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M.S. degree in Animal Science

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THE EFFECTS OF DEXAMETHASONE AND SUNFLOWER OIL ON ADIPOSE TISSUE DEVELOPMENT IN BEEF STEERS

By

Matthew Pierce McCurdy

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Submitted to
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ABSTRACT

THE EFFECTS OF DEXAMETHASONE AND SUNFLOWER OIL ON ADIPOSE TISSUE DEVELOPMENT IN BEEF STEERS

By

Matthew P. McCurdy

Experiments were conducted investigating the development of subcutaneous and intramuscular adipose tissue in beef steers in response to glucocorticoid. In the first experiment, twenty-four early weaned steers were randomly assigned to either a basal diet or a basal diet with 8% sunflower oil and intramuscular injections every 28 d of either dexamethasone or saline for a period of 112 d. There were no significant differences in carcass characteristics due to treatment. In the second experiment, four steers received either a single injection of saline or dexamethasone 24 h before harvest. Total RNA was extracted from subcutaneous and intramuscular adipose tissue. Analysis by cDNA microarray revealed that DEX up-regulated ($P < 0.01$) expression of urokinase receptor in both adipose depots, and that intramuscular adipose exhibited significantly greater expression of genes involved in cellular proliferation. Northern blot analysis revealed glucocorticoid receptor (**GR**) expression was lower ($P < 0.01$) in intramuscular than in subcutaneous adipose depots and administration of DEX lowered GR in both adipose depots ($P < 0.05$). Differential gene expression between depots may provide a way for manipulation of adipose tissue accumulation.

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I wish to thank my family, all of whom have supported me in my every endeavor, as well as all of my friends, both here and other places that have always been there to pull me through. Last, but far from least, I owe a debt of gratitude to the people who instilled in me a love for the beef business and a desire to set upon my chosen career path: the ranchers and cattlemen of Dewitt County, Texas, and my father, one of them.

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INTRODUCTION

Adipose tissue development in beef cattle production is an area that poses a challenge to the beef industry. A way must be found to reduce the fat content of meat carcasses to provide a nutritious product with minimum waste fat, while not affecting meat palatability (Smith and Crouse, 1984). Beef cuts with greater amounts of intramuscular fat or marbling have a higher probability of being juicy and flavorful, and of remaining tender when cooked to a greater degree of doneness. The National Beef Quality Audit (Smith et al., 2000) revealed that only 19.3% of the nation's beef supply contained at least a "Modest" degree of marbling as compared to a projected future consumer demand of 28%.

Within a carcass maturity classification, marbling is the major determinant of USDA Quality Grade. Because of this, subtle differences in the amount of visible intramuscular fat can dramatically change carcass value. Subcutaneous fat, however, has been shown to be less beneficial to the quality and palatability of beef and greater amounts of subcutaneous fat result in greater trim waste from beef carcasses. Cattle are often fed for extended periods of time to maximize marbling, because, it is generally accepted that the sequence of adipose accumulation from early to late maturity is: perirenal, subcutaneous, intermuscular and intramuscular. Cattle therefore often accumulate large amounts of external fat before marbling can develop. In addition, extended feeding

periods come at the expense of declining efficiency of converting feed to muscle protein.

Due to differences in the rate of accretion between subcutaneous and intramuscular adipose depots, research has focused on examining the two adipose depots on a cellular level. Evidence has accumulated to indicate that intramuscular adipocytes exhibit different cellular properties as opposed to more extensively investigated subcutaneous adipocytes. Adipocytes from subcutaneous depots are larger and more spherical in shape and contain larger quantities of triacylglycerides suggesting that adipocytes in subcutaneous adipose mature at a faster rate and reach terminal differentiation earlier than adipocytes in intramuscular depots. Additionally, intramuscular and subcutaneous adipocytes exhibit metabolic and physiological differences.

Glucocorticoids are routinely used to induce differentiation in adipocytes in vitro. In live animals, glucocorticoids have been shown to have a lipolytic effect in subcutaneous adipose stores. However, glucocortcoids have enhanced or not affected intramuscular adipose accumulation.

Due to the needs of the beef industry being in direct opposition of normal adipose tissue depot development, it is imperative that the molecular pathways that lead to development and terminal cellular differentiation of adipocytes be explored and that biological differences between adipose tissue depots be further characterized. Therefore, the

goal of the present study was to identify differences between subcutaneous and intramuscular adipose tissue and to characterize the differential response of the two depots to stimulation by a glucocorticoid.

CHAPTER I

REVIEW OF LITERATURE

Beef quality, marbling, and palatability

Importance of marbling. One of the greatest challenges facing the beef industry is the lack of sufficient marbling in carcasses of beef cattle to meet the demands of the consumer. Marbling is the amount and distribution of visible intramuscular fat in the muscle. According to the 2000 National Beef Quality Audit (**NBQA**), insufficient marbling/ low USDA Quality Grade ranked in the top five "Greatest Quality Challenges" identified by producers, packers, and wholesalers/retailers (3, 5, and 1 respectively) (Smith et al., 2000). The 2000 NBQA identified that the ideal Quality Grade consist for the U.S. beef industry in 2000 was 6% Prime, 27% Upper 2/3 Choice, 32% Low Choice, 35% Select, and 0% Standard. For the 2000 NBQA, researchers collected data from 9,396 carcasses in thirty packing plants from across the U.S. Quality Grade distribution from the collected data was shown to be 2.0% Prime, 17.3% Upper 2/3 Choice, 31.8% Low Choice, 42.3% Select, and 6.5% Standard or hardboned. Due to the shortcomings of the actual consist when compared to the ideal consist, the estimated economic losses to the beef industry caused by insufficient marbling were $20.96 for each fed steer and heifer harvested in 2000.

The standards for USDA Quality Grades set forth in 1975 (USDA, 1975) state that the quality grade of a steer, heifer, cow, or bullock is based on the evaluation of palatability-indicating characteristics of the lean. Marbling continues to receive primary consideration in the assessment of quality in the U.S. beef grading system (USDA, 1975; Tatum et al., 1982), and thus within a carcass maturity classification, marbling is the major determinant of USDA Quality Grade. In the current U.S. grading system, this makes marbling the dominant indicator of beef palatability.

Marbling and tenderness. Numerous studies have sought to link marbling with tenderness and overall palatability. McBee and Wiles (1967) reported that overall tenderness (determined by palatability panel and shear force), juiciness, and flavor increased with additional degrees of marbling in a linear fashion. Tatum et al. (1980) also reported an increase in palatability of steaks as marbling score increased. Additionally, Dolezal et al. (1982) found that marbling score was positively correlated with increased sensory panel ratings and decreased shear force values. Berry (1993) found that decreased shear force values existed for loin steaks from carcasses with a higher marbling classification.

Smith and Carpenter (1974) reviewed the literature concerning the link between fat content of meat and palatability. Additionally, they attempted to elucidate the theories behind the effects of marbling on beef palatability. Of the four theories presented in the paper, the first was the

bite theory. This theory suggests that with an increase in marbling, protein is replaced by lipid in each bite size piece of meat thus lowering the bulk density of each piece of meat. Fat has much less shear force resistance than protein and a consequent increase in tenderness occurs. Another theory presented was the lubrication theory. This idea states that intramuscular fat provides lubrication for the muscle fibers that it surrounds thus providing a juicier product. A third idea suggested is the strain theory. The strain theory suggests that marbling physically aids the tenderness of meat by disrupting the connective tissue structure of the muscle fiber walls. This would provide a decrease in the strength and thus the shear force resistance of the connective tissue associated with the muscle fibers and bundles. This theory is given relevance by a much more recent work by Nishimura et al. (1999) that evaluated changes in the connective tissue of intramuscular fat in Japanese Black steers. When examined by electron microscopy the longissimus muscle showed a partially broken connective tissue structure where adipose tissue had formed between muscle fiber bundles. Additionally, in the semitendinosus muscle, where fat content in the muscle was significantly lower, connective tissue structure remained rigid. It was concluded that the intramuscular adipose tissue development aids beef tenderization by disorganizing connective tissue structure (Nishimura et al., 1999). The last of the theories presented by Smith and Carpenter (1974) is the insurance theory. This theory concerns the tenderness of meat as it

relates to cooking method. The idea behind this theory is that increased marbling in the meat allows for cooking at higher temperatures and dry-heat methods of cooking. The fat tissue content allows the meat to endure higher cooking temperatures, thus providing that meat that is cooked by the wrong method or cooked too fast will still be palatable. The work of Wheeler et al. (1999) supports this theory. Their study found that steaks from carcasses that graded Top Choice (Upper 2/3 of the USDA Choice grade) were less affected by elevated degree of doneness than steaks from carcasses that graded Low Select. This suggests that beef cuts with greater amounts of marbling have a higher probability of being juicy and flavorful, especially when cooked well done by the consumer. Smith et al. (1987) also reported that higher USDA Quality Grades were associated with lessened variability in the palatability of cooked beef.

