INSULIN SENSITIVITY AND METABOLISM IN BOVINE MUSCLE TREATED WITH β-ADRENERGIC AGONISTS

A Thesis

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

MASTER OF SCIENCE

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August 2018

Animal Science

ABSTRACT

A limited number of ß-adrenergic agonists (ß-AA) are currently used for redirecting dietary energy from adipose tissue accretion towards muscle growth. At the end of typical feedlot finishing, cattle have depressed insulin sensitivity, similar to type II diabetes in humans. The objective of this study was to compare the effects of a known β_3-AA (BRL 37344) to a novel ß-AA (Experior) on muscle metabolism *in vitro*. We hypothesized that Experior would attenuate insulin insensitivity by promoting glucose metabolism in muscle. The *M. longissimus lumborum* (LL) was removed from the 9th to 11th rib from four steers. From the LL samples, muscle strips were dissected parallel to muscle fiber orientation. The conversion of glucose to glycogen, CO_2, and lactate was tested in muscle strips incubated for 90 min with 50 µM Experior, 50 µM BRL 37344, or no β-AA (Control), and different insulin concentrations. Incubation time was established by a preliminary experiment that determined the effect of different incubation times (30 min, 60 min, 90 min) on glycogen, CO_2, and lactate production. There were no ($P > 0.05$) insulin concentration x treatment effects, insulin concentration effects, and no significant effect of treatment on lactate production. There was a significant treatment effect of conversion of glucose to glycogen ($P < 0.001$) and CO_2 ($P = 0.018$). BRL 37344 caused 62% lower glycogen production compared to Control samples, and 50% lower glycogen production compared to Experior samples. Control samples produced 4.3 nmol/100 mg·90 min, Experior samples produced 3.1 nmol/100 mg·90 min glycogen, while BRL 37344 samples produced 1.6 nmol/100 mg·90 min. BRL 37344 elicited 23% more CO_2 than Experior, and 41% more CO_2 than Control

samples. Control samples produced 171.3 nmol/100 mg·90 min CO_2, Experior samples produced 222.9 nmol/100 mg·90 min CO_2, while BRL 37344 samples produced 292.6 nmol/100 mg·90 min CO_2. Experior did not have a consistent effect on glycogen or CO_2 production. We discovered the presence of $ß_3$- AR in muscle, since there was a distinct response of the muscle strips to BRL 37344. The relative insensitivity of bovine muscle to insulin is consistent with insulin resistance observed in feedlot cattle.

DEDICATION

I dedicate this thesis to my parents. Through it all, they always believed I could succeed and see this long process through to the end. I cannot thank them enough, and I will forever be indebted to them. M-I-Z-Z-O-U!

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jeffrey Savell, co-chair Dr. Kerri Gehring, and committee member Dr. Stephen Smith for their continuous support throughout the duration of this Master's program. Their patience and words of wisdom helped guide me through the process of learning to conduct a scientific experiment.

I would also like to thank my lab mates, and the members of the lab of Dr. Stephen Smith. Without their help, I would not have been able to complete this project and am forever grateful for their help.

Finally, I would like to thank my family and friends. Without their love and support, I would not have been able to complete this long journey. You all mean the world to me.

CONTRIBUTORS AND FUNDING SOURCES

This research experiment was supported by a thesis committee consisting of University Distinguished Professor Jeffrey Savell and Professor Kerri Gehring of the Department of Animal Sciences, and Reagents Professor Stephen Smith of the Department of Nutrition. Research was conducted with the financial assistance from Elanco Animal Health. Samples were collected at the Texas A&M Beef Center within the O. D. Butler Jr. Animal Science Complex located in College Station, TX. Cattle involved in this project were raised and fed out at the Texas A&M McGregor Research Center located in McGregor, TX. Lab work was conducted with the help of the students from the lab of Reagents Professor Stephen Smith or conducted independently by the student.

NOMENCLATURE

β-AA	β-adrenergic agonist
LL	*M. longissimus lumborum*
G6P	Glucose-6-phosphate
F6P	Fructose-6-phosphate
F-1, 6-P_2	Fructose 1-6 bisphosphate
TCA	Tricarboxylic acid cycle
ETC	Electron transport chain
G1P	Glucose-1-phosphate
PEP	Phosphoenol pyruvate
OAA	Oxaloacetate
PEPcarboxykinase	Phosphoenol pyruvate carboxykinase
ATP	Adenosine tripolyphosphate
ADP	Adenosine diphosphate
cAMP	Cyclic adenosine monophosphate

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CHAPTER I

INTRODUCTION

1.1 Background

Total U.S. beef consumption for the year 2017 was 56.8 billion pounds (USDA, 2017), increasing the pressure on beef cattle producers to maintain a consistent quantity of red meat. In an effort to increase red meat production in livestock in the United States, laboratories began developing chemical compounds that would redirect energy from adipose tissue deposition to muscle production (Anderson, Moody, and Hancock, 2004). The resulting compounds were β-AA. Since β-AA repartition energy from adipose tissue growth to muscle growth, they are referred to as repartitioning agents (Ricks et al., 1984). These compounds, however, do not change organ or bone mass (Beermann, 2002), resulting in heavier carcasses due to an increase in muscle mass. Most β-AA are sold as feed additives (Centner et al., 2014).

Along with being used as repartitioning agents, β-AA also have beneficial pharmacological effects. As energy is shunted to muscle production instead of fat production, glycogen (stored energy) is broken down and released as either glucose or glucose-6-phosphate (G6P). Increased blood glucose concentrations cause insulin to be released from the pancreas to regulate glucose homeostasis. If β-AA could indirectly increase blood glucose levels via glycogen breakdown, they could increase insulin sensitivity in cattle. One study with finishing cattle reported the cattle to be insulin

resistant (Schoonmaker et al., 2003). The laboratories of Dr. B. J. Johnson (Texas Tech University) and S. B. Smith (Texas A&M University) reported the novel finding that bovine muscle satellite cells express the β_3-AA receptor (AR) (Miller et al., 2012). This was the first study that associated β_3-AA with muscle. The original theory was that β_3-AR only resided on brown or white adipose tissue. However, no studies have documented the effects of specific β_3-AA on muscle metabolism. There could be potential health benefits in livestock resulting in a net increase in red meat production and a net decrease in adipose tissue production from these findings. In this study, the objective was to compare the effects of a known β_3-AA (BRL 37344) to a novel ß-AA (Experior) on muscle metabolism *in vitro*. We hypothesized that Experior would produce similar results to BRL 37344 and attenuate insulin insensitivity by promoting glucose metabolism in muscle.

1.2 Muscle ultrastructure

Muscle is a unique tissue in the body. In the living animal, muscle is the working tissue that turns chemical energy into mechanical energy, allowing movement of the skeletal system that translates into phenomena such as locomotion and breathing. In the post-mortem animal, muscle is the tissue that is converted to edible meat. Meat is composed of many essential nutrients, including the essential amino acids, the Vitamin B-complex, iron, and fatty acids (Lawrie and Ledward, 2006).

Basic muscle tissue structure consists of stromal, sarcoplasmic, and myofibrillar proteins. Stromal proteins are the connective tissues that have various jobs in the body,

including connecting muscle to bone, connecting bone to bone, and providing structure to organs and cells. The sarcoplasmic proteins, hemoglobin and myoglobin, are those that give the muscle its characteristic color depending on species (Gonzalez, 2015), although myoglobin is the main protein that determines color in meat and muscle.

Myoglobin is only present in muscle and will receive oxygen from hemoglobin to store for the muscle to use in oxidative metabolism. Myoglobin transports oxygen from the sarcolemma to the mitochondria (Wittenberg and Wittenberg, 1990), where oxygen will either receive carbon to make CO_2 to be transported to the lungs, or act as the final electron receptor in the electron transport chain, which will be discussed later. Myoglobin also functions at the highest efficiency in the ferrous state.

Myofibrillar proteins can be divided into three subcategories: regulatory, cytoskeletal, and contractile (Aberle et al., 2012). Regulatory proteins include troponin and tropomyosin (Aberle et al., 2012). There are three parts to troponin proteins; troponin C, troponin T, and troponin I. Troponin C binds calcium, and calcium binding changes the shape of the entire troponin protein, shifting tropomyosin from blocking the actin-myosin binding site. Troponin T physically binds to tropomyosin, so it is the protein that ultimately moves tropomyosin when calcium binds to troponin C. Troponin I binds to actin, and inhibits myosin from binding to the actin-myosin binding site (Aberle et al., 2012; Cohen, 1975). Two tropomyosin filaments are associated with each actin filament; each actin filament is made of two F-actin filaments (actin will be discussed later). One tropomyosin molecule per F-actin molecule. Tropomyosin molecules are made of two individual protein chains which form filaments, and two

filaments twist around each other to form an alpha helix. Tropomyosin is what lies over the actin-myosin binding site and is the physical barrier that prevents myosin from binding to actin (Cohen, 1975).

Cytoskeletal proteins are what provides the structure to the sarcomeres (Aberle et al., 2012). Some of the major cytoskeletal proteins include C-proteins, titin, nebulin, desmin, skelemin, M-filament, α-actinin, and β-actinin. C-proteins hold myosin molecules together by bundling myosin tails. Titin is an important protein and the most abundant cytoskeletal protein (Aberle et al., 2012). This protein connects myosin to the Z-disks and has a spring-like attachment to the Z-disk to allow for movement during contraction. Nebulin is the second-most abundant cytoskeletal protein in muscle (Aberle et al., 2012) and anchors the thin filament (actin, troponin, and tropomyosin) to the Z-disk, while α-actinin actually attaches the thin filament to the Z-disks. Desmin connects the Z-disks of a sarcomere to adjacent sarcomere Z-disks. M-filaments connect the M-lines of adjacent sarcomeres together. Finally, β-actinin caps the thin filaments, and regulates the length of actin in the sarcomere.

