PROMOTION OF LIPID ACCUMULATION IN ADIPOSE TISSUE BY GPR43 AND OLEIC ACID

A Dissertation

by

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ANIMAL SCIENCE

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ABSTRACT

Two experiments were conducted to demonstrate functional G-coupled protein receptor 43 (GPR43) and GPR120 activity in bovine intramuscular (i.m.) and subcutaneous (s.c.) adipose tissues. We hypothesized that media volatile fatty acids and long- chain fatty acids (LCFA) would affect cAMP concentration, glycerol concentration, and free fatty acid (FFA) concentration differently in i.m. and s.c. adipose tissues taken from steers at 20, 22, 24 mo of age. Fresh s.c. and i.m. adipose tissue from the 5th to 8th longissimus thoracic rib muscle section of Angus crossbred steers was transferred immediately to 6-well culture plates containing 3 mL of KHB/Hepes/5 mM glucose. Samples were preincubated with 0.5 mM theophylline plus 10 μM forskolin for 30 min, after which increasing concentrations of acetate or propionate (0, 1, 5, and 10 mM) in the absence or the presence of 100 μM oleic acid (18:1 n-9) or 100 μM palmitic acid (16:0) were added to the incubation media. In s.c. adipose tissue, cAMP production decreased in the presence of LCFA ($P < 0.0001$), suggesting that LCFA may induce lipid filling in s.c. adipose tissue, which is consistent with GPR120 receptor activity. At 22 mo of age, the addition of VFAs in the presence of LCFA effectively decreased cAMP production ($P = 0.03$), indicating increased lipid accumulation at 22 mo of age ($P = 0.03$). Free fatty acid concentration in the absence of LCFA was greater in i.m. adipose tissue at 22 mo of age ($P < 0.0001$). The relative expression of *GPR120* was greater in s.c adipose tissue than i.m. adipose tissue ($P = 0.05$). These results provide evidence for functional GPR43 receptors in i.m. adipose tissue and GPR120 receptors in s.c. adipose tissue, both of which suppress lipolysis.

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NOMENCLATURE

α-KG	αalpha-ketoglutarate
AC	adenylate cyclase
ACC	acetyl-CoA carboxylase
AFT	adjusted fat thickness
$ApoB_{48}$	apolipoprotein B-48
AR	adrenergic receptors
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
cAMP	cyclic AMP
CLA	conjugated linoleic acid
CPT_1	carnitine palmitoyl transferase I
DAG	diacylglycerol
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAD	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide
FAS	fatty acid synthase
FFA	free fatty acid
FFAR	free fatty acid receptor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCW	hot carcass weight
HDL	high-density lipoprotein
HSL	hormone sensitive lipase

i.m.	intramuscular
IP3	inositol triphosphate
KHB	krebs-henseleit buffer
KPH	kidney, pelvic, and heart fat
LCFA	long chain fatty acid
LDL	low-density lipoprotein
LPL	lipoprotein lipase
MAG	monoacylglycerol
MGL	monoacylglycerol lipase
mRNA	messenger ribonucleic acid
MUFA	medium chain fatty acids
NAD+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
PEP	phosphoenolpyruvate
PIP2	phosphatidylinositol 4,5-bisphosphonate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
REA	ribeye area
RhoGEF	Rho guanine nucleotide exchange factor
SREBP-1c	sterol regulatory element-binding proteins-1c
s.c.	subcutaneous
SCD	stearoyl-CoA desaturase
SCFA	short chain fatty acids
SFA	saturated fatty acid

TAG	triacylglycerol
TCA	tricarboxylic acid cycle
VFA	volatile fatty acid
7-TM	7-transmembrane

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1. INTRODUCTION

G-coupled protein receptors (GPCR) are the largest superfamily of transmembrane glycoproteins composed of intracellular and extracellular domains. The G protein-coupled receptor-43 (GPR43, also known as FFAR2) gene originally was cloned and sequenced as an orphan family A G-protein coupled receptor forming part of a cluster of 4 genes on human chromosome 19, also including GPR40, GPR41, and GPR43 (Sawzdargo et al., 1997). G-protein receptor 43 (GPR43) is found most abundantly in intramuscular (i.m.) and subcutaneous (s.c.) adipose tissues and is activated by volatile fatty acids (VFA) with acetate being the preferred ligand. The GPR41 and GPR43 receptors work via Class A G-protein complexes, inhibiting adenylate cyclase, which is one mechanism by which GPR41 and GPR43 reduce lipolysis (Moran et al., 2016). Fain and Shepherd (1975) first reported that oleic acid (18:1n-9) strongly inhibited norepinephrine-stimulated cAMP accumulation in isolated rat adipocytes. Long-chain fatty acid (LCFA), such as oleic acid are known to be potent ligands of GPR120, while volatile fatty acids (VFA) and short-chain fatty acids (C2–C8) stimulate GPR43 receptor activity (Brown et al., 2003; Tazoe et al., 2008; Moran et al., 2016). Binding of VFA to GPR43 receptors activates the extracellular signal-regulated kinase signaling pathway as a result of binding to the $G_{\alpha i}$ subunit (Moran et al., 2016). It is not clear if GPR43 depresses lipolysis (Daval et al., 2006; Moran et al., 2016) or promotes lipolysis in adipose tissue (Miyamoto et al., 2016). Our laboratory previously demonstrated that oleic acid increased *GPR43* mRNA in cultured bovine intramuscular (i.m.) preadipocytes but had no effect on *GPR43* mRNA in subcutaneous (s.c.) preadipocytes (Chung et al., 2016). As a ligand for GPR43 receptor, oleic acid may compete with acetate and other VFA for the GPR43 receptor or, possibly other GPR receptors. The objectives of this study included establishing the interactions among individual VFA (acetate and propionate) and

between VFA and LCFA palmitic acid (16:0) and oleic acid for the GPR43 receptor in short-term (1h) *ex vivo* incubations of bovine i.m. and s.c. adipose tissues. Additionally, we documented the effects of VFA and LCFA on cyclic AMP (cAMP), glycerol, and free fatty acid (FFA) concentrations, lipogenesis (short-term incubations), as well as establish age and tissue interactions on *GPR* mRNA in bovine i.m. and s.c. adipose tissues. We hypothesized that the binding of oleic acid and palmitic acid to GPR43 would promote triacylglycerol (TAG) accumulation in i.m. adipose tissue by antagonizing the increase in cAMP caused by acetate, thereby depressing lipolysis.

2. G-PROTEIN COUPLED RECEPTORS (GPCR)

2.1 GPCR

GPCR are the largest superfamily of trans-membrane proteins in the human genome with more than 800 unique GPCRs (El-Fakahany et al., 2022). Other names for GPCR include 7-transmembrane (7-TM) receptors and free fatty acid receptors (FFAR). GPCR are cell surface receptors that detect ligands (e.g., lipids, hormones, neurotransmitters) outside the cell and activate cellular responses. Upon activation, G-protein active the production of a variety of second messengers such as cAMP, inositol triphosphate (IP3), and diacylglycerol (DAG). These second messengers assist in the regulation of several body functions ranging from sensation to growth to hormone release.

2.1.1 Structure

Each GPCR is composed of 7 transmembrane helices connected by extracellular and intracellular loops. When a GPCR binds a ligand (a molecule that possesses an affinity for the receptor), the ligand triggers a conformational change in the seven-transmembrane region of the receptor. The receptors are coupled to intracellular guanosine triphosphate (GTP) binding proteins (G-proteins) and once activated, G-protein initiates the production of a variety of second messengers (e.g., cAMP, IP3, and DAG) (Yang et al., 2021).

2.1.2 Function

G-proteins are made of trimers – alpha (α), beta (ß), and gamma (ɣ). In a resting state, guanosine diphosphate (GDP) is attached to this trimer. Once the receptor is activated by a

specific agonist, the G-protein will be attracted to the receptor. This reaction will lead to GTP detaching from the GDP binding on the alpha subunit, resulting in the activation of the G-protein by dissociating the α subunit from the ßɣ dimer (Figure 1).

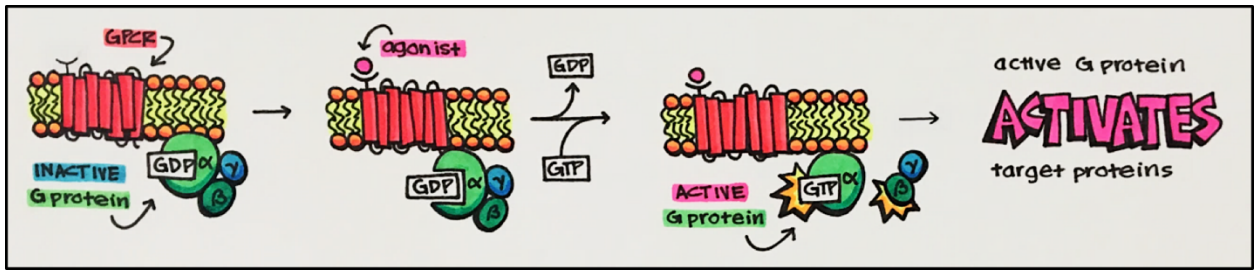


Figure 1. G-protein activation. Reprinted with permission from [El-Fakahany et al].

Ga subunits having several different subtypes, while the ßɣ subunit dimer activates potassium channels and some protein kinases. The Gα subunit include four main subtypes - Gαi, Gαs, Gαq, and G12/13. The Gαi subtype inhibits adenylyl cyclase to decrease intracellular cAMP formation, whereas Gαs stimulates adenylyl cyclase activity. Gαq activates phospholipase C (PLC), leading to the activation of IP3 (inositol triphosphate) and DAG. Activated IP3 and DAG activates protein kinase C (PKC), which increases calcium concentrations in the endoplasmic reticulum. G12/13 activates Rho guanine nucleotide exchange factor (RhoGEF), which goes on to activate Rho kinase (Figure 2). Lastly, beta and gamma subunits activate potassium channels and protein kinases.

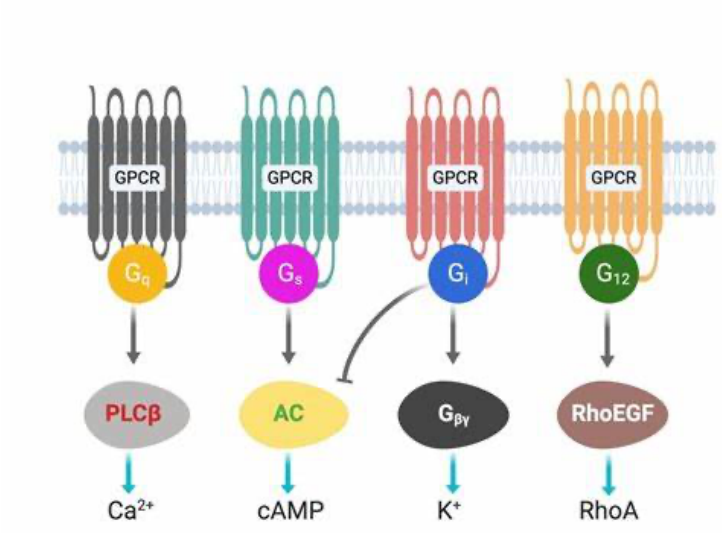


Figure 2. Main subtypes of the Gα subunit. Reprinted with permission from [Zhang].

2.1.3 Ligands

Ligands for the Gαi subtype include volatile fatty acid (VFA), short chain fatty acid (SCFA) (C2-C8), catecholamines, serotonin, histamine, opioid, and other receptors. Catecholamines, histamine, serotonin, and other receptors bind the Gαs subtype. Long-chain fatty acids (LCFA), catecholamines, peptides, VFA, and prostanoid receptors are known ligands for the Gαq subtype. Lastly, ligands for the G12/13 subtype include angiotensin II, serotonin, thromboxane, thrombin, and other receptors (El-Fakahany et al., 2022).

2.1.4 Cyclic AMP Pathway

GPCR is stimulated by an agonist, which activates the G-protein (Gαi or Gαs). Gαi will inhibit adenylate cyclase (AC), leading to a decrease in cAMP levels. Gαs stimulates adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cAMP.

Cyclic AMP binds the regulatory subunit of PKA, allowing the release of the catalytic subunit and thereby activating PKA. The activation of PKA can have a variety of effects including lipolysis, reduction in glycogen synthesis (glucose storage), and increased glycogenolysis (breakdown of glycogen to increase blood glucose levels). Protein kinases act to regulate the function of many different cellular proteins by controlling protein phosphorylation to regulate homeostasis (Figure 3).

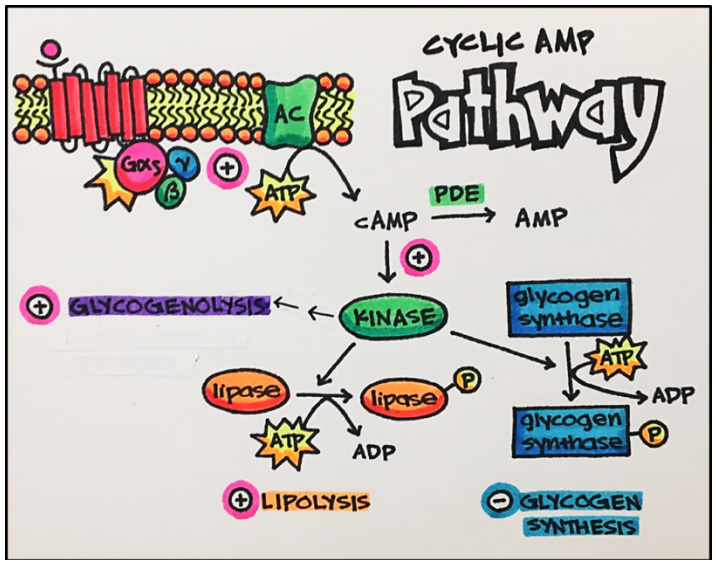


Figure 3. Cyclic AMP pathway. Reprinted with permission from [El-Fakahany et al].

2.2 G-Protein Receptor 43

GPR43 is expressed most abundantly in adipose tissue, but is also expressed in immune cells, skeletal muscle, intestine, spleen, and pancreas. The *GPR43* gene originally was cloned and sequenced as an orphan family A G-protein coupled receptor forming part of a cluster on chromosome 19. GPR43 is a 330-amino acid protein and is activated by VFA with acetate being the preferred ligand. GPR43 binds to the Gαi subunit, inhibiting AC and causing a decrease in

cAMP production. As a result, PKA activity is diminished, leading to the inactivation of hormone-sensitive lipase (HSL), and thereby the inhibition of lipolysis (Figure 4).

In adipocytes, VFA stimulation of the GPR43 receptor attenuates insulin signaling and thereby decreases lipid accumulation in adipose tissue (Kimura et al., 2013). However, this is in contrast to the promotion of adipogenesis via inhibition of lipolysis caused by GPR43 activation in adipocytes (Hong et al., 2005). Therefore, it is not clear if GPR43 activation promotes lipid filling in adipose tissue or antagonizes lipid filling. In the first report of *GPR43* gene expression from our laboratory (Smith et al., 2012), it was observed that *GPR43* gene expression was virtually undetectable in s.c. adipose tissues of grain-fed steers until 16 mo of age. These were the first data to suggest that GPR43 is important for carcass adipose tissue accretion during the late finishing phase in beef cattle.

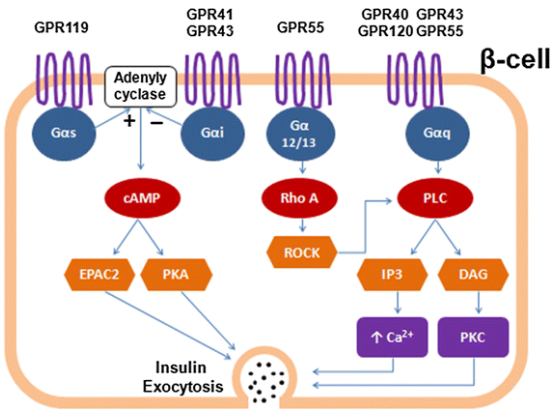


Figure 4. Proposed pathways for GPR. Reprinted with permission from [Moran et al].