Relation to beef flavor. Other studies have reported that an increase in marbling score and consequent increase in USDA Quality Grade is associated less with tenderness and more with overall palatability including flavor and juiciness. Francis et al. (1977) reported that between rib-eye steaks that possessed either Slight or Modest levels of marbling, flavor and juiciness of Modest marbled beef was significantly preferred by a group of over eight-hundred consumers. Neely et al. (1998) conducted a study on consumer satisfaction of beef. One component of the study examined differences in consumer preference for USDA Quality Grades of Top Choice, Low Choice, High Select, or Low Select. For overall like and

palatability consumers significantly preferred Top Choice to the other USDA grades.

Again referring to the 2000 NBQA (Smith et al., 2000), it was estimated that $38.30 per carcass was lost due to inadequate taste of beef. It was further estimated that approximately 74% of the losses associated with taste were caused by insufficient marbling.

Due to the reasons discussed above, marbling is not only the primary determinant of USDA Quality Grade, but also of beef carcass value. Because of this, subtle increases in the amount of visible intramuscular fat can change carcass value by 10% or more, resulting in greater revenues for all beef industry segments.

Implications of subcutaneous fat accumulation

Time-on-feed of beef cattle and subsequent accumulation of subcutaneous fat has been evaluated as an indicator of palatability attributes of beef by a number of studies. Dolezal et al. (1982) pointed out that while the time-on-feed concept appears to be valid, the usefulness of such a system would be limited by the complications of monitoring time-on-feed of all cattle entering the supply chain. An adjunct to the time-on-feed concept is a grading system based on the level of subcutaneous fat.

Subcutaneous fat and palatability. The fattening of animals has historically been accepted as a means of increasing beef quality (Dolezal et al., 1982). Smith et al. (1976) determined that subcutaneous fat aided tenderness by slowing the process of carcass chilling in lamb and

protecting the muscle tissue from the effects of sarcomere shortening. Tatum et al. (1982) investigated this effect on tenderness in beef and determined that steaks from carcasses having a subcutaneous fat thickness of less than 5.08 mm were lowest in palatability. However, there seemed to be little improvement in palatability beyond 7.62 mm of subcutaneous fat. It was concluded that fat thickness, as compared to marbling, was ineffective as a predictor of cooked beef palatability. Dolezal et al. (1982) compared subcutaneous fat thickness with marbling as a predictor of beef palatability. Results from this study found that steaks from carcasses with fat thickness of at least 5.08 mm were superior in palatability to steaks with 5.07 mm or less. Similarly, Shackelford et al. (1994) looked at the effect of a minimum fat thickness requirement of 5 mm on palatability within the USDA Select Quality Grade. It was found that shear force values were lower and overall tenderness was higher for carcasses that had fat thickness greater than 5 mm. The magnitude of the differences in this study was small, however, and it was concluded that a minimum fat thickness requirement for the Select grade would not greatly improve tenderness.

Even if significant differences in palatability exist for beef carcasses above or below the 5.08 mm level as indicated by Dolezal et al. (1982), the usefulness of such a predictor may be very limited. The 2000 National Beef Quality Audit showed that only 4.9% of the 9,396 carcasses evaluated had less than 5.08 mm (0.2 inches) of subcutaneous fat

thickness (Smith et al., 2000). With over 95% of the nation's beef supply exceeding this level, the contribution of subcutaneous fat thickness to beef palatability may already be close to maximization. Thus, the issues and concerns related to subcutaneous adipose development in beef become that of excess.

Economic losses. As stated previously, the insufficiency of marbling and tenderness ranked in the top five concerns of producers, packers and wholesalers/retailers as determined by the 2000 NBQA. In the same listing of concerns was the problem of excess external fat accumulation. It was estimated that $50.96 for each steer and heifer harvested in 2000 was lost due to excess fat. Considering that approximately 36.4 million cattle were harvested in the U.S. in 2000 (USDA, 2000), this translates to over 1.8 billion dollars in lost revenue annually. Therefore, in contrast to marbling, it is the reduction of subcutaneous fat that holds the key to increased profitability for the beef industry.

Comparative biology of adipose tissue depots

The current beef industry faces a difficult challenge in altering the fat content of its product. Because the desired future product requires a reduction in subcutaneous adipose tissue and an increase in the amount of intramuscular adipose tissue, the needs of the industry run in opposing directions. Thus, the goals of altering beef to achieve the desired fat

content can only be met if differences exist in adipose tissue due to location.

Growth and Development. As previously stated, fattening of cattle has historically been accepted as a way to improve the palatability of beef (Dolezal et al., 1982). It is also generally accepted that increasing subcutaneous fat in cattle to certain end points will result in adequate intramuscular fat levels. According to Hood (1983) fat is preferentially deposited in the following tissue sites: perirenal and omental > subcutaneous > intermuscular > intramuscular. However, the relationship between accumulation of subcutaneous and intramuscular adipose has been shown to be inconsistent (Zinn et al., 1970). Dubeski et al. (1997) conducted a study where cattle were fed to extreme slaughter weights. It was observed that while the heavy slaughter weights (680 kg) produced carcasses with extremely high amounts of external fat, they did not result in highly marbled beef. Cunha (1974) observed that many cattle reach the USDA Choice Quality Grade with only 0.2 to 0.3 inches of backfat and the 2000 NBQA determined that cattle grading USDA Choice spanned USDA Yield Grades 1 to 5.

In 1984, Smith and Crouse observed that previous studies indicated that marbling scores were less subject to manipulation through nutrition as compared to backfat thickness or total carcass fat. In this study, cattle fed a higher energy corn diet did not display significantly greater marbling scores than cattle fed a lower energy corn silage diet.

Cattle on the higher energy diet did, however, have greater backfat thickness and a greater percentage of kidney, pelvic, and heart fat. It was thus concluded that lipogenesis in the two depots was controlled by different means. Because adipose tissue metabolism appears to be regulated differentially due to location, it is necessary to examine differences between the two depots on a cellular and molecular level.

Cellularity. It has been observed that intramuscular adipocytes represent a unique cell population that exhibits different cellular and metabolic activity as compared to adipocytes from subcutaneous tissue (Lee and Kauffman, 1974; Lin et al., 1992). A number of studies have pointed to the fact that intramuscular adipocytes are smaller cells on average, with less cell volume and correspondingly less lipid content than subcutaneous adipocytes (Hood and Allen, 1973; Smith and Crouse, 1984; Miller et al., 1991; May et al., 1994). Hood and Allen (1973) observed that adipose tissue development in beef cattle was achieved by both hyperplasia and hypertrophy of adipocytes. After 14 months of age, cells in the subcutaneous adipose tissue had ceased to proliferate and developed solely by hypertrophy. However, intramuscular adipose tissue contained smaller cells (< 70 μm in diameter) that continued to actively engage in hyperplasia throughout the study. Similarly, Lee and Kauffman (1974) saw that hyperplasia in porcine intramuscular tissue never plateaued throughout the 24 wk duration of their trial. In cattle, as in other ruminant species, the majority of *de novo* fatty acid synthesis takes place

in adipose tissue. The smaller size and volume of adipocytes in the intramuscular adipose is associated with decreased lipogenic activity. Hood and Allen (1978) concluded that lipid was synthesized at a slower rate in intramuscular adipose than in subcutaneous adipose and that this was directly related to a smaller adipocyte volume.

Substrate utilization. In addition to differences in total lipogenic activity, differences exist between subcutaneous and intramuscular adipose tissue depots in their preferential uptake of substrates for fatty acid synthesis. Smith and Crouse (1984) demonstrated that in explants of bovine adipose tissue, subcutaneous adipocytes primarily used acetate-derived substrates as precursors for fatty acid synthesis. However, glucose-derived carbon units provided the main source of substrate for fatty acid synthesis in intramuscular adipose tissue. It was observed that acetate provided 70 to 80% of the acetyl units for lipogenesis in subcutaneous adipose tissue but only 10 to 25% for intramuscular tissue. Glucose provided only 1 to 10% of the acetyl units for fatty acid synthesis in subcutaneous adipose but provided 50 to 75% of the units for intramuscular fatty acid synthesis. Differences in esterification of exogenous fatty acids have also been demonstrated between depots. Lin et al. (1992) observed that subcutaneous adipose tissue more actively esterified fatty aids than intramuscular adipose. However, intramuscular adipose esterified a larger amount of palmitate as a percentage of total fatty acids.

