EFFECTS OF ZILPATEROL AND MELENGESTROL ACETATE ON BOVINE SKELETAL MUSCLE GROWTH AND DEVELOPMENT

by

ERIN KATHRYN SISSOM

B.S., California State University, Fresno, 2002
M.S., Kansas State University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Zilpaterol (ZIL) is a β-adrenergic receptor (β-AR) agonist that has been recently approved for use in feedlot cattle to improve production efficiencies and animal performance. One of the mechanisms through which this occurs is increased skeletal muscle growth. Therefore, two experiments were conducted to determine the effects of ZIL both *in vivo* and *in vitro*. In the first experiment, ZIL addition to bovine satellite cells resulted in a tendency to increase IGF-I mRNA and increased myosin heavy chain IIA (MHC) mRNA with 0.001 μ*M* and decreased MHC mRNA with 0.01 and 10 μ*M*. There were no effects of ZIL on protein synthesis or degradation. In myoblast cultures, there was a decrease in all three β-AR mRNA, and this was also reported in western blot analysis with a reduction in β2-AR expression due to ZIL treatment. In myotubes, there was an increase in β2-AR protein expression. In the second and third experiment, ZIL improved performance and carcass characteristics of feedlot steers and heifers. Additionally, ZIL decreased MHC IIA mRNA in semimembranosus muscle tissue collected from both steers and heifers. An additional part of the third study was conducted to determine the effects of melengestrol acetate (MGA) on bovine satellite cell and semimembranosus muscle gene expression. There were no effects of MGA on the expression of genes analyzed from semimembranosus muscle tissue collected. However, the addition of MGA to cultured bovine satellite cells resulted in increased β1 and β2-AR mRNA. These experiments aid in our understanding of the mechanism of action of MGA in heifers, as well as the effects of ZIL on both steers and heifers. Furthermore, they increase our knowledge and understanding of the mechanism of action of ZIL, as well as other β-agonists used to promote growth and efficiency in feedlot animals.

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Dedication

I would like to dedicate this to my husband Bill Miller, and my parents Ron and Barbara Sissom.

CHAPTER 1 - Review of Literature

Introduction

Growth has been defined as "the increase in size of an organism or of its parts due to synthesis of protoplasm or of apoplasmatic substances" (Balinsky, 1981). This process comes about through both anabolic and catabolic processes and can result in both negative and positive net growth rates. Animal growth is a very complex and regulated process that is of great interest to many people.

The regulation of growth and development of livestock species has been under investigation for over 60 years (Scanes, 2003). The ability to control or manipulate different aspects of tissue growth and development has great significance to the livestock industry due to the millions of dollars spent every year for the production of edible food products from domestic livestock species. There has been a vast amount of research completed investigating different products that can positively affect the growth and development process, which in turn leads to a more profitable system. Anabolic implants used in cattle can increase average daily gain 7-17% and improve feed efficiency 4-12% (Montgomery et al., 2001). The β-adrenergic receptor (β-AR) agonist ractopamine can improve feed efficiency 7-15% and increase average daily gain 6-20% in pigs (Kelly et al., 2003). In cattle, the response to ractopamine administration is less than that observed in pigs, which may be due to receptor populations in skeletal muscle which will be addressed later in this review.

Metabolic Modifiers

The most common compounds utilized for manipulation of growth in livestock species would be the group commonly referred to as "metabolic modifiers". Metabolic modifiers modify the metabolism and physiology of different species in ways that lead to improved efficiency of production of different products such as meat and milk (Beermann et al., 2005). There are four

groups of metabolic modifiers commonly used in animal agriculture today and they are: 1) anabolic implants, 2) somatotropin, 3) antiobiotics/ionophores, and 4) βAR agonists.

The steroid hormones that are utilized in anabolic implants have been used as growth promoters since the early 1950's when diethylstilbestrol (DES), a synthetic estrogen administered orally, was approved for use in beef cattle (Raun and Preston, 2002). Diethylstilbestrol was effective in improving growth and carcass characteristics of both cattle and sheep, and its oral activity was a beneficial tool (Raun and Preston, 2002). Diethylstilbestrol was subsequently removed from the market in 1979 by the Food and Drug Administration; however, this product paved the way for the future use of anabolic implants. These steroid containing implants are commonly utilized today as growth promoting tools, with the majority of feedlot cattle being implanted (Montgomery et al., 2001). The steroid hormones used in implants today are listed in the table below.

Table 1.1 Active ingredients in implants for beef cattle in the United States

Estradiol
Estradiol benzoate
Progesterone
Trenbolone acetate
Testosterone propionate
Zeranol

The second group of metabolic modifiers is somatotropin. Somatotropin, or classically known as growth hormone, has been widely used as a stimulator of milk production in lactating dairy cattle (Bauman, 1992). In pigs, growth hormone can increase average daily gain 9 to 15%, improve feed efficiency 3-23%, and increase carcass protein and reduce fat (Scanes, 2003). In cattle, the effects of exogenous growth hormone are inconsistent. When the growth hormone was delivered through an implant, steers had improved feed efficiency and increased protein in the rib section; however, there was no effect on carcass weight, dressing percentage, or

longissimus muscle area (Dalke et al., 1992). When the growth hormone was injected, increases in carcass weight, protein, and increased average daily gain and improved feed efficiency were observed (Early et al., 1990; Enright et al., 1990). Because of the variation of response in cattle, and the requirement for injection as the mode of administration, it is not a common practice. Additionally, it is not approved for enhancing growth and performance in the United States today.

The third group of metabolic modifiers includes antibiotics and ionophores. Antibiotics were some of the first compounds researched that showed growth stimulation of chickens and young pigs (Scanes, 2003). Today, sub-therapeutic levels of antibiotics are an effective tool to improve performance of both poultry and swine. Ionophores are a valuable instrument in influencing growth of ruminants as well. They work by transporting ions across the cell membrane which results in a shift in rumen fermentation with a decrease in acetate and butyrate, and an increase in propionate. The ionophore on the market today that is widely used is monensin. This product is marketed by Elanco Animal Health as Rumensin and has been demonstrated to improve feed efficiency and prevent coccidiosis in cattle (Goodrich et al., 1984).

The fourth and final group of metabolic modifiers is the β-AR agonists. Beta-agonists are synthetic organic compounds that are administered orally to livestock species in order to improve the efficiency through which tissues are deposited (Beerman et al., 2005). The first β-agonist approved for use in the United States was ractopamine-HCl, marketed under the name of Paylean™ which was approved in 1999 for use in finishing swine. In 2003, ractopamine-HCl was approved for use in beef cattle under the trade name Optaflexx™. The most recent β-agonist approved in 2007 for use in the United States is zilpaterol-HCl, which is sold under its trade name of Zilmax™.

Along with the recent approval of two different β-agonists for use in livestock species in the United States has come a need for a better understanding of the mode of action of these compounds. This review will focus on the specifics of the β-AR, their agonists, and the utilization of these products as growth promoters in livestock species.

β-Adrenergic Receptors

The β-AR are located embedded in the plasma membrane of almost every mammalian cell type. There are three different subtypes of the β-AR. These different receptor subtypes, the β1, β2, and β3 are distributed across different tissue types in mammals, and vary depending on the species (Strosberg, 1993; Liggett and Raymond, 1993). In cattle and sheep, the predominant form of βAR found in skeletal muscle and adipose tissue is the β2-AR (Sillence and Mathews, 1994). In pigs, the predominant form of β-AR found in skeletal muscle and adipose tissue is the β1-AR (McNeal and Mersmann, 1999). The distribution of β-AR subtype within species and specific tissues plays an important role in the effectiveness of different agonists. This is supported by data that suggests cattle are more sensitive to β2-AR agonists, whereas pigs are more sensitive to β1-AR agonists (Moody et al., 2000). Additionally, it has been demonstrated that in both finishing steers and heifers, there is a greater abundance of β2-AR mRNA in skeletal muscle compared to both β1 and β3-AR mRNA (Winterholler et al., 2006; Sissom et al., 2007).

The β-AR belong to a class of membrane-bound receptors that are commonly identified by their seven membrane-spanning domains. The sequences of the transmembrane domains are highly conserved between the different subtypes of β-AR compared to the intra- or extracellular loops (Mills and Mersmann, 1995). The receptors are coupled to G proteins and stimulated by the binding of different groups of specific ligands. The ligands for the receptor subtypes bind to the binding pocket that is formed from the transmembrane domains (Strosberg, 1993). The role

of this binding pocket is evident when changes in amino acid residues have altered the functionality of ligands attempting to bind to the receptor (Strosberg, 1993).

The interaction with G proteins is imperative to the function of β-AR (Strosberg, 1993). The third intracellular loop of the receptor is the main site for receptor interaction with the G proteins. Once an agonist is bound to the receptor and the stimulatory G protein is activated, the enzyme adenylate cyclase is activated which produces cyclic adenosine monophosphate (cAMP) (Mills and Mersmann, 1995; Mersmann, 1998). Cyclic adenosine monophosphate is an important intracellular signaling molecule that plays a role in many cellular functions. Its role in βAR signaling is binding to protein kinase A (PKA) to cause the release of the catalytic subunit that is then able to phosphorylate intracellular proteins. These proteins are either activated or inactivated via phosphorylation by PKA.

There are numerous proteins that can be affected by PKA phosphorylation in the signaling cascade. In livestock species, these enzymes are involved in protein synthesis, protein degradation, lipogenesis and lipolysis, to name a few. In adipose tissue, hormone sensitive lipase is activated via phosphorylation, which is considered the rate-limiting step in lipolysis (Mersmann, 1998; Strosberg, 1993; Liggett and Raymond, 1993). It is required for hydrolyzing triacylglycerol from adipocytes. Phosphorylation from PKA can also have an effect on gene transcription. When phosphorylated, the cAMP response element binding protein (CREB) binds to a cAMP response element in the regulatory part of a gene which results in the stimulation of the transcription of that particular gene (Mersmann, 1998, Strosberg, 1992). The actions of PKA phosphorylation also include enzyme inactivation. Acetyl-CoA carboxylase is one of the enzymes inactivated by phosphorylation. This enzyme is the rate-limiting enzyme for long-chain fatty acid biosynthesis. These different enzyme responses are a few of the specific modes in

which β-AR activation results in an increase in lipolysis via hormone sensitive lipolysis and a decrease in lipogenesis via acetyl-CoA carboxylase (Mersmann, 1998; Strosberg, 1993; Liggett and Raymond, 1993).

An important factor involved in β-AR function is the potential for receptor desensitization (Mills, 2002). There are two different modes of receptor desensitization, and they include uncoupling or down-regulation. Uncoupling occurs rapidly (within seconds) when agonist exposure occurs. This process is completely reversible. Down-regulation of β-AR is more of a concern because it is not rapidly overcome, and is not completely reversible. When down-regulation occurs, there is a decline in the number of β-AR present, thus leading to a reduction in response to an agonist (Mills, 2002). This response has been exhibited in both *in vitro* and *in vivo* systems (Pecquery et al., 1984; Hausdorff et al., 1990). Administration of clenbuterol to male rats for 10 d resulted in a 35% reduction in β2-AR density in skeletal muscle (Huang et al, 2000). This reduction in receptor density was also observed in lung tissue of the rats. Walker et al. (2007) observed reductions in the mRNA expression of both β1-AR and β2-AR mRNA in skeletal muscle from Holstein steers administered ractopamine for 28 d. Additionally, in pigs administered ractopamine, the number of β2-AR in adipose tissue was decreased at 1, 8, and 24 d following treatment, whereas there was no effect of ractopamine on receptor number in skeletal muscle (Spurlock et al., 1994). The ability for down-regulation of receptors can have an impact on the effectiveness of β-agonist and is a limitation that should be considered when utilizing these compounds.

Postnatal Skeletal Muscle Growth and the Muscle Satellite Cell

Skeletal muscle is a unique, complex tissue. In embryonic development, multi-nucleated muscle fibers are formed. These fibers are considered the cellular unit of muscle. The muscle

fibers, as well as nuclei within each fiber, are post-mitotic, thus leading to the inability to divide. Due to the post-mitotic nature, muscle fiber number is fixed at birth. In order to sustain postnatal muscle hypertrophy, the muscle fiber needs an external source of DNA. The DNA accumulation responsible for postnatal muscle hypertrophy is highly correlated to muscle growth rate (Trenkle et al., 1978). In fact, 60-90% of DNA located within mature skeletal muscle fibers is believed to be accumulated during postnatal growth (Allen et al., 1979). It is now known that muscle satellite cells are the source of DNA responsible for postnatal muscle hypertrophy.

Satellite cells are mononucleated cells located between the basal lamina and sarcolemma of the muscle fiber (Mauro, 1961). Upon initial discovery by Alexander Mauro in 1961, the true function of the satellite cell was unclear. Several theories were reported at that time, but it wasn't for another nine years that their functions as the postnatal muscle nuclei source were described. Moss and LeBlond (1970) determined there were two types of nuclei within the basement membrane of the muscle fiber that were distinguishable from one another. Following the labeling of nuclei by a single [^{3}H]-thymidine injection, male rats were sacrificed at different time intervals and the tibialis anterior muscle was removed for radioautography analysis. The results of this study showed that the true muscle nuclei were not labeled at 1 h following injection, indicating they were not actively dividing. However, the nuclei within the basement membrane were labeled, indicating the satellite cells were able to synthesize DNA and divide. Moss and LeBlond (1970) also reported that over the time course of 72 h, the number of labeled true muscle nuclei was increasing while the number of labeled satellite cells decreased. This lead to the conclusion that the source of labeled nuclei being counted within the fiber over the 72 h time frame were in fact that of satellite cells that were dividing and incorporating into the existing muscle fibers. Further research showed that once the satellite cells fused with the

existing fiber and donated their nuclei, they in turn lost their proliferative capacity (Moss and Leblond, 1971). These studies confirmed the postmitotic nature of true muscle nuclei and the importance of the muscle satellite cell in postnatal skeletal muscle growth.

The necessity of satellite cells in postnatal muscle growth is well understood, however, there are still limitations to the extent of DNA accretion at later stages of muscle growth. In a newborn animal, 30% of muscle nuclei are satellite cells, but the number reduces to 2 to 10% in adults, thus showing the actual number of satellite cells decrease with age (Cardasis and Cooper, 1975). Not only is there a reduction in satellite cell number, but those cells still present remove from the proliferative state of the cell cycle and enter G_0 (a state of quiescence) which leads to a growth plateau (Cardasis and Copper, 1975). In order to maintain the satellite cell population necessary to support muscle hypertrophy in mature animals, the cells in quiescence must be activated to allow them to progress through the cell cycle and contribute nuclei to the existing muscle fiber. Hepatocyte growth factor (HGF) is the necessary growth factor needed to activate quiescent satellite cells (Allen et al., 1995). Hepatocyte growth factor is the active agent in crushed muscle extract responsible for satellite cell activation needed for muscle regeneration (Bischoff, 1986; Tatsumi et al., 1998), and is the only known growth factor capable of activating satellite cells that exist in a state of quiescence. Interestingly, satellite cells express the receptor for HGF, c-met, at all times, but it isn't until HGF is produced and released that the cells are activated (Allen et al., 1995).

Once quiescent satellite cells have been activated, there is a need for growth factors capable of stimulating satellite cell proliferation and subsequent differentiation. Insulin-like growth factor-I (IGF-I) and fibroblast growth factor-2 (FGF-2) are known as progression factors due to their ability to aid in progressing cells through the cell cycle. Both growth factors are

potent stimulators of satellite cell proliferation (Johnson and Allen, 1990; Allen and Rankin, 1990). However, IGF-I is unique in that it is also a powerful stimulator of differentiation while FGF-2 inhibits differentiation (Allen and Boxhorn, 1989; Allen and Rankin, 1990).

The transforming growth factor-β (TGF-β) superfamily members are also capable of regulating satellite cell activity. These growth factors are considered negative regulators of skeletal muscle in that they inhibit both proliferation, as well as differentiation (Allen and Rankin, 1990). One member of the TGF-β superfamily responsible for negative regulation of skeletal muscle is myostatin, also known as growth and differentiation factor 8 (GDF-8) (McPherron et al., 1997). Myostatin is responsible for double muscling observed in cattle due to a mutation in the myostatin gene (McPherron and Lee, 1997). This embryonic mutation leads to a greater number of muscle fibers in each muscle, as witnessed in double muscle cattle. This recent discovery has lead to research surrounding the possible use of regulating myostatin in therapeutic settings such as muscular dystrophy, as well as future use in growth promoting systems for meat animals.

β-Adrenergic Receptor Agonists

There are both physiological β-AR agonists as well as synthetic agonists. The physiological agonists include the catecholamines norepinephrine and epinephrine. Norepinephrine is a neurotransmitter molecule that is synthesized from tryosine. Epinephrine is also a neurotransmitter and is a methylation product of norepinephrine (Mersmann, 1998). The synthetic agonists belong to a group of compounds classified as phenethanolamines. Both the physiological and synthetic agonists have similar chemical properties (Mersmann, 1998; Smith, 1998). The structure of the different agonists is directly linked to its proper function. One of the most important features of an agonist's structure is the aromatic rings that are attached to the β-

carbon (Smith, 1998). Each agonist contains the aromatic rings, but the function of each is determined by its aromatic substitution (Smith, 1998). These substitutions consist of hydroxyl groups, halogens, amines, hydroxymethyl groups, cyano groups, or combinations of each which affect the affinity of the agonist for its receptor (Smith, 1998).

The affinity for the β-AR subtypes is different between physiological and synthetic agonists, as well as within each specific group. For example, epinephrine binds to the β2-AR with a greater affinity than norepinephrine (Mills, 2002). There are many different synthetic agonists that have been investigated. The list includes ractopamine, zilpaterol, clenbuterol, cimaterol, and L-644-969. Many of these have been investigated thoroughly; however, only two have been approved for use in the United States. Those approved for use are ractopamine-HCl and zilpaterol-HCl. Ractopamine is approved for use in both finishing swine and cattle. In swine, ractopamine is fed up to 10 mg/kg of the diet per animal daily at the end of the finishing period. In cattle, ractopamine is administered between 200 and 400 mg per animal daily for the last 28 to 42 d of the finishing period. Zilpaterol is only approved for use in finishing cattle. It is recommended at 60 to 90 mg per animal daily the last 20 to 40 d of the finishing period. There is a 3 d withdrawal period with zilpaterol administration, but there is no withdrawal following ractopamine use.

β-Adrenergic Receptor Agonists and Muscle Growth

One of the most important and obvious responses to β-agonist administration is the resulting increase in skeletal muscle growth. Because of the financial gain due to increased muscle growth and efficiency with which this occurs, these agonists have been studied extensively. Administration of β-agonists results in increased muscle weights in multiple species across different muscle types. In steers administered L-644-969, there was a 22% increase in the

weight of the semitendenosous muscle (Wheeler and Koohmaraie, 1992). Additionally, in lambs administered the β-agonist cimaterol there was an increase in the weight of the semitendonosus, semimembranosus, and biceps femoris muscles (Beerman et al., 1987). These muscles are increased due to β-agonist administration are comprised of predominantly fast twitch muscle fibers. The fast twitch muscle fibers appear to have the greatest response to β-agonist treatment. However, other muscle types can also be affected by β-agonist treatment. For example, weights of the extensor hallucis longus, biceps femoris, gastrocnemius, and the pectoralis profundus were increased in chickens (Rehfeldt et al. 1997). In lambs, Bohorov et al. (1987) reported increased weight of the longissimus muscle with clenbuterol treatment.

The effects of β-agonist administration on different muscle types appear to be dictated by muscle fiber type. Individual muscle fibers are classified by energy metabolism for each fiber, and one of the characteristics for fiber classification is myosin heavy chain (MHC) isoform content. There are different isoforms of MHC that are important in determining muscle fiber type. Myosin heavy chain is a major protein in skeletal muscle that accounts for around 30% of all protein in skeletal muscle. Slow twitch fibers are predominantly MHC I, while fast twitch fibers are predominantly MHC IIA and IIX. It has been reported that β-agonist treatment can alter the expression of MHC isoforms, which can result in subsequent changes in muscle composition. In chickens fed clenbuterol there was a decrease in fast twitch type IIA muscle fibers and an increase in the fast twitch type IIB muscle fibers (Rehfeldt et al, 1997). Similarly, Depreux et al. (2002) observed a decrease in MHC IIA and IIX mRNA expression and an increase in MHC IIB mRNA in pigs fed ractopamine.

The effects of β-agonist administration on muscle hypertrophy appear to be dictated by muscle fiber composition, with the most significant response in fast twitch muscle fibers. There

also are data supporting the conversion of slow twitch to fast twitch with β-agonist administration. It is clear there are increases in muscle hypertrophy due to β-agonists, but the direct effects on skeletal muscle growth are still under investigation.

Postnatal skeletal muscle growth is a result of muscle fiber hypertrophy due to the post-mitotic nature of skeletal muscle fibers and the nuclei within each fiber. In order to sustain postnatal muscle hypertrophy, the fiber needs and external source of DNA. The source of DNA utilized to sustain postnatal muscle hypertrophy is the satellite cell (Moss and LeBlond, 1971). The ways through which postnatal muscle growth can be affected are by an increase in protein synthesis, a decrease in protein degradation and an increase in satellite cell fusion with the muscle fiber.

The majority of research suggests that β-agonists do not alter DNA accumulation in skeletal muscle fibers (Grant et al., 1990; O'Connor et al., 1991a). Chick breast muscle satellite cell proliferation increased, but there was no effect on the fusion of those cells as a result of treatment with ractopamine (Grant et al. 1990). Total DNA content of muscles from lambs fed 10 ppm cimaterol for 3 wk did not change in response to treatment (O'Connor et al. 1991a). However, there was a 30% increase in the total weight and protein content of the muscles, thus leading to an increase in the protein:DNA ratio of the muscle (O'Connor et al. 1991a). Similarly, there was no alteration in the DNA content of skeletal muscles of broiler chicks administered clenbuterol for 3 wk (Rehfeldt et al. 1997). In cull beef cows administered ractopamine for 35 d prior to harvest, satellite cell numbers and muscle fiber associated nuclei were not affected (Gonzales et al., 2007). These data provide evidence that satellite cell proliferation and DNA accumulation in skeletal muscle fibers was unaffected by β-agonists. These data suggest that there are other contributing factors that support the increase in muscle

hypertrophy that is observed with β-agonist administration. These factors have been shown to involve alterations in protein synthesis and degradation.

Skeletal muscle proteins are continually undergoing synthesis and degradation. The rates at which these processes occur are altered by β-agonist administration. For protein synthesis rates, they are often increased with β-agonist treatment, both *in vitro* and *in vivo*. Many different *in vitro* studies have been performed investigating different β-agonists utilizing many different cell types and culture conditions. There was an increase in protein synthesis rates in myotube cultures of ELC_5 (a subclone of rat L_6 cells) with ractopamine treatment (Anderson et al. 1990). The rate of protein synthesis was measure by [^{35}S]-methionine incorporation. The increase was observed at 24, 48, 72, and 96 h post-ractopamine treatment in both total protein and myosin heavy chain protein synthesis. In addition, there was an increase in total protein accumulation in the ELC_5 myotube cultures with ractopamine treatment. There were no differences in protein degradation reported in the cultures; therefore the increased protein accumulation was suggested to be primarily a result of the increased protein synthesis.

The β-agonist clenbuterol stimulated the fusion of neonatal muscle cells derived from rat muscle (McMillan et al, 1992). Within 24 h in those cultures, both fractional and absolute rates of protein synthesis were increased with clenbuterol. In contrast, the same authors reported no effects of clenbuterol on satellite cells isolated from mature rats, nor any affects on L_6 myoblast or myotube cultures. The β-agonist cimaterol did not have an effect on protein synthesis or degradation in primary muscle cell cultures derived from fetal tissue of lambs (Symonds et al., 1990). Conversely, cimaterol increased protein synthesis in satellite cells derived from 3 to 4 mo old pre-pubertal lambs. It can be concluded from these studies that the effects of β-agonist

treatment *in vitro* may be dependent upon cell source, type, time in culture, and the specific β-agonist used in the experiment.

There have also been reports of β-agonists altering skeletal muscle protein synthesis and degradation *in vivo*. There was a 27.1% reduction in fractional degradation rate of skeletal muscle myofibrillar protein in steers fed the β-agonist L-644,969 after 3 wk (Wheeler and Koohmaraie, 1992). Additionally, calpastatin activity was increased in muscle from those steers. The steers administered L-644,969 had improved feed efficiency, average daily gain, greater carcass weights, larger longissimus muscle area and lower yield grades. It was thus suggested that the β-agonist-induced muscle hypertrophy was due to reduced proteolytic capacity from increased calpastatin which in turn reduced protein degradation.

The rates of muscle myofibrillar protein synthesis are altered by ractopamine administration in pigs (Adeola et al., 1991). Barrows fed 20 mg/kg ractopamine had increased protein content in both the longissimus dorsi and biceps femoris muscles. Additionally, ractopamine increased the rates of synthesis of myofibrillar proteins in those muscles (Adeola et al., 1991). Bergen et al. (1989) reported an increase in semitendinosus muscle protein content in barrows administered 20 ppm ractopamine by d 21. Fractional protein synthesis rates were 1.7% higher in ractopamine fed pigs compared to control.