2.3 G-Protein Receptor 120

GPR120 is located on chromosome 10 and is expressed predominantly in adipose tissue, but also intestines, lungs, spleen, and proinflammatory macrophages. Hirasawa et al. (2005) initially identified LCFA as ligands for GPR120, which was confirmed by observing that 1% bovine serum albumin (a protein known to bind free fatty acids) inhibited the free fatty acid response at GPR120. Hirasawa et al. (2005) also reported a murine GPR120 construct fused to the G-protein Gαq, which activated a PLC-mediated increase in intracellular Ca^{2+}. This finding confirmed that saturated and unsaturated fatty acids were agonists of GPR120, with polyunsaturated fatty acids (PUFA; contain more than one double bond) being the most potent. GPR120 binds to Gαq subunit, activating PLC. PLC catalyzes the formation of two intracellular messengers, IP3 and DAG from phosphatidylinositol 4,5-bisphosphonate (PIP2)**.** IP3 increases Ca^{2+} by releasing Ca^{2+} from intracellular compartments. IP3 is an endogenous ligand for calcium ligand-gated channels imbedded in the endoplasmic and sarcoplasmic reticulum in smooth and skeletal muscle. Upon binding, IP3 opens the channel to release calcium into the cytosol. Calcium controls muscle contraction, secretion, enzyme activation, and membrane hyperpolarization. DAG activates PKC, which controls several cellular functions by phosphorylating a variety of target proteins (Figure 5).

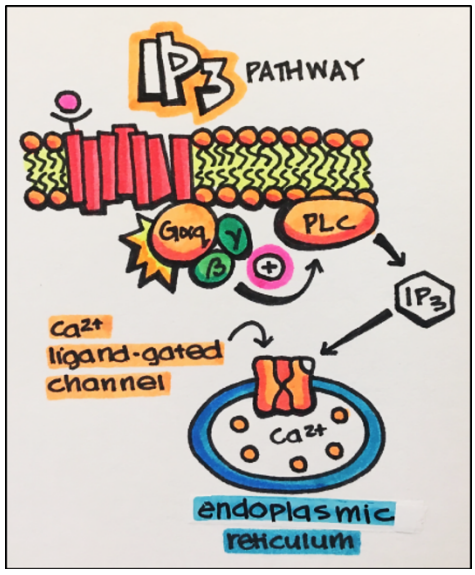


Figure 5. The pathway associated with GPR120. Reprinted with permission from [El-Fakahany et al].

3. LIPIDS AND FATTY ACIDS: STRUCTURE AND FUNCTION

3.1 Lipid Classification

Lipids, also known as fats, are components of plant (e.g., vegetable oils) and animal tissues (e.g., meat, eggs, milk). Lipids are described as chemically heterogenous organic compounds, which have a common characteristic of being insoluble in water, but soluble in organic solvents such as chloroform. Lipids are critical molecules that serve both structural and metabolic functions in animal systems. Lipids play an important role in animal diets, both nutritionally and physically. Nutritionally, lipids are an excellent source of energy and essential to the survival of animals. Physically, lipids are associated with improved feed quality, dust reduction in feed, increased palatability, decreased feed particle separation, digestive lubrication, and increased feed digestibility.

Lipids may be classified based on their physical properties at room temperature (solid or liquid), polarity, or their essentiality for animals and humans, but the preferable classification is based on their structure. Based on structure, lipids are classified into three major groups: simple, compound, and derived lipids.

Simple Lipids: These are simple esters of fatty acids and alcohol (1 glycerol + 3 fatty acids). This group of lipids are referred to as triglycerides or triacylglycerols (TAG). TAG have three fatty acids (often different) attached by the carboxyl end to a three-carbon glycerol backbone; and constitute most fatty acids in the diet and tissues of animals.

Compound Lipids: Compound lipids are comprised of a nonlipid molecule (e.g., protein). Phospholipids and glycolipids are examples of compound lipids and are used for lipid transport. Within the animal body, compound lipids are important in physiology and metabolism. Phospholipids molecules are constructed from four components, fatty acids (hydrophobic tails) that are attached to a hydrophilic head group containing glycerol and phosphate.

Derived Lipids: As implied by the name, derived lipids originate from simple and compound lipids through hydrolytic processes. Examples of derived lipids include fatty acids, steroids (e.g., cholesterol), and fat-soluble vitamins.

3.2 Fatty Acid Classification

Fatty acids are an important component of lipids in plants and animals. The molecular composition of a fatty acid includes a hydrophilic carboxyl group (-COOH) and a hydrophobic methyl group ($-CH_3$) at opposite terminals of a hydrocarbon backbone. Normally, three fatty acids are attached to a glycerol molecule, which are called TAG. Fatty acids are classified based on the number of carbons present in the chain as well as the number of double bonds. Fatty acid composition and structure determines the physical property and nutritional quality of fats. Short chain fatty acids (SCFA) normally contain less than 6 carbons in length (e.g., acetic acid, propionic acid, butyric acid). Medium chain fatty acids (MCFA) consist of 6-10 carbons in length (e.g., capric and lauric acid). Long-chain fatty acids (LCFA) contain 12 or more carbons and include fatty acids such as oleic acid (18:1n-9), α-linolenic acid (18:3n-3), and docosahexaenoic acid (DHA, 22:6n-3) (Figure 6).

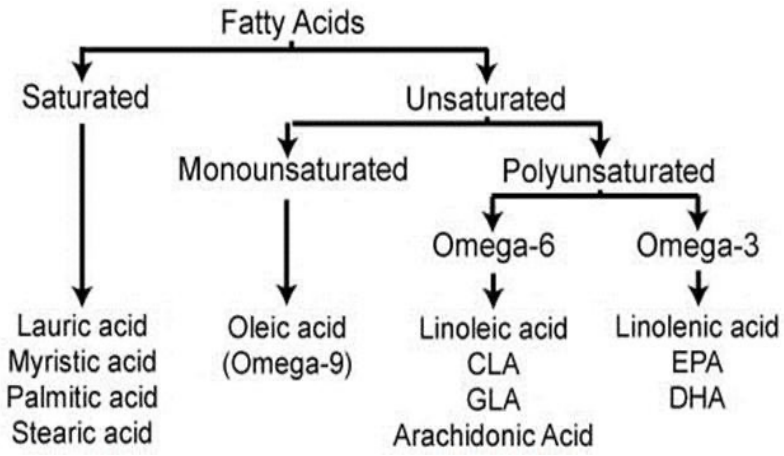


Figure 6. Fatty acid classification. Reprinted with permission from [Kesen et al].

3.2.1 Saturated vs. Unsaturated Fatty Acids

Fatty acids are classified into three families based on the presence (or absence) of double bonds in the hydrocarbon chain. These include saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Saturated fatty acids are "saturated" with hydrogen(s) with no double bonds present (e.g., stearic acid, 18:0) (Figure 7). Fat tends to be solid when there is a predominance of saturated fats in the TAG (e.g., tallow).

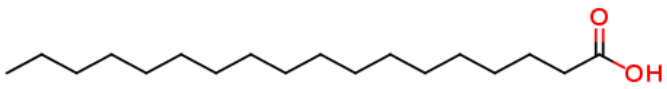


Figure 7. Stearic acid (18:0), a saturated fatty acid. Reprinted with permission from [Buckley et al].

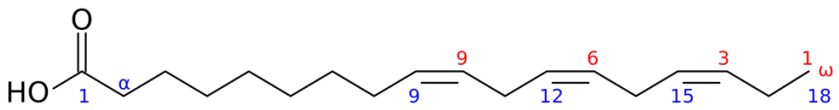


Figure 8. Alpha-linolenic acid (18:3 n-9,12,15), an PUFA. Reprinted with permission from [D'Antona et al].

Unsaturated fatty acids contain one or more double bonds between adjacent carbon atoms in the hydrocarbon chain (Figure 8). Unsaturated fatty acids can be either mono (one double bond) or polyunsaturated (more than two double bonds). Unsaturated fats tend to be liquid (i.e., oils) because unsaturation gives a "bend" in their structure and therefore the hydrocarbon chains cannot pack as tightly as saturated fats (e.g., oleic acid vs. stearic acid). The degree of saturation, length of carbon chain, and the position of the double bonds determine the physical property and nutritional quality of fats.

3.2.2 Fatty Acid Nomenclature

Scientists use the term omega (ω) or "n" to indicate the position of double bonds in an unsaturated fatty acid. The omega carbon is the first carbon with a double bond counting from the methyl end (CH_3) of the carbon chain. The location(s) of the double bond(s) are also indicated by Δ "delta", which denotes the position of the double bonds from the carboxyl end. There are two PUFA types – omega 6 (n-6, or ω-6) and omega 3 (n-3, or ω-3).

3.2.3 *cis* and *trans* Fatty Acids

Unsaturated fatty acids can form either *cis* or *trans* isomers, depending on the stereo-conformation of groups around a double bond. In the *cis* form, the two hydrogen substituents are

on the same side of the molecule, whereas in the *trans* form they are on the opposite sides. Most animals and plants fatty acids are in the *cis* conformation, whereas bacteria contain both *cis* and *trans* conformations. The *cis*-double bond introduces a kink in the hydrocarbon chain, thereby reducing the melting point of the fatty acid.

3.2.4 Melting point

Melting point (also known as slip point) is described as the temperature at which a substance (fat) changes from a solid to a liquid. For most animals, melting points of lipids are between 20°C and 40°C. Melting points are determined by fatty acid composition, and fatty acid composition is determined by diet, fat depot, and genetics. For example, lipid tissues from monogastric species fed unsaturated fatty acids closely resemble the fatty acid composition of the diet, whereas tissue desaturases control lipid tissue composition of monogastric species fed saturated fatty acids. Lipid composition of tissues from ruminant species is determined by diet, ruminal modification of fatty acids, and activity of tissue desaturases. Both carbon chain length and number of double bonds impact the physical properties of the fatty acid. As the number of carbons in a fatty acid chain increases, so does the melting point. More energy is needed to separate longer carbon chains than for shorter carbon chains, therefore Van der Waals forces and hydrophobic bonds increase as carbon chain length increases. A greater number of double bonds decreases the melting point because the Van der Waals forces are less effective. The efficacy of Van der Waals depends on the ability of molecules to pack closely together, and the presence of a carbon-carbon double bond disrupts this process. Melting point decreases as *cis*-double bonds increase, whereas *trans*-double bonds have less effect on melting point due to the absence of a

"bend" in the molecule. Melting point is often measured in lipids from beef cattle adipose tissue due to its relationship with meat quality.

3.2.5 Essential Fatty Acids

The term "essential fatty acid" refers to fatty acids that must be supplied in the diet of animals; essential fatty acids are required for biological processes but cannot be synthesized by animals. The two essential fatty acids are linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). These two fatty acids are considered essential due to the inability of animal cells to insert double bonds at the third and sixth carbon from the methyl end, i.e., in the n-3 and n-6 locations.

3.3 Lipid Metabolism

3.3.1 Lipid Digestion and Absorption in Ruminant Species

In ruminant species, the lipid content of the diet is low (typically under 5%) and comes from grass, leaves, oil seeds, and cereal grains. Lipid digestion begins in the rumen with the hydrolysis of TAG, phospholipids, and glycolipids. Hydrolysis of dietary lipids is done by microbial enzymes (TAG – lipase; phospholipids – phospholipase; glycolipids – galactolipase); these microbial enzymes release glycerol and free fatty acids (FFA). Glycerol is effectively fermented by the rumen bacteria to form VFA (acetate, propionate, butyrate). The unsaturated fatty acids are bio-hydrogenated, a process that adds hydrogens to form saturated fatty acids, mainly stearic acid. Too much unsaturated fatty acids are toxic to rumen microbes. Fatty acids such as linoleic acid are converted to "conjugated" fatty acids (e.g., conjugated linoleic acid, CLA), in which the double bonds are not separated by methylene (CH_2) groups; this process allows the fatty acids to become more stable "*trans*" fats (Figure 9).

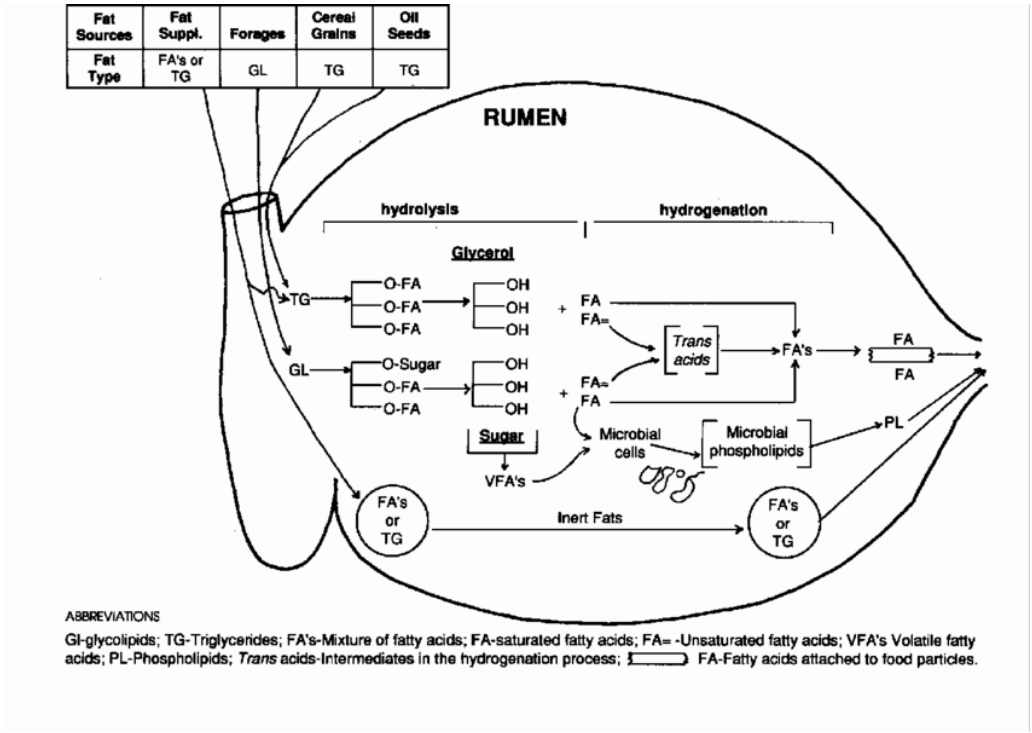


Figure 9. Lipid digestion in the rumen. Reprinted with permission from [Davis].

In ruminants, the first section of the small intestine, known as the duodenum, is very acidic, due to the low concentrations of bicarbonate. Pancreatic enzymes such as pancreatic lipase, phospholipase, and procolipase are secreted into the duodenum from the pancreas, while bile and lysolecithin are secreted from the liver (Cheeke et al., 2010). These compounds desorb (released from the surface) the fatty acids from the feed particles and microbes, allowing the formation of micelles. Fats are absorbed in the jejunum with the help of micelles, which facilitate the transfer of free fatty acids across the enterocytes (or intestinal mucosal cells). Once in the enterocytes, fatty acids less than 10 carbons in length exit the enterocyte and enter the portal blood, where they bind to albumin and are absorbed in the liver. Fatty acids greater than 12 carbons in length and glycerol undergo intracellular TAG reformation via the acyl-CoA

synthase. Seventy-five percent of TAG are synthesized via the 2-monoacylglycerol pathway located on the smooth endoplasmic reticulum, while 25% are synthesized via the standard TAG biosynthetic pathway located on the rough endoplasmic reticulum. Lipid droplets form within the endoplasmic reticulum and Golgi apparatus. These lipid droplets contain TAG, phospholipid, cholesterol, cholesterol ester, and apolipoprotein complexes. Apolipoprotein and phospholipids are formed in the rough endoplasmic reticulum, whereas TAG are formed in the smooth endoplasmic reticulum and are transferred to $ApoB_{48}$ to form a core for the chylomicron. After accumulating TAG, the Golgi vesicles form, and carbohydrate is added to the apolipoproteins, thus forming a chylomicron. The newly formed chylomicrons are exocytosed into lymphatic system circulation and then enters the blood. Chylomicrons deliver lipids to tissues other than the liver, with approximately 80% of lipids being delivered to muscle and adipose tissue. Chylomicrons produce diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acids (FFA) via lipoprotein lipase (LPL), which is associated with capillary endothelial cell of muscle and adipose tissue. As a result, the chylomicron remnant is formed and endocytosed by the liver.

