REGULATION OF LIPID DESATURATION AND TURNOVER IN ADIPOSE TISSUE

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2018

Major Subject: Animal Science

ABSTRACT

Mechanisms in the regulation of lipogenesis or lipolysis in adipose tissue significantly contribute to not only uncovering novel treatments for metabolic diseases in human health, but also creating economic profits to markets by improving livestock meat quality. This dissertation describes the investigation of lipid desaturation by ectopically expressed porcine stearoyl-desaturase 1 (*SCD1*) in non-adipocytes and the extent of lipolysis mediated by various kinds of selective ß-adrenergic receptor (ß-AR) agonists *ex vivo* and *in vitro*.

Inducible lentiviral expression vectors were generated for over-expression or knock-down of porcine *SCD1* in the swine kidney 6 (SK6) cells. *pSCD1*-transduced SK6 cells successfully overexpressed *pSCD1* expression as compared to uninduced SK6 ($P < 0.05$). *pSCD1*-transduced SK6 cells transfected with pSCD1shRNA significantly suppressed *pSCD1* expression. Furthermore, the *pSCD1*-transduced cells incubated with 50 µM palmitic acid increased the synthesis of palmitoleic acid nearly 4-fold, indicating that the *pSCD1*-transduced cells successfully can induce the Δ^9 desaturation of palmitic acid to palmitoleic acid.

ß-AR subtypes were characterized in bovine subcutaneous (s.c.) and intramuscular (i.m.) adipose tissues with the use of selective $ß_1$-, $ß_2$,- and $ß_3$-AR agonists. The most abundant ß-AR mRNA in both adipose tissues was the $ß_2$-AR ($P < 0.05$). Isoproterenol, ractopamine, and zilpaterol stimulated the release of glycerol and nonesterified fatty acid (NEFA) from s.c. adipose tissue, but BRL-37344 did not affect lipolysis in s.c. adipose tissue in *ex vivo* cultures. A novel ß-agonist suppressed the stimulation of cAMP production mediated by $ß_1$- and $ß_2$-AR agonists in s.c. adipose tissue ($P <0.05$). In contrast, these ß-AR agonists were not effective in i.m. adipose tissue.

Finally, we investigated mechanisms regulating ß-AR stimulation mediated by dobutamine, salbutamol, and a novel ß-agonist in primary bovine s.c. and i.m. adipocytes. The stimulation of $ß_1$-AR through dobutamine significantly activated adenylyl cyclase and protein kinase A, and concurrently increased glycerol release in s.c. adipocytes, more than salbutamol did ($P < 0.05$). The effects by ß-AR agonists were blocked by propranolol. A novel ß-agonist inhibited adenylyl cyclase and protein kinase A activation in s.c adipocytes ($P < 0.05$). In contrast, these ß-AR agonists were not effective in i.m. adipocytes. In conclusion, this research has suggested the opportunity not only to develop a non-rodent biomedical model of obesity and metabolic disease but also to contribute to the understanding of functionality of ß-AR subtypes in adipose tissue during cattle growth and maturity.

DEDICATION

This dissertation is dedicated to my family, friends and colleagues.

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Smith and Dr. Charles Long for giving me the opportunity to purse my research and for motivation, patience, support, and guidance with my research. I would also like to thank my committee members Dr. Chaodong Wu and Dr. Wes Osburn for their guidance and support throughout the course of this research.

Furthermore, I would like to thank Dr. Neetu Singh for her patience and guidance with the generation of lentiviral vectors. I would like to thank current and past members of laboratory. Without your friendship and help, this work would have not been possible. I would like to also thank the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, I would like to thank my beloved mother, father and brother for their endless encouragement and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Dr. Stephen B. Smith (advisor) and Dr. Wes Osburn of the Department of Animal Science, Dr. Charles R. Long (co-advisor) of the Department of Veterinary Physiology & Pharmacology, and Dr. Chaodong Wu of the Department of Nutrition & Food Science.

All work for the dissertation was completed by Jinhee Hwang, under the advisement of Dr. Stephen B. Smith of the Department of Animal Science and Dr. Charles R. Long and Dr. Neetu Singh of the Department of Veterinary Physiology & Pharmacology.

This work was supported by National Institutes of Health (NIH), Elanco Animal Health, and Texas A&M University Department of Animal Science Mini-Grant. Graduate study was also supported by a Tom Slick Graduate Research Fellowship from Texas A&M University.

NOMENCLATURE

AC	Adenylyl cyclase
ß-AA	Beta-adrenergic agonist
ß-AR	Beta-adrenergic receptor
cAMP	Cyclic adenosine monophosphate
DOX	Doxycycline
FAME	Fatty acid methyl esters
GFP	Green fluorescent protein
HEK	Human embryonic kidney
i.m.	Intramuscular
MUFA	Monounsaturated fatty acid
NEFA	Non-esterified fatty acid
PKA	Protein Kinase A
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SCD1	Stearoyl-Coenzyme A desaturase-1
SFA	Saturated fatty acid
shRNA	Short hairpin RNA
SK6	Swine kidney cells
TRE	Transcriptional response element
UCOE	Ubiquitous chromatin opening element

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CHAPTER I

INTRODUCTION

Adipose tissue is the body's largest repository of energy, and it plays an important role in total energy homeostasis. When energy production exceeds energy expenditure of the body, the remaining energy is stored as the form of triglycerides in adipose tissue (Krahmer et al., 2013). Hence, the excessive accumulation of adipose tissue can be a major risk factor triggering serious health problems such as obesity and obesity-related disorders.

Dietary fat is one of the most important sources of nutrients for supporting our body and can be derived from animal fat and vegetable oils. However, the consumption of a fat-rich diet has been responsible for the growing problem of obesity. In fact, many studies have demonstrated that a high-fat diet can easily induce obesity in human and laboratory animals (Hill et al., 2000; Schrauwen and Westerterp, 2000; Ghibaudi et al., 2002; Jequier, 2002; Buettner et al., 2007). Some studies have also reported that fatty acid composition of the diets is a more important factor in diet-induced obesity rather than the amount of energy from fat (Bourgeois et al., 1983; DeLany et al., 2000; Wang et al., 2002; Kien et al., 2005) because saturated fatty acids (SFA) are more obesogenic than either monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) (Takeuchi et al., 1995; Piers et al., 2003; Ailhaud et al., 2006; Silva et al., 2006).

Stearoyl-CoA desaturase 1 (SCD1) is a central lipogenic enzyme and is highly responsible for the obesogenic effect derived from dietary SFA since SCD1 catalyzes biosynthesis of MUFA from SFA that is ether derived from the diet or synthesized *de novo* (Paton and Ntambi, 2009). MUFA, especially, palmitoleic (C16:1) and oleic (C18:1) acids are then used not only as major substrates for the synthesis of membrane phospholipids and triglycerides (Tocher et al., 1998; Miyazaki et al., 2001a), but also serve as mediators in signal transduction and cellular

differentiation (Bradley et al., 2008; Yonezawa et al., 2008). Thus, the abnormal alteration of ratio SFA to MUFA has been implicated in a key metabolic pathogenesis such as obesity, cardiovascular disease, and diabetes (Paton and Ntambi, 2009). These points of view have been supported by studies that showed *Scd1* deficient mice have increased energy expenditure, reduced body adiposity, and are resistant to diet-induced obesity (Ntambi et al., 2002). Therefore, SCD1 could be an important metabolic control point, and the regulations of *SCD1* expression and catalytic activity seem to expect an important point for the treatment of obesity and obesity-related disease.

Epidemiological studies have reported a significant correlation between MUFA intake and a decrease in the risk factors for coronary heart disease (CHD) (Baggio et al., 1988; Grundy et al., 1988; Kris-Etherton et al., 2002; Mente et al., 2009). Our laboratory has also demonstrated this effect in the studies through beef consumption enriched in either SFA and *trans-fatty* acids or enriched in oleic acid (Adams et al., 2010; Gilmore et al., 2011; Gilmore et al., 2013). Our studies have provided information that the major MUFA, oleic acid, from red meat can also be a good source as dietary fat for heart-healthfulness.

As the amount of intramuscular (i.m.) lipid increases, total MUFA concentration is also elevated in the cattle (Smith et al., 2009). The formation of i.m. adipose tissue plays an important economic role in U.S., Japanese, Korean and Australia beef production as i.m. adipose tissue is considered an important index used to evaluate beef quality traits because of its contribution to organoleptic attributes such as juiciness, flavor, and tenderness (Savell and Cross, 1998).

Control of lean and excessive subcutaneous (s.c.) adipose tissue deposition in livestock and meat industries is important, for it is directly linked to improvement of product quality, thereby leading to economic profits to providers, and satisfaction of consumers that desire a lean, reliable healthy product of consistent quality. Various strategies have been proposed to manipulate the fat

and muscle ratio for livestock production (Sillence, 2004). As one of the strategies, using synthetic ß-agonists (ß-AA) has been applied in livestock production for more than three decades due to its repartitioning effects. These ß-AA redirect nutrients away from adipose tissue and toward muscle disposition, which results in improvement of feed utilization, lean growth rate, and carcass lean percentage in cattle, pigs, poultry and sheep (Anderson et al., 2005). The mechanism of repartitioning effects includes both increase in muscle protein synthesis and decrease in their muscle protein degradation, and stimulation of triacylglycerol degradation and inhibition of fatty acid synthesis in adipose tissue (Emery et al., 1984; Jones et al., 1985; Moloney et al., 1990; Schiavetta et al., 1990; Smith et al., 1995). However, the precise mechanisms how the ß-AA control adipose tissue growth remains unclear. At present, most ß-AA commercially available, such as zilpaterol, ractopamine, cimaterol, terbutaline, matuberol and salbutamol, target $ß_1$- and $ß_2$-adrenergic receptors (ß-AR). The $ß_3$-AA have limited application in livestock production.

Elucidating aspects of lipogenesis or lipolysis mechanisms not fully understood in adipose tissue will make significant contributions to human health, not only suggesting therapeutic insights to uncover novel treatments for metabolic diseases in human, but also creating economic profits to markets by improving product quality. Therefore, we hypothesized that SCD1 ectopically modulated can effectively regulate lipid desaturation in the body. We also hypothesized that ß-AR subtypes would exhibit different responses in bovine s.c. and i.m. adipose tissues. Taken together, the objective of this dissertation includes the following:

- To generate lentiviral expression vectors to overexpression or knock-down of porcine *SCD1* in the porcine kidney cells as a preliminary study to develop porcine biomedical models for obesity-associated disorders and pork products with healthy characteristics.

- To characterize ß-AR subtypes in bovine s.c. and i.m. adipose tissues with the use of selective ß-AR agonists and antagonists, including a novel β-agonist Experior™.
- To investigate mechanisms regulating ß-AR stimulation mediated by selective ß-AR agonists and Experior™ in primary bovine s.c. and i.m. adipocytes.

CHAPTER II

LITERATURE REVIEW

Structure and physiology of adipose tissue

The adipose tissue in mammals consists of one-third adipocytes and two-thirds a combination of small blood vessels, nerve tissue, fibroblasts and preadipocytes, referring to as the stromovascular fraction (Geloen et al., 1989). Adipocytes have two functionally different types of fat cells, white and brown. The brown adipose tissue (BAT) has polygonal, multiple small-scattered lipid droplets with variable diameters, central nuclei, and a large number of mitochondria. The BAT is specialized in the dissipation of energy through cold and diet induced thermogenesis via mitochondrial uncoupling protein 1 (UCP1) (Hassan et al., 2012; Saely et al., 2012). Therefore, it is abundant in neonates and hibernating animals due to their inability to shiver. In contrast, white adipose tissue (WAT) is diametrically opposed to physiological function of BAT. It functions as the central repository of energy storage and secretion of hormones and cytokines that regulate body metabolism. Unlike BAT, WAT has spherical, single lipid droplet with a larger diameter (100 μm or more), peripheral nuclei, and variable amounts of mitochondria. In mammals, the WAT is mainly located beneath the skin (subcutaneous fat), around the kidney (perinephric fat), gonads (inguinal/gonadal fat), GI track (omental/mesenteric fat), between muscle (intermuscular fat), and within muscles (intramuscular fat) (Mersmann and Smith, 2005; Saely et al., 2012).

Adipose tissue mass is determined by hyperplasia (cell proliferation) and hypertrophy (cell enlargement) through lipid filing. Adipocyte number and size differ from adipose depot regions. In cattle, subcutaneous (s.c.) adipose tissue hyperplasia is complete about 8 mo of age, but intramuscular (i.m.) adipose tissue hyperplasia is process at 14 mo of age, indicating that i.m. is a late developing depot. Further increase in both adipose tissue mass is progressed by hypertrophy

(Hood and Allen, 1973). Furthermore, s.c. adipocyte diameter increased before 13 mo of age, whereas, i.m. adipocyte diameter increased before 15 mo of age. The average adipocyte diameter unchanged after 17 mo of age, suggesting that adipocyte hypertrophy increased until 17 mo of age in cattle (Cianzio et al., 1985).

Adipogenesis

Adipogenesis defines as the overall process of preadipocyte determination, growth, and terminal differentiation. Preadipocyte differentiation begins in late prenatal and early postnatal development in different anatomical sites (Spiegelman et al., 1993). The adipocyte lineage is derived from multipotent mesenchymal stem cells (MSC) by sequential differentiation pathways. The MSCs are programmed to become committed adipocyte lineage when appropriate differential signals are triggered. This process, known as determination, results in the conversion of the MSCs to preadipocytes, but has lost the capacity to differentiate into other cell types. Although there have been lots of efforts to define distinct intermediate steps in determination of primitive MSCs to the adipocyte lineage, it is still unknown what early molecular factors are really involved (Gesta et al., 2006).

Preadipocytes have a fibroblast-like morphology, and express surface markers such as preadipocyte factor 1(Pref-1), adipose tissue specific secretory factor (ADSF), alpha 2 chain of type VI collagen (COL6A2) and secreted frizzled related protein 2 (SFRP2). The SFRP2 is particularly plentiful in subcutaneous fat (Gesta et al., 2006). All preadipocyte markers are highly expressed in preadipocytes, and markedly reduced in mature adipocytes, indicating that they enable to control fat cell differentiation and adipose tissue development (Ibrahimi et al., 1993; Hu et al., 1998; Villena et al., 2002).

Preadipocytes undergo the terminal differentiation, that is, preadipocytes turn into mature adipocytes. This process involves four distinct states: growth arrest, clonal expansion, early differentiation, and terminal differentiation. It has been extensively studied in mouse 3T3-L1 and 3T3-F442A cell lines and immortalized brown adipocytes (Rosen and Spiegelman, 2000; Rosen and MacDougald, 2006). The first stage in the differentiation of preadipocytes starts from growth arrest. In cultured cell model, initial growth arrest is induced by the addition of a prodifferentiative hormonal agent and undergoes at least one round of DNA replication and cell division known as clonal expansion. In very early stage of differentiation, *c-fos, c-jin, junB, c-myc* are expressed (Cornelius et al., 1994). *C-myc* has been shown to initiate mitogenesis in the process of differentiation in preadipocytes, suggesting that *c-myc* may act as branch point at which the growth arrested cells face either directly cell cycle reentry or terminal differentiation (Ntambi and Young-Cheul, 2000).

After clonal expansion, preadipocytes undergo a second and final period of growth arrest to reach terminal differentiation into mature adipose phenotype. Preadipocyte early and terminal differentiation is controlled by the sequential expression of key transcription factors, including the *CCAAT/enhance binding protein (C/EBP)*, the *peroxisome proliferator-activated receptor (PPAR)* families, and the *adipocyte determination and differentiation factor-1/sterol response element binding protein 1c (ADD1/SPEBP1c)* (Ailhaud et al., 1992; Mandrup and Lane, 1997; Rosen et al., 2000). *C/EBP*β and *C/EBP*δ are the first transcription factors, which are control of exogenous promoters of induction of differentiation and accumulation of terminal transcription factors of adipogenesis, *PPAR*γ and *C/EBP*α (Wu et al., 1995; Clarke et al., 1997; Lane et al., 1999).

Terminal differentiation is accompanied by dramatic increase in the expression of *C/EBP*α*, PPAR*γ*, glycerophosphate dehydrogenase (GPDH), fatty acid synthase (FAS), acetyl CoA*

carboxylase (ACC), malic enzyme (ME), fatty acid binding protein (FABP) (Spiegelman et al., 1993), which is followed by *de novo* or enhanced expression of the genes that specialize the adipocyte phenotype, along with huge triglyceride accumulation.

Lipid droplets

Lipid droplets (or vacuoles) are ubiquitous, dynamic cellular lipid-storage organelles, function as lipid reservoirs for energy production, membrane synthesis, viral replication, and protein degradation. Lipid droplets consist of a nonpolar, neutral lipid core (i.e., triglyceride (TAG) and sterol esters) coated with a protein-bounded phospholipid monolayer, composing primarily of phosphatidylcholine, with lesser amounts of phosphatidylethanolamine, phosphatidylinositol, and lyso-phosphatidylcholine. Lipid droplet sizes vary dramatically from < 1 μm in diameter in fibroblasts to > 100 μm in white adipocytes (Tauchi-Sato et al., 2002; Walther and Farese, 2012).

Lipid droplet formation could trigger either *de novo* TAG synthesis in the endometrial reticulum (ER) or the fusion of smaller lipoproteins. Several models of lipid droplet formation have been proposed such as ER budding, bicelle formation, vesicular budding and eggcup. Despite these models for explaining how to form lipid droplets, the molecular mechanisms of lipid droplet formation remain unclear.

Wilfling et al. (2014) recently proposed a step wise model of lipid droplet formation: 1) neutral lipid synthesis; 2) lens formation (intramembrane lipid accumulation); and 3) lipid drop formation. Neutral lipid synthesis is mediated by enzymes of the membrane-bound O-acyltransferase (MBOAT) family (i. e., *acyl CoA:cholesterol acyltransferase (ACAT1), ACAT2*, and *acyl-CoA: acyltransferase (DGAT1)*) and *DGAT2* gene families, generally localized to the ER. Specifically, TAG are formed with diacylglycerol and fatty acyl-CoA produced by acyl-CoA synthetase

(ACSL) via DGAT between leaflets of the ER bilayer. When neutral lipid accumulation reaches a crucial threshold, lipid lenses may form in the ER as the oil phase coalesces. Once lipid droplets achieve a certain size, the bilayer detaches and a nascent lipid droplet buds into the cytoplasm (Wilfling et al., 2014). Further, the growth of lipid droplets involves the local synthesis of TAG at the surface of lipid droplets. In an excess environments of fatty acids, the volume of lipid droplets rapidly expand their volumes, and several proteins are involved to regulate lipid droplet size and number, such as the PAT family proteins (i.e., perilipin1, perilipin2/adipophilin (ADRP), perilipin3/Tip47, perilipin4/S3-12) CIDE (Cell Death Including DNA Fragmentation factor) proteins, and several lipases (Krahmer et al., 2013).

Fatty acids

Fatty acids (FA) are the major components of TAG, phospholipid, cholesterol esters, and wax esters. Fatty acids are comprised of a hydrogen carbon and a terminal carboxyl group, which is either saturated or unsaturated. Saturated fatty acids (SFA) do not have double bonds in the hydrocarbon chain whereas unsaturated fatty acids contain at least one double bond. The double bonds of unsaturated fatty acids have two distinct configurations, *cis* or *trans*, and are divided two subclasses: monounsaturated fatty acids (MUFA) with a single double bond and polyunsaturated fatty acids (PUFA) with two or more double bonds. Furthermore, based on chain length, they are categorized as short-chain, medium-chain, long-chain, or very-long-chain fatty acids: Short-chain ($< C_6$); medium-chain (C_6-C_{12}); long-chain (C_{13}-C_{22}); and very-long chain ($> C_{24}$) (Wang et al., 2013). In animals, the number of carbon atoms in fatty acids is typically between C_{14} and C_{24}, with oleic acid (C18:1n-9) being the most abundant fatty acid (St. John et al., 1987b; Wood et al., 2004).

Fatty acid biosynthesis

Lipogenesis is a physiological process of endogenous fatty acid synthesis (*de novo*), and subsequent TAG synthesis. It mainly takes place both liver and adipose tissue but varies among the spices. In rat, both liver and adipose tissue are the major lipogenic organs; the liver dominates in chickens, fish, and humans; and, adipose tissue is the primary site in ruminants and pigs (Vernon et al., 1999). Additionally, in ruminants, most dietary carbohydrate is fermented to acetate, propionate, and butyrate in the rumen. Thus, acetate is used as the primary precursor for *de novo* fatty acid synthesis in adipose tissue (Vernon, 1980).

The precursors of *de novo* fatty acid synthesis are derived from catabolism of carbohydrates and their metabolites (i. e., glucose, lactate, acetate, and pyruvate), and to a lesser extent, amino acids. Cytosolic acetyl-CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are substrates for *de novo* synthesis. As lipogenesis takes place in the cytosol, mitochondrial acetyl-CoA itself is not directly used for *de novo* synthesis the mitochondrial membrane is not a permeable to acetyl-CoA. Instead, citrate, which is a condensation product of acetyl-CoA and oxaloacetate in the tricarboxylic acid (TCA) cycle, is transported to the cytosol, where it is cleaved into oxaloacetate and acetyl-CoA by the enzyme ATP-citrate lyase (Smith and Prior, 1981; Mersmann and Smith, 2005). NADPH is synthesized through pentose phosphate pathway, and NADP-malate dehydrogenase, and NADP-isocitrate dehydrogenase in ruminants (Smith, 1983) and via the pentose phosphate pathway and NADP-malate dehydrogenase in non-ruminants (Shingfield et al., 2010).

De novo fatty acid synthesis is initiated by the carboxylation of acetyl-CoA to form malonyl-CoA via ACC. Next, another acetyl-CoA is condensed with malonyl-CoA by the FAS complex through multiple serial enzyme reactions, resulting in the synthesis of long-chain fatty

acids. The major end product of *de novo* synthesis is palmitic acid (C16:0). The palmitic acid has two fates: elongation by adding two-carbon moieties in a chain-elongation reaction, yielding stearic acid (C18:0) and desaturation via stearoyl-CoA desaturase.

Insulin is considered as one of the important hormones for the regulation of fatty acid biosynthesis, as it activates enzymes such as acetyl-CoA carboxylase, ATP-citrate layase, and the pyruvate dehydrogenase complex (Potapova et al., 2000; Holness and Sugden, 2003; Brownsey et al., 2006).

Stearoyl-coenzyme A desaturase

Stearoyl-CoA desaturase (SCD) is a key enzyme in the biosynthesis of MUFA from SFA that are either synthesized *de novo* or derived from the diet. SCD catalyzes the Δ^9-*cis* desaturation of fatty acyl-CoA substrates. SCD is bound to the ER and contains four membrane-spanning domains with the NH_2 and COOH termini facing the cytoplasm. The SCD reaction is an aerobic process and involves a three-component enzyme system comprised of 11lavoprotein-NADH-dependent cytochrome *b*5 reductase, cytochrome *b*5, and SCD (Heinemann and Ozols, 2003; Man et al., 2006; Paton and Ntambi, 2009).

The *SCD* genes are ubiquitously expressed in higher organisms and have a high degree of variability in the gene complements of *SCD* in vertebrate species (Hodson and Fielding, 2013). Four isoforms (*Scd1-Scd4*) are present in the mouse, of which *Scd1* is the best documented isoform (Sampath and Ntambi, 2006); whereas only two isoforms (*SCD1* and *SCD5*) have been identified in humans, pigs, sheep, cattle, and chickens (Lengi and Corl, 2008). In mice, the *Scd1* is expressed in the liver, brown and white adipose tissue, and sebaceous glands (Ntambi, 1999; Heinemann and Ozols, 2003; Miyazaki et al., 2005). *Scd2* is universally expressed in most tissue except for liver

(Tabor et al., 1998), *Scd3* is only expressed in the skin, and *Scd4* is expressed solely in the heart (Zheng et al., 2001; Miyazaki et al., 2003). In humans and livestock species, *SCD1* is expressed in adipose tissue, liver, muscle, lung, heart, intestine, and mammalian gland, although expression in the liver is lower than the mouse (Cameron et al., 1994; Wang et al., 2006; Lengi and Corl, 2008; Rezamand et al., 2014). *SCD5* is mostly expressed in the brain and pancreas in humans, cattle, pigs, sheep, and chickens (Wang et al., 2005; Lengi and Corl, 2008).

Ntambi and his colleges, forefront in the study of SCD, have created the *Scd1* global and various tissue-specific knockout murine models for investigating the role of SCD1 for over two decades. The *Scd1* global knockout model (*SCD1*$^{-/-}$) was generated by Miyazaki et al in 2001 using C37BL/6 or SV129 mice (Miyazaki et al., 2001b). The *Scd1* global knockout model is similar growth to wild-type mice on a chow diet, but they are leaner, accumulating less adipose tissue, although they consume 25% more food than wild type mice (Ntambi et al., 2002). In addition, the *Scd1* global knockout mice had elevated levels of plasma ketones and greater insulin sensitivity but reduced levels of plasma insulin and leptin compared with the wild-type mice (Ntambi et al., 2002). Moreover, the *Scd1* global knockout had very low levels of TAG in the VLDL and low-density lipoprotein (LDL) fractions compared to wild-type mice (Attie et al., 2002). Liver-specific *Scd1* knockout mice were generated by Miyazaki et al. in 2007 using Cre-lox technology (Miyazaki et al., 2007). Liver-specific *Scd1* knockout mice have the tolerance to high-carbohydrate diet, but not high-fat diet, which induced obesity and liver steatosis. Additionally, this model triggered a marked decrease in lipogenesis and a severe impairment of gluconeogenesis, leading to hyperglycemia and a reduction of liver carbohydrate (Miyazaki et al., 2007). Adipose tissue-specific *Scd1* knockout mice were generated by Hyun et al. in 2010 using Cre-lox technology (Hyun et al., 2010). The adipose tissue-specific *Scd1* knockout mice displayed an increase in

GLUT1 and *TFN-α* expression but a decrease in adiponectin expression (Hyun et al., 2010). Combined liver and adipose tissue-specific *Scd1* knockout mice (Flowers et al., 2012) showed a robust reduction in SCDl MUFA products in both s.c. and WAT but were not protected from either genetic obesity or diet-induced obesity.

SCD gene expression is highly regulated by dietary lipids (PUFAs, cholesterol, and vitamin A), hormonal factors, developmental processes, temperature, metals, alcohol, and peroxisomal proliferation (Ntambi, 1999). The predominant products of SCD are palmitoleoyl-and oleaoyl-CoA (Ntambi, 1999). Palmitoleic and oleic acid serve as the major constituents of membrane phospholipids and TAG, cholesterol ester (CE), and wax ester found in fat depots (Miyazaki et al., 2000; Miyazaki et al., 2001a; Miyazaki et al., 2001b). The ratio of stearic to oleic acids directly influences cell membrane fluidity, cell-cell interaction, signal transduction, and cellular differentiation (Kates et al., 1984; Gyorfy et al., 1997). An increasing SCD activity and, thereby, abnormal alteration between the ratio of stearic to oleic acids results in physiological and disease states such as aging, diabetes, obesity, heart disease and cancer (Ntambi, 1999). Therefore, the regulation of the *SCD* gene expression is an important for human health (Spector and Yorek, 1985; Clandinin et al., 1991).

ß-Adrenergic receptor

The adrenergic receptors (AR) belong to the G-protein-coupled receptors (GPCR), and have been divided into two major isoforms, α and ß. To date, seven α-AR subtypes (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, and α_{2D}) and three ß-AR subtypes ($ß_1$, $ß_2$, and $ß_3$) have been identified in various tissues in the body, with the ß-AR subtypes predominating in cardiac, airway smooth muscle and adipose tissue (Woodcock, 2007; Lynch and Ryall, 2008).

The bovine ß$_1$-, ß$_2$-, and ß$_3$-AR genes are located on chromosome 26, 7, and 27, respectively. The ß$_1$- and ß$_2$-AR genes encode an intronless gene transcript, whereas the β_3-AR gene encodes a transcript containing a single intron. The bovine ß-AR consist of 405-467 amino acid residues with a protein molecular weight ranging from 42.9 to 50.1 kDa (Table 1). There is a 64 to 69% homology between ß$_3$-, ß$_2$, and ß$_1$-AR. Like all GPCR, the ß-AR have seven transmembrane-spanning α-helices, which are connected with three extracellular loops with an amino-terminus and with three intracellular loops with a carboxy-terminus (Liggett, 2002). The sequences of seven transmembrane-spanning α-helices are highly conserved between the ß-AR and have amino acid residues for either agonist- or antagonist-induced receptor trafficking. The third intracellular loop of the ß-AR has specific domains for central roles for G-protein coupling, desensitization, and downregulation (Liggett, 2002; Johnson, 2006).

ß-AR oscillate between inactivated and activated states, and these two states are in equilibrium under resting conditions. The predominant state is the inactivated state (Liggett, 2002). The activation of the ß-AR is initiated when ligands such as hormones or neurotransmitters bind to agonist binding sites within transmembrane-spanning α-helices. Ligand binding induces a conformational change in the ß-AR that leads to coupling with G-proteins that consist of α, ß, and γ subunits bound to the intracellular plasma membrane. Based on their amino acid sequences and function, Gα subunits are divided into four subfamilies, Gα_s, Gα_I, Gα_q and Gα_{12}. Upon the binding of a ligand to ß-AR, Gα subunits release G-protein-bound guanosine diphosphate (GPD), and subsequently bind to guanosine triphosphate (GTP). This activates the Gα subunits and dissociates it from G$_{ß\gamma}$ subunits (Liggett, 2002). ß-AR predominantly couple with Gα_s and Gα_I subunits (Wenzel-Seifert and Seifert, 2000). The ß$_1$-AR solely couples with Gα_s subunits, whereas the ß$_2$-AR has dual coupling with both Gα_s and Gα_I subunits (Xiao, 2001), which activates downstream

signaling pathways including adenylyl cyclase (AC), phosphoinositide 3-kinase/Ser and Thr kinase (PI3K/Akt), protein kinase A (PKA), ion-channel, extracellular-signal-regulated kinase (ERK), exchange protein activated by cAMP (Epac), and cyclic nucleotide-gated (CNG) signaling pathways (de Rooij et al., 1998; Murga et al., 1998; Dascal, 2001; Tasken and Aandahl, 2004; Craven and Zagotta, 2006; Robidoux et al., 2006). $G\alpha$ subunits bound to GTP drastically reduces the affinity of ß-AR for their ligands, which causes dissociation of $G\alpha$ subunits into the ß-AR and induces ß-AR to return to their inactive state (Johnson, 2006).