Altering protein synthesis and degradation rates play a key role in stimulating skeletal muscle growth; however, there are other important factors to be considered as well. Insulin-like growth factor-I is one that should be considered because it is a potent stimulator of postnatal skeletal muscle growth. The majority of research would suggest there is no demonstrable role for IGF-I in β-agonist induced skeletal muscle growth. O'Connor et al. (1991b) observed no change in IGF-I concentrations following administration of cimaterol for 3 wk. Similarly,

clenbuterol administration to growing lambs resulted in no change in IGF-I concentrations (Young et al., 1995). Grant et al. (1993) reported no change in IGF-I mRNA expression in both skeletal muscle and liver in pigs fed ractopamine. In addition to the data suggesting no effect of β-agonists on IGF-I, there is some data suggesting IGF-I can be reduced in both circulation and mRNA expression by β-agonist treatment. In Holstein steers administered ractopamine for 28 d prior to harvest there was a decrease in serum IGF-I (Walker et al., 2007). Additionally, there was a decrease in mRNA expression of IGF-I in the longissimus muscle tissue collected at harvest. In growing lambs fed cimaterol for 6 wk, there was a decrease in IGF-I level in circulation (Beermann et al., 1987). In contrast, it has been reported that clenbuterol administration to rats increased IGF-I mRNA and IGF-I content skeletal muscle without any change in serum IGF-I (Awede et al., 2002). Because of variability between studies investigating the role of IGF-I in β-agonist induced muscle hypertrophy, there are still many questions that should be explored.

It is evident that β-agonists can alter protein accumulation in skeletal muscle by altering the rates of protein synthesis, degradation, or a combination of the two. For the most part, there appears to be no effects of β-agonists on satellite cell proliferation, or DNA accumulation into muscle fibers. Additionally, there is some evidence that IGF-I can be altered by β-agonist treatment, but its role in β-agonist induced muscle hypertrophy is still unclear. There are still many unanswered questions regarding how each different β-agonist affects these important factors responsible for the muscle hypertrophy observed when β-agonists are used, and therefore further investigation into these is warranted at this time.

β-Adrenergic Receptor Agonist Use in Livestock Species

The effects of the administration of β-agonists to livestock species has been under investigation for many years. These compounds have proven effective tools in improving overall efficiency of growth in livestock (Anderson et al., 1991). The effects of β-agonists differ among species, and are more pronounced in cattle, pigs, and sheep compared to chickens (Anderson et al., 1991; Moody et al., 2000). The specific effects of β-agonists on skeletal muscle growth have already been mentioned in this review. The following focuses on the effects of β-agonists on performance and carcass characteristics on livestock.

There is significant published information about the effects of ractopamine on both pigs and cattle. There is a more profound effect in pigs, which may be associated with the abundance of β1-AR in porcine tissue, and the selectivity of ractopamine for the β1-AR (McNeel and Mersmann, 1999). In finishing pigs, ractopamine increased average daily gain, feed efficiency, dressing percentage, and carcass leanness (Watkins et al., 1990). Additionally, See et al. (2004) reported improved average daily gain and feed efficiency in barrows and gilts fed ractopamine.

There has also been some research conducted on the use of β-agonists in sheep. Lambs fed cimaterol had increased average daily gain and feed efficiency (Beerman et al. 1986). There was also improved carcass characteristics with increased longissimuss muscle area and decreased backfat. Additionally, Kim et al. (1987) reported increased gain, improved feed efficiencies, and increased hot carcass weight in lambs fed cimaterol.

Ractopamine also improves performance in both feedlot steers and heifers. In feedlot steers, ractopamine fed the final 28 d of the finishing period at 200 mg per steer daily resulted in improved average daily gain, feed efficiency, hot carcass weights, and larger longissimus muscle area (Gruber et al., 2007). Winterholler et al. (2007) reported similar findings in yearling steers fed 200 mg per animal daily of ractopamine the final 28 d of the feeding period. Steers fed

ractopamine had increased averaged daily gain and improved feed efficiency. They also had increased hot carcass weight and longissimus muscle area.

The effectiveness of ractopamine administration has also been observed in feedlot heifers (Walker et al., 2006; Sissom et al., 2007). Feedlot heifers fed ractopamine (200 mg per animal daily) 28 d prior to slaughter improved average daily gain, efficiency of gain, carcass-adjusted average daily gain and carcass-adjusted efficiency of gain (Walker et al., 2006). There were no large effects of ractopamine on carcass characteristics. In a similar manner, ractopamine (200 mg per animal daily) fed the last 28 d of the finishing period to feedlot heifers resulted in increased average daily gain, hot carcass weight, and longissimus muscle area (Sissom et al., 2007). Both feed efficiency and carcass yield grade were improved, and 12^{th} rib fat depth was decreased with ractopamine administration.

There are few studies investigating the effects of zilpaterol on animal performance because it has only recently been approved for use in the United States; however those that are available suggest zilpaterol is an effective tool in growth promotion of cattle (Plascencia et al., 1999; Avendano-Reyes et al., 2006). In feedlot steers administered 6 mg/kg of the diet daily of zilpaterol, there was an increase in average daily gain and improved feed efficiency (Plascencia et al., 1999). Zilpaterol supplementation also increased carcass weight by 4.5%, dressing percentage by 3.6%, and longissimus muscle area by 2.7%. There was no effect of zilpaterol on fat thickness or marbling score. Both zilpaterol (60 mg per head daily) and ractopamine (300 mg per head daily) were investigated in feedlot cattle to compare the two β-agonists used in cattle (Avendano-Reyes et al., 2006). Both β-agonists improved average daily gain, feed efficiency, hot carcass weight, and carcass yield. Additionally, zilpaterol increased longissimus muscle area. Both ractopamine and zilpaterol increased shear force values compared to control steers.

The authors commented that the β-agonists were effective in enhancing performance of the cattle without substantively compromising meat quality (Avendano-Reyes et al., 2006).

In conclusion, the effectiveness of β-agonists in improving animal performance is overwhelmingly supported by numerous published reports. These compounds can positively affect skeletal muscle growth and development, as well as overall performance when administered at the recommended dose.

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CHAPTER 2 - Effects of Zilpaterol on Cultured Bovine Satellite Cells

Abstract

Zilpaterol-HCl (**ZIL**) is a β-adrenergic receptor (**AR**) agonist recently approved to improve production efficiencies and dressing percentage in feedlot cattle. The purpose of these experiments was to determine the effect of various levels of ZIL (0, 0.0001, 0.001, 0.01, 0.1, 1.0, and 10 μ*M*) in culture media on bovine satellite cell proliferation and gene expression. Bovine satellite cells isolated from the semimembranosus muscle were plated on tissue culture plates coated with reduced growth factor matrigel or collagen. Total RNA was isolated from cells following 48-h ZIL exposure both in proliferating myoblast cultures at 96 h and in fused myotube cultures established after 192 h in culture. Real-time quantitative-PCR was performed to estimate mRNA abundance. There was no effect of ZIL dose on [^{3}H]-thymidine incorporation in proliferating myoblasts. Zilpaterol (1 μ*M*) addition to myoblasts resulted in a decrease ($P < 0.05$) in β1-AR mRNA. Similarly, ZIL (0.01 and 1μ*M*) decreased ($P < 0.05$) β2-AR and β3-AR mRNA. The expression of IGF-I mRNA tended to increase ($P = 0.07$) with ZIL (1 μ*M*) addition, and there was a tendency ($P = 0.07$) for ZIL (0.001 μ*M*) to increase myosin heavy chain (**MHC**) IIA mRNA, whereas 0.01 μ*M* and 1μ*M* ZIL reduced ($P < 0.05$) MHC IIA mRNA levels. There was no effect ($P > 0.05$) of ZIL dose on the expression of genes analyzed in fused myotube cultures at 192 h, or on fusion percentage in those cultures. The β2-AR antagonist ICI-118, 551 (0.1 μ*M*) blocked the effect of 0.1 μ*M* ZIL to reduce expression of β1 and β2-AR mRNA. The combination of ZIL (0.01 μ*M*) and ICI-118, 551 (0.1 μ*M*) resulted in an increase ($P < 0.05$) in β1-AR mRNA expression. There was no effect of the antagonist or ZIL on β3-AR or IGF-I mRNA in the antagonist specific experiment. There was an increase ($P < 0.05$) in MHC IIA mRNA expression with ICI-118, 551 (0.1 μ*M*) and the ZIL (0.01 μ*M*) + ICI-118, 551 (0.01 μ*M*) combination. Zilpaterol did not affect total protein accumulation or total protein synthesis

and degradation percent. Similar to changes in mRNA, western blot analysis revealed that the protein content of β2-AR in ZIL-treated myoblast cultures decreased ($P < 0.05$) relative to control. Additionally, ZIL increased ($P < 0.05$) β2-AR protein expression in myotube cultures as indicated by western blot analysis. Similar to previous work with other β-agonists, ZIL did not alter satellite cell proliferation but reduced both mRNA and protein levels of the various subtypes of β-AR in these cultures. The response to ZIL of IGF-I mRNA could be mediating changes in protein synthesis and degradation. These data indicate that ZIL addition can alter mRNA and protein concentrations of β-AR of muscle cell cultures which in turn could impact responsiveness of cells to prolonged ZIL exposure in vivo.

Key words: Beta-adrenergic receptor, bovine, satellite cell, zilpaterol.

Introduction

Zilpaterol-HCl (**ZIL**) is an orally active β-adrenergic receptor (**β-AR**) agonist approved for use in feedlot cattle in the United States. Administration of ZIL to feedlot steers and heifers the last 20 to 40 d on feed increased ADG, ribeye area, dressing percentage, and improved feed efficiency and carcass yield grade. Zilpaterol elicits a response through binding to β-AR which are membrane-bound receptors located on most mammalian cells (Mills and Mersmann, 1995; Strosberg, 1993). The binding of ZIL to its receptor results in enzyme phosphorylation that is responsible for changes in protein synthesis and degradation, particularly in skeletal muscle (Mersmann, 1998).

Satellite cells are mononucleated cells that are important in postnatal skeletal muscle growth (Mauro, 1961; Moss and Leblond, 1971). Because of the postmitotic nature of the muscle fiber, the fusion of satellite cells with muscle fibers is necessary to support postnatal muscle growth. These cells are the external source of DNA required for the muscle fiber to sustain muscle hypertrophy, and this DNA accumulation is highly correlated to muscle growth rate (Trenkle et al., 1978). Because of their role in postnatal muscle growth, satellite cells are useful tools in investigating the mode of action of different muscle growth promoting agents, such as β-AR agonists.

The effects of β-agonists have been extensively studied, but to our knowledge there is limited information on the direct effects of ZIL on bovine skeletal muscle growth. Thus, the purpose of these experiments was to investigate the direct effects of ZIL on cell proliferation, protein synthesis and degradation rates, protein accumulation, as well as the expression of mRNA for the β-AR subtypes, IGF-I, and myosin heavy chain (**MHC**) IIA in bovine satellite cell cultures.

Materials and Methods

All experimental procedures with animals were approved by the Kansas State University Institutional Animal Care and Use Committee.

Bovine Satellite Cell Isolation

Satellite cell isolation was conducted as described previously (Johnson et al., 1998). Cattle were sacrificed by bolting followed by exsanguination. Using sterile techniques, approximately 500 g of the semimembranosus muscle was dissected out and transported to the cell culture laboratory. Subsequent procedures were conducted in a sterile field under a tissue culture hood. After removal of connective tissue the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% pronase in Earl's Balanced Salt Solution (**EBSS**) for 1 h at 37° C with frequent mixing. Following incubation, the mixture was centrifuged at 1,500 x *g* for 4 min, the pellet was suspended in phosphate buffered saline (**PBS**: 140 m*M* NaCl, 1 m*M* KH_2PO_4, 3 m*M* KCl, 8 m*M* Na_2HPO_4), and the suspension was centrifuged at 500 x *g* for 10 min. The supernatant was centrifuged at 1500 x *g* for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated two more times. The resulting mononucleated cell preparation was suspended in cold (4°C) Dulbecco's Modified Eagle Medium (**DMEM**) containing 10% FBS and 10% (v/v) dimethylsulfoxide (**DMSO**) and frozen. Cells were stored frozen in liquid nitrogen.

[^{3}H]-Thymidine Incorporation

Satellite cells were plated on 2-cm^2 culture plates for thymidine incorporation. Culture plates were pre-coated with reduced growth factor basement membrane matrigel diluted 1:10 (v/v) with DMEM. Cells were plated in DMEM containing 10% fetal bovine serum (**FBS**) and incubated at 37°C, 95% CO_2 in a water-saturated environment. Plating density for cells was

empirically established so that all cultures were 25 to 50% confluent after the incubation period. This ensured that cell proliferation rate was not affected by contact inhibition. Cultures were rinsed three times with serum-free DMEM 24 h after plating the bovine satellite cells in 10% FBS/DMEM, and the appropriate levels of ZIL (provided by Intervet Inc., Millsboro, DE; 0, 0.0001, 0.001, 0.01, 0.1, 1.0, and 10 μ*M*) were added in 10% FBS/DMEM. At 72 h, cultures were rinsed three times with serum-free DMEM and 1 μCi/mL of [^{3}H]-thymidine (NEN Life Science, Boston, MA) was added to each well. Cells with [^{3}H]-thymidine were incubated at 37°C, 5% CO_2 in a water-saturated environment for 3 h. After incubation, satellite cells were rinsed three times with cold serum-free DMEM to remove free [^{3}H]-thymidine. Cold 5% trichloroacetic acid (**TCA**; Sigma, St. Louis, MO) was added to every well and incubated overnight at 4ºC. The following day, cells were rinsed two times with cold TCA to remove any remaining unincorporated [^{3}H]-thymidine. The precipitated cell material was dissolved in 0.5 mL of 0.5 *M* sodium hydroxide (**NaOH**; Sigma, St. Louis, MO) in a rocking incubator for 30 min at 37°C. The NaOH suspensions were transferred quantitatively into scintillation vials containing 10 mL of scintillation cocktail (Fisher Scientific, Hanover Park, IL). The samples were allowed to stand for a few hours in low light to reduce chemiluminescence before being counted in a scintillation counter. All treatments were measured in triplicate, with seven total assays included in the final analysis.

Markers of Differentiation

Satellite cells were plated as previously described on 9.62-cm^2 collagen coated culture plates for differentiation studies. At 48 h, cultures were rinsed three times with serum-free DMEM and 3% swine serum (**SS**)/DMEM was added. At 96 h, cells were rinsed three times with serum-free DMEM and 3% horse serum (**HS**)/1.5 μg/mL BSA-Linoleic acid/DMEM fusion

media was added. Zilpaterol (0, 0.0001, 0.001, 0.01, 0.1, and 1μ*M*) was added to the cultures at 144 h. After approximately 216 h in culture, cells fused into multinucleated myotubes and were stained using Hoechst 33342 stain. The stained nuclei were visible under blue fluorescent light, at which time, digital photos were taken of random fields in each well to determine extent of differentiation (fusion percentage; defined as myotube nuclei/total nuclei).

RNA Isolation on Proliferating Myoblasts

Satellite cells were plated in 10% FBS/DMEM as previously described on 9.62-cm^2 tissue culture plates. After 48 h, cultures were 25 to 50% confluent and were rinsed three times with serum-free DMEM and ZIL (0, 0.0001, 0.001, 0.01, and 1 μ*M*) was added to the cultures in 10% FBS/DMEM. At 96 h, total RNA was isolated using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). The concentration of RNA was determined by absorbance at 260_{nm}. One microgram of total RNA was reverse transcribed to produce first-strand complementary DNA (cDNA) using TaqMan Reverse Transcription Reagents, MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA), and the protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis.

In addition, satellite cells were plated as described above to assess the effects of zilpaterol alone, or in combination with a specific β2-adrenergic receptor antagonist, ICI-118, 551 (Sigma, St. Louis, MO). Treatments consisted of the following: 1) Control, 2) 0.01 μ*M* ZIL, 3) 0.1 μ*M* ICI, 4) 0.01 μ*M* ZIL + 0.1 μ*M* ICI, 5) 0.01 μ*M* ZIL + 0.01 μ*M* ICI. The concentration of inhibitor chosen to use in combination with ZIL was similar to that in previous experiments we conducted in our laboratory with comparable experiments in which receptor antagonists were used to try and inhibit the effect of its agonist (Sissom et al., 2006). Culture conditions and RNA isolation were as described above.

RNA Isolation on Myotubes

Bovine satellite cells were plated in 10% FBS/DMEM as previously described on 9.62 cm^2 tissue culture plates. After 48 h, cultures were rinsed three times with serum-free DMEM 3% SS/DMEM was added. Following a 96-h incubation, cells were rinsed three times with DMEM and 3% HS/1.5µg/mL BSA-Linoleic Acid/DMEM was added. At 120 h, ZIL (0, 0.0001, 0.001, 0.01, and 1 µ*M*) in 3% HS/1.5µg/ml BSA-Linoleic Acid/DMEM was added. At 168 h, total RNA was isolated using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). The concentration of RNA and cDNA synthesis was measured as previously described.

Real-Time Quantitative-PCR

Real-time quantitative-PCR (**RTQ-PCR**) was used to measure the quantity of β1-AR, β2-AR, and β3-AR, IGF-I, and MHC IIA gene expression relative to the quantity of 18S ribosomal RNA (**rRNA**) in total RNA isolated from bovine satellite cells treated with zilpaterol. Measurement of the relative quantity of cDNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), 900 n*M* of the appropriate forward and reverse primers, 200 n*M* of appropriate TaqMan detection probe, and 1 µL of the cDNA mixture. The bovine specific β1-AR, β2-AR, and β3-AR, IGF-I, and MHC IIA forward and reverse primers and TaqMan detection probes (Table 2.1) were designed using published GenBank sequences. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession #X03205). The ABI Prism 7000 detection system (Applied Biosystems) was used to perform the assay utilizing the recommended thermal cycling variables by the manufacturer (50 cycles of 15 s at 95ºC and 1 min at 60ºC). The 18S rRNA endogenous control was used to normalize the expression of β1-AR, β2-AR, and β3-AR, IGF-I and MHC IIA.

Preparation of Protein Extracts on Proliferating Myoblasts and Myotubes

Total protein was isolated using the Mammalian Protein Extraction Reagent (**M-PER**; Pierce Biotechnology, Rockford, IL). Bovine satellite cells were plated as previously described on 9.62 cm^2 collagen coated plates. For myoblast cultures, following a 48-h incubation, cells were rinsed three times with serum-free DMEM and ZIL (0, 0.001, 0.01, 0.1, and 1 μ*M*) in 10% FBS/DMEM was added. For myotube cultures, following 48 h of incubation, cells were rinsed three times with serum-free DMEM and 3% SS/DMEM was added. At 96 h, cells were rinsed three times with DMEM and 3% HS/1.5μg/mL BSA-Linoleic Acid/DMEM was added. After 24 h, ZIL (0, 0.001, 0.01, 0.1, and 1 μ*M*) in 3% HS/1.5μg/mL BSA-Linoleic Acid/DMEM was added. At 96 h for myoblasts and 168 h for myotubes, cells were rinsed with warm PBS and extraction reagent (350 μL/well) was added. The cultures were gently shaken for 5 min to ensure complete cell lysis, then centrifuged for 5 min at 14,000 x *g*. The supernatant was collected and protein concentration was determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Average protein concentration collected was 8 mg/mL.

Western Blot Analysis

Protein samples were denatured using equal volume of sodium dodecyl sulfate (SDS)-β-mercaptoethanol and boiled for 5 min. Total protein (30 μg) was then separated by gel electrophoresis using Pierce Precise Protein Gels (Pierce Biotechnology, Rockford, IL). Gels were run for 45 min at 150 V and 130 mA. The protein was then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The nitrocellulose membranes were blocked using Starting Block™ Buffer (Pierce Biotechnology, Rockford, IL) for 15 min at 37°C. The primary antibody against β2-AR (sc-569; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in

blocking buffer was added to the membrane and incubated overnight at 4ºC. Following overnight incubation, the membrane was washed three times with PBST. The secondary antibody (sc-3030; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for the β2-AR primary antibody was added to the membrane and incubated for 1h. Detection and quantification of proteins were analyzed using a Fluorchem 8800 Imaging System (AlphaInnotech, San Leandro, CA).

Measurement of Total Protein Synthesis Rate

Bovine satellite cell cultures were used to assess the rate of total protein synthesis. Cells were plated in 10% FBS/DMEM as previously described. Following a 48-h incubation, cultures were rinsed three times with serum-free DMEM and 3% SS/DMEM was added. At 144 h, cells were rinsed three times with DMEM and 3% HS/1.5μg/mL BSA-Linoleic Acid/DMEM was added. At 168 h ZIL (0, 0.001, 0.01, 0.1, 1, and 10 μ*M*) was added to the fused myotube cultures. Cultures were incubated in ZIL media for either 2 or 48 h. Following ZIL incubation, 2 μCi/mL of [^{3}H]-tyrosine was added to the cells for 2 h. Cultures were then rinsed five times with 2 mL PBS. Following the PBS rinse, satellite cells were incubated in 10% TCA overnight at 4°C, which allowed for proteins to be precipitated. Following the overnight incubation, precipitated proteins were removed from the plates using a rubber policeman. The plates were scraped and rinsed with 10% TCA an additional 5 times. The TCA collected from each well on were then sent through a glass fiber filter, which allowed for the precipitated proteins to be collected on the filter. The filter was then rinsed with 10% TCA, followed by 95% ethanol. Scintillation vials were used to place the fiber filters with 0.5 mL of tissue solubilizer (NCS tissue solubilizer, Amersham, Arlington Heights, IL) to solubilize the precipitated proteins. Scintillation cocktail (Fisher Scientific, Hanover Park, IL) was added to the vials, in addition to

0.1 mL of concentrated acetic acid. The rate of protein synthesis was determined by the rate of incorporation of label into TCA-precipitable proteins (Hembree et al., 1991).

Measurement of Total Protein Degradation Rate

The rate of protein degradation was determined by evaluating the release of radioactivity from total protein in bovine satellite cell cultures that were prelabeled with [^{3}H]-tyrosine. Satellite cells were labeled for 24 h with [^{3}H]-tyrosine (4 μCi/mL) in 1 mL of conditioned media after 168 h in culture. At 192 h, media containing the [^{3}H]-tyrosine was removed and satellite cells were incubated for 4 h in 1 mL of media containing 0.2 m*M* tyrosine. Following the 4 h incubation, satellite cell cultures were rinsed with DMEM and 2 mL of ZIL (0, 0.001, 0.01, 0.1, and 1μ*M*) test media was added for a period of 2 h. Following the 2 h ZIL dosing period, 0.75 mL of the media was removed from the cell culture plates and added to 0.094 mL of 90% TCA. The media in 90% TCA was then incubated for 30 min at 4°C. The media solubilized in TCA was centrifuged at 10,000 x g for 3 min. Supernatant (0.5 mL) was collected and added to liquid scintillation cocktail. This was then used to measure the release of TCA-soluble radioactivity from the cells. Test media (no TCA incubation) was counted to determine the dpm in the media collected from the cells. The dpm's from the cellular fraction were also evaluated by solubilizing cells in 1 mL of urea buffer (6 *M* urea, 20 m*M* Tris-HCl, 0.1% β-mercaptoethanol, pH 7.5 at 4° C). The solubilized material was then added to scintillation cocktail and counted. The percent degradation was determined by the following: TCA-soluble dpm released into media x 100/(total media dpm + cellular dpm).

Statistical Analysis

Data were analyzed as a completely randomized design using the PROC MIXED in SAS (SAS Inst. Inc., Cary, NC) The difference between control and treatment was evaluated($P <$

0.05) using the least significance difference procedure of SAS. Additionally, specific orthogonal contrasts were used to test for linear effects of ZIL dose.

Results and Discussion

Effect of ZIL on Cell Proliferation, Differentiation, and mRNA Expression of the β-Adrenergic Receptors, IGF-I, and MHC IIA mRNA in Bovine Satellite Cells

There was no effect of ZIL on bovine satellite cell rate of proliferation as measured by [^{3}H]-thymidine incorporation (Figure 2.1). Additionally, ZIL did not alter the extent of differentiation of bovine satellite cells as assessed by fusion percentage (data not shown). The lack of effect of ZIL on satellite cell proliferation and differentiation is in agreement with other data which investigated other βAR agonist's role in satellite cell proliferation and differentiation. O'Connor et al. (1991a) reported a decrease in DNA concentration in skeletal muscle of ram lambs that were fed cimaterol. The DNA concentration of the hind limb muscles was reduced by 42% during the 3 wk administration of cimaterol and remained 25% less after 6 wk of treatment. The total weight and protein content of the muscles from the hind limb of the lambs was increased by 30% during the 3-wk administration of cimaterol, suggesting that an increase in satellite cell proliferation is not necessary to support the β-agonist induced muscle hypertrophy. Rehfeldt et al. (1997) observed no alteration in the DNA content of skeletal muscles of broiler chicks administered clenbuterol for 3 wk. Similarly, cull beef cows administered ractopamine for 35 d prior to harvest showed no change in satellite cell numbers and muscle fiber associated nuclei (Gonzales et al., 2007). In contrast to our data, Grant et al. (1990) observed an increase in cell proliferation in muscle satellite cells isolated from chick breast muscle with ractopamine treatment; however, there was no effect on the fusion of those cells into the muscle fiber, and this is similar to the results from our research with ZIL. It is important to note the levels of ractopamine used in the experiments that resulted in increased cell proliferation were

pharmacological doses instead of the more physiological doses used in our experiments. Additionally, Shappell et al. (2000) observed a 30% increase in cell number and DNA concentration in C_2C_{12} cells treated with a high dose (10 μ*M*) of ractopamine. The results in that study may be due to the high level of ractopamine used or the source of the cells used because there was no effect of ractopamine in the cells utilized after later passages. The β2-agonist clenbuterol was reported to stimulate fusion of neonatal muscle cultures derived from rat muscle (McMillan et al, 1992). In those cultures, both fractional and absolute rates of protein synthesis were increased within 24 h of treatment with clenbuterol. In contrast, the same authors reported no effects of clenbuterol on satellite cells isolated from mature rats, nor any effects on L_6 myoblast or myotube cultures. There is some variability in the data reported with regard to β-agonists and skeletal muscle cell proliferation and differentiation in vitro; however, the majority of data suggest no alteration in DNA content of muscles from animals administered a β-agonist in vivo. The levels used in this study spanned from physiological to pharmacological and we observed no trends for differences in rate of proliferation as measured by [^{3}H]-thymidine incorporation. Therefore, the increased muscle growth reported in the majority of studies along with no effect on skeletal muscle DNA content support the theory that the increased muscle hypertrophy reported with β-agonists in supported by other processes, and an alteration of the DNA content may not be necessary.