The group of contractile proteins includes the major myofibrillar proteins (also called myofilaments), actin and myosin (Aberle et al., 2012). These are the most important proteins in the skeletal muscle because of their role in muscle contraction. In skeletal muscle, cells have the appearance of having stripes, which is termed striated. Striations occur because of the presence of sarcomeres, the subunits of myofibrils, which myofibrils are the tissue-specific organelles in muscle (Aberle et al., 2012). Sarcomeres have two different colored regions, the A-band and the I-band. The A-band is the darker

region and primarily contains overlapping myosin and actin filaments. The I-band primarily contains actin filaments, which is why it is the lighter band.

Structures called Z-disks form the boundaries of a sarcomere. There are two Z-disks per sarcomere, one at each end. In the center of the two Z-disks is the M-line. Z-disks are pulled towards the M-line by the myofilaments during contraction. Myofilaments are referred to as thick (myosin) or thin (actin, troponin, tropomyosin) filaments (Aberle et al., 2012). The M-line anchors the myosin proteins, which are oriented perpendicular to the M-line, and stretch on both sides of the M-line. Myosin proteins are the workhorses of the sarcomere. The myosin molecule is composed of a globular head, and a helical tail (Valin, Cross, and Smith, 1992). The tails, which can be composed of light chains and heavy chains (Biletter et al., 1981), are held together by C-proteins. C-proteins act like a clamp to keep the multiple tail chains bound together.

Proteins that compose thin filaments are actin, troponin, and tropomyosin. Actin monomers are globules that are called G-actin, which can form filamentous chains called F-actin. These polymers orient into double-stranded helices that connect to the Z-disks by the protein, nebulin. Actin monomers contain a binding site where myosin can bind and pull the F-actin, and subsequently the Z-disks, towards the M-line during contraction. Troponin complexes are positioned at regular intervals (every 7 G-actin) along actin filaments.

The sarcolemma, or the plasma membrane of a muscle cell, surrounds the entire muscle fiber and invaginates into transverse tubules (t-tubules) that serve a purpose in electrical conduction during contraction. An action potential travels across the

sarcolemma, which connects all of the muscle fibers in an entire muscle. After nerve cells receive a stimulus from the brain and conduct the impulse along nerve cells, the action potential will eventually reach the sarcolemma. Once the action potential travels through the t-tubules, the t-tubules eventually lead to longitudinal tubules, which is part of the same tube system as t-tubules. These tubules conduct the action potential through the muscle ultrastructure and will reach the fenestrated collar. The fenestrated collar lies over the terminal cisternae, which stores calcium. The membranous system that includes the series of t-tubules and terminal cisternae that are involved in calcium sequestering is called the sarcoplasmic reticulum (SR) (Aberle et al., 2012). The SR is similar to the endoplasmic reticulum in eukaryotic cells. This organelle is made of membrane sacs and proteins that hold calcium in the SR to maintain homeostasis in the sarcomere. Only an action potential can cause the release of calcium from the SR, which leads to contraction of the muscle.

1.3 Muscle contraction

At the beginning of each muscle contraction, an action potential begins the process. A signal to the brain initiates a nerve impulse. Neurons have a normal resting membrane potential of a negative intracellular charge, and a positive extracellular charge. A nerve impulse opens sodium-potassium pumps, causing a net influx of 3 Na^+ and a net efflux of 2 K^+ ions, switching the charges of the cell membranes to a positive intracellular charge, and a negative extracellular charge. This switching of membrane charges (the action potential) travels quickly from neuron to neuron until it reaches the

muscle cells. The reason the action potential travels so quickly is because neurons are surrounded by a lipid membrane called myelin sheaths. Impulse conduction speed is increased between 30 and 40-fold when the cells are myelinated (Aberle et al., 2012).

Neurons specifically associated with muscles are called motor neurons. After arriving at the muscle, the electrical charge stimulates the myoneural junction, which is the area that the dendrite of the motor neuron comes in close proximity to the muscle cell, but the two do not touch. The space between the nerve and the muscle is called the synaptic cleft. The electrical charge stimulates the dendrite of the motor neuron to release acetylcholine into the synaptic cleft.

Acetylcholine is the chemical stimulus that converts the neural action potential into an action potential that the muscle is able to react to. Acetylcholine binds to receptors on the sarcolemma, beginning the action potential in the muscle cell. Similar to neurons, muscle fibers normally have a net negative intracellular charge and a net positive extracellular charge. The acetylcholine switches the charges and causes an action potential in the muscle.

As previously stated, the action potential will travel through the t-tubules and longitudinal tubules, to the fenestrated collar which overlies the SR. A dihydropyridine receptor is embedded in the membrane of the t-tubule and is associated with a ryanodine receptor. The ryanodine receptor connects directly with the SR. When an action potential reaches the fenestrated collar, the action potential stimulates the dihydropyridine receptor, causing it to change shape and open. When the dihydropyridine receptor changes shape, it stimulates the ryanodine receptor causing it

to change shape as well, subsequently stimulating the SR membrane to open. Terminal cisternae will become permeable after being stimulated by the action potential, releasing calcium. Calcium is released into the t-tubules and enters the ultrastructure of the sarcomere and binds to troponin. Calcium alters the shape of troponin, causing the troponin to shift tropomyosin from the actin-myosin binding site.

Regardless of the presence of ATP, myosin will bind to actin if the actin-myosin binding site is exposed and will produce a power stroke. ATP is what breaks the bond between myosin and actin. When ATP is present to break the bond between actin and myosin, the myosin heads will repeatedly attach and reattach, and pull the actin filaments, causing the Z-disks to move closer to the M-line.

Once the muscle is in the contracted state, and impulses from the brain cease to stimulate contraction, steps to return to relaxation ensue. Immediately after calcium is released from the sarcoplasmic reticulum, the calcium pump begins to sequester calcium back into the terminal cisternae of the sarcoplasmic reticulum. The calcium pump uses ATP to re-sequester calcium back into the cisternae by using energy to pump against the established gradient. Calcium enters back into the terminal cisternae of the SR, and proteins called calsequestrin bind calcium to hold it in the SR. ATP breaks the bond between myosin and actin, allowing the Z-disks to slide back to resting state length. Calcium is released from Troponin C, causing tropomyosin to block the actin-myosin binding site. Also, acetylcholinesterase reacts with acetylcholine, breaking this molecule down into acetate and choline to stop the action potential in the muscle.

1.4 Insulin function

Insulin is a hormone that is important during digestion and energy utilization. This naturally-produced hormone increases the uptake of glucose into cells of peripheral tissues (Rhoades et al., 2007) allowing glucose to proceed through the glucose metabolism pathway, and be transformed into chemical energy in the form of ATP. Metabolism also increases deposition of energy reserves (glycogen).

Cells take up glucose with the help of glucose transporter (GLUT) receptors. The type of receptor that cells in the body mainly use are GLUT4 receptors. In the absences of insulin, GLUT4 receptors sit just inside the cell membrane. When insulin binds to its respective receptor, a multi-step pathway eventually causes the GLUT 4 receptors to travel to and fuse with the membrane, binding glucose and transporting it into the cell (Smith, 2017). Other phenomena have to occur in order to "trap" glucose into the cell, which will be discussed later in this report. Under normal feeding conditions (mostly pasture and forage diet), GLUT4 concentrations are highest in cattle at birth since they have a simple gastrointestinal system. As cattle age and begin eating a diet higher in grass and hay, GLUT4 receptors decrease significantly, disallowing the ability to digest high concentrate diets.

Insulin is produced in pancreas and is released by the Islet of Langerhans. Insulin is released when glucose concentrations are high in the blood. Rhoades et al. (2007) showed that muscle and adipose tissue collected from steers converted a significant amount of glucose to CO_2 and lactate after being treated with insulin, with muscle converting more glucose into CO_2 and adipose tissue converting more glucose

into lactate depending on diet. The difference in metabolic outputs means that glucose went through different cycles in the process of being oxidized to form chemical energy by the different tissues.

Insulin increases lipogenesis and reduce lipolysis (Matsuzaki, Takizawa and Ogawa, 1997; Rhoades et al., 2007). Rhoades et al. (2007) revealed subcutaneous adipose tissue in steers fed a corn diet had a higher *in vitro* rate of conversion of glucose to glyceride-fatty acid after being treated with insulin. Had this experiment tracked tissue in an *in vivo* system, the result would have been hypertrophy of adipose tissue, specifically subcutaneous adipose tissue.

However, blood-insulin concentrations tend to increase with age and body fatness in cattle (Matsuzaki et al., 1997; Rhoades et al., 2007; Schoonmaker et al., 2003; Trenkle and Topel, 1978) which can result in type II diabetes. High insulin concentrations in the blood associated with insulin resistance can lead to an increase in blood-glucose concentrations after feeding (Schoonmaker et al., 2003) which is a normal physiological and metabolic response. Insulin increases cell affinity to allow glucose to cross the membrane (with the help of glucose transporters) into the cell. Once blood glucose levels drop to a homeostatic level, a metabolic signal will tell the body to decrease circulating insulin levels, causing insulin to be cleared from the system.

High blood-insulin concentrations can also mean tissues are becoming insensitive to the hormone. A consequence for decreased sensitivity would be a need for increased circulating levels of insulin to get the same effect after blood glucose levels spiked. Matsuzaki et al. (1997) revealed Japanese Black cattle had high blood insulin

concentrations, yet low blood glucose concentrations as body weight increased. The diet these steers were fed was a normal high concentrate diet consistent with Japanese production conditions. This type of increase in blood insulin levels is abnormal, and normally mean the cattle are in a poor state of health. The increased plasma insulin levels in this study were associated with increased fatness in the steers, further reiterating the point that increased blood insulin levels are associated with obesity.

1.5 Glucose metabolism

When free glucose circulates in the blood, glucose is available to be utilized by body tissues. There are a few pathways that comprise glucose metabolism. These are glycolysis, the tricarboxylic acid cycle (TCA), glycogen metabolism, and the electron transport chain (ETC).