Brown adipose tissue. In many species (notably excluding swine) there exists at birth a population of adipocytes known as brown adipocytes (Smith, 1995). Most of the adipose tissue in newborn ruminant species is brown adipose tissue, although small amounts of white adipose tissue are present (Alexander et al., 1975; Alexander, 1978; Martin et al., 1997). Brown adipocytes are characterized by a profound abundance of mitochondria and the presence of specific gene products, such as the mitochondrial uncoupling protein (Casteilla et al., 1987; Klaus et al., 1991; Cousin et al., 1992; Brandler et al., 1993; Smith, 1995). Brown adipose tissue is present at birth and functions for thermogenesis in order to provide warmth for newborns. Shortly after birth however, it disappears or interconverts to white adipose tissue (Smith, 1995). Martin et al. (1999) observed that white subcutaneous adipose tissue from Angus and Brahman steers contained adipocytes with distinct brown adipocyte morphology, suggesting an involution of brown adipose tissue to white adipose tissue in utero. To our knowledge, data has not been reported to demonstrate whether or not intramuscular adipocytes originate from brown adipose tissue. Intramuscular adipocytes may have a unique origin as compared to adipocytes contained in subcutaneous adipose tissue.

Differential gene expression. Considering the observed differences between adipose tissue depots, it is reasonable to think that subcutaneous and intramuscular adipose may exhibit unique aspects of gene expression. It has been demonstrated in a number of studies by Northern

and Western blot analyses that differences do exist between the two depots at a molecular level.

It is well established that glucocorticoid induced differentiation takes place in the presence of insulin in experiments using in vitro cell culture models (Martin et al., 1998). This requires insulin-responsive glucose transporter (**GLUT-4**), which is considered to be a major glucose transporter in muscles and adipose tissue (Aso et al., 1995). However, in experiments using a clonal bovine intramuscular preadipocyte line, GLUT-4 protein was not detectable (Aso et al., 1995). This suggests that the differentiation of adipocytes in intramuscular tissue may take place through different means as compared to other fat tissue depots.

Kim et al. (2000) conducted a study to evaluate the expression of the *obese* gene (**ob**), which encodes for leptin, in fed and fasted states among adipose tissue depots in the bovine. They observed that *obese* mRNA was moderately expressed in subcutaneous and intermuscular adipose, and that fasting effectively decreased levels of transcript expression in these depots. In contrast, the *obese* gene was expressed only at very low levels in the intramuscular adipose and fasting did not elicit an expression change in this depot.

With variable behavior of cells in adipose tissue reflecting different locations and conditions, a thorough understanding of the growth and metabolism of adipose cells is critical for differential manipulation of subcutaneous and intramuscular adipose tissue accumulation.

Origin of adipose cells

In most species the development of white adipose tissue (**WAT**) begins prior to birth, although the developmental origin of adipocytes from embryonic cells is not well characterized (Gregoire, 1998). However, a number of studies using cloned cell lines suggest that the adipocyte lineage is derived from an embryonic stem cell precursor with the capacity to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts, and myocytes (Konieczny and Emerson, 1984). Ailhaud et al. (1992) states that the process of determination from the multipotential stem cells leads to the formation of unipotential adipoblasts. The adipoblasts then undergo commitment to form preadipocytes. These are cells that express early markers of differentiation but not late markers and have yet to begin lipid accumulation. It has not been clarified whether adipoblasts remain after birth or if only preadipocytes are present (Ailhaud et al., 1992). Postnatally, as preadipocytes undergo the process of terminal differentiation they begin to express late markers of adipose cell development as well as begin the accumulation of triacylglycerides. As terminal differentiation continues, very late markers of adipose development are expressed and the accumulation of lipid becomes more pronounced. Committed preadipocytes retain the ability to proliferate, whereas fully differentiated cells lose the capacity for mitosis (Ailhaud et al., 1992). The growth of WAT increases rapidly after birth as result of

both adipose cell size and number. Gregoire et al. (1998) states that the potential to acquire new fat cells from fat cell precursors throughout the life span is undisputed, suggesting the continued presence of preadipocytes. However, as previously cited, in cattle (Hood and Allen, 1973), adipose development appears to take place exclusively by an increase in cell size after approximately 14 months of age in subcutaneous tissue.

Adipocyte differentiation

During the growth phase, preadipocytes from cloned cell lines or from primary culture are morphologically similar to fibroblasts (Gregoire et al., 1998). As stated above, the preadipose cell has the ability to proliferate. However, for terminal differentiation to take place the cell must withdraw from the proliferative cycle. Terminal adipocyte differentiation is characterized by early, intermediate, and late markers of gene expression, as well as by the accumulation of lipid within the cell. Much of the available information regarding adipocyte differentiation comes from preadipose cell culture models. The process of differentiation appears to begin with the growth arrest of preadipose cells. Two transcription factors, CCAAT/enhancer binding protein α (**C/EBP** α) and peroxisome proliferator-activated receptor-γ (**PPAR-γ**), shown to activate adipocyte specific genes, also appear to have a role in the growth arrest required for adipocyte differentiation (Altiok et al., 1997).

Molecular events. Following the growth arrest of preadipocytes in cloned cell lines, committed cells undergo at least one round of mitosis

providing clonal expansion of committed cells. This has not, however, been shown to be the case with primary cell cultures and may indicate that the necessary clonal amplification for differentiation may have already taken place in vivo (Entenmann and Hauner, 1996). Clonal expansion appears to be a unique process as compared to preconfluent cell growth as indicated by the inactivation of growth promoting transcription factors by retinoblastoma proteins (pRB, p107, and p130) (Richon et al., 1997). The retinoblastoma family of proteins is known to inactivate the E2F family of transcription factors associated with cell growth and proliferation (Mulligan and Jacks, 1998).

During growth and differentiation, changes in cellular gene expression begin to take place. Lipoprotein lipase plays a key role in the accumulation of triacylglycerides and has been revealed to be an early marker of adipocyte differentiation (Ailhaud, 1996). Two families of transcription factors mentioned previously, C/EBPs (α, β, and δ isoforms) and PPARs (α and γ isoforms) are expressed during the early stages of adipocyte differentiation. Increased expression of C/EBP-β and C/EBP-δ appears to be followed by an increase in C/EBP-α, as well as PPAR-γ, which is associated with the activation of adipocyte specific genes (Altiok et al., 1997). The transcription factor PPAR-γ has been determined to have two isoforms (1 and 2). The expression of PPAR-γ2, which is specific to adipose tissue, appears to be a key step in the induction of adipocyte differentiation (Li and Lazar, 2002).

As changes in gene expression are induced, preadipose cells begin to lose their fibroblastic morphology and adopt a more spherical shape. Preadipocyte factor-1 (**pref-1**), a preadipose cell-specific protein, exhibits a dramatic decrease in expression during adipocyte differentiation and is not detectable in mature fat cells. Pref-1 may be responsible for maintenance of preadipocyte morphology and changes in cell shape during the differentiation process may be due to its down-regulation (Smas et al., 1993).

Enzymatic changes. As differentiation progresses in 3T3 cell cultures, many of the genes associated with lipid metabolism begin to be expressed. Lipoprotein lipase (**LPL**) and hormone sensitive lipase (**HSL**) expression becomes evident as well as that of GLUT-4 and fatty acid binding protein (**aP2**). In addition, acetyl-CoA carboxylase and fatty acid synthetase, two key enzyme markers for lipogenesis, begin to function (Ailhaud et al., 1992). As the enzymes necessary for lipogenesis including ATP citrate lyase, malic enzyme, stearoyl-CoA desaturase (**SCD1**), glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase, and glyceraldehydes-3-phosphate dehydrogenase begin to be expressed, triacylglyceride accumulation is initiated and lipid droplets form within the cell (Spiegelman et al., 1983; Paulauskis and Sul, 1988; Weiner et al., 1991).

Hormones and signaling in adipose

Hormonal effects. Growth hormone (**GH**) has known lipolytic activity and is inhibitory to differentiation. However, GH stimulates insulin-like growth factor-1 (**IGF-1**) gene transcription and IGF-1 is necessary for adipocyte differentiation. Growth hormone has been shown to be necessary for differentiation in cloned murine cell lines, possibly to sensitize the cells to mitogenic effects of IGF-1 (Corin et al., 1990). However, this has not been shown to be necessary in primary cultures of preadipocytes from rats (Wabitsch et al., 1995) and this may be attributed to the prior in vivo exposure of primary culture cells to GH (Gregoire et al., 1998).

IGF-1 is a requirement for adipocyte differentiation and has been shown to stimulate adipogenesis in both cloned cell lines and primary culture preadipocytes (Boney et al., 1994; Wabitsch et al., 1995). IGF-1 appears to be secreted from adipose cells and may act as an autocrine/paracrine regulator of differentiation although the specific mechanism of adipogenic stimulation remains unknown (Gregoire et al., 1998).

