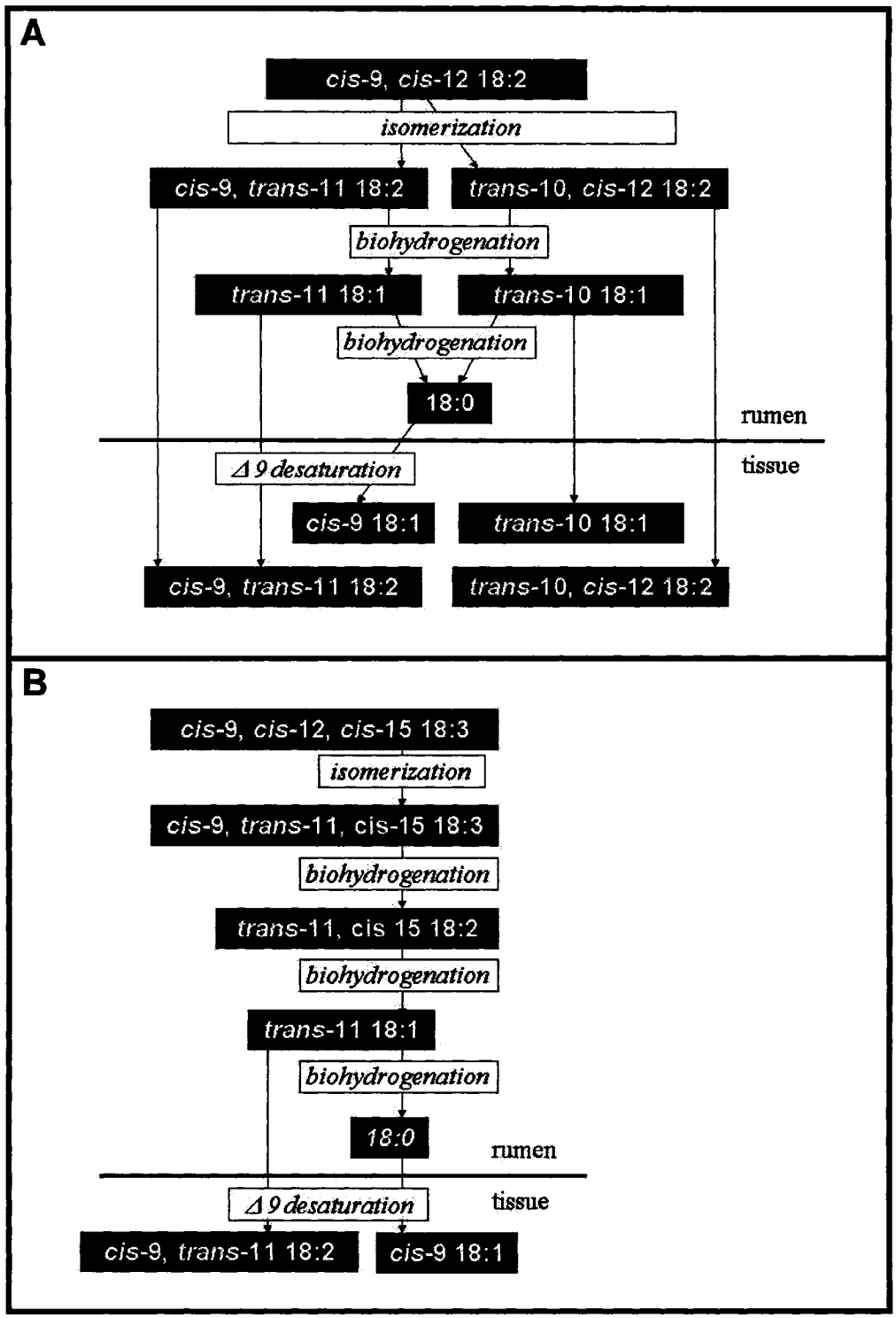

Figure 2. Schematic depicting the ruminal bioconversion pathways of linoleic and α-linolenic acid.

A: The conversion of linoleic acid. B: The conversion of α-linolenic acid.

De novo FA synthesis involves the carboxylation of acetyl-CoA to form malonyl-CoA, and is catalyzed by acetyl-CoA carboxylase (ACC, EC 6.4.1.2). Malonyl-CoA is used by fatty acid synthase (FAS, EC 2.3.1.15) as a carbon donor, resulting in the elongation of acetyl-CoA two carbons at a time, terminating with 16:0-CoA. Further elongation occurs to form 18:0-CoA (Wakil *et al.*, 1983). Premature termination results in the formation of 14:0-CoA. Glucose is also a substrate for *de novo* FA synthesis as it is converted into acetyl-CoA via pyruvate synthesis. In ruminants, however, there is a marked preference for the use of acetate carbon over glucose carbon for FA synthesis. Glucose can also be converted to ribulose-5-phosphate, which generates the NADPH required for FA synthesis (Neville and Picciano, 1997).

2.1.2. Triacylglycerol biosynthesis

The *sn*-glycerol-3-phosphate (G3P) pathway is the major pathway of TAG biosynthesis in the ruminant (Coleman and Lee, 2004)(Figure 3). The enzymes involved are embedded in the endoplasmic reticulum (ER) membrane. Glycerol-3-phosphate is the product of the reduction of dihydroxyacetone phosphate (DHAP) or the direct phosphorylation of glycerol catalyzed by glycerol kinase (GK, EC 2.7.1.30). Glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15) catalyzes the transfer of an acyl moiety from acyl-CoA to G3P at the *sn*-1 position to generate lysophosphatidate (LPA). Lysophosphatidate acyltransferase (LPAAT, EC 2.3.1.51) catalyzes the acyl-CoA dependent acylation of LPA at the *sn*-2 position, generating phosphatidate (PA). In adipocytes, a second pathway exists for the biosynthesis of PA. DHAP is esterified via acyl-CoA, generating acyl DHAP that is subsequently converted into LPA via acyl DHAP reductase (EC 1.1.1.101) activity (Coleman and Lee, 2004). The dephosphorylation of

PA is catalyzed by phosphatidate phosphatase (PAP EC 3.1.3.4) to generate *sn*-1,2-diacylglycerol (DAG). Diacylglycerol is also formed from the direct transfer of an acyl group from acyl-CoA to monoacylglycerol (MAG), a reaction catalyzed by monoacylglycerol acyltransferase (MGAT, EC 2.3.1.22). Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the transfer of an acyl-group from acyl-CoA to DAG, to produce TAG. This is the final and only committed step in the G3P and MAG pathways. It is believed that this step is rate-limiting in TAG synthesis (Mayorek *et al.*, 1989).

Acyl-CoA independent mechanisms of TAG synthesis have been identified. Diacylglycerol transacylase (DGTA), which catalyses the transfer of a fatty acyl moiety from one DAG molecule to another, was identified in rat tissue (Lehner and Kuksis, 1993). Dahlqvist *et al.* (2000) have also identified phospholipid: diacylglycerol acyltransferase (PDAT, EC 2.3.1.158) in yeast, which catalyzes the transfer of an acyl moiety from phosphatidylcholine to DAG. Although it has not been identified in mammalian species, PDAT shares sequence homology to lecithin: cholesterol acyltransferase (LCAT, EC 2.3.1.43).

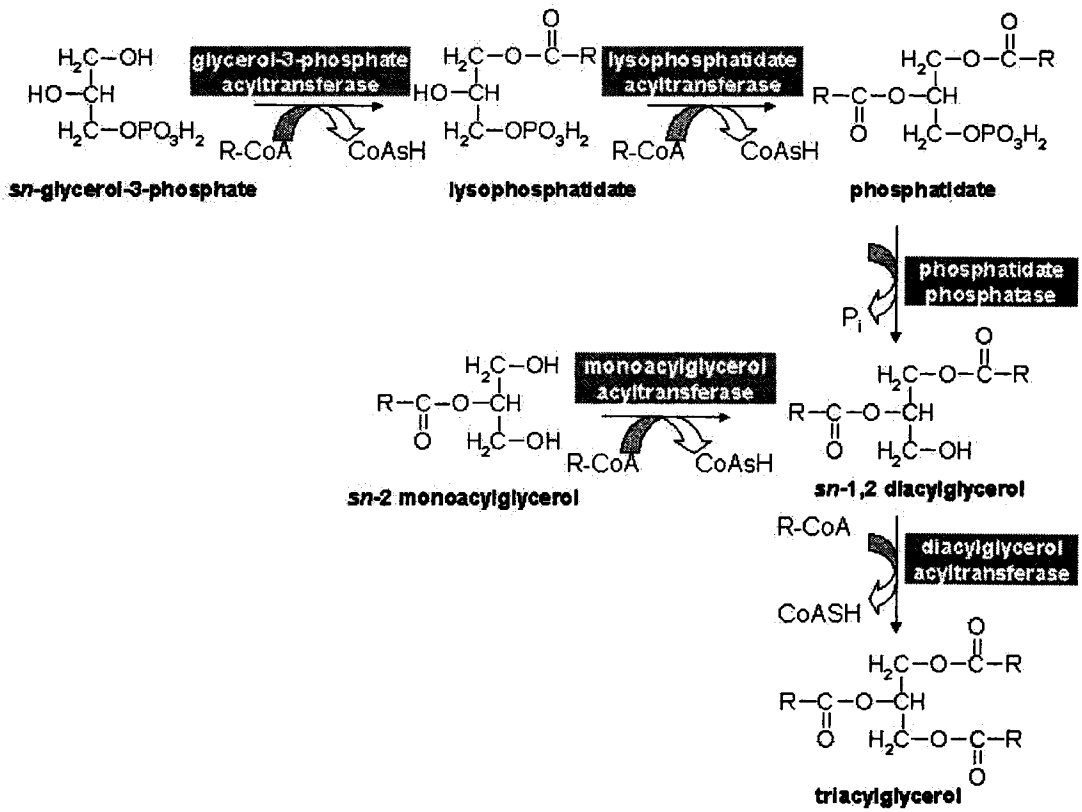

Figure 3. Schematic of mammalian triacylglycerol biosynthetic pathways.

2.2. Characteristics of mammalian diacylglycerol acyltransferase

Diacylglycerol acyltransferase was purified to near homogeneity from rat liver microsomes following gel filtration, Q-Sepharose and immunoaffinity chromatography (Andersson *et al.*, 1994). A 60 kDa protein was also obtained following chromatography of rat liver microsomes. Antibodies were generated against this protein, which were immunoreactive to both the DGAT containing fraction and the 60 kDa protein. Investigations with human DGAT suggest that the protein exists as a homotetramer (Cheng *et al.*, 2001). Owen and Zammit (1997) reported evidence for DGAT activity on both sides of the endoplasmic reticulum membrane, suggesting the existence of both latent and overt DGAT activities, which are differentially regulated. Upon incubation with Mg^{2+} and ATP, DGAT activity is modulated *in vitro*, suggesting that DGAT is activated/deactivated by a phosphorylation/dephosphorylation mechanism (Haagsman *et al.*, 1981). A protein tyrosine kinase was proposed to be responsible for the ATP dependent inactivation of DGAT (Lau and Rodriguez, 1996). Yu *et al.* (2002), however, reported that a base pair substitution at the conserved tyrosine phosphorylation site of human *DGAT1* did not result in enhanced DGAT activity, suggesting that tyrosine phosphorylation does not play a substantial role in regulating DGAT1 activity. It may be possible that there is a functional tyrosine phosphorylation site in another DGAT gene which would account for the previously observed inactivation of DGAT activity.

Coleman and Bell (1976) demonstrated that rat liver DGAT was able to effectively use a number of acyl-CoA substrates, with the exception of 18:0-CoA and that DGAT activity is directly dependent on acyl-CoA concentration. *sn*-1,2 Dioleoylglycerol appears to be the preferred substrate for DGAT in rat liver (Ide and Murata, 1993). Haagsman *et al.* (1981) noted a stimulatory effect of long chain FA on DGAT activity.

Incubation of microsomal DGAT from rat hepatocyte cells with longer chain FA enhanced DGAT activity compared to controls (Hillmar *et al.*, 1983). In rats fed a diet high in DAG, lipid accumulation was attenuated with a concurrent decrease in DGAT activity (Meng *et al.*, 2004). Addition of LPA to rat liver microsomal fractions increased DGAT activity in a dose dependant manner suggesting that DGAT is modulated by LPA and related phospholipids (PL) (Parthasarathy *et al.*, 1981).

Yu *et al.* (2002) suggest that *Dgat1* is post-transcriptionally controlled. Following differentiation of murine primary preadipocytes (3T3-L1) there was a 90-fold increase in DGAT1 protein with only a 7-fold increase in the abundance of *Dgat1* mRNA. A similar increase (~100-fold) occurred in DGAT activity. When abnormally high levels of mRNA are produced, however, protein expression is attenuated. When adipocytes were transduced with *Dgat1*, only a small increase in protein occurred. It was also suggested, based on analysis of the 5' untranslated region of *Dgat1* that it is under translational control (Yu *et al.*, 2002).

Murine *Dgat1* was first cloned by Cases *et al.* (1998) based on homology to acyl-CoA:cholesterol acyltransferase (ACAT EC 2.3.1.26). Mice with a disruption in the *Dgat1* gene (*Dgat1*$^{-/-}$) were leaner, resistant to diet-induced obesity, had smaller fat cells and reduced lipoprotein secretion (Smith *et al.*, 2000). *Dgat1*$^{-/-}$ mice had increased insulin and leptin sensitivity correlating to the decreases in adiposity (Chen *et al.*, 2002a). The decreased adiposity of the *Dgat1*$^{-/-}$ mice resulted from increased energy expenditure (Smith *et al.*, 2000). Female *Dgat1*$^{-/-}$ mice are unable to lactate as their mammary glands lack lipid droplets in epithelial cells (Smith *et al.*, 2000). These mice also have impaired mammary gland development (Cases *et al.*, 2004).The adipocytes of mice overexpressing *Dgat1* were up to twice as large and contained more TAG than those of

wild type mice (Chen *et al.*, 2002b). The degree of adiposity correlated with the degree of *Dgat1* overexpression. The mice were also more prone to weight gain in response to a high fat diet. Overexpression of *Dgat1* also increased the intramyocellular lipid content of skeletal muscles of rat (Roorda *et al.*, 2005). Yamazaki *et al.* (2005) overexpressed *Dgat1* in mice resulting in increased VLDL secretion from the liver. The same effect was reported in human cells (Liang *et al.*, 2004).