3.3.2 Lipogenesis and Fatty Acid Synthesis

Lipogenesis is the process of synthesizing lipids for storage. Adipocytes, or fat cells, are dispersed throughout the body and are considered a long-term energy depot. Lipogenesis encompasses fatty acid synthesis and synthesis of TAG and phospholipids. Glucose is the key signal for fat storage (fed-state). Acetyl-CoA carboxylase is stimulated when ATP begins to accumulate. Like β-oxidation, fatty acid synthesis begins with 2 carbon acetyl-CoA. Fatty acid synthesis occurs in the cytosol (or cytoplasm). Acetyl-CoA carboxylase uses one ATP to combine acetyl-CoA to form malonyl-CoA. Fatty acid synthesis begins with the addition of CO_2

to the 2-carbon acetyl-CoA to form the 3-carbon malonyl-CoA. Acetyl-CoA carboxylase is the enzyme required for this step. Malonyl-CoA reacts with acetyl-CoA to produce a 5-carbon intermediate compound, which is decarboxylated to form a 4-carbon butyryl-CoA. This combines with another malonyl-CoA and is decarboxylated to form the 6-carbon caproyl-CoA. Malonyl-CoA is added and loses CO_2 each time to produce 16-carbon palmitic acid as the end-product. Fatty acid synthase is the enzyme that catalyzes these steps.

3.3.3 Fatty Acid Oxidation

Fatty acids released from the hydrolysis of TAG are transported via blood. Oxidation occurs in the mitochondria of tissues such as liver tissue, adipose tissue, cardiac tissue, and other tissues by converting them to acetyl-CoA, which is then catabolized in the TCA cycle. Before oxidation begins, fatty acids are converted to fatty acyl-CoA via acyl-CoA synthase (ATP is needed for this step). Fatty acyl-CoA are transported across the mitochondrial membrane with the help of CPT_1 (carnitine palmitoyl transferase I). In ß-oxidation, two carbons at a time are cleaved off from the fatty acyl-CoA, starting from the carboxyl end. Oxidation of the α-carbon (by FAD) produced a *trans*-double bond. The *trans*-double bond is hydrated, producing a hydroxy-fatty acyl-CoA. The hydroxyl group is oxidized (by NAD+) to produce a keto-fatty acyl-CoA. The first two carbons are displaced to produce acetyl-CoA. The acetyl-CoA enters the TCA cycle and generates CO_2, NADH + H^+, and $FADH_2$. The electron transport system uses the protons (H^+) and electrons to produce ATP and metabolic water.

3.3.4 Fatty Acid Elongation and Desaturation

Palmitic acid can be elongated to 18-carbon stearic acid by elongases. The formation of unsaturated fatty acids, oleic acid (18:1n-9) is achieved by the introduction of double bonds by the Δ9 desaturase. Mammals cannot insert double bonds beyond Δ9 position, which is why essential fatty acids (linoleic acid, 18:2 Δ9,12; α-linolenic acid 18:3 Δ9,12,15) need to be provided in the diet. When essential fatty acids are consumed, mammals can further elongate and desaturate essential fatty acids to form longer chain fatty acids such as EPA (20:5 Δ5,8,11,14,17) and DHA (22:6 Δ4,7,10,13,16,19).

3.3.5 Lipolysis

Lipolysis is the process by which TAG are hydrolyzed to three free fatty acids and one glycerol. Hormone and physiological factors such as diet and exercise regulate the release of glycerol and free fatty acids from adipocytes. Catecholamines such as epinephrine and norepinephrine are the primary ß-agonists in adipose tissue (glucagon in liver). In situations of energy depletion, plasma free fatty acids increase and insulin production decrease. The regulation of lipolysis is unique as these hormones can both stimulate and inhibit lipolysis depending on their affinity for different adrenergic receptors (AR) (Nielsen et al., 2014). Therefore, the activation of ß-AR on the surface of the adipocyte is required to stimulate lipolysis, while antilipolytic signals are transmitted by the α_2-AR. Both AR belong to the GPCR family. G-protein coupled receptors are cell surface receptors that detect ligands (e.g., neurotransmitters, hormones, lipids) and activate cellular responses. GPCR associated with β-AR contain the stimulating G_s subunit, whereas α_2-AR contain the inhibitory G_i subunit. GPCR activate adenylate cyclase (AC) by interacting with the G_s subunit or inhibit using the G_i subunit. Once activated, AC converts ATP to cyclic AMP

(cAMP), leading to an increase in intracellular cAMP levels, which activates protein kinase A (PKA, also known as cAMP-dependent protein kinase A). Activated PKA phosphorylates and activates perilipin (a protein that coats lipid droplets) and HSL, causing HSL to migrate to the surface of the lipid droplet, where it initiates TAG hydrolysis (Figure 10). Adipose TAG lipase (ATGL) converts TAG to DAG, which is the rate-limiting enzyme in the pathway. DAG is hydrolyzed to MAG by HSL, and monoacylglycerol lipase (MGL) cleaves MAG into FFA and glycerol. Once released from adipocytes, glycerol is delivered to the liver for glucose or glycerol-3-phosphate (via glycerol kinase) synthesis, while the free fatty acids are released to other tissues for β-oxidation and ATP production. Lipolysis occurs in the cytosol/cytoplasm and can be activated by epinephrine (adipose tissue and muscle) or glucagon (liver) (Figure 11).

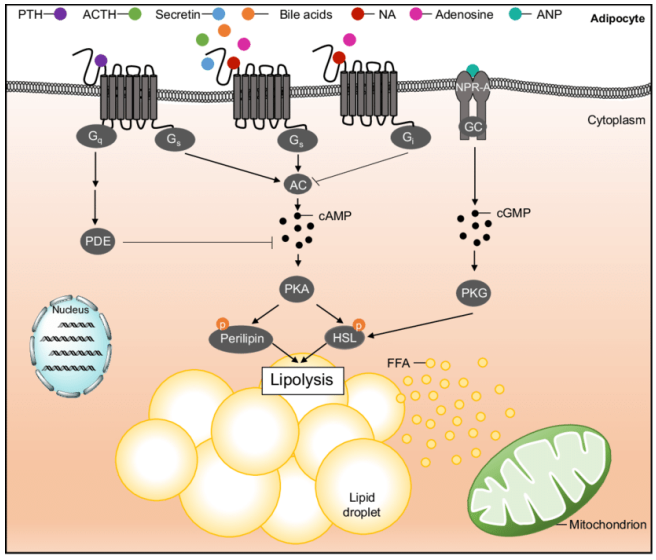


Figure 10. Pathway of lipolysis. Reprinted with permission from [Schnabl et al].

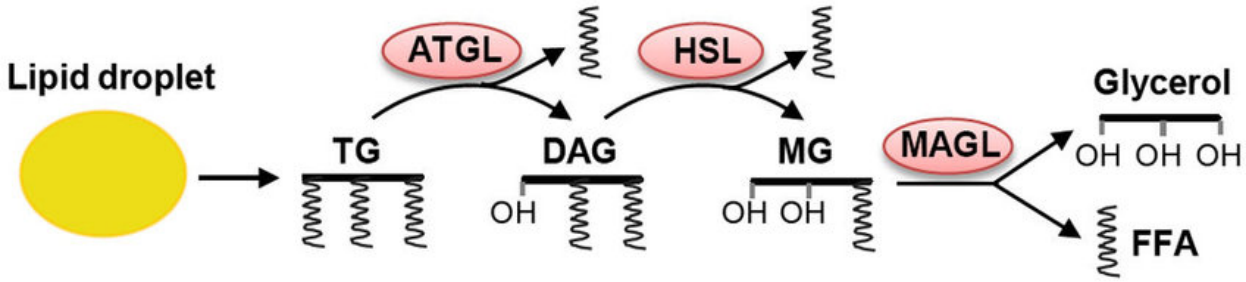


Figure 11. Lipolysis – TAG breakdown. Reprinted with permission from [Guo et al].

4. ADIPOSE TISSUE

Adipose tissue is described as loose connective tissue that is mostly composed of adipocytes (Figure 12). Adipocytes are the main cellular component of adipose tissue and play a vital role in the storage of TAG during energy consumption. Additionally, adipose tissue also serves as a cushion and insulates the body from hot and cold temperatures. Many physiological factors influence the amount and location of adipose tissue throughout mammalian tissues. The development and growth of adipose tissue is influenced by hyperplasia (cell numbers) and hypertrophy (cell size). The proliferation of preadipocytes and their differentiation into mature adipocytes is referred to as adipogenesis (Wandita et al., 2018).

Besides storing energy, adipose tissue has been identified as a major endocrine organ that regulates energy metabolism. This is achieved through endocrine, autocrine, and paracrine signals, allowing adipocytes to control the metabolism of other fat cells or cells located in the liver, muscle, pancreas, or brain (Kim et al., 2000). Adipose tissue produces hormones such as estrogen, leptin, adiponectin, resistin, in response to signals from other organs in the body (Ibrahim, 2010). Adipose tissue plays a major role in the regulation of glucose, cholesterol as well as sex hormones (Kim et al., 2000).

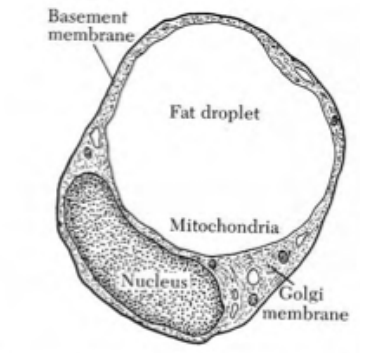


Figure 12. Adipocyte structure. Reprinted with permission from [Smith and Johnson].

4.1 Subcutaneous Adipose Tissue

The adipose tissue beneath the skin is referred to as subcutaneous (s.c.) adipose tissue, or white adipose tissue (Figure 13). The amount and distribution of s.c. adipose tissue (carcass fat) is an important component affecting beef carcass quality (Walker et al., 1990).

Researchers have demonstrated that bovine s.c. adipose tissue preferentially uses acetate as a substrate for *de novo* fatty acid biosynthesis (Smith and Crouse, 1984). Subcutaneous adipose tissue has approximately twice the Δ9 desaturase catalytic activity of i.m. adipose tissue, which is consistent with a higher MUFA concentrations in s.c. tissue than in i.m. adipose tissue (Archibeque, et al., 2005; Smith et al., 2006). Expression of the Δ9 desaturase gene greatly increases in Angus steers between weaning and 12 mo of age (Martin et al., 1999). Lee et al. (2005) demonstrated that SCD mRNA peaked at 12 mo of age in muscle from Hanwoo steers, suggesting that desaturase gene expression is highly expressed during adipocyte differentiation (Smith et al., 2006).

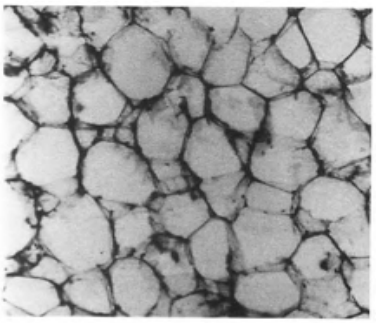


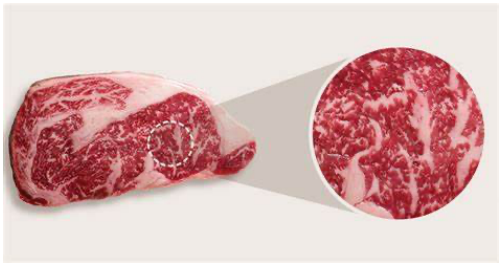
Figure 13. Histology of s.c. adipose tissue. Reprinted with permission from [Smith and Johnson].

4.2 Intramuscular Adipose Tissue

Intramuscular adipose tissue, also known as marbling, is distinguishable from other fat depots by its location within perimysial connective tissues adjacent to muscle fibers, with the greatest deposition occurring in the later stages of the growth process (Smith and Johnson, 2016) (Figure 14). The speckles of fatty tissues are composed of primarily neutral lipids, which consists of adipocytes (Troy et al., 2106). The presence of i.m. adipose tissue is one of the main factors associated with the consumer perception of beef eating quality (Hunt et al., 2016). Generally, i.m. fat content has been shown to influence flavor, juiciness, tenderness and overall acceptability of beef (Savell et al., 1987, 1989). The deposition of i.m. adipose tissue varies immensely between individual animals, due to differences in production and genetics (Mao et al., 2016). Numerous studies have demonstrated that the lipid profile and fatty acid composition of i.m. adipose tissue in beef can be altered based on three factors, genotype, time of feed, and finishing diet (Wood et al., 2008).

Previous studies have established that glucose is the preferred source of carbon for i.m. adipocytes (Smith and Crouse, 1984). Early weaning of beef steers has been shown to improve marbling development at slaughter than traditional weaning practices. This may be a result of increased glucose availability from concentrate-based diets at the early stages of marbling development (Smith and Johnson, 2016). Concentrate-based diets also increase MUFA (especially oleic acid) in marbling by stimulating the expression of SCD. Cell culture studies have established that oleic acid promotes *trans*-differentiation of muscle cells to marbling adipocytes (Li et al., 2019). Furthermore, there is a significant correlation between MUFA

concentration and the amount of i.m. lipid in the longissimus muscle of beef cattle when fed a concentrate-based diet for long periods of time (Smith and Johnson, 2016).


(A)

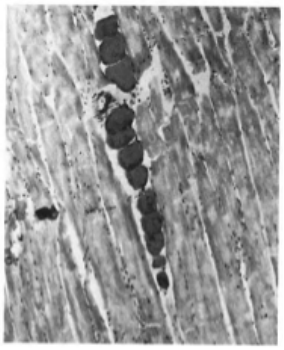

(B)

Figure 14. Beef marbling (A) and histology of i.m. adipocytes (B). Reprinted with permission from [Smith and Johnson].

4.3 Beef Quality

In the U.S., beef quality is evaluated by assessing factors that affect palatability (tenderness, juiciness, and flavor). These factors include carcass maturity, texture, firmness, and color as well as the amount and distribution of marbling (Savell et al., 1987, 1989). Beef carcass quality grades are determined based on two factors: degree of marbling and degree of maturity. Beef graders evaluate the amount and distribution of marbling in the longissimus dorsi muscle after the carcass has been ribbed between the 12^{th} and 13^{th} thoracic rib. Degree of marbling is the primary determination of quality grade. Each degree of marbling is divided into 100 subunits.

However, in general, marbling scores are analyzed in tenths within each degree of marbling. USDA quality grades such as Prime, Choice, and Select are the major factors that influence beef prices in the U.S.

5. OLEIC ACID AND PALMITIC ACID

5.1 Oleic Acid Structure and Function

Oleic acid is a MUFA that occurs naturally in the fats and oils of both animals and vegetables. It is a monounsaturated Δ-9 fatty acid, abbreviated with a lipid number of 18:1cis-9. Its name is derived from the Latin word oleum, which means oil. Oleic acid is referred to as a Δ-9 fat because it has a carbon-carbon double bond at the ninth carbon from the methyl end of the fatty acid (Figure 15). Oleic acid is the most common MUFA in nature. It is found in fats (TAG), the phospholipids that constitute membranes, and cholesterol esters. Oleic acid is the most abundant fatty acid in human adipose tissue, and second in human tissues overall, following palmitic acid.