Another important signaling hormone involved in adipose growth and function is leptin. Leptin is secreted specifically by adipose tissue and is thought to provide signals for regulation of body energy stores and nutritional intake (Fried et al., 2000). Leptin is the protein product of the *ob* gene, discovered by Zhang et al. (1994), and its expression is one of

the latest occurring markers of adipocyte differentiation (Gregoire et al., 1998).

Other hormones that may influence differentiation include triiodothyronine (T_3) and catecholamines. Triiodothyronine appears to be stimulatory to differentiation although the molecular mechanisms underlying this effect are not known (Gaskins et al., 1989). Gharbi-Chihi et al. (1981) observed, that T_3 stimulation of differentiation is only evident in certain cloned cell lines. Additionally, β-agonists or catecholamines are known to affect adipocyte growth and metabolism. The β-agonists exert their action through β-adrenergic receptors and are known to increase lipolysis in adipocytes, both in vitro and in vivo (Mersmann, 1998). The β-agonists include the physiological compounds of norepinephrine and epinephrine, as well as a large number of their synthetic analogs (Mersmann, 1998). The β-adrenergic receptor has three subtypes (**β1AR**, **β2AR**, and **β3AR**) and differences in the distribution of the three subtypes in adipose tissue vary according to stage of growth and development, as well as between species (Mersmann, 1998). However, it has been observed in in vitro cell cultures from 3T3 cloned cell lines that preadipocytes have higher levels of the β1AR subtype. As the process of adipocyte differentiation takes place, the expression of β1AR diminishes and expression of β2AR and β3AR is up-regulated (Feve et al., 1990; Feve et al., 1991).

Vitamins. Retinoic acid, the main active metabolite of Vitamin A has been shown to play a key role in differentiation and is recognized as a potent inhibitor of adipocyte differentiation (Villarroya et al., 1999). Retinoic acid has been hypothesized to reduce the expression of early gene markers in adipocyte differentiation including the C/EBP and PPAR families of transcription factors (Villarroya, et al., 1999). Additionally, vitamin C has been shown to enhance adipocyte differentiation in primary cultures of bovine adipocytes (Yano et al., 2000). However, the mode of action remains unknown.

Arachadonic acid metabolites. Various arachadonic acid metabolites may also play a role in adipose tissue development and significant amounts of prostaglandins (**PGs**) are excreted by mature adipocytes from 3T3 cloned cell lines (Hyman et al., 1982). Prostacyclin (**PGI_2**) released from mature mouse adipocytes has been demonstrated to have an adipogenic effect on neighboring preadipocytes by both the elevation of levels of cAMP and intracellular free calcium (Ailhaud et al., 1996) in the cell and as an activator of PPAR transcription factors (Brun et al., 1996). While PGI_2 appears to act on 3T3 preadipocytes to induce differentiation and has no effect on terminally differentiated adipose cells, prostaglandin E_2 (PGE_2) appears to act the opposite way (Ailhaud et al., 1996). Prostaglandin E_2 shows no effect on preadipocytes but behaves as an antilipolytic-hypertrophic effector of mature cells (Ailhaud et al., 1996). Another PG, prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**) has been shown to inhibit

adipocyte differentiation in both cloned cell lines and primary culture cells from multiple species and $PGF_{2\alpha}$ is thought to act through a calcium/calmodulin-dependent protein kinase, although the specific mode of action is not well understood (Gregoire et al., 1998). Additionally, prostaglandin J_2, a derivative of the prostaglandin D_2 has been shown to be a PPAR-γ agonist (Kliewer et al., 1995).

Additional signals. In addtion, two cytokines have been implicated in adipose cell endocrinology. Tumor necrosis factor-α (**TNF-α**), a mediator of apoptotis, and interleukin-6 (**IL-6**), a cytokine growth factor, are secreted from mature adipocytes and appear to play a regulatory role in adipose tissue growth and development by acting in a paracrine/autocrine fashion (Morrison and Farmer, 2000).

Glucocorticoids

Effects on adipocytes. It has been well established that glucocorticoids are a necessary part of the stimulation of differentiation in adipocytes. In studies involving cloned cell lines, the glucocorticoid analog dexamethasone has been observed to stimulate differentiation and is routinely used as an inducer of differentiation on preadipocyte cell lines in order to study mature adipocytes (Martin et al., 1998). Additionally, dexamethasone has been shown to enhance differentiation in the presence of insulin or serum in nearly every preadipocyte line studied (Martin et al., 1998). Dexamethasone, has been shown to increase the C/EBP-α isoform in embryonic mouse preadipocyes (Cao et al., 1991; Yeh

et al., 1995). This effect may be mediated through an interaction with the retinoblastoma family of proteins (Mulligan and Jacks, 1998). Additionally, it has been suggested by Wolf (2000) that the effects of the glucocorticoid are mediated through a direct down-regulation of pref-1. In other studies with mouse preadipocytes, glucocorticoid effects appear to be mediated through increased metabolism of arachidonic acid leading to an increase in production of prostacyclin, which in turn increases intracellular cAMP (Ailhaud et al., 1992). Aubert et al. (2000) recently demonstrated that activation of the prostacyclin receptor (IP receptor) via prostacyclin or cyclic AMP-elevating agents was sufficient to rapidly up-regulate the early expression of both transcription factors C/EBP-α and C/EBP-δ. These observations are consistent with the idea that PGI_2 is released from preadipose cells and behaves as a paracrine/autocrine effector of adipose cell differentiation.

Effects in live animals. Experiments involving glucocorticoids and their effects in live animals are limited. There is however some evidence to support the action of glucocorticoids on adipose tissue in vivo. Even though adrenal glucocorticoids readily stimulate mobilization of lipid from subcutaneous and other fatty depots, circulating levels of glucocorticoids have been positively correlated with intramuscular fat deposition in cattle (Cramer and Shahied, 1974).

Romans et al. (1974) observed that longissimus muscle lipid increased as a result of induced exercise stress in 21-month-old steers. It

was observed that increases in exercise stress appeared to cause lipolysis from adipose as evidenced by increases in circulating free fatty acids, but did not have detrimental effects on intramuscular lipid. This suggests that lipids may have been mobilized from specific adipose tissues and redeposited in muscle.

Brethour (1972) showed that acute injections of dexamethasone were effective at increasing intramuscular lipid when given from 30 to 90 d before harvest. In steers that received 10 to 20 mg of dexamethasone at 90, 76, or 33 d preslaughter, significant increases were observed in carcass marbling scores. Additionally, it was observed that marbling scores were not increased in steers that received injections of dexamethosone more than 90 d before harvest. Because steers were not adapted to full feed until less than 90 d from harvest, it was concluded that glucocorticoid effects may exhibit an interaction with body energy reserves. Nevertheless, adipose from unique depots appeared to display differential endocrine control in relation to glucocorticoids. A more recent attempt to increase marbling using low dosage dexamethasone implants was unsuccessful in cloned yearling Brangus steers (Corah et al., 1995). Due to the implant's slowed release over time (implants contained 100 mg of dexmethasone), this failure may be explained by the down-regulation of glucocorticoid receptors chronically exposed to elevated hormone levels (Preisler et al., 2000a, 2000b).

Additionally, it has been reported that intramuscular lipids were increased in rabbits that were infused with adrenocorticotropic hormone (**ACTH**) (Romans et al., 1974) or dexamethasone (Fredericks et. al., 1975). In addition, there is some evidence suggesting that exogenous glucocorticoid injected intramuscularly or subcutaneously can increase intramuscular fat deposition in sheep (Harter and Vetter, 1967).

Fatty acids

Circulating fatty acids may have a role in both initiating adipose differentiation and in initial filling of adipocytes. In cultured murine cell lines, arachidonic acid ($C_{20:4}$) and cyclic AMP-elevating agents, especially prostacyclin behave as mitogenic-adipogenic stimuli able to trigger growth-arrested cells to enter the terminal phase of differentiation, and thus appear to mimic the activity of glucocorticoids (Gaillard et al., 1991). Arachidonic acid is found in only very low concentrations in common feedstuffs. However, the essential fatty acid, linoleic acid ($C_{18:2}$), is a precursor of arachidonic acid biosynthesis and is relatively high in several oilseeds. One of the richest sources of linoleic acid is sunflower oil. Although extensive biohydrogenation of unsaturated fatty acids occurs in the reticulo-rumen, the degree of biohydrogenation is dependent upon factors such as form of dietary fat, passage rate, and microbial population (Allen, 2000). Ruminal biohydrogenation of sunflower oil fed to lactating cows was approximately 80% (Kalscheur, et al., 1997) and that of

unsaturated 18C fatty acids in corn oil fed to beef steers was approximately 70% (Kennington et al., 2000).