Surprisingly, the *Dgat1*$^{-/-}$ mice had normal serum TAG levels, and TAG was still relatively abundant in adipose tissue, suggesting the existence of additional TAG forming enzymes. The gene for a second DGAT isoform, *Dgat2*, was identified in *Mortierella ramanniana* (Lardizabal *et al.*, 2001) and later in mice (Cases *et al.*, 2001). DGAT2 does not share sequence homology to DGAT1, but rather belongs to a family of acyl-CoA: monoacylglycerol acyltransferase enzymes. Like *Dgat1*, *Dgat2* is ubiquitously expressed, except that *Dgat2* mRNA is the more abundant isomer in the liver while *Dgat1* mRNA is more abundant in adipose tissue (Meegalla *et al.*, 2002). Like DGAT1, DGAT2 is an intrinsic membrane protein, although it has only 1 or 2 putative transmembrane domains, while DGAT1 has between 6 and 12. DGAT2 also displays similar substrate specificity to DGAT1 although murine DGAT1 prefers 18:1-CoA over other acyl-CoA species (Cases *et al.*, 2001). The activity of DGAT2 is inhibited by high Mg^{2+} concentrations when assayed *in vitro* (Cases *et al.*, 2001). Waterman and Zammit (2002) found no correlation between overt and latent DGAT activities and the expression of the *Dgat1* and *Dgat2*.

Stone *et al.* (2004) generated *Dgat2* knockout mice (*Dgat2*$^{-/-}$). DGAT2 appears to be necessary for survival as the *Dgat2*$^{-/-}$ mice died soon after birth. Substrates for energy metabolism were rapidly depleted and the mice were incapable of increasing energy

stores. The mice also became rapidly dehydrated as the permeability barrier function of the skin was compensated, resulting in their death. DGAT2 might therefore be more important during early stages of development. Stone *et al.* (2004) found that DGAT1 was unable to compensate for the for the lack of DGAT2 while Cases *et al.* (2001) found that DGAT2 was unable to compensate for the lack of DGAT1 suggesting different functions of these enzymes. Yu *et al.* (2005) generated antisense oligonucleotides against *Dgat2* resulting in decreased TAG accumulation in the liver as well as an increase in FA oxidation.

Insect cells overexpressing murine *Dgat1* and *Dgat2* demonstrated wax synthase activity (Cheng and Russell, 2004). Yen *et al.* (2005) later found that DGAT1 demonstrated various additional activities including those of MGAT, and acyl-CoA:retinol acyltransferase (ARAT EC 2.3.1.76). Orland *et al.* (2005) treated colon cells with XP620, a selective inhibitor of DGAT1, finding that approximately 85% of the ARAT activity was inhibited. DGAT2 did not, however, possess any additional wax synthase activities (Yen *et al.*, 2005). The differences in the activities of the two enzymes might account for differences in their *in vivo* functions.

It is possible that DGAT has additional roles unrelated to lipid storage. For example, DGAT might help to regulate cellular levels of DAG, which is also a precursor of PL as well as a second messenger. To test this theory, Bagnato and Igal (2003) overexpressed *DGAT1* in human lung fibroblasts, resulting in decreased *de novo* synthesis of various PL, suggesting that DGAT used up substrate that was intended for PL synthesis.

2.3. Characteristics of bovine diacylglycerol acyltransferase

After Smith *et al.* (2000) reported that *Dgat1*$^{-/-}$ mice were incapable of lactation, bovine *DGAT1* became a functional candidate for regulating milk fat content. Through the use of systematic mapping, a quantitative trait locus (QTL) for milk fat was found on bovine chromosome 14, in the same region as the *DGAT1* gene (Winter *et al.*, 2002). Furthermore, Grisart *et al.* (2002) identified a missense mutation resulting in an amino acid substitution at position 232 in the bovine *DGAT1* gene, which has a major effect on milk fat content. The lysine (K) variant of *DGAT1* is associated with high milk fat, while the alanine (A) variant is associated with decreased milk fat percentage. The K232A substitution is in a region of the peptide that is not highly conserved across kingdoms, although it is conserved among mammals (Winter, 2003). This suggests that the essential motif of the protein is unchanged as a result of the mutation. The K232A substitution exists in several older breeds of cattle, suggesting that it took place before domestication (Kaupe *et al.*, 2004).

Certain individuals demonstrating high milk fat percentages did not, however, carry the lysine allele (Winter *et al.*, 2003). The regions flanking bovine *DGAT1* were assessed for polymorphisms (Winter *et al.*, 2004). Within these regions, 55 polymorphisms were discovered, and are currently being investigated to determine whether neighboring genes are related to alterations in milk fat. Polymorphisms in the promoter region of human *DGAT1* associated with body mass index were found in a population of Turkish women (Ludwig *et al.*, 2002). Bovine *DGAT2* was also investigated for polymorphisms associated with changes in milk fat (Winter *et al.*, 2003). Any polymorphisms detected were found in untranslated regions. Recently, Bennewitz *et al.*

(2004) provided evidence for at least a third allele at the *DGAT1* locus responsible for differences in milk fat production.

2.4. Fat secretion

Milk fat biosynthesis consists of three basic stages; the accumulation of FA, TAG assembly, and fat globule formation/secretion. Milk TAGs contain up to 400 different FA, although most are only present in trace amounts. A unique feature of ruminant milk TAGs is that they contain short chain FA such as 4:0 and 6:0 which are not present in appreciable amounts in TAGs of other ruminant tissues (Marshall and Knudsen, 1977). These, as well as medium chain FA are synthesized *de novo* in the mammary gland (Moore and Christie, 1979). Both short and medium chain FA are esterified to only the *sn*-2 and *sn*-3 position of the glycerol backbone (Parodi, 1982). The substrates for these FA include acetate (C2), propionate (C3), and β-hydroxybutyrate (C4), which are a result of ruminal fermentation of fiber. In ruminant mammary gland tissue, acetate from the blood is taken up by alveolar cells and converted to acetyl-CoA by acetyl-CoA synthetase (EC 6.2.1.1) located in the cytosol. Acetyl-CoA is subsequently converted into malonoyl-CoA by ACC. The activity of ACC in the mammary gland is substantially increased during lactogenesis (Clegg *et al.*, 2001).

A unique characteristic of milk TAGs, is saturated fatty acids (SFA) are predominantly esterified to the *sn*-2 position of the glycerol backbone (Parodi, 1982). In most other mammalian species, however, LPAAT has a greater affinity for unsaturated FA-CoA (e.g., 18:1-CoA) than SFA-CoA (e.g., 16:0-CoA and 18:0-CoA), resulting in a preponderance of unsaturated FA being esterified at the *sn*-2 position. Another exception to this is the pig which can have up to 70% 16:0 esterified at the *sn*-2 position

(Christie and Moore, 1970). It was suggested that there is a mammary gland specific isoform of LPAAT or that the specificity of LPAAT is regulated by metabolic factors during lactation. The activities of mammary gland GPAT and LPAAT are higher in lactating tissue than in non-lactating tissue (Morand *et al.*, 1998).

The majority of TAG synthesized in the mammary gland epithelia is incorporated into microlipid droplets that coalesce into larger droplets called cytoplasmic lipid droplets (Mather and Keenan, 1998). Two mechanisms of secretion are proposed; the apical mechanism and the secretory vesicle mechanism. In the apical mechanism, the cytoplasmic lipid droplets become enveloped in a plasma membrane, move towards the apical membrane of the cell and pinch off from the cell as milk lipid globules. The membrane of milk lipid globules in bovine consists of a unique assortment of proteins with possible roles in lipid secretion (Mather and Keenan, 1998). The membrane also protects the TAGs from lipolysis and oxidation. In the secretory vesicle mechanism, droplets are surrounded by vesicles and exocytosed. Following secretion, part of the membrane is shed, giving rise to membrane fragments (Mather and Keenan, 1998).

2.5. Conjugated linoleic acid and milk fat depression

2.5.1. Biological effects of conjugated linoleic acid

Pariza and Hargreaves (1985) first discovered that ground beef possessed anti-carcinogenic properties. The anti-carcinogenic agent was a mixture of CLA isomers. Individual isomers were tested for anti-cancer properties and both *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 appeared to be equally effective in preventing cancer in rat

models (Ip *et al.*, 2002). Corl *et al.* ((Corl *et al.*, 2003)) also provide evidence that *trans*-11 18:1 has anticarcinogenic properties in rat. Dietary supplementation with CLA also results in reduction in body fat mass and an increase in lean body mass in animal models. Mixtures of CLA rapidly reduced body fat content in mice (DeLany *et al.*, 1999). Mice fed diets enriched in *trans*-10, *cis*-12 18:2 gained substantially less body weight compared to controls (Park *et al.*, 1999). Supplementation with the *cis*-9, *trans*-11 18:2, however, failed to show similar effects. Conjugated linoleic acid has also demonstrated a number of other potential health benefits in animal models including enhancing immune function (Bassaganya-Riera *et al.*, 2003), increasing bone metabolism (Li and Watkins, 1998), normalizing glucose tolerance in diabetes (Houseknecht *et al.*, 1998), and reducing atherosclerosis (Truitt *et al.*, 1999)

Conjugated linoleic acid treatment is capable of inhibiting tumor growth in human cell lines (Maggiora *et al.*, 2004). Studies have not been done to demonstrate that direct treatment with CLA decreases the occurrence cancer in humans. Certain clinical studies, however, suggest that the weight loss effects of CLA carry over to humans. Overweight humans supplemented with up to 6.8 g of CLA per day had substantial reductions in body fat mass (Blankson *et al.*, 2000). Gaullier *et al.* (2004) also report that body fat mass is reduced in human adults following long term supplementation with CLA. In both studies subjects were given CLA in the form of Tonalin® capsules which contain a mixture of many isomers of CLA. Other studies however found no effects of CLA on body weight in humans Zambell *et al.*, 2000; Malpuech-Bruger *et al.*, 2004).

Because of the potential human health benefits of CLA, the dairy industry sees potential consumer demand for a dairy product with elevated levels of CLA. In Western

countries the typical daily intake of CLA is estimated to be between 150 and 210 mg (Ritzenthaler *et al.*, 2001). In sensory evaluations of dairy products with increased levels of CLA, consumers found no differences in acceptability or off-flavors compared with control milk (Ramaswamy *et al.*, 2001). Lynch *et al.* (2005) reported that CLA enhanced milk did not demonstrate increased susceptibility to oxidation when compared to controls, despite a higher content of polyunsaturated FA (PUFA). Surveys also show that consumers are willing to pay a premium for CLA enhanced milk and other dairy products, in particular cheese and ice cream (Ramaswamy *et al.*, 2001).

2.5.2. Strategies for increasing the conjugated linoleic acid content of milk

A number of factors influence the CLA content of milk fat such as breed, age, and diet with diet having the strongest influence. Cows grazing on fresh pasture tend to have higher CLA content than those fed mixed diets (Kelly *et al.*,1998). Fresh grass contains α-18:3 which is a substrate for biohydrogenation. Fermentation of the sugars in grass also helps to maintain a pH in the rumen conducive to the preservation CLA and *trans*-11 18:1 production (Dhiman *et al.*, 2005). The content of α-18:3 in grass undergoes seasonal variation which is reflected in seasonal variations in the milk fat content of CLA. There are often marked differences in the levels of CLA in milk fat of cattle fed the same diet. This is likely accounted for by differences in the expression of SCD (Bauman and Griinari, 2003). Differences in the activity of SCD might also account for breed differences in CLA production.

Oilseeds, particularly those that are abundant in *cis*-9, *cis*-12 18:2 (i.e., soybeans safflower, and sunflower) are effective in increasing CLA content of milk fat (Dhiman *et al.*, 2005). Oilseeds abundant in α-18:3 (i.e., flax) also increase CLA content, albeit to a lesser extent than oilseeds rich in *cis*-9, *cis*-12 18:2. The *cis*-9, *trans*-11 18:2 content of milk was substantially increased when fish oil was supplemented in combination with soybean oil, despite the fact that fish oil contains relatively small amounts of the precursors (Whitlock *et al.*, 2002). AbuGhazaleh and Jenkins (2004) suggest that docosahexaenoic acid (DHA, all-*cis*-4,7,10,13,16,19 22:6) in the fish oil promotes *trans*-11 18:1 accumulation by inhibiting the growth and activity of bacteria that reduce *trans*-11 18:1 to 18:0.

Biohydrogenation is altered by agents such monensin, an ionophore antibiotic that inhibits hydrogen producing bacteria resulting in increased CLA content in milk when supplemented to the diet of dairy cattle (Dhiman *et al.*, 1999). When used in combination with fish oil, monensin was able to increase the content of *cis*-9, *trans*-11 18:2 and *trans*-11 18:1 to a greater extent than when monensin was used alone (Wang *et al.*, 2005). Introduction of the oils as their calcium salts is effective in limiting interactions with microbes and reducing biohydrogenation (Chouinard *et al.*, 1998). The coat of oilseeds also offers some protection against biohydrogenation and accordingly different feed processing techniques have been investigated (Dhiman *et al.*, 2000). Amide-protected and lipid-encapsulated CLA (Perfield *et al.*, 2004) were also explored as a means of protecting the oils.

Another strategy for increasing the levels of CLA involves dietary supplementation of synthetic CLA. Post ruminal delivery of CLA mixtures (Chouinard *et al.*, 1999) and *trans*-11 18:1 (Corl *et al.*, 2001) resulted in increased concentrations in

milk fat of lactating dairy cattle. The various studies are difficult to compare to one another as treatments consisted of various ratios of CLA isomers, each of which might have different properties.

2.5.3. Milk Fat Depression

The dietary conditions that lead to elevated CLA in milk fat result in an overall decrease in milk fat, a phenomenon referred to as milk fat depression (MFD). In the milk fat of cattle fed a MFD diet, there is a decrease in the level of *cis*-9, *trans*-11 18:2 with increases in both *trans*-10, *cis*-12 18:2 (Piperova *et al.*, 2000) (Peterson *et al.*, 2003) and *trans*-7, *cis*-9 18:2 (Piperova *et al.*, 2000). There is also a predominance of *trans*-10 18:1 in the cattle with diet induced MFD compared to *trans*-11 18:1 in control cattle (Griinari *et al.*, 1998; Piperova *et al.*, 2000). Baumgard *et al.* (2000) noted that treatment of cattle with *trans*-10, *cis*-12 18:2 resulted in reduced milk fat synthesis, while *cis*-9, *trans*-11 18:2 had no effect on milk fat. The amount of CLA required to induce MFD is relatively low. Abomasal infusion of 0.1g/day of *trans*-10, *cis*-12 18:2 is sufficient to substantially reduce milk fat yield (Baumgard *et al.*, 2001). *Trans*-10, *cis*-12 18:2 induced MFD in a dose dependant manner (Giesy *et al.*, 2002; Viswanadha *et al.*, 2003). Cattle treated with salts of *trans*-10 18:1 also exhibited decreases in milk fat, albeit to a lesser extent than *trans*-10, *cis*-12 18:2 (Piperova *et al.*, 2004).