Continuous, short-term stimulation of agonists to ß-AR triggers a rapid attenuation of receptor responsiveness, known as desensitization. This mechanism includes three main processes: 1) uncoupling of the receptors from G-proteins in response to receptor phosphorylation; 2) internalization of uncoupled receptors; and 3) phosphorylation of internalized receptors (Ferguson, 2001; Johnson, 2006). The degree and duration of the ß-AR/agonists responses have various influences on the extent of desensitization. There are two principal mechanisms of desensitization, homologous and heterologous. Homologous desensitization is initiated when the $G_{ß\gamma}$ subunits of the activated G-protein bind with an activated G-protein-coupled receptor kinase (GRK). GRK selectively phosphorylates the C-terminus of the agonist-bound ß-AR, thereby recruiting and binding cytosolic cofactor proteins called ß-arrestins to ß-AR and resulting in dissociation of the receptors from G-proteins. This leads to limiting receptor functions. In contrast, heterologous desensitization begins when second messenger-dependent PKA phosphorylates not only the agonist-bound ß-AR but also agonist-unbound ß-AR (Madamanchi, 2007). ß-AR phosphorylation recruits ß-arrestins, and ß-arrestins interfere with further binding of G-proteins to receptors, ultimately releasing G-proteins from receptors. At more prolonged agonist exposure, some receptors are sequestered from the cell surface, termed sequestration, which leads to occurrence of

receptor internalization. Following internalization, receptors are transported to endosomes where the receptors are recycled back to the plasma membrane to receive new signals. In response to chronic agonist over-exposure, down-regulation of the cellular receptors occurs. This process reduces not only ß-AR mRNA and protein synthesis but degrades also pre-existing receptors in the liposome and plasma membrane. The time frames over which these processes take place are from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation) (Ferguson, 2001; Johnson, 2006; Madamanchi, 2007).

Table 1. Structural characteristics of the ß-Adrenergic receptors

Subtypes	Location	Transcript length (bp)	Translation length	M_r, Da
β_1	Chromosome 26	1404	467	50,137
β_2	Chromosome 7	1257	418	47,136
β_3	Chromosome 27	1959	405	42,903

ß-Adrenergic receptor agonists and antagonists

Agents such as drugs, hormones, or neurotransmitters that interact with ß-AR can be classified as agonists or antagonists based on their actions to ß-AR. Agonists are compounds that stimulate ß-AR to induce the chemical and physiological changes by only modulating functions or processes already existent without *de novo* effects. In contrast, antagonists bind specifically to the same receptor sites where agonists bind and block the ability of agonists to occupy and activate that receptor, thereby inactivating intracellular signaling pathways (Hershberger, 1994). There are two general types of antagonists, competitive and noncompetitive. Competitive antagonists trigger agonist dissociation by increasing concentration of agonist, whereas noncompetitive antagonists

do not allow receptors to be activated by agonists despite extremely high agonist concentrations because of their irreversible binding to receptors (Hershberger, 1994).

ß-agonists have an asymmetric center with aromatic group, hydroxyl group linked to the ß-carbon, and aliphatic nitrogen (Smith, 1998). Due to the presence of an asymmetric center, ß-agonists form a pair of optical isomers, the R- and S-enantiomers (or [-] and [+]). Some agonists, such as fentoterol, formoterol, and ractopamine, have two asymmetric centers, and there are four enantiomers, RR, SS, RS, and SR, present (Mills et al., 2003; Johnson, 2006). The molecular structure of ß-agonists is crucial for determining the way in which they interact with the ß-AR. Short-acting ß-agonists (e.g., salbutamol) are hydrophilic in nature, so they are able to access the active site of the ß-AR directly from the extracellular aqueous compartment, thereby provoking a rapid onset of action. However, as short-acting ß-agonists rapidly re-equilibrate, their duration at the receptor active site is short (4-6 h) (Johnson, 2001, 2006). In contrast, long-acting ß-agonists such as fermoterol and salmeterol are lipophilic in nature, and they are absorbed into the cell membrane in the form of a depot. The size of the depot depends on the concentration of long-acting ß-agonists. Therefore, the onset of action of these ß-agonists is slower than that of short-acting ß-agonists, but the duration of action is longer and concentration-dependent (Johnson, 2001, 2006).

ß-AR waver between inactivated and activated states, and these two states are in equilibrium under resting conditions. The inactivated state is dominant (Liggett, 2002). ß-agonists bind to the ß-AR and shifts the equilibrium to the active state. ß-agonists interchangeably behave as full or partial agonists in compliance with their potencies and receptor densities. When ß-agonists completely shift the equilibrium in the direction of active state of receptor, they are called full agonists. In contrast, ß-agonists that promote an intermediate state of receptor activation vs

inactivation are called partial agonists (Hershberger, 1994). For these reasons, full ß-agonists have a high efficacy (E_{max}), the maximum response reachable from a dosed agent; partial ß-agonists have an intermediate efficacy; and pure ß-antagonists have low or zero efficacy (Johnson, 2006). Most $ß_2$-agonists have an intermediate efficacy. If enough densities of ß-AR are present, then, $ß_2$-agonists behave as full agonists. However, receptor densities are too low, or receptor couplings are inadequate, they behave as partial agonists. Therefore, a partial agonist requires a higher occupation of receptors than a full agonist to reach its maximum effect (Johnson, 2006).

ß-agonists have been used for the treatment of chronic bronchitis, chronic obstructive pulmonary disease, asthma, uterine relaxants, and cardiac irregularities for more than 30 years (Barnes, 1999). Interestingly, some ß-agonists have identified "repartitioning effects", inducing increase in skeletal muscle mass and decrease in body fat (Emery et al., 1984). As a consequence of repartitioning effects, many studies on revealing the effects of ß-agonists have been conducted in human medicine and livestock industry with the aim of discovering new pharmaceuticals for muscle wasting disorders (Carter et al., 1991; Maltin et al., 1993; Kissel et al., 1998; Lynch et al., 2001), and of improving feed efficiency and meat quality (Hamby et al., 1986; Hoey et al., 1995; Bell et al., 1998; Mersmann, 1998).

In the livestock industry, many studies demonstrate that the oral administration of ß-agonists (e.g., clenbuterol, cimaterol, ractopamine, and zilpaterol) with high doses and/or extended days in cattle, pigs, chickens, and sheep resulted in a muscle hypertrophic response due to the increase in muscle protein synthesis (Smith et al., 1995) and reduction of their muscle protein degradation. Furthermore, they concomitantly reduce the adipose tissue mass due to the stimulation of TAG degradation and inhibition of fatty acid synthesis (Baker et al., 1984; Dalrymple et al., 1984; Ricks et al., 1984; Jones et al., 1985; Moser et al., 1986; Beermann et al.,

1987; Coleman et al., 1988; Miller et al., 1988; Schiavetta et al., 1990; Mersmann, 2002; Allen et al., 2009; Elam et al., 2009). In addition, some studies also demonstrate that certain ß-agonists such as clenbuterol and cimaterol increase the peripheral blood flow and influence the release of insulin, growth hormone, thyroid hormones, and corticosteroids (Bassett, 1970; Beermann et al., 1986; Beermann et al., 1987). For instance, clenbuterol upregulates the concentration of glucose, insulin, and free fatty acids in calves and increases activity of specific liver enzyme alanine aminotransferase and alkaline phosphatase in pigs (Luthman and Jacobsson, 1993; Gojmerac et al., 2002). The mechanism regulating tissue responsiveness to ß-agonists differs from species to species and even tissues within a species because of variations of each receptor subtype in species and tissues within a species (Hill et al., 1998).

Lipid turnover

In response to cellular signal cascades caused by ß-agonists in adipocytes, lipid droplets are subjected to lipolysis to release mobilized fatty acids and other metabolites derived from stored neutral lipids, which are used for energy generation, membrane biogenesis, protein modification, and secretion within lipoproteins (Barbosa et al., 2015). These processes are highly regulated by specific enzymes and hormones. In adipocytes, lipolysis of TAG in lipid droplets is initiated by the binding of ß-agonists such as epinephrine and isoproterenol to their receptors, which are coupled to stimulatory G protein $G_{\alpha s}$, and in turn, interacts with the membrane-bound AC to catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), the “intracellular second messenger”. The increased concentration of cAMP results in activation of PKA by binding to the PKA regulatory subunits. PKA phosphorylates perilipin and hormone-sensitive lipase (HSL), and phosphorylated perilipin undergoes a conformational change,

allowing HSL to gain access to stored lipid. Adipose tissue TAG lipase first cleaves the sn-1 fatty acyl group from the TAG. Then, HSL acts on diacylglycerol to hydrolyze the sn-3 fatty acyl group. Finally, the sn-2 fatty acyl group is hydrolyzed by monoacylglycerol lipase. Free fatty acids and glycerol, the net products of lipolysis, are liberated and released from cells. The liberated fatty acids may reenter the adipocyte intracellular fatty acid pool where they may either be reesterified to neutral lipids or be utilized for oxidation in mitochondria to generate ATP. However, adipocytes cannot metabolize the glycerol produced resulting from TAG lipolysis because adipocytes lack glycerol kinase, which is responsible for *de novo* synthesis of TAG and glycerol-phospholipids (Mersmann and Smith, 2005; Walther and Farese, 2009).

In cattle and sheep, isoproterenol, a non-selective ß-agonist, shows a strong lipolytic effect; terbutaline, a $ß_2$-agonist, shows a slight lipolytic effect; and dobutamine, a $ß_1$-agonist, has no effect on lipolysis in heifer and lambs (Ferlay and Chilliard, 1999; Ferlay et al., 2001). In addition, CL316,243, a β_3-agonist, has no effect on lipolysis in ewes (Ferlay et al., 2001).

CHAPTER III

THE LENTIVIRAL-SYSTEM CONSTRUCTION FOR HIGHLY EXPRESSED PORCINE SCD1 AND FUNCTIONAL CHARACTERIZATION IN STABLY TRANSDUCED PORCINE SK6 CELLS*

Introduction

Epidemiological studies and randomized controlled studies have provided conflicting evidence regarding dietary and risk for cardiovascular disease (CVD). Whereas epidemiological studies (Posner et al., 1991; Xu et al., 2006) indicated significant positive associations between the incidence of CVD and the proportion of dietary energy intake from monounsaturated fatty acids (MUFA), randomized controlled studies indicated that increasing dietary oleic acid (18:1n9) reduced risk factors for CVD (Kris-Etherton et al., 1999; Adams et al., 2010; Gilmore et al., 2011; Gilmore et al., 2013). However, endogenously produced oleic acid may promote obesity, hepatic steatosis, and lipid accumulation in muscle (Ntambi and Miyazaki, 2004; Hulver et al., 2005).

The conversion of saturated fatty acids (SFA) to MUFA by the fatty acid Δ^9 desaturase, stearoyl-CoA desaturase-1 (SCD1) accounts for the majority of MUFA in porcine muscle and adipose tissue (St. John et al., 1991; Klingenberg et al., 1995). SCD1 is also responsible for the conversion of *trans*-vaccenic acid to its corresponding conjugated linoleic acid (CLA) isomer, 18:2 *cis*-9, *trans*-11 CLA (Ntambi and Miyazaki, 2003). In laboratory rodents, *SCD1* is expressed in both liver (Ntambi, 1992; Waters and Ntambi, 1994) and adipose tissue (Kang et al., 2004), although SCD1 activity is at least two orders of magnitude higher in mouse liver than in adipose

* Reprinted from "The lentiviral-system construction for highly expressed porcine scd1 and functional characterization in stably transduced porcine sk6 cells" by Hwang et al., *Lipid*, In press, 2019.

tissue (Enser, 1979). Oleic acid is the preferred substrate for acyl-CoA:cholesterol acyltransferase (Landau et al., 1997; Miyazaki et al., 2000), and adipose tissue stores cholesterol primarily as cholesterol ester (Sweeten et al., 1990).

We have used the pig as a model to document the effects of dietary fatty acids on lipid metabolism (St. John et al., 1987a; Smith et al., 1996a; Smith et al., 1996b; Smith et al., 1999; Demaree et al., 2002; Smith et al., 2002; King et al., 2004; Go et al., 2012). Feeding palmitic acid (16:0) or a combination of myristoleic acid (14:1n5) plus palmitoleic acid (16:1n7) to pigs depressed lipid synthesis from glucose and subcutaneous adipocyte size (Smith et al., 1996b), whereas the myristoleic/palmitoleic acid combination increased plasma LDL cholesterol (Smith et al., 1996a). SCD1 activity in porcine adipose tissue increases when fed a starch-based diet, and is greater in obese pig adipose tissue than in lean pigs (Smith et al., 1999). In contrast to rodents, porcine adipose tissues exhibit substantially higher SCD1 catalytic activity than liver or intestinal mucosal cells (Klingenberg et al., 1995). However, we demonstrated that there were no differences in *SCD1* gene expression across liver, muscle, adipose tissue, and intestinal mucosal cells (Go et al., 2012), suggesting translational or post-translational control of activity.

SCD1 expression was previously demonstrated in mouse kidneys, which was depressed during the onset of diabetes (Wilson et al., 2003). *SCD1* is expressed in proximal kidney tubule cells, and *SCD1* expression is increased during uromodulin-associated kidney disease (Horsch et al., 2014). The predominant isoform of *SCD* in mouse kidneys is *SCD1* (Ntambi and Miyazaki, 2003), and *SCD1* is upregulated in the glomeruli of patients with diabetic nephropathy (Sieber et al., 2013). Palmitic acid induces glomerular podocyte death, whereas palmitoleic acid and oleic acid attenuate palmitic acid-induced lipotoxicity in podocytes (Sieber et al., 2010).

Measurement of SCD1 activity requires large amounts of microsomal protein and the assay inherently has high intra-sample variability (St. John et al., 1991; Yang et al., 1999; Smith et al., 2002; Chung et al., 2007). SCD1 activity has not been described in porcine kidney cells. The porcine SK6 cell line has been used to study viral infections such as hog cholera (Terpstra et al., 1990) and classical swine fever (van Gennip et al., 1999; Chen et al., 2015). However, to date, *SCD1* expression has not been documented in SK6 cells. We predicted that endogenous *SCD1* expression would be low in this kidney cell line, and hypothesized that *SCD1* expression would be upregulated in SK6 cells by exposure to palmitic acid. Therefore, one objective of this study was to establish an effective and highly reproducible means of estimating functional SCD1 catalytic activity. To accomplish this goal, we used SK6 cells, which do not contain detectable *SCD1* mRNA or protein and further, SK6 cells transduced with an inducible *pSCD1* lentiviral construct. *SCD1*-transduced SK6 cells effectively converted supplemental palmitic acid to palmitoleic acid, consistent with profound increases in *SCD1* mRNA and protein. The long-term goal of this research is to generate transgenic pigs for the study of obesity and muscle and liver steatosis using the lentiviral constructs utilized in this study.

Materials and Methods

Cell Lines

Swine kidney 6 cells (SK6) were obtained from the Foreign Animal Disease Diagnostic Laboratory (APHIS) at Plum Island Animal Disease Center (PIADC), Greenport, NY. Cells were cultured under standard tissue culture conditions, using minimum essential media (MEM) (Life Technologies/Invitrogen, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and supplemented with 1% antibiotics (Life Technologies/Invitrogen, Grand Island,

NY) and 1% non-essential amino acids. Lenti-X 293T cell line (Clontech Laboratories, Inc., Mountain View, CA) is a HEK cell line, transformed with adenovirus type 5 DNA that also expresses the SV40 large T antigen. The cell line was subcloned for high transfectability and high-titer virus production. This cell line was used to produce recombinant lentiviruses. These cells were also cultured under similar standard conditions as explained above.

Generation of all-in-one Tet Inducible bidirectional lentiviral vector for pSCD1 overexpression

The all-in-one bidirectional lentiviral vector system was derived from pLVX-Tre3G-IRES (Clontech Laboratories Inc., Mountain View, CA) (Figure 1a) and consisted of a *CMV*-driven rt-TA (Tet-On 3G transactivator) in the reverse orientation with gene of interest (GOI) under the influence of *Tre3G (TRE)* promoter in forward direction (Figure 1b). The promoters (*CMV* and *Tre3G*) in bidirectional orientation were separated by a *ubiquitous chromatin opening element (UCOE)* known to promote sustained and reliable transgene expression by resisting DNA methylation (Zhang et al., 2010). In the presence of doxycycline, the rt-TA (Tet-On 3G) is expressed, which in turn binds to *tetracycline responsive element* (*Tre3G*) to drive the expression of the transgene.

The full-length coding sequence of porcine *SCD1* was amplified from reverse transcribed porcine mRNA using primers listed in Table 2. The amplified *pSCD1* gene was inserted at BamHI-NotI sites of a bidirectional lentiviral vector, pLVX-*UCOE-Tre3G*-GOI, under the influence of *Tre3G* in forward orientation followed by IRES-*GFP* to create pLVX- *UCOE-Tre3G-pSCD1* (Figure 1c). The recombinant lentiviral vectors also consisted of a puromycin antibiotic selection marker driven by *PGK* promoter for selection of transduced cells. The correct orientation and

integrity of recombinant lentiviral vector was confirmed by restriction enzyme analysis followed by DNA sequencing.

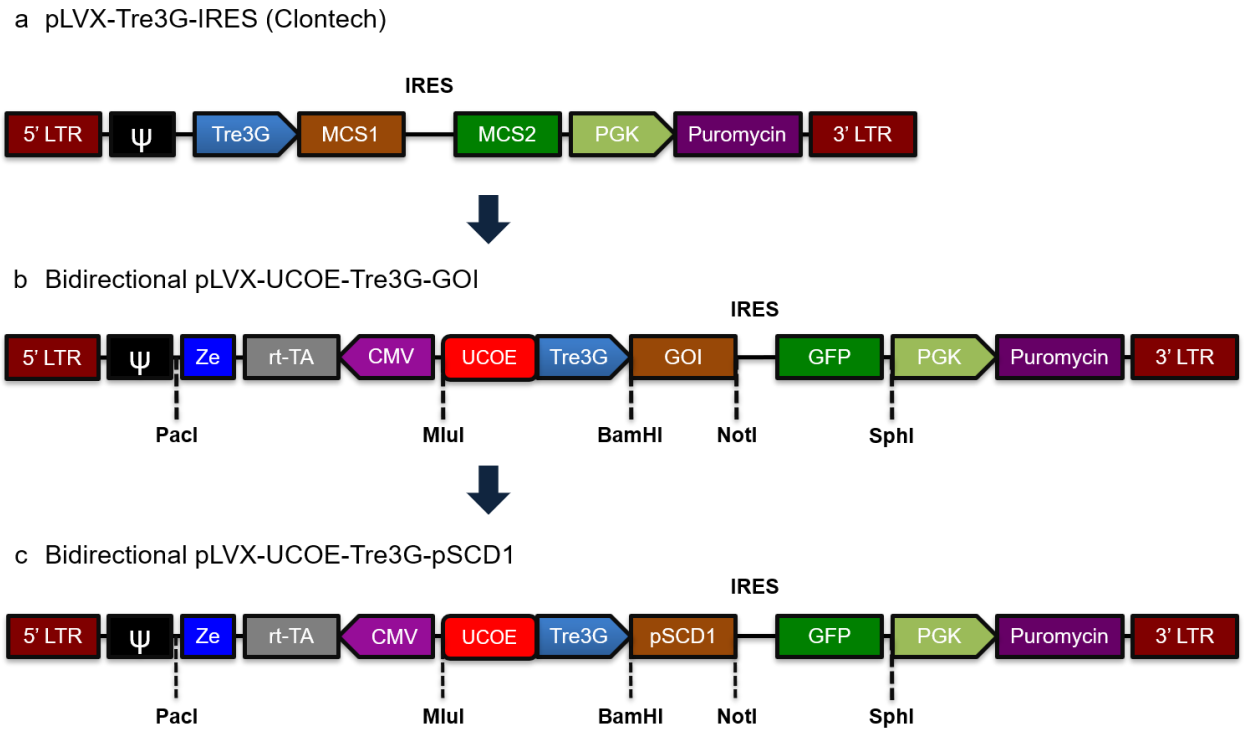

Figure 1. Schematics of the lentiviral vector constructs for *pSCD1* overexpression. SK6 were transduced with the bidirectional lentivector construct depicted in Figure 1c followed by puromycin selection to generate SK6-I-pSCD1 cells that could be induced with doxycycline to express pSCD1 and GFP. The transcription direction of the *CMV*, *Tre3G* and *PGK* promoters are indicated with arrows. The lentiviral bidirectional promoter constructs were packaged as recombinant lenti viruses in HEK293T cells. (a) Schematics of pLVX-*Tre3G*-IRES (Clontech). (b) Schematics of bidirectional pLVX-*UCOE-Tre3G*-GOI. (c) Schematics of bidirectional pLVX-*UCOE-Tre3G-pSCD1*. LTR, long-terminal repeat; ψ, packaging signal; Ze, zeocin; rt-TA, Tet responsive transactivator; Tre3G, Tet promoter; CMV, cytomegalovirus promoter; PGK, Phosphoglyceratekinase promoter; UCOE, ubiquitous chromatin opening element; pSCD1, porcine stearoyl-CoA desaturase-1; IRES, internal ribosome entry site; GFP, green fluorescent protein.

Table 2. Primers for RT-qPCR, cloning of *pSCD1*and *pSCD1*shRNA

Gene	Accession number	Sequence	Amplicon length (bp)
Primers for qPCR			
pSCD1[a]	NM_213781.1	F: 5'-ACACTTGGGAGCCCTGTATG-3'	152
		R: 5'-GGGCAGTCGAGCTTTGTAAG-3'	
pGAPDH[b]	NM_001206359.1	F: 5'-TCGGAGTGAACGGATTTG-3'	219
		R: 5'-CCTGGAAGATGGTGATGG-3'	
pYWHAG[c]	XM_005661962.3	F: 5'-TTTTTCCAACTCCGTGTTTCTCT-3'	75
		F: 5'-CCATCACTGAGGAAAACTGCTAA-3'	
pYWHAZ[d]	XM_021088756.1	F: 5'-ATGCAACCAACACATCCTATC-3'	178
		R: 5'-ATGCAACCAACACATCCTATC-3'	
Primers for Cloning			
pSCD1	NM_213781.1	F: 5'- ATGCCGGCCCACTTGCTGC-3'	1094
		R: 5'- AAGGGACCCCAAACTCAG-3'	
*pSCD1*shRNA1 oligo		TGCTGTTGACAGTGAGCGAGCCCAAGCTTGAATATGTTTG	
		TAGTGAAGCCACAGATGTACAAACATATTCAAGCTTGGG	
		CCTGCCTACTGCCTCGGA	
*pSCD1*shRNA2 oligo		TGCTGTTGACAGTGAGCGCGGAGTCACCGAACTTACAAA	
		GTAGTGAAGCCACAGATGTACTTTGTAAGTTCG	
		GTGACTCCATGCCTACTGCCTCGGA	

[a]pSCD1, porcine stearoyl-CoA desaturase-1; [b]pGAPDH, porcine glyceraldehyde 3-phosphate dehydrogenase; [c]pYWHAG, porcine tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma; [d]pYWHAZ, yrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Generation of all-in-one Tet-inducible bidirectional lentiviral vector for knockdown of pSCD1

To generate the all-in-one bidirectional lentiviral vector system for suppression of *pSCD1*, we utilized the same lentiviral backbone utilized for *pSCD1* overexpression, which was modified as shown in Figure 2b. Two different short hairpin RNAs (shRNA1 and shRNA2) were designed to target different regions of *pSCD1* and a scrambled shRNA was designed as a control. shRNAs for *pSCD1* were designed using a web-based tool (RNAi Central; http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA). Each shRNA was cloned using second-generation shRNA-mirs by the PCR-based strategy described previously (Silva et al., 2005) into a non-inducible lentiviral vector (PEG) consisting of a mir (miR30 microRNA) cassette (Figure 2a) (Golding and Mann, 2011) to create PEG-*pSCD1*shRNA1, PEG-*pSCD1*shRNA2, and PEG-scrambled shRNA. The shRNA-mir cassette was cloned into the 3' UTR of *GFP* under the influence of *elongation factor 1α (EF1α)* promoter for constitutive expression of hairpins (Figure 2a). The sequences for *pSCD1*shRNA oligos are listed in Table 2. Restriction enzyme analysis and DNA sequencing confirmed all cloned *pSCD1*shRNAs. The *GFP-pSCD1*shRNA fragment was cut from PEG-*pSCD1*shRNA and cloned at BamHI-sphI in bidirectional lentiviral vector pLVX-*UCOE-Tre3G* to create pLVX-*UCOE-Tre3G-pSCD1*shRNA (Figure 2b).

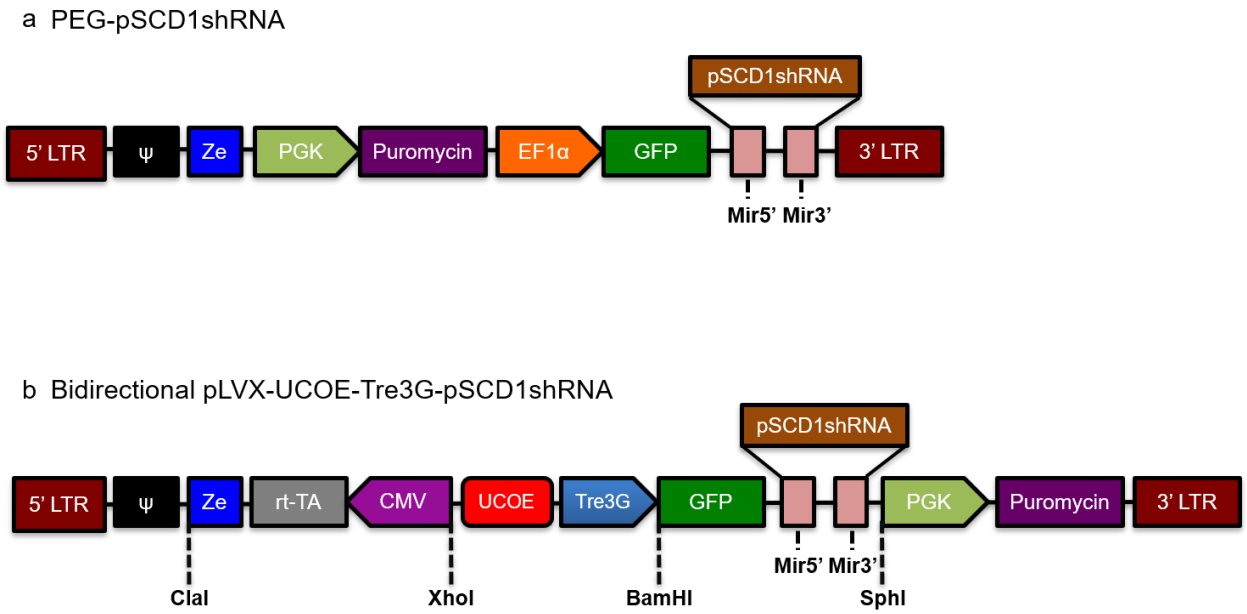

Figure 2. Schematics of the lentiviral vector constructs for *pSCD1* knockdown. The transcription direction of the *CMV*, *Tre3G*, *PGK* and *EF1α* promoters are indicated with arrows. The lentiviral bidirectional constructs (pLVX-*Tre3G-GFP-pSCD1*shRNA and pLVX-*UCOE-Tre3G-GFP-pSCD1*shRNA) and lentiviral unidirectional construct (PEG-*pSCD1*shRNA) were packaged as recombinant lentiviruses in HEK293T cells. (a) Schematics of PEG-*pSCD1*shRNA. (b) Schematics of bidirectional pLVX-*UCOE-Tre3G-pSCD1*shRNA. LTR, long-terminal repeat; ψ, packaging signal; Ze, zeocin; rt-TA, Tet responsive transactivator; Tre3G, Tet promoter; CMV, cytomegalovirus promoter; PGK, phosphoglyceratekinase promoter; UCOE, ubiquitous chromatin opening element; pSCD1, porcine stearoyl-CoA desaturase-1; EF1α, elongation factor ; GFP, green fluorescent protein ; miR , flanking and loop sequences from an endogenous miRNA which directs the excision of the engineered miRNA from a pri-miRNA.