Hood (1983) demonstrated that appreciable rates of fatty acid synthesis were occurring only in larger adipocytes in ovine adipose tissue, especially in intramuscular adipose tissue. Seemingly, preadipocytes need to be supplied with fatty acids for complex lipid biosynthesis, as endogenous synthesis from glucose is still undetectable in contrast with mature adipocytes (Ailhaud, 1996). Subsequently, fatty acids via fatty acid-activated receptor (**FAAR**) and adipogenic hormones participate in the formation of adipocytes from preadipocytes (Amri et al., 1994). Taken together, these reports indicate that preadipocytes and "young" adipocytes may be heavily dependent on the uptake of circulating fatty acids to provide substrate for triacylglyceride biosynthesis. However, to sufficiently establish a mode of action for these signals, it would be necessary to establish changes on a molecular or gene expression level.

cDNA microarray analysis

The bovine genome is composed of thousands of different genes that are responsive to the environment of the animal. Recent developments of cDNA microarray technologies offer the possibility of analyzing the responsiveness of vast numbers of genes to external stimuli (Yao et al., 2001). Although these technologies provide a means for examining gene responsiveness to treatment, nutrition, disease, and environment on a large scale, the resources to develop these technologies

for livestock species have been lacking (Yao et al., 2001). Recent advances in the creation of tissue, cell, or species-specific cDNA libraries for generation of thousands of expressed sequence tags (**ESTs**) make it possible to examine differential gene expression in tissues such as subcutaneous and intramuscular adipose. This is known as functional genomics.

To date, a number of studies have utilized cDNA microarray technology to examine differential expression in adipose tissue. Most of this work has been confined to rodent species and has dealt with gene expression differences between white adipocytes and brown adipocytes to examine their unique metabolism (Reue and Glueck, 2001; Boeuf et al., 2001; Boeuf et al., 2002).

Although functional genomics studies have been conducted with cattle utilizing differential display-polymerase chain reaction (Childs et al., 2002), the use of microarray analysis has been more limited due to lack of availability of cDNA libraries specific to the bovine species. However, in recent years the Center for Animal Functional Genomics at Michigan State University has worked to develop cDNA libraries for domestic livestock species. As a result of this work, a normalized cDNA library generated from bovine total leukocytes (**BOTL**) and containing 842 unique cDNAs has become available (Burton et al., 2001; Yao et al., 2001). Although originally designed for studies in bovine immunobiology, most of the differentiation and proliferation genes of leukocytes are ubiquitous in other

cells, expanding the usefulness of microarrays spotted with these cDNAs to the study of multiple bovine cell types, including adipocytes.

Adipose tissue development in beef cattle comprises the topic of a wide range of research within the animal industry. Although glucocorticoids have been studied extensively in relation to adipose cells, experiments with glucocorticoids and bovine adipose are limited. Additionally, most studies that have been carried out in this area do not fully explore the response of bovine adipose on a cellular or molecular level.

Despite its possible uses in animal species, cDNA technology and microarray analysis have seen only limited use in experiments with production livestock. With cDNA microarray analysis representing a new and powerful tool to examine gene expression and consequent phenotype in cattle, it seems logical to apply this technology to an area that has lacked conclusive research with conventional methods. Because glucocorticoids appear to act differently in subcutaneous and intramuscular depots, our hypothesis was that intramuscular and subcutaneous adipose tissue would exhibit differential response to dexamethasone treatment. Therefore, the objectives of these studies were to characterize the response of subcutaneous and intramuscular adipose in relation to glucocorticoids and to identify unique characteristics of the two depots on a molecular level.

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CHAPTER II

INFLUENCE OF SUPPLEMENTAL SUNFLOWER OIL AND DEXAMETHASONE THERAPY ON ADIPOSE TISSUE DEVELOPMENT AND PERFORMANCE IN EARLY WEANED BEEF STEERS

ABSTRACT

The purpose of this feedlot study was to investigate the effect of dexamethasone therapy and supplemental sunflower oil on the development of intramuscular adipose tissue in early weaned beef steers. Twenty-four early weaned steers (182 ± 21.7 kg) were randomly assigned to a 2 x 2 factorial arrangement of either basal diet only (**CON**) or basal diet with the addition of 8% high-linoleate sunflower oil (**OIL**) and intramuscular injections every 28 d either of dexamethasone (0.1 mg dexamethasone/kg body weight) (**DEX**) or an equal volume of physiological saline (**SAL**) over a period of 112 d. Steers were fed individually and amount fed was adjusted weekly so that treatment groups had isocaloric intake. Following the 112 d treatment period, all steers were fed a common basal diet for 138 d and then harvested. Accumulation of ultrasound measured intramuscular fat percentage, subcutaneous rib fat and rump fat was not significantly effected by treatment. Carcass marbling score, rib fat, and yield grade of treatment groups were not significantly different than control. Additionally, proximate

analysis of rib steaks from carcasses showed no differences in intramuscular lipid content and fatty acid analysis revealed few significant differences in fatty acid profile between treatment groups. However, DEX increased ($P < 0.05$) and OIL tended to increase ($P = 0.07$) kidney, pelvic, and heart fat. Dexamethasone lowered ADG ($P < 0.01$) and feed efficiency ($P < 0.05$) during the treatment period. However, during the finishing period, DEX tended to increase ADG ($P = 0.09$) and feed efficiency was increased by previous administration of DEX in combination with OIL ($P < 0.05$). Furthermore, over the entire trial, an interaction between DEX and OIL decreased feed efficiency ($P < 0.05$). In conclusion, neither treatment was successful in influencing the accumulation of adipose in intramuscular or subcutaneous depots. Additionally, dexamethasone administration negatively affected feedlot performance during the treatment period but increased performance during the finishing period.

Introduction

Intramuscular fat, or marbling, is the major determinant of USDA Quality Grade. Cattle are often fed for extended periods of time to maximize marbling, because the sequence of adipose maturation from early to late is: perirenal, subcutaneous, intermuscular and intramuscular (Hood, 1983). Therefore, cattle often accumulate large amounts of subcutaneous fat before marbling can reach desired levels. It is well established that glucocorticoids, or the glucocorticoid analog dexamethasone, enhance differentiation in the presence of insulin or serum in nearly every preadipocyte cell line studied (Martin et al., 1998). Even though adrenal glucocorticoids readily stimulate mobilization of lipid from subcutaneous and other fatty depots, circulating levels of endogenous glucocorticoids have been positively correlated with intramuscular fat deposition in cattle (Cramer and Shahied, 1974; Romans et al., 1974). In addition, there is limited evidence suggesting that intramuscular or subcutaneous injections of exogenous glucocorticoid can increase intramuscular fat deposition in cattle when administered late in the feeding period (Brethour, 1972).

In studies with mouse preadipocyte cell lines, glucocorticoid effects appear to be mediated through increased production of prostacyclins from arachidonic acid (Ailhaud et al., 1992). Arachidonic acid is found in only very low concentrations in common feedstuffs. However, linoleic acid

($C_{18:2}$) is a precursor of arachidonic acid ($C_{20:4}$) biosynthesis and is in relatively high concentration in several oilseeds. One of the richest sources of linoleic acid is sunflower oil. Therefore, the objectives of this study were to determine the influence of sunflower oil supplementation, dexamethasone, and their combination, on adipose development and performance in early weaned beef steers.

Materials and Methods

Twenty-four, spring-born, Simmental × Angus half-sibling steer calves were used to determine the effects of dexamethasone and sunflower oil supplementation on intramuscular fat accretion. The dams of these steers were maintained at the Michigan State University Upper Peninsula Experiment Station, Chatham, MI. Calves were weighed at birth and castrated. Two wks before weaning, steers were vaccinated against clostridial infections (UltraBac 7, Pfizer Inc., New York, NY) and common respiratory diseases (CattleMaster 4 plus L5, Pfizer Inc.). Steers were weaned at an average age of 110 d, weighed and transported 650 km to the Beef Cattle Teaching and Research Center, East Lansing, MI. Calves were given a 50 d adjustment period while being stepped up to a high concentrate diet. Calves were given booster vaccinations (Bovashield 4, Pfizer, Inc. and Ultrabac 7) 14 d after arrival at the feedlot, and were treated for internal and external parasites (IvomecPlus, Merial, Whitehouse Station, NJ) 21 d after arrival at the feedlot and before the initiation of treatments. Groups of six steers were housed in 13 × 3 m partially-covered outdoor pens and were adapted to individual feeders (American Calan Inc., Northwood, NH). None of the steers received anabolic implants during their lifetime to avoid possible confounding effects on intramuscular fat accretion.