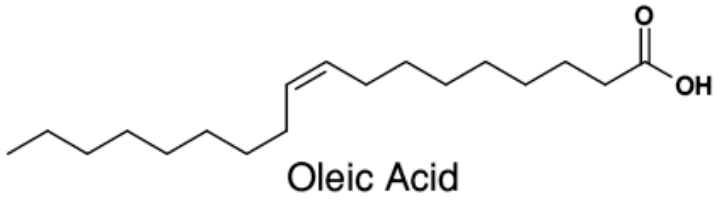


Figure 15. Structure of oleic acid. Reprinted with permission from [Buckley et al].

5.2 Oleic Acid Production

The biosynthesis of oleic acid involves the action of the enzyme stearoyl-CoA 9-desaturase (SCD, also referred to as Δ-9 desaturase), acting on stearoyl-CoA. As a result, stearic acid (18:0) is dehydrogenated, thereby giving rise to the monounsaturated derivative, oleic acid. Stearic acid is one of the main fatty acids affecting fat hardness; therefore, any dietary or

production factor that improves the conversion of stearic acid to oleic acid will increase fat softness (Smith et al., 1998). SCD gene expression greatly increases between weaning and 12 mo of age in s.c. adipose tissue of Angus steers (Martin et al., 1998), suggesting that SCD activity is vital for the subsequent development of subcutaneous adipose tissue in growing steers.

5.3 Oleic Acid in Beef Products

Oleic acid is the most abundant fatty acid in U.S. beef; it is especially elevated in beef with Japanese genetics, including the American Wagyu. Early research has established that the concentration of oleic acid in beef is positively correlated with improved palatability. Oleic acid is primarily responsible for fat softness in cattle, which is positively correlated with lower melting points and overall palatability of beef (Westerling and Hedrick, 1979). However, stearic acid is the main determining factor influencing fat hardness (Smith et al., 1998; Chung et al., 2006b). Therefore, any production or dietary factors that enhance the conversion of stearic acid to oleic acid will result in increased fat softness.

5.4 Health Benefits

Oleic acid is a common MUFA in human diets. Many studies have indicated that increasing the intake of oleic acid (e.g., olive or canola oil) reduces risk factors for metabolic diseases in humans. Furthermore, consumption of MUFA has been associated with decreased low-density lipoprotein cholesterol (LDL) and increased high-density lipoprotein (HDL) cholesterol (source). Our laboratory has demonstrated that consumption of beef products naturally enriched with oleic acid increases HDL cholesterol in men and women (reviewed in Smith et al., 2020).

5.5 Palmitic Acid Structure and Function

Palmitic acid is the most common SFA in animal tissues and, like oleic acid, can be provided in the diet or synthesized endogenously via *de novo* lipogenesis (Carta et al., 2017) (Figure 16). Palmitic acid accumulation is inhibited by enhanced Δ-9 desaturation to palmitoleic acid (16:1n-7) and/or elongation to stearic acid and eventually further desaturation via Δ-9 desaturase to form oleic acid (Strable and Ntambi, 2010). Nutritional factors and physiopathological conditions can activate *de novo* lipogenesis, leading to increased content of palmitic acid and homeostatic disruption (Wilke et al., 2009). Palmitic acid homeostatic disruption has been implicated in several different physiopathological conditions such as cancer, atherosclerosis, and neurodegenerative diseases. This disruption is often attributed to uncontrolled endogenous palmitic acid biosynthesis, regardless of dietary intake (Carta et al., 2017).

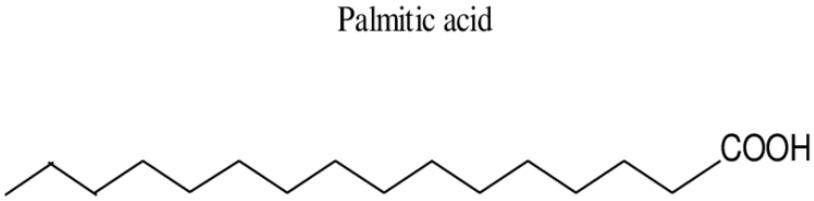


Figure 16. Structure of palmitic acid. Reprinted with permission from [Machado].

5.6 Palmitic Acid Production

Endogenous palmitic acid production, also known as fatty acid synthesis, begins with citrate being converted to acetyl-CoA and then malonyl-CoA, which is then elongated to form palmitate and other fatty acids. The key enzymes in this process are acetyl-CoA carboxylase (ACC), which catalyzes the *de novo* lipogenesis limiting step reaction, and the FA synthase (FAS). Glucose and glutamine-derived α-ketoglutarate (α-KG) are the main sources for *de novo* lipogenesis, especially under disruption of the mitochondrial oxidative machinery or hypoxia (Vernieri et al., 2016). High carbohydrate consumption, beyond the body's ability to store it as glycogen or use it as an energy substrate, promotes *de novo* lipogenesis by inducing an increase of insulin and substrate availability (Cohen et al., 2011). Insulin activates the transcription factor sterol regulatory element-binding proteins-1c (SREBP-1c), which is responsible for up-regulating the enzymes that catalyze lipogenesis (Horton et al., 2002).

6. VOLATILE FATTY ACIDS (VFA)

VFA, also known as SCFA (acetate, propionate, and butyrate) are metabolites produced by microbiota in the distal small intestine and the colon (in monogastrics) from complex dietary structural (fiber) and non-structural (resistant starch) carbohydrates in a fermentation process (Lin et al., 2012). Once produced, VFA are easily absorbed by colonocytes, with butyrate largely utilized by the colon epithelium as an energy source. Propionate is mainly utilized by the liver, while a considerable amount of acetate enters the circulation and reaches peripheral tissues (Lin et al., 2012). Acetate (the anion of acetic acid) is a major nutrient that supports acetyl-CoA metabolism, which results in several metabolic functions, including lipid synthesis, energy production and protein acetylation (Bose et al., 2019).

It is well documented that supplementing structural and non-structural carbohydrates, thereby increasing intestinal and circulating VFA, results in metabolic benefits. Supplementation of butyrate in the diet and oral administration of acetate suppressed weight gain independent of food intake suppression in rodent models of genetic or diet-induced obesity (Gao et al., 2009; Yamashita et al., 2007). However, the mechanism leading to obesity resistance remains unclear. Additionally, propionate was described to reduce food intake in humans, but the molecular mediators responsible have not yet been identified (Arora et al., 2011).

6.1 Carbohydrate Fermentation

In ruminant species, VFA are the main source of energy and are produced from microbial fermentation of dietary carbohydrates in the rumen. The goal of fermentation is to produce energy (as ATP) for bacteria to use for the synthesis and growth. Acetate, propionate, and

butyrate are absorbed through the ruminal epithelium. Acetate or propionate is thought to be required by the mesenchymal cells to evolve into preadipocytes and adipocytes (Wandita et al., 2018).

VFA are produced in a ratio that vary from approximately 75:15:10 to 40:40:20 (acetate: propionate: butyrate), depending on the diet (Bergman, 1990). In ruminants, the type of diet (forage vs concentrate), ruminal pH, and microbial species in the rumen are the primary variables that influence the proportion of each VFA produced. Acetate is the most abundant VFA and is produced from high forage-based diets. Elevated acetate concentrations ensure stable fermentation and balances ruminal pH (Wandita et al., 2018). When fed a high concentrate-based diet, propionate production increases, ruminal pH drops, feed intake decreases, and microbial reproduction is reduced (Russell, 1998). A high proportion of propionate in the rumen may lead to reduced fiber digestion and acidosis. A forage-fed ruminant will typically have VFA ratios of 70:20:10 for acetate, propionate, and butyrate. However, with concentrate-fed ruminants, the ratios will have a greater percentage of propionate at 50:40:10 (Remond et al., 1995).

Structural and non-structural carbohydrates from the diet are available for fermentation by rumen microbes (e.g., bacteria, protozoa, and fungi). Carbohydrates (cellulose and starch) are degraded to simple sugars (monosaccharides – glucose, fructose, and galactose) by microbial enzymes (Figure 17). These simple sugars are absorbed by the bacteria and converted to pyruvate through the glycolytic pathway. Pyruvate is then converted to the final products, acetate, propionate, and butyrate and these VFA are then released (by the bacteria) into the rumen and absorbed through the rumen wall (Ungerfeld, 2020).

There are many different species of bacteria present in the rumen and these different species may require or utilize different substrates and produce different products. Therefore, the

products of fermentation will depend on the composition of the rumen microflora. Diet will also directly influence how well microbial species grow and reproduce. The fermentation pattern, or the proportion of VFA can be affected by many factors. These factors include diet composition, physical nature of the diet (e.g., ground, pelleted), intake (e.g., restricted or ad libitum), species (e.g., cattle vs. sheep vs. deer), and additives (e.g., ionophores) (Weimer and Kohn, 2016).

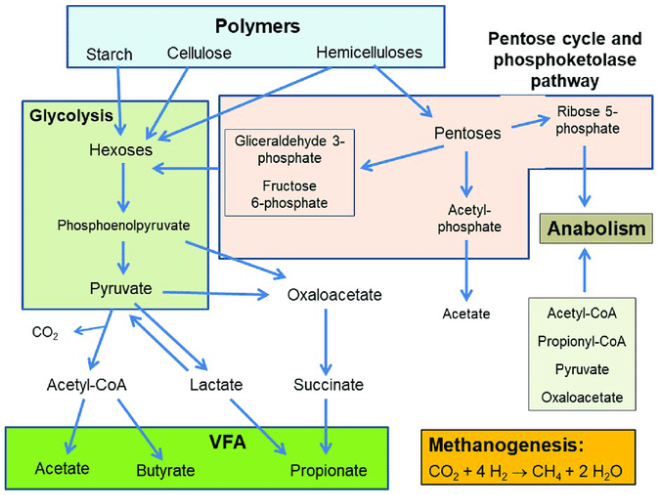


Figure 17. Simplified pathway of carbohydrate fermentation in the rumen. Reprinted with permission from [Ungerfeld].

7. HYPOTHESIS & OBJECTIVES

The purpose of this project was to better understand the role of GPR43 in the accumulation of lipid in bovine intramuscular (i.m., marbling) and subcutaneous (s.c.) adipose tissue. An increased understanding of the key differences between these two adipose tissues could allow for specific production practices and/or technologies to be developed that would increase marbling without concurrently increasing s.c. adipose tissue development.

Hypothesis

We hypothesized that the binding of oleic acid to GPR43 will promote TAG accumulation in i.m. adipose tissue by antagonizing the increase in cAMP caused by acetate, thereby depressing lipolysis.

The objectives for my doctoral research were:

1) Establish the interactions among individual VFA (acetate and propionate) and between VFA and LCFA palmitic acid (16:0) and oleic acid (18:1) for the GPR43 receptor in short-term (1h) *ex vivo* incubations of bovine i.m. and s.c. adipose tissues.

2) Establish age interactions on *GPR* mRNA in bovine i.m. and s.c. adipose tissues.

8. EXPERIMENT 1: EVIDENCE FOR FUNCTIONAL G-COUPLED PROTEIN RECEPTORS 43 AND 120 IN SUBCUTANEOUS AND INTRAMUSCULAR ADIPOSE TISSUE OF ANGUS CROSSBRED STEERS

8.1 Introduction

GPR43 gene originally was cloned and sequenced as an orphan family A G-protein coupled receptor forming part of a cluster of 4 genes on human chromosome 19, also including GPR40, GPR41, and GPR43 (Sawzdargo et al., 1997). The GPR41 and GPR43 receptors work via Class A G-protein complexes, inhibiting AC, which is one mechanism by which GPR41 and GPR43 reduce lipolysis (Moran et al., 2016).

Fain and Shepherd (1975) first reported that oleic acid strongly inhibited norepinephrine-stimulate cAMP accumulation in isolated rat adipocytes. Oleic acid binds to GPR120 receptors, and VFA and short-chain fatty acids (C2–C8) stimulate GPR43 receptor activity (Brown et al., 2003; Tazoe et al., 2008; Moran et al., 2016). Binding of VFA to GPR43 receptors activates the extracellular signal-regulated kinase signaling pathway as a result of binding to the $G_{\alpha i}$ subunit (Moran et al., 2016). It is not clear if GPR43 attenuates lipolysis (Daval et al., 2006; Moran et al., 2016) or promotes lipolysis in adipose tissue (Miyamoto et al., 2016). In the current study, we hypothesized that oleic acid would promote GPR43 activity (i.e., decrease cAMP production) to a greater extent in fresh i.m. ex vivo explants than in s.c. adipose tissue explants.

8.2 Materials and Methods

8.2.1 Institutional approval

All experimental procedures were approved by the Texas Tech University Institutional Animal Care and Use Committee, AUP #10032-05 (experiment 1), and performed at the Texas Tech University Animal and Food Sciences building, and the Texas A&M University Institutional Animal Care and Use Committee, AUP #2018-036A (experiments 2 and 3), performed in the Kleberg Center building.

8.2.2 Experimental animals and design

We hypothesized that the VFA acetate and propionate and the LCFA oleic acid and palmitic acid would differently affect forskolin-stimulated cAMP production in short-term incubations of s.c. and i.m. adipose tissue. Eight Angus × Brahman (≤ 75% Angus) calf steers were weaned at 6 mo of age and allowed to forage free choice on native pastures. At 8 mo of age, the steers were adapted gradually to a corn/milo-based finishing diet (Smith et al., 2012). Steers were slaughtered at ~20 mo of age. Steers were transported to the Texas A&M University Rosenthal Meat Science and Technology Center and fasted overnight with free access to fresh water. Cattle were slaughtered by standard industry practices. The 5th to 8th longissimus thoracic rib muscle section was removed immediately following exsanguination by cutting through the hide (~5 min post exsanguination; Miller et al., 1989; May et al., 1994). The rib section was placed in oxygenated, 37°C Krebs–Henseleit buffer (KHB; pH 7.35 to 7.40) plus 10 mM Hepes buffer and 5 mM glucose and transported immediately to the laboratory for dissection and incubation of i.m. adipose tissue. After removal of a portion of the s.c. adipose tissue overly the

longissimus muscle, slices (~1 cm thick) were cut from the longissimus muscle and i.m. adipose tissue was dissected and immediately placed in 37°C, oxygenated KHB containing 5 mM glucose. The samples were considered viable because the muscle remained at 37 to 38°C during dissection, and the muscle twitched during dissection.

8.2.3 Short-term s.c. and i.m. adipose tissue incubations

Fresh s.c. and i.m. adipose tissue pieces (50 to 150 mg) were transferred immediately after dissection to 6-well culture plates containing 3 mL of the KHB/Hepes/5 mM glucose media. Adipose tissue samples were preincubated with 0.5 mM theophylline plus 10 μM forskolin for 30 min in a CO_2 incubator, after which 0, 10^{-3}, $10^{-2.3}$, and 10^{-2} M acetate were added to the incubation medium in the absence or presence of 100 μM oleic acid or 100 μM palmitic acid. Subcutaneous and i.m. adipose tissues also were incubated with 0, 10^{-3}, $10^{-2.3}$, and 10^{-2} M propionate. Following an additional 30-min incubation, adipose tissue samples were transferred to test tubes containing 500 μL of cell lysis solution (Cell Signaling Technologies). Each adipose tissue sample was sonicated in lysis buffer for 15 s, the fatty acid layer was removed with a Pasteur pipet, and samples were stored at −80°C.

8.2.4 Tissue cAMP

Concentrations of cAMP in adipose tissue samples for the short- term incubations were measured in the tissue lysates using the Cyclic AMP XP Assay Kit (Cell Signaling Technologies). The highest concentrations of cAMP in adipose tissue lysates, ~20 pmol/mL, were in the lower to middle range of the standard curve, 0.3 to 80 pmol/100 mg adipose tissue. Results are reported as pmol cAMP/100 mg adipose tissue.