1.5.1 Glycolysis

During growth and development, or muscle contraction, chemical energy is required in order to be able to attain these outcomes. Energy in the form of ATP can be formed via glucose metabolism by way of glycolysis, glycogen metabolism, and gluconeogenesis. Glycolysis begins by glucose entering the cell due to the presence of insulin, which increases glucose uptake in the cell (Freedland and Briggs, 1977). Once in the cell, glucose is converted to glucose-6-phosphate (G6P) by hexokinase to keep the molecule in the cell. This traps glucose in the cell and begins the process of committing glucose to subsequent metabolism. After phosphoglucose isomerase converts G6P to

fructose-6-phosphate (F6P), 6-phosphofructokinase (6-PFK) will convert F6P into fructose 1-6 bisphosphate (F-1, 6-P_2). At this point, glucose is now "trapped" in glycolysis, and has to complete the glycolytic pathway. Glucose will eventually be transformed into the final product, pyruvate (Freedland and Briggs, 1977). Not only does glycolysis produce pyruvate, it also produces NADH and 2 ATP.

Pyruvate can be converted to lactate, which will travel to the liver via the bloodstream, and will be re-converted into glucose by gluconeogenesis.

1.5.2 Glycogen metabolism

Another pathway glucose can follow is glycogen metabolism. During glycogen synthesis, after G6P is produced during glycolysis, phosphoglucomutase transfers the phosphate group to the 1-carbon, producing glucose-1-phosphate (G1P). Combining with uracil-triphosphate (UTP), G1P uridyltransferase converts G1P and UTP into UDP-glucose plus two inorganic phosphates (Freedland and Briggs, 1977). The glucose residues, called glucosyl units, are attached to the non-reducing end of a glycogen molecule that is already stored in the body, by way of glycogen synthase. When glycogen synthase is activated, this enzyme attaches glucosyl residues at the reducing end of glycogen branches, storing excess energy in case of emergency situations. Glycogen synthase, like any other enzyme, can be regulated. There are three places on each glycogen synthase molecule that a phosphate group can bind to. When there are no phosphate groups attached to glycogen synthase, the enzyme is active. When a phosphate group is bound to all of the binding sites on glycogen synthase, the molecule

is inactive. Varying levels of activity are possible depending on how many phosphate groups are bound to glycogen synthase as well.

During glycogen degradation, glycogen phosphorylase is activated through a series of phosphorylation steps. Protein kinase A (PKA) phosphorylates phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase (active form). Activated glycogen phosphorylase releases glycosyl residues from glycogen stores at α- 1-4 linkages, producing G1P by adding a phosphate group to the 1- carbon as well as α- 1-6 branch points, the latter producing free glucose. Glucose-1-phosphate can be converted into G6P to be used in glycolysis.

1.5.3 Tricarboxylic acid cycle

Not only can PEP go into gluconeogenesis, it can also enter the tricarboxylic acid cycle (TCA) after being turned into pyruvate. The TCA cycle begins by pyruvate reacting with a Coenzyme A (CoA) and an NAD^+ molecule, forming acetyl Coenzyme A (acetyl CoA). Two metabolites produced from this reaction are a CO_2 and NADH molecule. Acetyl CoA combines with OAA, a 4-C molecule, to form Citrate, a 6-C molecule. An H^+ molecule is formed as well. Citrate is turned into Isocitrate with the help of the enzyme, Aconitase. Isocitrate is turned into α-ketoglutarate by way of the enzyme Isocitrate dehydrogenase. From this reaction, a CO_2 and an NADH molecule is formed.

Succinyl CoA is formed from α-ketoglutarate dehydrogenase removing a carboxyl group from α-ketoglutarate. A CO_2 and an NADH molecule are formed from

this reaction. Succinyl CoA is transformed into Succinate by the enzyme Succinyl CoA synthetase, and Succinate is turned into Fumarate by the action of Succinate dehydrogenase. An $FADH_2$ molecule is formed from this reaction. Fumarate is turned into Malate from the interaction of fumarate hydratase and H_2O. Finally, Malate is transformed into OAA by the action of Malate dehydrogenase. An NADH molecule and an H^+ is formed from that reaction. In total, the metabolites from the TCA cycle are 4 NAHD, 3 CO_2, 2 H^+, and 1 ATP (Hardin, Bertoni, and Kleinsmith, 2015).

1.5.4 Electron transport chain

After NADH and $FADH_2$ are produced from the TCA cycle, these are transported to the folds in the mitochondria. The folds are called cristae: this is where the electron transport chain (ETC) is located. NADH and $FADH_2$ donate their H^+ ions which exit the mitochondrial matrix, forming a gradient so ATP synthase can pull H^+ ions into the mitochondria matrix. This established gradient allows ATP synthase to produce ATP for the cell for energy usage. Once NADH and $FADH_2$ are turned into NAD^+ and FAD, respectively. H^+ ions will exit the mitochondria, causing a gradient to form. The established gradient will activate the proton pump that is embedded in the mitochondrial membrane. H^+ ions that have accumulated outside of the mitochondrial membrane are pumped into the cell, causing ADP to be turned into ATP. One molecule of glucose will form 34-36 molecules of ATP by way of the ETC (Hardin et al., 2015).

1.6 Introduction to β-AA

As previously stated, excessively obese cattle are being produced, which is causing a decrease in the amount of edible product being produced. Ricks et al. (1984) reported that β-AA would decrease the amount of fat accumulation in livestock species. β-AA are in a class of pharmaceutical drugs called phenethanolamines (Anderson et al., 2004; Scramlin et al., 2010). As previously mentioned, these chemicals repartition energy from adipose tissue accretion to muscle growth; which is why Ricks et al. (1984) coined the term "repartitioning agent" for β-AA.

Phenethanolamine drugs are laboratory produced chemicals. Some of the better known phenothanolamines are zilpaterol hydrochloride (ZH), clenbuterol, salbutamol, and ractopamine hydrocholoride. Some similarities between the structures of these two classifications of chemicals are, they all have at least one carbon ring, and an amine group. Compounds such as ractopamine hydrochloride and ZH are used in livestock production to increase lean muscle mass, and decrease excess subcutaneous and internal adipose tissue accretion. β-AA are also classified as sympathomimetic drugs when related to human medical use (Spratto and Woods, 2003). Uses of β-AA in the human medical industry include controlling contraction of the heart, increase lipolysis, stimulate vasodilation of the extremities, and control symptoms of certain diseases such as asthma and chronic obstructive pulmonary disorder (COPD).

1.7 Introduction to catecholamines

β-AA are chemically and structurally similar to the naturally occurring catecholamines (Bell et al., 1998). Catecholamines are a group of hormones that include norepinephrine, epinephrine, and dopamine. The name catecholamine was given to this group because of their common catechol group, which is a carbon ring with two hydroxyl groups projecting from the 2nd and 3rd carbon. Catecholamines and phenethenolamines are similar in structure due to the presence of at least one carbon ring, and an amine group. Epinephrine and norepinephrine are associated with the "fight or flight" response; also called the stress response. The main effects of this response are an increase in respiratory and circulatory rates, and a decrease in glycogen stores. These two hormones also increase during physically demanding situations, such as exercise.

Dimsdale and Moss (1980) showed the increase in epinephrine and norepinephrine levels in the systems of medical physicians during different tasks that they would complete on the job. During exercise (walking up flights of stairs), both hormones increased but norepinephrine almost tripled during exercise. During the first three minutes of a speech at a conference, both epinephrine and norepinephrine doubled to tripled from the baseline levels of these hormones. Clearly these two hormones are expressed in the greatest amounts during situations that induce stress.

Once the stress state is reached, and these hormones are released, different metabolic events occur. Blood circulation increases after the heart muscles are stimulated (Kaumann et al., 1999), increasing blood flow through the vessels, resulting in more nutrient rich blood that arrives to muscle and adipose tissue. Epinephrine and

norepinephrine will also increase the breakdown of glycogen and fatty acids (Cunningham and Friend, 1965) which can be utilized by the body as emergency energy.

After injecting 0.25 mg/80kg of epinephrine into pigs, Cunningham and Friend (1965) reported their pigs came off of their feed. After an epinephrine injection, blood-glucose levels had reached a point to where their pigs were receiving a signal from the brain that they were full. The injected epinephrine had broken down glycogen stores into glucose, and released into the blood stream. Similar results were reported for cattle, in that epinephrine injection significantly reduced muscle glycogen (Crouse and Smith, 1986). Body tissues could then utilize the free glucose for growth or energy production. Free fatty acid levels in the blood of these pigs acted similar to glucose, with the levels increasing a few hours after an epinephrine injection (Cunningham and Friend, 1965). Triacylglycerol was mobilized by epinephrine and formed free fatty acids that could have been used as a source of energy as well.

Epinephrine has an effect on both glycogen and fat stores. Norepinephrine behaved in a similar manner, although norepinephrine worked to a greater extent on fat stores than glycogen stores. Norepinephrine had about a 300% greater impact on free fatty acid levels in the blood compared to epinephrine (Cunningham and Friend, 1965). Thus, epinephrine and norepinephrine are catabolic hormones since clear evidence was presented that glycogen was broken degraded, and triacylglycerols were mobilized into blood metabolites (Cunningham and Friend, 1965).

1.8 β-AA receptors

β-AR are embedded in the membranes of muscle and adipocytes. Each β-AR has seven transmembrane domains, and three extracellular loops and three intracellular loops (Johnson, Smith and Chung, 2014; Mersmann, 1998). The extracellular end of the receptor contains an N-terminus, which is normally associated with stimulation of the receptor, while the intracellular end contains a C-terminus, which is normally associated with inhibition of the receptor (Johnson et al., 2014; Mersmann, 1998). Mersmann (1998) reported that β-AR interact with a G_S protein in the proximity of intracellular loop 2, 3, and 4. Recently, that location was isolated to loop 3 (Johnson et al., 2014). Each G_S protein is associated with adenylate cyclase. Also, a ligand binding site (the site where the β-AA would bind to the receptor) is associated with transmembrane domains 3, 4, 5, and 6.