Commercial preparations of CLA also contain *trans*-8, *cis*-10 18:2, and *cis*-11, *trans*-13 18:2. These isomers, like *trans*-10, *cis*-12 18:2 are naturally present in low concentrations in milk fat (Perfield *et al.*, 2004). Abomasal infusions of these isomers, however, had no effect on milk fat synthesis. Sæbø *et al.* (2005) also investigated the effects of other 10,12 18:2 isomers on milk fat synthesis. While *trans*-10, *trans*-12 18:2

had no effect, it appears that *cis*-10, *trans*-12 18:2 reduces milk fat synthesis. Although pure preparations of *cis*-10, *trans*-12 18:2 were unavailable, mixtures of 10,12 18:2 isomers containing substantial amount of this isomer were as capable of reducing milk fat synthesis as *trans*-10, *cis*-12 18:2 alone.

The most widely supported theory behind MFD is the 'biohydrogenation theory', which suggests that intermediates of biohydrogenation inhibit lipid biosynthetic enzymes (Bauman and Griinari, 2003). Abomasal treatment with *trans*-10, *cis*-12 18:2 reduced the mRNA abundance of ACC and FAS in lactating dairy cattle (Baumgard *et al.*, 2002) as well as the activities of GPAT and LPAAT (Baumgard *et al.*, 2000). Cattle fed a diet supplemented with plant oils or high concentrate exhibited MFD and also had reduced activities of ACC and FAS (Piperova *et al.*, 2000) as well as decreased mRNA abundance of GPAT, LPAAT, ACC, and FAS (Peterson *et al.*, 2003). Baumgard *et al.* (2000) observed that Holstein cattle treated with *trans*-10, *cis*-12 18:2 had increased ratios of 18:0 to 18:1 and 14:0 to 14:1 in milk TAG fractions, suggesting that this isomer had an effect on the activity and abundance of SCD. Treatment of 3T3-L1 cultures with *trans*-10, *cis*-12 18:2 decreased the expression of SCD (Choi *et al.*, 2000). A similar effect was observed in the bovine mammary gland (Baumgard *et al.*, 2002). In lactating mice, treatment with *trans*-10, *cis*-12 18:2 decreased the abundance of mammary gland SCD mRNA to a greater extent than *cis*-9, *trans*-11 18:2 (Lin *et al.*, 2004).

Peterson *et al.* (2004) investigated the effects of *trans*-10, *cis*-12 18:2 with respect to sterol response element binding protein 1 (SREBP1), a protein which acts to regulate genes involved in lipid metabolism. Most promoters for these genes contain a sterol response element (SRE). Sterol response element binding proteins are proteolytically cleaved and the N-terminal fragment migrates to the nucleus, activating

gene transcription (Brown and Goldstein, 1997). While the expression of SREBP1 mRNA or its precursor protein were unaffected by treatment with *trans-10, cis-12* 18:2, proteolytic activation was inhibited, coinciding with reductions in lipid synthesis.

2.6. Fat Accretion

While skeletal muscle depends largely on glycogen stores as an energy source, energy is also derived from fat, with a shift towards fat metabolism during prolonged exercise (Ikeda *et al.*, 2002). Higher vertebrates including ruminants have evolved adipocytes, which are specialized cells that store TAG. Adipose tissue consists of adipocytes, connective tissue, and pre-adipocytes (fibroblast-like mesenchymal cells) surrounding a blood capillary network (Hood and Allen, 1973).

Growth of adipose tissue occurs either via hyperplasia or hypertrophy. Hyperplasia refers to an increase in cell number as a result of preadipocyte differentiation. Hyperplasia, with respect to adipose tissue, is referred to as adipogenesis and occurs mainly during early development. As mammals mature, specialized cells including adipocytes lose the ability to replicate (Owens *et al.*, 1993). In the case of adipocytes, the fat droplet cannot be distributed between the resulting daughter cells. Later in life, growth of adipose tissue occurs mainly via hypertrophy, an accumulation of intracellular lipid. Adipogenesis, albeit to a lesser extent, does occur in mature cattle (May *et al.*, 1994).

2.7. Characteristics and determination of marbling in cattle

Marbling refers to the appearance of white adipocytes, or fat cells, between muscle fiber bundles in bovine skeletal muscle and is present in most muscle types to varying extents (Harper and Pethick, 2004). Marbling is thought to influence meat quality and, although this is a highly debated issue, minimum amounts of marbling are required for carcasses to attain top beef grades in current North American beef grading schemes. Nishimura *et al.* (Nishimura *et al.*, 1999) suggest that the development of adipose tissue disrupts the structure of the i.m. connective tissue and that this contributes to tenderization

Wheeler *et al.* (1994) found only a small positive association between marbling and palatability. Feuz *et al.* (2004) found that the marbling levels of steak had a positive association with consumer ratings of palatability, although their willingness to pay (as determined by experimental auction) was not significantly affected by marbling. Marbling nevertheless is an important consumer trait, particularly in markets such as Japan (Bindon, 2004).

2.8. Development of biochemical and molecular markers for predicting the extent of marbling

Marbling is measured visually in the carcass after it has been chilled for 24 h. Grading is performed on cross sections on the *longissimus dorsi* (rib eye) muscle. In Canada and the USA, the grading site is located between the 12th and 13th ribs. Marbling assessment takes into consideration quantity, distribution, and size of fat deposits. In Canada, marbling is scored on a scale of 10 to 1, where 10 represents a

carcass devoid of marbling and 1 represents a carcass with abundant marbling. There is relatively high correlation between marbling score and extractable fat (Park *et al.*, 1994).

Only a small percentage of beef carcasses produced in Canada have a desirable amount of marbling (Jones and Talbot, 1991). Carcasses with less than desirable marbling receive a lower grade and sell for a lower price. In response to high demand for sufficiently marbled cattle, producers have attempted to feed cattle high energy feeds rich in grain. Smith and Crouse (1984) studied the effects of high energy diet on cattle, finding that increases in marbling fat were negligible compared to the increases in back fat and excess seam fat. This excess fat is considered waste and represents extra cost to the producer.

The deposition of i.m. fat in cattle is a continuously varying trait and exhibits a high degree of heritability (Marschall, 1999). Furthermore, there is poor association between the deposition of (i.m.) fat and deposition in other depots (Smith and Crouse, 1984). This provides the opportunity to select for marbling via breeding. Selecting for cattle predisposed for high marbling can, however, require a great deal of time and expense as progeny must be slaughtered before any conclusions about marbling potential are made.

Marbling can be measured ultrasonically in live cattle. Marbling fat scatters the ultrasound waves and appears as white regions against a dark background. This method was effective in consistently predicting percentages of i.m. fat in cattle ranging from 52–63 weeks although at earlier ages many of the differences in i.m. fat are not due to genetic factors, making it difficult to use this as a selection tool. (Hassen *et al.*, 2003). Furthermore, differentiating fat from connective tissue is somewhat subjective.

Molecular and biochemical markers represent another possible selection tool for marbling. A SNP in the 5' leader sequence in the thyroglobulin (TG) gene was associated with marbling (Barendse *et al.*, 1997). Thyroglobulin is a precursor of several hormones that affect lipid metabolism. Cattle either homozygous or heterozygous for the '3' allele tend to have higher marbling scores than cattle homozygous for the '2' alleles. Because of its importance in fat synthesis, DGAT was also examined for its potential as a biochemical marker of marbling. Barendse *et al.* (2004) found a putative QTL for the marbling trait on bovine chromosome 14. The gene for TG is located within this QTL. The *DGAT1* gene is also located within the region of this QTL. Thaller *et al.* (2003) investigated the effects of the K232A polymorphism and the 5' polymorphism of TG on the fat content of two different muscle types in Holstein and Charolais cattle. Both polymorphisms have an effect on i.m. fat deposition, although the *DGAT1* effect was seen mainly in the *semitendinosus* muscle, while the TG polymorphism only affected the longissimus muscle.

Middleton *et al.* (1998) found an inverse relationship between DGAT activity in i.m. tissue and the quantity of i.m. fat, suggesting that DGAT activity is down-regulated once i.m. fat is deposited. The activities in both adipose and i.m. fat were also compared, showing strong correlation and suggesting that they might regulate each other. Lozeman *et al.* (2001) characterized microsomal DGAT activity from several bovine tissues including subcutaneous (SC) adipose, i.m. adipose, and muscle (*pars costalis diaphragmatis*). The substrate specificity of DGAT was examined in these tissues, and although 18:0 is relatively abundant in bovine TAG, DGAT specificity towards 18:0-CoA was relatively low likely because 18:0 is concentrated in the *sn*-2 position as opposed to the *sn*-3 position. Palmitoyl (16:0)- and *cis*-9 18:1-CoA were both

good substrates for DGAT in all tissues. In SC tissue, however, DGAT activity was somewhat different than DGAT activities form the other tissues. These results also support the existence of two different DGAT activities in the tissues.

The recent endeavors in the fields of bovine genomics and physiology discussed above have led to increased understanding of lipid biosynthesis, particularly with respect to the function of DGAT. This information may prove invaluable to dairy and beef producers in their attempts to enhance their respective products. Although a number of lipid biosynthetic genes and enzymes have been implicated in MFD depression, DGAT has not been investigated. This paper goes on to discuss how further knowledge of DGAT might help to further elucidate the mechanisms of MFD, which is a major hurdle in the development of CLA enriched dairy products. Further investigation of DGAT as potential biochemical marker as a follow up to the work of Middleton *et al.* (1998) will also be presented.

3. The effect of conjugated linoleic acid on the activity and expression of diacylglycerol acyltransferase in bovine mammary gland tissue and cell cultures

3.1. Introduction

Conjugated linoleic acid (CLA) refers to a group of isomers of linoleic acid (*cis*-9, *cis*-12 18:2) which is naturally present in ruminant fats. Various isomers of CLA, including *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 are produced as intermediates as dietary fatty acids (FA) including *cis*-9, *cis*-12 18:2 and linolenic acid (α-18:3) are hydrogenated by ruminal microbes. Other intermediates of ruminal biohydrogenation, including *trans*-vaccenic acid (*trans*-11 18:1), are desaturated in the small intestine and mammary gland to form *cis*-9, *trans*-11 18:2, a reaction catalyzed by stearoyl-CoA desaturase (SCD 1.14.99.5). Certain isomers of CLA have nutraceutic properties including reducing the occurrence of cancer (Ip *et al.*, 2002) and reduction of body weight (Park *et al.*, 1999). Neilson Dairies has recently introduced Dairy Oh!™ (http://www.dairy-oh.com/nutrition.htm), a milk beverage enriched in docosahexaenoic acid (DHA, all-*cis*- 4,7,10,13,16,19 22:6) another nutraceutic FA into the market. Milk producers have also shown interest in creating a similar product, enriched in CLA. The content of CLA can be readily increased in milk fat by supplementing cattle with oilseeds such as soybean, which are abundant in *cis*-9, *cis*-12 18:2 (Dhiman *et al.*, 2005). Monensin, an ionophore, may be supplemented to feeds resulting in increased CLA content in milk fat, as it disrupts transmembrane ion gradients, inhibiting the activity of ruminal microbes (Dhiman *et al.*, 1999). Another strategy is to supplement the cattle with

synthetic mixtures of CLA that are protected from ruminal bacteria by abomasal infusion (Chouinard *et al.*, 1999), preventing the further isomerization of CLA by ruminal bacteria.

Diets resulting in elevated levels of CLA also result in milk fat depression (MFD), an overall decrease in milk fat content. *Trans*-10, *cis*-12 18:2 has been suggested as the isomer responsible for MFD (Baumgard *et al.*, 2000). One theory behind MFD suggests that certain isomers of CLA, in particular *trans*-10, *cis*-12 18:2, may inhibit lipogenic enzymes (Bauman and Griinari, 2003). Cattle fed high-concentrate diets resulting in MFD had decreased expression of lipogenic genes including fatty acid synthase (FAS, EC 2.3.1.15), acetyl-CoA carboxylase (ACC EC 6.4.1.2) glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), and lysophosphatidate acyltransferase (LPAAT 2.3.1.51) (Peterson *et al.*, 2003). Cultured mammary gland epithelial cells treated with *trans*-10, *cis*-12 18:2 also had reduced abundance of FAS and ACC mRNA (Peterson *et al.*, 2004).

Conjugated linoleic acid may also mediate MFD by affecting the activity and/or expression of diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), an enzyme which catalyzes the transfer of an acyl moiety from acyl-CoA to *sn*-1,2 diacylglycerol to form triacylglycerol (TAG) and is believed to be rate-limiting in TAG biosynthesis (Mayorek *et al.*, 1989). Two isoforms of DGAT, DGAT1 and DGAT2, have been identified. Mice In which the *Dgat1*$^{-/-}$ has been disrupted (*Dgat1*$^{-/-}$) are incapable of lactation (Smith *et al.*, 2000) and have impaired mammary gland development (Cases *et al.*, 2004). Recently a missense mutation resulting in a K232A substitution has been identified in bovine DGAT1 (Grisart *et al.*, 2002) and individuals homozygous for the 'A' variant have a decreased milk fat content.

The focus of this study was to examine the effects of CLA isomers, namely *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2, as well as *cis*-9 18:1, *trans*-11, 18:1, *cis*-9, *cis*-12 18:2, and α-18:3 on the activity of DGAT in the mammary gland tissue of lactating dairy cattle. The effects of these isomers on DGAT activity, as well as the expression of *DGAT1* and *DGAT2,* were also investigated in cultured mammary gland epithelial cells (MAC-T), which serve as a model system of the mammary gland. Finally, the expression of *DGAT1* and DGAT2 were investigated in mammary gland biopsy samples from cattle fed CLA enhancing diets.

3.2. Materials and Methods

3.2.1 Animals and diets

All procedures involving the use of animals were approved by the University of Alberta Animal Policy and Welfare Committee. Cows were housed in tie-stalls with water available at all times. The diets were fed once per day at nine AM as total mixed rations consisting of 60% (w/w) forage and 40% (w/w) concentrate. Feed intake was recorded daily and adjusted to maintain 5 to 10% orts (feed remaining).

3.2.1.1 Biopsies

Primiparous and multiparous lactating Holstein cows were randomly assigned to either a control diet (CTL) or control diet including safflower oil at 6% w/w DM plus monensin supplemented at 24 ppm of DM (SAFF/M). All cows were fed the CTL diet initially for 10 days. The SAFF/M cows then received their diet for a period of nine weeks

while control animals remained on the CTL diet. Milking was carried out twice daily. A sample of mammary gland tissue was taken from 6 CTL and 6 SAFF/M cows during the ninth week using a method adapted from Knight *et al.* (1992). Mammary gland biopsies were also used in studies by Bell (2003).