8.2.5 Statistical analysis

The cAMP data for s.c. and i.m. adipose tissue were analyzed by split-plot (SuperAnova, Abacus Concepts, Berkeley, CA) with acetate concentration as the main plot and acetate in the absence and presence of LCFA (oleic acid or palmitic acid) as the subplot (SuperAnova). The cAMP data also were analyzed by split plot with propionate concentration as the main plot and adipose tissue (s.c. or i.m.) as the subplot. Differences between means were determined using the Fishers protected LSD procedure (SuperAnova). Means were considered significantly different at $P < 0.05$.

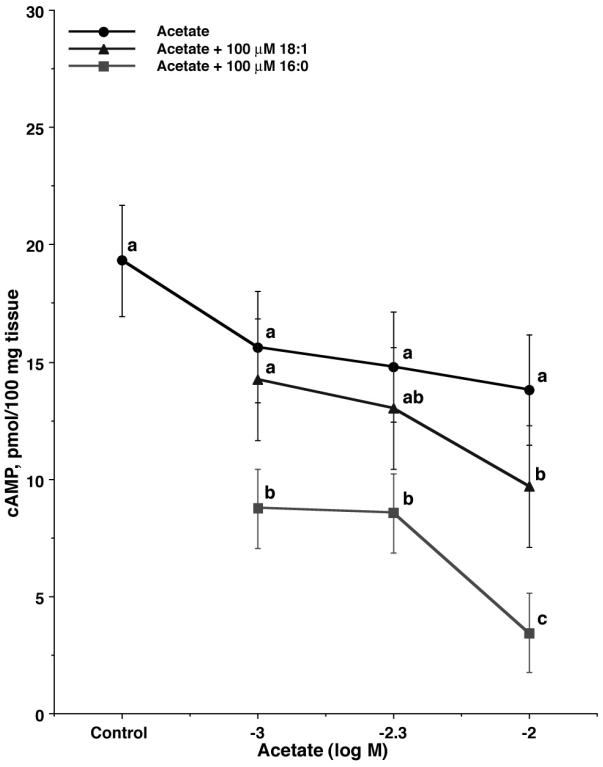
8.3 Results

Forksolin-stimulated cAMP production in the absence of VFA or LCFA was 4- to 6-fold greater in s.c. adipose tissue than in i.m. adipose tissue (Figure 18A). In s.c. adipose tissue, forskolin- stimulated cAMP production was not affected ($P > 0.05$) by media acetate, but the combination of 10^{-2} M plus 100 μM oleic acid depressed cAMP ($P < 0.05$; Figure 18B). Palmitic acid (100 μM) strongly depressed cAMP production at all concentrations of media acetate ($P < 0.05$). Split-lot analysis of variance indicated that the log M acetate × treatment interaction was not significant ($P = 0.92$), but the treatment effect was significant: acetate > acetate + oleate > acetate + palmitate ($P < 0.0001$).

In i.m. adipose tissue, $10^{-2.3}$ and 10^{-3} M acetate depressed cAMP production ($P < 0.05$), which was attenuated by 100 μM oleic acid (Figure 18B). Palmitic acid depressed cAMP production at 10^{-3} M acetate ($P < 0.05$) but had no effect on cAMP ($P > 0.05$) at higher concentrations of media acetate. Split-lot analysis of variance indicated that the log M acetate × treatment interaction was significant ($P = 0.05$), and the treatment effect also was significant:

acetate + oleate > acetate = acetate + palmitate ($P < 0.0001$). Propionate had no effect on cAMP production in s.c. or i.m. adipose tissue ($P > 0.05$).

A



B

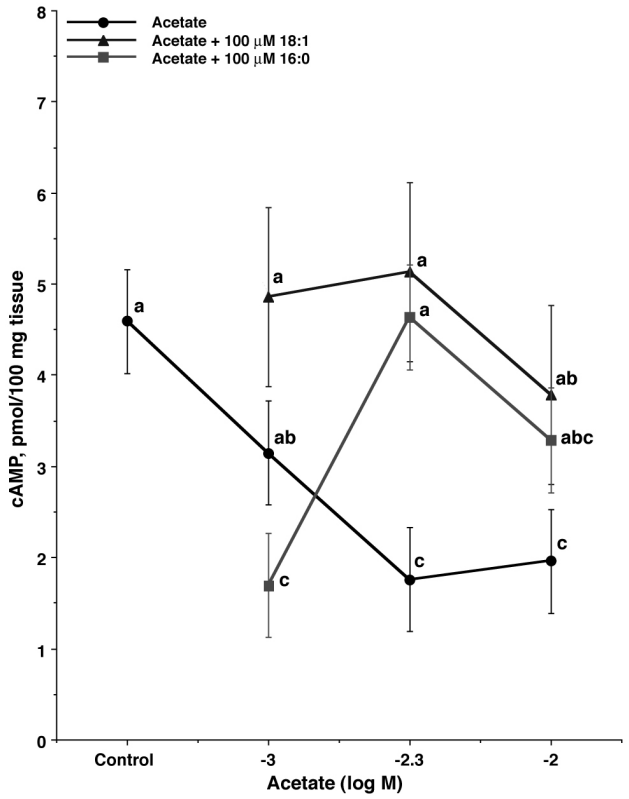


Figure 18. Forskolin-stimulated cAMP as a function of media acetate concentration in (A) s.c. and (B) i.m. adipose tissue explants at 20 mo of age. Media containing acetate also contained 100 μM oleic acid (18:1n-9) or palmitic acid (16:0). [abc] Means without a common superscript differ ($P < 0.05$). Reprinted with permission from [Westbrook et al].

8.4 Discussion

One of the more important findings of the current study is that forskolin, acetate, and LCFA affected cAMP production differently in s.c. and i.m. adipose tissue. Forskolin-stimulated cAMP production was nearly 4-fold greater in s.c. than in i.m. adipose, indicating greater AC activity in s.c. adipose tissue. We recently demonstrated that isoproterenol (nonspecific β-adrenergic agonist), salbutamol (β_1-adrenergic 2 agonist), and dobutamine β_2-adrenergic agonist) effectively increased cAMP in s.c. adipose tissue but not i.m. adipose tissue (Hwang et al., 2021), which is consistent with apparently greater AC activity in s.c. adipose tissue (i.e., greater forskolin-stimulated cAMP production) than in i.m. adipose tissue in the current study.

Brown et al. (2003) transfected *Xenopus laevis* (frog) oocytes with GPR43 constructs and demonstrated that GPR43 had equal affinity for acetate and propionate. In the current study, whereas acetate caused a clear depression of cAMP production in i.m. adipose tissue, propionate had no effect on cAMP production. This indicates a greater affinity of the GPR43 receptor for acetate than for propionate in bovine i.m. adipose tissue. Neither acetate nor propionate significantly affected cAMP production in s.c. adipose tissue, which may reflect the lesser *GPR43* expression in s.c. than in i.m. adipose tissue. We cannot with certainty explain the lack of effect of acetate on cAMP. However, the demonstrable effect of acetate on cAMP production in conjunction with lack of responsiveness of i.m. adipose tissue to β -adrenergic agonists (Hwang et al., 2021) underscores the importance of GPR receptors in regulating lipid metabolism in i.m. adipose tissue.

In i.m. adipose tissue, oleic acid effectively reversed the acetate-induced depression of cAMP production. Also, palmitic acid attenuated the effects of acetate on cAMP production at

$10^{-2.3}$ and 10^{-2} M acetate. More than 4 decades ago, Fain and Shepherd (1975) demonstrated that oleic acid strongly depressed norepinephrine/theophylline-stimulated cAMP production in rat adipocytes, which occurred within 1 min of exposure of adipocytes to oleic acid. Much more recently, Husted et al. (2020) exposed murine and human adipose tissues to isoproterenol and demonstrated vast increases in media fatty acids, including oleic acid. Also, the synthetic GPR120 agonist compound A (CpdA) depressed isoproterenol-induced cAMP production by ~50%.

Oleic acid binds to the GPR120 receptor (Widmayer et al. 2019), and Husted et al. (2020) demonstrated that treatment of cultured murine adipocytes with CpdA (a specific activator of GPR120) strongly upregulated Gai protein production, similar to the downstream effects of GPR43. In s.c. adipose tissue, at 10^{-2} M acetate, oleic acid depressed cAMP production by ~30% and palmitic acid depressed cAMP production by ~75%. These results suggest that s.c. adipose tissue expresses *GPR120*, thereby decreasing cAMP in s.c. adipose tissue exposed to LCFA. However, we did not anticipate these results and therefore did not measure *GPR120* mRNA in the current study. The expression of *GPR120* was reported in 3T3-L1 adipocytes, but only once the adipocytes had begun to differentiate (Gotoh et al., 2007). Agrawal (2017) reported that *GPR120* expression was expressed in s.c. adipose tissue of Holstein steers, which is consistent with the results of the current study, i.e., depression of cAMP production by LCFA in s.c. adipose tissue.

As stated above, in i.m. adipose tissue, acetate depressed cAMP production, an effect that was attenuated by palmitic and oleic acid, but the upregulation Gai protein production associated with the GPR120 receptor does not explain these effects. The results of the current study suggest

that, in intact i.m. adipose tissue explants, oleic acid could actually increase lipolysis. We have not documented the effects of oleic acid on the p-AMPKα/AMPKα protein ratio in intact i.m. or s.c. adipose tissues, but it is possible that oleic has different effects on the p-AMPKα/AMPKα ratio (hence, lipid filling) in cultured, differentiating adipocytes than in fully differentiated adipose tissue explants.

Upregulation of Gai protein production associated with the GPR43 receptor can explain the depression of cAMP production caused by acetate in i.m. adipose tissue. Previous research by others demonstrated that GPR43 activation promotes lipid filling (Daval et al., 2006; Moran et al., 2016) or antagonizes lipid filling (Miyamoto et al., 2016) in adipose tissue. The attenuation of cAMP production by acetate in i.m. adipose tissue suggests a promotion of lipid filling via the GPR43 receptor.

There is a growing interest in GPR receptors in tissues of livestock species. Whereas *GPR43* or *GPR120* gene expression has been measured previously, the current study is unique in that is has provided functional evidence for GPR43 receptors in i.m. adipose tissue and GPR120 receptors in s.c. adipose.

9. EXPERIMENT 2: CHANGES IN GPR43 ACTIVITY BETWEEN 20 AND 22 MO OF AGE IN ANGUS CROSSBRED STEERS

9.1 Introduction

The presence of intramuscular fat, or marbling, has been widely reported to be one of the main factors associated with consumer perception of beef eating quality. GPR43 in intramuscular (i.m.) and subcutaneous (s.c.) adipose tissues and is activated by volatile fatty acids (VFA), such as acetate and propionate (Westbrook et al., 2021). Both GPR43 and GPR41 receptors inhibit AC, thereby depressing lipolysis (Moran et al., 2016). LCFA such as oleic acid and palmitic acid, are known to be potent ligands of GPR120 and GPR40 (Hirasawa et al., 2005). Previous studies have indicated that oleic acid strongly inhibited norepinephrine-stimulated cAMP accumulation in isolated rat adipocytes (Fain and Shepherd, 1975). In the previous experiment, we demonstrated that acetate and LCFA (oleic acid and palmitic acid) affected cAMP production differently in s.c. and i.m. adipose tissue (Westbrook et al., 2021). The decrease in cAMP production caused by acetate plus LCFA suggests that s.c. adipose tissue expresses *GPR120,* thereby depressing cAMP production in s.c. adipose tissue exposed to LCFA, thereby reducing lipolysis. Furthermore, acetate depressed cAMP production, but only in the absence of LCFA, indicating a reduction in lipid accumulation in i.m. adipose tissue. In the current study, we hypothesized that LCFA would promote GPR43 activity by decreasing cAMP, glycerol, and free fatty acid production more effectively in fresh i.m. ex vivo explants than in s.c. adipose tissue explants.

9.2 Materials and Methods

9.2.1 Institutional approval

All experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee, AUP #2018-036A. Twenty-four Angus crossbred steers were used in this study.

9.2.2 Experimental animals and design

Twenty-four Angus crossbred calf steers were weaned at 6 mo of age and allowed to forage free choice on native pastures. At 8 mo of age, the steers were adapted gradually to a corn/milo-based finishing diet (Smith et al., 2012). Steers were slaughtered at approximately 20 and 22 mo of age. Steers were transported to the Texas A&M University Rosenthal Meat Science and Technology Center and fasted overnight with free access to fresh water. Cattle were slaughtered by standard industry practices. The 5^{th}-8^{th} longissimus thoracic rib muscle section was removed immediately following exsanguination by cutting through the hide (approximately 5 min post-exsanguination) (Miller et al., 1989; May et al., 1994). The rib section was placed in oxygenated, 37^{o}C KHB (pH 7.35-7.40) plus 10 mM Hepes buffer and 5 mM glucose and transported immediately to the laboratory for dissection and incubation of i.m. and s.c. adipose tissues. Slices (approximate 1 cm) were cut from the muscle and i.m. adipose tissue was dissected immediately and then placed in 37°C, oxygenated KHB solution containing 5 mM glucose. Adipose tissue samples are considered viable if the muscle remains at 37^{o}C during dissection, and the muscle twitches during dissection.

9.2.3 Short-term s.c. and i.m. adipose tissue incubations

Fresh s.c. and i.m. adipose tissue pieces (50 to 150 mg) were transferred immediately after dissection to 6-well culture plates containing 3 mL of the KHB/Hepes/5 mM glucose media. Adipose tissue samples were preincubated with 0.5 mM theophylline plus 10 μM forskolin for 30 min in a CO_2 incubator, after which 0, 1, 5, and 10 mM acetate were added to the incubation medium in the absence or presence of 100 μM oleic acid or 100 μM palmitic acid. Subcutaneous and i.m. adipose tissues also were incubated with 0, 1, 5, and 10 mM propionate. Following an additional 30-min incubation, adipose tissue samples were transferred to test tubes containing 500 μL of cell lysis solution (Cell Signaling Technologies, Danvers, MA). Each adipose tissue sample was sonicated in lysis buffer for 15 s, the fatty acid layer was removed with a Pasteur pipet, and samples were stored at −80°C.

9.2.4 Tissue cAMP

The concentration of cAMP was determined based on the principle of competitive binding using the Cyclic AMP XP Assay Kit (Cell Signaling Technologies) according to the manufacturer's instructions. Adipose tissue explants were incubated at room temperature for 3 hrs. in 96-well plates with the HRP-linked cAMP substrate coated onto an immobilized rabbit monoclonal cAMP antibody using a horizontal orbital plate shaker. After the reaction, color development was measured at 450 nm using an Epoch microplate reader (Biotek Instruments, Winooski, VT). All samples were analyzed in duplicate. A standard curve and cAMP concentrations were calculated using Microsoft Excel. Results are reported as pmol cAMP/100 mg adipose tissue.

9.2.5 Tissue glycerol and free fatty acids

Concentrations of glycerol and FFA in adipose tissue samples for the short- term incubations were measured in the tissue lysates using the Glycerol Assay Kit (Sigma-Aldrich, St. Louis, MO). The highest concentrations of glycerol was in adipose tissue lysates. Results are reported as pmol glycerol/100 mg adipose tissue. Concentrations of FFA in adipose tissue samples for the short- term incubations were measured in the tissue lysates using the Free Fatty Acid Assay Kit (Cell Biolabs Inc, San Diego, CA). Results are reported as pmol free fatty acid/100 mg adipose tissue.