Production of recombinant lentiviral vector stock

The lentiviral vector stocks were generated by triple plasmid co-transfection of HEK293T cells, with a Calcium Phosphate Transfection Kit (Life Technologies, Grand Island, NY) or X-Fect Transfection Reagent (Clontech Laboratories Inc., Mountain View, CA). Briefly, the HKE293T cells were co-transfected with bidirectional lentiviral vectors expressing the *pSCD1* or *pSCD1*shRNA cassettes along with envelope plasmid pMD.G and packaging plasmid pCMV8.91 described previously (Case et al., 1999). A total of 13.8 µg of vector, 10.2 µg of pCMV8.91 and 6 µg of pMD.G plasmids were used to transfect a 10 cm tissue culture dish. The transfection

efficiency was determined by green fluorescent protein (GFP) expression by fluorescence microscopy. The medium was replaced with DMEM after 24 h of transfection. The supernatant fractions were harvested 48 and 72 h after transfection, centrifuged at 1,000 x g for 10 min and filtered through a 0.45 µm polyethersulfone (PES) (low protein binding) filter. The recombinant lentiviral vector stocks were concentrated using Lenti-X™ Concentrator (Clontech Laboratories Inc., Mountain View, CA) as per manufacturer's protocol. Briefly, the lentiviral vector particles were concentrated by combining 1 volume of Lenti-X Concentrator with 3 volumes of clarified supernatant fraction followed by incubation at 4°C for 60 min and centrifugation at 1,500 x g for 45 min. The supernatant fraction was removed carefully, and pellet was resuspended in 1/100th of the original volume using complete DMEM.

Viral titers were determined by standard viral titration protocol which consists of transducing SK6 cells with serial dilutions of these recombinant lentivirus stocks and then selecting for stable transductants with antibiotic (3 µg/mL of puromycin) and counting the resulting cell colonies. This dose of puromycin was selected based on the kill curve in unmodified SK6 cells. The titer of virus corresponds to the number of colonies generated by the highest dilution. Viral titers were 4.5×10^5 colony forming units (CFU).

Generation of transgenic SK6 expressing pSCD1

SK6 cells were transduced with recombinant lentiviral stocks at the multiplicity of infection (MOI) of 1 along with 4 µg/mL of polybrene. Media was replaced 24 h after transduction with DMEM supplemented with 10% tetracycline free heat inactivated FBS. After 48 h, transduced cells were subjected to puromycin drug selection at the dose of 3 µg/mL for 7-14 d to obtain stable transductants. Puromycin-resistant colonies were picked using cloning cylinders and expanded in

presence of puromycin at a maintenance dose of 0.25 μg/mL. These colonies were selected and expanded to create SK6-I-*pSCD1* cells. SK6-I-*pSCD1* cells were induced with dox at a dose of 4 μg/mL for transgene (*pSCD1* and *GFP*) expression. Transduction efficiency in SK6 cells upon induction with dox was estimated based on GFP fluorescence. This dose of dox was optimized in SK6 cells by a dose response experiment. Dox was replenished in media every 48-72 h.

Testing shRNA knockdown of pSCD1

Inserting shRNA into the mir cassette ensured efficient processing of the expressed hairpins (Manjunath et al., 2009). The efficiency of the hairpins was validated in SK6-I-*pSCD1* cells which were overexpressing *pSCD1*, since SK6 cells exhibited very low or undetectable levels of *pSCD1*. SK6-I-*pSCD1* cells in 6-well plates were induced *pSCD1* and *GFP* expression 48 h before transfection by induction with dox. Following 48 h, the cells were mock transfected or with 2 μg/mL of PEG-*pSCD1*shRNA1, PEG-*pSCD1*shRNA2 and PEG-scrambled shRNA using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). The medium was replaced with DMEM after 24 h of transfection. The cells were harvested 48 h post-transfection for RNA and protein analysis.

Quantitative real-time RT-qPCR

Total RNA was isolated from cells using the RNAeasy kit (Qiagen, Valencia, CA) as per manufacturer's protocol followed by DNAseI (Sigma-Aldrich, St. Louis, MO) treatment. The DNAseI treated RNA was quantified and used to produce cDNA with the qScript kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Relative mRNA levels were determined by comparative threshold cycle (CT) analysis (Livak and Schmittgen,

2001) for pSCD1 using the PerfeCTa® SYBR® Green FastMix, ROX (Quanta Biosciences, Gaithersburg, MD) on a ABI Prism 7500 thermocycler (Applied Bio systems, Carlsbad, CA). Porcine *GAPDH*, *YWHAZ* and *YWHAG* were used as endogenous controls for these experiments. Relative mRNA levels were expressed as fold change over transfection control. The primers used in these studies are listed in Table 2.

Western blot

Protein concentrations in samples were measured using Pierce™ BCA Protein Assay Kit (Life Technologies, Grand Island, NY). Total protein (30 µg) was separated on a 12% SDS-PAGE gel at constant current. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane using Mini Trans-Blot (Bio-Rad, Hercules, CA). Porcine SCD1 and ß-actin were detected using a polyclonal anti-SCD1 (2 µg/mL) and anti-ß-actin antibodies (0.2 µg/mL) (Abcam, Cambridge, MA). For quantification of SCD1 protein, the pixel intensity of SCD1 signal was normalized to that of β-actin for each sample using Image J software.

Palmitic acid treatment and fatty acid analysis

SK6 and SK6-I-*pSCD1* cells (dox+ and dox-) in T-175 flasks were mock treated or treated with a SCD1 inhibitor (ab 142089, Abcam, Cambridge, MA) at a dose of 2 µM. Twenty-four hours later, cells were exposed to 50 µM palmitic acid or ethanol (control). Six hours after palmitic acid treatment, cells were harvested for fatty acid, RNA, and protein analyses. The extraction of fatty acids was conducted by a modification of the method of (Folch et al., 1957). Total lipids from SK6 cells were extracted in chloroform/methanol (2:1, vol/vol) and then methylated by 14% (wt/vol) boron trifluoride-methanol (Sigma-Aldrich Corp, St. Louis, MO). The fatty acid methyl esters

(FAME) were analyzed using a gas chromatography equipped with a CP-8200 auto sampler and flame ionization detector (FID) (Varian CP-3800 GC system, Varian Inc., Walnut Creek, CA). FAME were separated on a CP-Sil88 fused silica capillary column (100 m x 0.25 mm internal-diameter with 0.2-mm film thickness), with hydrogen as the carrier gas at a flow rate of 35 mL/min (split ratio 20:1) (Chrompak Inc., Middleburg, Netherlands). The oven temperature was programmed to increase from 150°C at 5°C/min to 220°C and held for 22 min. Front inlet and FID temperature were at 270°C and 300°C, respectively. Individual fatty acid peaks were identified by genuine external standard GLC-68D (Nu-Chek Prep, Inc., Elysian, MN) and calculated as the ratio of individual areas to that of total identified fatty acids.

Statistical analyses

Statistical analysis was performed using either Student's t-test or one-way analysis of variance followed by Tukey's Multiple Comparison Test (Graph Pad Prism 6.0, Graph Pad Software, La Jolla, CA). Means for fatty acid percentages were compared by analysis of variance and when significant ($P < 0.05$), means were separated by Fisher's Protected LSD method. All the experiments were performed in triplicates with at least two independent runs. The data are presented as mean ± SE. The treatment means were considered significantly different when $P < 0.05$.

Results

SCD1 is a key enzyme in lipid metabolism and plays a major role in health and disease states of animals and humans. The main aim of this study was to generate porcine cell culture

models for sustained over expression or suppression of porcine SCD1 in a controlled manner (dox inducible) and to assess their functionality in lentiviral-transduced SK6 cells.

Over expression of pSCD1 in transduced SK6 cells

The expression of *pSCD1* in lentiviral-transduced SK6 cells (SK6-I-*pSCD1* cells) was validated by both RT-qPCR and western blot. SK6-I-*pSCD1* cells were subjected to puromycin selection at a dose of 3 μg/mL, which resulted in death of majority of the SK6 cells within 3-4 days, with only SK6-I-*pSCD1* cells surviving in colonies. These colonies were expanded in presence of puromycin (3 μg/mL) for 10-14 days and thereafter they were grown in maintenance dose of puromycin (0.5 μg/mL). Two colonies (Cl 1 and Cl 2), seeded in 6-well plates were induced with different doses of dox as indicated in Fig. 3a to test the dose response. Twenty-four hours after dox induction (dox+), GFP expression was monitored under a microscope. Both Cl 1 and Cl 2 exhibited GFP expression upon induction with dox. Cells were harvested for RNA 48 h post-dox induction for RT-qPCR. A dose-dependent increase in *pSCD1* transcripts was seen in Fig. 3a. There was a significant increase in *pSCD1* mRNA levels in both Cl 1 and Cl 2 upon induction with dox at 2 μg/mL (> 600-fold increase) and 4 μg/mL (> 800-fold increase) as compared to uninduced (dox-) SK6-I-*pSCD1* cells (Figure 3a). The mRNA levels of *pSCD1* Cl 2 at either level of dox was not different than Cl 1 (Figure 3a). However, in Cl 1, 4 μg/mL dox increased the level of *pSCD1* mRNA compared to the lower dose ($P < 0.05$). To determine the optimum time after dox induction for harvesting and analyzing our samples for transgene (*pSCD1*) expression, we performed a time-response study.

Cells from two transgenic colonies, Cl 1 and Cl 2, were seeded in 6-well plates and induced with dox at a dose of 4 μg/mL. The samples were harvested at indicated time points after dox

induction (Figure 3b). Total RNA was extracted for RNA analysis by RT-qPCR. There was a significant increase in *pSCD1* transcripts in transduced Cl 1 and Cl 2 cells upon induction with dox as compared to induction without dox in SK6-I-*pSCD1* cells at all time points (Figure 3b). The increase in *pSCD1* mRNA levels in SK6-I-*pSCD1* cells (dox+) was detected as early as 24 h post-dox induction and maintained until 72 h (Figure 3b). There was a substantial decrease, though not significant, in the *pSCD1* mRNA levels 96 h after addition of dox to culture media (Figure 3b), suggesting that fresh dox has to be replenished after every 48-72 h in the culture media.

The expression of pSCD1 also was confirmed by western blot analysis (Figure 3c), wherein a pSCD1-specific band corresponding to 37 kDa was seen in cell lysates of SK6-I-*pSCD1* cells induced with dox (Figure 3c). Interestingly, no band was observed in cell lysates of normal SK6 cells, indicating that pSCD1 is expressed at very low or undetectable levels in these cells (Figure 3c).

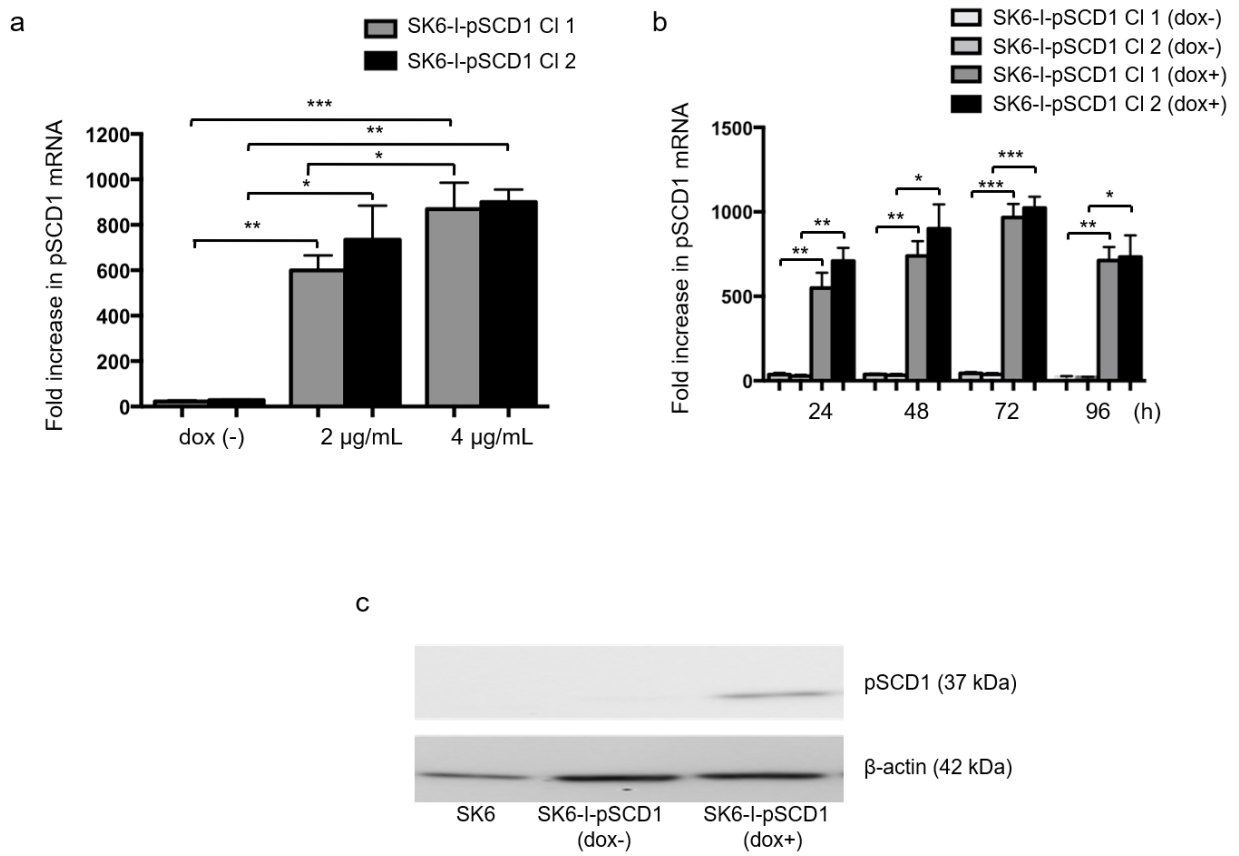

Figure 3. Doxycycline-induced expression of porcine *SCD1* in SK6-I-*pSCD1* cells. SK6 cells were transduced with recombinant lentivirus, pLVX-*UCOE-Tre3G-pSCD1*, selected with puromycin (3 µg/ml) and clonally expanded to create SK6-I-pSCD1 cells which is overexpressing *pSCD1*. (a) Dose response in two colonies of SK6-I-*pSCD1* cells. (b) SK6-I-*pSCD1* cells were induced with dox at the dose of 4 µg/ml and total RNA was extracted at indicated time points from two colonies of SK6-I-*pSCD1* cells to measure mRNA levels of *pSCD1* by RT-qPCR. mRNA levels were normalized to the geometric mean of endogenous porcine *GAPDH*, *YWHAG* and *YWHAZ* and are represented as fold increase compared to control. (c) pSCD1 protein levels were detected by western blot using anti-SCD1 or anti-ß-actin antibodies. dox, doxycycline; SK6-I-*pSCD1*, SK6 cells overexpressing *pSCD1*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

shRNA effectively suppressed expression of pSCD1

Transfection of SK6-I-*pSCD1* cells (overexpressing *pSCD1*) with PEG-*pSCD1*shRNA1 or PEG-*pSCD1*shRNA2 led to significant knockdown of *pSCD1* as compared to PEG-scrambled shRNA (Figure 4a). A similar trend was observed with western blot analysis (Figure 4b). PEG-*pSCD1*shRNA2 showed a better knockdown efficiency of *pSCD1* as compared to PEG-

*pSCD1*shRNA1. A significant decrease in pSCD1 protein expression was observed in cell lysates of PEG-*pSCD1*shRNA2 as compared to PEG-scrambled shRNA (Figure 4c). Therefore, we used PEG-*pSCD1*shRNA2 in our inducible all-in-one lentiviral system and for further experiments. To generate the inducible all-in-one lentiviral system for knocking down *pSCD1*, we cloned the GFP-*pSCD1*shRNA2-mir fragment from PEG-*pSCD1*shRNA2 in bidirectional pLVX-*UCOE-Tre3G*-GOI at BamHI-SphI sites replacing GOI-IRES-*GFP* to create bidirectional pLVX-*UCOE-Tre3G-GFP-pSCD1*shRNA2-mir (Figure 2b).

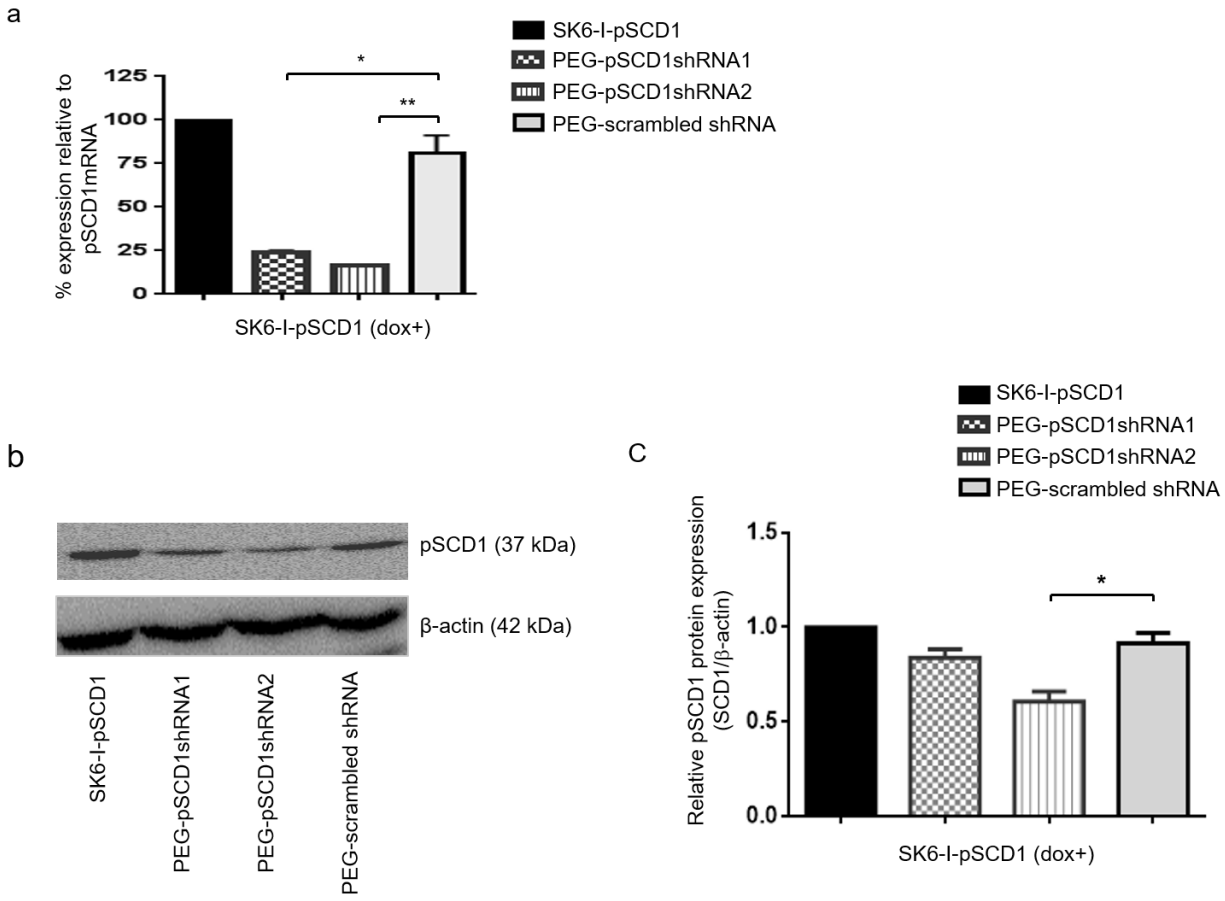

Figure 4. Knockdown of *pSCD1* in SK6 and SK6-I-*pSCD1* cells overexpressing *pSCD1*. SK6 cells (6-well plates) or SK6-I-*pSCD1* cells overexpressing *pSCD1* were mock transfected or transfected with lentivector shRNA constructs, PEG-*SCD1*shRNA1, PEG-*SCD1*shRNA2 or PEG-Scrambled shRNA at a dose of 2.0 µg using lipofectamine 3000. Transfection efficiency was determined next day by GFP expression. Cells were harvested 48 h post-transfection for RNA and protein analysis by RT-qPCR and western blot. (a) *pSCD1* mRNA levels were normalized to the geometric mean of endogenous porcine *GAPDH*, *YWHAG* and *YWHAZ* and are represented as percentage knockdown. The data represent means ± SE from three independent experiments performed in duplicates. (b) Knockdown of pSCD1 protein by western blot analysis using anti-SCD1 or anti-ß-actin antibodies. (c) pSCD1 protein levels detected by western blot were quantified and normalized to ß-actin from three independent experiments and expressed as means ± SE (Image J). dox, doxycycline; SK6-I-*pSCD1*, SK6 cells overexpressing *pSCD1*. **P* < 0.05, ***P* < 0.01, determined by two-tailed Student's t-test.

Functional assessment of pSCD1 in SK6-I-pSCD1cells

The functionality of *pSCD1* in SK6 and SK6-I-*pSCD1* cells was assessed by fatty acid analysis in absence or presence of supplemental palmitic acid and a SCD1 inhibitor. We previously had demonstrated that supplemental palmitic acid enhanced bovine *SCD1* gene expression, putatively through interaction with the intrinsic *SCD1* promoter (Choi et al., 2016). Therefore, we first established the effect of supplemental palmitic acid and SCD1 inhibitor treatment in SK6 and SK6-I-*pSCD1*cells on *pSCD1* gene expression. We found no significant difference in *pSCD1* transcript (Figure 5a) in absence or presence of palmitic acid (50 µM) and SCD1 inhibitor (2 µM), indicating that supplemental palmitic acid has no effect on *pSCD1* gene expression in the SK6-I-*pSCD1* cell model. However, SCD1 inhibitor significantly decreased pSCD1 protein level in SK6-I-pSCD1 cells induced with dox ($P < 0.05$). In the absence of supplemental palmitic acid, palmitic acid, stearic acid (18:0), and oleic acid were the most abundant fatty acids in SK6 cells and comprised approximately 19, 21, and 29% of total fatty acids (Table 3). Less abundant fatty acids, palmitoleic acid and *cis*-vaccenic acid (18:1n7), comprised approximately 4 and 5%, respectively. With the addition of 50 µM palmitic acid, cellular palmitic acid increased to from 19 to 27% of total lipids (Table 3).

The base-catalyzed fatty acid methylation procedure used in this study methylates only esterified fatty acids (Smith et al., 1998), so any changes in the proportions of fatty acids with treatment reflected alterations in cellular neutral lipids and phospholipids. Proportions of palmitoleic acid and *cis*-vaccenic acid in cellular lipids were highest in SK6-I-*pSCD1* cells (dox+), and incubated with supplemental palmitic acid (Figure 6a). Transfection with PEG-*pSCD1*shRNA2 followed by treatment with SCD1 inhibitor strongly depressed the proportions of palmitoleic acid and *cis*-vaccenic acid (Figure 6b). Palmitoleic acid is produced endogenously

from the Δ^9 desaturation of palmitic acid, which subsequently is elongated to *cis*-vaccenic acid. Therefore, depression in the proportion of these n-7 fatty acids under these conditions represents inhibition of *pSCD1* gene expression plus reduction in pSCD1 catalytic activity.

In the absence of dox, supplemental palmitic acid increased the proportion of cellular palmitic acid ($P < 0.05$) and induction with dox followed by treatment with the SCD1 inhibitor further increased palmitic acid (Table 3), indicating low pSCD1 activity under both conditions. The highest concentration of cellular palmitoleic acid was observed in SK6-I-*pSCD1* cells (dox+) supplemented with palmitic acid.

Palmitoleic acid is inversely proportional to stearic acid (Figure 6c), as the concentration of each is reciprocally established by SCD1 activity. Therefore, the palmitoleic:stearic acid ratio (an index of SCD1 activity), was highest in SK6-I-*pSCD1* cells (dox+), incubated with supplemental palmitic acid (Table 3). Similarly, the fold increase in palmitoleic acid was greatest in SK6-I-*pSCD1* cells (dox+) incubated with supplemental palmitic acid (Figure 6d). The fold increase in palmitoleic acid in SK6-I-*pSCD1* cells was depressed by transfecting these cells with PEG-*pSCD1*shRNA2 followed by treatment with SCD1 inhibitor (Figure 6d).

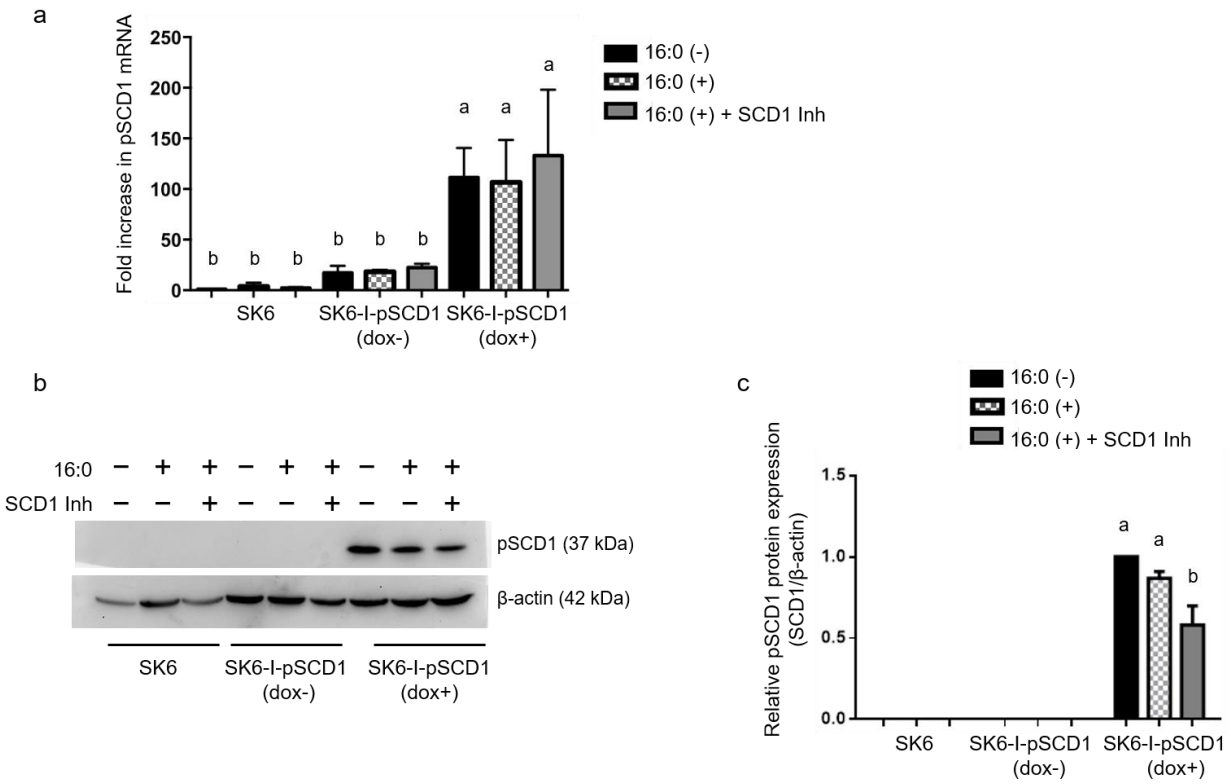

Figure 5. *pSCD1* in SK6 and SK6-I-*pSCD1* cells after palmitic acid treatment. SK6 cells or SK6-I-*pSCD1* cells were treated with 50 µM palmitic acid or ethanol (control). Six hours after palmitic acid treatment, cells were harvested for RNA and protein analyses. (a) mRNA levels were normalized to the geometric mean of endogenous porcine *GAPDH*, *YWHAG* and *YWHAZ* and are represented as fold increase compared to control. (b) western blot analysis using anti-ß-actin or anti-SCD1 antibodies. (c) SCD1 protein levels detected by western blot were quantified and normalized to ß-actin from three independent experiments and expressed as means ± SE (Image J). [abc]Means within with common superscripts are not different ($P > 0.05$). 16:0, palmitic acid; dox, doxycycline; SCD1Inh, SCD1 inhibitor.