The animals were sedated by administration of xylazine hydrochloride (10 mg/50 kg body weight) and ketamine (2 mg/kg body weight) via jugular catheter. The animals were maintained in a state of sedation with an intravenous drip of glycerol guaiacolate and ketamine (50 g glycerol guaiacolate, 1000 mg ketamine, 1000 mL 50% (w/v) dextrose and 900 mL sterile water). The drip rate was set at 20 mL/min and maintained during surgery. The udder was shaved around the biopsy site and swabbed with iodine and 70% v/v ethanol. A region of the basal portion of the udder, free of major subcutaneous (SC) blood vessels was chosen for the biopsy site. The area was frozen with local anaesthetic (10–30 mL of 2% v/v lidocaine with epinephrine). An 8 cm incision was made in the skin, a 5–10 g piece of mammary gland tissue was excised using a scalpel and snap frozen in N_2 (l) and then stored at -80°C.

3.2.2. Chemicals

Both *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 were obtained from Matreya (Pleasant Gap, Pennsylvania, U.S.A.). [1-^{14}C] *cis*-9 18:1 was from GE Healthcare (Baie d'Urfé, Quebec, Canada). [1-^{14}C] *cis*-9 18:1-CoA was synthesized from [1-^{14}C] *cis*-9 18:1 as described by Taylor *et al.* (1990). Silica gel 60 H was from VWR Canlab (Missisauga, Ontario, Canada) High performance liquid chromatography (HPLC) grade solvents were from Fisher Scientific (Ottawa, Ontario, Canada). Ecolite(+) biodegradable scintillation cocktail was from ICN Biomedicals (Irvine, California, U.S.A.). Bovine serum albumin

(BSA), N-[2-hydroxyethyl]peperazine-N'-[2-ethanesulfonic acid] (HEPES), 2-(N-Morpholino)ethanesulfonic acid (MES), digitonin, bovine insulin, FA, diethylpyrocarbonate (DEPC), dithiothreitol (DTT), ethylene diamine tetraacetate (EDTA) and all other biochemicals were from Sigma (Oakville, Ontario, Canada) Fatty acid methyl ester standards and *sn*-1,2 diolein were from Nu Chek Prep Inc. (Elysian, Minnesota, U.S.A.). Dulbecco's Modified Eagle Media (DMEM), fœtal bovine serum (FBS), antibiotic-antimycotic liquid, TRIzol® reagent, M-MLV reverse transcriptase, oligo (dT)12–18 primer, Rnase OUT Rnase inhibitor, dNTPs, 5X First Strand Buffer (250 mM TRIS-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), and custom primers were from Invitrogen (Burlington, Ontario, Canada). rDNase, 10X buffer (100 mM TRIS-HCl pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$), and DNase inactivation agent were included in the Ambion DNA-*free*™ kit (Cat #1906, Austin, Texas, U.S.A.). FAM and VIC labeled probes and TaqMan® Universal PCR Mastermix were from Applied Biosystems (Foster City, California, U.S.A.).

3.2.3. Maintainance and treatment of cultured mammary gland epithelial cells

Mammary gland epithelial cells (MAC-T) were originally established by Huynh *et al.* (1991) and were kindly provided by Nicholas Lemee of Nexia Biotechnologies Inc. (Montreal, Quebec, Canada). The cells were cultured in DMEM high glucose already containing L-glutamine, sodium pyruvate, and pyroxidine-HCl. This was supplemented with 10% (v/v) FBS, 1% (v/v) antibiotic-antimycotic liquid and 5 µg/mL bovine insulin. Cells were routinely passaged every 3–4 days by treating with trypsin- ethylene diamine tetraacetate (EDTA) (0.05% w/v trypsin, 0.53 mM EDTA) to remove adherent cells. Cells

were maintained in a humidified incubator at 37°C and 5% CO_2. Cells were typically grown in untreated 75 cm^2 polystyrene flasks with vented caps (Corning Inc., Corning, New York, U.S.A.).

To 12 mg of FA was added 1.2 mL of hexane, which was subsequently evaporated under N_2 (g). To this was added 1 mL of 0.1 M KOH. The mixture was vortexed and incubated at 50°C for 10 min, mixing often. The mixture was then added dropwise to 9 mL of 7.5% (w/v) BSA. The 4 mM FA-BSA stock solution was incubated at room temperature for 3 h and overnight at 4°C. The solution was stored at -20°C when not in use. The FA-BSA stock solution was added to DMEM to give the appropriate concentration. It was then filtered through a 0.22 µM syringe filter (Fisher Scientific) before being added to the tissue culture flask.

Fatty acid salts were prepared in the same manner as the FA-BSA solution, with the omission of BSA.

3.2.4. Lipid accumulation in cultured mammary gland epithelial cells treated with exogenous fatty acid

MAC-T cells were grown in 75 cm^2 flasks in DMEM containing 20 µM FA-BSA and incubated at 37°C. Upon confluence, cells were treated with trypsin-EDTA and harvested and transferred to acid washed glass tubes. The vials were centrifuged at 1500 x *g*. The supernatant was removed and the cells were resuspended in 1 mL phosphate buffered saline (PBS). The resuspended cells were probe sonicated for 30 s and a sample was removed for protein determination. Lipids were extraction using a

modified Folch protocol. The following were added in sequence to the cells suspension, mixing by vortex for 30 s after each addition: 0.1 M KCl to bring suspension to total volume of 1.6 mL, 0.8 mL methanol, 2 mL chloroform/methanol (1:1, v/v), 2.7 mL chloroform, 2.5 mL chloroform/methanol (2:1, v/v). The samples were incubated overnight at 4°C. Phase separation was achieved by centrifugation and the chloroform layer was transferred to a glass vial and dried under N_2 (g). Prior to extraction, 100 µg of tritricosanoin (tri-23:0) were added to the cell suspension as an internal standard.

Methylation was performed based on methods described by Christie *et al.* (1982). To the dried extract was added 100 µg of trinonadecanoin (tri-19:0), 1.7 mL of hexane, 40 µL of methyl acetate, 100 µL of $NaOCH_3$ (0.5 M in methanol) followed by mixing for 2 min and incubation at room temperature for 20 min. The vial was then cooled at -20°C for 20 min and 60 µL of oxalic acid (saturated in diethyl ether) was added. The vials were centrifuged to collect the oxalate precipitate and the supernatant was spotted to a TLC plate. The plate was developed in one ascension of hexane/diethyl ether (80:20, v/v) using methyl-oleate as a standard. The plates were then visualized by spraying with 0.2% (w//v) 2,7' dichlorofluorescein. The area corresponding to methyl-oleate was scraped and transferred to a column containing sodium sulfate. The fatty acid methyl esters (FAME) were eluted using 2 mL of chloroform and 50 µg of methyl-hexacosanoate (methyl-26:0) were added.

FAMEs were analyzed by gas chromatography (GC) with a Varian 3800 GC (Varian Inc., Mississauga, Ontario, Canada), equipped with splitless injection port flushed after 0.3 sec, a flame ionization detector (FID), autosampler (Model 8200, Varian Inc.), 100m CP-Sil 88 fused capillary column (Varian Inc.), and a Hewlett-Packard ChemStation software system (Version A.07). Operating conditions included: injector

and detector temperatures both at 250°C; H_2 as carrier gas (1 mL/min) and for the FID (40 mL/min), N_2 (g) as makeup gas (100 mL/min), and purified air (250 mL/min). The initial temperature of 45°C was held for 4 min, increased at a rate 13°C/min to 175°C and held for 27 min, and finally increased at a rate 4°C/min to 215°C and held for 35 min. FAME were identified by comparison with a reference standard.

3.2.5. Ribonucleic acid isolation, reverse transcription and real time polymerase chain reaction

MAC-T cells were grown in 75 cm^2 tissue culture flasks in DMEM containing 20 µM FA-BSA and incubated at 37°C. When the cells were confluent, the media was removed and 5 mL of TRIzol® reagent was added to the flask and allowed to incubate at room temperature for 2–3 min. The mixture was transferred to a polycarbonate culture tube and 0.2 volumes of chloroform was added for every one volume of TRIzol® reagent added. The mixture was shaken and allowed to incubate at room temperature for 5 min. The mixture was then centrifuged at 12 000 x *g* and 4°C for 15 min in an Avanti J-F Centrifuge (Beckman Coulter). The aqueous layer was removed and 0.5 volumes of isopropanol was added for every one volume of TRIzol®. The tubes were stored at -20°C overnight and centrifuged at 12 000 x *g* and 4°C for 10 min. The supernatant was removed and the pellet was washed with one volume of 70% (v/v) ethanol for every 1 volume of TRIzol® reagent. The pellet was allowed to air dry and was dissolved in DEPC treated H_20. The RNA was quantified in 96-well Costar® UV plates (Corning) using a SpectraMax® 190 multiplate reader (Molecular Devices, Sunnydale, California, U.S.A.). For the extraction of mRNA from mammary gland biopsies, the same procedure was

used except that one volume of TRIzol® reagent was added per 100 mg of tissue and the mixture was homogenized using a PowerGen 700 homogenizer (Fisher Scientific).

To a 500 µL microfuge tube was added 10 µg RNA, 1 µL rDNase, 5 µL 10X buffer and DEPC treated water to give a final volume of 50 µL. The reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 5 µL of inactivation agent and incubating for 2–3 min at room temperature with occasional mixing. The mixture was centrifuged at 10 000 x *g* for 1.5 min and the supernatant was transferred to a new tube. To the supernatant was added 6 µL of 3 M sodium acetate and 125 µL of absolute ethanol. The reaction mixture was stored at -80°C for 60 min. The mixture was centrifuged at 17 000 X *g* for 10 min and the supernatant was removed. The pellet was washed with 70% (v/v) ethanol and resuspended in DEPC treated water.

cDNA was synthesized from 1 µg of total RNA in a 20 µL reaction volume with a final concentration of 0.5 mM dNTP mix, 0.5 µg Oligo $(dT)_{12-18}$, 5 µM DTT, 4µL 5X first strand buffer, 40 U RNase out RNase inhibitor, and 200 U of M-MLV reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min, heated for 70°C for 15 min and stored at -20°C until use.

Quantitative real-time PCR (RT-PCR) was performed using the ABI Prism® 7900HT FAST RT-PCR system with 9600 Emulation (Applied Biosystems) in a total volume of 25 µl reaction mixture following the manufacturer's protocol, using TaqMan® 2X Universal PCR Mastermix, 0.1 µM each of forward and reverse primer and 0.2 µM of probe. Primers and probes were designed using based on *DGAT1*, *DGAT2*, and *LPAAT* from *Bos taurus* using Primer Express® v 2.0 Software (Applied Biosystems, Table 1).

Table 1. Primer and probe sequences for real time polymerase chain reaction

primers/detection probes	sequence
DGAT1 forward primer	5'-GAACTCCGAGTCCATCACCTACTT-3'
DGAT1 reverse primer	5'-TCTGATGCACCACTTGTGAACA-3'
DGAT1 detection probe	5'-6FAM-TGGCAGAACTGGAACAT-MGBNFQ-3
DGAT2 forward primer	5'-GCCCTGCGCCATGGA-3'
DGAT2 reverse primer	5'-TACACCTCATTCTCCCCAAAGG-3'
DGAT2 detection probe	5'-6FAM-CCTGGTTCCCACCTAC-MGBNFQ-3
LPAAT forward primer	5'-GGACGCAACGTCGAGAACA-3'
LPAAT reverse primer	5'-CCGTACAGGTATTTGATGTGGAGTA-3'
LPAAT detection probe	5'-6FAM-AAGATCTTGCGTCTGATG-MGBNFQ-3
GAPDH forward primer	5'-TGCCGCCTGGAGAAACC-3'
GAPDH reverse primer	5'-CGCCTGCTTCACCACCTT-3'
GAPDH detection probe	5'-6FAM-CCAAGTATGATGAGATCAA-MGBNFQ-3

Relative gene expression for *DGAT1*, *DGAT2*, or *LPAAT* mRNA was normalized to a calibrator that was chosen to be the basal condition (BSA control) for each treatment. Results were calculated with the ΔΔCt method and expressed as n-fold differences in gene expression relative to GAPDH mRNA and calibrator (Equation 1). ΔΔCt values were calculated by subtracting the ΔCt value of the calibrator (BSA) from the ΔCt value of the treated sample (Equation 2). ΔCt values were determined by subtracting the average Ct value of the target gene from the average Ct value of GAPDH rRNA, for each sample (Equation 3) where the parameter Ct (threshold cycle) is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold.

$$n - fold = 2^{-\Delta\Delta CT} \quad (1)$$

$$\Delta\Delta Ct = \Delta Ct_{\ treated\ sample} - \Delta Ct_{\ calibrator} \quad (2)$$

$$\Delta Ct = Ct_{\ GAPDH} - Ct_{\ target\ gene} \quad (3)$$

Standard errors of the average target gene Ct and average GAPDH Ct were pooled (Equation 4)

$$S_{pooled} = \sqrt{S_{target}^{\ 2} + S_{GAPDH}^{\ 2}} \quad (4)$$

Upper and lower intervals were calculated as ΔΔCt – s and ΔΔCt + s , respectively.

3.2.6. Digitonin based assay of diacylglycerol acyltransferase

DGAT activity was measured in MAC-T cells based on the protocol described by Geelen (2003). MAC-T cells were grown in 6 well plates in DMEM containing 20 µM FA-BSA and incubated at 37°C. When confluent, the cells were treated with trypsin-EDTA and harvested. The cells were centrifuged for 10 minutes at 1500 x *g* and the pellet was washed with PBS. To the cells was added 60 µL of preheated assay buffer to containing assay medium which consisted of 96 mM MES (pH 6.5), 9.6 mM EDTA, 1.9 mM DTT, 125 mM NaCl, 15 µM [1-^{14}C]*cis*-9 18:1-CoA, 30 µg BSA, and 18 µg of digitonin. The mixtures were incubated for 10 min at (37°C) and terminated with 10 µL of 12.4 M HCl. Chloroform and methanol were added and lipids were extracted according to the method of Bligh and Dyer (1959). Lipids were spotted to TLC plates and developed in one ascension of hexane/diethyl ether (80:20, v/v) using 50 µL of 10 mg/mL triolein as a standard. The standard spot was visualized using iodine vapor. The areas corresponding to the standard were scraped and transferred to 20 mL scintillation vials. To the vials was added 5 mL Ecolite. The incorporation of [1-^{14}C] *cis*-9 18:1 into TAG was measured using a scintillation counter. For protein measurements, the resuspended pellets were suspended in PBS and probe sonicated for 30 s and assayed using the Bio-Rad protein microassay (Bio-Rad, Hercules, California, U.S.A.) based on the method of Bradford (1976), using BSA as a standard

3.2.7. Assay of microsomal diacylglycerol acyltransferase from mammary gland tissue

Microsomes were prepared from the mammary gland tissue of two cows. Animal 2041 was a Holstein in her fourth lactation and was five years and months old. Mammary gland tissue was homogenized using a PowerGen 700 homogenizer in three volumes homogenization buffer which consisted of 100 mM HEPES-NaOH (pH 7.0), 250 mM sucrose, 4M EDTA, and 1mM DTT which was added immediately prior to homogenization. The homogenate was then centrifuged for 10 min at 4°C and 700 x *g* in a Sorvall RC-SB Refrigerated Superspeed centrifuge (Thermo Electron Corporation, Asheville, North Carolina, U.S.A.). The supernatant was filtered through four layers of cheesecloth, added to another tube and centrifuged for 2 h at 4°C and 100 000 x *g* in an L8-70M ultracentrifuge (Beckman Coulter, Mississauga, Ontario, Canada). The pellet consisting of the microsomal fraction was resuspended in resuspension buffer (100 mM HEPES-NaOH pH 7.0, 4 mM EDTA, and 1 mM DTT added directly before use). The protein content of the microsomes was determined using the Bio-Rad protein microassay. The microsomes were then flash frozen in N_2 (l) and stored at -80°C.