There are three β-AR subtypes; β_1, β_2, and β_3. β_1-AA receptors are typically embedded in slow twitch, or Type I and Type IIA muscles, while β_2-AR are associated with fast twitch, or Type IIB and Type IIX muscles. Kim et al. (1987) concluded type II fibers are more sensitive to β_2-AA. Also, Sillence et al. (1991) and Sillence and Matthews (1994) reported in their two separate studies that β_1-AR were not detectable on certain bovine muscles. These findings agree with the study conducted by Miller et al. (1988) which determined through histochemical testing that type II fibers increased in size via hypertrophy, while type I fiber size decreased significantly. Therefore, β-AA should increase the amount of edible muscle in livestock species.

1.8.1 β_3-AA receptors

Prior to the 1970's, the only two receptor subtypes that were known to be present in animal tissues were the β_1, and β_2-AR. These two receptor subtypes were thought to predominantly be embedded in muscle cell walls. The β_3-AR were not discovered until the beginning of the 1970's (Mersmann, 1998). These receptor subtypes were first discovered embedded in brown and white adipose tissue in rats. There was a possibility that β_3-AR could be present in skeletal muscle and cardiac muscle (Mersmann, 1998). Up until 2012, the status quo remained that β_3-AR were only present in the two different adipose tissue types.

Miller et al. (2012) extracted mRNA from semimembranosus muscles from cattle treated with different concentrations of ZH. Graphs from this study revealed that the treated muscle was composed of β_1-, β_2-, and β_3-AR. Before this study, there was no previous evidence of β_3-AR being present in the membrane of skeletal muscle. However, these scientists graphed the amount of β-AR relative to the β-AA receptors in the control, so the abundance of the β_3-AR embedded in the semimembranosus muscles were not determined.

1.9 β-AA mechanisms

β-AA work in the same manner as epinephrine and norepinephrine. The specific series of metabolic events begins when a β-AA binds to a β-AR. The G_S protein directly interacts with adenylate cyclase (Anderson et al., 2004; Johnson et al., 2014) after being activated by a β-AR. Adenylate cyclase activation causes a series of phosphorylation

events ultimately leading to the activation of phosphorylase kinase. This causes the repartitioning of energy from adipose tissue accretion to muscle growth.

Binding of a β-AA to a receptor changes metabolic pathways and signals, causing a cascade of events that alter glucose, muscle, and fat metabolism towards muscle accretion (Anderson et al., 2004). Immediately after a β-AA binds to a receptor, the G_S protein interacts with, and simultaneously activates adenylate cyclase, converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Many metabolic events are controlled by the presence of cAMP (Smith, 1987).

There are four binding sites on the regulatory protein that controls the activity of PKA. Increasing cAMP concentrations cause enough of this intracellular metabolite to bind to the regulatory protein. Once all four places are bound, the regulatory protein will release PKA, activating it (not phosphorylating it). PKA phosphorylates, and simultaneously activates phosphorylase kinase. Subsequently, increasing cAMP concentrations inhibit glycogen synthase, causing phosphate groups to become bound to the binding sites, and ultimately inhibiting the functionality of this enzyme.

1.9.1 β-AA mechanisms on glucose metabolism

There are three functions of phosphorylase kinase during glycogen metabolism. The first is to phosphorylate a specific region of glycogen synthase, decreasing the activity of glycogen synthase slightly. Glycogen synthase has three regions that can be phosphorylated. One site is phosphorylated by glycogen phosphorylase, as previously stated. Another site is bound by a cAMP-dependent kinase, and the third by a glycogen

synthase-dependent kinase. If all sites are bound by phosphate, glycogen synthase will become inactivated. The function of active glycogen synthase is to add glucosyl residues to stored glycogen chains to the non-reducing end of the glycogen chain. This function will cease when a β-AA binds a receptor, disallowing glycogen synthesis.

The second function of phosphorylase kinase is to activate glycogen phosphorylase. After glycogen synthase is deactivated, glycogen phosphorylase will break glucosyl residues from the non-reducing end of the α-1, 6 branch points on stored glycogen. The third function of phosphorylase kinase is to add a phosphate group to these free glucosyl residues. The phosphate group will be added to the 1-carbon, creating G1P. Phosphoglucomutase will transfer the phosphate group from the 1-carbon to the 6-carbon, producing G6P. Once G6P is formed, the normal glycolysis cycle can ensue to utilize chemical energy.

1.9.2 β-AA mechanism on insulin sensitivity

Traditionally, β-AA are known to decrease carcass fatness in steers after being fed during the finishing phase. In rats treated with one dose of 10 μg/kg compared to a saline injection, glucose utilization index for specified muscles doubled on average in treated rats (Liu and Stock, 1995). In this experiment, the glucose utilization index indicated that glucose was being taken up into the muscles, meaning the β-AA in this experiment caused insulin sensitivity to increase (caused an insulin response). Liu and Stock (1995) also revealed in their study, treated rats produced a significant, 2.5 fold increase in plasma insulin. One effect of β-AA action on muscle tissue, as previously

mentioned, is the breakdown of glycogen stores into glucose residues, ultimately increasing blood glucose levels. Excess glucose in the blood signals the pancreas to release insulin into the blood. These data agree with the metabolic events caused by β-AA.

1.9.3 β-AA mechanisms on muscle tissue

As previously stated, β-AA redirect chemical energy from accretion of fat muscle growth. Muscle mass is thought to be increased in livestock by decreasing protein degradation, and increasing protein synthesis (Johnson et al., 2014). Ergo, the body is decreasing protein turnover to increase leanness and mass. Muscle response to β-AA begins when a β-AR is activated and cAMP is produced (Mersmann, 1998). Muscle mass increases via hypertrophy of muscle fibers (Anderson et al., 1991; Beermann, 2002; Johnson et al., 2014; Mersmann, 1998; Smith, 1987). Specifically hypertrophy of type II muscle fibers (Beermann, 2002; Johnson et al., 2014; Mersmann, 1998; Smith, 1987).

Schiavetta et al. (1990) proposed in their study that muscle hypertrophy was induced by an increase in protein synthesis, or a decrease in proteolysis. Both protein synthesis and protein degradation occurring simultaneously (Smith et al., 1995; Mersmann, 1998; Wheeler and Koohmaraie, 1992) has been the suggested mechanism of β-AA function on muscle metabolism as well. Protein synthesis in animals treated with β-AA was induced by an increase in protein transcription, leading to mRNA production (Hod, Morris, and Hanson, 1984; Smith et al., 1989). Myofibrillar mRNA specifically

(Anderson et al., 1991; Hod et al., 1984; Smith et al., 1989) provides a template for new muscle fibers to be formed and would likely be a result of β-AA induced up-regulation of myofibrillar protein gene transcription (Smith, Garcia and Anderson, 1989; Smith et al., 1995). Accumulation of protein mRNA would lead to an increase in protein synthesis and accretion, causing muscle hypertrophy.

The implication that muscle grows via hypertrophy is because of the observation that there was not a change in the amount of DNA produced resulting from feeding cattle different concentrations of ZH (Miller et al., 2012). Muscle hypertrophy can occur only if there is a repartitioning of nutrients away from adipose tissue. After β-AR are stimulated, increasing cAMP concentrations inhibit lipogenic enzymes, and stimulate lipolysis enzymes, causing adipose tissue degradation via lipolysis, releasing free fatty acids into the blood. These fatty acids will then be used as an alternative source of energy for amino acid production (Ricks et al., 1984) if the need arises in the animal.

As previously stated, β-AA are chemically related to catecholamines, which can elicit a "fight or flight" response. β-AA increase circulation of blood in the same manner as catecholamines. As glycogen and adipose tissue stores are catabolized, nutrient concentrations increase in the blood. A simultaneous increase in circulation and respiration occurs as well. A higher circulation rate causes more nutrient-rich blood to flow to the muscles after treatment with β-AA (Smith, 1987). Not only will this increase chemical energy flow to muscles, studies show that specific nutrients that become more readily available to cattle, including nitrogen uptake (Smith, 1987) and retention in the hindlimbs of cattle. Nitrogen is an essential building block for amino acids, which go on

to produce proteins. Increased nitrogen accumulation near growing muscles would increase amino acids available for protein accretion.

An increase in muscle mass and size is be a consequence of decreased protein catabolism. Ricks et al. (1984) proposed that β-AA caused muscle hypertrophy as a result of decreased protein degradation. Myofibrillar proteins can become damaged or too old to function during the normal turnover cycle, and need to be replaced. Calpains are molecules located in muscle tissues that function in protein degradation.

Cessation of protein degradation is thought to occur by inhibiting calpains through an increase in calpastatin concentration (Wheeler and Koohmaraie, 1992; Sensky et al., 1996; Killefer and Koohmaraie, 1994). Wheeler and Koohmaraie (1992) and Killefer and Koohmaraie (1994) reported that a β_2-AA reduced protein degradation in steers, and that calpastatin was elevated in these steers as well. During the normal aging period of livestock carcasses, calpains break down the Z-disks of the sarcomeres. calpastatin decreases Z-disk degradation by inhibiting calpain activity. Data suggest in pigs infused with epinephrine, an increase in calpastatin concentration was exhibited (Sensky et al., 1996) as well.