9.2.6 RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was extracted from ~ 200 mg of i.m. and s.c. tissues following slaughter using Qiazol (Qiagen, Hilden, Germany) as per manufacturer's instructions and treated with RNase-Free DNase (Qiagen). The RNA was further purified using the RNeasy Lipid Mini Kit (Qiagen) as per manufacturer's instructions. The purity, concentration, and integrity of the total RNA from each sample were quantified using a NanoDrop spectrophotometer (Thermo Scientific, 2000C, Washington, DE) and the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA) assessed the RNA integrity. The purity of the RNA (A260/A280) was > 1.85, and the A260/A230 ratio was > 2.0 in all samples. Only samples with an RNA integrity number (RIN) greater than 7 were used. First-strand cDNAs were synthesized from 1μg of total RNA using oligo (deoxythymidine) primers and SuperScript II Reverse Transcriptase (Invitrogen, Waltman, MA) as per the manufacturer's instructions. Negative controls without reverse transcriptase were included to verify a lack of genomic contamination. Quantitative polymerase chain reaction (qPCR) was performed using the ABI prism 7900HT system (Applied Biosystems, Foster City,

CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) as specified by the manufacturer to determine the levels of expression of mRNA encoding for genes of interest. Primer sequences are listed in Table 1. Primer efficiency and specificity were tested by generating a standard curve from pooled cDNA and by the inclusion of a dissociation curve for the qPCR reaction, respectively. Serial dilutions of pooled cDNA in nuclease-free water ranging from 1:2 to 1:256 (50, 25 12.5, 6.25, 3.125, 1.56, 0.781, 0.39 uM) were used as standards. All primer sets used amplified a single product (i.e., a dissociation curve with a single peak) and had an efficiency of between 95% and 100%. Each well contained 10% diluted cDNA, 30% nuclease-free water, 10% primer, and 50% SYBR Green reaction mix in a 10 µL reaction volume. Each sample was run in triplicate using the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed at an annealing temperature of 60°C. For primers of interest with lower expression (i.e., Ct values above 30; AMPKα), 1 µL of cDNA was used in a modified pre-amplification step using a Thermocycler (Eppendorf AG) (Vermeulen et al., 2009). Briefly, cDNA, nuclease-free water, forward and re verse primer, and SYBR were combined as described above in a 20-µL volume. The reaction was performed with the following conditions for 15 cycles: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. *GPR120*, *SCD*, and *AMPKα* gene expression was measured in i.m. and s.c. adipose at 20, 22, and 24 mo of age (Table 1). We were unable to detect GPR40, GPR41, GPR43, FASN, PPARγ, and C/EBPb. Relative gene expression levels were calculated by the cycle threshold (CT) deviation of samples vs. *40S ribosomal protein S9* (*RPS9*) reference gene and were quantified by using the $2^{-\Delta\Delta CT}$ method.

Table 1. Forward and reverse primers for real-time PCR.

Item	Accession no.	Sequence (5' to 3')
GPR120	NM_001328657	Forward: TCCAGAACTTCAAGCAGAAC
		Reverse: AAACAGGGACATGTTGTAGAG
SCD	AB075020	Forward: TGCCCACCACAAGTTTTCAG
		Reverse: GCCAACCCACGTGAGAGAAG
AMPKa	NM_001109802	Forward: ACCATTCTTGGTTGCTGAAACTC
		Reverse: CACCTTGGTGTTTGGATTTCTG
RPS9	DT860044	Forward: GAGCTGGGTTTGTCGCAAAA
		Reverse: GGTCGAGGCGGGACTTCT

9.2.7 Statistical analysis.

Data were analyzed by 2-factor analysis of variance (SuperNova, Abacus Concepts, Berkeley, CA). Differences between means were determined using the Fisher's LSD procedure (SuperNova). Means were considered significantly different at $P < 0.05$. Means were considered at trend from $P > 0.06$ to $P < 0.10$.

9.3 Results

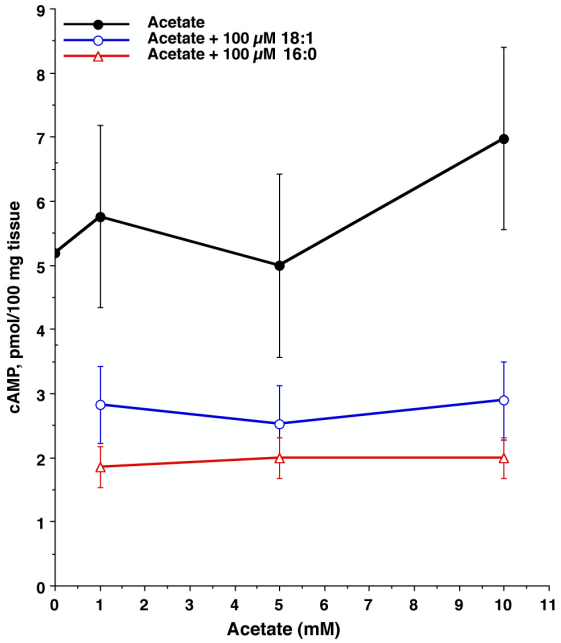
9.3.1 Tissue cAMP

At 22 mo of age, forskolin-stimulated cAMP production in the presence of VFA and LCFA was depressed in s.c. adipose tissue (Figure 19). In i.m. adipose tissue, treatments with propionate and LCFA demonstrated the greatest reduction in cAMP production in steers at 22 mo of age.

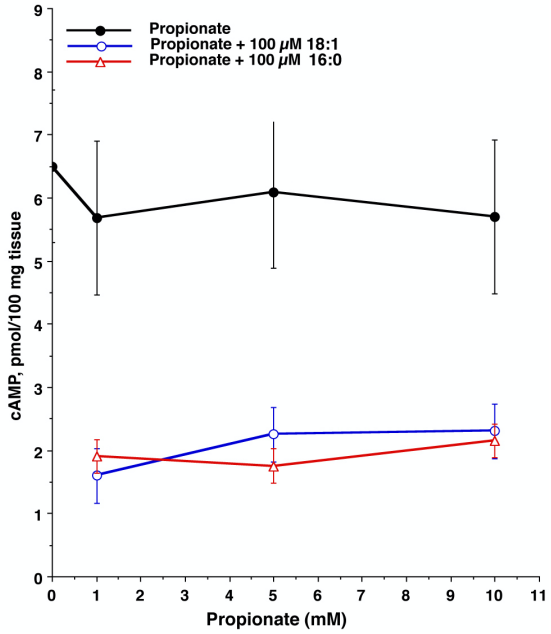
Main effects of cAMP, glycerol, and free fatty acids are shown in Table 2. Forskolin-stimulated cAMP production in the absence of VFA or LCFA was greater in s.c. adipose tissue at 20 mo of age as compared to i.m. adipose tissue at 20 mo of age ($P < 0.0001$) (Table 3) (Figure 20). In i.m. adipose tissue, forskolin-stimulated cAMP production was not affected by age ($P > 0.05$). In s.c. adipose tissue, the addition of acetate and propionate in the presence of LCFA greatly depressed cAMP production as compared to the control ($P < 0.0001$) (Figure 21). Forskolin-stimulated cAMP increased in the presence of both acetate and propionate in s.c. adipose tissue. Treatment had no effect on cAMP production in i.m. adipose tissue ($P > 0.05$).

Forskolin-stimulated cAMP production in the presence of VFA were greater in s.c. adipose than i.m. adipose tissue. In i.m. adipose tissue, the addition of acetate with or without oleic acid decreased cAMP production as compared to s.c. adipose tissue. The addition of VFA in the presence or absence of LCFA depressed cAMP production in steers at 20 mo of age as compared to the control ($P = 0.021$) (Table 4). At 22 mo of age, VFA in the presence of LCFA effectively decreased cAMP production when compared to other treatments. Forskolin-stimulated cAMP production decreased from 20 mo of age to 22 mo of age in treatments with acetate and oleic acid (Figure 22).

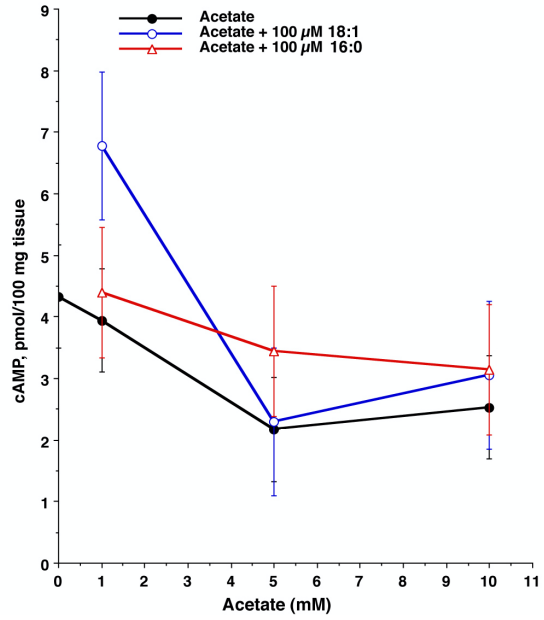
A


Acetate
Acetate + 100 μM 18:1
Acetate + 100 μM 16:0
cAMP, pmol/100 mg tissue
Acetate (mM)

B


Propionate
Propionate + 100 μM 18:1
Propionate + 100 μM 16:0
cAMP, pmol/100 mg tissue
Propionate (mM)

C



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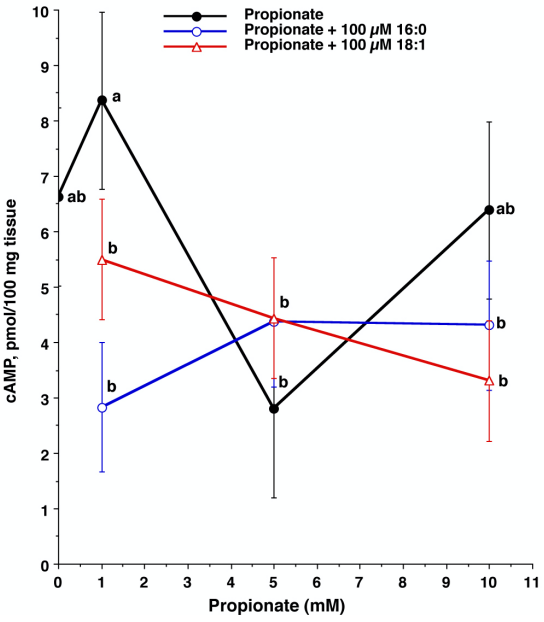


Figure 19. Forskolin-stimulated cAMP as a function of media acetate (A) and propionate (B) concentration in s.c. and acetate (C) and propionate (D) concentration in i.m. adipose tissue explants at 22 mo of age. Media containing acetate and propionate also contained 100 µM oleic acid (18:1) or palmitic acid (16:0). [ab]Means without a common superscript differ ($P < 0.05$).

Table 2. Main and interaction effects for cAMP, glycerol, and FFA production from Experiment 1 and 2 pooled across all concentrations in i.m. and s.c. adipose tissue in steers at 20 and 22 mo of age.

	P-value		
Main Effects	cAMP	Glycerol	FFA
Tissue	< 0.0001	0.441	< 0.0001
Treatment	< 0.0001	0.585	0.617
Concentration	0.0004	0.398	0.789
Age	< 0.0001	< 0.0001	N/A
Tissue x treatment	< 0.0001	0.012	< 0.0001
Tissue x concentration	0.156	0.502	0.029
Tissue x age	< 0.0001	0.019	N/A[1]
Treatment x Concentration	0.979	0.954	0.006
Age x treatment	0.021	0.313	N/A
Age x concentration	0.066	0.273	N/A
Tissue x treatment x age	0.131	0.034	N/A
Tissue x age x concentration	0.022	0.218	N/A
Tissue x treatment x concentration	0.953	0.894	0.839

[1]N/A = not applicable

Table 3. Tissue x age and tissue x treatment interactions for cAMP in i.m. and s.c. adipose tissue in steers at 20 and 22 mo of age.

			Tissue Pooled SEM	P-value
	I.M.	S.C.		Tissue x Age
Age (mo)			0.37	< 0.0001
20	3.35[b]	12.55[a]		
22	4.73[b]	4.88[b]		
				Tissue x Treatment
Treatment			0.37	< 0.0001
Control	5.97[cd]	11.88[a]		
Acetate	3.18[d]	9.96[ab]		
Acetate + oleic acid	4.37[d]	7.75[bc]		
Acetate + palmitic acid	4.68[cd]	4.09[d]		
Propionate	4.26[d]	10.13[ab]		
Propionate + oleic acid	3.00[d]	3.27[d]		
Propionate + palmitic acid	2.92[d]	4.98[cd]		
				Tissue x Concentration
VFA concentration (mM)			0.37	0.156
0	5.97	11.57		
1	4.26	8.70		
5	3.61	6.93		
10	3.83	6.07		

[abcd]Tissue x age and tissue x treatment interactions; means without a common superscript differ ($P < 0.05$).

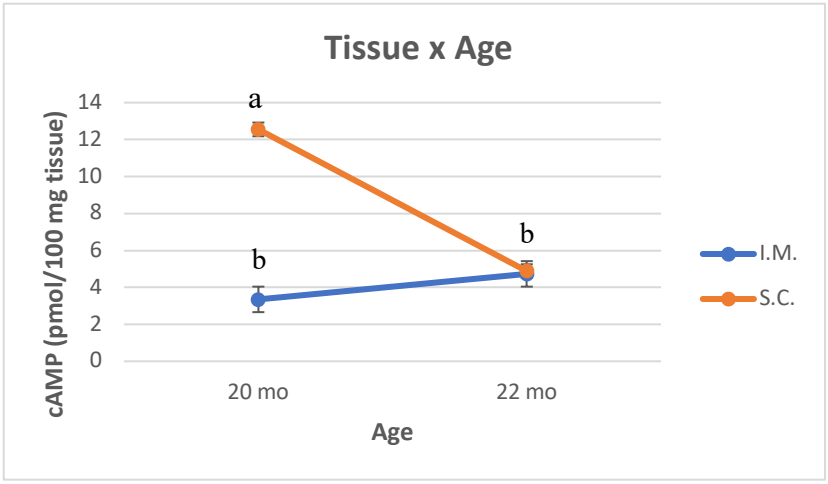


Figure 20. Forskolin-stimulated cAMP production in i.m. and s.c. adipose tissue at 20 and 22 mo of age. Data were pooled across treatment and VFA concentration. [ab]Means without a common superscript differ ($P < 0.05$).

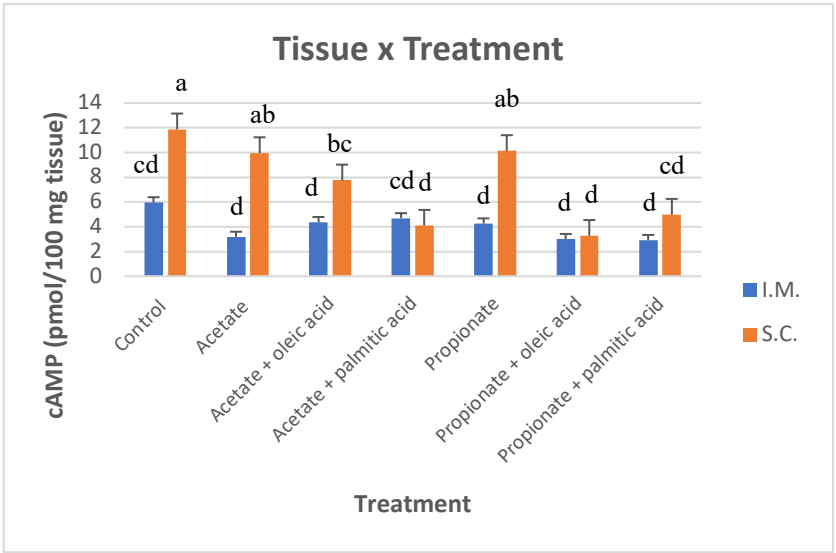


Figure 21. Tissue and treatment effects on cAMP production in i.m. and s.c. adipose tissues. Data were pooled across age and VFA concentration. [abcd]Means without a common superscript differ ($P < 0.05$).

Table 4. Treatment and age interactions for cAMP and glycerol production (pooled across concentrations) in steers at 20 and 22 mo of age.