Changes in the mRNA levels of the βAR in skeletal muscle may be an important method for the enhanced muscle growth reported with β-agonists. We observed changes in the expression of βAR mRNA in bovine satellite cell cultures treated with ZIL. Zilpaterol addition (1 μ*M*) to proliferating myoblasts resulted in a decrease ($P < 0.05$) in β1-AR mRNA (Figure 2.2). Similarly, ZIL (0.01 and 1μ*M*) decreased ($P < 0.05$) β2-AR and β3-AR mRNA (Figures

2.3 and 2.4). The reduction in mRNA of the βAR is similar to that of other studies demonstrating alterations in density of receptor number with β-agonist treatment. Administration of clenbuterol to male rats for 10 d resulted in a 35% reduction in β2-AR density in skeletal muscle (Huang et al, 2000). This reduction in receptor density was also observed in lung tissue of the rats. Walker et al. (2007) observed reductions in the mRNA expression of both β1-AR and β2-AR mRNA in skeletal muscle from Holstein steers administered ractopamine. Additionally, in pigs administered ractopamine, the number of β2-AR in adipose tissue was decreased at 1, 8, and 24 d following treatment, whereas there was no effect of ractopamine on receptor number in skeletal muscle (Spurlock et al., 1994). In contrast, Winterholler et al. (2007) observed a tendency for increased β2-AR mRNA in skeletal muscle isolated from yearling steers administered ractopamine for 28 d prior to harvest. Additionally, we previously observed a tendency for ractopamine administration to feedlot heifers to increase β2-AR mRNA in semimembranosous muscle collected at harvest (Sissom et al., 2007). It is evident from our current results and previous research that β-agonists can alter the mRNA expression and density of β-AR in skeletal muscle as well as other tissues. These changes may play an important role in regulating the response to β-agonist treatment and warrant further investigation.

In addition to the alteration of βAR mRNA in proliferating bovine satellite cell cultures, there was a tendency ($P = 0.07$) for the expression of IGF-I mRNA to be increased with ZIL (1 μ*M*) addition (Figure 2.5); however, there were no linear effects detected. The change in IGF-I mRNA with ZIL treatment is interesting and may be an important factor when considering the mode of action of ZIL in increasing skeletal muscle growth. Insulin-like growth factor-I is a potent stimulator of postnatal skeletal muscle growth. In cultured satellite cells, IGF-I is a potent stimulator of both cell proliferation and differentiation (Johnson and Allen, 1990; Allen and

Rankin, 1990). There is also an important role of IGF-I in stimulating protein synthesis and decreasing protein degradation in muscle cell cultures (Hembree et al., 1991; Forsberg and Hong, 1994). Because of the tendency for increased expression of IGF-I mRNA, we would expect to see increased cell proliferation or differentiation. We did not detect differences in proliferation and differentiation, but one important factor to note is the potential role of IGFBP-3 in the media used. Kamango-Sollo et al. (2004) reported that IGFBP-3 in media containing swine or fetal bovine serum exhibits antagonistic action on IGF-I bioavailability to proliferating myogenic cells. They observed that utilizing media containing swine serum or fetal bovine serum resulted in no effect on [^{3}H]-thymidine incorporation following steroid treatment. They reported that the IGFBP-3 was masking the steroid effect on proliferation and was removed to eliminate any interference it may cause. This supports the theory that the IGFBP-3 found the in the media we used may have attenuated the differences in IGF-I production caused by ZIL addition. However, the majority of research would suggest there is little effect of IGF-I in β-agonist induced skeletal muscle growth. O'Connor et al (1991b) observed no change in IGF-I concentrations following administration of cimaterol for 3 wk. Similarly, clenbuterol administration to growing lambs resulted in no change in IGF-I concentrations (Young et al., 1995). Grant et al. (1993) reported no change in IGF-I mRNA expression in both skeletal muscle and liver in pigs fed ractopamine. In addition to the data suggesting no effect of β-agonists on IGF-I, there is some data suggesting IGF-I can be reduced in both circulation and mRNA expression by β-agonist treatment. In Holstein steers administered ractopamine for 28 d prior to harvest there was a decrease in serum IGF-I (Walker et al., 2007). Additionally, there was a decrease in mRNA expression of IGF-I in the longissimus muscle tissue collected at harvest. In growing lambs fed cimaterol for 6 wk, there was a decrease in IGF-I level in circulation (Beermann et al., 1987). Our results are in

contrast to much of the research available. However, it has been reported that clenbuterol administration to rats increased IGF-I mRNA and IGF-I content skeletal muscle without any change in serum IGF-I (Awede et al., 2002). This data would support the increase in IGF-I mRNA we observed; however, there are still many questions as to the role of IGF-I in β-agonist induced muscle hypertrophy because of the variability observed between different studies.

There was a tendency ($P = 0.07$) for 0.001 μ*M* ZIL to increase myosin heavy chain mRNA, whereas 0.01 and 1μ*M* ZIL reduced ($P < 0.05$) MHC IIA mRNA levels (Figure 2.6). The change in MHC IIA mRNA in the bovine satellite cell cultures is intriguing because of the observed increase with the lower level of ZIL and a decrease in the higher levels of ZIL. The increase in MHC IIA observed may be an important factor in ZIL stimulated muscle growth. Mysoin is the most abundant protein in skeletal muscle, and it is widely reported that β-agonists can alter protein synthesis and degradation, thus altering MHC. Anderson et al. (1990) reported increased protein synthesis rates of both total and MHC in myotube cultures of ELC_5 cells with ractopamine treatment. This may be the reason for the observed increase in MHC IIA mRNA with the lower level of ZIL. However, we speculate the decrease in MHC IIA mRNA with the higher levels of ZIL may be a result of a shift towards protein degradation. A reduction in protein degradation can result in an overall net increase in skeletal muscle, and this has been observed with β-agonist treatment. Wheeler and Koohmaraie (1992) reported a 27.1% reduction in fractional degradation rate of skeletal muscle myofibrillar protein in steers fed the β-agonist L-644,969 after 3 wk. Additionally, Reeds et al. (1986) reported a decrease in protein degradation in skeletal muscle of rats fed clenbuterol. Additionally, MHC is also important in determining muscle fiber type. Slow twitch fibers are predominantly MHC I, whereas fast twitch fibers are predominantly MHC IIA and IIX. It has been reported that β-agonist treatment can alter the

expression of MHC isoforms, which can result in subsequent changes in muscle composition. The increase in MHC IIA mRNA with the lower level of ZIL and the decrease with the higher levels may be a result of muscle fiber conversion. In chickens fed clenbuterol there was a decrease in fast twitch type IIA muscle fibers and an increase in the fast twitch type IIB muscle fibers (Rehfeldt et al, 1997). Similarly, Depreux et al. (2002) observed a decrease in MHC IIA and IIX mRNA expression and an increase in MHC IIB mRNA in pigs fed ractopamine. These data demonstrate the possible role for alteration of MHC IIA mRNA concentration observed in the satellite cell cultures following treatment with ZIL.

There was no effect ($P > 0.05$) of ZIL dose on the expression of genes analyzed in fused myotube cultures at 192 h (Figures 2.7-2.11). There have been differences in response to β-agonist due to culture conditions reported, similar to that observed in our study. Shappell et al. (2000) reported increased cell number, protein, and DNA concentrations in C_2C_{12} myoblasts following ractopamine treatment; however, no differences were reported in fused myotube cultures treated with ractopamine. Unfortunately, there is little information on the direct effects of β-agonists on skeletal muscle cells *in vitro*, particularly that of ZIL. Therefore we can only report that in our culture conditions, ZIL administration to bovine satellite cell myotube cultures had no effect on the expression of the β-AR, IGF-I, and MHC IIA mRNA.

Effect of ZIL and the β2-Adrenergic Receptor Antagonist ICI-118, 551 on the mRNA Expression of the β-Adrenergic Receptors, IGF-I, and MHC IIA mRNA in Proliferating Bovine Satellite Cells

We utilized the specific β2-AR antagonist ICI-118, 551 to determine if the effects of ZIL in myoblast cultures were mediated through the β2-AR. The β2-AR antagonist ICI-118, 551 was able to block the reduced expression of β1-AR mRNA by ZIL (0.01 μ*M*) when used at a dose of

0.1 μ*M* (Figure 2.12). Interestingly, the combination of ZIL (0.01 μ*M*) and IC-118, 551 (0.1 μ*M*) resulted in an increase ($P < 0.05$) in β1-AR mRNA expression. For the expression of β2-AR, both levels (0.01 μ*M* and 0.1 μ*M*) of ICI-118, 551 were able to block the reduction in mRNA as a result of ZIL (0.01 μ*M*) treatment (Figure 2.13). There was no effect ($P > 0.05$) of the antagonist on β3-AR mRNA, nor was there an effect of ZIL in the doses we used for this specific experiment (Figure 2.14). In addition, there was no effect ($P > 0.05$) of ICI-118, 551 alone on the expression of the βAR. There was no effect ($P > 0.05$) of ICI-118, 551 alone or in combination with ZIL on the expression of IGF-I mRNA (Figure 2.15). There was an increase ($P < 0.05$) in MHC IIA mRNA expression with ICI-118, 551 (0.1 μ*M*) and the ZIL (0.01 μ*M*) + ICI-118, 551 (0.01 μ*M*) combination. The use of the antagonist in this culture system was to determine if the effects on the expression of the receptors, IGF-I, and MHC IIA mRNA was mediated through the β2-AR because it is reported that ZIL is a β2-AR agonist. Multiple studies utilizing similar methods have been reported looking at β-agonists and antagonists in different cell types. In one study, ICI-118, 551 was used to block the effect of ZIL in lipopolysaccharide-exposed u937 macrophages (Verhoeckx et al, 2005). The effects of ZIL were blocked by ICI-118, 551, and to a lesser extent by atenolol, a β1-AR specific antagonist. In a similar manner, ractopamine-stimulated increases in cell number, protein, and DNA concentrations were blocked by propanolol, an antagonist for both the β1 and β2-AR (Shappell et al., 2000). The stimulation of lipogenesis by ractopamine in rat adipocytes was partially inhibited by propranolol as well (Hausman et al., 1989). These studies demonstrate the effectiveness of specific antagonists in determining if the β-agonist induced response is mediated through a specific receptor, or in some cases, multiple receptors. In our study, the results indicate the reduction in β-AR mRNA by ZIL was mediated through the β2-AR and ICI-118, 551 attenuated the effect of ZIL through the β2-

AR. However, we also observed an increase in MHC IIA mRNA with ICI-118, 551 treatment. This response was interesting because it was also observed in the combination (0.01 μ*M* ZIL + 0.01 μ*M* ICI-118, 551) treatment as well. Unfortunately, there is no information available on the direct effects of ICI-118, 551 on skeletal muscle cells, or other β-AR antagonists regarding MHC IIA or other muscle specific proteins. Therefore, we can only conclude that in our culture system, ICI-118, 551 has a stimulatory effect on MHC IIA mRNA at the levels used.

Effect of ZIL on Bovine Satellite Cell Total Protein Accumulation, Protein Synthesis and Protein Degradation

There was no effect ($P > 0.10$) of ZIL on total protein accumulation in both proliferating myoblasts (Figure 2.17) and fused myotube cultures (Figure 2.18). There was no effect ($P > 0.10$) of ZIL exposure for 2 h (Figure 2.19) or 48 h (Figure 2.20) on protein synthesis in fused myotube cultures. Similarly, there was no effect ($P > 0.10$) of ZIL on proliferating myoblast protein degradation percent (Figure 2.21). Our lack of response on protein synthesis and accumulation are in contrast to much of the data available which suggest alterations in total protein accumulation which result from either increased protein synthesis, decreased protein degradation, or a combination of the two. These changes have been reported both *in vitro* and *in vivo*, with much variation between studies. In satellite cells isolated from lambs, cimaterol increased protein synthesis, but had no effect on protein degradation in the cell cultures (Symonds et al., 1990). In ELC_5 cells, ractopamine increased both total and MHC protein synthesis rates in myotube cultures (Anderson et al., 1990). They also reported that ractopamine had no effect on the rate of protein degradation. In clenbuterol fed rats, muscle protein accretion was increased, with no change in protein synthesis, thus the authors speculated the response was due to decreased protein degradation (Reeds et al., 1986). Additionally, Wheeler and

Koohmaraie (1992) reported a 27.1% reduction in fractional degradation rate of skeletal muscle myofibrillar protein in steers fed the β-agonist L-644,969 after 3 wk. It has been reported that the agonists specific for β2-AR have more of an effect on protein degradation, which would be supported by the research reported (Reeds et al., 1986; Wheeler and Koohmaraie, 1992). However, there is not an abundance of research investigating effects of β-agonists on protein degradation rate because of difficulty associated with its measurement. However, there is some research in support of our data showing no effect of β-agonists on protein metabolism in muscle cell cultures. McMillan et al. (1992) observed no effect of clenbuterol on satellite cell cultures derived from rats. There was no effect on protein accretion, creatine kinase activity, or protein synthesis rates. Similarly, they reported no effect of clenbuterol on protein synthesis and accretion in L6 myoblast or myotube cultures. The lack of effect on protein synthesis in our experiments may be due to ZIL specificity for the β2-AR, thus having more of an effect on protein degradation. However, we were unable to detect differences in protein degradation percent. Further investigation is needed to determine if ZIL addition to fused myotube cultures has an effect of protein degradation.

Effect of ZIL on Bovine Satellite Cell and β2-Adrenergic Receptor Protein Expression

The protein isolated from the cell cultures was used to assess the effects of ZIL on β2-AR protein content using western blot analysis. Similar to the changes in mRNA expression, western blot analysis revealed the protein content of β2-AR in ZIL-treated myoblast cultures decreased ($P = 0.05$) relative to control (Figure 2.23). Additionally, ZIL increased ($P < 0.05$) β2-AR protein expression in myotube cultures (Figure 2.24). These changes in receptor protein expression are important because they support the evidence of the mRNA expression with regards to the proliferating myoblasts. Interestingly, we were unable to detect differences in the mRNA expression of β2-AR mRNA in myotube cultures, but there was an increase in protein in

the western blot analysis for β2-AR. This suggests that the increase in β2-AR protein expression due to ZIL treatment may be in response to a posttranscriptional event due to the lack of difference in β2-AR mRNA. The reduction in protein expression from myoblasts supports much of the research suggesting β-agonists decrease receptor density (Spurlock et al., 1994; Huang et al, 2000; Walker et al, 2007). In contrast, ractopamine administration has led to a tendency for an increase in β2-AR mRNA in skeletal muscle isolated from yearling steers and feedlot heifers collected at harvest (Sissom et al., 2007; Winterholler et al., 2007). These data support the increase in β2-AR protein expression we observed in the western blot analysis. This has also been observed in chicken skeletal muscle cells treated with isoproterenol (a β-agonist) that had an increase in β-AR population by 40% between days 7 and 10 in culture (Young et al., 2000). These data suggest that the expression of β2-AR may increase in more mature muscle, as indicated by our myotube culture data. Additionally, they would support previous research suggesting the expression of β-AR in bovine skeletal muscle cells can vary due to variables such as treatment with β-agonists, as well as time in culture (Bridge et al., 1998). These data further support the important role the β2-AR plays in modulating the function of ZIL on skeletal muscle growth. Additionally, these data aid in our understanding of the direct effects of β-AR agonists, and may help to better understand the mode of action of these growth promoting agents.

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Table 2.1. Sequences for β-1, β-2, and β-3 adrenergic receptors, IGF-I, and myosin heavy chain IIA specific PCR primers and TaqMan probes

β-1 adrenergic receptor (Accession #AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
β-2 adrenergic receptor (Accession #NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
β-3 adrenergic receptor (Accession #XF86961)	
Forward	AGGCAACCTGCTGGTAATCG
Reverse	GTCACGAACACGTTGGTCATG
TaqMan probe	6FAM-CCCGGACGCCGAGACTCCAG-TAMRA
IGF-1 (Accession #X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan probe	6FAM-TGCCCATCACATCCTCCTCGCA-TAMRA
Myosin heavy chain IIA (Accession #AB059398)	
Forward	CCCCGCCCCACATCTT
Reverse	TCTCCGGTGATCAGGATTGAC
TaqMan probe	6FAM-TCTCTGACAACGCCTATCAGTTCAT-TAMRA

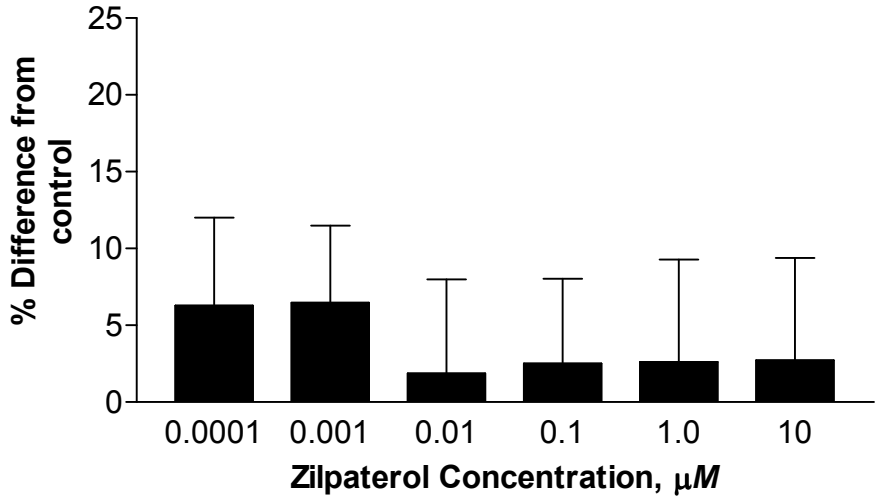

Figure 2.1 Effect of zilpaterol on [^{3}H]-thymidine incorporation, n=7. Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h, and [^{3}H]-thymidine was added at 96 h. Bars represented as percent difference from control ± SEM. All data points in individual assays were the average values obtained from 3 wells on each culture dish. There was no effect of zilpaterol on [^{3}H]-thymidine incorporation.

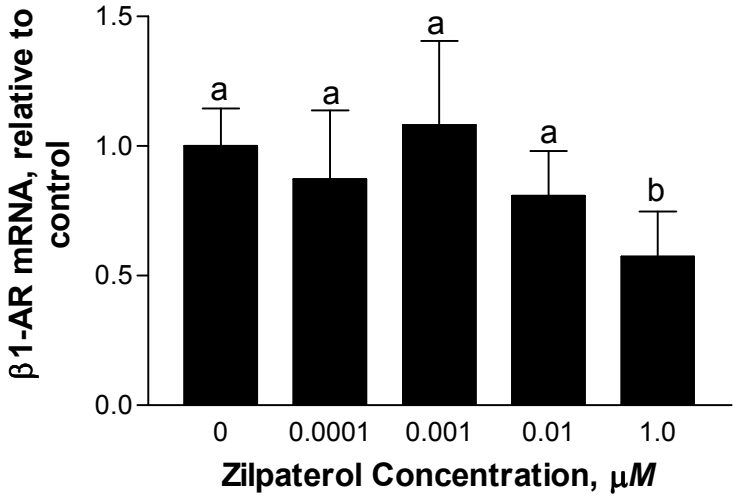

Figure 2.2 Zilpaterol decreased myoblast β1-AR mRNA ($P = 0.07$, n=7). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by real-time polymerase chain reaction (RT-PCR). Data points represented as relative to control. Bars not bearing a common letter differ ($P = 0.07$). Bars are mean values relative to control ± SEM.

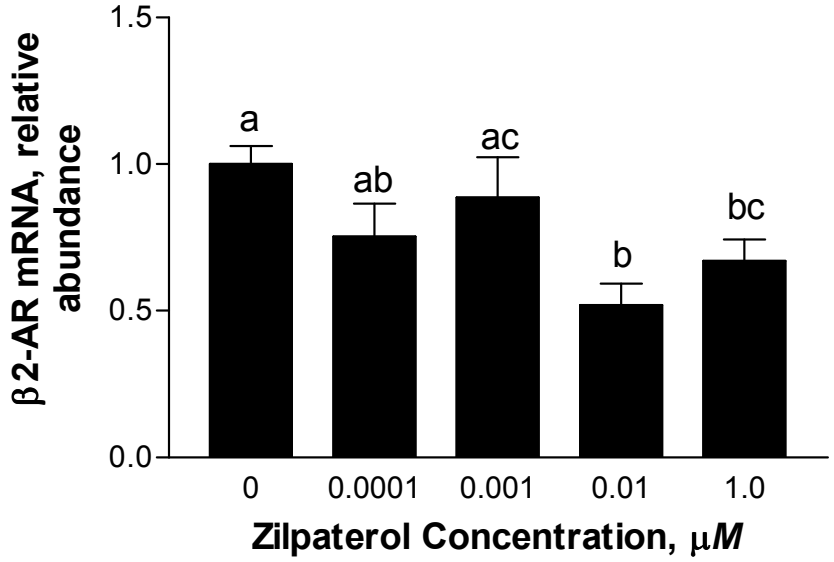

Figure 2.3 Zilpaterol decreased myoblast β2-AR mRNA ($P < 0.05$, n=7). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Bars not bearing a common letter differ ($P < 0.05$). Data points represented as relative to control. Bars are mean values relative to control ± SEM.

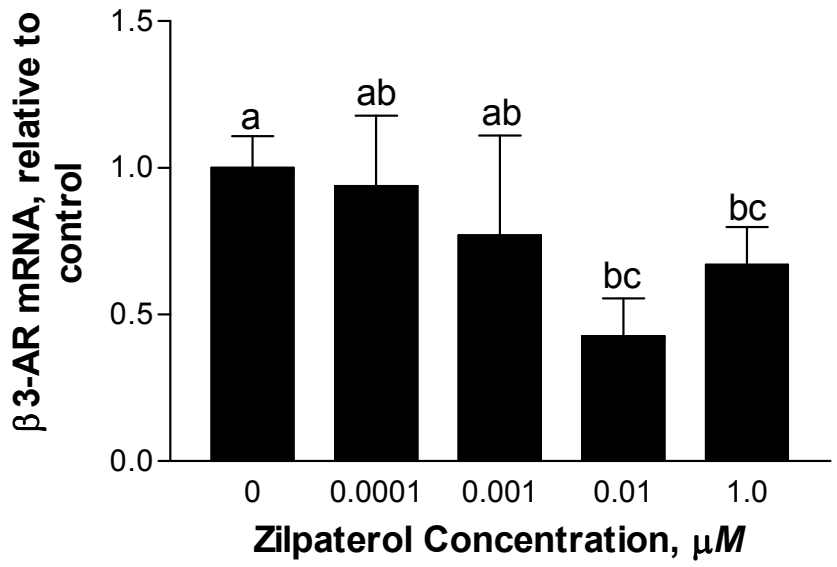

Figure 2.4 Zilpaterol decreased myoblast β3-AR mRNA (0.01 μ*M*, $P < 0.01$; 1.0 μ*M*, $P = 0.06$, n=7). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars not bearing a common letter differ ($P < 0.05$). Bars are mean values relative to control ± SEM

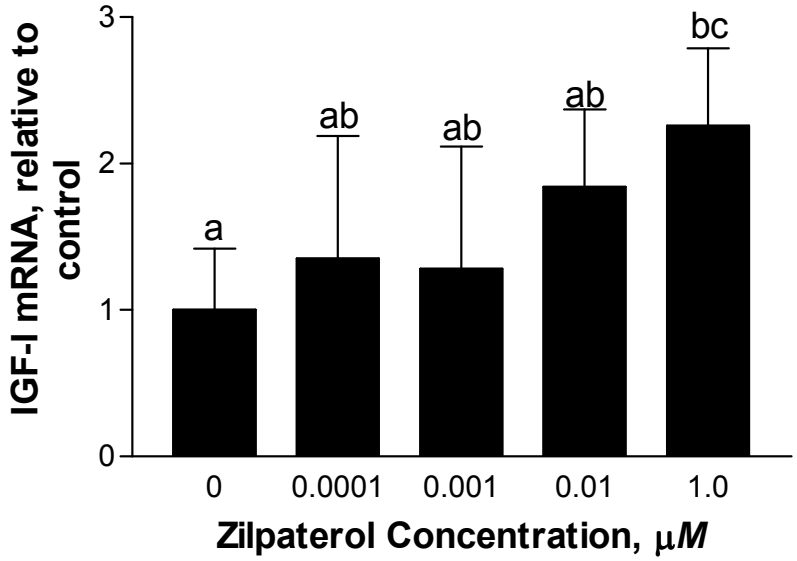

Figure 2.5 Zilpaterol increased myoblast IGF-I mRNA ($P = 0.07$, n=7). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars not bearing a common letter differ ($P = 0.07$). Bars are mean values relative to control ± SEM.