1.9.4 β-AA mechanism on adipose tissue

Because energy production is redirected to muscle production, this means adipose tissue production is decreased in livestock fed β-AA. As previously described, in the early stages of the metabolic pathway during glucose uptake, adenylate cyclase activation (due to interaction with the G_S protein) increases cAMP production. Protein

kinase A is activated after four cAMP molecules bind to the regulatory protein that keeps PKA activity dormant. Protein kinase A not only activate phosphorylase kinase, but it can also activate lipase and deactivate lipogenic enzymes (Anderson et al., 1991). Thornton et al. (1985) tended to agree with these statements, revealing in their study a distinct decrease of lipogenesis and a three-fold increase in lipolysis rate as the level of clenbuterol increased from 0 to 0.1 μg, and an even larger increase in lipolysis after increasing clenbuterol concentrations from 0.1 to 10 μg. With such a drastic change in lipid metabolism, there is no surprise when studies revealed these metabolic events cause significant decreases in marbling scores (Miller et al., 1988; Miller et al., 1989) and in external fat cover (Miller et al., 1988; Miller et al., 1989; Ricks et al., 1984; Thornton et al., 1985).

1.10 Radioactive material in *in vitro* experiments

In most experiments that study *in vitro* metabolism of muscle tissue, the element that is used is ^{14}C. This is a radioactive element that can be manually incorporated into glucose metabolism during the incubation periods of these experiments. Radiolabeled elements, known as tracers, make it possible to conduct metabolism and biochemistry-based experiments. The common elements used are ^{3}H, ^{14}C, ^{32}P, and ^{35}S. These elements are called radioisotopes. In the current study, ^{14}C is the radioisotope used because of ease of incorporation into metabolites produced during glucose metabolism in adipose tissue and muscle.

^{14}C is an unstable isotope of the stable form of carbon, which is ^{12}C. The instability is useful when a liquid scintillation counter is utilized. ^{14}C degrades after one neutron converts to a proton, changing the chemical identity of the element. Nitrogen and a radioactive electron are produced after ^{14}C disintegrates.

The unit used in metabolism studies is the μCi. A Curie (Ci) is the base unit when measuring radioactive elements (similar to the gram or the meter). One Ci is equal to 2.22×10^{12} dpm, meaning in one minute, 2.22×10^{12} radioactive degradations occur, such as the example of ^{14}C degrading into Nitrogen and one electron. In most laboratories, the μCi is what is used, which is equal to 2.22×10^{6} dpm.

^{14}C acts well as a tag during metabolism studies. Only a small amount of the radiolabeled element is added (1 μCi) to media, therefore a ratio can be determined of radiolabeled glucose to unlabeled glucose. This ratio is multiplied to dpm picked up by a scintillation counter to give a Depending on which metabolite is tested for the tag (i.e., glycogen, and CO_2), measured dpm can be multiplied by a certain number to get the amount of carbon incorporated into the metabolites. For example, if the scintillation counter detects 99.47 dpm from a glycogen sample from an *in vitro* experiment, that number will be multiplied by 6, since there are 6 carbons to a glucose carbon ring, and only one of those carbons are tagged with the μCi of glucose.

Scintillation liquid that the electrons (β-particle) are dissolved in help to enumerate disintegrations of ^{14}C to detect metabolites. Suspended in the scintillation liquid are molecules that can fluoresce, called fluors, due to electrical excitation after interacting with β-particles. β-particles become surrounded by the fluors after being

mixed with the scintillation liquid. Electrical energy is passed to the fluors after disintegration, and excites the fluors, causing them to fluoresce and give off light. Scintillation counters detect the light with the help of a photomultiplier, which can be translated into a numeric count of radioactive ^{14}C as disintegrations per minute (dpm).

CHAPTER II

MATERIALS AND METHODS

2.1 Animals and facilities

Four Angus or Angus-cross steers (with no Brahman influence) were used in this study. All steers were fed a finishing diet at the Texas A&M University Research Center at McGregor, TX. Steers were transported to the Texas A&M Beef Center within the O. D. Butler Jr. Animal Science Center, located in College Station, TX until muscle samples were ready to be collected. When muscle samples were collected, they were prepared for *in vitro* metabolism analysis in the adipose tissue and muscle tissue laboratory, located within the Kleberg Animal & Food Sciences Center at Texas A&M University in College Station, TX. These cattle were harvested under the AUP AACUC #2016-009A. Tissue samples used in this study were obtained through tissue sharing from cattle under this same AUP.

2.2 Harvest procedures, sample collection, and sample preparation

On scheduled slaughter days, each steer was haltered and restrained, exposing the right jugular vein. All animals were stunned using a captive bolt stunner and were tested for reflexes of the eye and nose before exsanguination. The right jugular vein was severed to exsanguinate each steer. Immediately after exsanguination, the LL muscle on the right side of each animal, stretching from approximately the 9^{th} to 11^{th} rib was removed from each carcass for muscle samples.

LL muscles were transported to the laboratory by being placed in a container with media consisting of KHB (pH 7.4) and 5 m*M* glucose, warmed to 37°C. These are similar transporting methods used by Coleman, Eckeren, and Smith (1988), Espinal, Dohm, and Newsholme (1983), Miller et al. (1989), and Schiavetta et al. (1990), with the latter methods being adapted from Prior and Jacobson (1979). Once at the laboratory, LL samples were cut into sections, and from the sections, muscle strips were cut using sterile scalpels and forceps. The muscle strips were between 100-300 mg, and ran parallel to the muscle fiber orientation.

2.3 Experimental design

Muscle strips were placed in individual flasks for analysis of metabolism. Flasks were placed in the shaking water bath. Each flask contained 3 mL of KHB, 5 m*M* glucose, a single muscle strip, and 1μCi [U-^{14}C]glucose. Each flask was capped with a rubber stopper, perforated to hold a hanging center well. A folded piece of filter paper was placed in each hanging center well, and each piece filter paper saturated with 2 N NaOH. All flasks were subjected to a 90 min incubation period in the water bath.

2.3.1 Experiment one and two

For experiment one, 45 muscle strip samples were used. Three treatment groups made up this experiment; a Control group, a group containing Experior (Elanco Animal Health, Greenfield, IN), and a group containing BRL 37344 (Tocris Bioscience, Bristol, UK). Five different insulin concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) were tested in

triplicate (this makes the three groups of 15 for a total of 45 samples). These treatment groups directly tested for a β_3-AA receptor response in the muscle strips. The Control group flasks contained only 5 m*M* glucose, 1μCi [U-^{14}C]glucose in Krebs-Hensleit buffer (pH 7.35-7.4), one muscle strip, and insulin (either 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M). The second treatment group contained all of the materials named in the Control group, plus 50 μM Experior. The third set of flasks contained all of the materials specified for the Control group, plus 50 μM BRL 37344.

2.3.2 Experiment three

Thirty-six muscle strip samples were used in this experiment. There were two treatment groups: a Control group and a group that contained Experior. Six different insulin concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) were tested in triplicate (this makes up the two groups of 18, for a total of 36 samples). Each flask in the control group contained one concentration of insulin, 5m*M* glucose, 1 μCi [U-^{14}C]glucose, 3 mL KHB, and one muscle strip. The treatment group included all materials specified in the Control group, plus 50 μM Experior.

2.3.3 Experiment four

Fifty-four samples were used in this experiment. This experiment tested three incubation times: 30, 60, and 90 min. Six different concentrations of insulin (either 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) were tested in triplicate (this makes up the three groups

of 18, totaling 54 samples). Each flask in this experiment contained one concentration of insulin, 5m*M* glucose, 1 μCi [U-^{14}C]glucose, 3 mL KHB, and one muscle strip.

2.3.4 Muscle CO_2 synthesis

After the initial incubation time was complete, 0.5 mL 2 N H_2SO_4 was injected into the flasks to stop metabolic reactions. Muscle strips were not placed back into the flasks once the H_2SO_4 was injected so as to not interfere with enzyme activity in the muscle strip. The flasks containing the media and injected with H_2SO_4 was subjected to another 60 min incubation period in the shaking water bath so CO_2 could react with the NaOH, forming $NaHCO_3$ which represents CO_2 produced during glucose metabolism from the muscle strips. Once the second incubation period was complete, the filter paper was placed into individual scintillation vials, followed by an addition of 1 mL of water and 10 mL of scintillation cocktail. The recovery of ^{14}C-labeled glucose carbon in glucose was measured by a Beckman L53000 liquid scintillation counter.

2.3.5 Muscle glycogen synthesis

Alternatively to being placed back into the flasks, muscle strips were placed in glass test tubes containing media to obtain weights for glycogen, lactate, and CO_2 radiolabeled carbon calculations. Muscle strips were transferred to individual 15-mL screwcap test tubes and kept at 4°C when chemical analyses were not being performed. Muscle strips were rinsed with 0.5 mL of water, and 3 mL of 75% ethanol, and vortexed to dilute the [U-^{14}C]glucose. This process was repeated three times. The mixture caused

the coagulation of glycogen synthesized during the initial incubation period. The coagulated glycogen is called a glycogen pellet. After the third dilution series, pellets sat overnight, and glycogen pellet were transferred to individual scintillation vials. Each pellet was mixed with 1 mL of distilled water, and 10 mL of scintillation cocktail. Each vial was gently swirled in order to dissolve each pellet. The recovery of ^{14}C-labeled glucose carbon in glucose was measured in a liquid scintillation counter.

2.3.6 Muscle lactate synthesis

After the initial 90 min, and the additional 60 min incubation period were completed, media from each flask was transferred to individual 15 mL screw cap test tubes. Lactate Assay Kits were purchased from Eton Bioscience (Eton Bioscience, Inc., San Diego, CA) and utilized to determine lactate production from the metabolism of muscle strips. A standard curve was established in order to calculate lactate production. The standard curve was determined by mixing 50, 40, 30, 20, 10, 5, 1, and 0 µL of L-lactate Standard with double distilled water to produce a total volume of 50 µL in each well that holds the standard curve media.