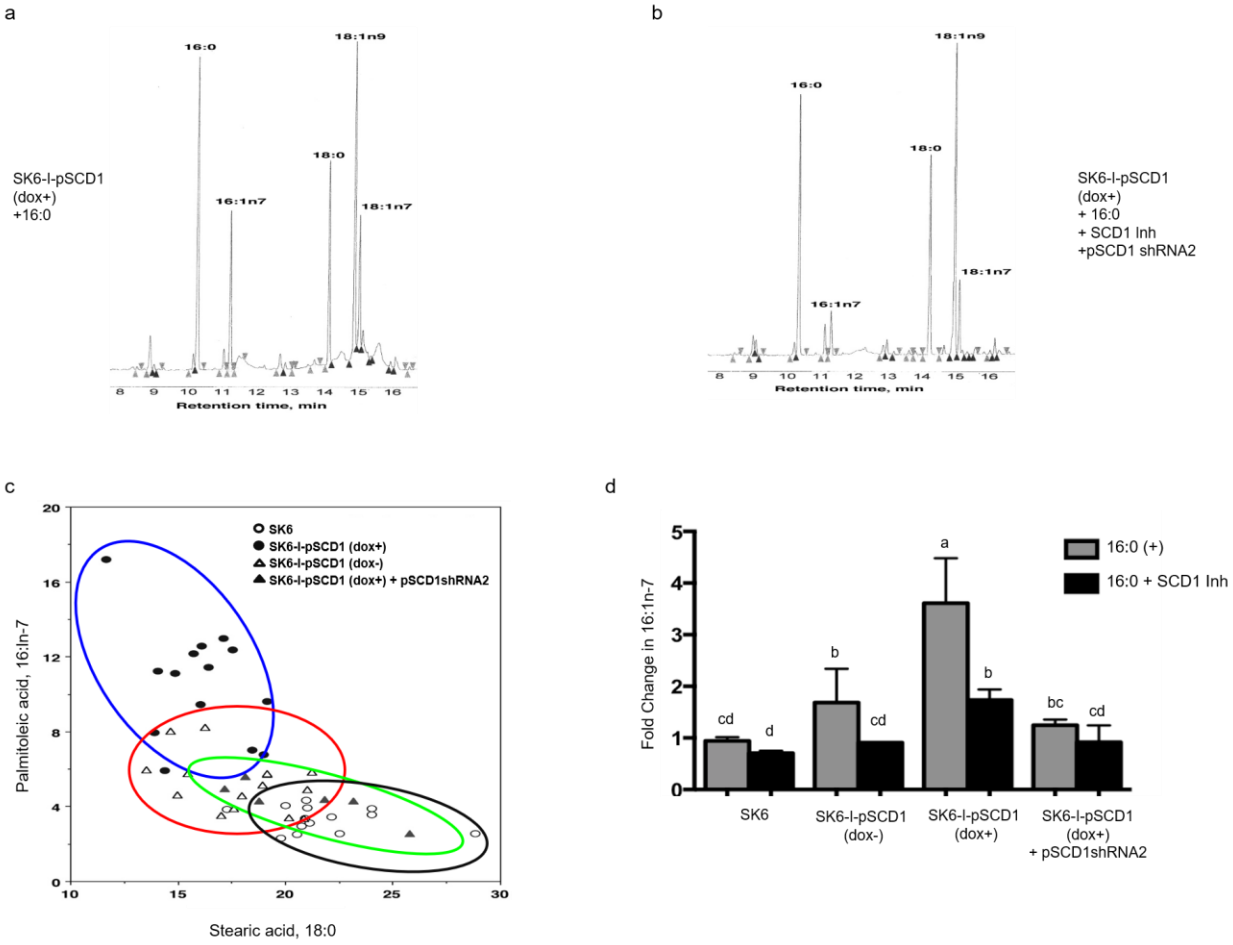

Figure. 6. Partial gas/liquid chromatograms showing FAME profiles of total cellular lipids of SK6-I-*pSCD1* cells. SK6 or SK6-I-*pSCD1* cells were mock treated or treated with SCD1 inhibitor at a dose of 2 µM. Twenty-four hours later, cells were exposed to 50 µM palmitic acid or ethanol (control). Following 6 h after palmitic acid treatment, cells were harvested for fatty acid analysis. Total lipids from cells were extracted in chloroform/methanol (2:1, vol/vol) and then methylated by 14% (wt/vol) boron trifluoride-methanol. The fatty acid methyl esters (FAME) were analyzed using a gas chromatography equipped with a CP-8200 auto sampler and flame ionization detector (FID). (a) FAME from SK6-I-*pSCD1* cells treated with palmitic acid. (b) FAME from SK6-I-*pSCD1* cells transfected with PEG-*pSCD1*shRNA2 and treated with palmitic acid plus SCD1 inhibitor. The peaks in A and B reflect FAME detector signals (mEV). (c) Relationship between cellular palmitoleic acid and stearic acid. Data are proportions of palmitoleic acid (16:1n7) as a function proportion of stearic acid (18:0). (d) Fold change in palmitoleic acid**.** The data represent means ± SE from three independent experiments. [abc]Means within with common superscripts are not different ($P > 0.05$). 16:0, palmitic acid; dox, doxycycline;SCD1 Inh, SCD1 inhibitor.

Table 3. Fatty acid composition of SK6 cells and SK6-I-*pSCD1* cells. SK6 or SK6-I-*pSCD1* cells (with or without dox) were incubated in the absence and presence of palmitic acid (16:0) and SCD1 inhibitor.

Treatment	Fatty acid composition, mg/100 mg total fatty acids				16:1n7/ 18:0 ratio
	16:0	16:1n7	18:0	18:1n9	
SK6 -16:0	19.14^{def}	3.74^{de}	21.63^{ab}	31.33^{bc}	0.174^{bc}
SK6 + 16:0	27.03^{b}	3.49^{de}	20.70^{abc}	29.15^{cd}	0.169^{bc}
SK6 + 16:0 + SCD1 inhibitor	26.26^{bc}	2.64^{e}	23.07^{a}	26.80^{de}	0.117^{c}
SK6-I-*pSCD1*/dox(-) - 16:0	20.73^{cde}	4.89^{cd}	18.31^{bc}	36.50^{a}	0.271^{bc}
SK6-I-*pSCD1*/dox(-) + 16:0	24.67^{bcd}	6.30^{c}	16.30^{cd}	29.21^{cd}	0.401^{b}
SK6-I-*pSCD1*/dox(-) + 16:0 +SCD1 inhibitor	29.91^{ab}	3.40^{de}	20.53^{abc}	24.38^{de}	0.166^{b}
SK6-I-*pSCD1*/dox(+) - 16:0	13.45^{f}	10.17^{b}	17.20^{cd}	32.08^{abc}	0.596^{a}
SK6-I-*pSCD1*/dox(+) + 16:0	25.00^{bcd}	13.49^{a}	14.08^{d}	25.97^{de}	0.999^{a}
SK6-I-*pSCD1*/dox(+) + 16:0 + SCD1 inhibitor	34.28^{a}	6.48^{c}	16.41^{cd}	22.27^{e}	0.397^{b}
SK6-I-*pSCD1*/dox(+) -16:0 + pSCD1shRNA2	15.19^{ef}	4.95^{cd}	20.65^{abc}	35.25^{ab}	0.247^{b}
SK6-I-*pSCD1*/dox(+) + 16:0 + pSCD1shRNA2	22.75^{bcde}	4.67^{cd}	19.50^{abc}	32.79^{abc}	0.244^{b}
SK6-I-*pSCD1*/dox(+) + 16:0 + *pSCD1*shRNA2 + SCD1 inhibitor	23.97^{bcd}	3.43^{de}	22.29^{ab}	28.71^{c}	0.164^{b}
Pooled SE	1.10	0.57	0.60	0.76	0.047
P-values	0.0012	0.0001	0.0136	0.004	0.0001

^{abcd}Means within a column with common superscripts are not different ($P > 0.05$).

Discussion

The conventional TET On/TET Off inducible lentiviral vector systems generally used to produce transgenic animals are based on two separate lentiviral vectors (Koponen et al., 2003; Park, 2007; Sheng et al., 2010). One vector encodes the inducible transcriptional activator (tetracycline-controlled transactivator protein (rt-TA)) and the other vector encodes the gene of interest under the influence of a *tetracycline-responsive element (TRE)*. The expression of the transgene can thus be regulated in a quantitative and reversible manner by exposing the transgenic animal to varying amounts of tetracycline or its derivatives (dox) (Sheng et al., 2010). In the presence of dox, the rt-TA (Tet-On 3G) gets expressed, which in turn will bind to *tetracycline responsive element (Tre3G)* to drive the expression of transgene. However, the efficiency of the two-vector system is low as co-transduction of the target cells with both vectors is required. In that regard, our combination of the two vectors into one contributed to improved transduction efficiency and, in turn, increased porcine *SCD1* expression in transduced SK6 cells (SK6-I-*pSCDl* cells).

The *SCD1* isoform is the most abundant in lipogenic tissues and is common to most species. The *pSCD1* gene has been mapped to chromosome 14 (Uemoto et al., 2012), and has > 80% homology with other mammalian *SCD1* genes (Ren et al., 2004). As in humans, the only other *SCD* isoform identified in livestock species is *SCD5*, which has 90% homology with human *SCD5* (Lengi and Corl, 2007, 2008). *SCD5* gene expression is primarily limited to the brain, although low levels of *SCD5* mRNA have been detected in liver and muscle (Lengi and Corl, 2007, 2008).

Proportions of the endogenously produced fatty acids in SK6 cells were similar to fatty acid proportions observed in porcine tissues (St. John et al., 1987b; Klingenberg et al., 1995; Go

et al., 2012). Thus, oleic acid was the most abundant MUFA and palmitic acid the most abundant SFA in SK6 cells. The high relative abundance of stearic acid indicated an active fatty acid elongase under all incubation conditions. This was supported by the relative increases in *cis*-vaccenic acid in SK6-I-*pSCD1* cells, which provided evidence that some portion of the palmitoleic produced by the Δ^9 desaturation of supplemental palmitic acid was elongated. However, in spite of the relative abundance of oleic and *cis*-vaccenic acid, in SK6 cells we detected only small amounts of *pSCD1* mRNA, and pSCD1 protein was below detection limits by western blot. Porcine SCD1 protein also was not detectable in SK6-I-*pSCDl* cells in the absence of dox, even though *pSCD1* mRNA levels were detectable. The small increases in *pSCD1* mRNA and the significant increases in palmitic and *cis*-vaccenic acid in SK6-I-*pSCD1* cells (dox-) indicates that, even in the absence of dox, there was some functional *pSCD1* gene expression. We interpret this to mean that post-transcriptional regulation of *pSCD1* mRNA may control protein levels and that even undetectable levels of pSCD1 are catalytically active.

The SCD1 inhibitor effectively decreased proportions of palmitoleic acid and *cis*-vaccenic acid. In previous research we have used the palmitoleic:stearic acid ratio as an index of SCD1 catalytic activity (Smith et al., 2006). Only small amounts of palmitoleic and stearic acid naturally occur in diets of animals, and dietary factors that promote SCD1 activity increase tissue concentrations of palmitoleic acid and concomitantly decrease stearic acid. Similarly, in the current study, SK6 cells had the highest proportions of stearic acid and lowest proportions of palmitoleic acid; the converse was true for SK6-I-*pSCD1* cells incubated with dox. Essentially identical results were obtained when data were expressed as fold increase in palmitoleic acid.

Previous researchers have transfected cells with *SCD1* with varying results. Lu et al. (2014) demonstrated that transfection of bone marrow mesenchymal stem cells with *SCD1* enhanced

SCD1 gene expression, but did not document changes in fatty acid composition (Lu et al., 2014). Nakaya et al. (2013) transfected 293A macrophages with mouse *Scd1* and demonstrated small but significant increases in HDL-mediated cholesterol efflux (Nakaya et al., 2013). However, neither palmitoleic nor oleic acid proportions were increased in the *SCD1* transgenic macrophages. Wu et al. (2010) demonstrated that *SCD1* transfection of human embryo kidney (HEK) 293 cells increased palmitoleic acid, *cis*-vaccenic acid, and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (all products of SCD activity) by two- to three-fold (Wu et al., 2010). Wang et al. (2014) reported that an *SCD1* mammary-specific vector caused a 50% increase in palmitoleic acid and an 11% increase in oleic acid in goat ear skin-derived fibroblastic cells (Wang et al., 2014). We attribute the profound increases in palmitoleic acid (four-fold) in the current study to the stability of our SK6-I-*pSCD1* cells.

Collectively, these data indicate that our lentiviral expression system was successfully established, and thereby stably and functionally expresses *pSCD1* in SK6 cells. The lentiviral constructs utilized in this study can be further utilized to generate transgenic animals or other cell lines to enhance our understanding of the contribution of fatty acid desaturation to the promotion of disease states such as obesity.

CHAPTER IV

CHARACTERIZATION OF ß-ADRENERGIC RECEPTORS IN BOVINE INTRAMUSCULAR AND SUBCUTANEOUS ADIPOSE TISSUE

Introduction

For more than three decades, the mechanism of action of ß-adrenergic receptor (ß-AR) agonists (i.e., cimaterol, clenbuterol, ractopamine, and zilpaterol) has been studied extensively in livestock species (i.e., cattle, pigs, chickens, and sheep). Depending on species and compounds, ß-AR agonists can cause a considerable increase in carcass muscle mass and a decrease in fat accumulation, thus promoting the production of lean meat as a source of high quality protein (Dalrymple et al., 1984; Jones et al., 1985; Moser et al., 1986; Coleman et al., 1988; Miller et al., 1988; Schiavetta et al., 1990; Allen et al., 2009; Elam et al., 2009). Decreased lipid in adipose tissue occurs through the ß-adrenergic receptors (ß-AR)/adenylyl cyclase (AC)/cAMP-dependent protein kinase A (PKA) signaling cascade, in turn activating perilipin and hormone-sensitive lipase (Wallukat, 2002).

Three subtypes of ß-AR ($ß_1$-AR, $ß_2$-AR, and $ß_3$-AR) are expressed in tissues of most species, and the distribution and specificity for synthetic ligands in adipose tissue differs widely among species. For example, the most predominant subtype is $ß_2$-AR in bovine adipose tissue, whereas $ß_1$-AR is the primary subtype in porcine adipose tissue (Sillence and Matthews, 1994; McNeel and Mersmann, 1999). For example, the synthetic ligand, ICI118,551 is a selective antagonist for $ß_2$-AR in both bovine skeletal muscle and adipose tissue, while it has no effect on $ß_2$-AR in porcine adipose tissue (Sillence and Matthews, 1994; Mersmann, 1998).

Recently, a new synthetic ß-AR ligand, lubabegon fumarate (as known as Experior™) has been developed by Elanco Animal Health. Preliminary data indicated that Experior™ acts as $ß_3$-AR agonist and a $ß_1$-AR and $ß_2$-AR antagonist in Chinese hamster ovary cells (M. E. Spurlock, unpublished data). In this study, we tested the hypothesis that bovine adipose tissues express $ß_3$-AR, and therefore Experior™ would exert similar effects in bovine adipose tissue. Furthermore, we hypothesized subcutaneous (s.c.) adipose tissue would exhibit grater response to $ß_3$-agonists and antagonists than intramuscular (i.m.) adipose tissue. Therefore, the aim of this study was to characterize ß-AR in bovine s.c. and i.m. adipose tissues with the use of selective ß-AR agonists and antagonists, including Experior™.

Materials and Methods

Materials

Lubabegon fumarate (Experior™) and ractopamine hydrochloride (RH) were provided by Elanco Animal Health (Elanco Animal Health, Indianapolis, IN). Other drugs and reagents were purchased from the following companies: dobutamine, salbutamol, zilpaterol hydrochloride (ZH), propranolol, Glycerol Assay Kit and DNAseI (Sigma-Aldrich, St. Louis, MO); L-748,337 and BRL-37344 (R&D system, Minneapolis, MN); Cyclic AMP XP® Assay Kit (Cell Signaling Technology, Danvers, MA); nonesterified fatty acid (NEFA) kit (Wako Life Sciences, Inc., Mountain View, CA); qScript™ cDNA synthesis kit and Perfecta® SYBR® Green fastmix® (Quanta Biosciences, Gaithersburg, MD).

Animals

Angus crossed bred steers (BW = 498 ± 59 kg) (n = 20) were fed a standard, corn-based finishing diet at the Texas A&M University McGregor Research Center, McGregor, TX, and then transported approximately 190 km to the Texas A&M Animal Science Teaching Research and Extension Complex (ASTREC), College Station, TX.

Bovine adipose tissue sampling

Bovine s.c. and i.m. adipose tissues were obtained as described previously (Miller et al., 1988; Miller et al., 1989; Miller et al., 1991; Brooks et al., 2011). Briefly, cattle were stunned using a captive bolt followed by exsanguination. Immediately after exsanguination, the longissimus muscle (LM) overlying s.c adipose tissue from the 8^{th} to 10^{th} thoracic region was removed by cutting through the hide immediately lateral to the spinal process. The muscle was immediately transported to the laboratory in oxygenated Krebs-Henselheit Ca^{2+}-free bicarbonate buffer (KHB) (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$; 37°C; pH 7.4) plus 5 mM glucose and 10 mM HEPES. The time elapsed between the stunning and arrival of the LM muscle at the laboratory was less than 20 min.

Adipose tissue explant culture

Fresh s.c. adipose tissue was cut into small pieces (50 to 100 mg) and i.m. adipose tissue was dissected from the LM as described previously (Smith and Crouse, 1984; Miller et al., 1988). The s.c. and i.m. adipose tissues were incubated according to three different sets of experimental conditions. **Experiment 1:** small pieces of s.c. and i.m. adipose tissues were pre-incubated in 6-well tissue culture plates for 30 min at 37°C, 5% CO_2 in KHB plus 5 mM glucose, 10 mM HEPES,

and 0.5 mM theophylline. After the 30 min pre-incubation, isoproterenol (non-selective ß-AR agonist), RH, ZH, Experior™, and BRL-37344 were added to the culture plate wells in a dose-dependent manner (10^{-9} to 10^{-4} M) for an additional 30 min. **Experiment 2:** the s.c. and i.m. adipose tissues were pre-incubated in KHB plus 5 mM glucose, 10 mM HEPES, and 0.5 mM theophylline in the presence or absence of either 10 µM L-748,337 or 50 µM propranolol. After the 30 min pre-incubation, Experior™ was added to the culture plate wells in a dose-dependent manner (10^{-9} to 10^{-4} M) for an additional 30 min. **Experiment 3:** the s.c. and i.m. adipose tissues were pre-incubated in KHB plus 5 mM glucose, 10 mM HEPES, and 0.5 mM theophylline in the presence or absence of 1 µM Experior™ or 1 µM Experior™ plus 10 µM L-748,337. After the 30 min pre-incubation, either dobutamine ($ß_1$-AR agonist) or salbutamol ($ß_2$-AR agonist) was added to the culture plate wells in a dose-dependent manner (10^{-9} to 10^{-4} M) for an additional 30 min. For all experiments, there were at least three wells per treatment condition. After a total 1 h incubation, s.c. and i.m. adipose tissues were homogenized in 1 mL cell lysis buffer (Cell Signaling Technology, Danvers, MA) with 1 mM phenymethylsulfony fluoride and centrifuged at 14,000 x g for 30 min to remove tissue debris. The supernatant fractions were stored at -80°C until subsequent analyses were performed.

Viability of adipose tissue

To confirm viability of adipose tissue samples following incubation with agonists/antagonists, lipogenesis in vitro in s.c. and i.m. adipose tissues was measured at sample collection and after 60 min incubation as described previously (May et al., 1995). Briefly, s.c and i.m. adipose tissue pieces (~100 mg) were incubated for 0 or 60 min at 37°C with oxygenated (95%:5% O_2:CO_2) KHB (pH 7.35-7.40) plus 5 mM glucose, 5 mM acetate, 10 mM HEPES, 1 µCi

[1-^{14}C]acetate (sodium salt) (American Radiolabeled Chemicals, Inc.). After incubation, neutral lipids in adipose tissues were extracted (Folch et al., 1957). Total lipids were resuspended in 10 mL of scintillation cocktail (Bio-safe2, Research Product International Corp., Mount Prospect, IL). Radioactivity of lipid extracts was counted with a scintillation counter (Packard 1600TR Liquid Scintillation Analyzer, Downers Grove, IL). Results are reported as nmol acetate converted to fatty acids/(1 h•100 mg adipose tissue).

Cyclic AMP

The concentration of cAMP was determined based on the principle of competitive binding using the Cyclic AMP XP® Assay Kit according to the manufacturer's instructions. Briefly, cytosolic extracts from s.c and i.m adipose tissue explants were co-incubated in 96-well plates with the HRP-linked cAMP substrate coated onto an immobilized rabbit monoclonal cAMP antibody at room temperature for 3 h on 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 analyzed in duplicate. A standard curve and cAMP concentrations were calculated using GrapPad Prism 6.04 software (GraphPad Software Inc., San Diego, CA).

Lipolysis

Lipolysis was measured with a glycerol assay kit and a nonesterified fatty acid (NEFA) kit according to the manufacturer's procedures. In brief, to determine glycerol released from tissue, 10 µL of the cell extracts was reacted with 100 µL glycerol reaction reagent for 20 min at room temperature and the absorbance was read at 570 nm. The glycerol standard from the kit was used for the calibration curve. To analyze the level of NEFA released from the adipose tissues, 25 µL

of each cell fraction plus 200 µL of the first reagent were incubated for 5 min at 37°C, then 100 µL of the second reagent was incubated for another 5 min at 37°C. The absorbance was read at 550 nm. Oleic acid was used to plot a standard curve and to calculate concentration of NEFA in the supernatant fractions.

Quantitative real-time RT-qPCR

Approximately 200 mg of s.c. and i.m. adipose tissues (n = 10) stored at -80°C were used to isolate the total RNA using a combination of the Trizol reagent (Invitrogen, Carlsbad, CA) and HiBind® RNA mini column (Omega Bio-tek, Inc., Norcross, GA) followed by DNAseI treatment. Total RNA was quantified on the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and reverse transcription was performed with the qScript™ cDNA synthesis kit. The gene expressions of ß-AR subtypes (*ADRB*) in s.c. and i.m. adipose tissues were analyzed in a CFX384™ Real-Time System (Bio-rad, Hercules, CA) using the Perfecta® SYBR® Green fastmix® kit. The different efficacy of cDNA synthesis between samples was normalized with three reference genes (*RSP9*, *GAPDH*, and *SDHA*). The relative expression of mRNA was determined by the cycle threshold (CT) deviation of an unknown sample versus geometric mean of three reference genes and data were presented as $2^{-\Delta CT}$. The primers used in this assay are listed in Table 4.

Table 4. Primers for RT-qPCR

Gene	Accession Number	Sequence	Amplicon Length (bp)
ADRB1	NM_194266.1	F: 5'-CAGAAGGCACTCAAGACGCT-3' R: 5'-CACCACGTTGGCTAGGAAGA-3'	81
ADRB2	NM_174231.1	F: 5'-TGATCGCTGTGGATCGCTAC-3' R: 5'-CCGGTACCAGTGCATCTGAA-3'	149
ADRB3	NM_174232.2	F: 5'-ACCTTCATTCTGTTCCTTCTG-3' R: 5'-CTGTGAGGTAGGTGTGTCTA-3'	145
GAPDH	NM_001034034.2	F:5'-CTGCCCGTTCGACAGATAG-3' R: 5'-CTCCGACCTTCACCATCTTG-3'	76
RPS9	NM_001101152.2	F: 5'-GAGCTGGGTTTGTCGCAAAA-3' R: 5'-GGTCGAGGCGGGACTTCT-3'	65
SDHA	NM_174178.2	F:5'-ACCTGATGCTTTGTGCTCTG-3' R: 5'-TCGTACTCGTCAACCCTCTC-3'	106

Statistical analysis

The data are expressed as means ± SEM. Statistical analysis of the change in gene expression obtained by RT-qPCR was tested with a two-sided, unpaired student's t-test and Tukey's honest significant difference test using JMP Pro 12 software (SAS Institute Inc., Cary, NC). A $P < 0.05$ was considered significant. Otherwise, nonlinear regression model was used to test effects on either ß-AR agonists or ß-AR antagonists against lipolytic response. Non-parametric Friedman test and one-way analysis of variance (ANOVA) were used where it was appropriate for statistical comparison of drug effects with the control and different ß-AR agonists or antagonists using GraphPad prism 6.0.

Results

ß-adrenergic receptor gene profiling in subcutaneous (s.c.) and intramuscular (i.m.) adipose tissue

All three *ADRB* genes were detected in bovine s.c. and i.m. adipose tissues (Figure 7). Regardless of adipose tissue type, the predominant *ADRB* was *ADRB2* ($P < 0.05$). The gene expression of *ADRB3* was not different from *ADRB1* expression in either s.c. or i.m. adipose tissue ($P > 0.05$). The expression of *ADRB* m*RNA* were 5.3, 2.9, and 8.3 times higher in s.c. adipose tissue than in i.m. adipose tissue for *ADRB1*, *ADRB2*, and *ADRB3*, respectively ($P < 0.05$). The gene expression of the *ADRB2* in the s.c. adipose tissue was 5.3 and 3.1 times higher than that of *ADRB1* and *ADRB3*, respectively. The *ADRB2* mRNA level was 9.6 and 10 times higher compared to *ADRB1* and *ADRB3*, respectively, in i.m. adipose tissue.

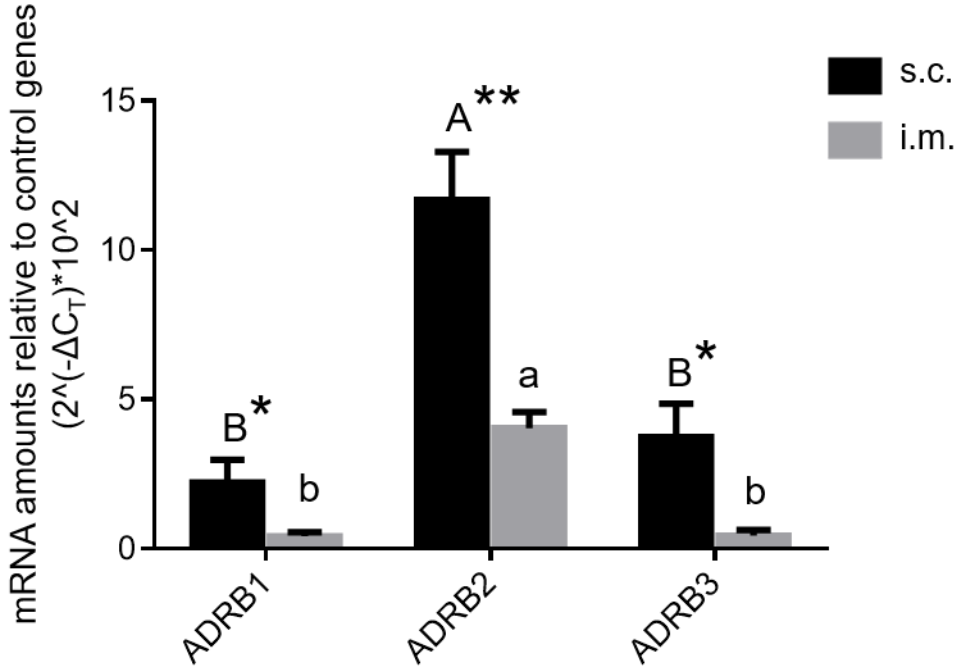

Figure 7. ß-Adrenergic receptor gene populations in growing steers. The data are expressed as means ± SEM (n = 10). ^{AB}means within s.c. adipose tissue not sharing common superscripts differ ($P < 0.05$). ^{ab}means within i.m. adipose tissue not sharing common superscripts differ ($P < 0.05$). * $P < 0.05$, **< $P < 0.01$ s.c vs i.m. adipose tissue.

Lipogenesis

Lipogenesis from acetate increased ($P < 0.05$) in s.c. adipose tissue was greater following incubation for 60 min compared to rates observed in fresh samples (time 0) (Table 5). Rates of lipogenesis did not differ ($P > 0.05$) between fresh and incubated i.m. adipose tissue. We conclude that there was no indication of loss of viability during the pre-incubation/incubation period.

Table 5. Fatty acid synthesis from acetate in s.c. and i.m. adipose tissue[1]

Incubation time, min	0	60
s.c.	45.56 ± 8.57^{b}	89.63 ± 17.79^{a}
i.m.	26.65 ± 8.46^{b}	39.21 ± 9.91^{b}

[1]Values are means ± SEM for n = 16 steers. Rates are nmol acetate converted to fatty acids per 100 mg adipose tissue per 1 h incubation. [ab]Means with common superscripts do not differ ($P > 0.05$). The main effect of tissue (s.c. vs i.m.) was significant ($P = 0.003$).

Accumulation of cAMP accumulation following treatment with ß-adrenergic receptor agonists in s.c. and i.m. adipose tissues

The non-selective ß-AR agonist, isoproterenol, the $ß_1$-and $ß_2$-AR agonist RH, and the $ß_2$-AR agonist ZH were used to assess the ß-AR-cAMP signaling cascade in s.c. and i.m. adipose tissue. Isoproterenol increased cAMP production in s.c. adipose tissue, indicating that the test system effectively activated adenylyl cyclase (Figure 8a). Stimulation of production of cAMP by isoproterenol in s.c. adipose tissue reached a plateau at 10^{-6} M. The EC_{50} of isoproterenol in s.c. adipose tissue was 0.22 µM. The concentration of cAMP in i.m. adipose tissue was slightly higher than in s.c. adipose tissue (Figure 8b). The EC_{50} of isoproterenol in i.m. adipose tissue was 2.1 nM. However, the cAMP accumulation was not dose-dependent in i.m. adipose tissue. Neither RH nor ZH affected cAMP production in s.c. and i.m. adipose tissue at low concentrations (10^{-8} to 10^{-6} M) ($P > 0.05$) (Table 6).

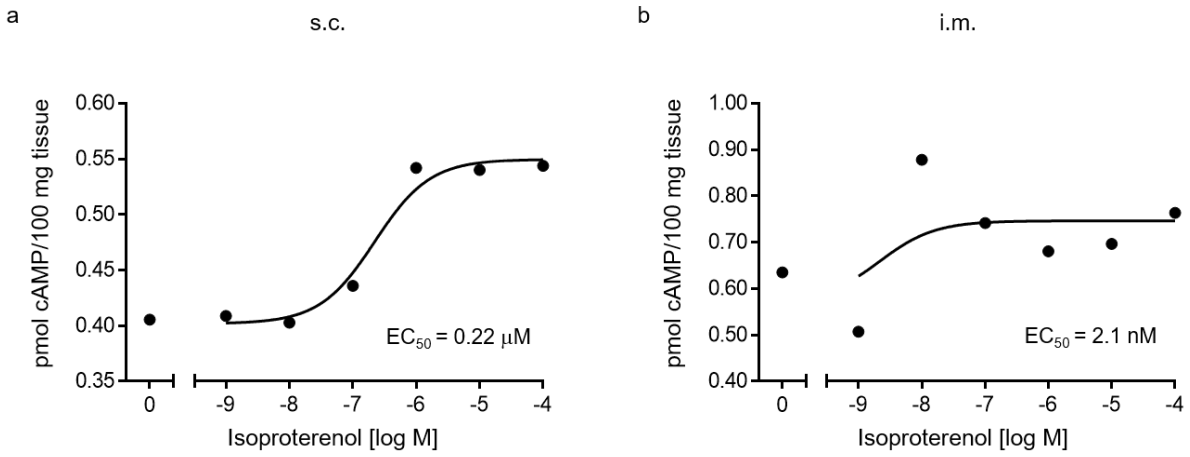

Figure 8. cAMP production in s.c. (a) and i.m. (b) adipose tissue in response to isoproterenol. Tissues were incubated with isoproterenol for 30 min at 37˚C. Data are expressed as mean values (n = 5). The results at each concentration of isoproterenol were fitted together by nonlinear regression.