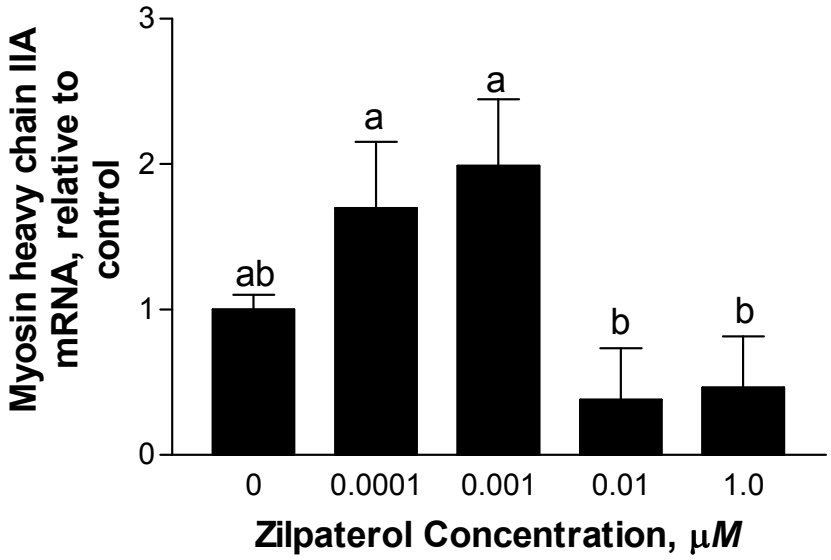

Figure 2.6 Zilpaterol decreased myoblast MHC IIA mRNA ($P < 0.05$, n=7). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars not bearing a common letter differ ($P < 0.05$). Bars are mean values relative to control ± SEM.

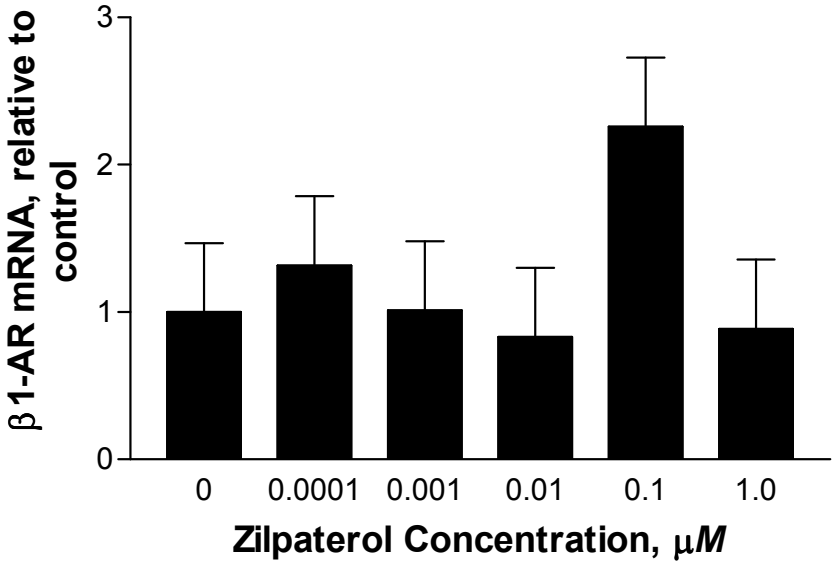

Figure 2.7 Effect of zilpaterol on myotube β1-AR mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Total RNA was isolated at 216 h, and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM. There was no effect ($P > 0.10$) of treatment on the expression of β1-AR mRNA, n=5.

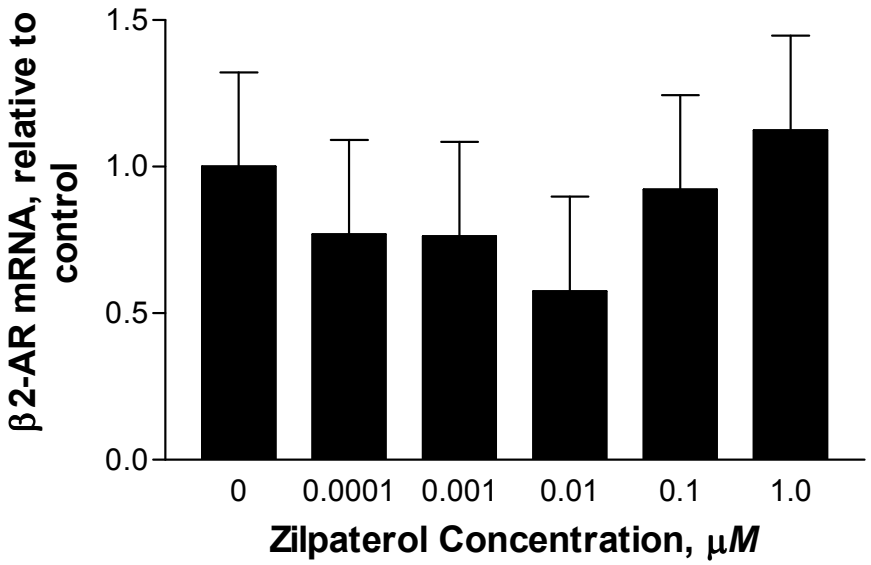

Figure 2.8 Effect of zilpaterol on myotube β2-AR mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Total RNA was isolated at 216 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM. There was no effect ($P > 0.10$) of treatment on the expression of β2-AR mRNA, n=5**.**

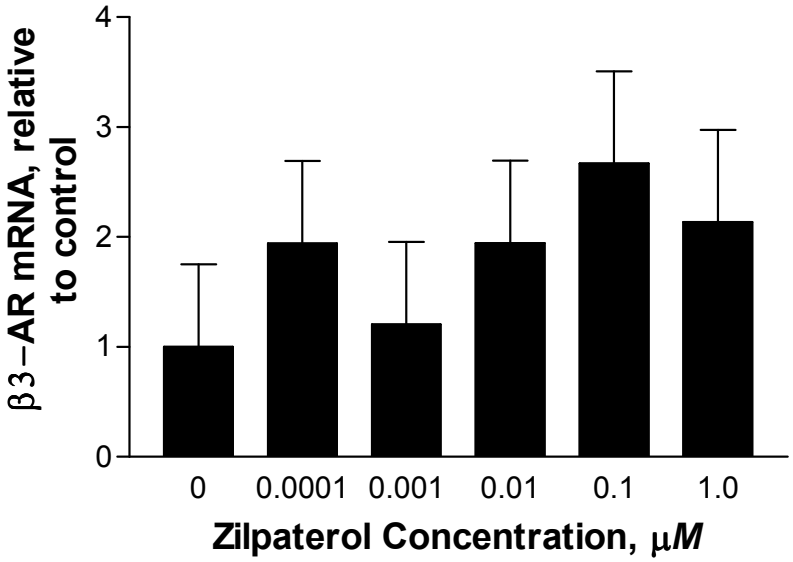

Figure 2.9 Effect of zilpaterol on myotube β3-AR mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Total RNA was isolated at 216 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM. There was no effect ($P >$ 0.10) of treatment on the expression of β3-AR mRNA, n=5.

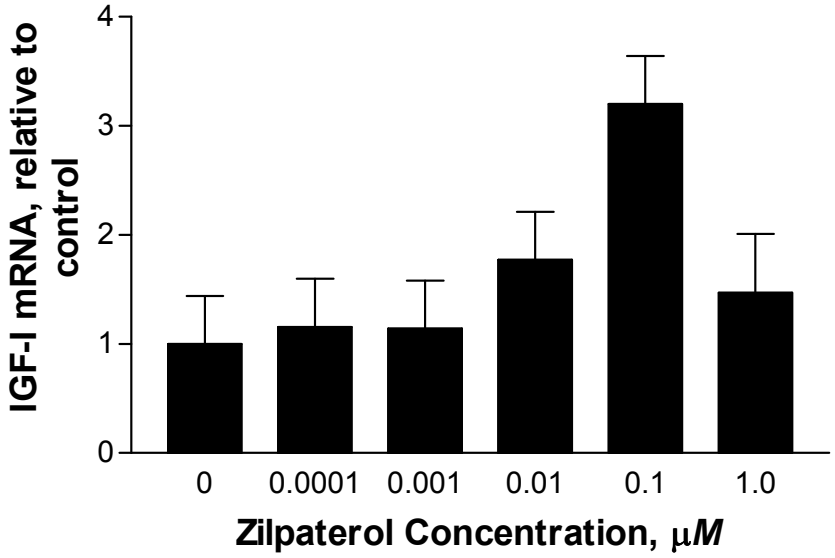

Figure 2.10 Effect of zilpaterol on myotube IGF-I mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 14 h. Zilpaterol was added at 168 h to fused myotubes. Total RNA was isolated at 216 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM. There was no effect ($P > 0.10$) of treatment on the expression of IGF-I mRNA, n=5.

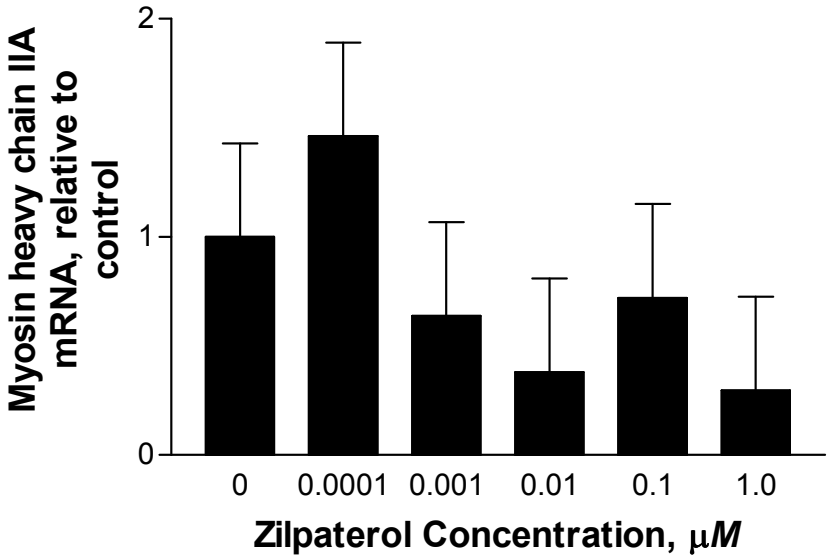

Figure 2.11 Effect of zilpaterol on myotube MHC IIA mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Total RNA was isolated at 216 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM. There was no effect ($P > 0.10$) of treatment on the expression of myosin heavy chain mRNA, n=5.

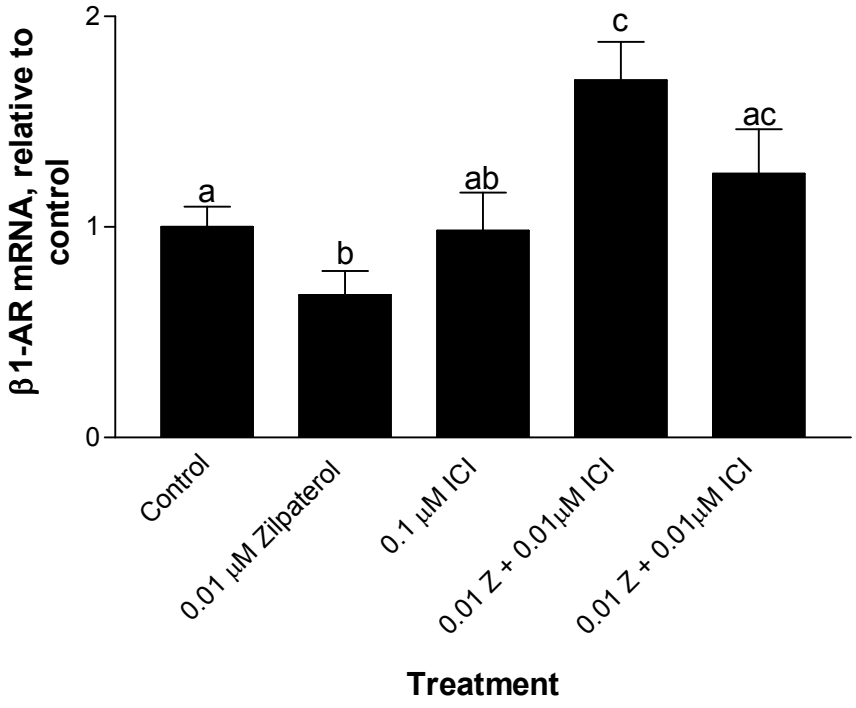

Figure 2.12 Zilpaterol decreased and zilpaterol + ICI-118, 551 combination increased myoblast β1-AR mRNA ($P < 0.05$, n=4). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars not bearing a common letter differ ($P < 0.05$). Bars are mean values relative to control ± SEM.

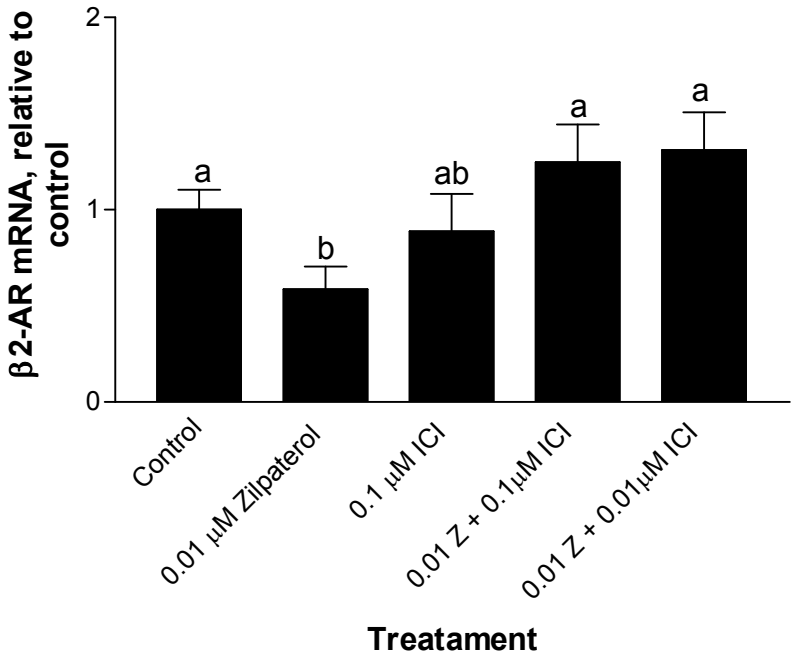

Figure 2.13 Zilpaterol decreased myoblast β2-AR mRNA, and ICI-118, 551 blocked the reduction ($P < 0.05$, n=4). Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 48 h. Total RNA was isolated at 96 h, and relative mRNA abundance was determined by RT-PCR. Bars not bearing a common letter differ ($P < 0.05$). Data points represented as relative to control. Bars are mean values relative to control ± SEM.

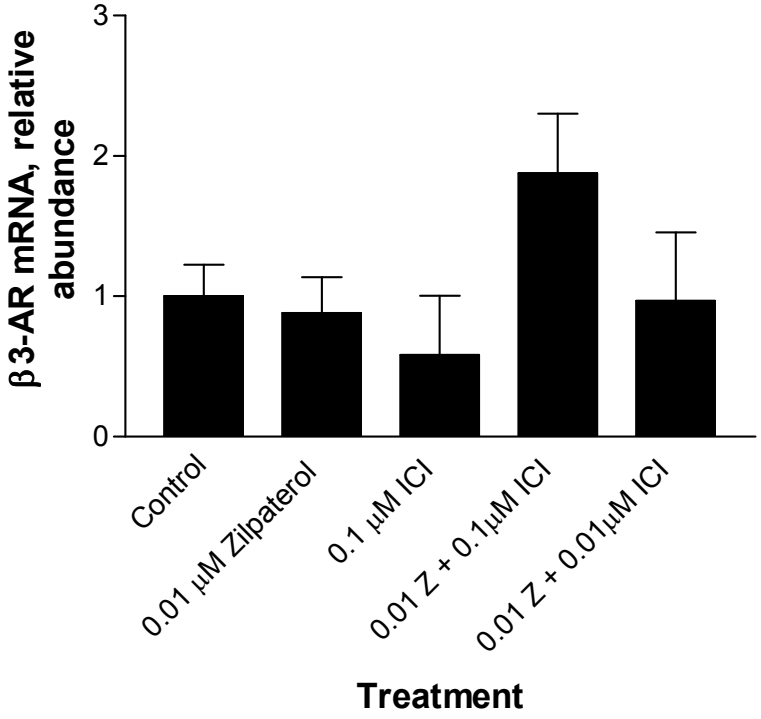

Figure 2.14 Effect of zilpaterol and ICI-118, 551 alone, or combination on myoblast β3-AR mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol and ICI-118, 551 was added at 48 h. Total RNA was isolated at 96 h, and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM, n=4.

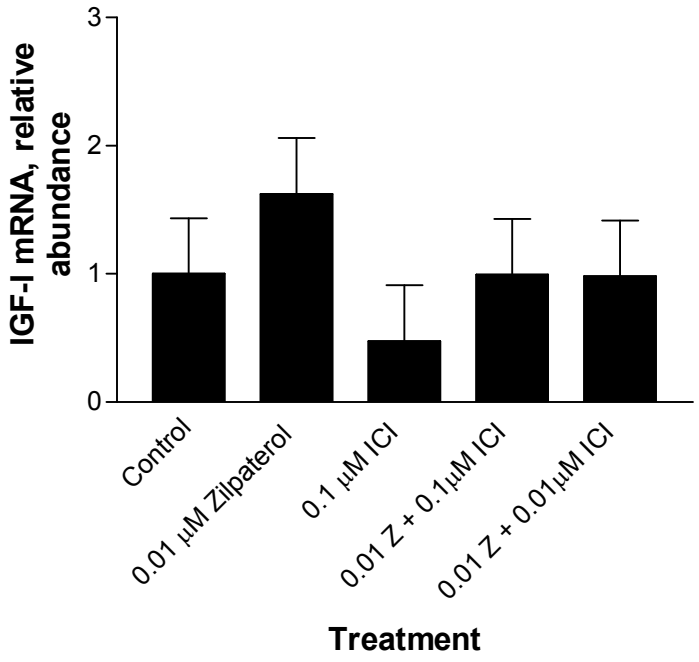

Figure 2.15 Effect of zilpaterol and ICI-118, 551 alone, or combination on myoblast IGF-I mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol and ICI-118, 551 was added at 48 h. Total RNA was isolated at 96 h and relative mRNA abundance was determined by RT-PCR. Data points represented as relative to control. Bars are mean values relative to control ± SEM, n=4.

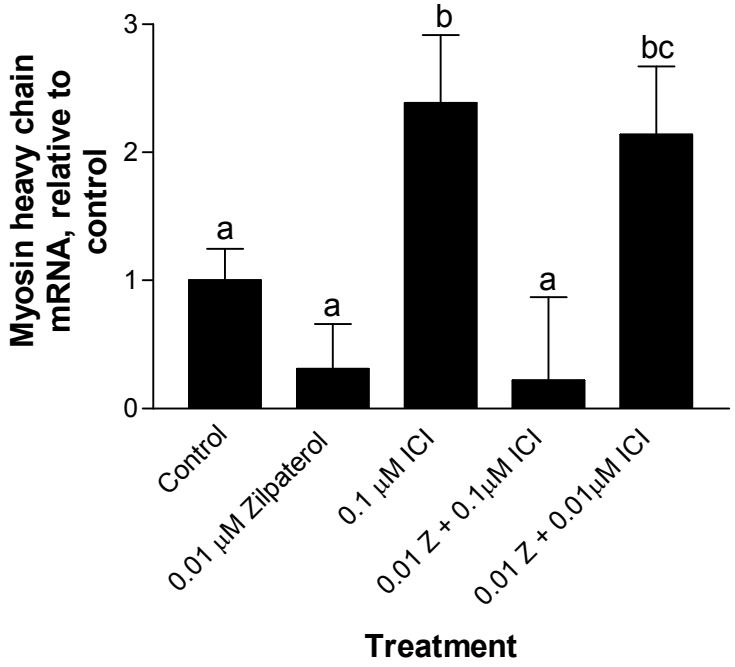

Figure 2.16 Effect of zilpaterol and ICI-118, 551 alone, or combination on myoblast MHC IIA mRNA. Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol and ICI-118, 551 was added at 48 h. Total RNA was isolated at 96 h, and relative mRNA abundance was determined by RT-PCR. Bars not bearing a common letter differ ($P < 0.05$). Data points represented as relative to control. Bars are mean values relative to control ± SEM, n=4.

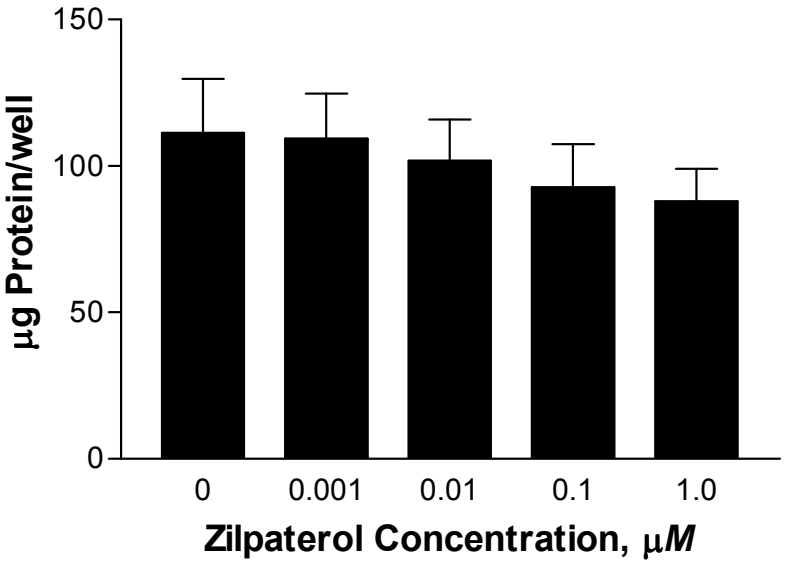

Figure 2.17 Effect of zilpaterol on total protein accumulation in proliferating myoblasts. Bovine satellite cells were plated in 10% FBS/DMEM. Zilpaterol was added at 24 h to proliferating myoblasts. Protein was isolated after 96 h in culture. Bars are mean values ± SEM. There was no effect ($P > 0.10$) of treatment on total protein accumulation, n=10.

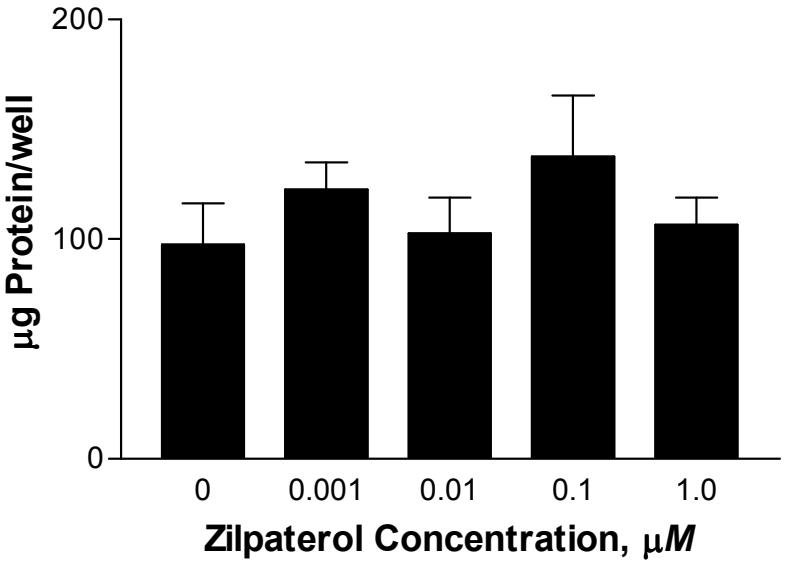

Figure 2.18 Effect of zilpaterol on total protein accumulation in fused myotube cultures. Bovine satellite cells were plated in 10% FBS/DMEM. After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Protein was isolated after 216 h in culture. Bars are mean values ± SEM. There was no effect ($P > 0.10$) of treatment on the total protein accumulation, n=6.