Media from the flasks had to be neutralized (pH of 7.4-8) in order for the lactate assay solutions to work. 50 µL of media from each screw cap test tube were placed in individual wells. Media was tested in duplicate. Fifty µL of L-Lactate Assay Solution was added to each well containing media to initiate the reaction. Plates were wrapped in aluminum foil and incubated at 37°C for 30 min. After the incubation period was complete, 50 µL of 0.5 M acetic acid was added to each well containing media stop the

reaction. The well plates were gently agitated for 20 sec. Absorbance was measured at 490 nm using a BioTek Epoch 96-well plate reader.

2.4 Statistical analysis

Data were analyzed as a two-way analysis of variance (SuperANOVA®: Abacus Concepts, Inc., Berkeley, CA). Main effects were treatment (insulin, Experior, and ß$_3$-AA); the model also tested theinsulin x Experior or ß$_3$-AA interaction. Main effects and interactions were considered significant at ($P < 0.05$).

CHAPTER III

RESULTS

A preliminary experiment was conducted to determine the effect of incubation time (30 min, 60 min, 90 min) on insulin sensitivity; glycogen, CO_2, and lactate production was linear over time. Experiments were conducted to measure to conversion of glucose to glycogen, CO_2, and lactate in muscle strips incubated with either 50 μM Experior, 50 μM BRL 37344, or no β-AA (Control). All flasks contained increasing amounts of insulin to specifically test the interaction between insulin and $ß_3$-adrenergic agonists ($ß_3$-AR).

For glucose conversion to glycogen, there was a significant treatment effect ($P < 0.001$) (Figure 1). BRL 37344 caused lower levels of glucose conversion to glycogen than either the Control or Experior. BRL 37344 caused 62% lower amounts of glycogen synthesis compared to the Control samples (insulin only), and produced 50% lower amounts of glycogen synthesis compared to Experior samples. The Control samples produced the highest amount of glycogen (4.3 nmol/100 mg·90 min), while Experior produced an intermediate amount of glycogen (3.1 nmol/100 mg·90 min), between Control and BRL 37344 (1.6 nmol/100 mg·90 min). Experior did not have a consistent effect on glycogen production as insulin concentrations changed.

There was a significant treatment effect for CO_2 production ($P = 0.018$; Figure 2 and Table 1). BRL 37344 caused the highest amount of glucose conversion to CO_2 compared with the other treatments. BRL 37344 elicited 23% more glucose conversion

to CO_2 than Experior, and 41% more CO_2 than Control samples. The Control samples produced the lowest amount of CO_2 (171.3 nmol/100 mg·90 min), whereas Experior produced an intermediate amount of CO_2 (222.9 nmol/100 mg·90 min) between the Control and BRL 37344 (292.6 nmol/100 mg·90 min). There was not a significant main effect of treatment on lactate production ($P = 0.419$; Figure 2 and Table 1).

The insulin x treatment interactions were not significant glycogen ($P = 0.221$; Figure 3), CO_2 ($P = 0.641$; Figure 4), or lactate ($P = 0.748$; Figure 5). There was not a significant main effect of insulin concentration on glycogen production ($P = 0.634$), CO_2 production ($P = 0.953$), or lactate production ($P = 0.953$).

Table 1. Main effects means for the conversion of glucose to glycogen, CO_2, and lactate in vitro.

	Treatment				
Metabolite	Insulin	Experior	ß$_3$-AA	Pooled SEM	*P*-values
Glycogen	4.3[a]	3.1[b]	1.6[c]	0.3	0.001
CO_2	171.3[b]	222.9[ab]	292.6[a]	23.5	0.018
Lactate	638.5	622.3	640.1	40.4	0.419

Units are nmol/(100 mg·90 min)

[abc]Means with common superscripts are not different ($P > 0.05$).

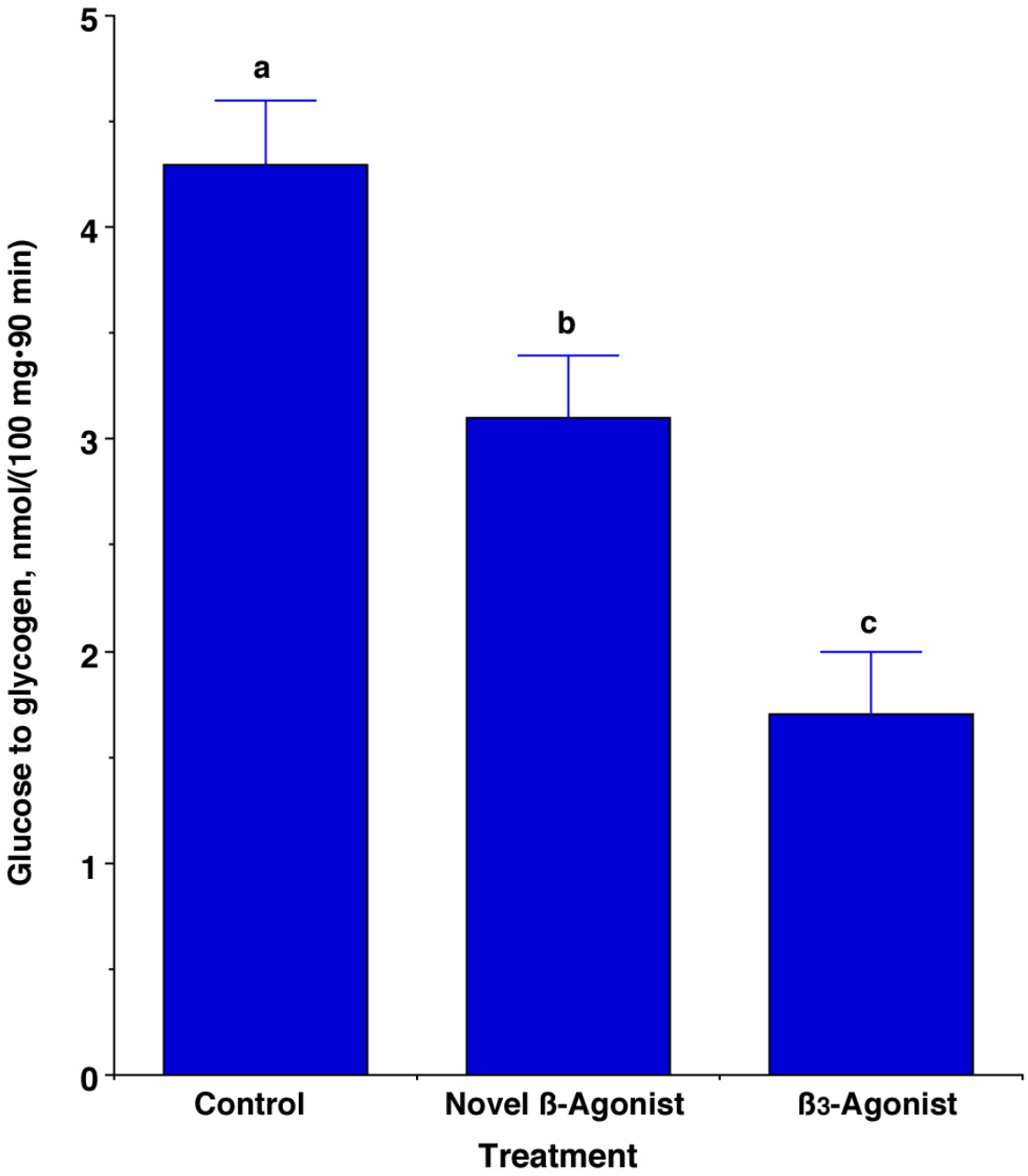

Fig 1: Glucose conversion to glycogen (nmol/100 mg·90 min) between treatment groups.
[abc]Means with common superscripts are not different ($P > 0.05$)