Isoproterenol, RH, and ZH-stimulated lipolysis in s.c. and i.m. adipose tissue

To determine the effects isoproterenol, RH, and ZH on lipolysis, the release of glycerol and NEFA from s.c. and i.m. adipose tissues was measured. Isoproterenol increased glycerol concentrations in a dose-response manner in s.c. adipose tissue (EC_{50} = 5.1 µM), whereas it had no effect on glycerol release in i.m. adipose tissue (Figures 9a and 9b). RH and ZH increased glycerol concentrations in a dose-response manner in s.c. adipose tissue, but RH and ZH did not affect glycerol release in i.m. adipose tissue (Table 6). Likewise, isoproterenol increased glycerol release in s.c. adipose tissue, and isoproterenol also increased NEFA concentrations in a dose-dependent manner in s.c. adipose tissue (EC_{50} = 0.18 µM) (Figure 9c). However, isoproterenol decreased the NEFA release in a dose-dependent manner in i.m. adipose tissue and (Figure 9d). Although RH and ZH increased NEFA release from s.c. adipose tissue at all concentrations compared to the basal level, NEFA release was not dose-dependent (Table 6). None of the ß-AR agonists affected NEFA release from i.m. adipose tissue ($P > 0.05$).

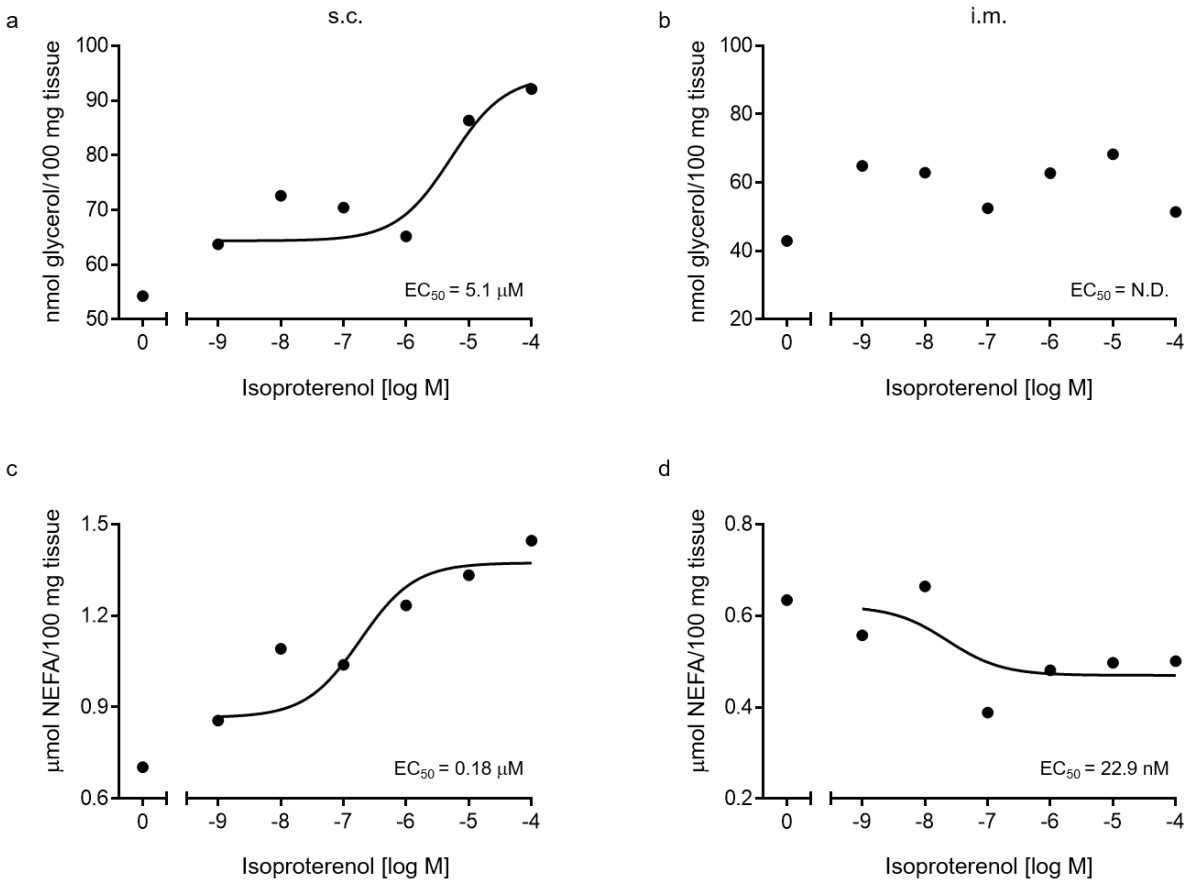

Figure 9. Glycerol and NEFA release in s.c. (a and c) and i.m. (b and d) adipose tissue in response to isoproterenol. Tissues were incubated with isoproterenol for 30 min at 37˚C (n = 5). Glycerol (a and b) and non-esterified fatty acid (NEFA) (c and d) released in tissue supernatant were determined. Data are expressed as mean values (n = 5). The results at each concentration of isoproterenol were fitted together by nonlinear regression.

Table 6. Tissue cAMP, glycerol, and NEFA release stimulated by RH and ZH

	RH[1]		ZH[2]	
	s.c.	i.m.	s.c.	i.m.
cAMP (pmol/ 100 mg tissue)				
Baseline	0.42 ± 0.06	0.64 ± 0.19	0.42 ± 0.06	0.64 ± 0.19
10^{-8}	0.39 ± 0.09	0.86 ± 0.22	0.76 ± 0.37	0.79 ± 0.30
10^{-7}	0.38 ± 0.08	0.76 ± 0.18	0.61 ± 0.27	0.51 ± 0.13
10^{-6}	0.44 ± 0.14	0.70 ± 0.18	0.62 ± 0.27	0.78 ± 0.39
Glycerol (nmol/ 100 mg tissue)				
Baseline	50.0 ± 12.8	48.6 ± 10.3	50.0 ± 12.8	48.6 ± 10.3
10^{-8}	50.3 ± 7.60	52.4 ± 16.4	54.8 ± 8.00	67.4 ± 21.6
10^{-7}	52.6 ± 11.1	81.7 ± 25.5	59.3 ± 15.4	48.6 ± 13.7
10^{-6}	63.3 ± 9.20	42.6 ± 11.4	65.9 ± 12.6	46.0 ± 14.5
NEFA (μmol/ 100 mg tissue)				
Baseline	0.99 ± 0.40	0.65 ± 0.20	0.99 ± 0.40	0.65 ± 00.20
10^{-8}	1.10 ± 0.15	0.75 ± 0.21	1.06 ± 0.30	0.78 ± 0.28
10^{-7}	1.20 ± 0.21	1.18 ± 0.50	1.25 ± 0.31	0.91 ± 0.30
10^{-6}	1.06 ± 0.13	0.56 ± 0.13	1.17 ± 0.35	0.50 ± 0.16

Values are means ± SEM; n = 6 steers. Means within treatment and tissue were not different ($P > 0.05$). [1]RH; Ractopamine Hydrochloride, [2]ZH; Zilpaterol Hydrochloride.

Lipolytic response to BRL-37344 and Experior™ for ß3-AR

To determine if binding of Experior™ to ß3-AR might contribute to its lipolytic response, the selective ß3-AR agonist, BRL-37344, was used as a positive control to compare the effects on Experior™ in s.c. and i.m. adipose tissue. The pD_2 value for Experior™ was similar to the BRL-37344 pD_2 value in s.c. adipose tissue, whereas the pD_2 value for Experior™ was not applicable in i.m. adipose tissue (Table 7). No lipolytic responses for cAMP or NEFA were observed in s.c. adipose tissue for BRL-37344 or Experior™ (Figures 10a and 10c). Total cAMP production from i.m. adipose tissue was significantly less in BRL-37344-treated i.m. adipose tissue than in Experior™-treated in i.m. adipose tissue at 10^{-6} and 10^{-5} M ($P < 0.05$), but no difference in NEFA release was observed in i.m. adipose tissue for BRL-37344 or Experior™ (Figures 10b and 10d).

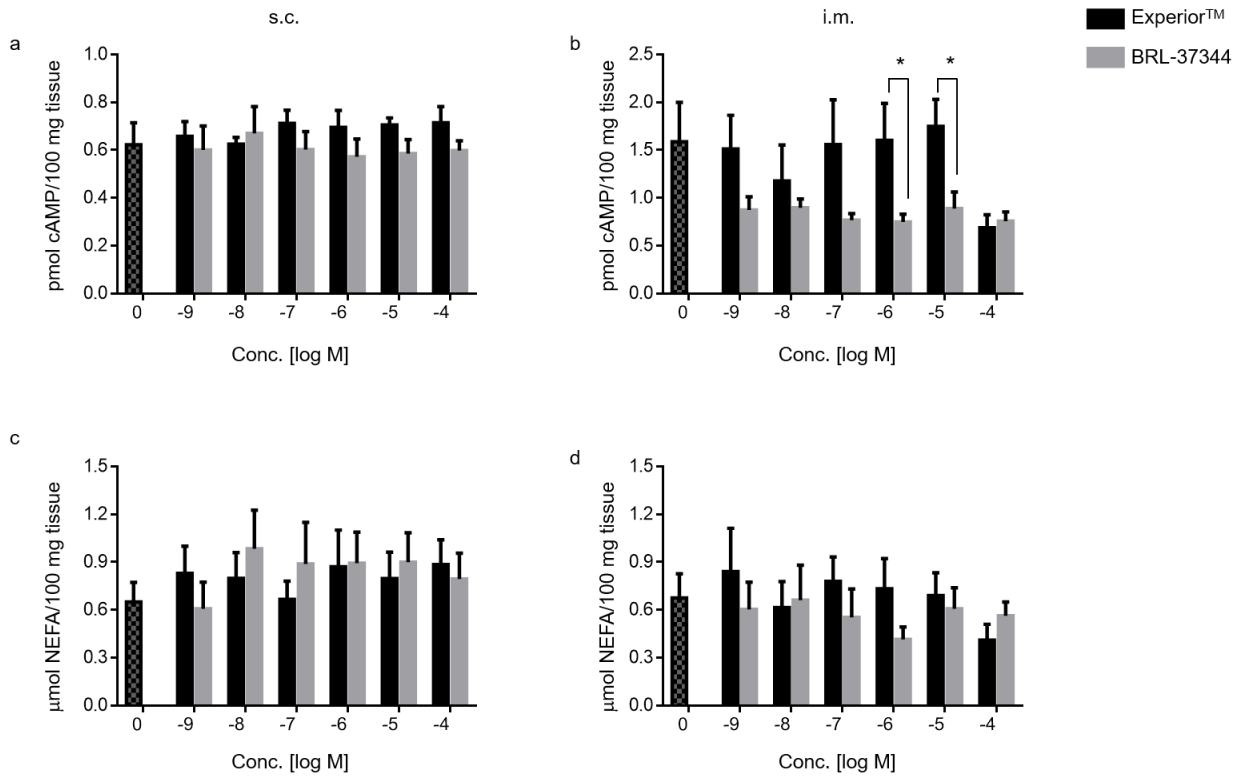

Figure 10. cAMP and NEFA release in response to BRL-37344 and Experior™ in s.c. (a and c) and i.m. (b and d) adipose tissue. Tissues were incubated with either BRL-37344 or Experior™ for 30 min at 37˚C. Data are expressed as means ± SEM (n = 6). **P* < 0.05 Experior™ vs BRL-37344.

Table 7. Adipose tissue lipolytic sensitivity to ß-AR agonists

	s.c.	i.m.
Experior™	7.40± 1.78	[1]N.A.
BRL-37344	7.00 ± 3.27	N.A.
Salbutamol	6.23 ± 0.35	4.59 ± 4.42
Salbutamol + Experior™	3.34 ± 0.59 ***	8.07 ± 4.90
Salbutamol + Experior™ + L-748.337	4.04 ± 0.34 ***	N.A.
Dobutamine	5.50 ± 0.68	6.52 ± 1.23
Dobutamine + Experior™	6.44 ± 3.20	7.29 ± 1.44
Dobutamine + Experior™ + L-748.337	5.87 ± 0.81	6.12 ± 1.70

Values are means ± SEM of sensitivity (pD_2) of adipose tissue = -log EC_{50}; n = 12 cattle. pD_2 was calculated from the individual concentration-response to agonists fitted together by nonlinear regression. EC_{50}, half maximum effective response. [1]N.A., not applicable. It was not possible to calculate pD_2 from the data. ***P < 0.001, vs salbutamol.

Experior™ as a ß1-and ß2-AR antagonist in s.c. adipose tissue

We set the optimal concentrations of propranolol (50 µM), a non-selective ß-AR antagonist, and L-748,337 (10 µM), a selective $ß_3$-AR antagonist based on preliminary tests, which caused depression of cAMP production in s.c. adipose tissue. Subcutaneous and i.m. adipose tissues were preincubated with either 50 µM propranolol or 10 µM L-748,337 for 30 min before adding Experior™ in a dose-response manner. The production of cAMP was reduced by Experior™ in a dose-response manner in s.c. adipose tissue pretreated with either 50 µM propranolol or 10 µM L-748,337 (Figures 11a and 11c). There was no effect of Experior™ on cAMP production in i.m adipose tissue preincubated with propranolol (Figure 11b). However, Experior™ depressed cAMP production in i.m. adipose tissue preincubated with L-748,337 (Figure 11d).

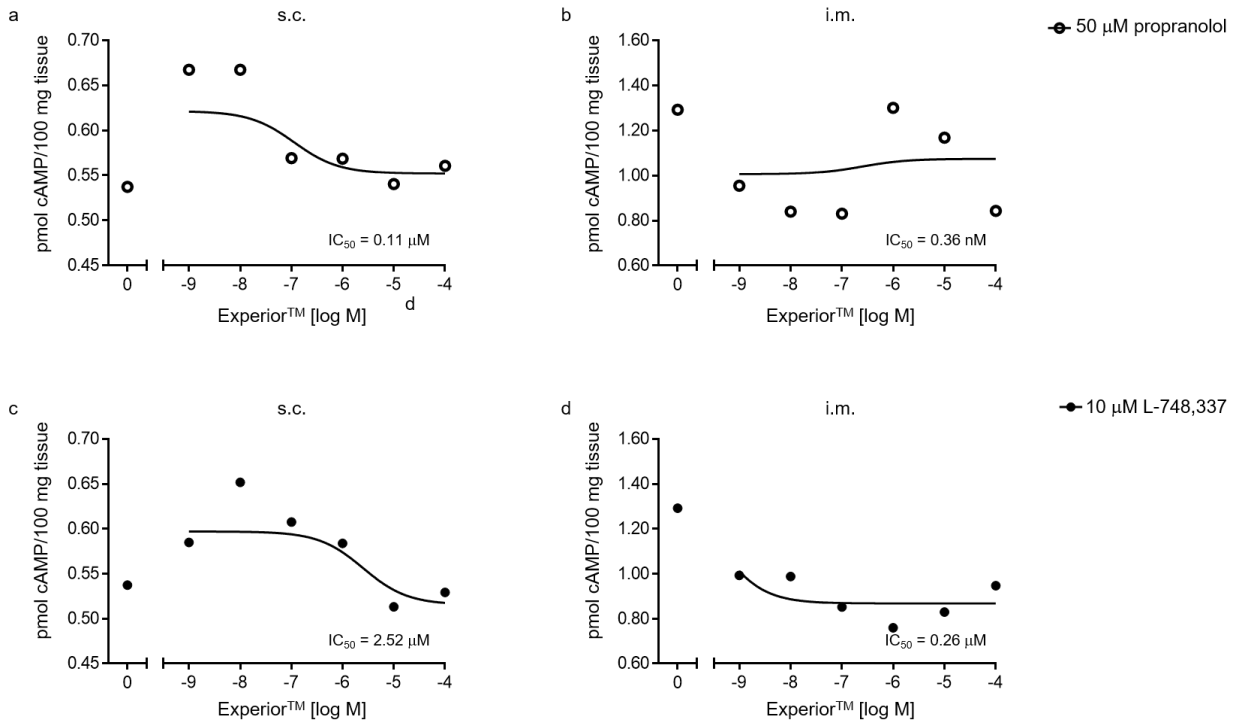

Figure 11. Depression of cAMP production in response to propranolol (a and b) and L-748,337 (c and d) in s.c. and i.m. adipose tissue. Tissues were pre-incubated with 10 µM L-748,337 or 50 µM propranolol, and then Experior™ was added for an additional 30 min. Data are expressed as mean values (n = 8). The results at each concentration of Experior™ were fitted together by nonlinear regression.

Antagonism of ß$_1$-and ß$_2$-AR by Experior™

In s.c. adipose tissue, Experior™ blunted the production of cAMP in the presence of ß-AR antagonists, suggesting that Experior™ is also a ß-AR antagonist (Figure 11). To address this hypothesis, a selective ß$_1$-AR agonist, dobutamine and a selective ß$_2$-AR agonist, salbutamol, were used to stimulate cAMP production mediated directly by individual ß$_1$-AR and ß$_2$-AR subtypes. Dose-response curves for cAMP production induced by either dobutamine or salbutamol in s.c. and i.m. adipose tissue were indicated in Figure 12, and pD_2 values are summarized in Table 7. Both salbutamol and dobutamine increased cAMP production in s.c. adipose tissue in a dose-dependent manner (Figures 12a and 12c) but were without effect in i.m. adipose tissue (Figures 12b and 12d). To investigate the antagonism of Experior™ to ß$_1$- and ß$_2$-AR, s.c. and i.m. adipose

tissues were pretreated with 1 µM Experior™ in the presence or absence of 10 µM L-74,337 before adding dobutamine or salbutamol. Experior™ and Experior™ plus 10 µM L-748,337 pre-treatment to salbutamol significantly shifted the EC_{50} in s.c. adipose tissue (Figure 12a) ($F(2, 99) = 19.21$, $P < 0.0001$). Furthermore, Experior™ with or without L-748,337 significantly blunted cAMP production mediated by salbutamol in s.c. adipose tissue and the cAMP reduction was remarkable from 10^{-6} to 10^{-4} M of salbutamol ($P < 0.05$) (Figure 12a). There was no significant change in EC_{50} shift in response to dobutamine in the presence of Experior™ and Experior™ plus L-748,337 in s.c. adipose tissue ($P > 0.05$). However, Experior™ plus L-748,337 significantly inhibited cAMP production mediated by dobutamine in s.c. adipose tissue ($P < 0.05$) (Figure 12c). Neither dobutamine nor salbutamol had any effect on cAMP production in i.m. adipose tissue. In addition, there was no antagonistic effect of Experior™ in i.m. adipose tissue (Figures 12b and 12d).

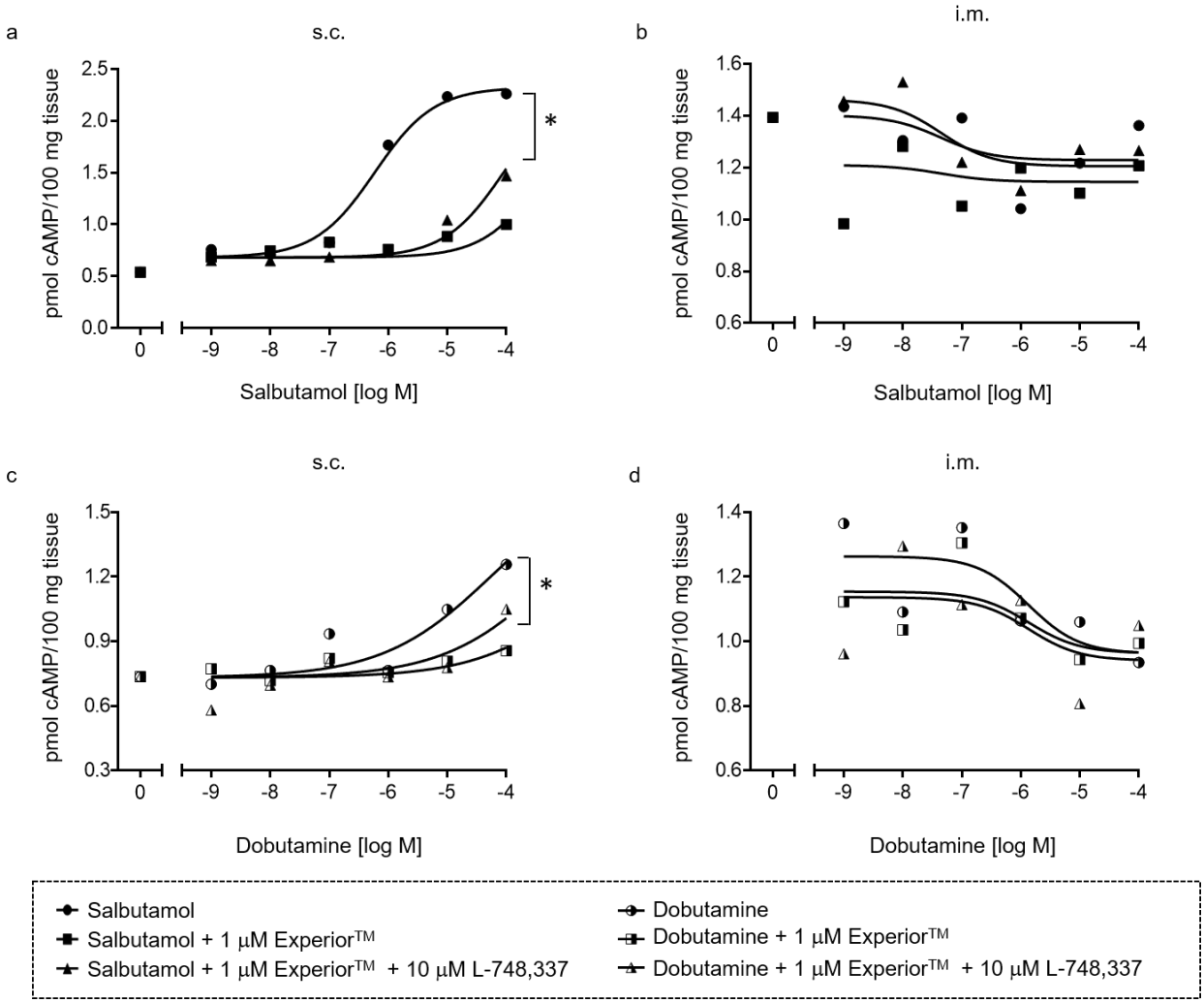

Figure 12. cAMP production in response to selective ß$_1$- and ß$_2$-AR agonists in s.c. (a and c) and i.m. adipose tissue (b and d). With 1 μM Experior™ or 1 μM Experior™ + 10 μM L-748,337, salbutamol and dobutamine reduced cAMP production in s.c. and i.m. adipose tissues. Tissues were pre-incubated with 1 μM Experior™ or 1 μM Experior™ + 10 μM L-748,337, and then salbutamol (a, b) or dobutamine (c, d) was added for an additional 30 min. The results of each individual concentration-response to salbutamol and dobutamine were fitted together by nonlinear regression. Data are expressed as means (n = 5). *$P < 0.05$.

Discussion

In the present study, s.c. and i.m. adipose tissues were used to investigate the effects of different ß-AR agonists and antagonists along with the novel ß-AR ligand Experior™ on cAMP production induced by adenylyl cyclase/PKA/hormone sensitive lipase cascade. The results of this

study indicated that s.c. and i.m. adipose tissue have physiologically different responses to ß-AR agonists. Isoproterenol, RH and ZH stimulated glycerol and NEFA release from s.c. adipose tissue. In contrast, those ß-AR agonists were not effective in elevation of cAMP production or in release of glycerol or NEFA in i.m. adipose tissue. Experior™ significantly inhibited stimulation of cAMP production mediated by interaction of $ß_1$-AR and $ß_2$-AR and adenylyl cyclase in s.c. adipose tissue, whereas Experior™ did not have any effect in i.m. adipose tissue.

ß-adrenergic receptors are G protein-coupled receptors, and three ß-AR subtypes ($ß_1$-AR, $ß_2$-AR, and $ß_3$-AR) are expressed in most mammalian cells. The cellular proportion of each subtype present on the surface of membranes varies among tissues and across species. Furthermore, ß-AR subtypes, even in single cells, have different distributions among adipocytes (Seydoux et al., 1996). This suggests that each ß-AR subtype may be transcriptionally regulated to vary the proportion of their receptor subtypes on the cell surface. Early *ADRB* gene expression was reported in bovine oocytes and preimplantation embryos (Cikos et al., 2014). The first transcription gene of *ADRB* was the $ß_2$-AR in the morula, and then all *ADRB* genes were expressed in the blastocyst, indicating that transcription of these genes begins early after embryonic genome activation. The current study confirmed *ADRB* expression in s.c. and i.m. adipose tissue from growing cattle and reported the distribution of *ADRB* in bovine i.m. adipose tissue for the first time. Both s.c. and i.m. adipose tissues expressed all three ß-AR, and the most abundant *ADRB* was the *ADRB2*. Interestingly, the $ß_1$-AR and $ß_3$-AR subtypes showed similar levels of gene expression in both s.c. and i.m. adipose tissues. In the past, *ADRB3* was believed to be expressed primarily on the surface of white and brown adipocytes in rodents (Strosberg, 1997). For two decades, the expression of *ADRB3* had been identified and confirmed in various tissues of livestock spices. The *ADRB3* gene is expressed in brown adipose tissue in cattle, s.c. adipose tissue in pigs,

most of the tissues of sheep, and s.c. adipose tissue, mammary gland, gastrointestinal tracts, and liver of dairy cattle (Casteilla et al., 1994; Pietri-Rouxel et al., 1995; McNeel and Mersmann, 1999; Inderwies et al., 2003; Meylan et al., 2004; Carron et al., 2005; Kobel et al., 2006; Sumner and McNamara, 2007; Wu et al., 2010). However, there were no reports for the identification *ADRB3* gene expression in s.c. and i.m. adipose tissue of beef cattle. Therefore, our findings are noteworthy in that we confirmed *ADRB3* gene expression in adipose tissues of beef cattle for the first time.

ß-Adrenergic agonists act as repartitioning agents that lead to the redirection of nutrients from lipid synthesis to protein synthesis, modulating animal growth in various species of mammals and birds, including cattle, pigs, poultry, and sheep (Jones et al., 1985; Moloney et al., 1990; Schiavetta et al., 1990; Smith et al., 1995). Oral administration of these ß-AR agonists (e.g., cimaterol, clenbuterol, L-644,969, and RH) increased muscle mass by increasing muscle protein synthesis and depressing adipose tissue accretion in livestock species by directly stimulating the triacylglycerol degradation and by inhibiting fatty acid and triacylglycerol synthesis in adipose tissue. The effects of ß-AR agonists begin with the stimulation of ß-AR through the G-coupled proteins to activate adenylyl cyclase which, in turn, stimulates production of cAMP. In current study, isoproterenol was chosen as the non-specific agonist for all ß-AR subtypes, to compare the effects of ZH and RH on cAMP-dependent lipolysis in response to ß-AR stimulation. After 30 min incubation, isoproterenol increased cAMP production and, consequently, induced increases in glycerol and NEFA release from s.c. adipose tissue in a dose-response manner. However, we prolonged the incubation time 60, 90, and 270 min, cAMP production was dramatically reduced (data not shown). This suggests that extended exposure to isoproterenol causes ß-AR to be sequestered and degraded, resulting in diminished sensitivity to agonists due to desensitization to ß-AR agonists.

Although RH and ZH had no effect on cAMP production, the release of glycerol by RH and ZH in s.c. adipose tissue was increased. This suggest that the lipolytic response is controlled by even a very small range of cAMP levels (Allen and Quesenberry, 1988). The release of NEFA in response to RH and ZH was similar at individual concentrations, but the ZH effect was not significant. Activation of ß-AR stimulated by ß-AR agonists, including isoproterenol, cimaterol, clenbuterol, and RH in porcine adipose tissue increased the release of glycerol and NEFA (Peterla and Scanes, 1990). Meanwhile, oral administration of RH to pigs reduced fat accretion owing to suppression of the activity of lipogenic enzymes and, in turn, the depression of *de novo* fatty acid synthesis (Mills et al., 1990). Additionally, gene expression associated with lipid synthesis including *sterol regulatory element binding protein-1(SREBP1), fatty acid synthase (FAS),* and *proliferator-activated receptor-γ2 (PPARγ2)* was also reduced in pigs by RH (Reiter et al., 2007; Halsey et al., 2011). Page et al. (2004) postulated that RH can trigger apoptosis in mouse adipose tissue (Page et al., 2004). Unfortunately, knowledge of altering lipogenic gene expression induced by ß-AR agonists is limited in cattle. Taken together, reduction in fat accretion induced by RH may be through increased lipolysis and depressed lipogenesis.