	Age (mo)			P-value
Treatment	20	22	Pooled SEM	Age x Treatment
cAMP, nmol/100 mg			0.37	0.021
Control	12.01[a]	6.44[bc]		
Acetate	8.05[b]	5.18[c]		
Acetate + oleic acid	8.78[b]	3.50[d]		
Acetate + palmitic acid	4.21[c]	4.55[cd]		
Propionate	6.92[bc]	7.39[bc]		
Propionate + oleic acid	ND	3.14[d]		
Propionate + palmitic acid	ND	3.95[d]		
Glycerol, nmol/10 mg			0.002	0.313
Control	59	87		
Acetate	70	87		
Acetate + oleic acid	62	78		
Acetate + palmitic acid	56	76		
Propionate	65	83		
Propionate + oleic acid	ND[1]	74		
Propionate + palmitic acid	ND	78		

[abcd]Age x treatment interactions; means without a common superscript differ ($P < 0.05$). [1]ND, not determined. Data were pooled across tissue and VFA concentration.

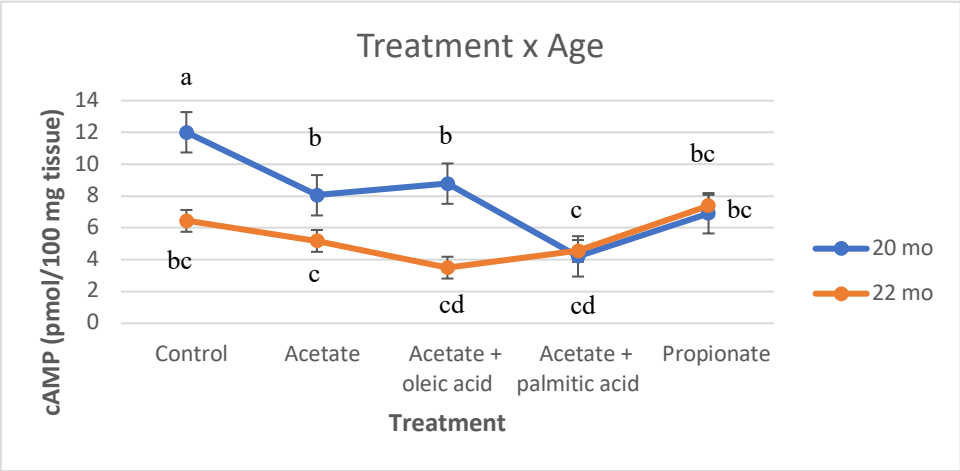


Figure 22. Treatment and age effects on cAMP production in steers at 20 and 22 mo of age. Data were pooled across treatment and VFA concentration. [abcd]Means without a common superscript differ ($P < 0.05$).

9.3.2 Tissue glycerol and free fatty acids

At 22 mo of age, glycerol concentrations increased in i.m. and s.c. adipose tissues. At 20 and 22 mo of age, glycerol release was not affected by tissue type ($P < 0.019$) (Table 5) (Figure 23). In i.m. adipose tissue, glycerol release was depressed in treatments containing acetate plus LCFA as compared to the control. Glycerol concentrations were elevated in treatments containing propionate plus LCFA in i.m. adipose tissue. In s.c. adipose tissue, the combination of propionate in the presence of LCFA decreased the release of glycerol as compared to the control ($P = 0.012$). In i.m. adipose tissue, the addition of acetate plus oleic decreased the release of glycerol when compared to s.c. adipose tissue. Propionate while in the presence of LCFA reduced glycerol release in s.c. adipose but increased glycerol in i.m. adipose tissue (Figure 24).

Table 5. Tissue x age and tissue x treatment interactions for glycerol production (nmol/100 mg tissue) in i.m. and s.c. adipose tissue in steers at 20 and 22 mo of age.

	Tissue			P-value
	I.M.	S.C.	Pooled SEM	Tissue x Age
Age (mo)			0.016	0.413
20	68	65		
22	79	81		
				Tissue x Treatment
Treatment			0.016	0.063
Control	0.70[bc]	0.79[b]		
Acetate	0.75[bc]	0.83[ab]		
Acetate + oleic acid	0.65[c]	0.76[b]		
Acetate + palmitic acid	0.59[c]	0.75[bc]		
Propionate	0.78[bc]	0.72[bc]		
Propionate + oleic acid	0.85[ab]	0.64[c]		
Propionate + palmitic acid	0.89[ab]	0.68[c]		
				Tissue x VFA Concentration
VFA concentration (nM)			0.016	0.539
0	0.70	0.78		
1	0.82	0.76		
5	0.72	0.75		
10	0.74	0.73		

[abc]Tissue x age and tissue x treatment interactions; means without a common superscript differ ($P < 0.05$).

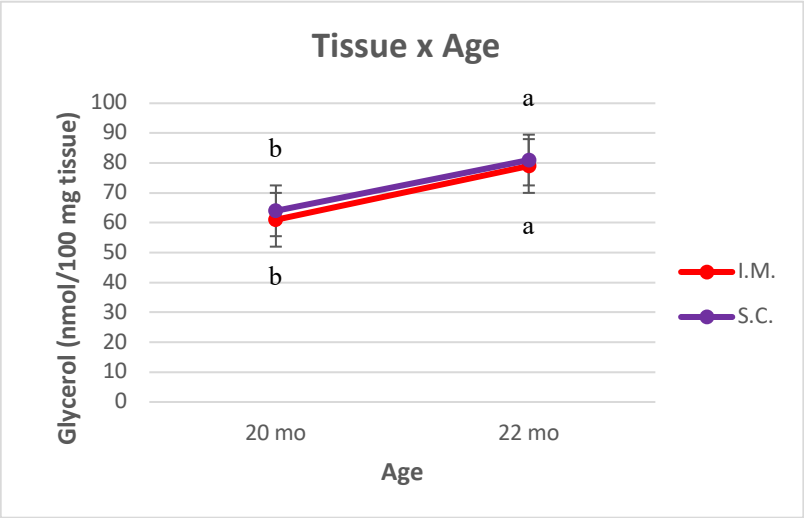


Figure 23. Glycerol production in i.m. and s.c. adipose tissue at 20 and 22 mo of age. Data were pooled across treatment and VFA concentration. [ab]Means without a common superscript differ ($P < 0.05$). Age effect $P < 0.0001$.

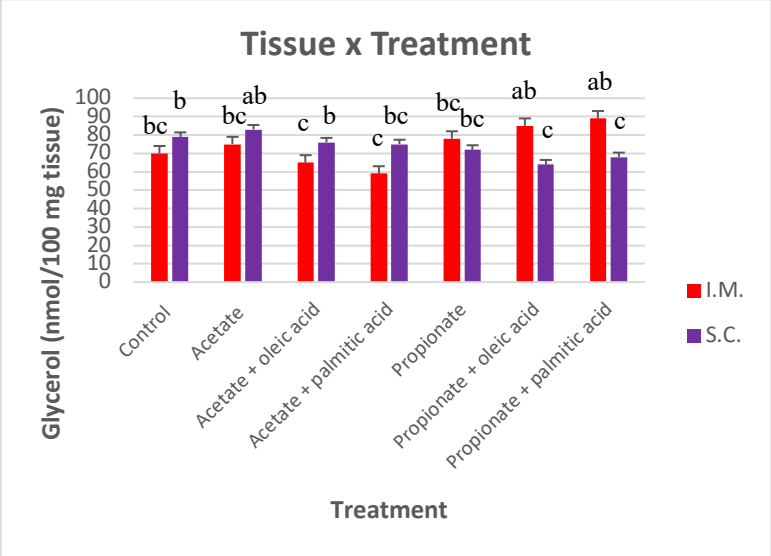


Figure 24. Treatment effects of glycerol production in i.m. and s.c. adipose tissues. Data were pooled across age and VFA concentration. [abc]Means without a common superscript tended to be different ($P < 0.063$).

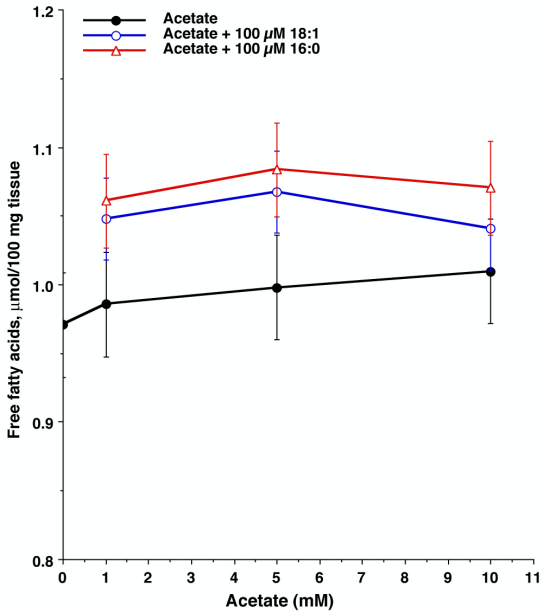
In the s.c. adipose tissue, acetate depressed FFA release as compared to all propionate treatments at 22 mo of age ($P < 0.0001$) (Table 6) (Figure 25A). In i.m. adipose tissue, acetate increased the release of FFA when compared to all other treatments at 22 mo of age ($P < 0.0001$) (Figure 25B). In i.m. adipose tissue, acetate in the presence or absence of LCFA elevated FFA release when compared to s.c. adipose tissue. At 22 mo of age, the combination of propionate and LCFA decreased FFA release in i.m. adipose tissue. In i.m. adipose tissue, all media concentrations (0, 1, 5 and 10 nM) increased the release of FFA as compared to s.c. adipose tissue ($P = 0.029$).

Table 6. Tissue x treatment and tissue x concentration interactions for free fatty acid production (umol/100 mg tissue) in i.m. and s.c. adipose tissue in steers at 22 mo of age.

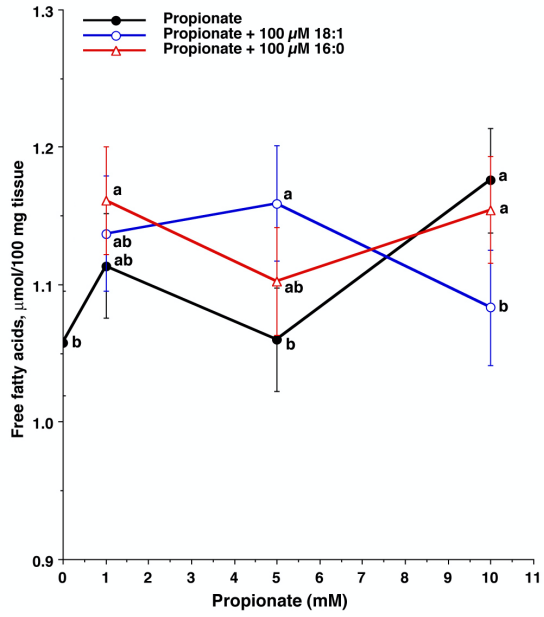
	Tissue			P-value
	I.M.	S.C.	Pooled SEM	Tissue x Treatment
Treatment			0.008	< 0.0001
Control	1.29[a]	1.01[d]		
Acetate	1.26[a]	1.00[d]		
Acetate + oleic acid	1.20[b]	1.05[cd]		
Acetate + palmitic acid	1.22[b]	1.07[cd]		
Propionate	1.22[b]	1.11[c]		
Propionate + oleic acid	1.15[c]	1.12[c]		
Propionate + palmitic acid	1.17[bc]	1.13[c]		
				Tissue x VFA Concentration
VFA Concentration (nM)			0.008	0.029
0	1.29[a]	1.01[c]		
1	1.27[b]	1.08[c]		
5	1.20[b]	1.07[c]		
10	1.24[b]	1.08[c]		

[abcd] Tissue x age, tissue x treatment, and tissue x concentration interactions; means without a common superscript differ ($P < 0.05$).

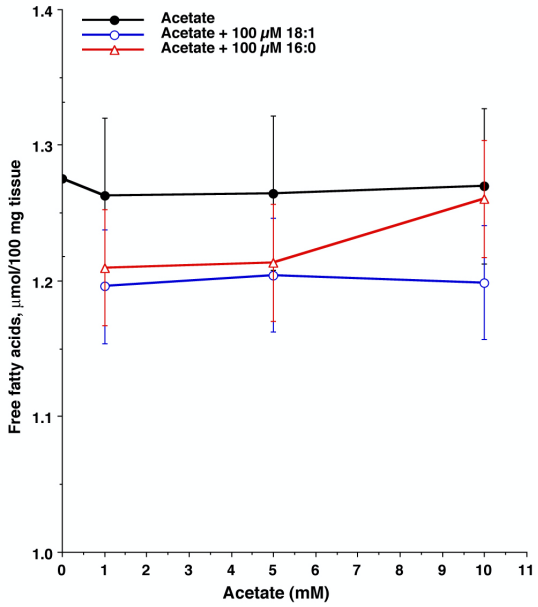
A


Acetate
Acetate + 100 μM 18:1
Acetate + 100 μM 16:0
Free fatty acids, μmol/100 mg tissue
Acetate (mM)
1.2
1.1
1.0
0.9
0.8
0
1
2
3
4
5
6
7
8
9
10
11

B


Propionate
Propionate + 100 μM 18:1
Propionate + 100 μM 16:0
Free fatty acids, μmol/100 mg tissue
Propionate (mM)
1.3
1.2
1.1
1.0
0.9
a
a
ab
ab
ab
b
b
a
a
b
0
1
2
3
4
5
6
7
8
9
10
11

C



D

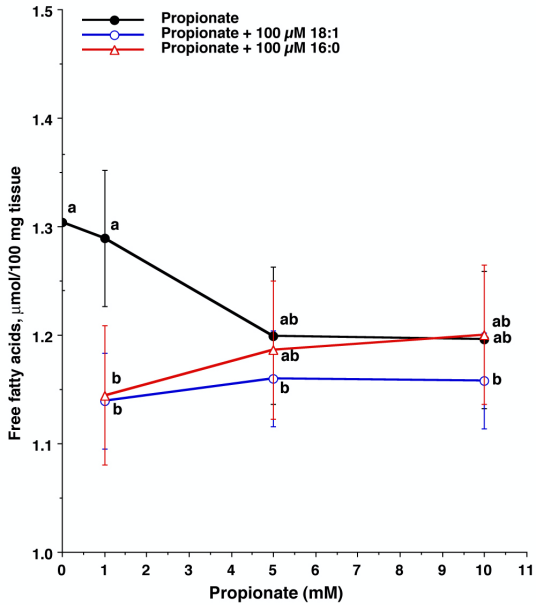


Figure 25. FFA release as a function of media acetate (A) and propionate (B) concentration in s.c. and acetate (C) and propionate (D) concentrations in i.m. adipose tissue explants at 22 mo of

age. Media containing acetate and propionate also contained 100 µM oleic acid (18:1) or palmitic acid (16:0). [ab]Means without common superscript differ ($P < 0.05$).

9.3.3 Gene expression

Main and tissue x age effects for adipogenic gene expression are shown in Table 7. *GPR120*, *SCD*, and *AMPKα* gene expression was measured in i.m. and s.c. adipose at 20, 22, and 24 mo of age. We were unable to detect *GPR40, GPR41, GPR43, FASN, PPARγ*, and *C/EBPb*. In s.c. adipose, *GPR120* and *SCD* expression were greater than in i.m. adipose tissue ($P = 0.05$ and $P < 0.0001$, respectively) (Table 8). Additionally, *AMPKα* gene expression tended to increase gene in s.c. adipose tissue ($P = 0.081$). Age did not significantly affect AMPKα, GPR120, or SCD ($P > 0.05$). GPR120 expression tended to increase from 20 mo of age to 22 mo of age in both i.m. and s.c. adipose tissues ($P = 0.078$). SCD expression tended to increase from 20 mo of age to 24 mo of age in s.c. adipose tissue ($P = 0.088$) (Figure 26).

Table 7. Main and tissue x age effects for adipogenic relative gene expression.

	P-value		
Effects	*AMPKα*	*GPR120*	*SCD*
Tissue	0.081	0.050	< 0.0001
Age	0.209	0.314	0.198
Tissue x Age	0.608	0.078	0.088

Table 8. Tissue means for adipogenic relative gene expression.

	Tissue			
Gene	IM	SC	Pooled SEM	P-value
AMPKα	0.70	0.76	0.018	0.081
GPR120	1.02[b]	1.62[a]	0.153	0.05
SCD	0.75[b]	2.43[a]	0.218	< 0.0001

[ab]Means without a common superscript differ ($P < 0.05$).