Fatty acyl-CoAs were quantified with an Ultrospec™ II UV-visible spectrometer (Biochrom LKB) based on the absorbance maximum of CoA at 259 nm. The concentration of unknown fatty acyl-CoAs was interpolated from a concentration curve based on the absorbance of *cis*-9 18:1-CoA.

Ten microliters of microsomal fraction were added to glass test tubes and the liquid was removed under vacuum for 2–3 h in a cooled dessicator. Fifty microliters of 1.5 mg/mL diolein in benzene were added to each tube and the benzene was evaporated under N_2 (g). To each tube was added 55 µL buffer consisting of 53 mM sodium citrate and 53 mM sodium phosphate (pH 6.7). The tubes were placed in a sonicating water bath for 10 min. The reaction was initiated with 10 µL of 180 µM [1-^{14}C]

cis-9 18:1-CoA (56 Ci/mol) and incubated at 30°C for 10 min. The reaction was terminated by the addition of 10 µL 10% (w/v) SDS. Thin layer chromatography of the mixture was performed as described in section 3.2.6 above.

3.2.8. Statistical analysis

Statistical analysis was performed using JMP® IN statistical software (version 4, Duxbury Press, Toronto, ON, Canada) or SAS statistical software (version 9.1). For the mammary gland biopsies, measures of association between *DGAT1*, *DGAT2* and *LPAAT* expression and various measurements of milk yield and composition were determined the Spearman's Rho ranked product-moment correlation method (JMP® IN). Differences between the various FA treatments or diets were assessed using ANOVA (JMP® IN). Remaining analyses were performed using the mixed procedure (SAS) with fixed random parameters.

3.3. Results

3.3.1. Triacylglycerol accumulation and fatty acid composition of cultured mammary gland epithelial cells treated with exogenous fatty acid

MAC-T cells were treated with 20 µM of FA-BSA and grown to confluence. Lipid was extracted and FAME were generated and analyzed using GC. The amount of cellular TAG was expressed in relation to the amount of cellular protein (Figure 4). None

of the exogenous FA treatments appeared to have a significant effect on the accumulation of TAG.

The mole percentage of certain FA and FA classes was calculated (Table 2). Treatment with *trans*-10, *cis*-12 18:2 significantly increased the levels of SFA and 18:0 ($P < 0.01$) and decreased the levels of *cis*-9 18:1.

3.3.2. The effect of exogenous fatty acid treatment on the expression of lipogenic enzymes in cultured mammary gland epithelial cells

mRNA was extracted from MAC-T cells which had been treated with various FA. The expression of *DGAT1* and *DGAT2* was monitored using RT-PCR (Figure 5). In general, *DGAT1* had a lower Ct than *DGAT2,* meaning that the transcript is more abundant in the MAC-T cells. None of the isomers appear to have had a substantial effect on the abundance of *DGAT1* relative to the control. None of the treatments resulted in significant change in the expression of DGAT1. Treatment with *cis*-9 18:1, *trans*-11 18:1, *cis*-9, *cis*-12 18:2, *cis*-9, *trans*-11 18:2, *trans*-10, *cis*-12 18:2, and α-18:3 resulted in significant increases in DGAT2 expression ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.001$ respectively). All isomers significantly enhanced LPAAT expression ($P < 0.001$).

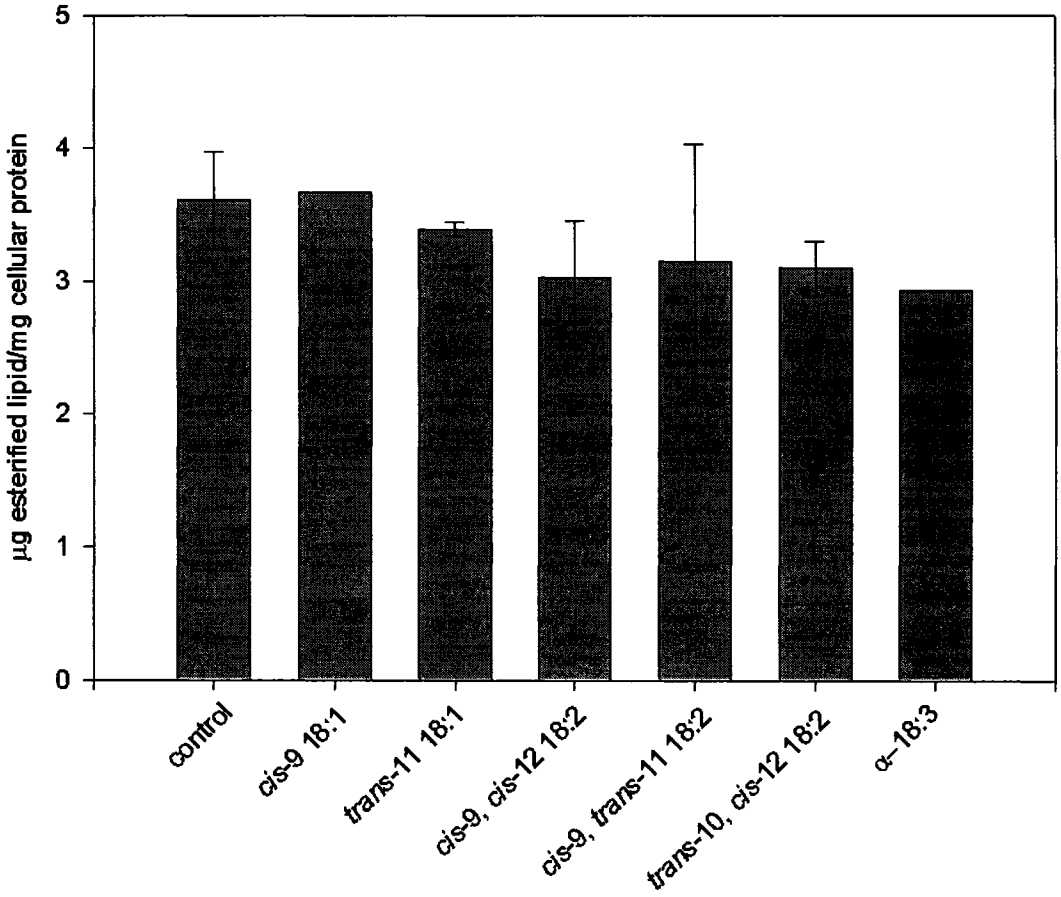

Figure 4. The effect of exogenous fatty acid treatment on the accumulation of esterified lipid in cultured mammary gland epithelial cells.

Values represent means ± range

Table 2. The effect of various fatty acid treatments on the mole percentages of various fatty acids and fatty acid groups in lipid fractions of cultured mammary gland epithelial cells treated with exogenous fatty acid

	Treatment						
	BSA	***cis*-9 18:1**	***cis*-9, *cis*-12 18:2**	**α-18:3**	***trans*-11 18:1**	***cis*-9, *trans*-11 18:2**	***trans*-10, *cis*-12 18:2**
SFA	38.4 ± 1.1	38.5 ± 0.7	39.9 ± 0.4	45.7 ± 2.7*	37.8 ± 2.0	40.6 ± 0.8	52.2 ± 1.1***
MUFA	42.2 ± 2.4	43.9 ± 0.7	19.9 ± 1.1***	26.5 ± 2.6**	45.5 ± 1.8	34.9 ± 1.2*	25.4 ± 1.5**
PUFA	19.3 ± 1.7	17.7 ± 1.0	40.2 ± 0.7***	27.8 ± 0.6**	16.6 ± 1.2	24.6 ± 0.8*	22.4 ± 2.1
18:0	12.3 ± 1.0	10.0 ± 0.5	12.0 ± 0.5	14.6 ± 1.2	10.6 ± 1.9	11.6 ± 0.3	17.7 ± 0.4**
***cis*-9 18:1**	25.0 ± 1.4	32.1 ± 0.3**	10.9 ± 0.5***	14.7 ± 1.0**	21.4 ± 1.0	20.0 ± 0.5*	15.6 ± 1.4**
cis-9, *cis*-12 18:2	4.2 ± 0.6	4.0 ± 0.3	24.1 ± 0.2***	4.4 ± 0.4	3.4 ± 0.1	3.9 ± 0.1	3.1 ± 1.0
α-18:3	0.4 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	9.2 ± 1.7***	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
***cis*-9, *trans*-11 18:2**	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	1.9 ± 0.7	3.6 ± 1.7	0.3 ± 0.0
***trans*-10, *cis*-12 18:2**	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	3.0 ± 1.5

Values represent means ± SEM (n=4)
*, **, *** represent significant differences between treatment and control within a row $^{*}(P < 0.05)$ $^{**}(P < 0.01)$ $^{***}(P < 0.001)$

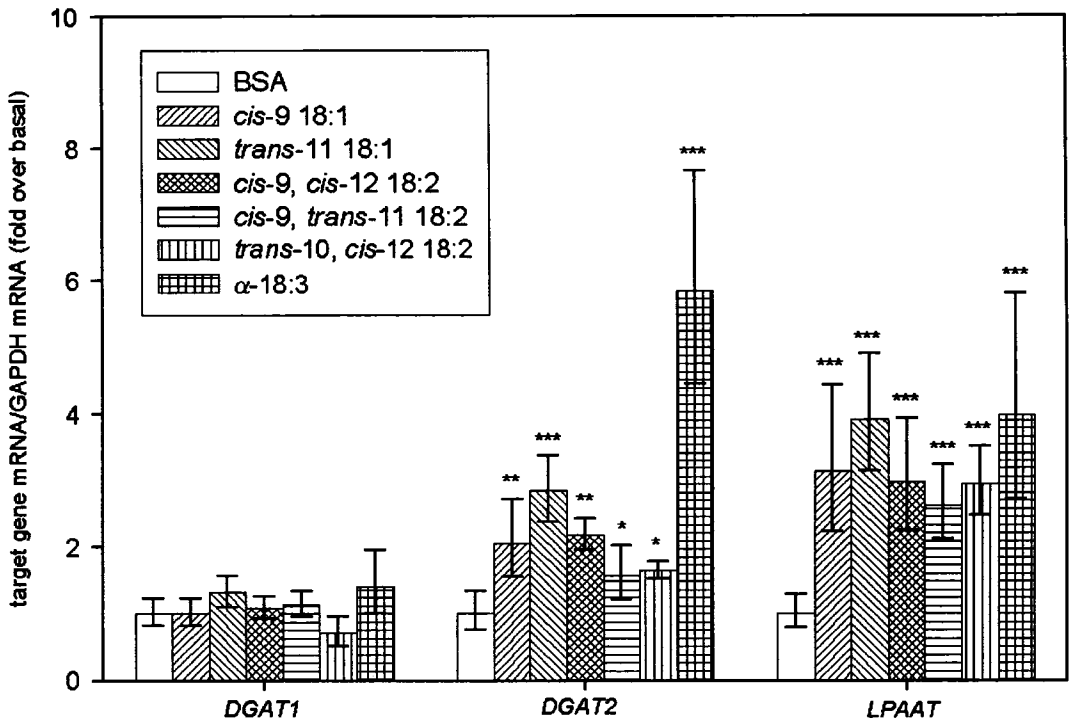

Figure 5. The effect of exogenous fatty acid on the expression of lipogenic genes in cultured mammary gland epithelial cells treated with exogenous fatty acid

Data points were calculated using the ΔΔCt method (See section 2.3.5). Duplicates from three separate trials were used. Error bars represent upper and lower intervals.

*, **, *** represent significant differences between treatment and control *($P < 0.05$) **($P < 0.01$) ***($P < 0.001$)

3.3.3. The effect of fatty acid treatment on diacylglycerol acyltransferase activity in cultured mammary gland epithelial cells

MAC-T cells were treated with various FA complexed with BSA (20 μM) and grown to confluence. The activity of DGAT was assayed by incubating the cells in the presence of digitonin (0.3 μg/μL) for 10 min at 37°C and monitoring the incorporation of [1-14C] *cis*-9 18:1-CoA (56 Ci/mol) into TAG, relying on endogenous DAG. Compared to control treated cells (BSA only), the specific DGAT activity of cells treated with *trans*-10, *cis*-12 18:2, and α-18:3 was significantly decreased ($P < 0.05$, Figure 6).

3.3.4. Acyl-coenzyme A competition assays of microsomal mammary gland diacylglycerol acyltransferase

Microsomal fractions prepared from the mammary gland tissue of a lactating cow were incubated with 15 μM [1-^{14}C]*cis*-9 18:1-CoA (56 Ci/mol) and a 15 μM of unlabeled *cis*-9, *trans*-11 18:2-CoA *trans*-10, *cis*-12 18:2-CoA, or *cis*-9 18:1-CoA for 10 min at 30°C. The incorporation of [1-^{14}C]*cis*-9 18:1 into TAG was monitored. (Figure 7). At 15 μM, none of the acyl donors had a significant effect on DGAT activity.

Microsomes from the same preparation were then incubated with 15 μM [1-^{14}C] *cis*-9 18:1-CoA (56 Ci/mol) and increasing concentrations (0–20 μM) of unlabeled *cis*-9, *trans*-11 18:2-CoA *trans*-10, *cis*-12 18:2-CoA, *cis*-9, *trans*-12 18:2-CoA or *cis*-9 18:1-CoA for 10 min at 30°C (Figure 8). The incorporation of *cis*-9, *trans*-11 18:2-CoA *trans*-

10, *cis*-12 18:2-CoA, and *cis*-9, *trans*-12 18:2-CoA were all significantly ($P < 0.001$) decreased compared to *cis*-9 18:1-CoA at all concentrations.