Steers were allotted by age (average of 110 d) and weight (182 ± 21.7 kg), and randomly assigned to a 2 x 2 factorial arrangement of dietary and drug therapy treatments for a period of 112 d. Dietary treatments (Table 2-1) were basal diet only (**CON**) or basal diet with the addition of 8% high-linoleate, refined, bleached, and deodorized sunflower oil (**OIL**) (Columbus Foods Co., Chicago, IL). Fatty acid composition of the sunflower oil is presented in Table 2-2. Calves were also assigned to saline (**SAL**) or dexamethasone (**DEX**) treatment bouts every 28 d during the treatment period. Following the 112-d treatment period, all steers were fed a common basal diet until harvest at approximately 15 mo of age.

Because the composition of diets during the treatment period was not isocaloric, steers were organized into feeding groups to control calorie intake. Steers were organized into groups of four by DMI during the adjustment period so that each treatment group was represented once within each feeding group. Orts were removed, sampled, and weighed weekly, and fresh feed was offered daily to each steer. At the beginning of each wk the steer with the lowest energy intake from the previous wk was given ad libitum access to feed. The other three steers in the group were fed to meet the ad libitum fed steer's energy intake from the previous wk. The purpose of the feeding group system was to maintain isocaloric intake among treatments. Steers were switched from a diet containing an

average of 12.8% CP to one containing 11.7% CP (Table 2-1) at the end of the treatment period.

Injections of saline or dexamethasone were given at 0, 12, and 24 h after the start of each 28-d period. Dexamethasone-treated calves received intramuscular injections of 0.1 mg dexamethasone/kg body weight (Azium®, Schering Corp, Kenilworth, NJ; 2 mg dexamethasone/mL). Control calves received an equivalent volume of physiological saline. Consecutive injections were given on alternating sides of the neck.

Blood samples were collected by jugular venipuncture into 5-mL evacuated tubes containing citrate-dextrose on d 0, 1, and 7 of each 28-d period. Whole blood samples were used for immunostaining and flow cytometric analysis of L-selectin on blood leukocytes as described by (Burton et al., 1995). L-selectin is a neutrophil adhesion molecule that is down-regulated in dexamethasone treated cattle (Burton and Kehrli, 1995). L-selectin level was used to verify the effectiveness of the dexamethasone treatment and to monitor the return of normal immune function in the calves before the following bout of injections was administered.

Steers with rectal temperatures over 40°C (103.5°F) during the trial were considered to be morbid and those with clinical signs of bacterial infection were treated with Micotil (Elanco Animal Health, Indianapolis, IN),

Baytril (Bayer Animal Health, Shawnee Mission, KS), or Nuflor (Schering-Plough Corp., Union, NJ) per label instructions.

Initial and final steer body weights were calculated by averaging two weights taken on consecutive days. Interim weights and ultrasound measures were taken every 28 d throughout the finishing period. Percentage of lipid in the longissimus dorsi muscle, 12th-rib and rump subcutaneous fat, and 12th-rib longissimus dorsi area was evaluated using real-time ultrasound (Pie 200 SLC, Pie Medical, Tequesta, FL) by an Animal Ultrasound Practicioners Assoc. certified technician.

Experimental procedures in this trial were conducted according to those approved by the Michigan State University All University Committee on Animal Use and Care (AUF No. 09/00-131-00).

Feedstuffs were sampled every 21 d. Samples for each of the feedstuffs were ground through a Wiley mill fitted with a 1 mm screen (Arthur H. Thomas, Philadelphia, PA). After grinding, samples were combined into a single composite sample for each feedstuff. Dry matter was determined by drying samples in a forced air oven for 24 h at 102°C. Combustion method 990.03 (AOAC, 1995; Leco FP-2000, Leco Corp., St. Joseph, MI) was used to determine CP. Total lipid was determined by solvent extraction method 991.36 (AOAC, 1995; Tecator Soxtec System HT 1046 service unit and Tecator Soxtec System HT 1043 extraction unit, Tecator, Inc., Herndon, VA). Neutral detergent fiber was determined using an ANKOM 200/220 Fiber Analyzer (ANKOM Technology, Macedon, NY).

Steers were harvested at Murco Foods, Plainwell, MI. Carcass data were collected 48 h postmortem. Carcass measurements collected included hot carcass weight, longissimus muscle area, adjusted 12th rib subcutaneous fat thickness, marbling score, USDA Quality Grade, and skeletal maturity. Kidney, pelvic, and heart fat was trimmed from the carcass and weighed.

An 11-12 rib section was removed from the right side of each carcass and then frozen. The frozen rib section was fabricated into 2.5 cm thick steaks. Each steak was numbered sequentially starting from the anterior end of the rib section. Moisture, CP, lipid (Steak #1), and fatty acid composition (Steak #2) were determined in duplicate from powdered samples. Powdered samples were prepared by denuding frozen steaks of the spinalis dorsi muscle, external fat and connective tissue, so that only the longissimus dorsi muscle remained. Then, the frozen longissimus dorsi was sliced (0.5 cm thick) and cut into small pieces. Pieces of chopped muscle, along with dry ice, were powdered using a Tecmar grinder (Tecmar Co., Cincinnati, OH). Dry ice was allowed to evaporate before powdered samples were stored at -20°C. Moisture was determined by drying powdered muscle samples in a forced air oven for 18 h at 102°C (method 950.46; AOAC, 1995). Combustion method 992.15 (AOAC, 1995; Leco FP-2000, Leco Corp., St. Joseph, MI) was used to determine crude protein. Total lipid was determined as described for feed samples.

Dietary ingredients as well as the longissimus muscle (Steak #2) from each steer were homogenized and used for FA analysis. Samples were prepared for FA analysis as methyl esters (Sukhija and Palmquist, 1988) with nervonic acid ($C_{24:1}$) as the internal standard. Concentrations of FA were determined by using a gas chromatograph (Varian 3800, Varian Chromatography Systems, Wallnut Creek, CA) on a fused silica capillary column (100 m × 0.32 mm [SP 2560]; Supelco, Inc., Bellefonte, PA). The separation was achieved by a temperature gradient program beginning at 50°C and increased to 200°C at 15°C/min, held at 200°C for 25 min, raised to 225°C at 1°C/min, and finally held at 225°C for 27 min. The temperatures of the injection port and the flame ionization detector were set at 180 and 230°C, respectively. Helium was used as the carrier gas (flow rate of 1.5 mL/min with a total column pressure of 25 psi) with a split ratio of 100:1. Peaks were identified by comparisons with authentic standards and quantified by peak area integration.

Statistical analyses. Performance and tissue composition data were analyzed using the General Linear Model procedure of SAS (SAS Inst. Inc., Cary, NC) with individual steer as the experimental unit. The model included a random effect of steer, and fixed effects of diet, dexamethasone-treatment, and diet × dexamethasone-treatment interaction as independent variables. Data variables collected over time such as weight and ultrasound measures were analyzed using the appropriate repeated measures procedures. Two steers, both from the

dexamethasone-treated group, died during the course of the 112 d treatment period. Neither of the steers were observed to be morbid before death and no steers in the DEX group showed clinical signs of morbidity during the trial. Data from these two steers were not included in the analysis. The level of probability at which main effects and interactions were considered significant was $P < 0.05$ and $P < 0.10$, respectively.

Results and Discussion

Feedlot performance. Dexamethasone resulted in a decrease in DMI ($P < 0.05$), ADG ($P < 0.01$), and feed efficiency ($P < 0.05$) during the 112 d treatment period (Table 2-3). The decreased performance during this period of the trial may have been the result of a down-regulation of the immune system due to the acute dexamethasone treatment bouts. Results from blood samples of steers confirmed a down regulation of neutrophil adhesion molecules in DEX treated steers (Weber et al., 2001). Dexamethasone has been shown to down regulate key immune regulatory molecules on neutrophils (Burton et al., 1995). This results in decreased white blood cells being targeted to infected tissues and a consequent decrease in immune response. During the trial steers treated with DEX were observed to have more instances of fever that required treatment with antibiotics. Steers that received DEX were more likely to need one antibiotic treatment ($P < 0.05$) and were also more likely to need two or more treatments ($P < 0.01$). Thus, steers receiving DEX appear to have been fighting subclinical infections, decreasing ADG and feed efficiency.

The Mcal of NEg consumed during the treatment period as well as DMI were not different between treatments, indicating that the strategy of maintaining isocaloric intake between dietary treatment groups was successful.