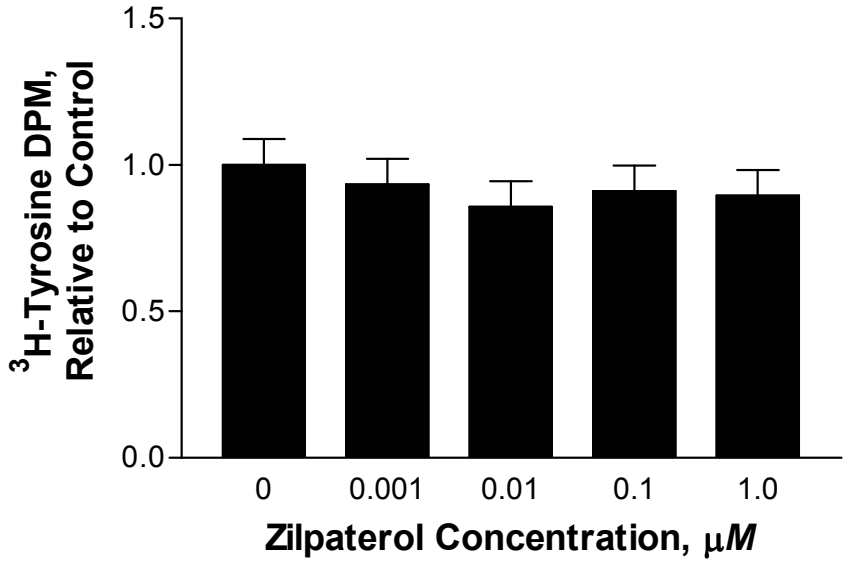

Figure 2.19 Effect of 2h zilpaterol treatment on total protein synthesis, measured by [^{3}H]-tyrosine incorporation. Bovine satellite cells were plated in 10% FBS/DMEM. After 48 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h. [^{3}H]-Tyrosine incorporation was measured at 170 h in culture. Bars are mean values ± SEM. There was no effect ($P > 0.10$) of treatment on the total protein synthesis, n=3.

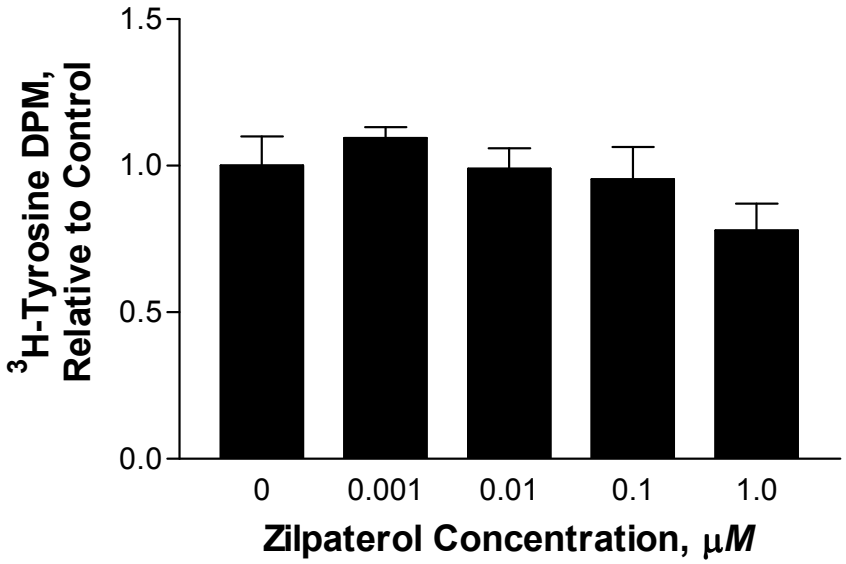

Figure 2.20 Effect of 2-h zilpaterol treatment on total protein synthesis, measured by [^{3}H]-tyrosine incorporation. Bovine satellite cells were plated in 10% FBS/DMEM. After 48 h, 3% swine serum/DMEM was added, and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h. [^{3}H]-Tyrosine incorporation was measured at 216 h in culture. Bars are mean values ± SEM. There was no effect ($P > 0.10$) of treatment on the total protein synthesis, n=6.

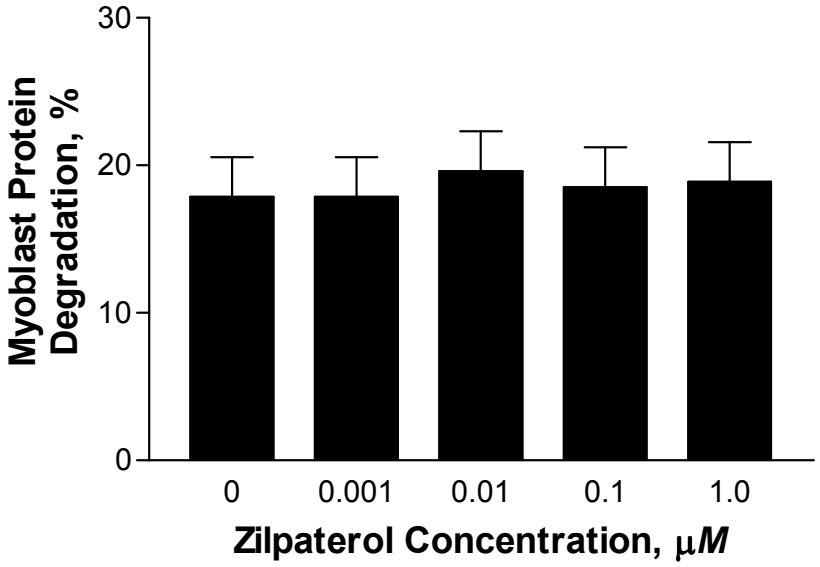

Figure 2.21 Effect of zilpaterol on protein degradation. Bovine satellite cells were plated in 10% FBS/DMEM. After 48 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. At 168 h in culture, cells were labeled for 24 h with [^{3}H]-tyrosine in 1 mL of conditioned media. At 192 h, the [^{3}H]-tyrosine media was removed and satellite cells cultures were incubated for 4 h in 1 mL of media containing 0.2 m*M* tyrosine. Following the 4 h incubation, cultures were rinsed with DMEM and zilpaterol was added. Degradation was expressed as a percentage of total radioactivity in prelabeled proteins as follows: TCA-soluble dpm released into media x 100/(total media dpm + cellular dpm). Bars are mean values ± SEM. There was no effect ($P > 0.10$) of treatment on the total protein degradation, n=4.

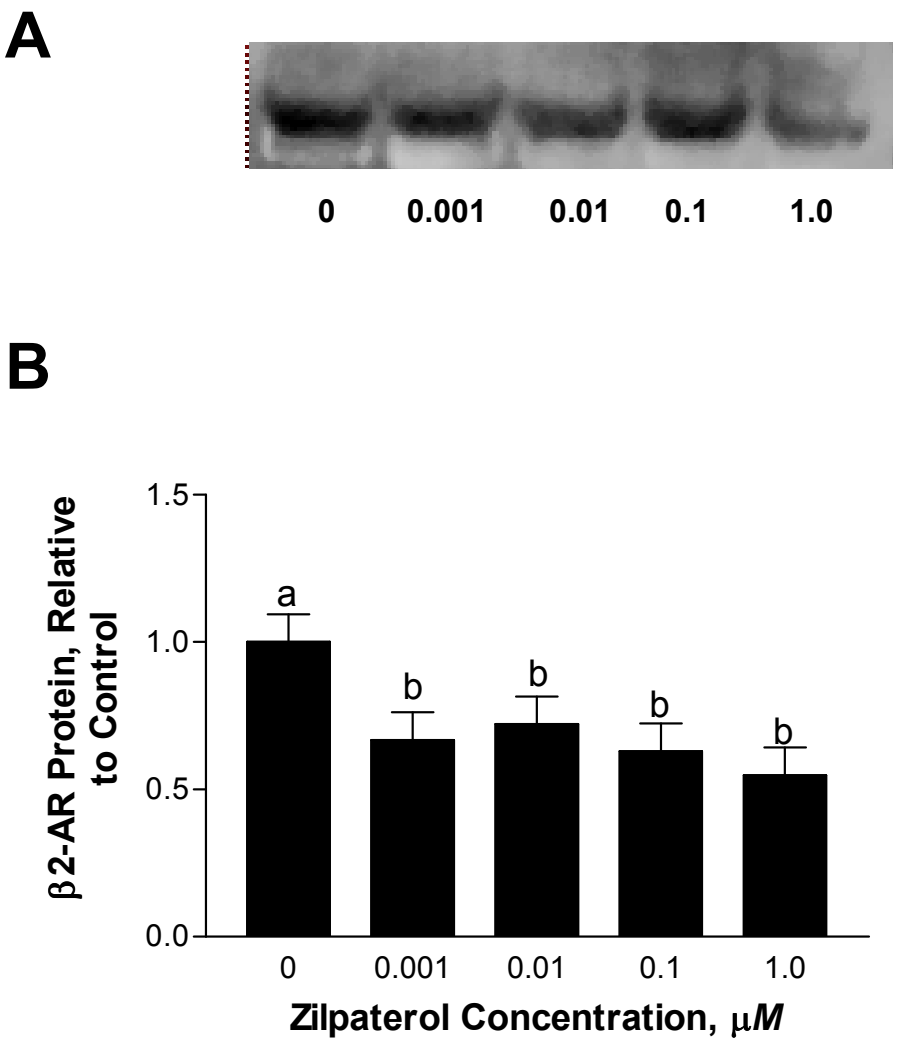

Figure 2.22 (A) Western blot image of zilpaterol induced decrease in β2-AR in myoblast cultures. (B) Zilpaterol decreased β2-AR protein expression in myoblast cultures ($P < 0.05$, n=3). Bovine satellite cells were plated in 10% FBS/DMEM. Total protein was isolated at 96 h and used in western blot analysis. Bars are mean values ± SEM.

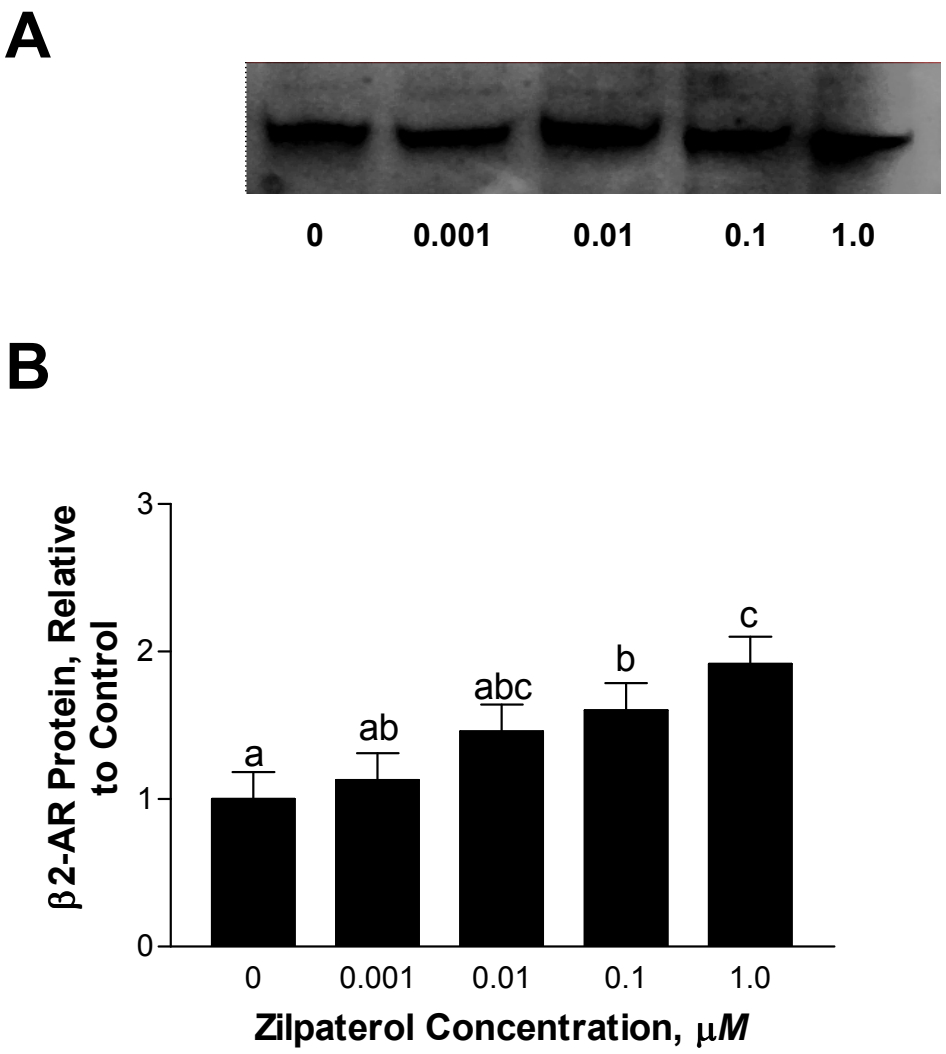

Figure 2.23 (A) Western blot image of zilpaterol induced increase in β2-AR in myotube cultures. (B) Zilpaterol increased β2-AR protein expression in myotube cultures ($P < 0.05$, n=3). After 24 h, 3% swine serum/DMEM was added and 3% horse serum/DMEM was added at 144 h. Zilpaterol was added at 168 h to fused myotubes. Total protein was isolated and used for western blot analysis. Bars are mean values ± SEM.

CHAPTER 3 - Effect of Zilpaterol on Feedlot Steer Performance, Carcass Characteristics, and Skeletal Muscle Gene Expression

Abstract

The objectives of this experiment were to determine the effects of zilpaterol-HCl (**ZIL**) supplementation on growth performance, carcass characteristics, and skeletal muscle gene expression in feedlot steers. British and British x Continental steers calves (n = 2,229) with an initial BW of 330 kg were used in a randomized complete block design. Steers were administered ZIL for 0, 20, 30, or 40 d prior to slaughter. During zilpaterol treatment time, the diet fed for the prescribed duration contained 8.32 mg/kg of diet DM of zilpaterol HCl, but no monensin or tylosin. Additionally, there was a 3-d withdrawal period prior to harvest. Treatments were randomly assigned to 16 pens. At harvest, semimembranosus muscle tissue was collected (2 animals per pen) for RNA isolation to determine the expression of β1, β2, and β3-adrenergic receptor (**AR**), IGF-I, and myosin heavy chain (**MHC**) IIA mRNA. There was no effect ($P > 0.05$) of ZIL administration on feed intake, but there was an increase ($P < 0.01$) in ADG and G:F in ZIL-fed steers compared to the control animals. Zilpaterol also increased ($P < 0.01$) LM area, HCW, and dressing percentage. Zilpaterol decreased ($P < 0.01$) marbling score, 12^{th} rib fat, and improved ($P < 0.05$) yield grades. There was no effect ($P > 0.05$) of ZIL administration on KPH fat. There was no effect ($P > 0.10$) of ZIL administration or duration of ZIL feeding ($P > 0.10$) on the expression of the β1, β2, and β3-AR mRNA in semimembranosus muscle. Additionally, there was no effect ($P > 0.10$) of ZIL administration or duration of ZIL feeding ($P > 0.10$) on the expression of IGF-I mRNA in semimembranosus muscle. Zilpaterol administration did decrease ($P < 0.05$) the expression of myosin heavy chain MHC IIA mRNA in semimembranosus muscle tissue. These data suggest that administration of ZIL to steers can improve animal performance. Zilpaterol administration also reduced the expression of MHC IIA mRNA in skeletal muscle, which may be a result of reduced protein degradation or of up-

regulation of other myosin heavy chain mRNA isoforms. These data aid in our understanding of the effects of ZIL administration to feedlot steers. Furthermore, they increase our knowledge and understanding of the mechanism of action of ZIL, as well as other β-agonists used to promote growth and efficiency in feedlot animals.

Key words: β-Adrenergic receptor, carcass, heifer, myosin, steer, zilpaterol

Introduction

In recent years, there has been a considerable amount of research investigating the effects of compounds that can alter the rate of growth in livestock animals. One group of growth promoters used today is the β-adrenergic receptor (**AR**) agonists. There are two β-agonists approved for use in the United States in feedlot cattle, ractopamine-HCl and zilpaterol-HCl (**ZIL**). Ractopamine has been approved for over 4 yr, whereas ZIL has only been approved for use since 2006.

Zilpaterol-HCl administration the last 20 to 40 d on feed results in increased ADG, ribeye area, dressing percentage, and improved feed efficiency and carcass yield grade (Plascencia et al., 1999; Avendano-Reyes et al., 2007). It is thought that zilpaterol elicits a response through binding to one of the β-AR which are membrane-bound receptors located in most mammalian cells (Strosberg, 1993; Mills and Mersmann, 1995). The binding of ZIL to β-AR results in enzyme phosphorylation that is responsible for changes in protein synthesis and degradation, particularly in skeletal muscle. These changes result in increased animal performance and lean tissue deposition as demonstrated by improved carcass characteristics.

The β2-AR is the most abundant subtype of β-AR in bovine skeletal muscle, and it is believed that ZIL mediates its growth promoting response through the β2-AR. The receptor-mediated response can be altered by the number of receptors in specific tissues, and β-agonist administration can alter the number of receptors in different tissues (Spurlock et al., 1994; Sissom et al., 2007; Winterholler et al., 2007).

The purpose of these experiments was to investigate the effects of ZIL administration to feedlot steers on animal performance and carcass characteristics. Additionally, we evaluated the

effects of ZIL administration in steers on the expression of β1, β2, and β3-AR, as well as IGF-I, and myosin heavy chain (**MHC**) IIA mRNA in skeletal muscle.

Materials and Methods

The following experiments were a collaboration between Intervet, Inc. (Millsboro, DE), Cactus Research, LTD (Amarillo, TX) and Kansas State University. Research conducted at commercial research facilities followed the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999, Savoy, IL).

Animals

British and British x Continental steers calves (n = 2,229) with an initial BW of 330 kg were used in a randomized complete block design with 4 treatments. Treatments consisted of feeding 8.32 mg/kg of zilpaterol-HCl for 0, 20, 30, or 40 d prior to slaughter. Treatments were randomly assigned to each pen (n = 24) with approximately 93 animals per pen. At arrival, steers were implanted with Revalor-IS (80 mg trenbolone acetate and 16 mg estradiol), and reimplanted with Revalor-IS approximately 73 d prior to harvest. Animals were fed 3 times daily during the transition period and 2 times daily for the remainder of the study, and they were allowed ad libitum access to feed. The finishing diet was based on steam-flaked corn (Table 3.1). Feed additives (Rumensin, Tylan, Zilmax and vitamins A, D and E) were added using a micro-weigh machine (Micro Beef Technologies, Amarillo, TX). During the zilpaterol-treatment time for each group, the diet fed for the prescribed duration contained 8.32 mg/kg of Zilpaterol HCl, but no Rumensin or Tylan.

For determination of final BW, pens were weighed and the industry standard 4% pencil shrink was applied, following a 3-d withdrawal period. Animals were transported 96 km to a

commercial slaughter facility (Tyson Fresh Meats Inc., Amarillo, TX). Carcass characteristics were obtained from chilled carcasses 36 h after slaughter.

Sample Preparation and RNA Isolation

Within 10 min of slaughter, a muscle sample was collected from the semimembranosous muscle of 2 randomly selected animals per pen. The samples were snap frozen in liquid nitrogen and delivered to Kansas State University for analysis. Total RNA was isolated from muscle samples using TRI Reagent (Sigma, St. Louis, MO). Semimembranosous muscle tissue (100 mg) was transferred to a steel mortar bowl cooled by liquid nitrogen. The samples were homogenized using a sterile pestal in liquid nitrogen. TRI Reagent (3 mL) was then added to the ground tissue sample. One mL of muscle tissue in Tri Reagent was incubated at room temperature for 5 min. Following incubation, chloroform (Sigma, St. Louis, MO) was added and samples were centrifuged for 15 min at 12,000 x g at room temperature. Following centrifugation, the top layer was removed and transferred to a new microcentrifuge tube. Isopropanol (Sigma, St. Louis, MO) was added and samples were centrifuged for 10 min at 12,000 x g to isolate the RNA pellet. The RNA pellet was then treated to remove any contaminating genomic DNA using the DNA-*free* kit (Ambion, Austin, TX). The RNA concentration was determined by absorbance at $260_{nm.}$ The integrity of the RNA was determined by gel electrophoresis. Total RNA with ethidium bromide was loaded onto a 1% agarose gel to separate and visualize the 28S and 18S rRNA.

Real-time quantitative-PCR

Real-time quantitative-PCR was used to measure the quantity of β1-AR, β2-AR, β3-AR, IGF-I, and MHC IIA gene expression relative to the quantity of 18S ribosomal RNA (**rRNA**) in total RNA isolated from muscle tissue. Measurement of the relative quantity of cDNA was

performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 n*M* of the appropriate forward and reverse primers, 200 n*M* of appropriate TaqMan detection probe, and 1 μL of the cDNA mixture. The bovine specific β1-AR, β2-AR, and β3-AR, IGF-I, and MHC IIA forward and reverse primers and TaqMan detection probes (Table 3.1) were synthesized using published GenBank sequences. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession #X03205). The ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA) was used to perform the assay utilizing the recommended thermal cycling variables by the manufacturer (50 cycles of 15 s at 95ºC and 1 min at 60ºC). The 18S rRNA endogenous control was used to normalize the expression of β1-AR, β2-AR, β3-AR, IGF-I, and MHC IIA.

Statistical Analysis

Data were analyzed as a randomized complete block design with PROC MIXED (SAS Inst. Inc., Cary, NC). Pen served as the experimental unit for all feedlot and carcass characteristics. For all analysis, specific orthogonal contrasts were used to test 1) control vs. the average of the 3 duration of ZIL feeding groups and 2) linear and quadratic effects of duration of ZIL feeding.

Results and Discussion

Effect of ZIL Administration on Performance and Carcass Characteristics in Finishing Steers

The data for performance over the entire finishing period and carcass characteristics are shown in Table 3.3. There was no effect ($P > 0.05$) of ZIL administration on feed intake in steers. Regardless of the duration of ZIL feeding, there was an increase ($P < 0.01$) in ADG and G:F in ZIL fed animals compared to the controls. Additionally, ZIL increased ($P < 0.01$) LM

area, dressing percentage, HCW, and improved ($P < 0.05$) yield grades regardless of ZIL duration. Additionally, ZIL administration decreased ($P < 0.01$) marbling score and 12th rib fat and improved ($P < 0.05$) yield grades regardless of ZIL duration. There was no effect ($P > 0.05$) of ZIL administration on KPH fat. These data are consistent with other studies utilizing β-AR agonists such as ractopamine and ZIL as growth promoters. In feedlot steers administered 6 mg/kg of zilpaterol in the diet daily, there was an increase in average daily gain and improved gain efficiency (Plascencia et al., 1999; Vasconcelos et al., 2008). Zilpaterol supplementation also increased carcass weight by 4.5%, dressing percentage by 3.6%, and longissimus muscle area by 2.7% compared to control steers. There was no effect of zilpaterol on fat thickness or marbling score (Vasconcelos et al., 2008). Both zilpaterol (60 mg/d) and ractopamine (300 mg/d) were investigated in feedlot cattle in a study to compare the two β-agonists (Avendano-Reyes et al., 2006). Both ractopamine and ZIL improved ADG, feed efficiency, and HCW. Additionally, ZIL increased longissimus muscle area; however, both ractopamine and ZIL increased shear force values compared to control steers. The authors commented that the β-agonists were effective in enhancing performance of the cattle without having a substantial effect on meat quality (Avendano-Reyes et al., 2006; Vasconcelos et al., 2008). Similar improvements in animal performance have been reported with ractopamine administration. In feedlot steers, ractopamine fed the final 28 d of the finishing period at 200 mg/d resulted in improved ADG, feed efficiency, HCW, and larger LM area (Gruber et al., 2007). Winterholler et al. (2007) reported similar findings in yearling steers fed 200 mg/d of ractopamine the final 28 d of the feeding period. Steers fed ractopamine had increased ADG and improved gain efficiency, as well as increased HCW and LM area.

Effect of ZIL Administration on Semimembranosus Muscle β1, β2, and β3-AR mRNA Concentrations in Finishing Steers

Total RNA was isolated from semimembranosus muscle tissue collected from steers to evaluate the effects of ZIL on the expression of muscle β1, β2, and β3-AR mRNA. There was no effect ($P > 0.10$) of ZIL administration or duration of ZIL feeding ($P > 0.10$) on the expression of the β1, β2, and β3-AR mRNA in semimembranosus muscle tissue (Figures 3.1 to 3.3). The administration of other β-agonists has resulted in altered mRNA expression of β-AR, as well as receptor number and density. Administration of clenbuterol to male rats for 10 d resulted in a 35% reduction in β2-AR density in skeletal muscle (Huang et al., 2000). This reduction in receptor density was also observed in lung tissue of the rats. Walker et al. (2007) observed reductions in the mRNA expression of both β1-AR and β2-AR in skeletal muscle from Holstein steers administered ractopamine. Additionally, in pigs administered ractopamine, the number of β2-AR in adipose tissue was decreased at 1, 8, and 24 d following treatment, whereas there was no effect of ractopamine on receptor number in skeletal muscle (Spurlock et al., 1994). In contrast, we previously observed tendency for increased β2-AR mRNA in skeletal muscle isolated from yearling steers administered ractopamine for 28 d prior to harvest (Winterholler et al., 2007). Similarly, we observed a tendency for ractopamine administration to feedlot heifers to increase β2-AR mRNA in semimembranosus muscle collected at harvest (Sissom et al., 2007). It is evident that β-agonists can alter the mRNA expression and density of β-AR in skeletal muscle as well as other tissues; however, we were unable to detect differences in the current study. These changes may play an important role in regulating the response to β-agonist treatment and warrant further investigation.