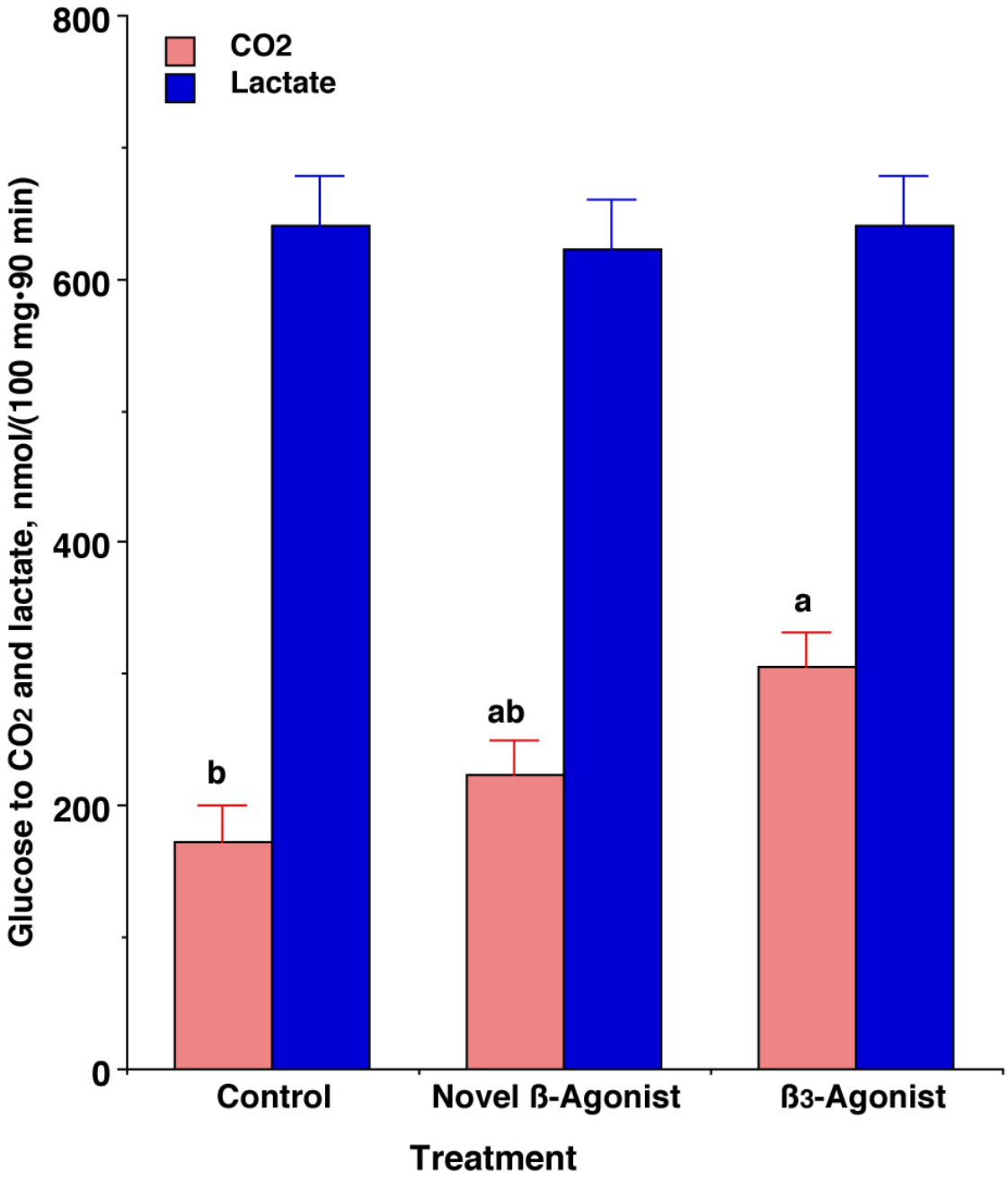

Fig 2: Glucose conversion to lactate and CO_2 nmol/100 mg·90min between treatment groups.
[abc]Means with common superscripts are not different ($P > 0.05$)

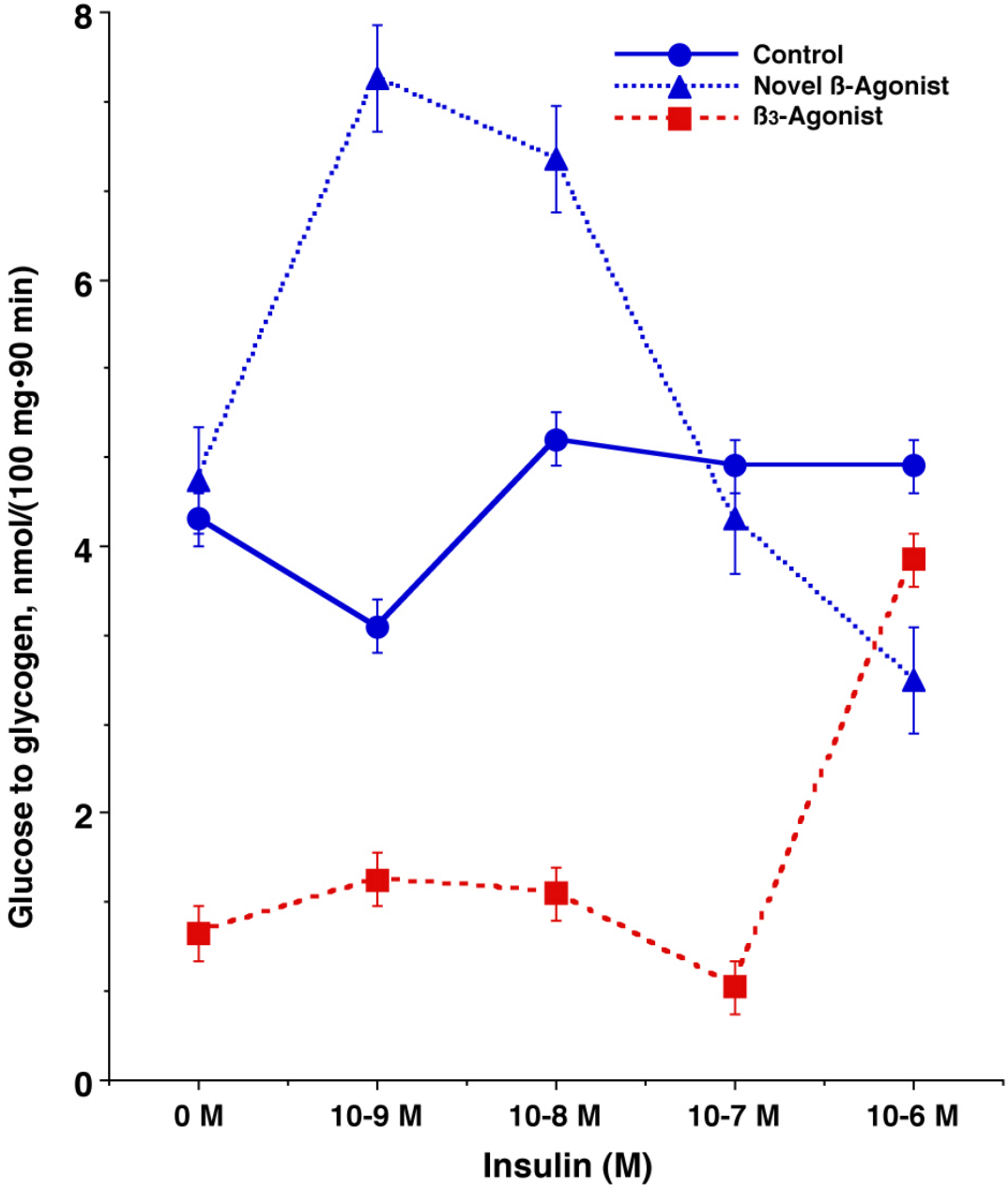

Fig 3: Insulin x treatment interaction graph for glucose conversion to glycogen (nmol/100 mg·90min). n = 3. The insulin x treatment interaction was not significant (P = 0.221)

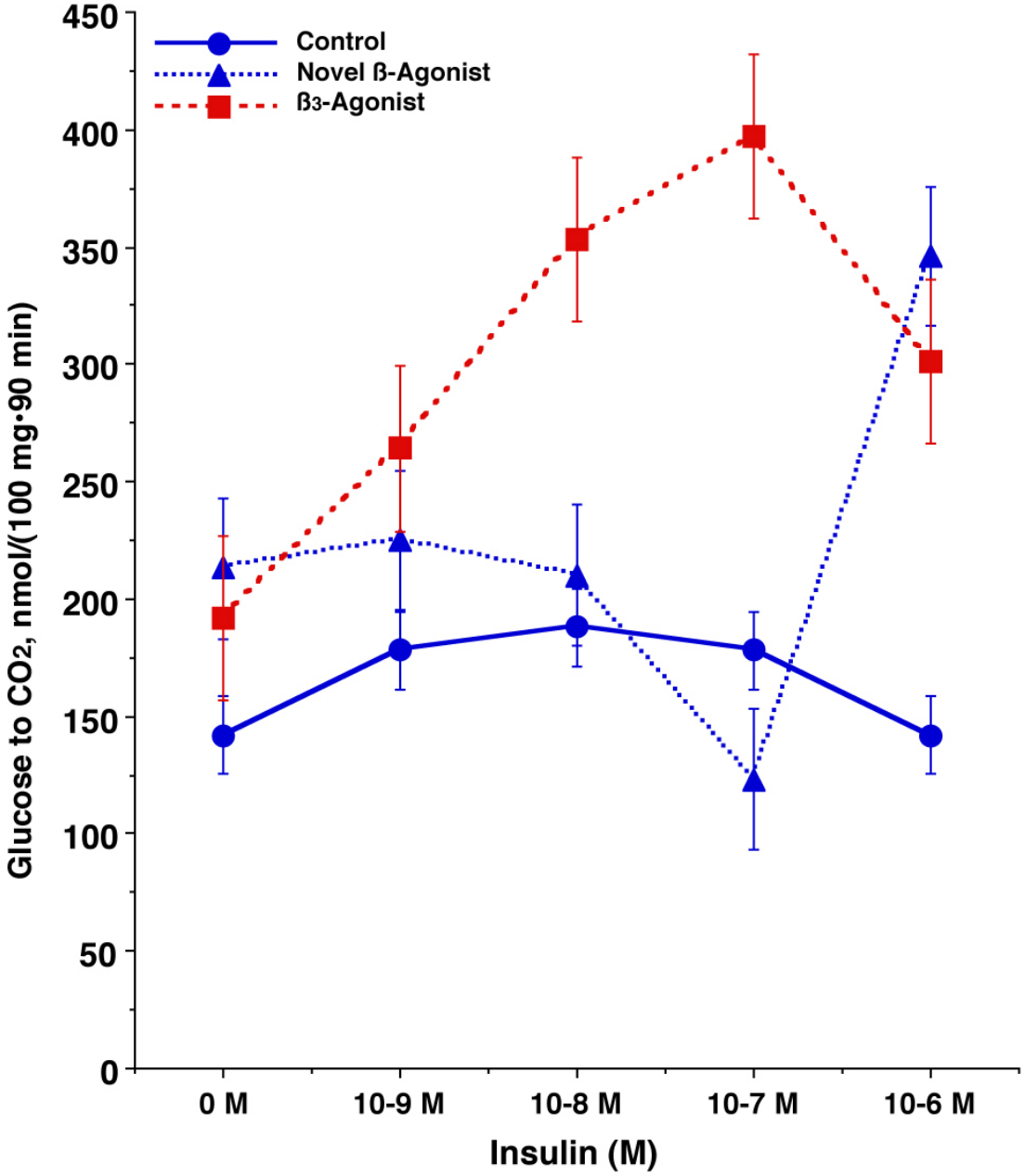

Fig 4: Insulin x treatment interaction graph for glucose conversion to CO_2 (nmol/100 mg·90min). n = 3. The insulin x treatment interaction was not significant (P = 0.641).