Species have different expression and functional roles for ß-AR subtypes because of their potential redundancy and complexity in signaling responses, and the function of the $ß_3$-AR is species-specific (Langin et al., 1991). The $ß_3$-AR predominantly mediates the lipolytic response through selective $ß_3$-AR agonists such as CL-316243 and BRL-37344 in rodents, rabbits, and dogs, whereas it is poorly responsive to $ß_3$-AR agonists in humans, primates, guinea pigs, and pigs (Bousquet-Melou et al., 1994; Carpene et al., 1994; Langin et al., 1995; Mills, 2000). To date, there is no clear evidence for the existence of a functional $ß_3$-AR in bovine adipose tissue from physiologically mature cattle. In our present study, we attempted to examine the presence of a

functional ß$_3$-AR in response to BRL-37344, a selective ß$_3$-AR agonists, and Experior^{TM}, a newly synthesized, putative ß$_3$-AR agonists in bovine adipose tissues. Both BRL-37344 and Experior^{TM} failed to stimulate cAMP production and NEFA release from s.c. and i.m. adipose tissues. Similar results were previously reported in porcine adipose tissue by Mills (2000), which tested ß$_3$-mediated lipolysis in response to BRL-37344 in porcine adipose tissue, in which BRL-37344 did not increase cAMP production or increase lipolysis (Mills, 2000). Our *ADRB* gene expression data showed that ß$_1$-AR and ß$_3$-AR were expressed similar levels in bovine s.c. and i.m. adipose tissues, which may propose a possibility of low G_s coupling with ß$_3$-AR. Moreover, Soeder et al. (1999) demonstrated that ß$_3$-AR can be constitutively coupled to G_s as well as G_i protein to control lipid metabolism (Soeder et al., 1999). This result raises another possibility as to why the concentrations of cAMP or NEFA produced by ß$_3$-AR activation remained unchanged with increasing in ß$_3$-AR agonist concentrations. In addition, Experior^{TM} exhibited similar sensitivity (pD_2) to adipose tissue as BRL-37344 in both adipose tissues, suggesting that Experior^{TM} may function as a ß$_3$-AR agonist in rodents or rabbits to same extent of BRL-37344. Taken together, ß$_3$-AR may not be functional in bovine adipose tissue and the tissue distribution of ß$_3$-AR in cattle is very different from that of rodents.

Individual ß$_1$-AR or ß$_2$-AR subtypes have different abilities to evoke the cAMP production. Dobutamine, a ß$_1$-AR agonist, modestly increased cAMP production, while salbutamol, a ß$_2$-AR agonist, strongly evoked cAMP production in s.c. adipose tissue, suggesting that ß$_2$-AR is the primary regulator of lipolysis in cattle, and that the ß$_1$-AR has a lesser function in lipolysis.

To investigate the antagonism of Experior^{TM}, we co-incubated Experior^{TM} with tissues pre-treated propranolol, a non-selective ß-AR antagonist. cAMP production was depressed strongly by increasing Experior^{TM} concentrations. Further, following pre-treatment with Experior^{TM},

Experior™ inhibited the ability for salbutamol and dobutamine to increase cAMP accumulation. This supports the concept that Experior™ functions better as a selective antagonist for ß$_1$-AR or ß$_2$-AR than as a ß$_3$-AR agonist in bovine adipose tissue. Backer et al. (2003) proposed the existence of two separate binding sites of the human ß$_1$-AR: 1) one for classic agonists and ß-antagonists and 2) the other for another agonist (i.e., CGP 12177) (Baker et al., 2003). CGP 12177 is an agonist that is relatively resistant to inhibition by propranolol and CGP 20712A (Konkar et al., 2000). The results of the current study demonstrated that dobutamine and salbutamol had agonistic effects on individual ß$_1$-AR and ß$_2$-AR, respectively, and also depressed cAMP production by Experior™ at higher concentrations of dobutamine and salbutamol. Furthermore, the combination of propranolol plus Experior™ decreased cAMP production. This suggests that bovine ß$_1$-AR or ß$_2$-AR may have two separate binding sites, and each ß-AR agonist and Experior™ may act on different binding sites in bovine adipose tissue.

In the current study, i.m. adipose tissue did not show a reproducible lipolytic response to ß-AR agonists, although cAMP production was greater in i.m. adipose tissue than in s.c. adipose tissue for all experiments. Adipocyte diameter and volume in i.m. adipose tissue are less than in s.c. adipose tissue (Smith and Crouse, 1984; Miller et al., 1989). Subcutaneous adipose tissue may develop initially as brown adipose tissue, subsequently dedifferentiating and redifferentiating to white adipose tissue (Landis et al., 2002). The current study demonstrated that the levels of gene expression of ß-AR were much lower in i.m. adipose tissue than that of s.c. adipose tissue, which indicates small amounts of ß-AR populations in i.m. adipose tissue. Therefore, i.m. adipose tissue apparently would be less responsive to lipolysis induced by synthetic or parasympathetic stimulation than s.c. adipose tissue.

In conclusion, our results indicate the potential specificity of adipose tissue in the expression of particular ß-AR subtypes during cattle growth and different physiological responses of ß-AR subtypes to ß-AR agonists administration in s.c. and i.m. adipose tissue. We also investigated cAMP accumulation and lipolysis mediated by the interaction of ß-AR and ß-AR agonist, resulting in increased lipolysis in s.c. adipose tissue. Experior^{TM}, a novel ß-AR agonist, may be both a $ß_3$-AR agonist and a $ß_1$-AR and $ß_2$-AR antagonist. These unique combinations of agonistic and antagonistic effects may have confounding impacts on lipolysis and muscle hypertrophy compared to traditional ß-AR agonist supplementation to cattle.

CHAPTER V

ASSESSMENT OF ANTAGONISTIC EFFECTS ON $B_{1/2}$-ADRENERGIC RECEPTORS USING A NOVEL B-AR LIGAND IN BOVINE ADIPOCYTES

Introduction

The adrenergic receptors (α and ß) are a class of G protein-coupled receptors, and three ß-AR subtypes have been identified in the most mammalian tissues, $ß_1$-AR, $ß_2$-AR, and $ß_3$-AR (Strosberg, 1997). The proportions of ß-AR subtypes in adipose tissue varies with species. For example, $ß_2$-AR is the most predominant subtype in bovine adipose tissue and human adipose tissue, $ß_1$-AR is the primary subtype in porcine adipose tissue, and $ß_3$-AR is the most abundant subtype in rodent adipose tissue (Sillence and Matthews, 1994; McNeel and Mersmann, 1999; Johnson et al., 2014). These ß-AR subtypes either stimulate or inhibit the physiological response in binding to ß-agonists (ß-AA) or ß-antagonists (Hershberger, 1994; Woodcock, 2007; Lynch and Ryall, 2008), which the stimulation of ß-AR by ß-AA signals through G-protein $G_{\alpha s}$ to activate the adenylate cyclase (AC)-cAMP-protein kinase A (PKA) signaling cascade (Mersmann, 1998; Wallukat, 2002).

Adipose tissue is uniquely sensitive to ß-AA. The activation of ß-AR by ß-AA promotes lipolysis of triglyceride in lipid droplets, thereby releasing mobilized fatty acids and other precursors derived from stored triglyceride, which are used for energy generation (Barbosa et al., 2015). The lipolysis is known to be induced by $ß_1$-AR and $ß_2$-AR in mammals except for rodents, as the $ß_3$-AR in rodent white and brown adipocytes mainly mediates lipolysis. Nevertheless, lipolysis mediated by $ß_3$-AR has also been reported in various mammals, but the effects were low (Bousquet-Melou et al., 1994). For instance, $ß_3$-AR agonists induced lipolysis in white adipocytes

from marmosets, dogs, and rats, while they were ineffective in stimulating lipolysis in white adipocytes from baboons, macaques and humans. Lipolysis induced by ß$_3$-AR on bovine white adipose tissue remain unclear. In addition, specificity between synthetic ß-AA and ß-AR reveals species-specific differences. Ractopamine and clenbuterol are partial ß$_2$-agonists in porcine adipocytes (Liu et al., 1989), and they are reacted as antagonists in porcine adipocytes under certain conditions (Liu and Mills, 1989). BRL- 37,344 and CL-316,243, selective ß$_3$-agonists, had full agonistic effects in rat and dog white adipocytes, whereas another ß$_3$-agonists such as CGP-12,177 and SR-56,811A were partial agonists in rat and dog white adipocytes (Bousquet-Melou et al., 1994).

ß-Adrenergic agonists traditionally have been used for the treatment of chronic bronchitis, chronic obstructive pulmonary disease, asthma, uterine relaxants, and cardiac irregularities for more than 30 year (Barnes, 1999). Interestingly, some ß-AA (e.g., zilpaterol, ractopamine, cimaterol, terbutaline, matuberol and salbutamol) in livestock industry revealed repartitioning effects, which promotes muscle hypertrophy through increase in muscle protein synthesis and decrease in adipose tissue mass by stimulation through triacylglycerol degradation and inhibition of their synthesis and fatty acid synthesis (Emery et al., 1984; Jones et al., 1985; Moloney et al., 1990; Schiavetta et al., 1990; Smith et al., 1995). Therefore, these ß-AA contribute to improvement of feed utilization, lean growth rate, and carcass lean percentage in cattle, pigs, poultry and sheep. At present, most ß-AR agonists commercially available, such as zilpaterol, ractopamine, cimaterol, terbutaline, matuberol and salbutamol target ß$_1$-AR and ß$_2$-AR. Recently, a new ß-AR modulator, lubabegon fumarate (as known as Experior^{TM}) has been synthesized by Elanco Animal Health. Preliminary data indicated that Experior^{TM} acted as ß$_3$-AR agonist and a ß$_1$-AR and ß$_2$-AR antagonist in Chinese hamster ovary cells (M. E. Spurlock, unpublished data). Our previous study

(chapter 4) revealed that Experior™ showed strong antagonistic effects on ß$_1$-AR and ß$_2$-AR in bovine subcutaneous (s.c.) adipose tissue explant culture. Based on these observations, we used primary bovine s.c. and intramuscular (i.m.) adipocytes to investigate the mechanisms regulating ß-AR stimulation mediated by Experior™.

Materials and Methods

Materials

Lubabegon fumarate (Experior™) was provided by Elanco Animal Health (Elanco Animal Health, Indianapolis, IN). Other drugs and reagents were purchased from the following companies: dobutamine, salbutamol, propranolol, insulin, dexamethasone, gentamicin, Glycerol Assay Kit, DNAseI, bovine serum albumin (BSA), phenymethylsulfony fluoride (PMSF), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO); troglitazone, rosiglitazone, ciglitazone, 3-isobutyl-1-methylxanthine (IBMX), and forskolin (Cayman Chemical, Ann Arbor, MI); Cell Counting kit-8 (Dojindo, Rockville, MD); Cyclic AMP XP® Assay Kit, Hormone Sensitive Lipase (HSL) antibody and phospho-HSL (Ser563) antibody (Cell Signaling Technology, Danvers, MA); antibiotic-antimycotic, Dulbecco's modified Eagle's Medium, nutrient mixture Ham's F12 (DMEM/F12), Dulbecco's phosphate-buffer saline (DPBS), collagenase II, gentamicin, amphotericin B, Protein Kinase A Colorimetric Activity Kit, Pierce BCA Protein Assay Kit, and protease inhibitor (Thermo Fisher Scientific, Waltham, MA); nonesterified fatty acid (NEFA) kit (Wako Life Sciences, Inc., Mountain View, CA); qScript™ cDNA synthesis kit and Perfecta® SYBR® Green fastmix® (Quanta Biosciences, Gaithersburg, MD); HiBind® RNA mini column (Omega Bio-tek, Inc., Norcross, GA); polyvinylidene fluoride (PVDF) membrane (GE Healthcare

Bio-Sciences, Pittsburgh, PA); anti-ß-actin (Santa Cruz Biotechnology, Inc., Dallas, TX); horseradish peroxidase-conjugated rabbit anti-goat IgG (Abcam, Cambridge, MA).

Bovine adipose tissue sampling at slaughter

Angus crossed bred steers were fed a standard, corn-based finishing diet at the Texas A&M University McGregor Research Center, McGregor, TX, and then transported approximately 190 km to the Texas A&M Rosenthal Meat Science and Technology Center, College Station, TX. Cattle were stunned using a captive bolt followed by exsanguination. The longissimus muscle (LM) and overlying s.c. adipose tissue from the 8^{th} to 10^{th} thoracic region was removed by cutting through the hide immediately lateral to the spinal process. The muscle was immediately transported to the laboratory in pre-warmed DPBS with 3% antibiotic-antimycotic within 30 min.

Primary preadipocyte isolation from bovine adipose tissues

Fresh LM and overlying s.c. adipose tissue were rinsed with pre-warmed DPBS containing 1% antibiotic-antimycotic to remove blood clots. Subcutaneous adipose tissue and i.m. adipose tissues (dissected fresh from the LM muscle) were minced into small pieces using surgical scissors and incubated in pre-warmed digestion buffer containing DMEM/F12 (1:1 vol/vol), 0.15% collagenase II, and 1% BSA (essentially fatty acid free) for 45 min at 37°C in a shaking incubator. Following incubation, the digest was filtered through a 100-μm nylon mesh to eliminate undigested adipose tissues, mature adipocytes and large cell aggregates. The filtered stromal vascular fractions were centrifuged at 1,500 rpm for 10 min at room temperature, and then were incubated in erythrocyte lysis buffer for additional 5 min at room temperature to lyse red blood cells. The suspension was centrifuged at 1,500 rpm for 10 min, and resuspended in fresh growth

medium (DMEM/F12, 10% FBS and 1% antibiotic-antimycotic). The preadipocytes were counted and directly cultured at 37°C in a humidified incubator with 5% CO_2 or were frozen in lipid nitrogen until further use.

Cell viability assay

Subcutaneous preadipocytes were seeded at a density of 5×10^3 cells/well in 96-well microplates. Cells were incubated with or without dobutamine, salbutamol, or Experior™ for varying time periods and with various concentrations. Viabilities were evaluated by the cell counting kit-8 (CCK-8) according to the manufacturer's instructions. Eight wells were set in each group. Following treatment, 10 μL of CCK-8 was added into each well, and then the cells were incubated for additional 2 h in an incubator at 37°C. The absorbance of the formazan product was measured at 450 nm using an Epoch microplate reader (Biotek Instruments, Winooski, VT).

Preadipocytes differentiation

Subcutaneous and i.m. preadipocytes were cultured in normal growth media until 80% confluent. Cells were differentiated for 14 d into mature adipocytes in DMEM/F12 containing 2% FBS, 1 μM dexamethasone, 20 μg/mL insulin, 0.5 mM IBMX, 50 μg /mL gentamicin, 2.5 μg/mL amphotericin B, 1% antibiotic-antimycotic supplemented with PPARγ agonists (10 μM troglitazone, 10 μM ciglitazone, and 1 μM rosiglitazone) for the first 8 d. From d 8 to d 14, maintenance medium supplemented only with 20 μg/mL insulin and 1 μM rosiglitazone was used. The medium was replaced every 48 h. Morphologic changes of the s.c. and i.m. adipocytes were monitored under a microscope.

Production of cAMP

The level of cAMP production in s.c. and i.m. adipocytes was determined by using the Cyclic AMP XP® Assay Kit according to the manufacturer's instructions. Briefly, s.c and i.m preadipocytes were seeded at a density of 2×10^4 cells/well in 24-well microplates. At d 14 of differentiation, s.c. and i.m. adipocytes were starved for 1 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, 10 μM forskolin and 1% antibiotic-antimycotic at 37°C. After starvation, the cells were pre-stimulated in the presence or absence of either 1 μM Experior™ or 10 μM propranolol for 5 min. Following 5 min, the cells were stimulated with 10 μM dobutamine, 10 μM salbutamol, or 1 μM Experior™ for 15 min. The cells in 24-well plates were lysed in 200 μL of cell lysis buffer provided by the kit with 1 mM PMSF and 10 μL/ mL of protease inhibitor. The cell lysate was centrifuged at 14,000 x g for 10 min at 4 °C to remove cell debris. Fifty microliters of cell lysates from s.c. and i.m. adipocytes or standards were co-incubated in 96-well plates with the horseradish peroxidase (HRP)-linked cAMP substrate coated onto an immobilized rabbit monoclonal cAMP antibody at room temperature for 3 h on a horizontal orbital plate shaker. Next, 100 μL of the TMB substrate were added to each well and incubated at room temperature for 30 minutes. Afterward, 100 μL of stop solution was added to each well. After the reaction, color development was measured at 450 nm using an Epoch microplate reader. A standard curve was generated by a four-parameter logistic regression, and cAMP concentrations were calculated using GrapPad Prism 6.04 software (GraphPad Software Inc., San Diego, CA). Protein concentrations were determined by Pierce BCA Protein Assay Kit. Levels of cAMP were normalized to cellular protein content.

Protein Kinase A activity

Protein Kinase A activity was measured using PKA Colorimetric Activity Kit following manufacturer's protocol. Briefly, cells in 24-well plates were lysed in 200 μL of activated cell lysis buffer provided by the kit with 1 mM PMSF, 1 μL/mL protease inhibitor and 1 μL/mL phosphatase inhibitor cocktail and incubated for 30 min on ice with occasional vortexing. The lysates were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant fraction was used as substrates for the PKA enzymes. All supernatant fractions were diluted 1:1 in kinase assay buffer provided by the kit, adding 1 mM PMSF and 0.5 μL/mL protease inhibitor. Forty microliters of samples or standards and 10 μL of ATP were co-incubated in 96-well plates at 30°C for 90 min on a horizontal orbital plate shaker. The wells then were aspirated and washed 4 times with wash buffer. Twenty-five microliters of rabbit phospho PKA substrate antibody and 25 μL of secondary goat anti-rabbit IgG-HRP were co-incubated at room temperature for 60 min with shaking. Next, 100 μL of the TMB substrate were added to each well and incubated at room temperature for 30 min. Afterward, 50 μL of stop solution was added to each well. Color development was measured at 450 nm using an Epoch microplate reader. A standard curve was generated by a four-parameter logistic regression, and the PKA activity was calculated using GrapPad Prism 6.04 software.

Lipolysis assay

To test lipolysis, s.c and i.m preadipocytes were seeded at a density of 2×10^4 cells/well in 24-well microplates. At day 14 of differentiation, s.c. and i.m. adipocytes were starved for 2 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, and 1% antibiotic-antimycotic at 37°C. Following starvation, the cells were pre-stimulated in the presence or absence of either 1 μM Experior™ or 10 μM propranolol for 30 min. After 30 min, the cells were stimulated with 10 μM

dobutamine, 10 μM salbutamol or 1 μM Experior™ for 6 h. After 6 h, media were harvested for measuring concentration of glycerol and NEFA. The cells were harvested for protein quantification. Lipolysis was measured with a glycerol assay kit and a NEFA kit according to the manufacturer's procedures. In brief, to determine glycerol released from cells, 10 μL of media was reacted with 100 μL glycerol reaction reagent for 20 min at room temperature and the absorbance was measured at 570 nm. The glycerol standard was used to generate a calibration curve. To analyze the level of NEFA released from the cells, 10 μL of media plus 200 μL of the first reagent was incubated for 5 min at 37°C, then 100 μL of the second reagent was incubated for another 5 min at 37°C. The absorbance was read at 550 nm. Oleic acid was used to plot a standard curve and to calculate concentration of NEFA in the media. Glycerol and NEFA release were normalized to cellular protein content.

Quantitative real-time RT-qPCR

Subcutaneous and i.m preadipocytes were seeded at a density of 5×10^5 cells in T-25 flasks. At d 14 of differentiation, the cells were stimulated with 10 μM dobutamine, 10 μM salbutamol, 1 μM Experior™, dobutamine plus Experior™, and salbutamol plus Experior™ for 3 d in DMEM/F12 containing 2% FBS, and 1% antibiotic-antimycotic at 37°C. Total RAN was isolated from s.c. and i.m. cells using HiBind® RNA mini columns followed by DNAseI treatment. Total RNA was quantified on the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and reverse transcription was performed with the qScript™ cDNA synthesis kit. The gene expressions of lipid metabolism related genes in s.c. and i.m. cells were analyzed in a CFX384™ Real-Time System (Bio-rad, Hercules, CA) using the Perfecta® SYBR® Green fastmix® kit. The different efficacy of cDNA synthesis between samples was normalized with

three reference genes (*ribosomal protein 9* (*RSP9*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *succinate dehydrogenase* (*SDHA*)). The relative expression of mRNA was determined by the cycle threshold (CT) deviation of an unknown sample vs geometric mean of three reference genes and data were presented as either $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$. The primers used in this assay are listed in Table 8.

Table 8. List of primers used in RT-qPCR

Gene	Accession number	sequence	Amplicon length (bp)
ADRB1	NM_194266.1	F: 5'-AGAAGGCACTCAAGACGCT-3' R: 5'-CACCACGTTGGCTAGGAAGA-3'	81
ADRB2	NM_174231.1	F: 5'-TGATCGCTGTGGATCGCTAC-3' R: 5'-CCGGTACCAGTGCATCTGAA-3'	149
ADRB3	NM_174232.2	F: 5'-ACCTTCATTCTGTTCCTTCTG-3' R: 5'-CTGTGAGGTAGGTGTGTCTA-3'	145
ARRB1	NM_174243.3	F: 5'-TTCAACACAGCCCAGTACAA-3' R: 5'-GTAGACCTTGCAGAACGTAGAG-3'	87
ARRB2	NM_001205277.2	F: 5'-GGACCAGGGTCTTCAAGAAAT-3' R: 5'-CACTAGCACCACACCATCTAC-3'	116
BARK1	NM_174710.2	F: 5'-GAGATCTTCGACACGTACATCAT-3' R: 5'-CACCTGCTTCTTCACCAGAT-3'	105
BARK2	NM_174500.2	F: 5'-GCCTTCCACACTCCAGATAAA-3' R: 5'-AAACCGCATCTCCTTCTCAG-3'	111
ATGL	NM_001046005.2	F: 5'-TCTGCCTGCTGATTGCTATG-3' R: 5'-GCAGACATTGGCCTGGATAA-3'	130
HSL	NM_001080220.1	F: 5'-GAGACTGGCATCAGTGTGAC-3' R: 5'-TGCACGTCTAGGTTCTGAATG-3'	113
MGLL	NM_001206681.1	F: 5'-AAAGTTCTGAACCTCGTCCTG-3' R: 5'-GATGTCCACCTCCGTCTTATTC-3'	87
PLIN1	NM_001083699.1	F: 5'-CTGAAGGACACCATCTCCAC-3' R: 5'-CATACTCGGCAGTGTCTCTC-3'	145
ABHD5	NM_001076063.2	F: 5'-CATCCAGGGTTAGTCATCTCATT-3' R: 5'-CCAAGGCTCTGATCCAAACT-3'	105
FABP4	NM_174314.2	F: 5'-GGAAAGTCAAGAGCATCGTAAAC-3' R: 5'-TGGCAGTGACACCATTCAT-3'	141
FASN	NM_001012669.1	F: 5'-CCATCCTTCTGACCAAGAAGTC-3' R: 5'-AGGTGACGCCTTTCTCTTTG-3'	99
CEBPA	NM_176784.2	F: 5'-ATCGACATCAGCGCCTACAT-3' R: 5'-GCCCGGGTAGTCAAAGTCG-3'	141
PPARG	NM_181024.2	F: 5'-ATCTGCTGCAAGCCTTGGA-3' R: 5'-TGGAGCAGCTTGGCAAAGA-3'	76
GAPDH	NM_001034034.2	F: 5'-CTGCCCGTTCGACAGATAG-3' R: 5'-CTCCGACCTTCACCATCTTG-3'	76
RPS9	NM_001101152.2	F: 5'-GAGCTGGGTTTGTCGCAAAA-3' R: 5'-GGTCGAGGCGGGACTTCT-3'	65
SDHA	NM_174178.2	F: 5'-ACCTGATGCTTTGTGCTCTG-3' R: 5'-TCGTACTCGTCAACCCTCTC-3'	106

Immunoblotting

To measure HSL activity, at d 14 of differentiation, s.c. and i.m. adipocytes in T-25 flasks were starved for 1 h in DMEM/F12 containing 2% FBS, 0.5mM IBMX, and 1% antibiotic-antimycotic at 37°C. After starvation, the cells were pre-stimulated in the presence or absence of 1 μM Experior™ for 30 min. After 30 min of pre-treatment, the cells were stimulated with 10 μM dobutamine, 10 μM salbutamol or 1 μM Experior™ for 6 h, then cells were harvested for protein extraction. Protein concentrations in samples were quantified using Pierce™ BCA Protein Assay Kit. Total protein (10 μg) was separated on a 10% SDS-PAGE gel at constant current. Proteins in the gel were transferred onto a PVDF membrane using a Trans-blot® SD Semi-dry Transfer Cell (Bio-Rad, Hercules, CA) for 1 h. The membrane was immediately blocked with ether a 5% non-fat milk solution or 5% BSA (for phosphor-protein detection) at room temperature for 1 h. The membrane was then incubated overnight at 4°C with anti-HSL (1:500), phospho-HSL (Ser563) (1:500), anti-ß-actin (1:10,000). The membranes were incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1,000) for 2 h at room temperature. The proteins on the membrane were visualized using an enhanced chemiluminescence detection kit (Bio-Rad, Hercules, CA). Bands were quantified using Image Studio Lite version 5.2 software (LI-COR Biotechnology, Lincoln, NE) and protein levels were normalized to ß-actin on the same membrane.

Statistical analysis

All the experiments were performed in triplicates with at least three independent runs. The data are expressed as means ± SEM. Statistical analyses were tested either the two-sided, unpaired student's t-test or one-way analysis of variation (ANOVA) with either LSMeans Student's *t* or

Dunett's test using JMP Pro 13 software (SAS Institute Inc., Cary, NC). A *P*-value < 0.05 was considered significant.

Results

Induction of differentiation in bovine s.c. and i.m. preadipocyte and validation of concomitant gene expression

In this study, we have successfully induced adipogenic differentiation in bovine primary cultured preadipocytes (Figure 13). Before inducing adipogenic differentiation, s.c. and i.m. preadipocytes were phenotypically fibroblasts-like morphologies (Figures 13a and 13b). With the introduction of differentiation media, small lipid droplets were starting to become visible under a light microscope from d 3. Exposure to the differentiation media for 8 d led to an enhanced adipogenic differentiation of s.c and i.m. preadipocytes. For six additional days, cultured in maintenance medium supplemented only with 20 μg/mL insulin and 1 μM rosiglitazone, these s.c. and i.m. preadipocytes had further formations of medium to large lipid droplets (Figures 13c and 13d).

To further validate the adipogenic differentiation of s.c. and i.m preadipocytes, differentiation was confirmed by genes expressed in the commitment (*C/EBPα* and *PPARγ*) and terminal phase (*FABP4*) of adipogenesis through RT-qPCR. There were significant increases in expression of adipogenic genes in differentiated s.c. and i.m adipocytes ($P < 0.05$) (Figures 13e and 13f). The expression of *C/EBPα* and *PPARγ* was 2- and 6-fold higher expression in differentiated s.c. adipocytes than in s.c. preadipocytes, respectively (Figure 13e). Unlike s.c adipocytes, the expression of *C/EBPα* was 1.7-fold upregulated, but the expression of *PPARγ* was not different in differentiated i.m adipocytes compared with i.m. preadipocytes (Figure 13f). The

FABP4 gene was highly expressed in both s.c. adipocytes (50-fold) and i.m. adipocytes (14-fold) compared to s.c. and i.m. preadipocytes, respectively even though levels of *FABP4* expression in i.m. adipocytes were less than in s.c. adipocytes.

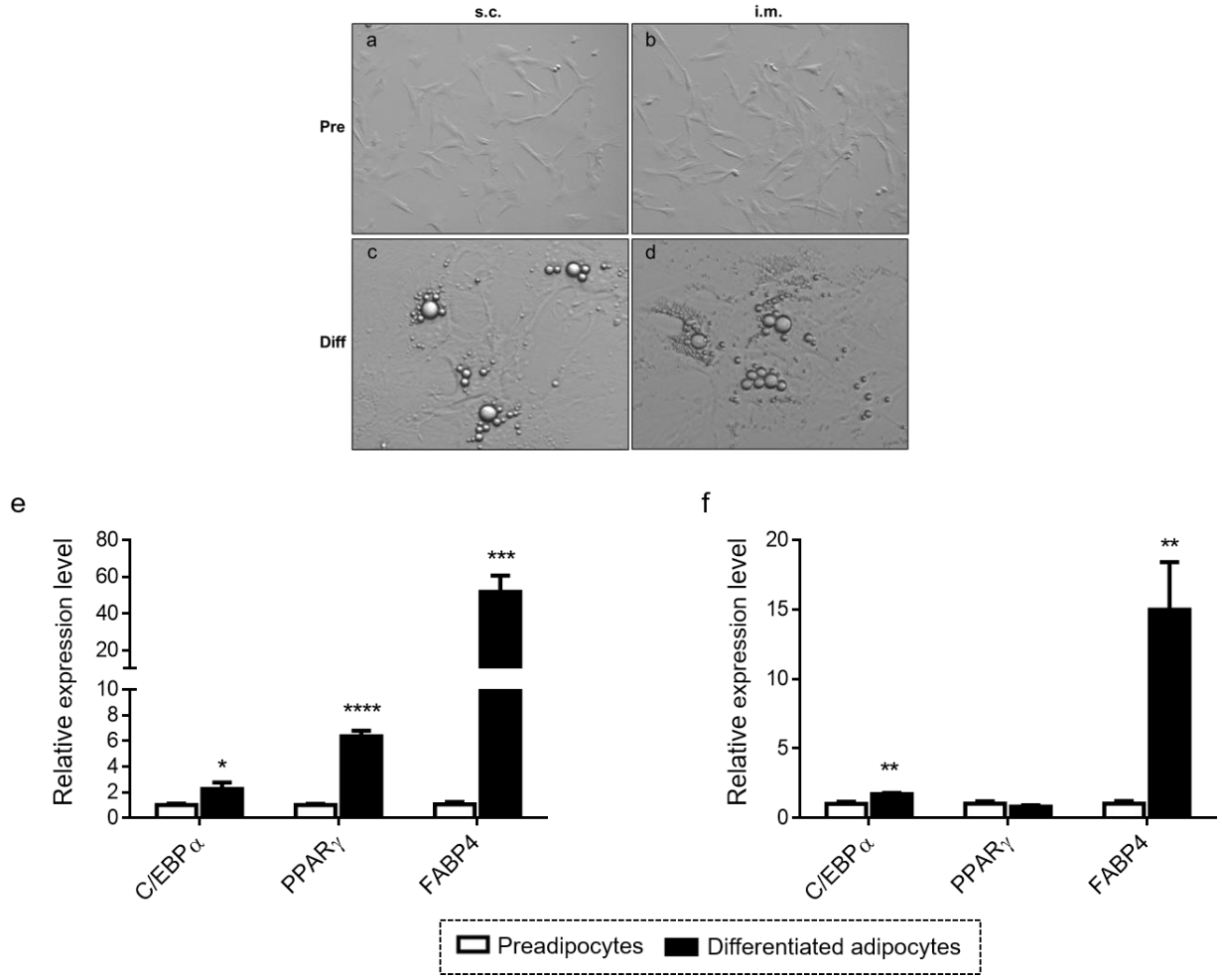

Figure 13. Morphological changes after induction of differentiation in bovine s.c. and i.m. preadipocyte and validation of concomitant gene expression. (a) s.c. preadipocytes. (b) i.m. preadipocytes. (c) Differentiated s.c. adipocytes. (d) Differentiated i.m. adipocytes. (e) relative gene expression levels in s.c. adipocytes. (f) relative gene expression in i.m. adipocytes. Data are expressed as means ± SEM (n = 5). **P* < 0.05, ***P* < 0.01, ****P* <0.001, differentiated adipocytes vs preadipocytes.