Effect of ZIL Administration on Semimembranosus Muscle IGF-I, and MHC IIA mRNA Concentrations in Finishing Steers

The expression of IGF-I mRNA in semimembranosus muscle tissue collected from steers (Figure 3.4) was not altered ($P > 0.10$) by ZIL administration or duration ($P > 0.10$) of ZIL feeding. The lack of effect of ZIL administration on the expression of IGF-I mRNA in semimembranosus muscle tissue is similar to that of other studies. The majority of research would suggest there is no role for IGF-I in β-agonist induced skeletal muscle growth. O'Connor et al. (1991) observed no change in IGF-I concentrations following administration of cimaterol for 3 wk. Similarly, clenbuterol administration to growing lambs resulted in no change in IGF-I concentrations (Young et al., 1995). Grant et al. (1993) reported no change in IGF-I mRNA expression in both skeletal muscle and liver in pigs fed ractopamine. In addition to the data suggesting no effect of β-agonists on IGF-I, there is some data suggesting IGF-I can be reduced in both circulation and mRNA expression by β-agonist treatment. In Holstein steers administered ractopamine for 28 d prior to harvest there was a decrease in serum IGF-I (Walker et al., 2007). Additionally, there was a decrease in mRNA expression of IGF-I in the longissimus muscle tissue collected at harvest. In growing lambs fed cimaterol for 6 wk, there was a decrease in IGF-I level in circulation (Beermann et al., 1987). In contrast, it has been reported that clenbuterol administration to rats increased IGF-I mRNA and IGF-I content in skeletal muscle without any change in serum IGF-I (Awede et al., 2002). There is a lot of variability in the information regarding the role of IGF-I in β-agonist induced muscle hypertrophy. Much of the data available suggest no effect of β-agonists on IGF-I in both muscle tissue and in circulation, and our results are in agreement with the data that is currently available.

The expression of MHC IIA was decreased ($P < 0.05$) by ZIL administration in steers (Figure 3.5). Myosin heavy chain is a major protein in skeletal muscle that accounts for around 30% of all protein in skeletal muscle. There are different isoforms of MHC that are important in determining muscle fiber type. Slow twitch fibers are predominantly MHC I, whereas fast twitch fibers are predominantly MHC IIA and IIX. It has been reported that β-agonist treatment can alter the expression of MHC isoforms, which can result in subsequent changes in muscle composition (Rehfeldt et al., 1997; Depreux et al., 2002). We observed a decrease in MHC IIA mRNA with the administration of ZIL steers. In chickens fed clenbuterol there was a decrease in fast twitch type IIA muscle fibers and an increase in the fast twitch type IIB muscle fibers (Rehfeldt et al., 1997). Similarly, Depreux et al. (2002) observed a decrease in MHC IIA and IIX mRNA expression and an increase in MHC IIB mRNA in pigs fed ractopamine. These findings are similar to our data. However, we did not measure other isoforms of MHC so we can only report on the effects of ZIL on the reduction in MHC IIA. It is also important to note that MHC IIB mRNA is not detected in bovine skeletal muscle tissue (Chikuni et al., 2004). The reduction in MHC IIA in skeletal muscle may also be an indirect response to decreases in protein degradation due to ZIL administration. β-Agonists, especially those specific for the β2-AR have been shown to decrease protein degradation in skeletal muscle. Wheeler and Koohmaraie (1992) reported a 27.1% reduction in fractional degradation rate of skeletal muscle myofibrillar protein in steers fed the β-agonist L-644,969 after 3 wk. Additionally, calpastatin activity was higher in muscle from treated steers. It was suggested that the β-agonist-induced muscle hypertrophy observed in their study was a result of the reduced proteolytic capacity due to increased calpastatin which in turn reduced protein degradation. Additionally, Parr et al. (1992) observed an increase in both m-calpain and calpastatin activity in Friesian steers fed the β-agonist

cimaterol. There was also an increase in mRNA for both m-calpain and calpastatin. We did not measure the expression of either m-calpain or calpastatin in the present study. However, these data provide a possible explanation for the reduction in MHC IIA mRNA concentration observed in the semimembranosus muscle tissue collected from the steers fed ZIL.

Overall, the data from our study suggest that the β-AR agonist ZIL can be an effective tool used to improve animal growth and efficiency. Zilpaterol administration improved feedlot steer performance and carcass characteristics. Additionally, ZIL reduced the expression of MHC IIA in semimembranosus muscle tissue. These data aid in our understanding of the mechanism through which ZIL improves animal performance, and enhance our overall knowledge of β-agonists.

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Table 3.1 Composition of the diet fed to steers[1]

	Amount, % of DM	
Item	Control	Zilpaterol[2]
Corn, steam-flaked	72.9	72.9
Corn DDGS[3]	9.9	9.9
Corn silage	10.1	10.1
Tallow	3.0	3.0
Supplement	4.0	4.0
Micro-ingredients[4]		
Rumensin, mg/kg	37.2	
Tylan, mg/kg	10.8	
Zilmax, mg/kg		8.32
Vitamin A, IU/kg	3,080	3,080
Vitamin D, IU/kg	208	208
Vitamin E, IU/kg	11	11

[1]Fed through the end of the trial, including the 3 d withdrawal following zilpaterol feeding.
[2]Fed to zilpaterol treatments for 20, 30 or 40 d.
[3]Corn dried distillers grains with solubles.
[4]Added using a micro-weigh machine (Micro Beef Technologies, Amarillo, TX).

Table 3.2. Sequences for β-1, β-2, and β-3 adrenergic receptors, IGF-I, and myosin heavy chain IIA specific PCR primers and TaqMan probes

β-1 adrenergic receptor (Accession #AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
β-2 adrenergic receptor (Accession #NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
β-3 adrenergic receptor (Accession #XF86961)	
Forward	AGGCAACCTGCTGGTAATCG
Reverse	GTCACGAACACGTTGGTCATG
TaqMan probe	6FAM-CCCGGACGCCGAGACTCCAG-TAMRA
IGF-1 (Accession #X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan probe	6FAM-TGCCCATCACATCCTCCTCGCA-TAMRA
Myosin heavy chain IIA (Accession #AB059398)	
Forward	CCCCGCCCCACATCTT
Reverse	TCTCCGGTGATCAGGATTGAC
TaqMan probe	6FAM-TCTCTGACAACGCCTATCAGTTCAT-TAMRA

Table 3.3 Effects of zilpaterol (Z) administration on performance of feedlot steers

	Zilpaterol[1]					*P*-values[2]		
Item	0	20	30	40	SEM	0 vs. Z	L	Q
Pens	6	6	6	6	-	-	-	-
Starting weight, kg	330	333	328	331	4.2	0.74	0.4	0.77
DMI, kg/d	9.44	9.45	9.49	9.36	0.32	0.94	0.70	0.86
ADG, kg	1.66	1.73	1.76	1.75	0.08	0.03	0.03	0.89
G:F	0.178	0.182	0.186	0.187	0.003	<0.01	<0.01	0.74
Hot carcass weight, kg	390	406	409	410	4.1	<0.01	<0.01	0.27
LM area, cm^2	87.95	96.65	99.01	98.98	1.376	0.01	<0.01	0.08
12th rib fat, cm	1.60	1.53	1.52	1.51	0.035	0.05	0.11	0.48
Marbling score[3]	464	444	436	434	7.1	<0.01	<0.01	0.48
Dressing percentage	64.05	65.58	65.99	66.09	0.399	<0.01	<0.01	0.01
KPH, %	1.97	1.97	1.97	1.97	0.013	0.21	0.43	0.86
Calculated final yield grade	3.43	3.07	2.99	2.97	0.078	0.03	<0.01	0.14

[1]Treatments diets were formulated to provide no Z (0 d) or Z (8.32 mg/kg, DM basis) for the last 20, 30, or 40 d of the finishing period

[2]Observed significance levels for orthogonal contrasts: 0 vs. Z = control vs. Z-fed steers; L = linear effects of Z treatment; Q = quadratic effects of Z treatment.

[3]300 = Slight^{00}; 400 = Small^{00}.

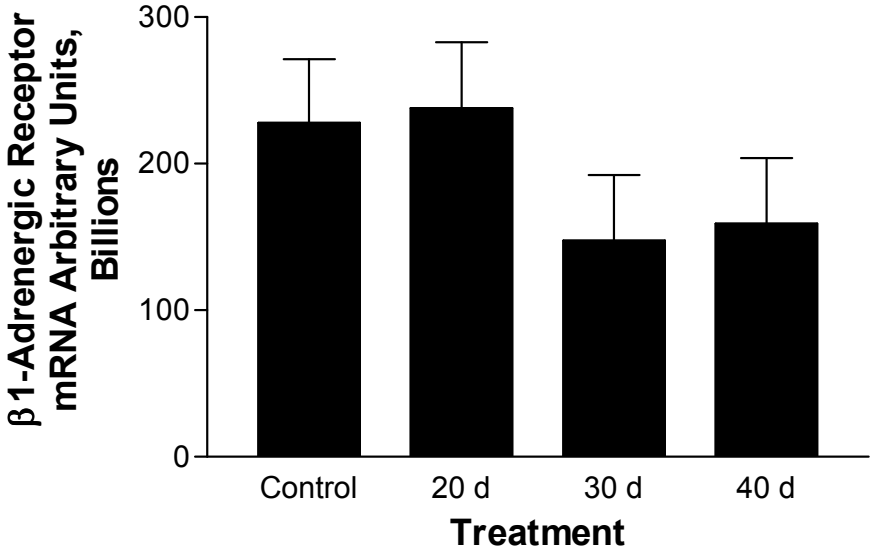

Figure 3.1 β1-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.33$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β1-AR mRNA abundance.

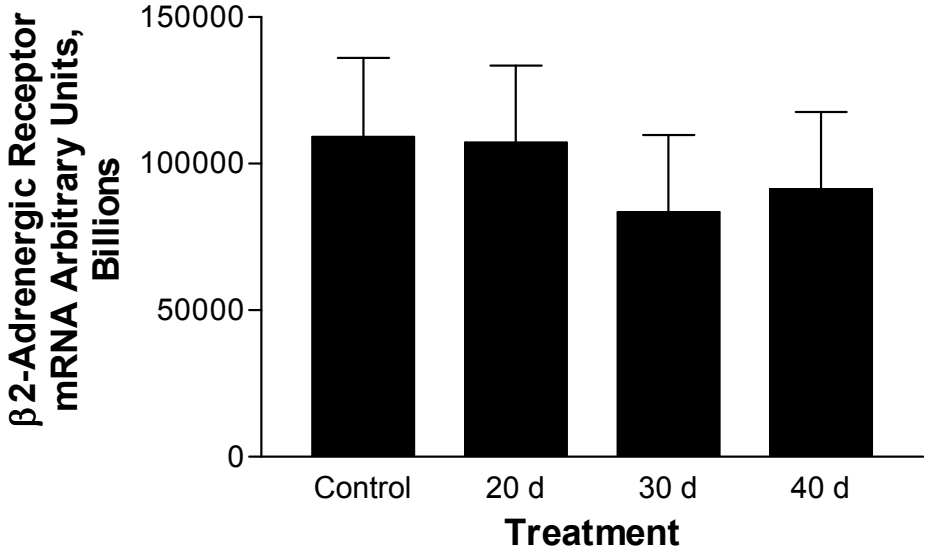

Figure 3.2 β2-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.46$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β1-AR mRNA abundance.

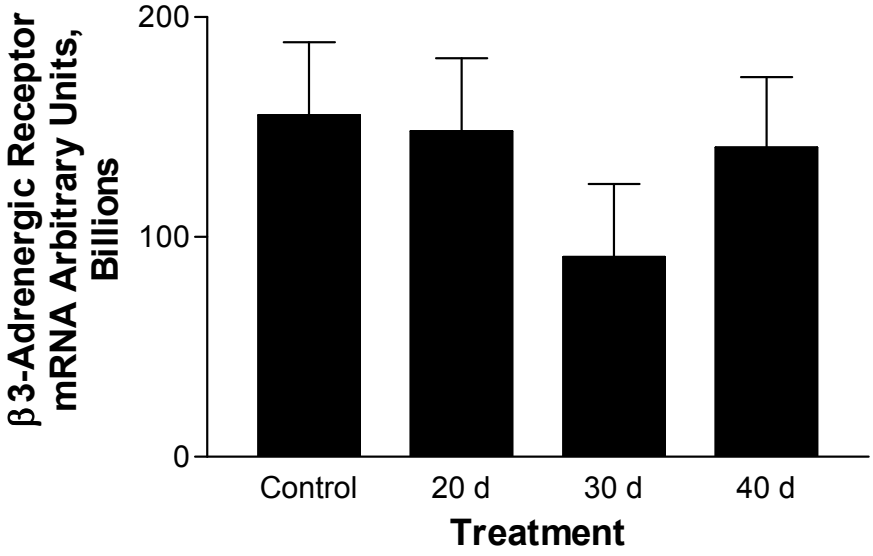

Figure 3.3 β3-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.47$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β1-AR mRNA abundance.

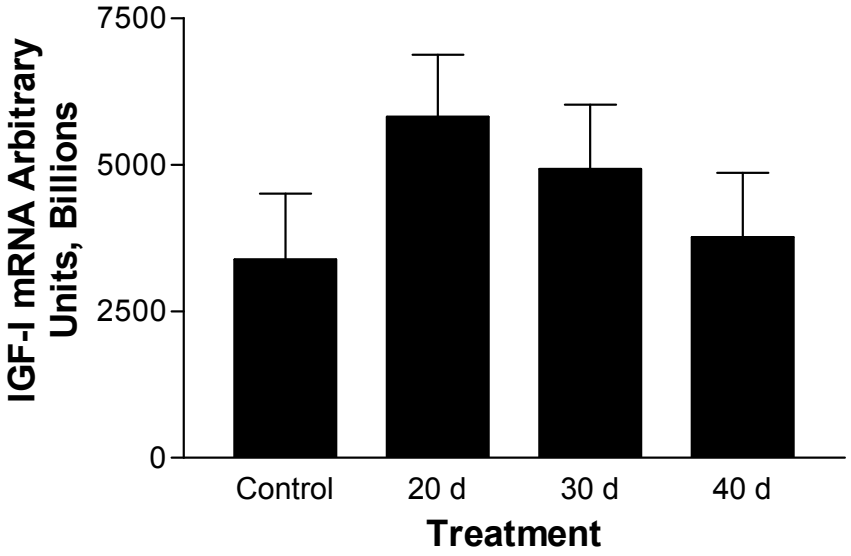

Figure 3.4 Relative abundance of IGF-I mRNA in bovine semimembranosus muscle collected from feedlot steers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.23$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β1-AR mRNA abundance.

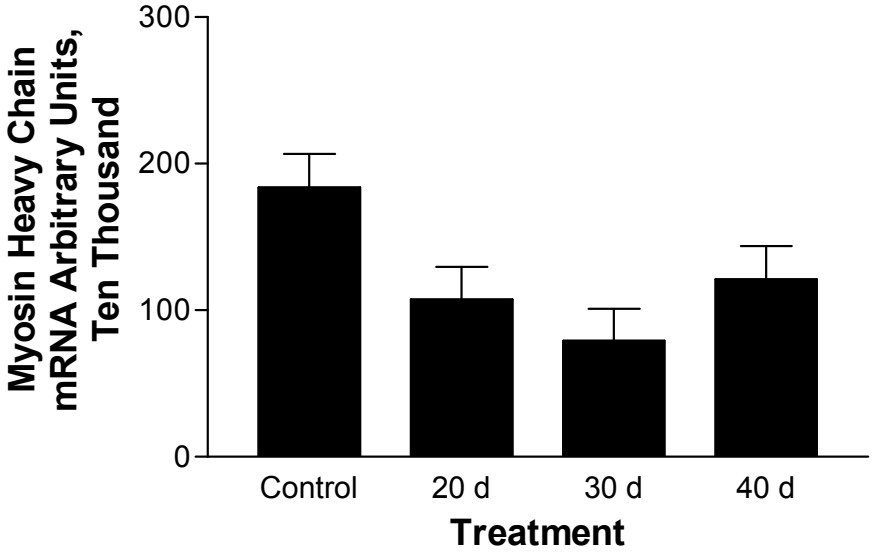

Figure 3.5 Relative abundance of myosin heavy chain (**MHC**) IIA mRNA in bovine semimembranosus muscle collected from feedlot steers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Zilpaterol administration decreased ($P < 0.05$) the expression of myosin mRNA abundance.

CHAPTER 4 - Effect of Zilpaterol and Melengestrol Acetate on Performance, Carcass Characteristics, and Skeletal Muscle Gene Expression in Feedlot Heifers and Cultured Bovine Satellite Cells

Abstract

Two experiments were performed to determine the effects of zilpaterol (**ZIL**) and melengestrol acetate (**MGA**) on feedlot heifer performance, skeletal muscle growth, and muscle gene expression, one *in vivo* and one *in vitro*. British and British x Continental heifer calves (n = 2,660) with an initial BW of 317 kg, were used in a randomized complete block design with 5 treatments. Treatments consisted of control (no MGA or ZIL), ZIL the last 20, 30, or 40 d, or feeding MGA (0.4 mg/hd) with no ZIL. At slaughter, semimembranosus muscle tissue was excised (2 heifers per pen) for RNA isolation. Real-time polymerase chain reaction was used to determine the expression of the β1, β2, β3-adrenergic receptor (**AR**), IGF-I, and myosin heavy chain (**MHC**) IIA mRNA concentrations. There was no effect ($P > 0.05$) of ZIL administration on feed intake or ADG. Regardless of the duration of ZIL feeding, there was an increase ($P < 0.01$) in G:F and LM area in ZIL fed heifers compared to the controls. Zilpaterol increased ($P = 0.03$) HCW, dressing percentage, and decreased ($P < 0.01$) marbling score. Zilpaterol also improved ($P < 0.01$) yield grades and decreased ($P < 0.01$)12th rib fat. There was no effect ($P > 0.05$) of ZIL administration on KPH fat. Melengestrol acetate administration to heifers had no effect on DMI, ADG, G:F, HCW, dressing percentage, and KPH fat; however, MGA decreased ($P < 0.01$) LM area, increased ($P < 0.01$) 12th rib fat and calculated yield grade, and increased ($P = 0.03$) marbling score. Melengestrol acetate, ZIL, or duration of ZIL feeding had no effect on the expression of β1, β2, and β3-AR mRNA. Similarly, they had no effect on the expression of IGF-I mRNA. Melengestrol acetate had no effect of MHC IIA mRNA, but ZIL decreased ($P < 0.05$) the expression of MHC IIA mRNA. Bovine satellite cells were used to assess the effects of MGA (0 and 10 n*M*) on β1, β2, and β3-AR mRNA levels. Cells were plated in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum on tissue culture plates coated with reduced growth factor matrigel. The MGA was added directly onto cell cultures at 0 and 48 h

after plating. At 72 h, total RNA was isolated from the cells and reverse transcribed for complimentary DNA (**cDNA**) synthesis. Real-time quantitative-PCR was performed on the cDNA to measure βAR mRNA abundance. Melengestrol acetate addition (10 n*M*) increased (3.1-fold, $P = 0.01$) β1-AR mRNA abundance. There was also a tendency (3.2 fold, $P = 0.06$) for MGA addition to increase β2-AR mRNA; however, there was no significant effect ($P > 0.10$) on the level of β3-AR mRNA. These results indicate that ZIL can improve heifer performance, and decrease expression of MHC IIA mRNA. Also, MGA can increase the expression of β1-AR mRNA in bovine muscle satellite cell cultures. There was also a tendency for MGA to increase β2-AR levels in cell cultures. These data may aid in our understanding of potential effects of MGA in bovine skeletal muscle growth and development, as well as provide some insight into some possible responses when utilizing ZIL in combination with MGA in feedlot heifers.
Key words: β-adrenergic receptor, bovine, melengestrol acetate, satellite cell

Introduction

Melengestrol acetate (**MGA**) is a synthetic progestin administered to feedlot heifers to inhibit the estrous cycle. It has been used in the United States for over 35 yr to improve feed efficiency, average daily gain, and suppress estrus when administered at the recommended dose range of 0.25 to 0.50 mg/d. It is an effective tool in inhibiting estrus in feedlot heifers; however, there has been unclear evidence as to the effects of MGA on heifer performance and carcass characteristics (Lauderdale, 1983; Adams et al., 1990; Hutcheson et al., 1993; Sissom et al., 2006).

Research suggests steroids, such as progestins, can affect the levels and sensitivity of β-adrenergic receptor (**β-AR**) in different tissue types (Engstrom et al., 2001; Kam et al., 2001). In rats treated with estradiol benzoate, desensitization of the β2-AR function in the rat myometrium was observed (Engrstom et al., 2001). Additionally, estrogen administration to ventricular myocytes was accompanied by reduced protein expression of the β1-AR (Kam et al., 2001). The potential for alterations in β-AR expression due to steroids can have significance to the livestock industry. There are currently two β-AR agonists used to promote growth and efficiency in feedlot cattle, zilpaterol-HCl and ractopamine-HCl. Potential effects, if any, of the use of MGA in conjunction with these compounds have not been investigated. Due to the potential for steroids to alter the β-AR in different tissues, it is important to determine if MGA can have an effect on the expression of the β-AR in bovine skeletal muscle.

The purpose of these experiments was to investigate the effects of ZIL and MGA administration to feedlot heifers on performance, carcass characteristics, and β1, β2, β3-AR, IGF-I, and myosin heavy chain (**MHC**) IIA mRNA concentrations in semimembranosus muscle

tissue. Additionally, we evaluated the effect of MGA on β1, β2, and β3-AR mRNA in cultured proliferating bovine muscle satellite cells.

Materials and Methods

The following experiments were a collaboration between Intervet, Inc. (Millsboro, DE), Cactus Research, LTD (Amarillo, TX) and Kansas State University. Research conducted at commercial research facilities followed the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999, Savoy, IL).

Animals

British and British x Continental heifer calves (n = 2,660) with an initial BW of 317 kg, were used in a randomized complete block design with 5 treatments. Treatments consisted of control (no MGA or ZIL), ZIL the last 20, 30, or 40 d, or feeding MGA (no ZIL, 0.4 mg/hd). Treatments were randomly assigned to each pen (n = 30) with approximately 88 heifers per pen. At arrival, heifer calves were implanted at with Revalor-IH (80 mg trenbolone acetate and 8 mg estradiol) and reimplanted with Revalor-H (140 mg trenbolone acetate and 14 mg estradiol) 81 d prior to harvest. Heifers were fed 3 times daily during the transition period and 2 times daily for the remainder of the study, and allowed ad libitum access to feed. The finishing diet was based on steam-flaked corn (Table 4.1). Feed additives (Rumensin, Tylan, MGA, and vitamins A, D and E) were added using a micro-weigh machine (Micro Beef Technologies, Amarillo, TX). In the MGA ration, 0.4 mg/hd was administered. During the zilpaterol-treatment time for each group, the diet fed for the prescribed duration contained 8.32 mg/kg of Zilpaterol HCl, but no Rumensin or Tylan. For determination of final BW, pens were weighed and the industry standard 4% pencil shrink was applied. Animals were transported 96 km to a commercial

slaughter facility (Tyson Fresh Meats Inc., Amarillo, TX). Carcass characteristics were obtained from chilled carcasses 36 h after slaughter.

Sample Preparation and RNA Isolation

Within 10 min of slaughter at the abattoir, a muscle sample was collected from the semimembranosus muscle of 2 randomly selected heifers per pen. The samples were snap frozen in liquid nitrogen and delivered to Kansas State University for analysis. Total RNA was isolated from muscle samples using TRI Reagent (Sigma, St. Louis, MO). Semimembranosus muscle tissue (100 mg) was transferred to a steel mortar bowl cooled by liquid nitrogen. The samples were homogenized using a sterile pestal in liquid nitrogen. TRI Reagent (3 mL) was then added to the ground tissue sample. One mL of muscle tissue in Tri Reagent was incubated at room temperature for 5 min. Following incubation, chloroform (Sigma, St. Louis, MO) was added and samples were centrifuged for 15 min at 12,000 x g at room temperature. Following centrifugation, the top layer was removed and transferred to a new microcentrifuge tube. Isopropanol (Sigma, St. Louis, MO) was added and samples were centrifuged for 10 min at 12,000 x g to isolate the RNA pellet. The RNA pellet was then treated to remove any contaminating genomic DNA using the DNA-*free* kit (Ambion, Austin, TX). The RNA concentration was determined by absorbance at $260_{nm.}$ The integrity of the RNA was determined by gel electrophoresis. Total RNA with ethidium bromide was loaded onto a 1% agarose gel to separate and visualize the 28S and 18S rRNA.