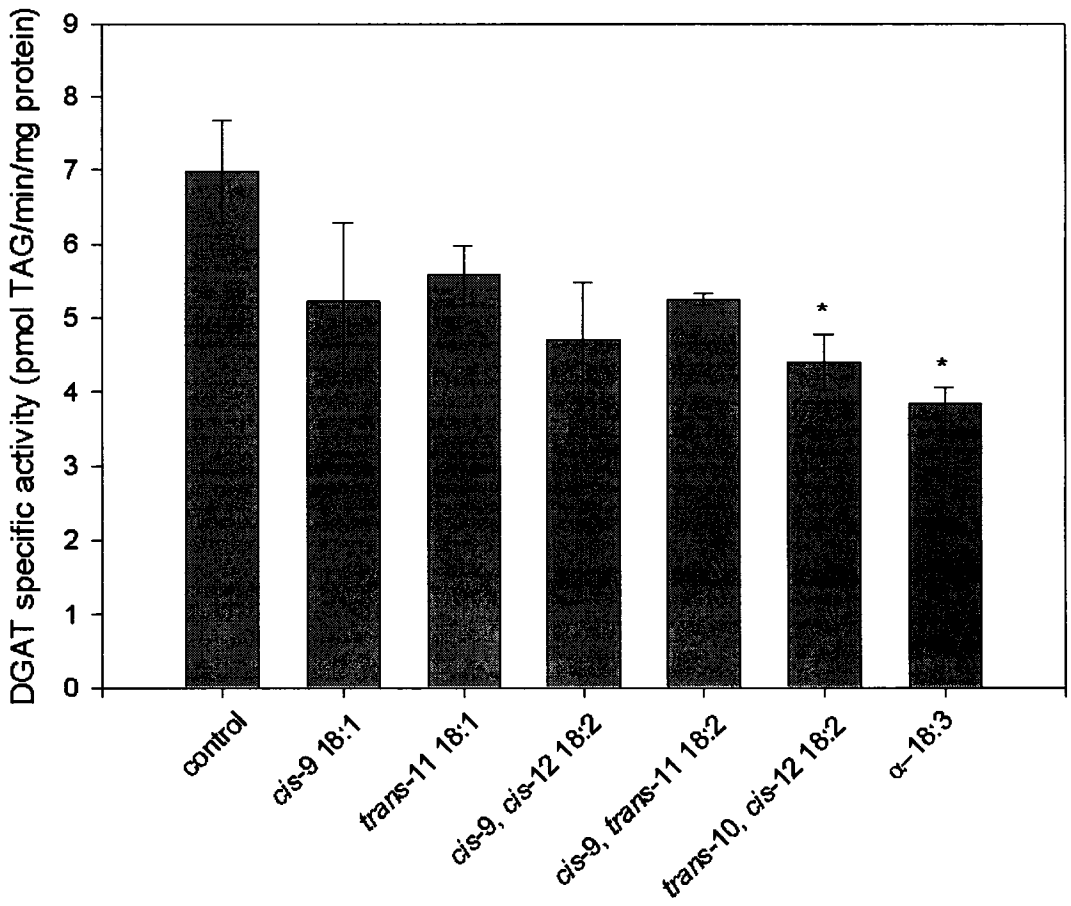

Figure 6.The effect of various exogenous fatty acid treatments on the activity of diacylglycerol acyltransferase in cultured mammary gland epithelial cells

Values represent means ± SEM (n=6)

*Values are significantly different from control ($P < 0.05$)

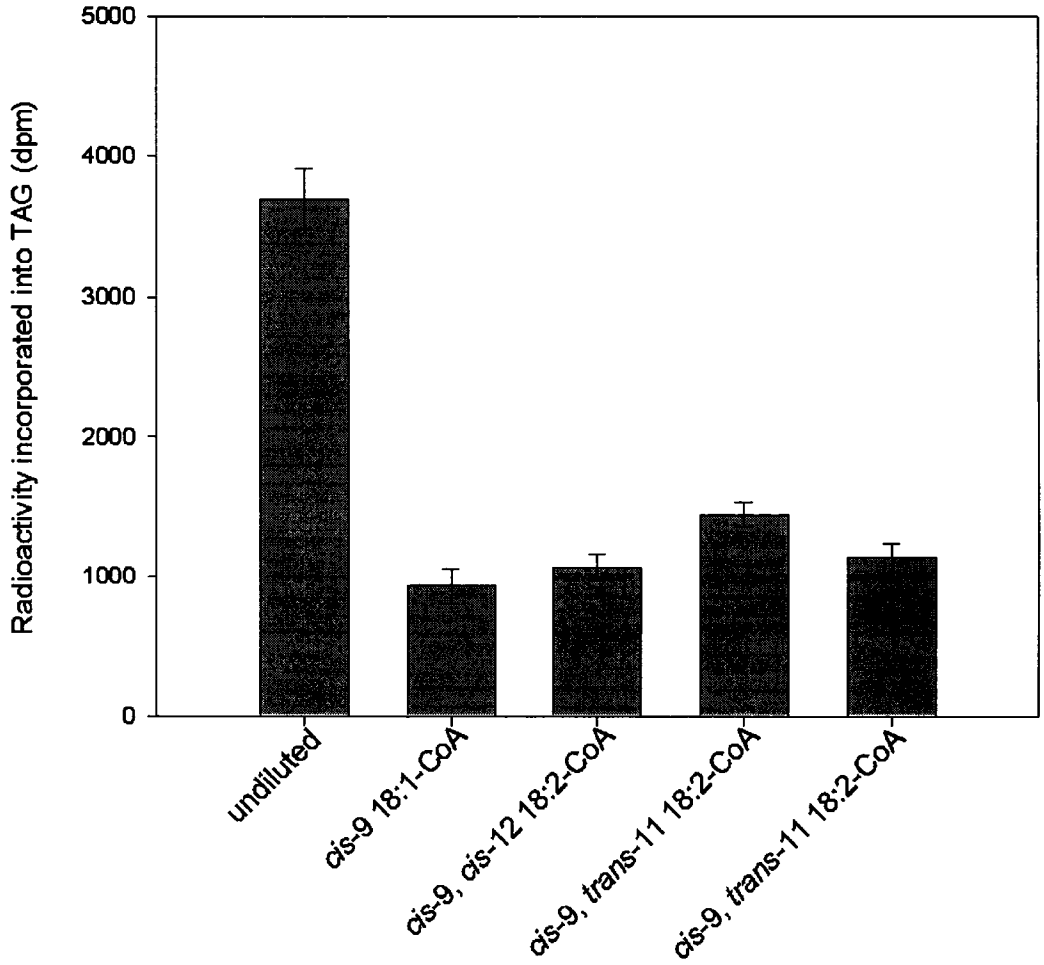

Figure 7. The effect of competing acyl donors on the incorporation of radiolabelled oleate into triacyglycerol in microsomal fractions of mammary gland tissue from Holstein cow

Values represent means ± SEM (n=3)

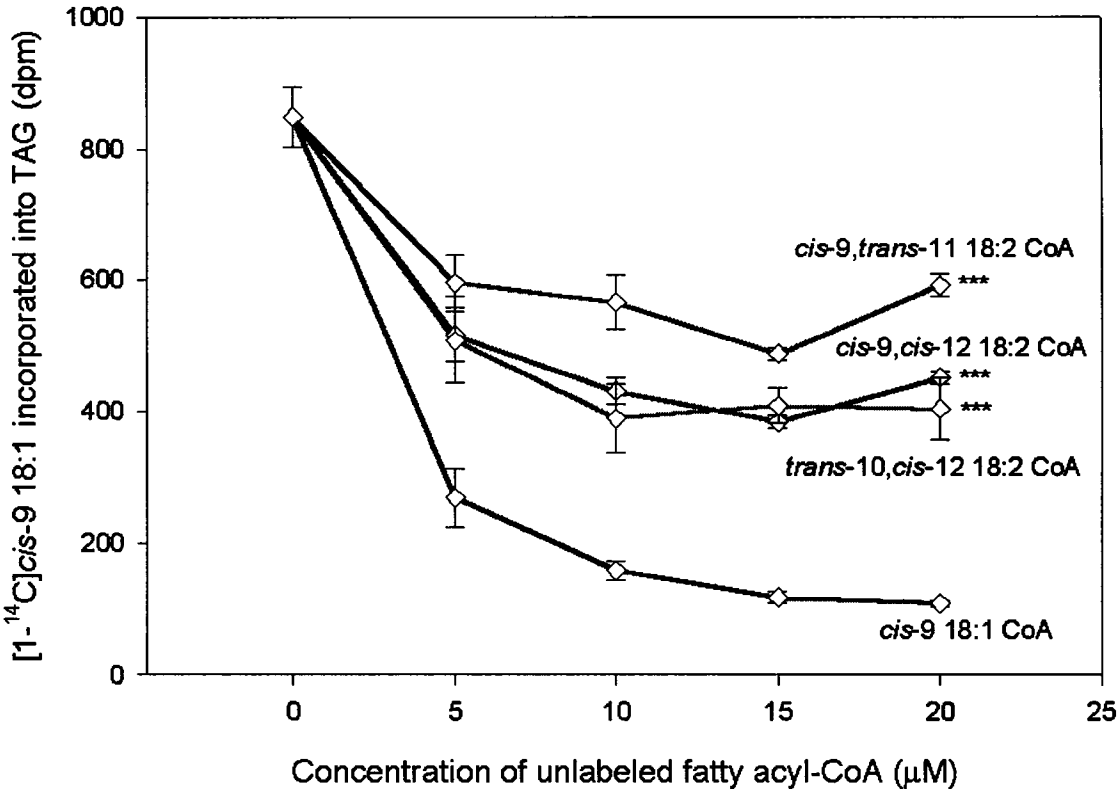

Figure 8.The effect of competing acyl donors of various concentrations on the incorporation of radiolabeled oleate into triacylglycerol in microsomal fractions of mammary gland tissue from Holstein cow

Values represent means ± SEM (n=3)

*, **, *** represent significant differences between *cis*-9 18:1-CoA and other acyl donors at all concentrations *($P < 0.05$), **($P < 0.01$) ***($P < 0.001$)

3.3.5. The effect of fatty acid salts on the activity of microsomal mammary gland diacylglycerol acyltransferase

Microsomal fractions from a lactating cow were incubated with 15 μM [1-^{14}C] *cis*-9 18:1-CoA (56 Ci/mol) and 15 μM FA salt for 10 min at 37°C and the incorporation of [1-^{14}C]*cis*-9 18:1 into TAG was monitored. None of the salts had a significant effect on DGAT activity (Figure 9).

3.3.6. Effect of diet on the expression of lipogenic enzymes in mammary gland biopsies

The ΔCt values of *DGAT1*, *DGAT2* and *LPAAT* were determined using RT-PCR. There were no differences in these parameters between the two treatment groups, although the safflower/monensin significantly decreased percentage of milk fat at 8 weeks ($P < 0.05$) and increased the occurrence of *cis*-9, *trans*-11 18:2 ($P < 0.001$) (Table 3.).

Spearman's Rho was calculated to determine correlations between expression of these enzymes and milk characteristics such as percentage of *cis*-9, *trans*-11 18:2, *trans*-10, *cis*-12 18:2 and milk fat yield (Table 4.). There were no correlations between any of the genes and any milk characteristics. There was a small positive association ($P < 0.05$) between *LPAAT* and *DGAT2*

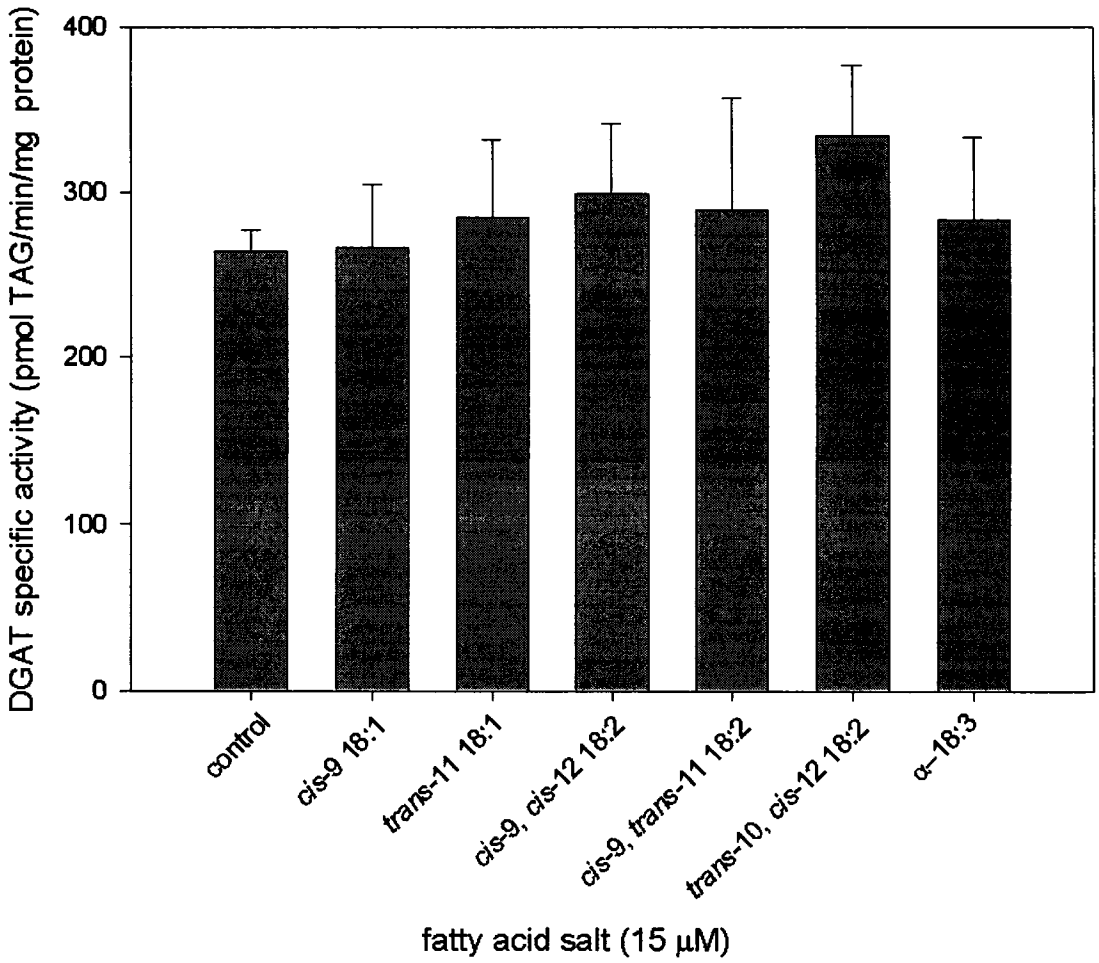

Figure 9.The effect of various fatty acid salts on the activity of diacyglycerol acyltransferase activity in microsomal fractions of mammary gland tissue from Holstein cow

Values represent means ± SEM (n=3). The final concentration of FA salt in the reaction mixture was 15 μM.