ß-adrenergic receptor genes (ADRB) profiling in primary s.c. and i.m. adipocytes

We have successfully detected all three ß-adrenergic receptor (*ADRB*) genes in both preadipocytes and differentiated adipocytes (Figures 14a and 14b). All *ADRB* genes were observed at least 2.5 times higher in differentiated s.c. adipocytes than in s.c. preadipocytes ($P < 0.05$). The predominant ß-AR subtype was $ß_2$-AR in s.c. preadipocytes, whereas the proportions of *ADRB* genes were not different in s.c. adipocytes upon differentiation (Figure 14a). In contrast, the expressions of *ADRB* genes did not differ in i.m. adipocytes compared to preadipocytes except for $ß_2$-AR. The *ADRB2* gene was highly expressed in i.m preadipocytes ($P < 0.05$). After induction of differentiation, the expression of *ADRB2* gene was significantly depressed in i.m. adipocytes (Figure 14b).

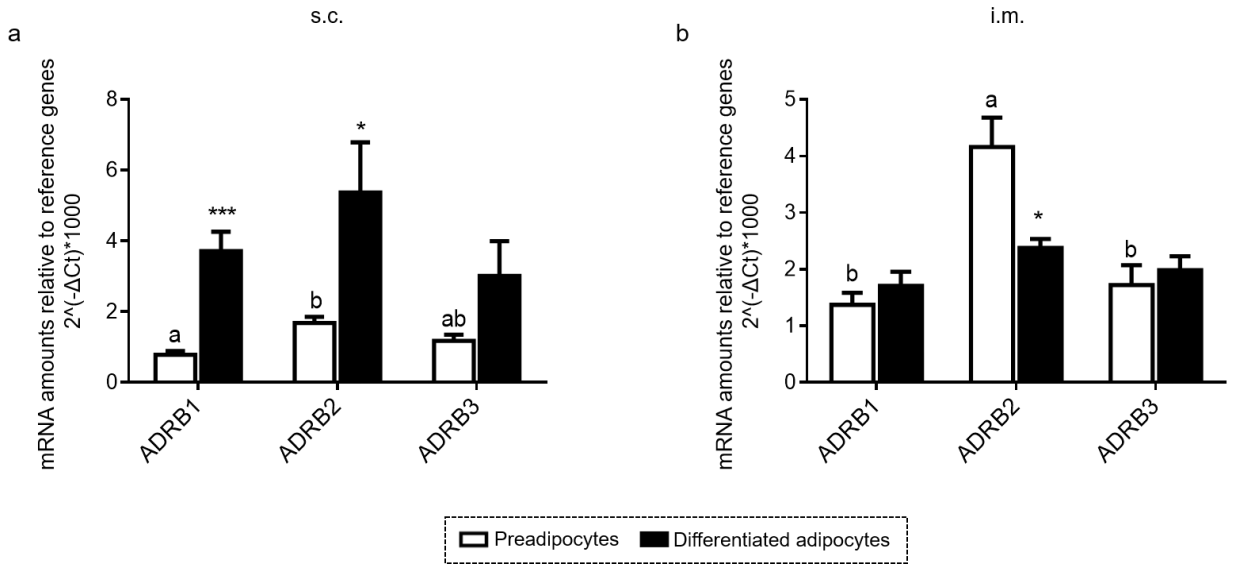

Figure 14. Investigation of ß-adrenergic receptor subtypes by RT-qPCR in bovine primary preadipocytes and differentiated adipocytes. (a) ß-AR gene profiling in s.c. preadipocytes and differentiated adipocytes. (b) ß-AR gene profiling in i.m. preadipocytes and differentiated adipocytes. Data are expressed as means ± SEM (n = 5). *$P < 0.05$, ***$P < 0.001$, differentiated adipocytes vs preadipocytes. ^{ab}Means across each *ADRB* receptors within each preadipocyte sharing a common superscript do not differ ($P < 0.05$).

Changes in s.c. preadipocytes proliferation after exposure to ß-AR agonists

We have evaluated the proliferation of s.c. preadipocytes to select optimal concentrations of ß-AR agonists for further studies. The cells were treated with various concentrations of dobutamine, salbutamol, and Experior^{TM} for various time points, and cell proliferation was measured by cell counting kit-8 (CCK-8). The CCK-8 assay indicated that individual ß-AR agonists showed different proliferative influences on s.c preadipocytes, especially at high concentrations after 1h incubation of these ß-AA (Figure 15). Following 1 h incubation, the treatment of dobutamine, a selective $ß_1$-AA, promoted the proliferation of preadipocytes by 59% and 200% at concentrations of 10 µM and 100 µM, respectively ($P < 0.05$) (Figure 15a). However, the exposure of various concentrations of salbutamol, a selective $ß_2$-AA, did not alter the proliferation of preadipocytes (Figure 15d). In contrast, the treatment of Experior^{TM}, a novel ß-AR modulator, inhibited the proliferation of preadipocytes by 46% and 63% at concentrations of 10 µM and 100 µM, respectively ($P < 0.05$) (Figure 15g). Based on these results, we decided to prolong the exposure time up to 6 h for dobutamine and salbutamol. Furthermore, we selected 1 µM Experior^{TM} to evaluate proliferation of preadipocytes for extended time points (3 to 72 h) and 10 µM Experior^{TM} for testing toxic effect on preadipocytes. Dobutamine treatment promoted cell proliferation up to 126% and 293% at concentrations of 10 µM and 100 µM, respectively, by increase in time ($P < 0.05$) (Figures 15b and 15c). In addition, 1 µM dobutamine also significantly increased cell proliferation by 67% at 6 h incubation (Figure 15c). In contrast, no changes in the proliferation of the preadipocytes were observed after treatment with serial concentration ranges of salbutamol until 3 h (Figure 15e). However, salbutamol promoted at least 50% cell proliferation at all concentrations at 6 h incubation ($P < 0.05$) (Figure 15f). There was no change in cell proliferation treated with 1 µM Experior^{TM} until 24 h but a significant increase in cell proliferation

after 48 h ($P < 0.05$) (Figure 15h). Experior™ (10 µM) reduced proliferation of preadipocytes by 21% after 5 min incubation. After 45 min, the rate of proliferation of preadipocytes was reduced by 38% (Figure 15i). Taken together, we selected 10 µM dobutamine and salbutamol and 1 µM Experior™ for further studies.

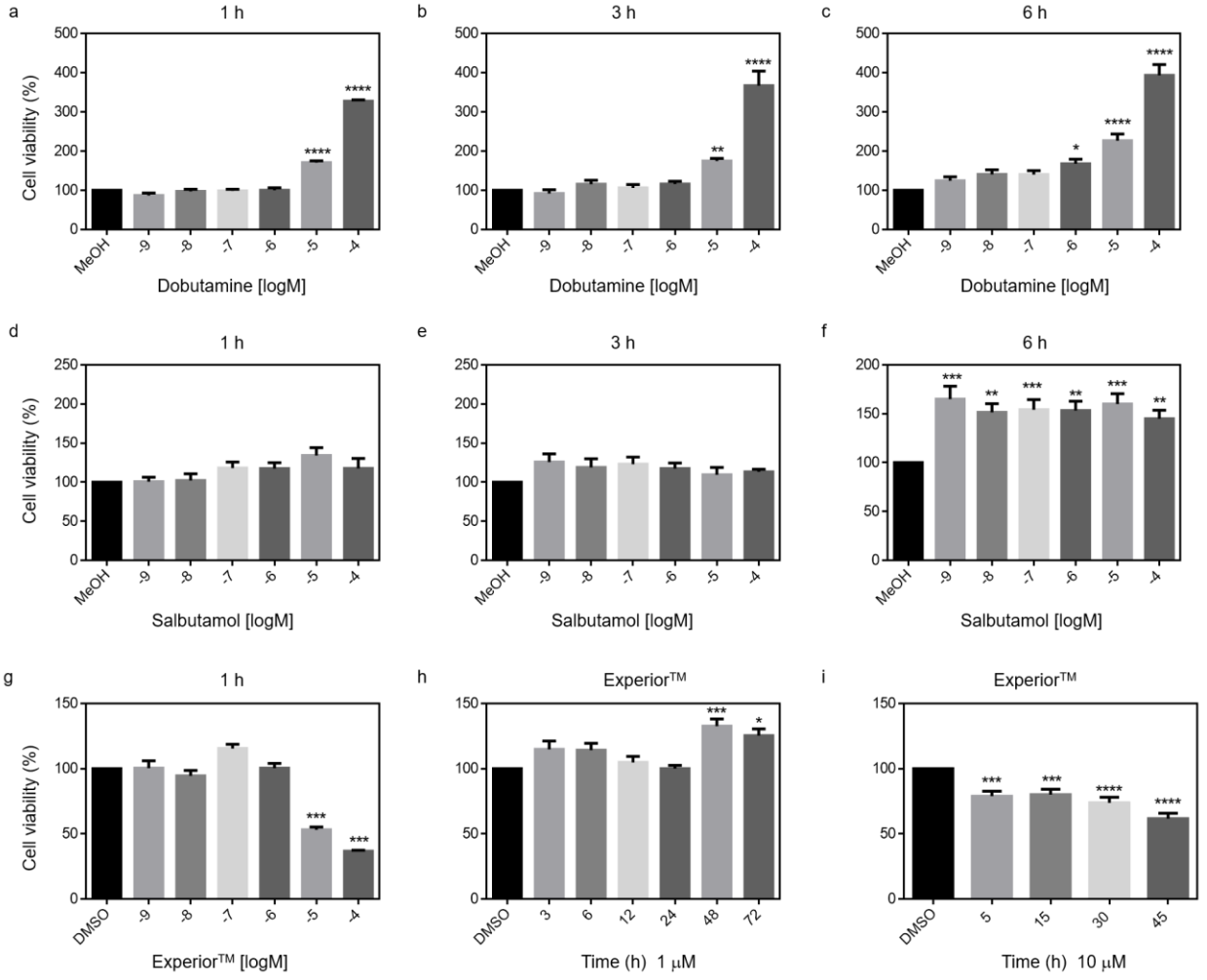

Figure 15. Cell viability after treatment with dobutamine, salbutamol, or Experior™ in bovine preadipocytes. Dose responses for cell viability after exposure to dobutamine at 1 h (a), 3 h (b), and 6 h (c). Dose responses for cell viability after exposure to salbutamol at1 h (d), 3 h (e), and 6 h (f). Dose responses for cell viability after exposure to Experior™ (g). Cell viability after exposure to 1 µM Experior™ at different time points (f). Cell viability after exposure to 10 µM Experior™ at different time points (i). Data are expressed as means ± SEM (n = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, vs control (either MeOH or DMSO).

Experior™ as a ß1-and ß2-AR antagonist in s.c and i.m. adipocytes

Our previous study demonstrated that Experior™ inhibited the activity of AC in bovine s.c. adipose tissue. Based on these results, we hypothesized that Experior™ acts as a ß-AR antagonist. To address this hypothesis, we used a selective $ß_1$-AA, dobutamine, and a selective $ß_2$-AA, salbutamol, to stimulate individual $ß_1$-AR and $ß_2$-AR in s.c. and i.m. adipocytes. As an index of activation of $ß_1$-AR and $ß_2$-AR, we estimated the activity of AC by measuring cAMP production. We treated 10 µM dobutamine, 10 µM salbutamol, and 1 µM Experior™ for 15 min in s.c. and i.m adipocytes that were pre-starved for 1 h in starvation media supplemented with 0.5 mM IBMX and 10 µM foreskin at d 14 of differentiation. The activity of AC was significantly increased by dobutamine treatment ($P < 0.05$), somewhat increased by salbutamol treatment, but was not altered by Experior™ treatment as compared to control s.c adipocytes (Figure 16a). In contrast, AC activity was not changed by these ß-AA and Experior™ treatments in i.m. adipocytes (Figure 17a). To investigate the antagonism of Experior™ against $ß_1$-AR and $ß_2$-AR in s.c. and i.m. adipocytes, the cells were pretreated with 1 µM Experior™ for 5 min before adding dobutamine or salbutamol. In addition, as a positive control, 10 µM propranolol, a non-selective ß-AR antagonist was prior to assess dobutamine or salbutamol. Following pre-treatment with either Experior™ or propranolol, they strongly blocked the potency for dobutamine and salbutamol to stimulate $ß_1$-AR and $ß_2$-AR, resulting in less stimulation of AC, and thereby less cAMP production than either dobutamine or salbutamol alone in s.c. adipocytes produced cAMP ($P < 0.05$) (Figures 16b and 16c). In contrast, dobutamine did not activate $ß_1$-AR in i.m. adipocytes (Figure 17a), and neither Experior™ nor propranolol treatment also exhibited antagonistic effects on $ß_1$-AR (Figure 17b). However, Experior™ effectively blunted the stimulation of $ß_2$-AR in i.m. adipocytes ($P < 0.05$) (Figure 17c).

The results on PKA activity were similar to that of AC activity in s.c. and i.m. adipocytes. PKA activity was significantly increased in dobutamine treatment ($P < 0.05$), somewhat increased in salbutamol treatment, but was not different from control with Experior™ treatment in s.c adipocytes (Figure 16d). There were no differences of PKA activity among treatments in i.m. adipocytes (Figure 17d). The activity of PKA was strongly reduced with co-treatment of Experior™ or propranolol with either dobutamine or salbutamol when compared to treatments of dobutamine or salbutamol alone in s.c. adipocytes ($P < 0.05$) (Figures 16e and 16f). However, PKA was not activated by either dobutamine alone or combined treatments in i.m. adipocytes (Figure 17e). In salbutamol treatment group, PKA activity tended to be reduced by Experior™ and propranolol, but no statistical differences were observed in i.m. adipocytes (Figure 17f).

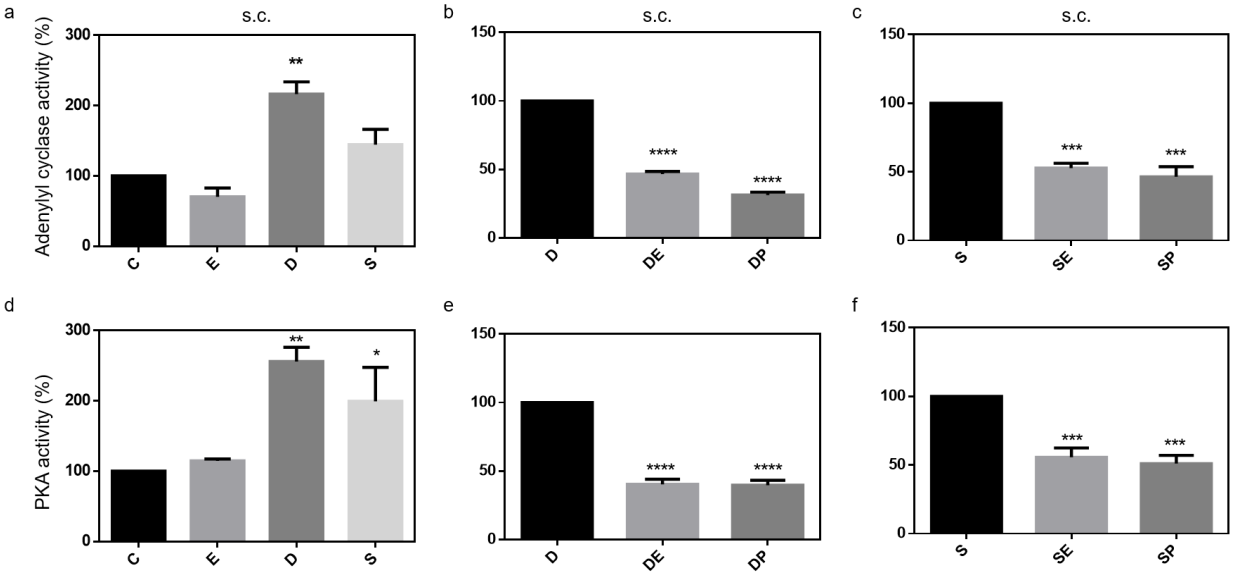

Figure 16. The effect of dobutamine, salbutamol, and Experior™ on adenylyl cyclase and PKA activity in differentiated s.c. adipocytes. At d 14 of differentiation, s.c. adipocytes were starved for 1 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, 10 µM forskolin and 1% antibiotic-antimycotic at 37°C. After starvation, cells were pre-stimulated in the presence or absence of either 1 µM Experior™ or 10 µM propranolol for 5 min. Following 5 min, the cells were stimulated with 10 µM dobutamine, 10 µM salbutamol, or 1 µM Experior™ for 15 min. (a-c) Adenylyl cyclase activity in the presence or absence of either 1 µM Experior™ or 10 µM propranolol. (d-f) PKA activity in the presence or absence of Experior™ or propranolol. Data are expressed as means ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol.

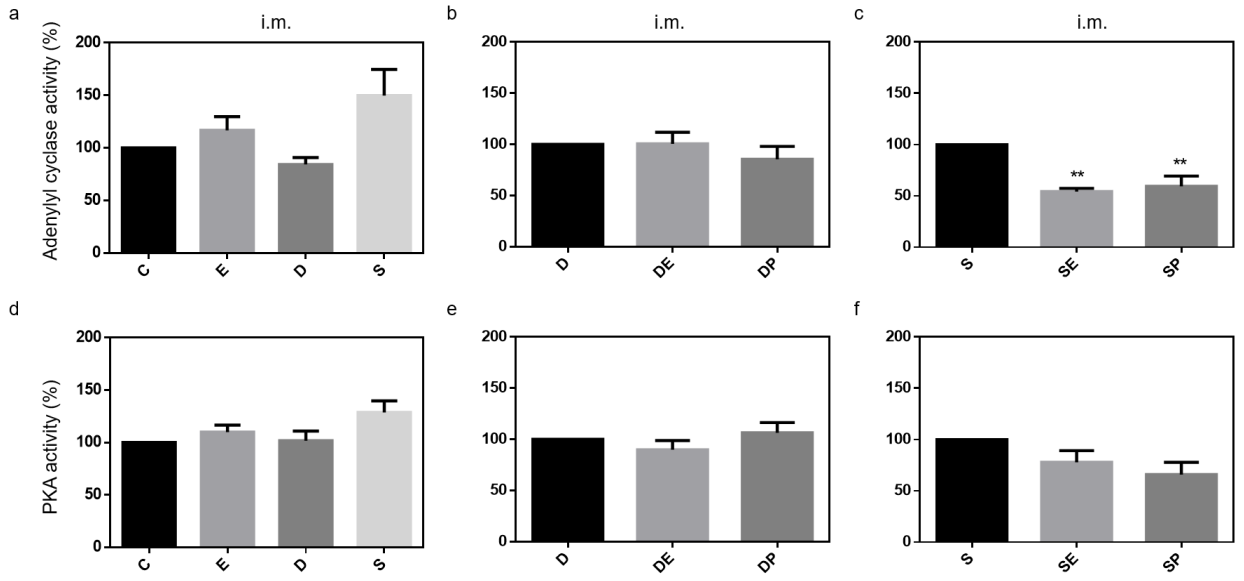

Figure 17. The effect of dobutamine, salbutamol, and Experior™ on adenylyl cyclase and PKA activity in differentiated i.m. adipocytes. At d 14 of differentiation, s.c. adipocytes were starved for 1 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, 10 µM forskolin and 1% antibiotic-antimycotic at 37°C. After starvation, cells were pre-stimulated in the presence or absence of either 1 µM Experior™ or 10 µM propranolol for 5 min. Following 5 min, the cells were stimulated with 10 µM dobutamine, 10 µM salbutamol, or 1 µM Experior™ for 15 min. (a-c) Adenylyl cyclase activity in the presence or absence of either 1 µM Experior™ or 10 µM propranolol. (d-f) PKA activity in the presence or absence of Experior™ or propranolol. Data are expressed as means ± SEM (n = 3). $^{**}P < 0.01$. C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol.

The effects of Experior™ on lipolysis in s.c. and i.m. adipocytes

Previous results revealed that Experior™ repressed the ß-AR/AC/PKA signaling cascades in s.c and i.m adipocytes. Based on these results, we hypothesized that binding of Experior™ to $ß_1$-AR and $ß_2$-AR would inhibit lipolysis in s.c and i.m adipocytes. To test this hypothesis, we measured the levels of glycerol and NEFA release from s.c. and i.m. adipocytes as an index of lipolysis. The cells were treated with 10 µM dobutamine, 10 µM salbutamol, and 1 µM Experior™ for 6 h after 2 h in starvation media supplemented with 0.5 mM IBMX at d 14 of differentiation. Glycerol release was increased by dobutamine ($P < 0.05$) but not altered by Experior™ and salbutamol treatments as compared to control s.c adipocytes (Figure 18a). Glycerol release was not altered by dobutamine, salbutamol, or Experior™ in i.m. adipocytes (Figure 19a). To test the effects of Experior™ on lipases in s.c. and i.m. adipocytes, adipocytes were pretreated with either 1 µM Experior™ or 10 µM propranolol for 30 min before adding dobutamine or salbutamol. Propranolol was used as positive control. Following pre-treatment with either Experior™ or propranolol, propranolol reduced glycerol release compared to dobutamine alone ($P < 0.05$) in s.c adipocytes (Figure 18b). Experior™ had no effect on glycerol release. There was also not a significant effect with Experior™ or propranolol with salbutamol treatment (Figure 18c). Neither Experior™ nor propranolol treatments exhibited antagonistic effects on lipolysis for dobutamine or salbutamol treatments in i.m. adipocytes (Figures 19b and 19c). The release of NEFA release was markedly decreased by dobutamine treatment in both s.c. and i.m adipocytes ($P < 0.05$) (Figures 18d and 19d). With dobutamine treatment, NEFA release was not reduced by Experior™ or propranolol in s.c adipocytes (Figure 18e). Overall NEFA results indicated that either ß-AA alone or combination did not affect NEFA release for 6 h incubations in either s.c. or i.m. adipocytes (Figures 18 and Figure 19).

Based on these data, HSL activity was measured using the ratio of phospho-HSL to total HSL using western blots. We successfully detected both phospho-HSL and HSL in s.c. and i.m. adipocytes (Figure 20). The activity of HSL did not differ ($P > 0.05$) between treatments in s.c. and i.m. adipocytes.

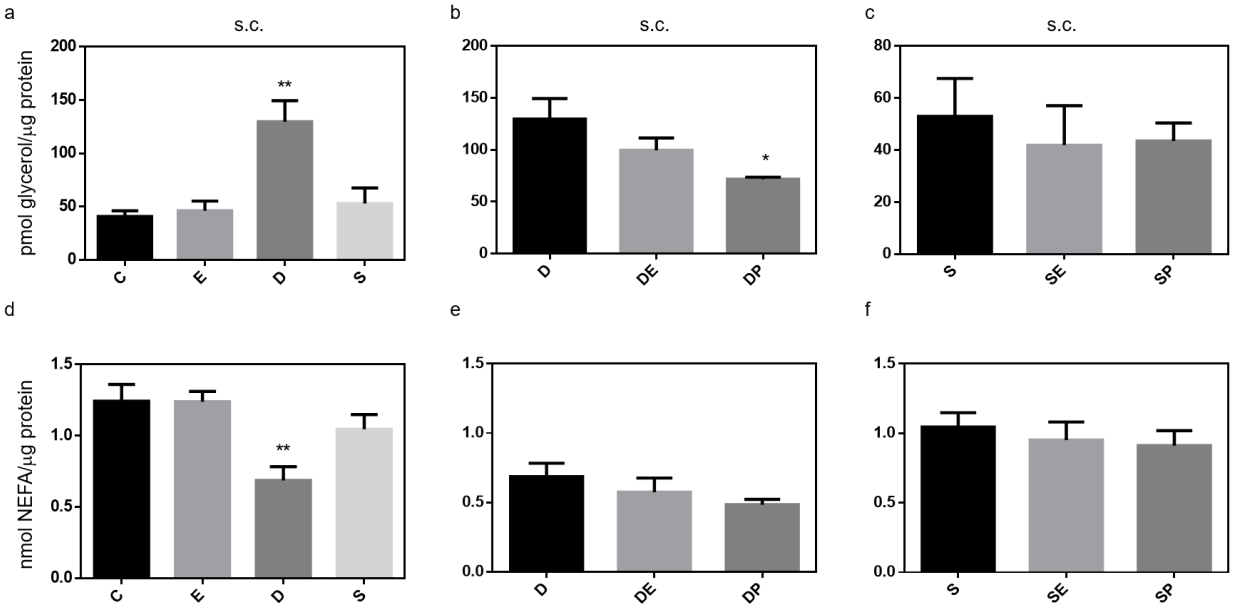

Figure 18. Glycerol and NEFA release in differentiated s.c. adipocytes in response to dobutamine, salbutamol, and Experior™. At d 14 of differentiation, s.c. adipocytes were starved for 2 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, and 1% antibiotic-antimycotic at 37°C. Following starvation, cells were pre-stimulated in the presence or absence of either 1 µM Experior™ or 10 µM propranolol for 30 min. After 30 min, the cells were stimulated with 10 µM dobutamine, 10 µM salbutamol or 1 µM Experior™ for 6 h. (a-c) Glycerol release in the presence or absence of Experior™ or propranolol. (d-f) NEFA release in the presence or absence of Experior™ or propranolol. Data are expressed as means ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$. C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol.

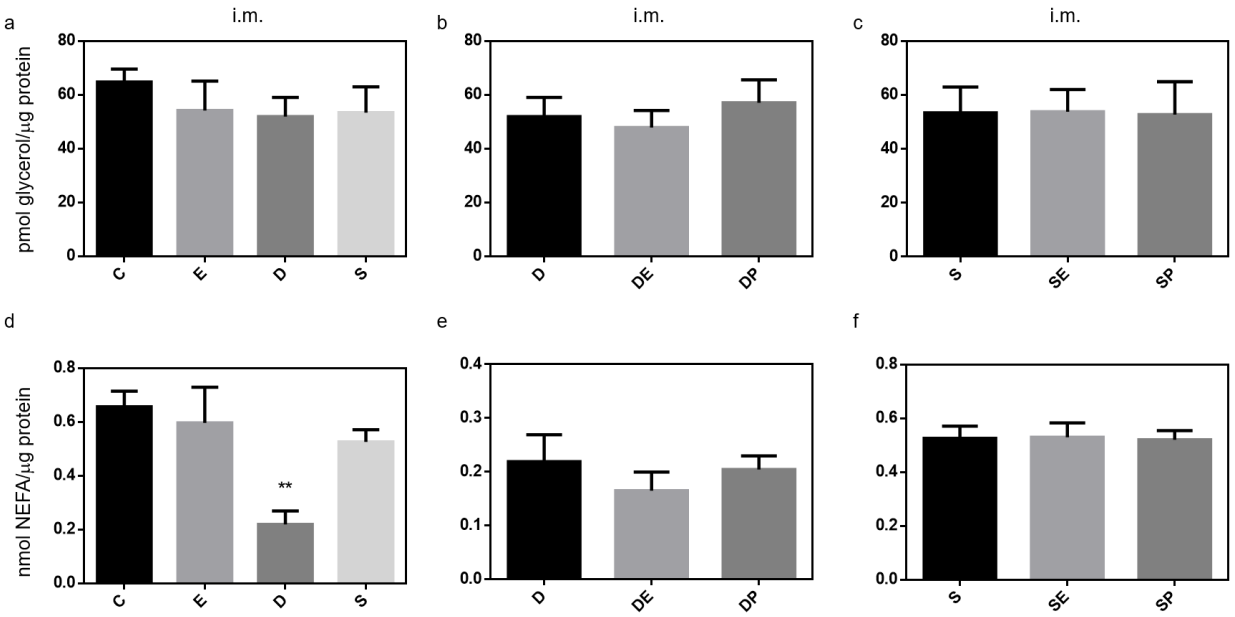

Figure 19. Glycerol and NEFA release in differentiated i.m. adipocytes in response to dobutamine, salbutamol, and Experior™. At d 14 of differentiation, i.m. adipocytes were starved for 2 h in DMEM/F12 containing 0.5% BSA, 0.5 mM IBMX, and 1% antibiotic-antimycotic at 37°C. Following starvation, cells were pre-stimulated in the presence or absence of either 1 µM Experior™ or 10 µM propranolol for 30 min. After 30 min, the cells were stimulated with 10 µM dobutamine, 10 µM salbutamol or 1 µM Experior™ for 6 h. (a-c) Glycerol release in the presence or absence of Experior™ or propranolol. (d-f) NEFA release in the presence or absence of Experior™ or propranolol. Data are expressed as means ± SEM (n = 3). $^{**}P < 0.01$. C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol.