During d 113 to 251, DEX tended to increase ADG ($P = 0.09$) and increased feed efficiency ($P < 0.05$). The increases in ADG and feed efficiency during d 113 to 251 of the finishing period for the DEX group may be attributed to compensatory gain as steers in the DEX group were lower performing in gain and efficiency during the 112 d treatment period. The increased feed efficiency during the 113 to 251 d finishing period was diminished however, in combination with OIL ($P < 0.05$). Over the entire trial (0 to 251 d), DEX appeared to increase feed efficiency, but increases in feed efficiency were reduced by an interaction between DEX and OIL. Because high lipid content of feed may serve to decrease ruminal digestibility (Doreau and Chilliard, 1997), the decreases in feed efficiency that were caused by OIL may be attributed to decreased digestibility of the diet.

Ultrasound measures are presented in Table 2-4. Subcutaneous fat thickness in DEX treated steers remained lower for the entire trial, even after DEX treatment was terminated, however it was not a significant decrease in accumulation. Overall DEX did not significantly affect intramuscular lipid content. This agrees with the work of Romans et al. (1974) in which it was observed that increases in exercise stress caused lipolysis and increased circulating free fatty acids without detrimental effects on intramuscular lipid deposition. If subcutaneous tissue contains a larger percentage of mature adipocytes per gram of tissue than intramuscular adipose in cattle as observed by May et al. (1994), the

subcutaneous depot may experience a more acute reaction to the lipolytic effect of glucocorticoids than intramuscular tissue, however in this study not significant differences were seen in either depot. The failure of dexamethasone to increase intramuscular lipid, however, is in contradiction of the results of Brethour (1972). Brethour (1972) reported that injections of exogenous glucocorticoids significantly raised intramuscular fat content and marbling scores in steers that were administered dexamethasone before harvest.

Differences in intramuscular fat content for the treatment groups as determined by ultrasound every 28 d are plotted in (Figure 2-1). Supplemental sunflower oil increased ultrasound intramuscular fat over time compared to control but not to a significant degree. If the increased availability of linoleic acid from sunflower oil supplementation resulted in increased arachadonic acid synthesis and metabolism, a subsequent increase in prostacyclins and cAMP-elevating agents may have resulted. Aubert et al. (2000) concluded that similar conditions could result in an adipogenic response. It is also possible that increases in intramuscular lipid may have been elicited by the availability of readily esterifiable fatty acids provided by the sunflower oil.

Dexamethsone may have exerted opposite effects on marbling at different points during the trial. The apparent increase in intramuscular lipid caused by DEX after the first treatment may have had the effect of causing differentiation of preadipocytes thus maturing adipose cells in the

intramuscular tissue. With the second treatment, DEX caused a decrease in intramuscular lipid. DEX may have exerted an opposite effect on the mature cells, causing lipolysis and a subsequent decrease in intramuscular adipose tissue with later injections. Another explanation for DEX failing to increase intramuscular fat over the entire trial period may be due to the large, acute exposure to glucocorticoids resulting in a down-regulation of glucocorticoid receptors and a decreased response (Preisler et al., 2000). Corah et al. (1995) reported that dexamethasone did not result in an increase in intramuscular lipid but only increased the external fat depth of steers. The method of administration for dexamethasone in the Corah et al., (1995) study was a long lasting implant instead of acute injections, which may have resulted in a similar down-regulation of the glucocorticoid receptor.

Differences in subcutaneous 12th rib backfat and subcutaneous rump fat are plotted in Figure 2-2 and Figure 2-3, respectively. Subcutaneous fat thickness did not show the same erratic increases and decreases in response to DEX treatments that were observed in intramuscular adipose.

Carcass marbling score and overall USDA Quality Grade of treatment groups were not significantly different from control (Table 2-5), and there were not significant differences in lipid content of the longissumus dorsi muscle of steers measured by proximate analysis (Table 2-6). Although ultrasound rib and rump fat were decreased by DEX

over the entire trial period, there were no significant differences in 12th rib fat thickness of carcasses or final yield grade. However, kidney, pelvic, and heart fat percentage was increased by DEX ($P < 0.05$) and tended ($P = 0.07$) to be increased by OIL.

Results of the fatty acid (**FA**) analysis on the longissimus dorsi muscle from the rib steaks of steers are presented in Table 2-7. Percentages of individual FA as compared to total FA were generally not significantly different and different treatment groups exhibited similar profiles.

Conjugated linoleic acid (**CLA**), a product of incomplete microbial biohydrogenation of linoleic acid did not significantly increase with the supplementation of the high-linoleate sunflower oil. This disagrees with previous work (Kennington et al, 2000; Mir et al., 2002) in which significant increases in CLA formation were realized due to increased linoleic acid in the diets of cattle. However, the 10,12 isomer of CLA, which is the less abundant isoform, was increased in a highly significant fashion by OIL ($P < 0.01$). The failure of sunflower oil to increase CLA content in the intramuscular lipid of steers may be due to the extended finishing period (138 d) that followed the end of sunflower oil supplementation. Because ruminant FA composition is affected by dietary FA composition (Doreau and Chilliard, 1997), the profile of intramuscular lipid may have been altered over the finishing period. This may partially explain the differing CLA content from this trial as compared to previous work.

Implications

Intramuscular and subcutaneous adipose tissue may be subject to manipulation by glucocorticoids. Stimulation of intramuscular adipose tissue by dexamethasone may be able to increase marbling at a particular time or stage of tissue development. However, additional investigations would be required to discover the optimum time and dosage of dexamethasone administration to exert these effects. Additionally, dexamethasone may be a useful tool in reducing subcutaneous adipose tissue.

Supplementation of sunflower oil increased ultrasound measured intramuscular lipid content in beef cattle, but not to a significant degree and did not increase carcass marbling score. Furthermore, sunflower oil supplementation did not significantly change fatty acid profile in rib steaks or increase levels of CLA. As this is in contradiction with previously cited work, it is most likely the effect of an extended finishing period with the absence of sunflower oil. This suggests that the effects of sunflower oil on adipose may only be realized when the oil is supplemented to harvest.

Table 2-1. Composition of finishing diets (DM basis)

Ingredient	Diet d 0-112		Diet d 113-251
	CON	OIL	
	--% of diet --		
High moisture shelled corn	65.7	56.0	74.0
Corn silage	15.0	15.0	15.0
Soybean meal	11.9	14.2	5.8
Sunflower oil	0.0	8.0	0.0
Protein-mineral-vitamin supplement[a]	5.0	5.0	5.0
Calcium carbonate	0.52	0.52	0.0
Magnesium oxide	0.30	0.30	0.0
Components			
Protein	13.2	12.3	11.7
NDF	15.2	13.3	15.4
Lipid	1.2	9.0	1.3
NE_g, Mcal/kg	1.39	1.56	1.41

[a]Protein-mineral-vitamin supplement composition -- soybean meal, 47.7%; calcium carbonate, 22.1%; urea, 7.5%; potassium chloride, 5.6%; dicalcium phosphate, 2.2%; ground corn, 3.3%; selenium 90 (198 mg/kg) 1.06%; Vitamin A (30,000 IU/g), 0.16%; Rumensin 80 (176 g monensin/kg), 0.28%; Trace mineral salt (NaCl, 94%; Zn, 3500 ppm; Fe, 2000 ppm; Cu, 300 ppm; I, 70 ppm; Co, 50 ppm), 10.0%.

Table 2-2. Fatty acid profile of sunflower oil provided to steers during the 112 d treatment period

Fatty acid	% of Sunflower oil
16:0 palmitic acid	5.8
16:1 palmitoleic acid	0.1
18:0 stearic acid	3.0
18:1 oleic acid	0.4
18:1ω-9 oleic acid	18.4
18:1ω-7 oleic acid	0.7
18:2ω-6 linoleic acid	68.4
18:3ω-3 linolenic acid	0.8
Other	2.4

Table 2-3. Finishing performance of steers that received saline (SAL) or dexamethasone (DEX) treatments and received diets containing no supplemental oil (CON) or sunflower oil (OIL) for a 112-d treatment period

	SAL		DEX			Prob.		
	CON	OIL	CON	OIL	SEM[a]	DEX	OIL	DEXxOIL
No. of steers	6	6	5	5				
DMI, kg/d								
d 0-112	6.2	5.5	5.3	5.6	0.29	0.17	0.38	0.04
d 113-251	7.9	7.8	7.0	7.8	0.41	0.34	0.39	0.28
d 0-251	7.1	6.7	6.3	6.8	0.27	0.22	0.73	0.11
NEg Intake, Mcal/d[b]								
d 0-112	8.66	7.68	7.51	7.94	0.33	0.17	0.38	0.04
d 113-251	11.3	11.1	10.1	11.20	0.58	0.34	0.39	0.27
d 0-251	10.1	9.54	8.90	9.70	0.41	0.23	0.72	0.11
ADG, kg/d								
d 0-112	1.24	1.09	0.98	1.00	0.06	0.01	0.26	0.16
d 113-251	1.29	1.31	1.47	1.32	0.06	0.09	0.24	0.13
d 0-251	1.27	1.22	1.27	1.19	0.05	0.76	0.13	0.71
Gain/feed								
d 0-112	0.20	0.20	0.18	0.18	0.01	0.02	0.54	0.84
d 113-251	0.19	0.19	0.25	0.19	0.01	0.02	0.09	0.03
d 0-251	0.19	0.20	0.22	0.19	0.01	0.22	0.06	0.03

[a]Standard error of the mean.
[b]Based on energy value of feedstuffs according to 1996 NRC.