Table 3. Gene expression of lipogenic enzymes and milk related characteristics in Holstein cattle fed control and CLA enhancing diets

	Diet	
	control	**safflower/monensin**
DGAT1 [a]	3.8 ± 0.5	4.0 ± 0.4
DGAT2 [a]	8.1 ± 0.5	8.4 ± 0.8
LPAAT [a]	3.3 ± 0.4	4.4 ± 0.6
Milk yield [b]	31.3 ± 1.8	28.3 ± 2.6
Fat % [c]	3.7 ± 0.3	2.6 ± 0.1*
***% cis*-9, *trans*-11 18:2** [c]	0.7 ± 0.1	5.8 ± 0.5***
***% trans*-10, *cis*-12 18:2** [c]	0.0 ± 0.0	0.1 ± 0.0***

Values represent means ± SEM (n=6).

[a] average ΔCt value (i.e., $Ct_{target\ gene} - Ct_{GAPDH}$)

[b] average milk yield (kg/day) for the final two weeks.

[c] average Fat % at week 8

[d] average mol % at week 8.

*, **, *** Means of safflower/monensin treated group differ significantly from the control within a row

*($P < 0.05$), **($P < 0.01$) ***($P < 0.001$)

Table 4. Spearman Rho coefficients for gene expression and milk related characteristics in Holstein cattle fed control and conjugated linoleic acid enhancing diets

	DGAT2[a]	*LPAAT*[a]	Milk yield[b]	Fat %[c]	*cis*-9,trans-11 18:2[d]	*trans*-10,cis-12 18:2[d]
DGAT1[a]	0.37	0.37	0.06	0.13	0.44	0.33
DGAT2[a]		0.59*	0.56	0.23	0.12	-0.02
LPAAT[a]			0.04	0.13	0.27	0.36
Milk yield[b]				0.43	-0.34	-0.38
Fat %[c]					-0.69*	-0.57
cis-9,trans-11 18:2[d]						0.71**

[a] ΔCt value (i.e., $Ct_{target\ gene} - Ct_{GAPDH}$)

[b] average milk yield (kg/day) for the final two weeks.

[c] measured at week 8

[d] mol % measured at week 8.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.4. Discussion

In an early study, Hansen and Knudsen (1987) found that the addition of long chain FAs, namely 18:0 and *cis*-9, *cis*-12 18:2, inhibited the incorporation of FA into the TAG of mammary gland epithelial cells. Peterson *et al.* (2004) reported that treatment of mammary gland epithelial cells with *trans*-10, *cis*-12 18:2 resulted in reduced lipid synthesis in MAC-T cells. Our results, however, do not show that this isomer had a significant effect on lipid accumulation. It should be noted that Peterson *et al.* (2004) measured lipid synthesis by monitoring the incorporation of ^{14}C acetate into total lipid, and that inhibition was noted at increasing concentrations of *trans*-10, *cis*-12 18:2. The effects of other isomers were not investigated. It is possible that these other isomers at increasing concentrations might also result in inhibition of lipid accumulation and that the effect may not be specific to *trans*-10, *cis*-12 18:2. That treatment of MAC-T cells with *trans*-10, *cis*-12 18:2 resulted in increased levels of SFA and 18:0 and decreased levels of *cis*-9, 18:2 compared to the control treatment (Table 2) is consistent with evidence that this isomer results in decreased expression and activity of SCD (Baumgard *et al.*, 2002). The decreased production of oleate and ultimately *cis*-8 18:1-CoA, a preferred substrate of DGAT is likely to be a factor of MFD.

Degrace *et al.* (2004) report that feeding *trans*-10, *cis*-12 18:2 to mice increased the level of hepatic *DGAT1* mRNA. In the current study, however, treatment of the MAC-T cells with various isomers did not have any significant effect on the expression of *DGAT1.* Treatment with α-18:3 increased the expression of *DGAT2* 6-fold compared to basal levels. Peterson *et al.* (2003) reported that a MFD inducing diet (i.e., high concentrate/low forage) resulted in decreased expression of LPAAT, while our data

suggest that *trans*-10, *cis*-12 18:2 increased the expression of LPAAT. It should be noted that different methodologies and systems were used, which could account for the discrepancy.

Thomas-Yeung *et al.* (2000) found that hamsters supplemented with CLA isomers had decreased levels of intestinal acyl-CoA: cholesterol acyltransferase (ACAT EC 2.3.1.26) activity, suggesting that CLA may act to down regulate ACAT activity. DGAT shares 20% sequence homology with ACAT, and it is possible that isomers of CLA may interact with a conserved region of ACAT resulting in decreased activity. It is therefore possible that CLA may interact with the same region in DGAT leading to a decrease DGAT in activity. Petersen *et al.* (2003) found that treating murine primary preadipocyte cultures (3T3-L1) with arachidonic acid (all-*cis*-5,8,11,14 20:4) resulted in repressed DGAT activity which was unrelated to decreased FA synthesis. In rats fed mixtures of CLA, the activity of hepatic DGAT was decreased compared to a control group given the same amount of FA (Giudetti *et al.*, 2005). Previous studies had found no difference in DGAT activity in rats fed similar amounts of *cis*-9 18:1 versus 18:0 (Giudetti *et al.*, 2003). In our study, incubation of MAC-T cells with *trans*-10, *cis*-12 18:2 and α-18:3 resulted in significant decreases ($P < 0.05$) in DGAT activity. The addition of *cis*-9 18:1-CoA decreased the incorporation of radiolabeled [1-^{14}C] *cis*-9 18:1-CoA to a greater extent than the other acyl-CoA substrates used. Although *cis*-9 18:1 may be preferentially incorporated into DGAT, it is possible that certain acyl-CoA species may be inhibitory. Berge *et al.* (1999) incubated rat liver microsomes in the presence various acyl-CoA derivatives in the presence of 20 μM *cis*-9 18:1-CoA. They found that in the presence of the other acyl-CoAs, including *cis*-9, *cis*-12 18:2-CoA, TAG synthesis was decreased. Rustan *et al.* (1988) also found that eicosapentaenoyl-CoA (all *cis*-

5,8,11,14,17 20:5-CoA) caused a substantial decrease in hepatic DGAT activity. Other FA-CoAs, however, were not shown to have an inhibitory effect.

Park *et al.* (2000) suggested that a *cis*-12 bond was a structural requirement of the inhibition of SCD activity. *Trans*-10, *cis*-12 18:2, as well as other analogs such as *cis*-12 18:1 decreased SCD activity murine hepatic microsomes to a greater extent than FA not containing a *cis*-12 bond. In our study, treatment of cells with FA containing a *cis*-12 bond (i.e., α-18:3 and *trans*-10, *cis*-12 18:2) resulted in lower DGAT activities. Emken *et al.* (1980) studied the distribution of *cis*-12 18:1 and *trans*-12 18:1 in human plasma lipids and found that these isomers were less likely to be incorporated into neutral lipids (i.e., TAG) than other isomers such as *cis*-9 18:1. It is possible that the presence of the *cis*-12 bond might affect the spatial orientation of the molecule, decreasing the ability of the corresponding acyl-CoA to fit into the active site of DGAT.

Although DGAT activity was decreased in the MAC-T cells as a result of incubation with *trans*-10, *cis*-12 18:2 and other isomers, it is unclear at which level (i.e., transcriptional, translational) DGAT may have been affected. CLA may not directly affect the expression and/or activity of DGAT, but rather the apparent CLA mediated decrease in DGAT activity may be the result the action of CLA on other lipogenic enzymes which generate substrates for DGAT. Cattle treated with *trans*-10, *cis*-12 18:2 have been shown to have decreased expression of SCD (Baumgard *et al.*, 2002). In SCD-/- mice, decreased SCD activity resulted in the accumulation of SFA which had an inhibitory effect on ACC (Ntambi and Miyazaki, 2002) Decreased ACC activity has been shown to result in decreased production of malonyl-CoA and ultimately a decrease in FA synthesis which would mean decreased substrates available for DGAT. In cattle fed a MFD inducing diet (i.e., high concentrate/low forage), Peterson *et al.* (2003) noted a

decreased mRNA abundance of FA binding protein (FABP). Brown *et al.* (2003) also found that treatment of human primary preadipocytes with *trans*-10, *cis*-12 18:2 resulted in decreased expression of acyl-CoA binding protein (ACBP). A decrease in these proteins might affect the availability of substrates to DGAT.

Fatty acids are known to regulate gene transcription either directly or indirectly. Fatty acids can directly affect transcription by binding to transcription factors. For example, it is well known that the peroxisome proliferator-activated receptor (PPARγ) is regulated by FA. PPARγ activation results in the induction of many genes involved in lipid oxidation and storage. Brown *et al.* (2003) found that in human preadipocytes, *trans*-10, *cis*-12 18:2 resulted in decreased expression of PPARγ. It is possible that CLA may influence the splicing of DGAT mRNA. Grisart *et al.* (2004) showed in baculovirus expressing recombinant bovine *DGAT1* that the K232A mutation increases the occurrence of an alternative splicing variant of *DGAT1* that produces a DGAT1 isoform which is devoid of DGAT activity.

Incubation with *trans*-10, *cis*-12 18:2 may have affected DGAT activity by altering the physical properties of the membrane environment via alterations in phospholipids (PL) composition. Mathur *et al.* (1983) found that altering PL in incubations of rat liver microsomes altered ACAT activity. Ma *et al.* (2002) also showed that *trans*-10, *cis*-12 18:2 is rapidly incorporated into membrane PL. Changes in SCD activity, induced by *trans*-10, *cis*-12 18:2 have also been shown to result in altered membrane composition.

Cattle fed a MFD inducing diet did not exhibit any differences in the expression of *DGAT1*, *DGAT2*, and *LPAAT* compared to those animals fed a control diet. The sample

size of each group, however, was particularly small (n=6). The expression of lipogenic genes (e.g., SCD) has been known to vary substantially between individual cattle. It would be interesting to run a pair-wise experiment comparing the expression of the genes in biopsies taken from the same cow before and after being subjected to the MFD inducing diet.

In conclusion, CLA does not appear to mediate MFD depression by decreasing the expression of either isoform of DGAT. Our results suggest that, upon exposure to *trans*-10, *cis*-12 18:2, DGAT activity in bovine mammary cells is decreased. The mechanism by which the activity is decreased is, however, unclear. This isomer may inhibit DGAT activity either directly, or indirectly as a result of altered substrate pools which in turn are a result of the action of *trans*-10, *cis*-12 18:2 on enzymes which are located upstream of DGAT in lipid biosynthetic pathways. If CLA induced MFD is in fact mediated by DGAT, is likely that it is only one of many lipogenic genes that are affected.

4. Diacylglycerol acyltransferase (DGAT) activity in relation to muscle fat content and *DGAT1* genotype in two different breeds of *Bos taurus*

4.1. Introduction

Marbling is an evaluation of the amount of intramuscular (i.m.) fat, or the visible adipocytes between the fibers of most bovine skeletal muscles. Marbling is incorporated into North American grading schemes, illustrating the value of this trait to both producers and consumers. The degree of marbling varies among and within breeds of cattle, making it possible to breed cattle for high marbling. Molecular markers (e.g., a polymorphism in the leader sequence of thyroglobulin) have been developed which can assist in determining which cattle may be predisposed for the marbling trait (Barendse *et al.*, 2004). A greater understanding of the mechanisms behind these differences could lead to the development of additional molecular and biochemical markers for use in selection strategies to enhance marbling. Fat deposition is largely a result of hypertrophy of adipocytes, (i.e., fat cells), which is the result of increased storage lipid accumulation. Thus, enzymes involved in fat synthesis may be ideal candidates as biochemical markers. For example, Chakrabarty and Romans (1972) investigated lipogenic enzymes in bovine tissues and found that the specific activity of acetyl-CoA carboxylase (ACC, EC 6.4.1.4), which catalyzes the first step in fatty acid synthesis, was directly proportional to the amount of marbling.

Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the terminal step in triacylglycerol (TAG) synthesis via the acyl-CoA–dependent acylation of *sn*-1,2-diacylglycerol (Coleman and Lee, 2004). The level of DGAT activity in adipose tissues

may have a substantial effect on the quantity of triacylglycerol (TAG) deposited (Mayorek *et al.*, 1989). Middleton *et al.* (1998) found a negative correlation between lipid content of the *pars costalis diaphragmatis* muscle and DGAT specific activity within the i.m. adipose tissue in Japanese Black hybrid cattle, which suggested DGAT was being down-regulated as this depot became larger. Two genes, *DGAT1* and *DGAT2*, which do not share sequence homology, are known to encode DGAT (Cases *et al.*, 2001). Recently, a non-conservative substitution involving a lysine residue versus an alanine residue at position 232 in DGAT1 has been linked to decreased i.m. fat content of *semitendinosus* (ST) muscle in cattle (Thaller *et al.*, 2003). Furthermore, *in vitro* assays in a baculovirus expression system indicated that DGAT1 was more catalytically active with the lysine residue at position 232 when compared with the protein with an alanine substitution at the same position (Grisart *et al.*, 2004).

Previous investigations on the relationship between the activity of lipid metabolising enzymes and the amount of muscle lipid have been conducted using tissues from Japanese Black hybrid cattle which are known to exhibit a high propensity for fat deposition (Middleton *et al.*, 1998; Kazala *et al.*, 2003). The current study extends the investigation of the relationship between TAG synthesis and muscle lipid content using cattle that produce considerably less lipids in muscle. For this purpose, we used the *longissimus dorsi* (LD) and ST muscles from Holstein and Charolais bulls. Both DGAT activity and *DGAT1* genotype were taken into consideration in this investigation.

4.2. Materials and Methods

4.2.1. Chemicals

[1-^{14}C]*cis*-9 18:1 (60 Ci/mol) was obtained from Amersham Biosciences Europe GmbH (Freiburg, Germany). [1-^{14}C]*cis*-9 18:1-CoA was synthesized from radiolabeled fatty acids as described by Taylor *et al.* (1990). Pre-coated G-25 thin layer chromatography (TLC) plates were from Machery-Nagel GmbH & Co. (Düren, Germany), adenosine triphosphate (ATP) was from Boehringer-Mannheim (Germany). ethylene diamine tetraacetate (EDTA) was from Fluka Chemie AG (Buchs, Switzerland).N-[2-hydroxyethyl]peperazine-N'-[2-ethanesulfonic acid] (HEPES), acyl-CoA synthetase, Coenzyme A, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), triolein, and *sn*-1,2-diolein were from Sigma-Aldrich (Taufkirchen, Germany). Dithiothreitol (DTT), Rotiszint scintillation cocktail, solvents, and all other chemicals were from Carl Roth GmbH & Co. (Karlsruhe, Germany).