Bovine Satellite Cell Isolation

Satellite cell isolation was conducted as described previously (Johnson et al., 1998). Cattle were sacrificed by bolting followed by exsanguination. Using sterile techniques, approximately 500 g of the semimembranosus muscle was dissected out and transported to the

cell culture laboratory. Subsequent procedures were conducted in a sterile field under a tissue culture hood. After removal of connective tissue the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% pronase in Earl's Balanced Salt Solution (**EBSS**) for 1 hour at 37° C with frequent mixing. Following incubation, the mixture was centrifuged at 1500 x *g* for 4 min, the pellet was suspended in phosphate buffered saline (**PBS**: 140 m*M* NaCl, 1 m*M* KH_2PO_4, 3 m*M* KCl, 8 m*M* Na_2HPO_4), and the suspension was centrifuged at 500 x *g* for 10 min. The supernatant was centrifuged at 1500 x *g* for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated two more times. The resulting mononucleated cell preparation was suspended in cold (4° C) Dulbecco's Modified Eagle Medium (**DMEM**) containing 10% fetal bovine serum (**FBS**) and 10% (v/v) dimethylsulfoxide (**DMSO**) and frozen. Cells were stored frozen in liquid nitrogen.

RNA Isolation from Satellite Cells

Bovine satellite cells were plated in 10% FBS/DMEM. MGA (0, and 10 n*M*) was added to the cultures immediately following plating. Previous work in our laboratory showed that 10 n*M* MGA was the lowest level to elicit a response on cell proliferation and was used in subsequent experiments. We further showed that levels lower than 10 n*M* were also effective in reducing cell proliferation, but we chose to continue with 10 n*M* in the present study due to the results reported in earlier work (Sissom et al., 2006). At 48 h, cultures were rinsed three times with serum-free DMEM and fresh 10% FBS/DMEM. At 72 h, total RNA was isolated using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). The concentration of RNA was determined by absorbance at 260_{nm}. One microgram of total RNA was reverse transcribed to produce first-strand complementary DNA (**cDNA**) using TaqMan Reverse Transcription Reagents, MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA), and the

protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis.

Real-time quantitative-PCR

Real-time quantitative-PCR was used to measure the quantity of β1-AR, β2-AR, β3-AR, IGF-I, and MHC IIA gene expression relative to the quantity of 18S ribosomal RNA (**rRNA**) in total RNA isolated from muscle tissue and satellite cells. Measurement of the relative quantity of cDNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 n*M* of the appropriate forward and reverse primers, 200 n*M* of appropriate TaqMan detection probe, and 1 μL of the cDNA mixture. The bovine specific β1-AR, β2-AR, and β3-AR, IGF-I, and MHC IIA forward and reverse primers and TaqMan detection probes (Table 4.2) were synthesized using published GenBank sequences. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession #X03205). The ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA) was used to perform the assay utilizing the recommended thermal cycling variables by the manufacturer (50 cycles of 15 s at 95ºC and 1 min at 60ºC). The 18S rRNA endogenous control was used to normalize the expression of β1-AR, β2-AR, β3-AR, IGF-I, and MHC IIA.

Statistical Analysis

The heifer data were analyzed as a randomized complete block design with PROC MIXED (SAS Inst., Cary, NC). For the MGA data, pen served as the experimental unit, and means were separated ($P < 0.05$) with the least significance difference procedure of SAS. For all ZIL analysis, specific orthogonal contrasts were used to test 1) control vs. the average of the 3 duration of ZIL feeding groups and 2) linear and quadratic effects of duration of ZIL feeding.

Cell culture data were analyzed as a completely randomized design using the MIXED model (SAS Inst., Cary, NC). For the *in vitro* experiment, the difference between control and MGA treatment was determined using the least significance difference procedure. Means were determined to be significantly different at $P < 0.05$.

Results and Discussion

Effect of MGA Administration to Feedlot Heifers on Performance and Carcass Characteristics

The data for performance and carcass characteristics are shown in Tables 4.3 and 4.4. Melengestrol acetate administration to heifers had no effect on DMI, ADG, G:F, HCW, dressing percentage, and KPH fat; however, MGA decreased ($P < 0.01$) LM area, increased ($P < 0.01$) 12th rib fat and calculated yield grade, and increased ($P = 0.03$) marbling score. Melengestrol acetate is an effective tool in inhibiting estrus in feedlot heifers; however, there has been unclear evidence as to the effects of MGA on heifer performance and carcass characteristics (Bloss et al., 1966; Lauderdale, 1983; Hutcheson et al., 1993; Sissom et al., 2006). Some of the earliest work done with MGA in feedlot heifers showed an improvement in weight gain and feed efficiency in intact heifers with doses ranging from 0.35 to 0.50 mg/d (Bloss et al., 1966). This study also reported no significant effect on carcass weights, grades or dressing percentage. In a review of numerous studies with data from around 10,000 heifers, MGA showed to be effective in improving ADG by 10% and feed efficiency by 6.5% (Lauderdale, 1983). On the other hand, carcass quality in these studies was not altered by the administration of MGA in the diet (Lauderdale, 1983). There have been studies that suggest MGA alters carcass characteristics as we observed in the current study (Hutcheson et al., 1993). In a review of various trials, MGA was not effective in improving gain or feed efficiency; however, fat thickness and the percentage

of heifers with yield grades 4 & 5 increased (Hutcheson, 1993). This data is in agreement with our results that suggest MGA may be responsible for earlier maturing heifers, thus resulting in increased fat thickness, smaller rib eye area, and increased yield grade.

One possible mechanism through which early maturity may occur is through early satellite cell withdrawal form the cell cycle possibly through regulation of growth factors important in this process. Satellite cells are necessary for supporting postnatal muscle hypertrophy; however, as an animal ages, the number of the satellite cells present is greatly reduced, causing a growth plateau. If these cells are pushed into the differentiation pathway early, this will reduce the number available for continued muscle growth, possible causing early-maturity in animals. Once the satellite cells available have fused and muscle growth has ceased, this shifts calories to adipose tissue thus causing a greater accumulation of fat, possible leading to the increased fat thickness often observed in MGA heifers. We previously reported that MGA addition to cultured bovine satellite cells and C_2C_{12} cells reduces the rate of cell proliferation (Sissom et al., 2006). The addition of MGA also increased IGF-I mRNA and the addition of progesterone to bovine satellite cells increased myogenin mRNA. These data suggested that MGA may have been causing the cells to withdrawal from the cell cycle early and terminally differentiate with muscle fibers. That data would support the early maturity phenomenon reported with the decreased LM area, increased 12^{th} rib fat, and increased calculated yield grade we observed.

Effect of ZIL Administration to Feedlot Heifers on Performance and Carcass Characteristics

The data for performance over the entire finishing period and carcass characteristics showing the effect of ZIL is shown in Table 4.4. There was no effect ($P > 0.05$) of ZIL administration on feed intake or ADG. Regardless of the duration of ZIL feeding, there was an

increase ($P < 0.01$) in G:F, HCW, LM area, and dressing percentage in ZIL fed animals compared to the controls. Zilpaterol administration decreased ($P < 0.01$) marbling score, 12th rib fat, and improved ($P < 0.01$) yield grades regardless of ZIL duration. There was no effect ($P > 0.05$) of ZIL administration on KPH fat. These data are consistent with other studies utilizing β-AR agonists such as ractopamine and ZIL as growth promoters. In feedlot steers administered 6 mg/kg of zilpaterol in the diet daily, there was an increase in average daily gain and improved gain efficiency (Plascencia et al., 1999; Vasconcelos et al., 2008). Zilpaterol supplementation also increased carcass weight by 4.5%, dressing percentage by 3.6%, and longissimus muscle area by 2.7% compared to control steers. There was no effect of zilpaterol on fat thickness or marbling score (Vasconcelos et al., 2008). Both zilpaterol (60 mg/d) and ractopamine (300 mg/d) were investigated in feedlot cattle in a study to compare the two β-agonists (Avendano-Reyes et al., 2006). Both ractopamine and ZIL improved ADG, feed efficiency, and HCW. Additionally, ZIL increased longissimus muscle area; however, both ractopamine and ZIL increased shear force values compared to control steers. The authors commented that the β-agonists were effective in enhancing performance of the cattle without having a substantial effect on meat quality (Avendano-Reyes et al., 2006; Vasconcelos et al., 2008). Similar improvements in animal performance have been reported with ractopamine administration. In feedlot steers, ractopamine fed the final 28 d of the finishing period at 200 mg/d resulted in improved ADG, feed efficiency, HCW, and larger LM area (Gruber et al., 2007). Winterholler et al. (2007) reported similar findings in yearling steers fed 200 mg/d of ractopamine the final 28 d of the feeding period. Steers fed ractopamine had increased ADG and improved gain efficiency, as well as increased HCW and LM area.

Effect of MGA and ZIL Administration to Feedlot Heifers on Semimembranosus Muscle β1, β2, and β3-AR mRNA Concentrations

There was no effect ($P > 0.05$) of MGA or ZIL administration, or duration of ZIL feeding ($P > 0.10$) on semimembranosus muscle β1, β2, and β3-AR mRNA concentrations (Figures 4.1 to 4.6). The expression of β-AR in bovine skeletal muscle is of importance because of the utilization of β-AR agonists such as ZIL in the feedlot industry today. The number of receptors can impact the response to such compounds, therefore having an effect on animal performance. It is important to understand any potential interactions or synergistic effects steroids may have on β-AR number or expression if MGA or steroid implants are used in conjunction with β-agonists. Steroid hormones have been shown to affect the sensitivity and number of β-AR in numerous tissues (Engstrom et al., 2001; Malo and Puerta, 2001). In the rat myometrium, estradiol benzoate treatment resulted in desensitization of β2-AR function, while progesterone had no effect on the β2-AR (Engstrom et al., 2001). Additionally, the treatment of ventricular myocytes with estrogen resulted in reduced protein expression of the β1-AR (Kam et al., 2004). In a similar manner, estradiol and progesterone administration reduced the density of β3-AR in interscapular brown adipocytes of male Wistar rats (Malo and Puerta, 2001). Additionally, the binding affinity for the β3-AR in the brown adipocytes was increased with estradiol and progesterone administration (Malo and Puerta, 2001). In male Sprague-Dawley rats, castration resulted in a decrease in β-AR numbers, and this reduction was returned to normal levels with testosterone treatment (Xu et al., 1991). These data demonstrate that steroid hormones can differentially affect β-AR; however, we were unable to detect any changes in semimembranosus muscle β-AR mRNA expression due to MGA administration.

Effect of MGA and ZIL Administration to Feedlot Heifers on Semimembranosus Muscle IGF-I and MHC IIA mRNA Concentrations

There was no effect ($P > 0.05$) of MGA or ZIL administration, or duration of ZIL feeding ($P > 0.10$) on semimembranosus muscle IGF-I mRNA concentrations (Figures 4.7 and 4.8). There is an abundant amount of information as to the effects of estrogens and androgens on skeletal muscle growth in animals. Conversely, there is little information on the effects of progestins, or specifically MGA, on skeletal muscle growth. We previously reported that MGA addition to cultured bovine satellite cells and C_2C_{12} cells reduces the rate of cell proliferation (Sissom et al., 2006). The addition of MGA also increased IGF-I mRNA and the addition of progesterone to bovine satellite cells increased myogenin mRNA. The majority of research suggests estrogens and androgens increase skeletal muscle growth through up-regulation of IGF-I in both circulation and local skeletal muscle production (Dunn et al., 2003; Pampusch et al., 2003). Dunn et al. (2003) observed increased muscle IGF-I mRNA in steers implanted with a combined trenbolone acetate (**TBA**)/estradiol (**E_2**) implant. The implanted steers also had greater circulating IGF-I by d 14. Pampusch et al. (2003) observed similar increases in IGF-I of both the liver and skeletal muscle in steers implanted with TBA/E_2. In steers implanted with TBA/E_2, circulating IGF-I concentrations were increased compared to control (Johnson et al., 1996). The effects of MGA appear to be different than that of estrogens and androgens. In heifers administered MGA, circulating IGF-I was not different compared to heifers not receiving MGA (Mader and Kreikmeier, 2006). As mentioned previously, we have shown that MGA increased IGF-I mRNA in bovine satellite cell cultures (Sissom et al., 2006). We did not, however, detect any differences in semimembranosus muscle tissue from MGA fed heifers on IGF-I mRNA in the current study. The lack of effect of ZIL administration on the expression of

IGF-I mRNA in semimembranosus muscle tissue is similar to that of other studies. The majority of research would suggest there is no role for IGF-I in β-agonist induced skeletal muscle growth. Grant et al. (1993) reported no change in IGF-I mRNA expression in both skeletal muscle and liver in pigs fed ractopamine. Additionally, O'Connor et al. (1991) observed no change in IGF-I concentrations following administration of cimaterol for 3 wk. Similarly, clenbuterol administration to growing lambs resulted in no change in IGF-I concentrations (Young et al., 1995). Additionally, there was a decrease in mRNA expression of IGF-I in the longissimus muscle tissue collected at harvest. In growing lambs fed cimaterol for 6 wk, there was a decrease in IGF-I level in circulation (Beermann et al., 1987).

There was no effect ($P > 0.05$) of MGA administration on semimembranosus muscle MHC IIA (Figure 4.9) mRNA concentrations. There is very little information on the effects of steroids on MHC expression in skeletal muscle, and there is no information available on the direct effects of MGA on MHC in skeletal muscle. Other steroids have been shown to have effects on protein synthesis and degradation (Roeder et al., 1986; Desler et al., 1996), therefore it would be suggested that they would affect MHC due to it being the most abundant skeletal muscle protein. However, in young male Wistar rats, nandrolone (nortestosterone decanoate) treatment for 25 d had no effect on the level of MHC in the soleus and extensor digitorum longus muscles (Noirez and Ferry, 2000). There is no information suggesting MGA has direct growth promoting effects on skeletal muscle growth like other steroid hormones; therefore we were not surprised we were unable to detect changes in MHC expression in skeletal muscle from MGA fed heifers in the present study.

The expression of MHC IIA was decreased ($P < 0.05$) by ZIL administration, but as the duration of ZIL feeding increased, MHC IIA mRNA increased ($P = 0.05$) in heifers (Figure

4.10). Myosin heavy chain is a major protein in skeletal muscle that accounts for around 30% of all protein in skeletal muscle. There are different isoforms of MHC that are important in determining muscle fiber type. Slow twitch fibers are predominantly MHC I, whereas fast twitch fibers are predominantly MHC IIA and IIX. It has been reported that β-agonist treatment can alter the expression of MHC isoforms, which can result in subsequent changes in muscle composition (Rehfeldt et al., 1997; Depreux et al., 2002). Depreux et al. (2002) observed a decrease in MHC IIA and IIX mRNA expression and an increase in MHC IIB mRNA in pigs fed ractopamine. These findings are similar to our data. However, we did not measure other isoforms of MHC so we can only report on the effects of ZIL on the reduction in MHC IIA. It is also important to note that MHC IIB mRNA is not detected in bovine skeletal muscle tissue (Chikuni et al., 2004). The reduction in MHC IIA in skeletal muscle may also be an indirect response to decreases in protein degradation due to ZIL administration. β-Agonists, especially those specific for the β2-AR have been shown to decrease protein degradation in skeletal muscle. Wheeler and Koohmaraie (1992) reported a 27.1% reduction in fractional degradation rate of skeletal muscle myofibrillar protein in steers fed the β-agonist L-644,969 after 3 wk. These data provide a possible explanation for the reduction in MHC IIA mRNA concentration observed in the semimembranosus muscle tissue collected from the heifers fed ZIL.

Effect of MGA on Bovine Satellite Cell β1, β2, and β3-AR mRNA Concentrations

Melengestrol acetate addition (10 n*M*) increased (3.1-fold, $P = 0.01$) β1-AR mRNA abundance in bovine satellite cell cultures (Figure 4.11). There was also a tendency (3.2 fold, $P = 0.06$) for MGA addition to increase β2AR mRNA (Figure 4.12); however, there was no significant effect ($P > 0.10$) on the level of β3AR mRNA (Figure 4.13). As previously mentioned, steroid hormones can alter both the sensitivity and number of β-AR in different

species and specific tissues (Engstrom et al., 2001; Malo and Puerta, 2001). This has been shown with estrogen, progesterone, and testosterone. MGA is a synthetic progestin that is more potent than progesterone. We previously reported that MGA increased IGF-I mRNA in bovine satellite cells 2.2 times that of control cultures, whereas progesterone had no effect of IGF-I mRNA. Additionally, MGA reduced cell proliferation in a dose-dependent manner, while only one level of progesterone decreased cell proliferation. We did not observe an effect of MGA on β-AR in semimembranosus muscle tissue in the current study. This lack of effect of MGA *in vivo* may be a result of a multitude of different factors. The heifers had been on MGA for a long duration before the muscle tissue was collected for analysis, while the *in vitro* experiments were only exposed to MGA for a period of 72 h. The change in expression of β-AR may occur at an earlier time point and decrease over a longer period of time. This theory is also supported by the IGF-I data. We did not observe any changes in IGF-I mRNA from semimembranosus muscle tissue of heifers fed MGA. However, we previously reported increased IGF-I mRNA in bovine satellite cell cultures with 48 h MGA exposure (Sissom et al., 2006). The previous increase of IGF-I mRNA reported from *in vitro* experiments like the ones observed in the present study with the β-AR support the theory that the shorter exposure to MGA may be responsible for the changes observed *in vitro* with a lack of changes in the corresponding *in vivo* study. However, the increased β1-AR and β2-AR mRNA *in vitro* accompanied by other data still suggest a possibility for steroids such as MGA to alter the expression of these receptors in skeletal muscle, even though we did not detect any differences in the current heifer experiment.

Further investigation is needed to determine if steroids used in feedlot cattle such as MGA, TBA, or E_2 have an effect on the β-AR number or expression because of the use of β-AR agonists as tools for increased animal performance and efficiency. Steroids and β-AR agonists

have different modes of action; therefore it is possible that if these two different classes of compounds are used together, there may be interactions or synergistic responses. One of the important factors is duration of feeding of MGA and implantation. Melengestrol acetate is administered throughout the entire finishing period whereas β-AR agonists such as ZIL are fed at the end of the finishing period. Steroid containing implants are used at different time points during the finishing period and the implant release patterns and payout times are different between implant types (Preston, 1999). These differences can impact animal performance and can make it more difficult to discern interactions or synergistic responses between different classes of compounds. There is however more recent evidence of steroid hormones working through second messenger systems such as cyclic adenosine monophosphate and intracellular calcium, similar to that of the β-AR agonists mode of action (Falkenstein et al., 2000). These responses are called nongenomic actions, and we have previously reported this with MGA in bovine satellite cells (Sissom et al., 2006). This recent information suggests a potential point for interaction with steroid hormones and β-AR agonists. Additionally, we have observed that the response to ractopamine, a β1-AR agonist, is altered by different implant strategies in heifers (Sissom et al., 2007). Unfortunately, all heifers in the previous study received MGA; therefore we were unable to ascertain any effects of MGA on the expression of β-AR mRNA in that study. We did not see any effects of MGA in the present study on β-AR expression; however with the vast use of steroid implants today to promote growth and efficiency, more investigation with other steroid hormones is warranted at the present time.

Overall, the results from our study suggest that MGA can decrease LM area while increasing 12th rib fat leading to increased calculated yield grades. However, zilpaterol administration can positively affect heifer performance and carcass characteristics. Additionally,

MGA can alter the expression of β-AR *in vitro*, but there are no effects of MGA or ZIL on skeletal muscle gene expression of β1, β2, β3-AR, and IGF-I mRNA concentrations in semimembranosus muscle tissue collected from feedlot heifers fed MGA. However, ZIL decreased the expression of MHC IIA which may be an indicator of decreased protein degradation. These data suggest that MGA does not have the effects that other steroids such as TBA and E_2 have on skeletal muscle growth and development in heifers. This information aids in our understanding of the effects of MGA and ZIL on heifer growth skeletal muscle and development.

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Table 4.1 Composition of the diet fed to heifers[1]

	Amount, % of DM		
Item	Control	MGA	Zilpaterol[2]
Corn, steam-flaked	72.9	72.9	72.9
Corn DDGS[3]	9.9	9.9	9.9
Silage, Corn	10.1	10.1	10.1
Tallow	3.0	3.0	3.0
Supplement	4.0	4.0	4.0
Micro-ingredients[4]			
Rumensin, mg/kg	37.2	37.2	
Tylan, mg/kg	10.8	10.8	
Zilmax, mg/kg			8.32
MGA, mg/hd/d		0.4	
Vitamin A, IU/kg	3,080	3,080	3,080
Vitamin D, IU/kg	208	208	208
Vitamin E, IU/kg	11	11	11

[1]Fed through the end of the trial, including the 3 d withdrawal following zilpaterol feeding.
[2]Fed to zilpaterol treatments for 20, 30 or 40 d.
[3]Corn dried distillers grains with solubles.
[4]Added using a micro-weigh machine (Micro Beef Technologies, Amarillo, TX).

Table 4.2. Sequences for β-1, β-2, and β-3 adrenergic receptors, IGF-I, and myosin heavy chain IIA specific PCR primers and TaqMan probes

β-1 adrenergic receptor (Accession #AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
β-2 adrenergic receptor (Accession #NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
β-3 adrenergic receptor (Accession #XF86961)	
Forward	AGGCAACCTGCTGGTAATCG
Reverse	GTCACGAACACGTTGGTCATG
TaqMan probe	6FAM-CCCGGACGCCGAGACTCCAG-TAMRA
IGF-1 (Accession #X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan probe	6FAM-TGCCCATCACATCCTCCTCGCA-TAMRA
Myosin heavy chain IIA (Accession #AB059398)	
Forward	CCCCGCCCCACATCTT
Reverse	TCTCCGGTGATCAGGATTGAC
TaqMan probe	6FAM-TCTCTGACAACGCCTATCAGTTCAT-TAMRA

Table 4.3 Effects of MGA administration on performance of feedlot heifers

	Treatment			
Item	Control	MGA	SEM	*P*-value
Pens	6	6	-	-
Starting weight, kg	318	316	11	0.62
DMI, kg/d	7.85	7.96	0.12	0.49
ADG, kg	1.31	1.34	0.03	0.43
G:F	0.167	0.168	0.002	0.61
Hot carcass weight, kg	341	344	1.2	0.22
LM area, cm^2	92.09	87.87	0.502	<0.0001
12th rib fat, cm	1.34	1.46	0.026	<0.001
Marbling score	429	440	3.5	0.03
Dressing percentage	64.53	64.70	0.162	0.40
KPH, %	1.98	1.98	0.005	0.89
Calculated final yield grade	2.52	2.86	0.034	<0.0001

Table 4.4 Effects of zilpaterol (Z) administration on performance of feedlot heifers

	Zilpaterol[1]					*P*-values[2]		
Item	0	20	30	40	SEM	0 vs. Z	L	Q
Pens	6	6	6	6	-	-	-	-
Starting weight, kg	318	316	317	315	12	0.61	0.81	0.71
DMI, kg/d	7.85	7.81	7.87	7.69	0.11	0.64	0.89	0.70
ADG, kg	1.31	1.33	1.36	1.36	0.04	0.23	0.24	0.88
G:F	0.167	0.171	0.173	0.177	0.003	<0.01	0.06	0.88
Hot carcass weight, kg	341	349	354	355	3.8	0.03	0.04	0.85
LM area, cm^2	92.1	99.9	100.5	102.1	1.043	<0.01	<0.01	<0.01
12th rib fat, cm	1.34	1.22	1.28	1.22	0.033	<0.01	0.14	0.01
Marbling score	429	417	406	405	3.6	<0.01	<0.01	0.81
Dressing percentage	64.53	65.71	65.99	66.17	0.235	<0.01	<0.01	0.05
KPH, %	1.971	1.974	1.977	1.983	0.014	0.12	0.18	0.42
Calculated final yield grade	2.52	2.06	2.14	2.05	0.037	<0.01	<0.01	<0.01

[1]Treatments diets were formulated to provide no Z (0 d) or Z (8.32 mg/kg, DM basis) for the last 20, 30, or 40 d of the finishing period

[2]Observed significance levels for orthogonal contrasts: 0 vs. Z = control vs. Z-fed heifers; L = linear effects of Z treatment; Q = quadratic effects of Z treatment.

[3]300 = $Slight^{00}$; 400 = $Small^{00}$.

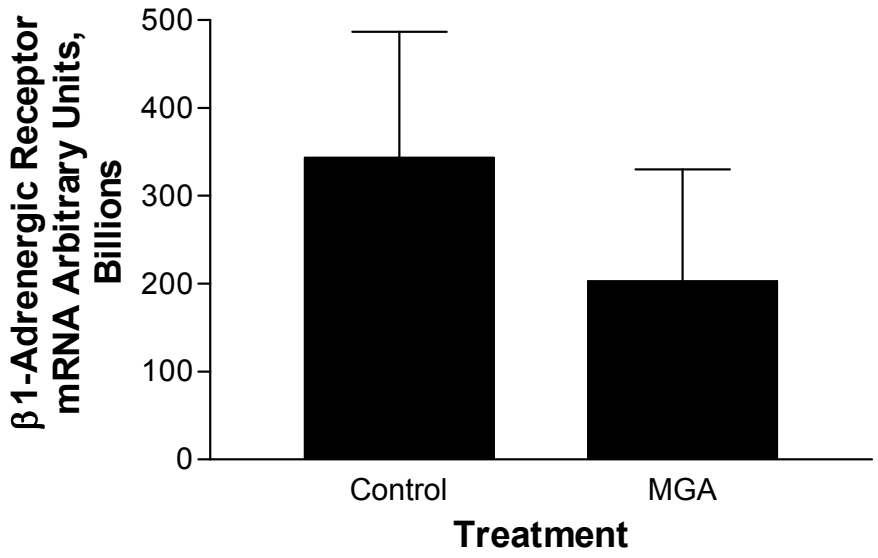

Figure 4.1 β1-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/ treatment). Bars are means ± SEM relative to control. There was no effect ($P > 0.05$) of MGA on β1-AR mRNA.