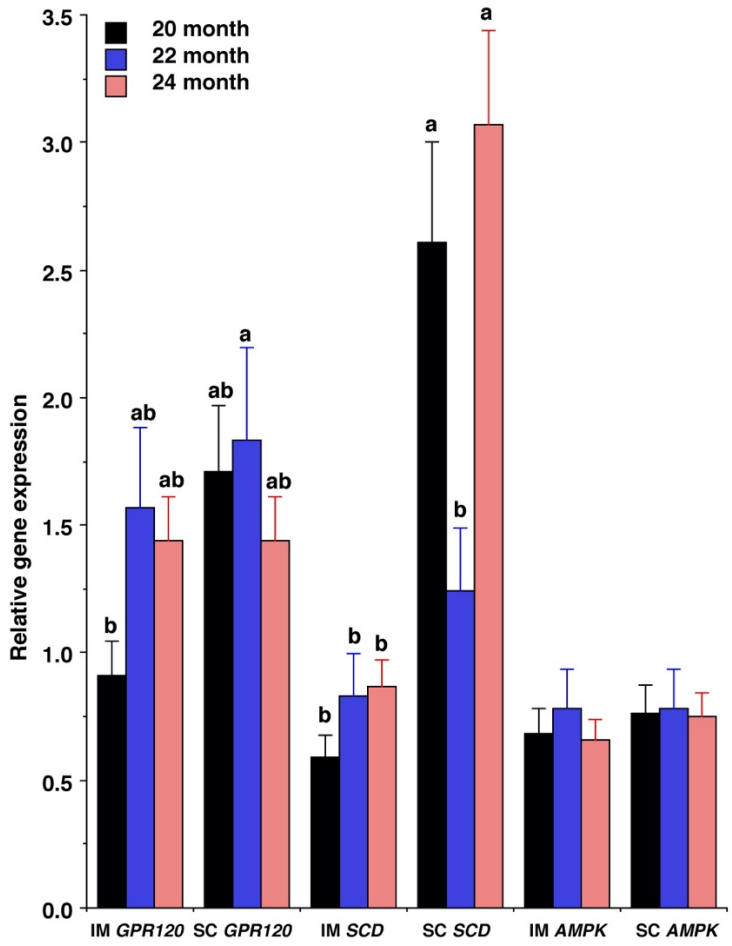


Figure 26. Tissue by age interactions for gene expression in i.m. and s.c. adipose tissues. [ab]Means within a gene without common superscript tended to be different ($P < 0.09$). There were no significant tissue x age interactions for *AMPKα* ($P = 0.608$).

Hot carcass weight significantly increased with steer age (24 > 22 > 20 mo of age) (Table 9). Ribeye area and kidney, pelvic, and heart fat (KPH) were not significantly different at 20 and 22 mo of age but increased at 24 mo of age. Yield grade increased from 20 mo of age to 22 mo of age but was not significantly different between 22 and 24 mo of age. There was a significant increase in marbling score from 20 mo of age to 22 mo of age. Quality grade and adjusted fat thickness (AFT) also increased from 20 mo of age to 22 mo of age.

Table 9. Carcass values for Angus cross bred cattle slaughtered at 20, 22, and 24 months of age.

	Age, mo				
Item	20	22	24	SEM	*P*-value
Hot carcass weight, kg	254.0[c]	297.7[b]	347.3[a]	7.3	< 0.001
Overall maturity[2]	46.3[b]	69.4[a]	63.8[a]	3.5	< 0.001
Ribeye area, cm^2	67.3[b]	69.2[b]	79.9[a]	2.2	< 0.001
Adjusted fat thickness, cm	1.14[b]	2.05[a]	2.32[a]	0.14	< 0.001
Kidney, pelvic, heart fat, %	2.31[b]	2.25[b]	2.94[a]	0.14	0.004
Yield grade	2.87[b]	4.02[a]	4.31[a]	0.15	< 0.001
Marbling score[3]	108.8[b]	243.8[a]	191.3[a]	39.2	0.029
Quality grade[4]	86.8[b]	140.5[a]	127.9[a]	16.5	0.026

[1]Values are means for n = 8 steers per slaughter age. [2]Overall maturity: 0 – 100, A maturity. [3]Marbling score: 0 – 99 = Slight; 100 - 199 = Small; 200 – 299 = Modest; 300 – 399 = Moderate. [4]Quality grade: 0 – 99 = Select; 100 – 199 = Choice. [ab]Means without common superscripts differ ($P > 0.05$).

9.4 Discussion

9.4.1 Tissue cAMP

We previously demonstrated the absence of VFA or LCFA affected cAMP production differently in s.c. and i.m. adipose tissue. Similar, to our previous experiment, forskolin-stimulated cAMP production was greater in s.c. adipose tissue than i.m. adipose tissue, indicating greater AC activity in s.c. adipose tissue. Additionally, acetate in the absence of LCFA decreased cAMP production in i.m. adipose tissue at 22 mo of age, suggesting a greater GPR43 receptor in i.m. adipose tissue than s.c. adipose tissue. The presence of LCFA did not significantly influence cAMP production in i.m. adipose tissue, whereas the presence of LCFA decreased cAMP production in s.c. adipose tissue. These results suggest that LCFA have a greater affinity for GPR120 receptors in s.c. adipose tissue or s.c. adipose tissue has a greater abundance of GPR120 receptors. Consistent with our results, Widmayer et al. (2019) reported GPR120 receptor activation in the presence of LCFA, such as oleic acid, in culture murine adipocytes. A recent study by Husted et al. (2020) reported that LCFA-activated GPR120 inhibited lipolysis by decreasing intracellular cAMP through Gi-mediated inhibition of adenylate cyclase.

Volatile fatty acids alone did not affect cAMP concentrations in s.c. adipose tissue. However, all s.c. treatments demonstrated a substantial decrease in forskolin-stimulated cAMP from 20 mo of age to 22 mo of age, whereas acetate plus LCFA caused the greatest depression in cAMP at 22 mo of age, which would cause increased lipid accumulation at that age. Unlike Experiment 1, the addition of propionate in the presence of LCFA caused a clear depression of cAMP production in s.c. adipose tissue. This may indicate a greater affinity of the GPR43 receptor for propionate in s.c. adipose tissue than observed in the 20-mo-old steers.

In experiment 1, oleic acid reversed the acetate-induced decrease of cAMP production in i.m. adipose tissue, which is also consistent with results observed in experiment 2. However, in experiment 2, propionate in the presence or absence of LCFA effectively depressed cAMP production in i.m. adipose tissue, also indicating an affinity of the GPR43 receptor for propionate at 22 mo of age. Propionate is a major precursor of glucose synthesis in ruminant species. Subcutaneous adipose tissue preferentially uses acetate as a substrate for *de novo* fatty acid biosynthesis in s.c. adipose tissue, whereas glucose is the primary fatty acid substrate in i.m. adipose tissue (Smith and Crouse, 1984).

9.4.2 Tissue glycerol and free fatty acids

Glycerol release was low to undetectable, indicating incomplete hydrolysis of TAG. Treatment with VFA and LCFA decreased FFA release in i.m. adipose tissue as compared to s.c. adipose tissue. A human study by Akanji et al. (1989) reported a reduction in plasma FFA levels during intravenous acetate infusions, suggesting the inhibition of lipolysis via the GPR43 receptor. Aberdein et al. (2014) demonstrated that murine 3T3-L1 adipocytes treated with sodium acetate decreased FFA release. Ge et al. (2018) reported a reduction in plasma FFA levels in murine adipocytes treated with acetate, indicating that GPR43 activation in adipocytes can lead to the suppression of FFA release. Similar to cAMP results, propionate in the presence or absence of LCFA reduced FFA release in i.m. adipose tissue, whereas acetate in the presence or absence of LCFA depressed FFA release in s.c. adipose tissue, suggesting tissue-specific preferences for VFA.

9.4.3 Gene expression and carcass characteristics

The relative expression of *GPR120* and *SCD* was greater in s.c. adipose tissue than i.m. adipose tissue in Angus steers. Choi et al. (2014) also reported greater *SCD* gene expression in s.c. adipose tissue than i.m. adipose tissue in cattle. Furthermore, Agrawal (2017) reported that *GPR120* was expressed in s.c. adipose tissue of Holstein steers, which is consistent with the results of Experiment 1 (decrease in cAMP production by LCFA in s.c. adipose tissue). Previous cAMP data suggested that GPR43 and GPR120 respond differently to s.c. adipose tissue when exposed to LCFA. Volatile fatty acids (mainly acetate) and LCFA depresses cAMP via the GPR43 receptor, thereby inhibiting lipolysis. Our data indicate that GPR120 also inhibits lipolysis by decreasing cAMP through Gi-mediated inhibition of adenylate cyclase. These results provide evidence for functional GPR43 receptors in i.m. adipose tissue and GPR120 receptors in s.c. adipose tissue, both of which suppress lipolysis.

AMPKα a tended to be elevated in s.c. adipose tissue, indicating increase lipolysis in s.c. adipose tissue, and thereby leading to a decrease in carcass fatness. Generally, age did not significantly influence AMPKα, GPR120, or SCD. However, GPR120 tended to increase from 20 mo of age to 22 mo of age in both tissues, with i.m. adipose tissue demonstrating the greatest increase between 20 and 22 mo of age, which may suggest elevated GPR120 receptor activity in i.m. adipose tissue from 20 to 22 mo of age. SCD tended to increase with age (20 mo vs 24 mo of age) in s.c. adipose tissue, indicating that SCD gene expression may increase from 20 mo to 24 mo of age in cattle adipose tissue.

Yield grade increased more than a full grade between 20 and 22 mo of age, while AFT and KPH increased in steers from 20 mo of age to 24 mo of age, indicating an overall increase in

carcass fatness between 20 mo of age and 24 mo of age. Steers at 22 mo of age had a numerically higher marbling score (Modest) and quality grade (Choice). Steers at 24 mo of age also graded as Choice but had a lower marbling score (Small). Furthermore, *GPR120* gene expression tended to be greater at 22 mo of age for both i.m. and s.c. adipose tissue, suggesting that fat accumulation may peak in steers at 22 mo of age. There was a relationship between marbling score and i.m. GPR120, but no pattern for AFT and s.c. GPR120. Despite this, 18:1 and 16:0 clearly had more profound effects on cAMP and glycerol concentrations in s.c. adipose tissue.

10. SUMMARY & CONCLUSION

Data from experiments 1 and 2 strongly indicate that VFA and LCFA affected cAMP production differently in i.m. and s.c. adipose tissues. The production of cAMP is greater in s.c. than in i.m. adipose, indicating greater AC activity in s.c. adipose tissue. Similarly, the relative expression of GPR120 was greater in s.c. adipose tissue than i.m. adipose tissue, which is consistent with data reported by Agrawal (2017). Long-chain fatty acids have been well-documented to be potent ligands for GPR120. GPR120 recognizes several different LCFA, but the receptor is more responsive to unsaturated fatty acids (Oh et al., 2014). Fain and Shepard (1975) first reported the antilipolytic and cAMP decreasing effect of FFA in adipocytes more than forty years ago, but the mechanism behind it has remained unclear. Recently, Husted et al. (2020) demonstrated that GPR120 decreased cAMP production in white murine adipocytes through Gi-mediated signaling by functioning as an FFA-activated, autocrine, negative feedback regulator of lipolysis (Figure 27). LCFA such as oleic and palmitic acid are some of the main FFA released from adipocytes upon lipolysis stimulation. Although, omega-3 fatty acids are more potent GPR120 agonists, a wide range of saturated and unsaturated LCFA, including the ones used in the present study, are potent and effective GPR120 agonists. In conclusion, these results provide evidence for functional GPR43 receptors in i.m. adipose tissue and GPR120 receptors in s.c. adipose tissue, both of which suppress lipolysis through Gi-mediated signaling.

The results of the current study indicate that fatty acids can be autocrine or paracrine regulators of lipolysis. During saturation or long-term aerobic exercise, adipose tissue releases fatty acids for fuel for those tissue rich in mitochondria (e.g., cardiac muscle). When plasma fatty

acids reach concentrations in excess of body requirements, depression of lipolysis by LCFA, via the GPR120 receptor, would attenuate lipolysis.

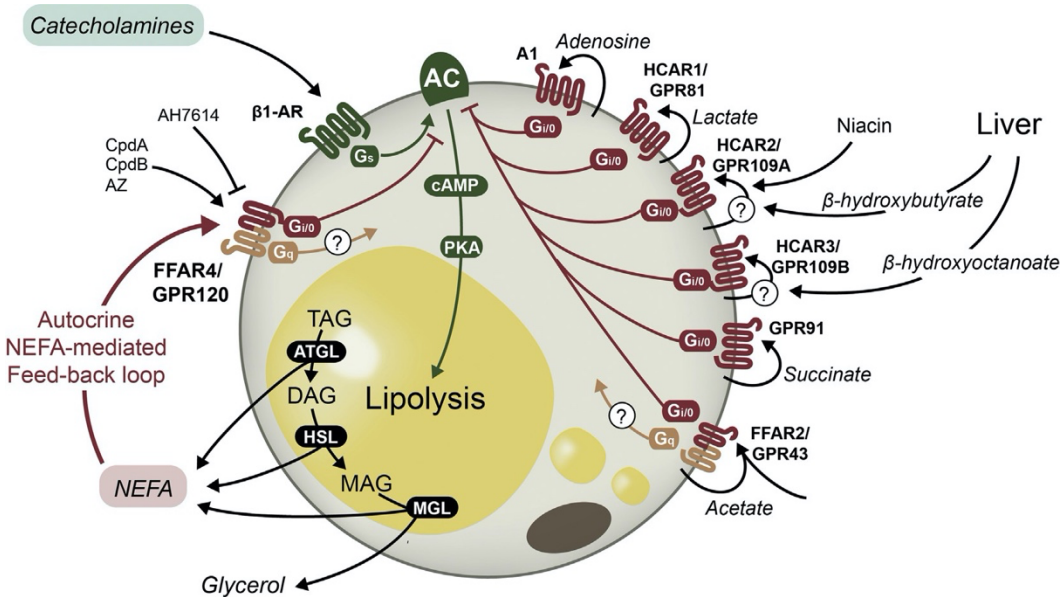


Figure 27. Simplified overview of control of lipolysis by GPR120. Reprinted with permission from [Husted et al].

The current study did not document short-term incubations with LCFA in the absence of VFA. Even though we expect the results to be consistent with previous data from experiment 1 and 2, it would be worthwhile to demonstrate possible similarities or differences between i.m. and s.c. adipose tissue when exposed to only LCFA. In conclusion, these data confirm that i.m. and s.c. adipose tissues respond differently to VFA and LCFA.

11. FUTURE RESEARCH AND GOALS

Never before have beef producers faced so many challenges and opportunities. The global demand for high protein animal products, especially beef has increased in response to rapid population growth, which has put additional pressure on the availability of land, water, and energy needed for beef and crop production. A significant increase in per capita beef consumption is also anticipated due to the rise of urbanization and wealth accumulation in developing countries. Feed costs have also increased dramatically partly due to the competition between bioenergy companies and beef producers for corn and other carbohydrate rich grains. The efficiency of animal production will need to be substantially greater to meet the increased global demand for animal products by 2050. Diverse and productive research programs will be essential to overcome potential threats to animal agriculture and food security.

Beef sustainability is at the forefront of many research programs. Therefore, the overarching goal for animal scientists is to obtain higher marbling scores earlier in the finishing period with lower overall cost of gains. Researchers have established that the embryonic and fetal stages are vital for the formation of muscle fibers, adipose (especially intramuscular fat), and connective tissues. Maternal nutrition during specific stages of gestation may alter muscle fiber composition and intramuscular fat, leading to more marbled carcasses (Costa et al., 2021a). Thus, future research studies are needed to better understand specific stages of gestation that affect carcass composition of the offspring; this will help improve meat quality and beef production efficiency.

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