4.2.2. Animals

Charolais (n=18) and German Holstein (n=18) bulls were raised using a tethering system with individual feeding. All animal experimentation was done in compliance with the animal care guidelines of the state of Mecklenburg-Vorpommern, Germany. Calves were fed with a milk replacer diet up to 4 mo. After weaning, the bulls received a body weight-related diet consisting of concentrates (6 kg dry matter) based on barley, beet pulp and soybean extraction meal (92.8% organic matter, 15% crude protein, and 9% crude fiber) and roughage (2 kg dry matter). Feed intake was adjusted weekly according to eating habits. The weight of uneaten feed was recorded. Protein accretion and energy demand for protein accretion were calculated. All bulls were weighed monthly and

slaughtered at 18 mo in the abattoir of the Research Institute for the Biology of Farm Animals in Dummerstorf, Germany. Muscle tissue from LD and ST muscle was collected within 30 min after slaughter. Samples were immediately frozen in liquid nitrogen, and stored at -70°C.

4.2.3. Determination of muscle lipid content, microsomal preparation and diacylglycerol acyltransferase activity assay

The lipid content of LD and ST whole muscle was obtained via the Soxhlet extraction (Association of Official Agricultural Chemists, 1984). Marbling scores were assigned based on the German standard using photographic standards in a six-point scale with 1 being extremely low marbled and 6 being extremely high marbled. Scoring was performed by only one experienced operator to eliminate subjective influences. Microsomes were prepared from muscle tissue as described in section 3.2.7 above. The microsomal fraction was assayed for DGAT activity as described in section 3.2.7 above

4.2.4. Genotyping at the D*GAT1* K232 mutation

To account for the effects of the *DGAT1* K232A alleles previously shown to exhibit an effect on i.m. fat content (Thaller *et al.*, 2003) and on *in vitro* efficiency of TAG synthesis (Grisart *et al.*, 2004), all bulls were genotyped for the non-conservative *DGAT1* K232A mutation according to Winter *et al.* (2002).

4.2.5. Statistical analysis

The CORR and TTEST procedures for SAS Windows Version 8 (SAS Institute Inc., 1999) were used in statistical analyses. A general linear model was applied (PROC GLM, SAS Institute Inc., 1999) for the analysis of the association between the *DGAT1* genotype of the bulls and DGAT activity, which included fixed effects of breed, sire of the bulls and *DGAT1* genotype.

4.3. Results

4.3.1. Muscle DGAT activity, muscle lipid content and marbling score

Data for Charolais and Holstein are presented in Table 5. Holstein bulls had a higher ($P < 0.05$) percentage of lipid and higher ($P < 0.10$) DGAT activity in the LD muscle than Charolais. Although Holstein had a slightly greater ($P <0.10$) percent lipid content in ST muscle than Charolais, there were no breed differences in DGAT activity for this muscle. Marbling scores were higher ($P < 0.001$) for Holstein than Charolais. In both Charolais and Holstein, DGAT activity was higher ($P < 0.05$ and $P < 0.001$, respectively) in LD muscle than in the SD muscle. The percentage of lipid in LD muscle was also higher than for ST muscle in Charolais and Holstein bulls ($P < 0.05$ and $P < 0.001$, respectively).

4.3.2. Associations involving muscle DGAT activity and fat-related characteristics of muscle

Pearson's correlation coefficients were calculated to examine possible relationships involving DGAT activity and lipid content of the investigated muscles (Table

6). In Charolais, highly significant positive associations were observed for the lipid content of LD and ST muscle in relation to marbling score ($P < 0.001$). The DGAT activity of both muscle types showed no significant associations with muscle fat characteristics in Charolais. In Holstein, the lipid content of LD muscle also showed a significant positive association ($P < 0.01$) with marbling score but the lipid content of ST muscle in this breed did not (Table 6). In Holstein, however, there appeared to be a positive relationship between DGAT activity and the lipid content of ST muscle.

4.3.3. Muscle DGAT activity in relation to *DGAT1* genotype

Muscle DGAT activity versus *DGAT1* genotype is shown for all bulls, regardless of breed, in Table 7. Marked differences were observed for enzyme activity between the two muscles. For LD muscle, the DGAT activity of the K/K *DGAT1* genotype was more than five-fold greater than for either the K/A or A/A genotype. In contrast, no differences in DGAT activity among all three genotypes were observed in ST muscle.

Table 5. Muscle DGAT activities and fat related characteristics in Charolais and Holstein bulls

	Charolais (n=18)	Holstein (n=17)
DGAT activity[a] in LD muscle	$44.8^{g} \pm 32.5$	$74.9^{i} \pm 64.5$
DGAT activity[a] in ST muscle	$21.0^{h} \pm 19.7$	$16.5^{j} \pm 10.4$
Marbling score in LD muscle	$2.1^{c} \pm 0.6$	$2.8^{d} \pm 0.6$
Lipid content in LD muscle (%)	$2.6^{e,k} \pm 1.8$	$4.1^{f,m} \pm 1.7$
Lipid content in ST muscle (%)	$1.4^{l} \pm 0.9$	$1.9^{n} \pm 0.9$

Values represent means ± SD
[a] pmol TAG/min/mg protein
[b] as determined by German grading standards
Means with these pairs of superscripts within the same row differ significantly
$^{cd}(P< 0.001)$, $^{ef}(P < 0.05)$
Means with these pairs of superscripts within the same column differ significantly
$^{gh}(P <0.05)$,$^{ij}(P <0.001)$,$^{kl}(P<0.05)$,$^{mn}(P <0.001)$

Table 6. Pearson correlation coefficients for muscle specific DGAT activity and fat related characteristics in Charolais and Holstein bulls

	Lipid content		DGAT specific activity[b]	
	ST muscle	LD muscle	LD muscle	ST muscle
Marbling score[a]				
Charolais	0.78***	0.72***	0.32	-0.10
Holstein	0.25	0.65**	0.07	0.11
Lipid content in ST muscle				
Charolais		0.63**	0.22	-0.12
Holstein		0.36	0.20	0.41[†]
Lipid content in LD muscle				
Charolais			-0.18	-0.04
Holstein			0.03	0.14
DGAT specific activity[b] in LD muscle				
Charolais				-0.38
Holstein				0.29

[a] as determined by German grading standards
[b] pmol TAG/min/mg protein
[†] $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 7. Muscle DGAT activity in individuals with different genotypes for the non-conservative *DGAT1* K232A mutation

	K/K (n = 4)	K/A (n = 13)	A/A (n = 19)	*P* value
DGAT activity in LD muscle (pmol TAG/min/mg)	184.9 ± 36.8	35.3 ± 15.4	38.5 ± 14.0	0.004
DGAT activity in ST muscle (pmol TAG/min/mg)	21.1 ± 12.6	14.7 ± 5.8	18.6 ± 5.2	0.8

Values represent means ± SEM

4.4. Discussion

The two breeds of cattle used in the current study are known to differ metabolically in terms of utilization of energy stores (Bellmann *et al.*, 2004). Holstein cattle generally accumulate greater amounts of fat compared to Charolais. Subsequently, we hypothesized that these two cattle breeds would be particularly useful for comparative studies of lipid deposition. Measurements of the lipid content of LD and ST muscle, and marbling score were consistent with the higher fat producing potential of Holstein cattle. Furthermore, the increased fat accumulation observed in the muscle tissues from Holstein was accompanied enhanced in DGAT activity.

There was also a positive relationship ($P < 0.10$) between DGAT activity and the lipid content of ST muscle from Holstein. In contrast, Middleton *et al.* (1998) reported a negative relationship between DGAT activity of adipose tissue from *pars costalis diaphragmatis* muscle and the lipid content of this muscle, and postulated that DGAT activity is down-regulated as muscle lipid depots increase. It should be noted, however, that different muscles and different breeds were used in the two studies. In the study of Middleton *et al.* (1998), the i.m. adipose tissue was dissected from the muscle and used to prepare microsomes for the assay of DGAT activity. This was not feasible in our study because the amount of i.m. fat was considerably less than for the Japanese Black hybrid cattle used by Middleton *et al.* (1998). For example, the average lipid content of the *pars costalis diaphragmatis* muscle from the herd of Japanese Black hybrid cattle was over four-fold greater than for the lipid content of LD muscle from the Holstein bulls used in the current study. Since the muscle of Japanese Black hybrid cattle accumulates substantially greater levels of i.m. fat compared to muscles from Charolais and Holstein,

DGAT might be down-regulated at an earlier stage in the development of the Japanese Black hybrid cattle.

The assay used in this study to measure DGAT activity did not distinguish between DGAT1 and DGAT2 isoforms. Cases *et al.* (2001) have assessed DGAT activity in mice deficient in the *Dgat1* gene. The residual DGAT activity was substantially reduced compared to control mice, demonstrating that the contribution to DGAT2 activity was small in comparison to DGAT1. Furthermore, female mice *Dgat1* knockouts failed to lactate. DGAT2, however, has not been characterized in bovine tissues. Contributions of the two DGAT isoforms to TAG accumulation in different tissues at various stages of development needs to be assessed in the bovine system. The observation of an increased DGAT activity in LD muscle in individuals with the *DGAT1* K/K genotype is in agreement with the observation of the increased *in vitro* efficiency for TAG synthesis catalyzed by DGAT1 carrying a lysine residue at position 232 compared to the allele product which has an alanine residue at this position. The two alleles of *DGAT1*, which encode gene products differing in catalytic efficiency, were first reported in studies of milk fat production in dairy cattle (Grisart *et al*, 2002; Grisart *et al.*, 2004; Winter *et al.*, 2002). It should be noted, however, that there were no significant differences in DGAT activity between heterozygous K/A and homozygous A/A individuals in the current study. A similar observation was described for the effects of *dgat1* genotypes on lipid content of muscle (Thaller *et al.*, 2003) and was interpreted to be a potentially recessive gene effect. Due to the low number of homozygous K/K bulls in our study, further interpretation of the effects of the *DGAT1* genotypes has to be tentative. This also includes the marked differences observed between DGAT activity in LD and ST muscle in relation to *DGAT1* genotype. The relative contributions of DGAT1 and DGAT2

activities and/or differences in expression and regulation of *DGAT1* might account for the differences in enzyme activity observed for the K/K genotype.

In conclusion, measurement of the DGAT activity of LD muscle might represent a useful approach to assess the potential of cattle displaying the K/K genotype to accumulate i.m. fat. It is encouraging that enhanced DGAT activity of the K/K genotype is associated with the LD muscle because this muscle represents the grading site used to determine marbling score. In order to fully assess the use of DGAT activity as a biochemical predictor of i.m. fat deposition, future work should be aimed at assessing the relationship between the DGAT activity of LD muscle biopsy samples from younger cattle and marbling score of carcasses following slaughter.

5. Conclusions and Future Directions

The aim of these studies was to gain further knowledge of bovine diacylglycerol acyltransferase (DGAT) so that this knowledge may be applied to the enhancement of ruminant products such as dairy products with enhance conjugated linoleic acid (CLA) content and beef with enhanced marbling.

The effects of various isomers of CLA and precursors on the activity and expression of DGAT in bovine mammary cells was examined to determine if DGAT has a role in milk fat depression (MFD) induced by CLA. Our studies suggest that treatment of cultured mammary gland cells with *trans*-10, *cis*-12 18:2 has an effect on DGAT activity, although the exact mechanisms behind this are unclear. Decreased DGAT activity in response to interactions with CLA isomers such as *trans*-10, *cis*-12 18:2 might explain in part MFD, but it is likely that a number of lipogenic factors are affected by CLA and the additive effects result in MFD. The interactions of CLA with other lipogenic enzymes may affect substrate availability for DGAT.

DGAT activity was also examined in the muscle tissue of two metabolically different breeds of bulls and relationships between activity, intramuscular fat (i.m.) fat, and occurrence of the K232A mutation were examined. No significant relationship between DGAT activity and i.m. fat content was found, although occurrence of the K232A mutation was reflected in the DGAT activity.

New methods are continually being applied to increase the resolution of CLA isomers in GC and HPLC columns. Chromatograph peaks once thought to consist solely

of *trans*-10, *cis*-12 18:2 also contain quantities of *trans*-7, *cis*-9 18:2. Milk samples from cattle fed MFD inducing diets have increased levels of this isomer. It is therefore possible that *trans*-7, *cis*-9 18:2 may also be responsible for MFD and the effects of *trans*-7, *cis*-9 18:2 on the expression and activity of lipogenic enzymes including DGAT should be investigated. Currently, commercial preparations of *trans*-7, *cis*-9 18:2 are unavailable, although methods for the synthesis of this isomer have been described (Delmonte *et al.*, 2003). Metabolites of CLA containing a conjugated diene structure (e.g., *cis*-11, *trans*-13 20:2) have been identified which demonstrate a number of biological activities including the reduction of body fat (Park *et al.*, 2005). These metabolites may have an effect on mammary gland DGAT expression or activity that might be revealed in future studies involving highly purified isomers. Pure isomers of CLA intermediates, in particular *trans*-10 18:1 should also be investigated.

Yamasaki *et al.* (2004) found that there were slight differences in the immune function of rats given a diet supplemented with either FA-CLA or TAG-CLA. The bioavailability of CLA may also have an effect on mammary gland DGAT and studies involving structured TAG and PL that contain a CLA moiety should be performed.

de Roos *et al.* (2005) found that in transgenic mice, treatment with *trans*-10, *cis*-12 18:2 resulted in the upregulation of proteins involved in β-oxidation and ketogenesis pathways. A proteomic approach (i.e., 2 dimensional polyacrylamide gel electrophoresis, liquid chromatography-mass spectroscopy) should also be applied to tissue to determine the effects of CLA isomers on the regulation of lipogenic proteins. The effects of CLA on *Dgat1*$^{-/-}$ and/or *Dgat2*$^{-/-}$ mice could also be studied.

It would be advantageous to further investigate the role of DGAT2 in *Bos taurus* as only a limited amount of research has been done in this area (Winter *et al.*, 2003). DGAT2 may have a substantial role in i.m. fat deposition, and prove to be a viable a marker of marbling. It might also be of interest to examine the relationship between DGAT activity and/or expression and i.m. fat content over the developmental period of beef cattle.

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