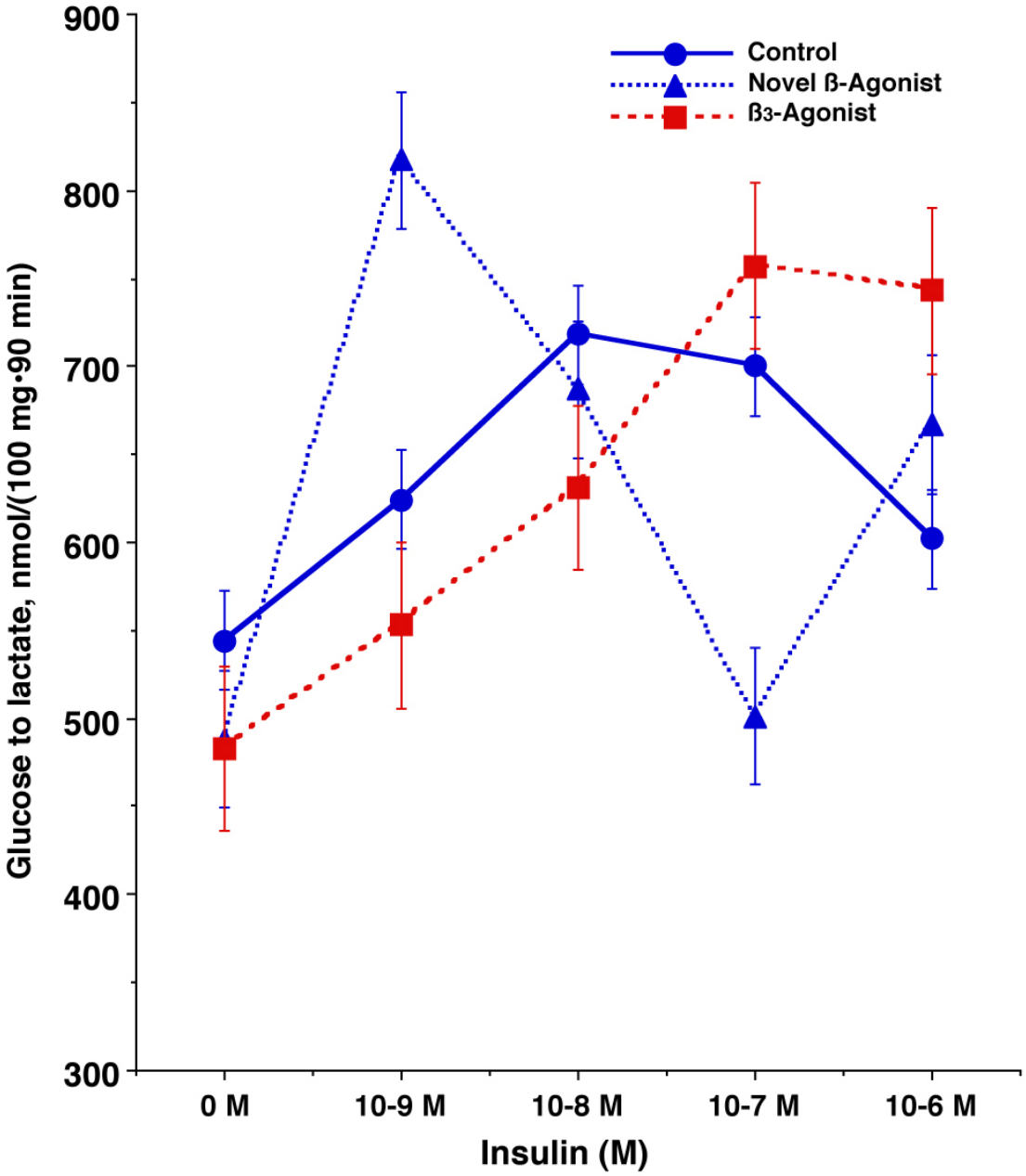

Fig 5: Insulin x treatment interaction graph for glucose conversion to lactate (*nmol/100 mg·90min)*. n = 3. The insulin x treatment interaction was not significant ($P = 0.748$)

CHAPTER IV
DISCUSSION

The most important finding from this study is that muscle strips reacted to BRL 37344 in a manner that supported our hypothesis that muscle does contain β_3-AR. These results refute the accepted theory that β_3-AR are present only on brown and white adipose tissue membranes.

Stimulation of β-AR increases glycolysis and strongly depresses glycogen synthesis, thereby increasing glucose metabolism to lactate and CO_2. If a specific β-AA is effective, then it should elicit an increase in lactate and CO_2 production. However, the known β_3-AA, BRL 37344, only increased CO_2 production *in vitro*, and did not affect lactate.

During the 90-minute incubation period, the muscle strips released approximately 600 nmol lactate per 100 mg muscle. Because 1 nmol glucose yields 2 nmol of lactate, this represents 300 nmol glucose that was metabolized to lactate during the incubation period. Bovine muscle contains approximately 70 µmol glycogen (as glucosyl units) per g, or 7 µmol (7,000 nmol) per 100 mg muscle (Crouse, Smith, and Prior, 1984; Crouse and Smith, 1986). However, the incubation media contained 5 µM glucose, or 15,000 nmol per flask. Thus, it is possible that the contribution of increased glycogen degradation to lactate production (caused by the β_3-AA) was masked lactate production from media glucose.

As indicated above, the ß$_3$-AA BRL 37344 strongly increased CO_2 production. A recent study demonstrated that an ERK-dependent pathway phosphorylates pyruvate dehydrogenase (PDH) and thereby inhibits its activity (Li et al., 2016). PDH catalyzes the first step in the Krebs cycle, converting pyruvate to acetyl-CoA. Therefore, activation of the ERK-dependent pathway would decrease CO_2 production from glucose. The mechanism by which the ß$_3$-AA increased CO_2 is unknown, but may represent antagonism of the ERK-dependent pathway. Hamby et al. (1986) also demonstrated that muscle from lambs treated with the ß-AA clenbuterol had greater CO_2 production from glucose than muscle from control lambs.

Also, if β-AA affects muscle metabolism, it should elicit a decrease in glycogen synthesis in the muscle. Hamby et al. (1986) reported that clenbuterol treatment of lambs actually increased the *in vitro* conversion of glucose to glycogen (as well as to lactate). However, the 35-d exposure to clenbuterol was sufficient to cause an increase in type II (glycolytic) myofibers (Hamby et al., 1986), explaining the increase in overall glucose metabolism.

The findings in the current study demonstrate for the first time that the LL muscle contains β_3-AR. These results suggest that in live animal studies utilizing a β_3-AA (assuming that bovine adipose tissue contains β_3-AR), there should be an increase lean muscle and decrease undesirable adipose tissue.

In the study of Espinal et al. (1983), soleus muscles of exercised and exercise-trained rats were incubated in different concentrations of insulin and a set amount of ^{14}C glucose to detect metabolism. Assuming norepinephrine was released during exercise,

the catecholamine caused the soleus muscles to increase lactate production, which is an indicator of increased glycolysis. Hatefi et al. (2015) collected blood samples from castrated male goats fed ZH, a β_2-AA, and reported a significant decrease in free glucose and free triacylglycerol concentrations in blood. The decrease in blood triacylglycerol concentrations most likely represented decreased hepatic VLDL-cholesterol output.

We would have expected an increase in blood glucose since these results would indicate that there was an increase in glycogen due to exposure of ZH. However, data from Hatefi et al. (2015) indicated that carcasses from goats treated with ZH showed increased muscle mass and decreased adipose tissue deposition, possibly indicating that low blood glucose was due to glycose being used for energy production for muscle metabolism. These data are in accordance with the results from the current study that β-AA increased muscle metabolism, and the response of muscle strips to BRL 37344 were similar to the β_2-AA ZH.

Another significant finding is that Experior did not behave like a β_3-AA. Experior produced intermediate effects compared with the other two treatments. The hypothesis for this study was to compare Experior and BRL 37344 function on muscle strips, and ideally would have expected similar results from the two β-AA. If Experior is a β_3-AA, it is not nearly as potent as BRL 37344.

Viewing the results from the current study, the idea that Experior might actually be a β- antagonist should be discussed. Mersmann (1998) proposed β_1- and β_2-antagonists could be agonists for β_3-AR, suggesting a bidirectional pathway of β_3-AA and antagonists.

Although glycogen and CO_2 outputs from Experior were intermediate to the control and BRL 37344 samples, glycogen outputs were higher, and CO_2 outputs were lower compared to BRL 37344. Propranolol, a known β-antagonist, was given to adult men with type II diabetes mellitus. Propranolol caused non-significant increase in fasting serum glucose levels, and significantly increased fasting serum glucose levels when propranolol was combined with hydrochlorothiazide treatments (Dornhorst, Powell, and Penskey, 1985). Different outputs were collected from the latter compared to the current study, but both sets of data concluded that the metabolic condition of the men and muscle strips worsened because of propranolol and Experior, respectively.

In an experiment conducted by McVeigh and Tarrant (1983), Friesian bulls received a treatment of propranolol immediately after they were mixed with other bulls that they were unfamiliar with socially. Social mixing is extremely stressful for cattle since a hierarchy must be established. Mixing caused an increase in epinephrine release in these bulls. The two scientists did not see complete β-blocking by propranolol on the effects of epinephrine, but they did observe a depression in the effects of epinephrine. According to McVeigh and Tarrant (1983), there was a 63% breakdown of muscle glycogen in untreated bulls, while glycogen breakdown in treated bulls was only 55%. These data are similar to the data presented in the current study.

CHAPTER V

SUMMARY

Additional experiments that utilize *in vitro* metabolism and treating muscle strips are needed to confirm whether Experior is a β_3-AA. Comparison to a known β-antagonist such as propranolol, along with β_1-AA are needed as well. Finally, an *in vivo* experiment comparing Experior, a known β_3-AA such as BRL 37344, a known β_2-AA, and a known β-antagonist is needed to confirm the mechanisms of Experior on metabolism in a living system.

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