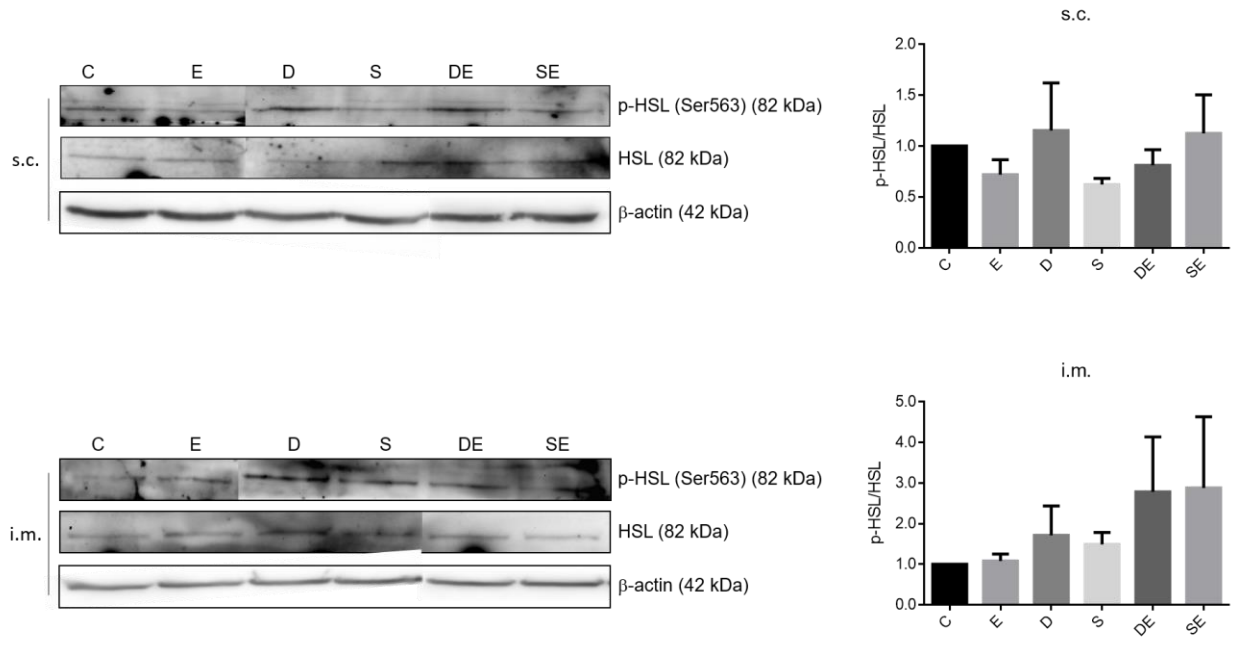

Figure 20. The ratio HSL Ser563 phosphorylation to total HSL in differentiated s.c. and i.m. adipocytes. Data are expressed as means ± SEM (n = 3). C, basal; E, Experior^{TM}; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior^{TM}; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior^{TM}; SP, salbutamol plus pre-treated with propranolol.

Gene expression associated with lipolysis and lipogenesis by ß-AA and Experior^{TM}

Gene expressions associated with lipolysis and lipogenesis were measured following the treatment of ß-agonists and Experior^{TM}. At d 14 of differentiation, adipocytes were treated with 10 µM dobutamine, 10 µM salbutamol, 1 µM Experior^{TM}, dobutamine plus Experior^{TM}, and salbutamol plus Experior^{TM} for 3 d in DMEM/F12 containing 2% FBS, and 1% antibiotic-antimyotic at 37°C. The mRNA levels associated with lipolysis (*adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), monoacylglycerol lipase (MGLL), perilipin 1 (PLIN1), and abhydrolase domain containing 5 (ABHD5)*) were measured by RT-qPCR (Figures 21a and 21b). The expression of *HSL* was increased by salbutamol, dobutamine plus Experior^{TM}, and salbutamol plus Experior^{TM} compared to control (basal expression) in s.c. adipocytes ($P < 0.05$).

The expression of *PLIN1* was upregulated by dobutamine, salbutamol, dobutamine plus Experior™, salbutamol plus Experior™ in differentiated s.c. adipocytes ($P < 0.05$). However, expression of *ATGL*, *MGLL*, and *ABHD5* were not affected by ß-AA and ß-AA plus Experior™ in s.c. adipocytes (Figure 21a). Expression of *ATGL* was depressed by salbutamol plus Experior™ as compared to control ($P < 0.05$) in differentiated i.m. adipocytes. Expression of *HSL* was increased by dobutamine and dobutamine plus Experior™ in i.m. adipocytes ($P < 0.05$). There were no differences in *MGLL*, *PLIN1*, or *ABDH5* gene expression by these treatments in i.m. adipocytes (Figure 21b).

We also measured the expression of genes associated with lipid synthesis and adipocyte differentiation: *fatty acid-binding protein 4 (FABP4)*, *fatty acid synthase (FASN)*, and *PPARγ* (Figures 21c and 21d). The expression of *FABP4* was increased by salbutamol plus Experior™ as compared to control differentiated s.c. adipocytes ($P < 0.05$) (Figure 21c). The gene expression of *FASN* and *PPARγ* were not altered by ß-AA or ß-AA plus Experior™ in s.c. adipocytes. There were no differences in *FABP4*, *FASN*, or *PPARγ* gene expression with any the treatments in i.m. adipocytes (Figure 21d).

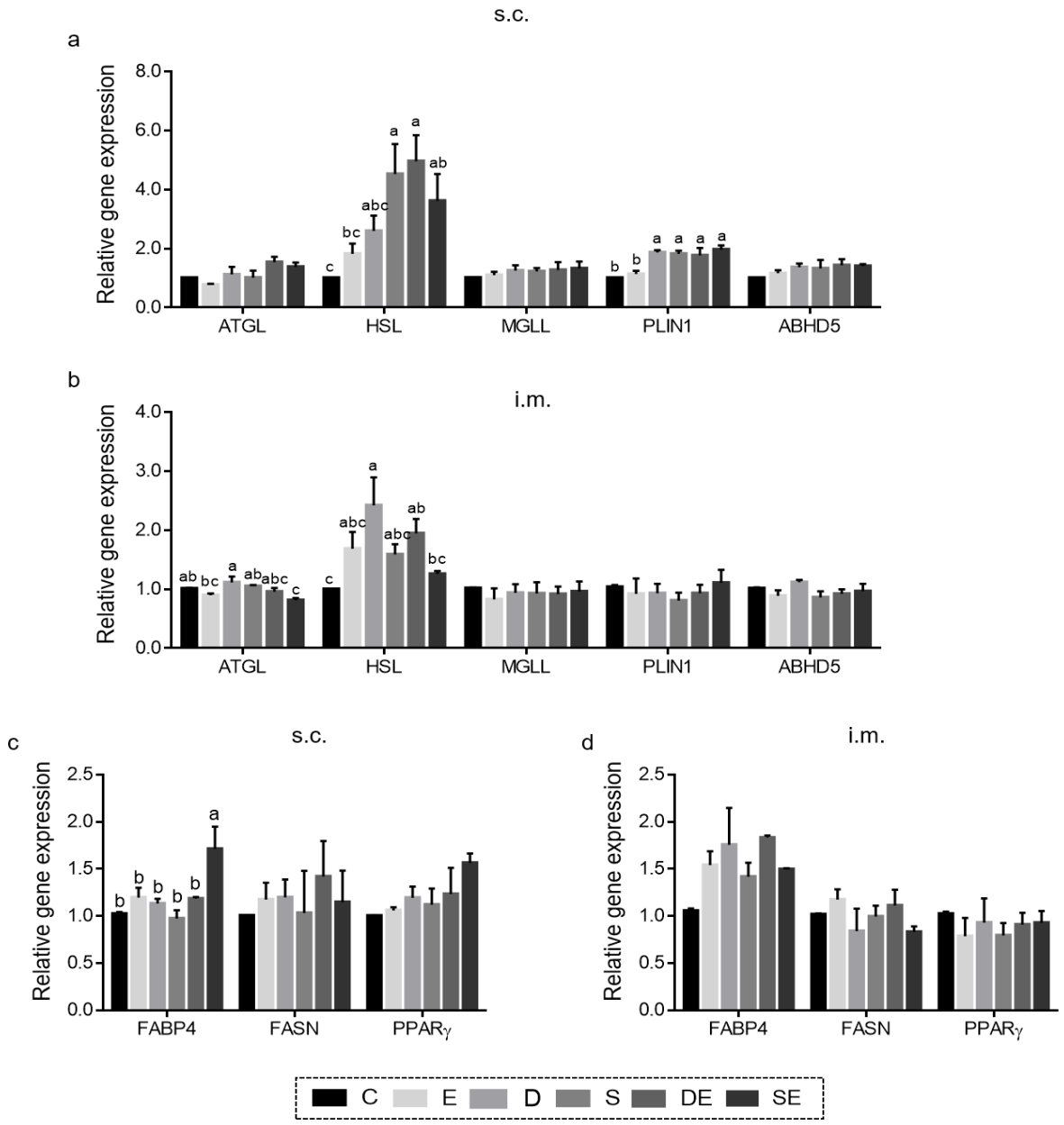

Figure 21. Gene expression associated with lipolysis and lipogenesis after 3 d treatment with ß-adrenergic agonists and Experior™ in differentiated s.c. (a and c) and i.m. (b and d) adipocytes. Data are expressed as means ± SEM (n = 3). [abc]Means within each treatment sharing common superscripts are not different ($P < 0.05$). C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol; *ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MGLL, monoacylglycerol lipase; PLIN1, perilipin-1; ABHD5, abhydrolase domain containing-5; FABP4, fatty acid-binding protein-4; FASN, fatty acid synthase; PPARγ, peroxisome proliferator-activated receptor gamma.*

Expression of ADRB genes after prolonged exposure of ß-AA and Experior™

Prolonged stimulation of ß-AA to ß-AR triggers a rapid attenuation of receptor responsiveness, known as desensitization. In response to chronic agonist over-exposure, down-regulation of the cellular receptors occurs (Ferguson, 2001; Johnson, 2006). Thus, we hypothesized that *ADRB* gene expression would be decreased by ß-AA treatments. The mRNA levels of *ADRB, ß-adrenergic receptor kinase (BARK)* and *ß-arrestins (ABBR)* were measured after chronic exposure of ß-AA and Experior™ for 3 d in s.c. and i.m. adipocytes. Subcutaneous and i.m. adipocytes exhibited different responses to ß-AA (Figure 22). The expression of *ADRB* was not affected by either ß-AA or ß-AA plus Experior™ in in differentiated s.c. adipocytes (Figure 22). In contrast, the mRNA levels of *ADRB2* were down-regulated by all treatments except dobutamine alone in differentiated i.m. adipocytes (P = 0.07). The expression of *ADRB3* was decreased by all treatments expect salbutamol plus Experior™ (Figure 22b). The expression of *BARK1*, *BARK2*, *ARRB1*, and *ARRB2* was not altered by ß-AA and ß-AA plus Experior™ in both s.c. and i.m. adipocytes (Figures 22c and Figure 22d).

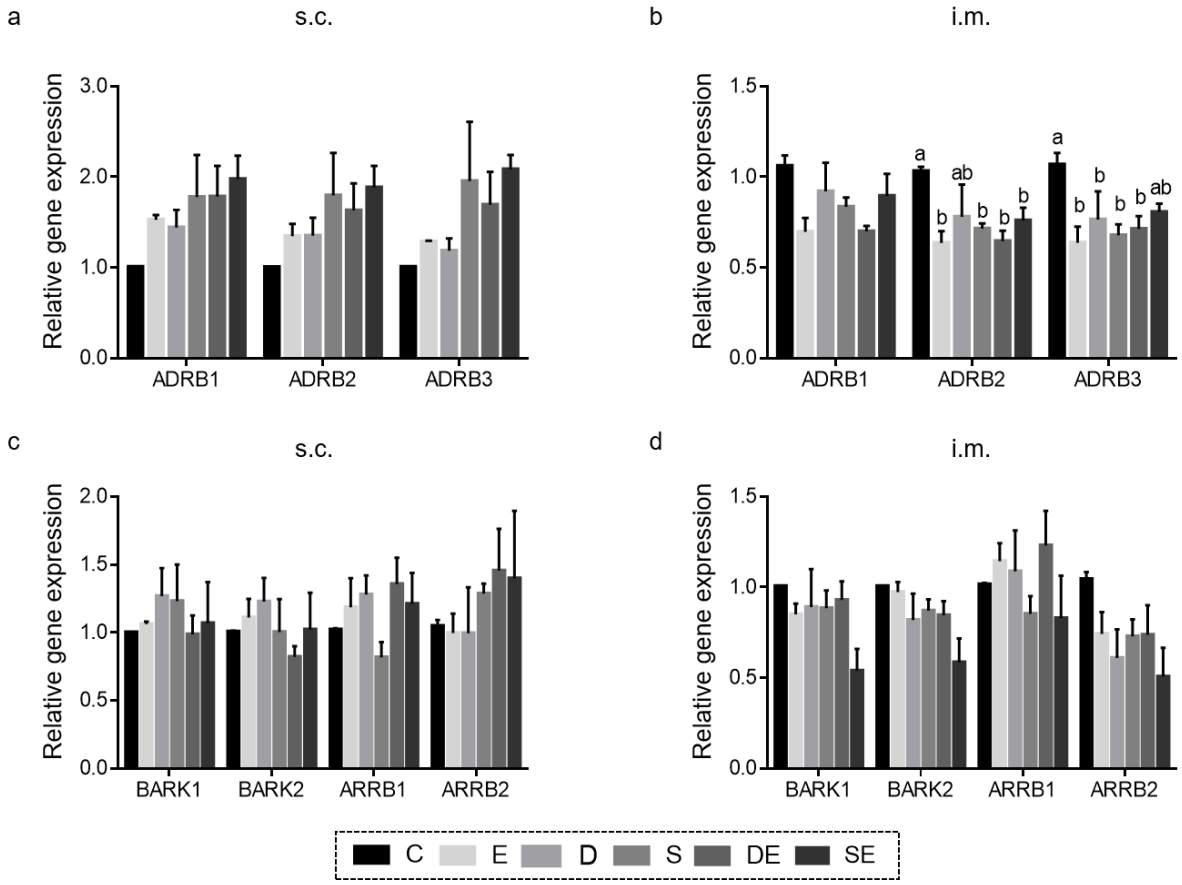

Figure 22. Relative mRNA expression of ADRB and gene associated with receptor-desensitization after 3 d treatment with ß-adrenergic agonists and Experior™ in differentiated s.c. (a and c) and i.m. (b and d) adipocytes. Data are expressed as means ± SEM (n = 3). [ab]Means within each treatment sharing common superscripts are not different ($P < 0.05$). C, basal; E, Experior™; D, dobutamine; S, salbutamol; P, propranolol; DE, dobutamine plus pre-treated with Experior™; DP, dobutamine plus pre-treated with propranolol; SE, salbutamol plus pre-treated with Experior™; SP, salbutamol plus pre-treated with propranolol; *BARK1, ß-adrenergic receptor kinase-1; BARK2, ß-adrenergic receptor kinase-2; ABBR1, ß-arrestin-1; ABBR2, ß-arrestin -2.*

Discussion

This study was designed to examine the effects of a novel ß-AR ligand Experior™ on $ß_1$- and $ß_2$-AR. Also, for the first time we have demonstrated the relative importance of $ß_1$-AR and $ß_2$-AR in the AC/ PKA/HSL cascade in s.c. and i.m. adipocytes. The current study demonstrated that s.c. and i.m. adipocytes have physiologically different responses to ß-AR stimulation by ß-AA.

Adenylyl cyclase was highly stimulated by dobutamine (a ß$_1$-AA) and somewhat activated by salbutamol (a ß$_2$-AA), but was not affected by Eexperior^{TM} in s.c. adipocytes. The data for PKA activity and glycerol release were similar to results for AC activation in s.c adipocytes. In contrast, these ß-AA were not effective in the activation of AC and concomitant PKA activation or in glycerol release in i.m. adipocytes. Experior^{TM} significantly antagonized the effects of dobutamine and salbutamol with in turn suppressed the activations of AC and PKA in s.c. adipocytes. The antagonistic efficacy of Experior^{TM} was equivalent the effects propranolol, which antagonized the stimulation of ß$_1$-AR and ß$_2$-AR in s.c. adipocytes, even though the concentration of Experior^{TM} was 10-fold lower (1 µM) than that of propranolol (10 µM), indicating that Experior^{TM} is a powerful antagonist of ß-AR.

During the differentiation of adipocytes, the expression of ß-AR is especially important, because catecholamine sensitivity to ß-AR plays a central role in the mechanism for the regulation of the transmembrane signaling system that controls energy balance and thermogenesis (Collins, 2011; Cypess et al., 2015). Adipocytes acquire their responsiveness to catecholamine during differentiation (Lai et al., 1982; Feve et al., 1990). Augmentation of ß-AR numbers is paralleled by increased levels of ß-AR mRNA because development of catecholamine sensitivity results from *de novo* ß-AR biosynthesis (Feve et al., 1990). There are several reports that changes in ß-AR numbers can be also exerted by the action of glucocorticoids like dexamethasone during murine 3T3 adipocyte differentiation (Lai et al., 1982; Nakada et al., 1987; Feve et al., 1990; Guest et al., 1990). Upon differentiation, ß$_1$-AR and ß$_3$-AR expression was totally depressed, but ß$_2$-AR expression was up-regulated by dexamethasone. Furthermore, in fully mature adipocytes, ß$_2$-AR expression was increased, but ß$_1$-AR and ß$_3$-AR expression remained depressed. In contrast, ß$_2$-AR mRNA expression was not changed upon induction by dexamethasone in bovine skeletal

muscle cells (Bridge et al., 1998). In rat C6 glioma cells, ß$_2$-AR mRNA was increased, but there was no alteration in ß$_1$-AR mRNA levels after exposure to dexamethasone (Zhong and Minneman, 1993). Glucocorticoid treatment increased ß$_1$-AR mRNA levels in preterm piglet hearts (Kim et al., 2014). Our current study demonstrated that all ß-AR mRNA levels increased during differentiation of s.c. adipocytes without a depression of ß$_1$-AR mRNA, even though the cells were exposed to dexamethasone during differentiation. These observations illustrate that dexamethasone may differently act among species, and other mechanism, including modifying membrane composition and the coupling properties of cellular G-proteins, and post-transcriptional modifications of ß-AR may be involved in increasing ß-AR numbers during differentiation of bovine adipocytes (Lai et al., 1981; Gierschik et al., 1986; Benovic et al., 1987; Storch et al., 1989).

The current study tested the viability of s.c. preadipocytes after exposure to dobutamine, salbutamol, and Experior^{TM} in different doses and for different time points using CCK-8 kit. Subcutaneous preadipocytes responded differently to individual ß-AA treatments, especially at high concentrations (10 µM and 100 µM). Dobutamine highly promoted the proliferation of the cells at 10 µM and 100 µM, salbutamol did not induce proliferation at any concentration, and Experior^{TM} showed toxicity 10 µM and 100 µM after 1 h incubation. In addition, prolonged exposures (6 h) to salbutamol significantly induced proliferation. The CCK-8 kit is an enzyme based-method that measures mitochondrial dehydrogenase activity. Dobutamine plays an important role in mitochondrial bioenergetic function by enhancing the efficiency of mitochondrial respiration and by regulating mitochondrial activity in response to increases in energy demands (Mukae et al., 1997; Porta et al., 2006; Radhakrishnan et al., 2013). The data of the current study suggest the possibility that high concentrations of dobutamine may promote stimulation of mitochondrial dehydrogenase in bovine preadipocytes. These results indicate that the chronic ß$_1$-

AA and ß$_2$-AA exposures potentially stimulates proliferation of s.c. adipocytes via stimulation of ß-AR.

Dobutamine strongly activated AC activity in differentiated adipocytes, whereas salbutamol did not affect AC activity. Similar results were obtained for PKA activity and glycerol release in s.c adipocytes. Although both dobutamine and salbutamol are potent ß-AA, they may modulate different levels of receptor occupation because very small populations of receptors could produce large responses (Kenakin, 1995). Therefore, data of the present study suggest that agonist-receptor efficacy between ß$_1$-AR and dobutamine may be much stronger than agonist-receptor efficacy between ß$_2$-AR and salbutamol in bovine s.c. adipocytes differentiated in culture, which would cause greater $G_{\alpha s}$ coupling with AC via the ß$_1$-AR.

Pre-treatment with Experior^{TM} strongly antagonized individual ß$_1$-AR and ß$_2$-AR and subsequently depressed the AC and PKA activity stimulated by dobutamine and salbutamol in s.c. adipocytes differentiated in culture. The antagonism of the ß$_2$-AR with Experior^{TM} was also observed in i.m. adipocytes. Although, Experior^{TM} significantly suppressed PKA activity, neither glycerol nor NEFA release was influenced in s.c. and i.m adipocytes by Experior^{TM}. In addition, Experior^{TM} had no effect on gene regulation such as *ADRB*, or genes associated with lipid filing and lipolysis. Hence, Experior^{TM} may function only in modulating ß-AR activation. The antagonistic efficacy of Experior^{TM} was similar to propranolol, a non-selective ß-AR antagonist. These findings support the concept that Experior^{TM} works as an antagonist for ß$_1$-AR or ß$_2$-AR in bovine adipocytes. According to Freddolino et al. (2004) the agonists epinephrine, norepinephrine, salbutamol, and isoproterenol strongly bind to Ser-203, Ser-204, and Ser-207 at transmembrane 5 (TM5). Antagonists such as propranolol and butoxamine bind to Ser-203 but not to Ser-207 at TM5, which leads to more flexible binding to TM5 not to transit to the activated state (Freddolino

et al., 2004). This suggests the possibility that Experior™ may have similar binding properties as propranolol in bovine ß$_1$-AR or ß$_2$-AR. Further, the activities of antagonists can be completely dependent upon the tissues or cell types (Kenakin, 1987). This also would explain why both Experior™ and propranolol strongly blocked individual both ß$_1$-AR and ß$_2$-AR in s.c. differentiated adipocytes, but only antagonized ß$_2$-AR in i.m. adipocytes.

An increased catabolism and a decreased anabolism of lipid in the adipocytes by ß-AR stimulation would decrease hypertrophy of the adipocyte through a consequent decrease in formation of lipid droplets (Smith, 1987). In lipid catabolism, ATGL, HSL and MGL are the most important lipases. Upon ß-AR stimulation, PKA phosphorylates PLIN on the surface of lipid droplets, leading to ABHD5 release, which then binds and activates ATGL to hydrolyze triglycerides. PKA also phosphorylates HSL in the cytoplasm, after which HSL translocates to lipid droplets where it binds to PLIN to hydrolyzes diacylglycerides, and MGL hydrolyzes monoglycerides (Zimmermann et al., 2009; Zechner et al., 2012). Thus, ATGL activity is indirectly induced by PKA stimulation by ß-AR whereas HSL activity is induced by direct HSL phosphorylation. Our current study examined the expression of these five genes after exposure to dobutamine, salbutamol, and Experior™ for 3 d. Salbutamol and dobutamine increased *HSL* gene expression in s.c. adipocytes and i.m. adipocytes, respectively, and both dobutamine and salbutamol increased *PLIN* gene expression in s.c adipocytes. Taken together, the data suggest that the expression of *HSL* and *PLIN* contributes to an increase in lipolysis by increasing these lipolytic proteins. Because we only measured mRNA abundance, we can only speculate the importance of the role of the ß-AR in the current study. These contributions of individual ß-AR likely involve multiple factors such as ß-AR function, transcription factors, and the rate of mRNA decay.

In conclusion, we have demonstrated for the first time the pattern of *ADRB* expression in i.m. adipocytes. In spite of expressing all three ADRB subtypes, i.m. adipocytes were refractory of specific ß-AA, suggesting that treatment of cattle with commercial ß-AA would not directly affect lipid filling in i.m. adipose tissue. This study also demonstrated different physiological responses of ß-AR subtypes to selective $ß_1$-AA, $ß_2$-AA, and Experior™ in differentiated s.c. and i.m. adipocytes. Even though the $ß_2$-AR is considered the predominant ß-AR subtype in the adipose tissue, the three ß-AR mRNA levels in differentiated adipocytes were not different. Dobutamine, a selective $ß_1$-AA, significantly stimulated AC/PKA activity and lipolysis in s.c. adipocytes and was more potent than salbutamol, a selective $ß_2$-AA. These findings imply that the efficacious ligands for regulating adipose tissue accretion could effectively signal through both the $ß_1$-AR and $ß_2$-AR in bovine species. The antagonistic efficacy of Experior™ was similar that of propranolol even the concentration of propranolol was 10-fold higher than that of Experior™. Our findings also indicate that Experior™ may function as a potent antagonist for both $ß_1$-AR and $ß_2$-AR in bovine adipose tissue.

CHAPTER VI

SUMMARY

The objectives of this research were to investigate lipid desaturation by ectopically expressed *pSCD1* in non-adipocytes and to investigate the extent of lipolysis mediated by ß-AR subtypes through various kinds of selective ß-AR agonists *ex vivo* and *in vitro*.

The first major point of this research was to successfully generate inducible lentiviral expression vectors to overexpression or knock-down of *pSCD1* and to validate its stably expression and functional catalytic activity in SK6-I-*pSCD1* cells through RT-qPCR, western blot and fatty acid analysis. SK6-I-*pSCD1* cells incubated with palmitic acid increased the synthesis of palmitoleic acid nearly 4-fold, indicating that the *pSCD1*- transduced cells successfully can induce the Δ^9 desaturation of palmitic acid to palmitoleic acid. Based on these results, we confirmed that *pSCD1* expression was ectopically controlled using inducible lentiviral system. Further, it implies that the lentiviral constructs utilized in this study can be utilized to generate transgenic pigs or other cell lines to enhance our understanding of the contribution of fatty acid desaturation and concomitant to lipogenesis to the promotion of disease states such as obesity and metabolic disease.

The second major point of this research was to characterize ß-AR subtypes in bovine s.c. and i.m. adipose tissues with the use of selective $ß_1$-, $ß_2$- and $ß_3$-AR agonists. This study documented the distribution of *ADRB* in bovine s.c. and i.m. adipose tissues from growing cattle. In addition, the distribution of *ADRB* in i.m. adipose tissue was demonstrated for the first time. The interesting findings of this study were that *ADRB1*and *ADRB3* mRNA showed similar levels of expression in both s.c. and i.m. adipose tissues; *ADRB3* in cattle had been believed to be expressed only in BAT.

We also demonstrated that s.c. and i.m. adipose tissues have physiologically different responses to ß-AR agonists. $ß_{1/2}$-AR agonists including isoproterenol, RH, and ZH, increased lipolysis in s.c. adipose tissue but were not effective in i.m. adipose tissue, even though cAMP production was greater in i.m. adipose tissue than in s.c. adipose tissue for all experiments. We speculated the reasons that the levels of gene expression of ß-AR were much lower in i.m. adipose tissue than that of s.c. adipose tissue, which indicated a small amount of ß-AR populations in i.m. adipose tissue. Therefore, ß-AR would be easily occupied even at very low concentrations of ß-AR agonist in i.m. adipose, which would lead to low efficacy to signal downstream inducing lipolysis.

The $ß_3$-AR predominantly mediates the lipolytic response through selective $ß_3$-AR agonists such as CL-316243 and BRL-37344 in rodents, rabbits, and dogs, but there is no evidence for the existence of a functional $ß_3$-AR in bovine adipose tissue from physiologically mature cattle. Our study examined the presence of a functional $ß_3$-AR in response to BRL-37344, and Experior™ revealed that neither $β_3$-AR agonists stimulated cAMP production or NEFA release from s.c. and i.m. adipose tissues. The data suggested that $ß_3$-AR may not be functional in bovine adipose tissue, regardless of the fact that the tissue distribution of $ß_3$-AR in cattle was similar $ß_1$-AR in bovine adipose tissue.

The final major objective of this research was to investigate antagonistic effects on ß-AR mediated by Experior™ in primary bovine s.c. and i.m. adipocytes. The results of this study demonstrated that Experior™ depressed cAMP production in the presence of either propranolol or L-748,337 in s.c. adipose tissue. Further, Experior™ strongly depressed the cAMP accumulation stimulated by dobutamine and salbutamol in a dose-dependent manner. These findings suggested that Experior™ may function as a competitive antagonist for $ß_1$-AR or $ß_2$-AR in bovine adipose

tissue. Thus, we further investigated antagonistic effects on ß-AR/AC/PKA/HSL signaling cascade through Experior™ in bovine s.c and i.m. mature adipocytes. The data demonstrated that Experior™ strongly repressed the ß-AR/AC/PKA signaling cascades in s.c and i.m adipocytes. However, neither glycerol and NEFA release nor HSL activity was not influenced by Experior™ in s.c. and i.m adipocytes. In addition, Experior™ did not affect gene expression such as *ADRB* and lipogenesis- and lipolysis- associated gene expression. Hence, these results suggested that Experior™ may function by only modulating receptor activation. The antagonistic efficacy of Experior™ was similar to that of propranolol, even though the concentration of Experior™ was 10-fold lower than that of propranolol in our experiments. Through these finding, we confirmed again that Experior™ acts as an antagonist for $ß_1$-AR or $ß_2$-AR in bovine adipose tissue.

Taken together, this dissertation has suggested the opportunity not only to develop a non-rodent biomedical model of obesity and metabolic disease but also to produce beef products with heart healthy characteristics, which can contribute to human health and economic profits to the beef cattle industry. It has also contributed to the understanding of functionality of ß-AR subtypes in adipose tissue during cattle growth and maturity. This research also demonstrated different physiological responses of ß-AR subtypes to ß-AR agonists in bovine s.c. and i.m. adipose tissue.

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