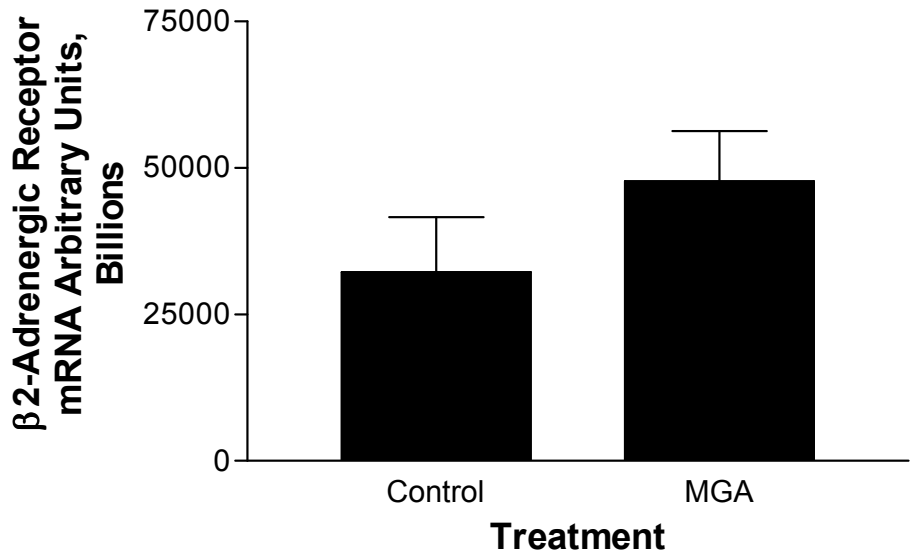

Figure 4.2 β2-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/ treatment). Bars are means ± SEM relative to control. There was no effect ($P > 0.05$) of MGA on β2-AR mRNA.

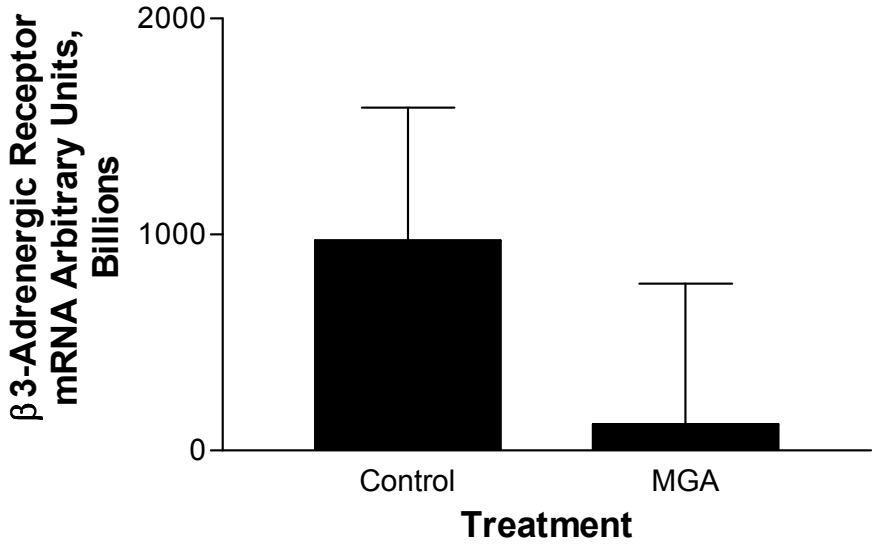

Figure 4.3 β3-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/ treatment). Bars are means ± SEM relative to control. There was no effect ($P > 0.05$) of MGA on β3-AR mRNA.

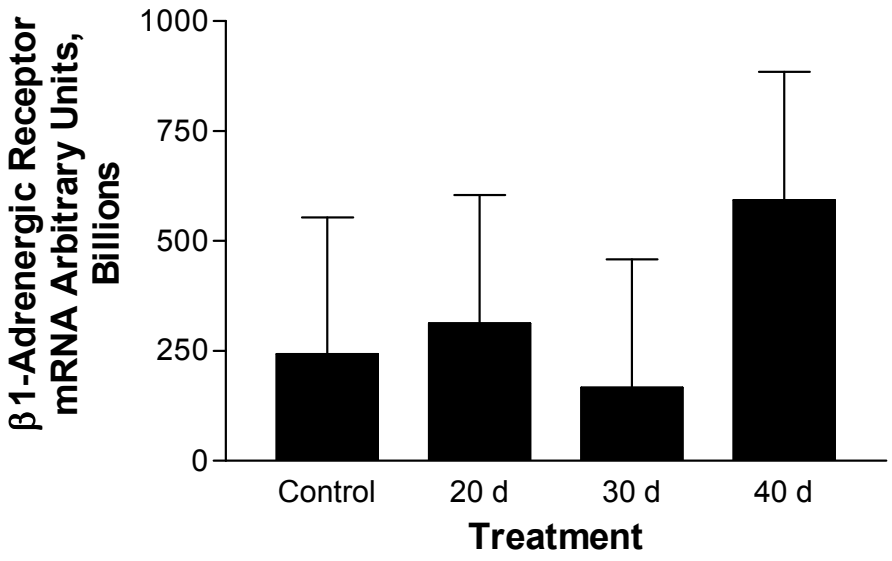

Figure 4.4 β1-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.73$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β1-AR mRNA abundance.

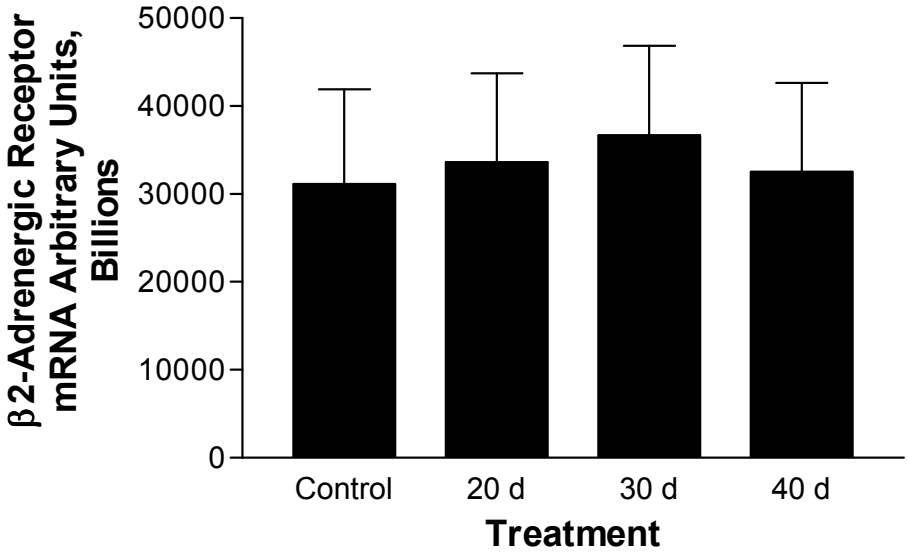

Figure 4.5 β2-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.77$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β2-AR mRNA abundance.

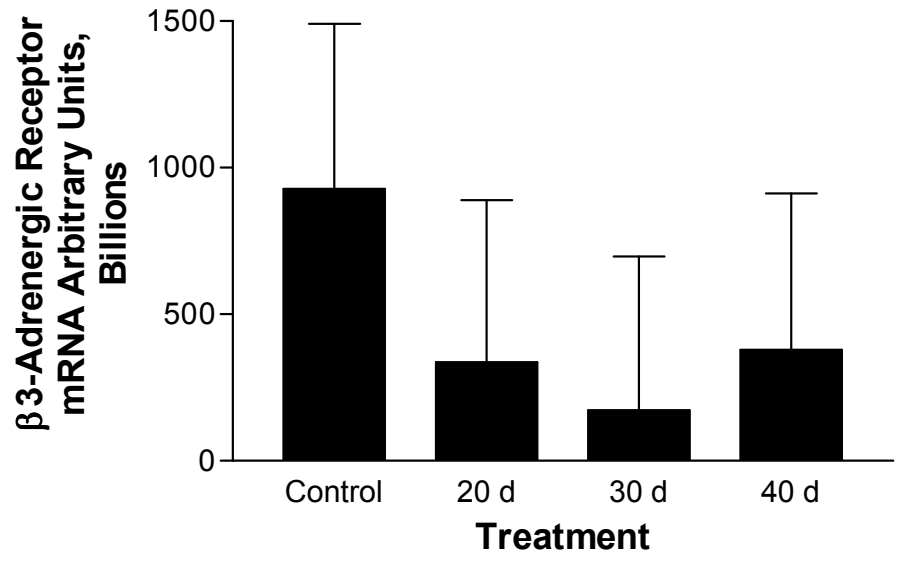

Figure 4.6 β3-Adrenergic receptor (**AR**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.93$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of β3-AR mRNA abundance.

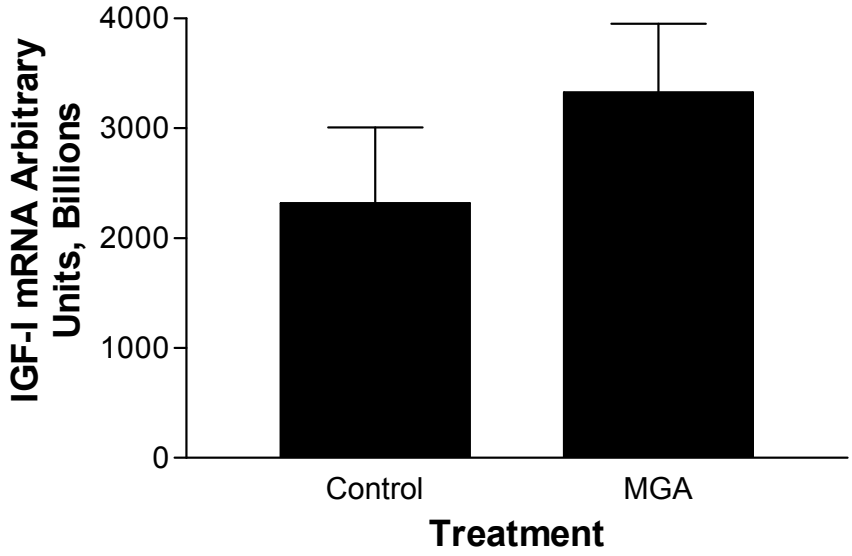

Figure 4.7 Insulin-like growth factor I (**IGF-I**) mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/ treatment). Bars are means ± SEM relative to control. There was no effect ($P > 0.05$) of MGA on IGF-I mRNA.

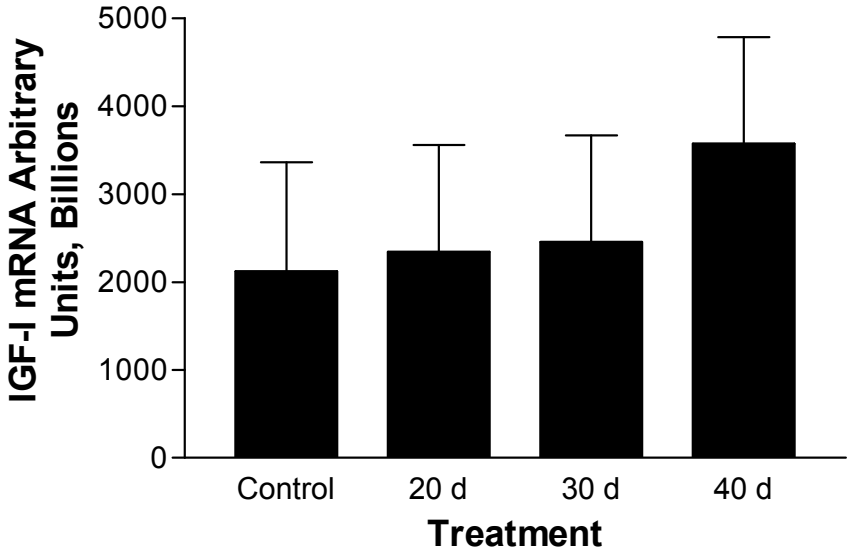

Figure 4.8 Relative abundance of IGF-I mRNA in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. Neither the effect of adding ZIL ($P = 0.46$) nor the duration of ZIL feeding ($P > 0.10$) affected the expression of IGF-I mRNA abundance.

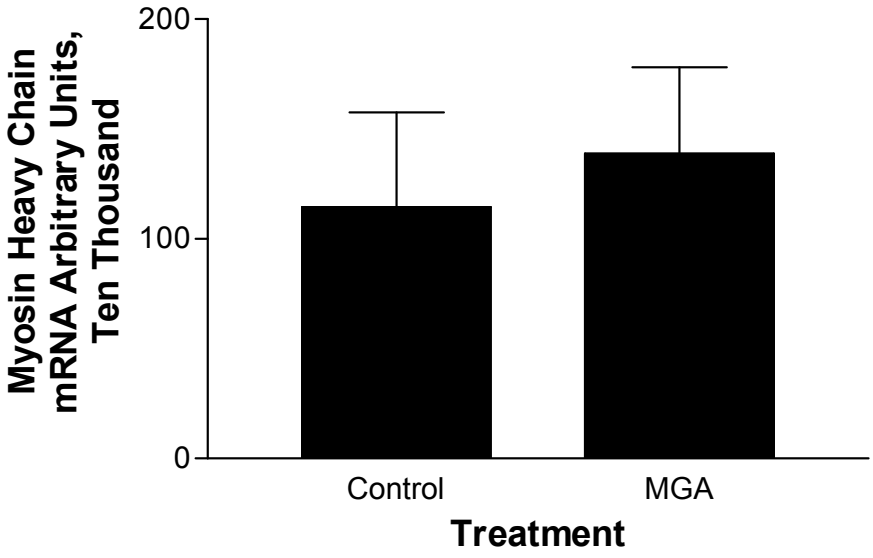

Figure 4.9 Myosin heavy chain (**MHC**) IIA mRNA abundance in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/ treatment). Bars are means ± SEM relative to control. There was no effect ($P > 0.05$) of MGA on MHC IIA mRNA.

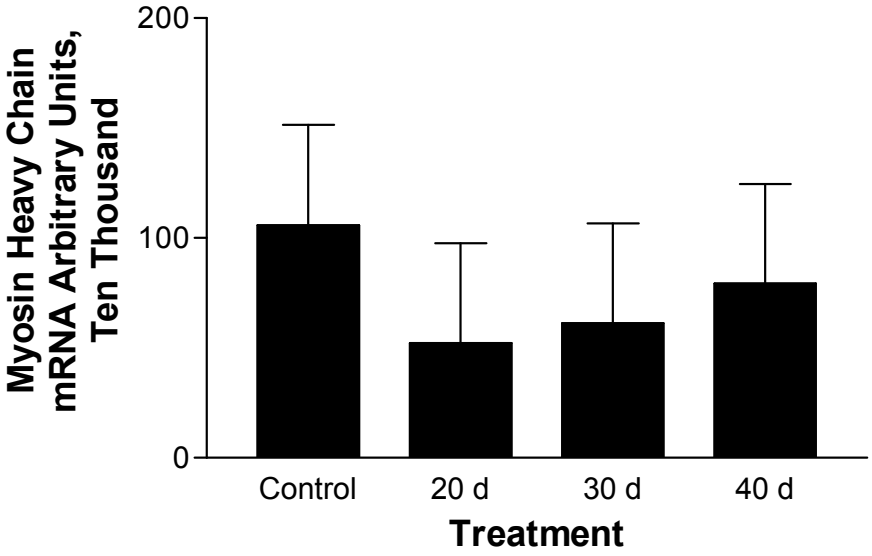

Figure 4.10 Relative abundance of myosin heavy chain (**MHC**) IIA mRNA in bovine semimembranosus muscle collected from feedlot heifers 10 min postslaughter. Two animals per pen were used in the analysis (6 pens/treatment). Treatments consisted of: 1) no zilpaterol (**ZIL**; control), 2) 20 d ZIL, 3) 30 d ZIL, or 4) 40 d ZIL. The expression of MHC IIA was decreased ($P < 0.05$) by ZIL administration, but as the duration of ZIL feeding increased, MHC IIA mRNA increased ($P = 0.05$) in heifers.

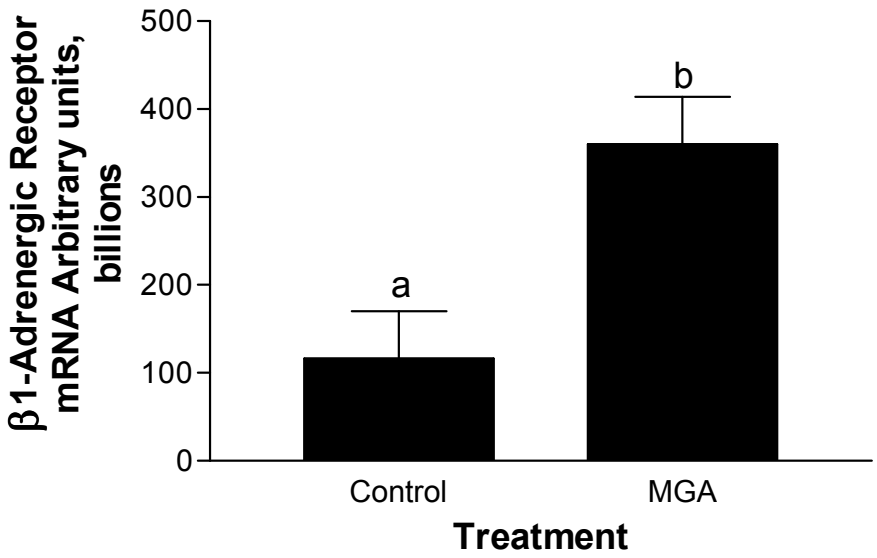

Figure 4.11 Effect of MGA on proliferating bovine satellite cell β1-adrenergic receptor (**AR**) mRNA abundance. Bovine satellite cells were plated in 10%FBS/DMEM. After 24 h, MGA (0 or 10 n*M*) was added. After 48 h exposure, total RNA was isolated from the cells, and relative mRNA abundance was determined using real-time quantitative PCR. Bars are means ± SEM relative to control. Bars with different letters differ ($P < 0.05$). Values are means from 11 culture dishes derived from 6 animals.

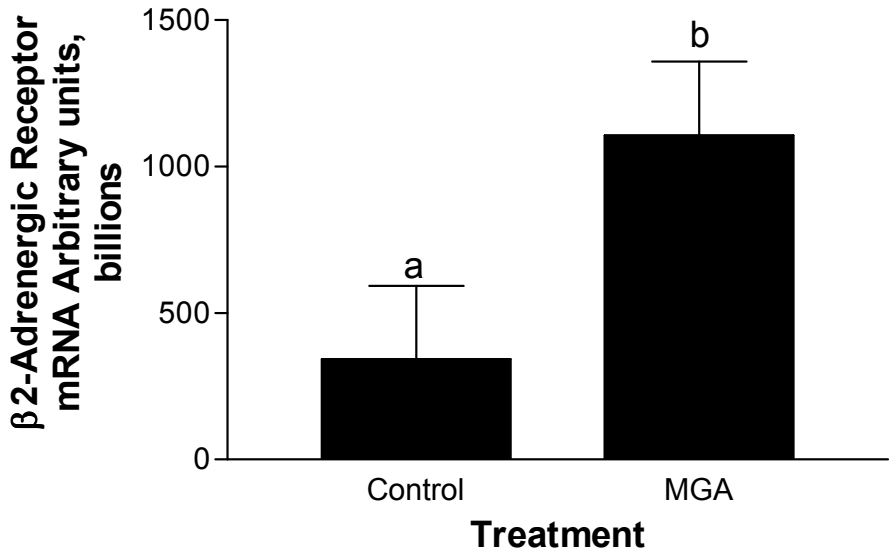

Figure 4.12 Effect of MGA on proliferating bovine satellite cell β2- adrenergic receptor (**AR**) mRNA abundance. Bovine satellite cells were plated in 10%FBS/DMEM. After 24 h, MGA (0 or 10 n*M*) was added. After 48 h exposure, total RNA was isolated from the cells, and relative mRNA abundance was determined using real-time quantitative PCR. Bars are means ± SEM relative to control. Bars with different letters differ ($P < 0.05$). Values are means from 11 culture dishes derived from 6 animals.

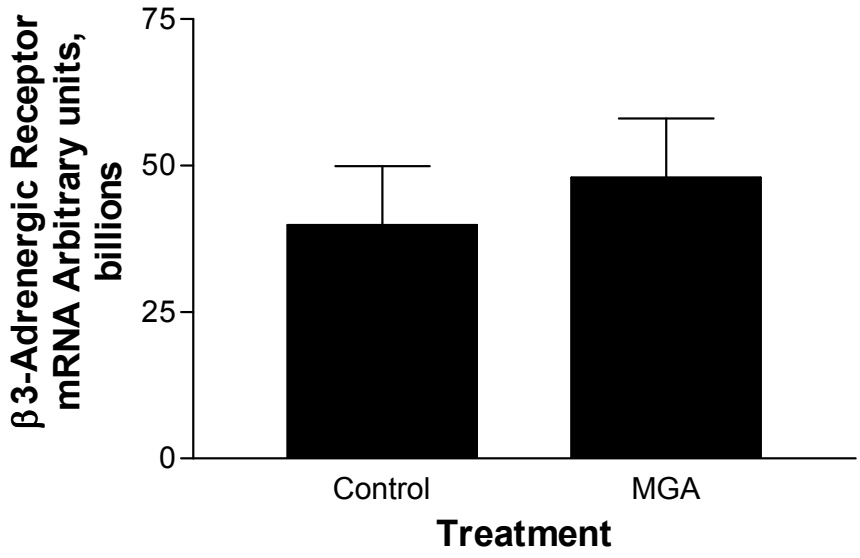

Figure 4.13 Effect of MGA on proliferating bovine satellite cell β3-adrenergic receptor (**AR**) mRNA abundance. Bovine satellite cells were plated in 10%FBS/DMEM. After 24 h, MGA (0 or 10 n*M*) was added. After 48 h exposure, total RNA was isolated from the cells, and relative mRNA abundance was determined using real-time quantitative PCR. Bars are means ± SEM relative to control. Values are means from 11 culture dishes derived from 6 animals.

Dissertation Summary

The data reported in this dissertation demonstrate the ability of ZIL to improve feedlot animal performance and carcass characteristics, as well as impact gene expression in bovine satellite cells and semimenbranosus muscle tissue. In chapter 1, it was reported that the β2-AR plays an important role in modulating the function of ZIL on skeletal muscle growth. The expression of β2-AR was decreased in proliferating myoblasts, and increased in fused multinucleated myotubes. This data should be considered when utilizing ZIL in feedlot animals. It is understood that administration of β-agonists leads to a decrease in receptor number, which in turn can impact the effect of the β-agonist on animal performance and muscle growth. The number of receptors available for ZIL may have an impact on the ability of ZIL to impact animal performance.

In chapter 3 and 4, it was established that ZIL can be an effective tool when utilized to improve animal growth and efficiency. Zilpaterol administration improved feedlot steer and heifer performance and carcass characteristics. Additionally, ZIL reduced the expression of MHC IIA in semimembranosus muscle tissue. This reduction in MHC IIA mRNA may be an indicator of decreased protein degradation. This decrease in protein degradation may be one of the factors involved in the increased muscle growth observed in these animals. As previously mentioned, growth of muscle tissue can come about from either an increase in protein synthesis, a decrease in protein degradation, or a combination of the two that results in a net increase in protein accretion. These factors are imperative when determining the specific mode of action of metabolic modifiers such as ZIL. It is evident from this research that ZIL affects muscle growth; however, further research is needed to determine the specific mode of action of this growth process. Understanding the effect of ZIL as confirmed by our data, should aid in better

utilization of ZIL in a commercial setting. Our data suggests that ZIL can be a useful tool in improving animal performance and carcass characteristics. However, more information is needed in order to understand the dose and duration required to achieve the optimum level of performance in feedlot animals.

Additional research was reported on the effects of MGA on heifer performance and skeletal muscle growth. MGA decreased skeletal muscle growth while increasing 12^{th} rib fat in heifers. This leads to an overall increase in calculated yield grades. Additionally, MGA altered the expression of β-AR *in vitro*, but there were no effects *in vivo*. Due to the widespread use of MGA in feedlot heifers, this information may be a valuable tool to those who are looking to improve feedlot heifer carcass characteristics. Because of the utilization of MGA in conjunction with ZIL in feedlot heifers, the potential ability of MGA to alter the expression of β-AR should be considered because of the potential for interactions or synergistic effects these two compounds may have. These data suggest that MGA does not have the effects that other steroids such as TBA and E_2 have on skeletal muscle growth and development in heifers.

In conclusion, our results demonstrate the ability of MGA administration to result in smaller longissimus muscle area with increased fat thickness, which may be due in part to early cell cycle withdrawal of satellite cells. This information can be useful due to the high utilization of MGA in feedlot heifers today. Additionally, we demonstrated the ability of ZIL to improve overall animal performance and efficiency. This positive response to muscle growth and efficiency can lead to increased profitability when ZIL is administered to feedlot cattle the last 20 to 40 d.