Table 2-4. Ultrasound measures of steers that received saline (SAL) or dexamethasone (DEX) treatments and received diets containing no supplemental oil (CON) or sunflower oil (OIL) for a 112 d treatment period

	SAL		DEX			Prob.		
	CON	OIL	CON	OIL	SEM[a]	DEX	OIL	DEXxOIL
No. of steers	6	6	5	5				
Increase in intramuscular fat, % lipid								
0-112 d	0.72	0.70	0.37	0.57	0.24	0.32	0.70	0.62
113-251 d	0.83	0.68	0.56	1.23	0.36	0.69	0.45	0.24
0-251 d	1.55	1.38	0.92	1.81	0.44	0.81	0.41	0.22
Increase in 12th rib fat thickness, cm								
0-112 d	0.20	0.15	0.15	0.20	0.043	0.96	0.77	0.32
113-251 d	0.43	0.46	0.36	0.36	0.071	0.22	0.80	0.86
0-251 d	0.63	0.61	0.51	0.56	0.101	0.38	0.96	0.76
Increase in rump fat thickness, cm								
0-112 d	0.23	0.13	0.12	0.13	0.056	0.33	0.31	0.23
113-251 d	0.31	0.38	0.22	0.25	0.048	0.02	0.24	0.59
0-251 d	0.55	0.51	0.33	0.38	0.074	0.03	0.99	0.56

[a]Standard error of the mean.

Table 2-5. Carcass data for steers that received saline (SAL) or dexamethasone (DEX) treatments and received diets containing no supplemental oil (CON) or sunflower oil (OIL) for a 112 d treatment period

	SAL		DEX			Prob.		
	CON	OIL	CON	OIL	SEM[a]	DEX	OIL	DEXxOIL
No. of steers	6	6	5	5				
Hot carcass wt., kg	333	333	336	334	10.9	0.86	0.94	0.90
KPH fat, %	1.63	2.16	2.19	2.76	0.29	0.05	0.07	0.93
Longissimus muscle area, cm^2	88.4	88.4	87.7	80.7	3.9	0.28	0.34	0.34
Adj. 12^{th} rib fat thickness, cm	1.24	1.19	1.12	1.30	0.20	0.96	0.78	0.55
Yield grade	2.47	2.52	2.51	3.11	0.34	0.35	0.33	0.40
Marbling score[b]	450	393	398	448	26.8	0.96	0.90	0.05
Quality grade	Ch-	Se+	Se+	Ch-				

[a]Standard error of the mean.
[b]300=Slight^0, 400=Small^0.

Table 2-6. Proximate analysis of longissimus dorsi muscle from rib steaks of steers that received saline (SAL) or dexamethasone (DEX) treatments and received diets containing no supplemental oil (CON) or sunflower oil (OIL) for a 112 d treatment period

	SAL		DEX			Prob.		
	CON	OIL	CON	OIL	SEM[a]	DEX	OIL	DEXxOIL
No. of steers	6	6	5	5				
Moisture, %	69.77	68.77	71.36	71.48	1.58	0.17	0.77	0.71
Intramuscular lipid, %	5.39	4.32	3.64	4.46	0.61	0.19	0.83	0.12
Protein, %	30.74	29.36	26.10	27.83	2.10	0.14	0.93	0.45

[a]Standard error of the mean.

Table 2-7. Fatty acids as a percentage of total fatty acids for steers that received saline (SAL) or dexamethasone (DEX) treatments and received diets containing no supplemental oil (CON) or sunflower oil (OIL) for a 112 d treatment period

	SAL		DEX			Prob.		
	CON	OIL	CON	OIL	SEM[a]	DEX	OIL	DEXxOIL
No. of steers	6	5	5	5				
Myristic acid (14:0)	2.930	2.647	2.829	3.039	0.239	0.54	0.88	0.31
Myristoleic acid (14:1)	0.043	0.369	0.502	0.090	0.255	0.72	0.87	0.16
Palmitic acid (16:0)	27.57	26.87	27.50	27.36	0.566	0.70	0.45	0.62
Palmitoleic acid (16:1)	3.552	2.783	3.210	3.352	0.172	0.51	0.08	0.02
Stearic acid (18:0)	12.41	14.33	13.25	12.78	0.470	0.45	0.14	0.02
Oleic acid (18:1n9)								
-cis-isomer	40.81	38.27	39.67	39.52	1.370	0.96	0.24	0.30
-trans isomer	2.372	3.610	2.293	3.603	0.337	0.90	<0.01	0.91
Linoleic acid (18:2n6)								
-cis-isomer	3.329	4.305	3.745	3.552	0.340	0.62	0.26	0.10
-trans-isomer	0.059	0.051	0.049	0.050	0.011	0.62	0.81	0.67
Cojugated linoleic acid								
-9,11 isomer	0.236	0.200	0.224	0.283	0.026	0.19	0.65	0.08
-10,12 isomer	0.007	0.047	0.002	0.037	0.011	0.52	<0.01	0.84
Arachadonic acid (20:4n9)	0.001	0.004	0.003	0.003	0.001	0.72	0.56	0.46

[a]Standard error of the mean.

Figure 2-1. Ultrasound readings of intramuscular fat taken at 28 d increments for steers that were control, received supplemental sunflower oil in the diet (OIL), received treatments of dexamethasone (DEX), or received supplemental sunflower oil in the diet and received treatments of dexamethasone (OIL+DEX).

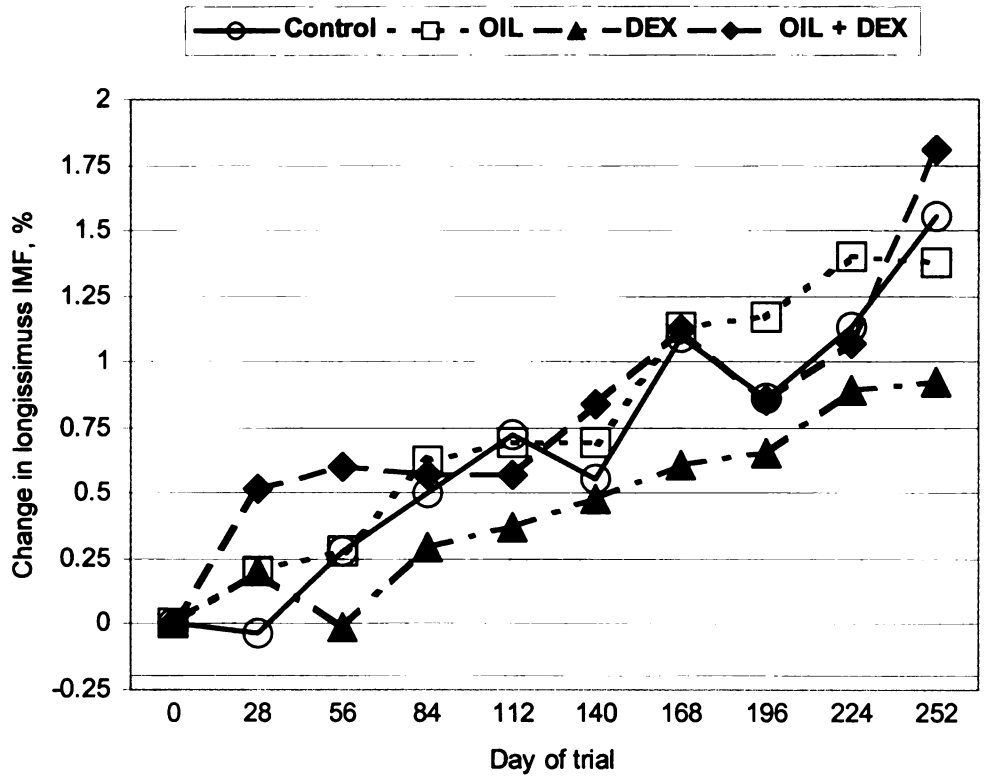


Figure 2-2. Ultrasound readings of subcutaneous 12th rib fat taken at 28 d increments for steers that were control, received supplemental sunflower oil in the diet (OIL), received treatments of dexamethasone (DEX), or received supplemental sunflower oil in the diet and received treatments of dexamethasone (OIL+DEX).

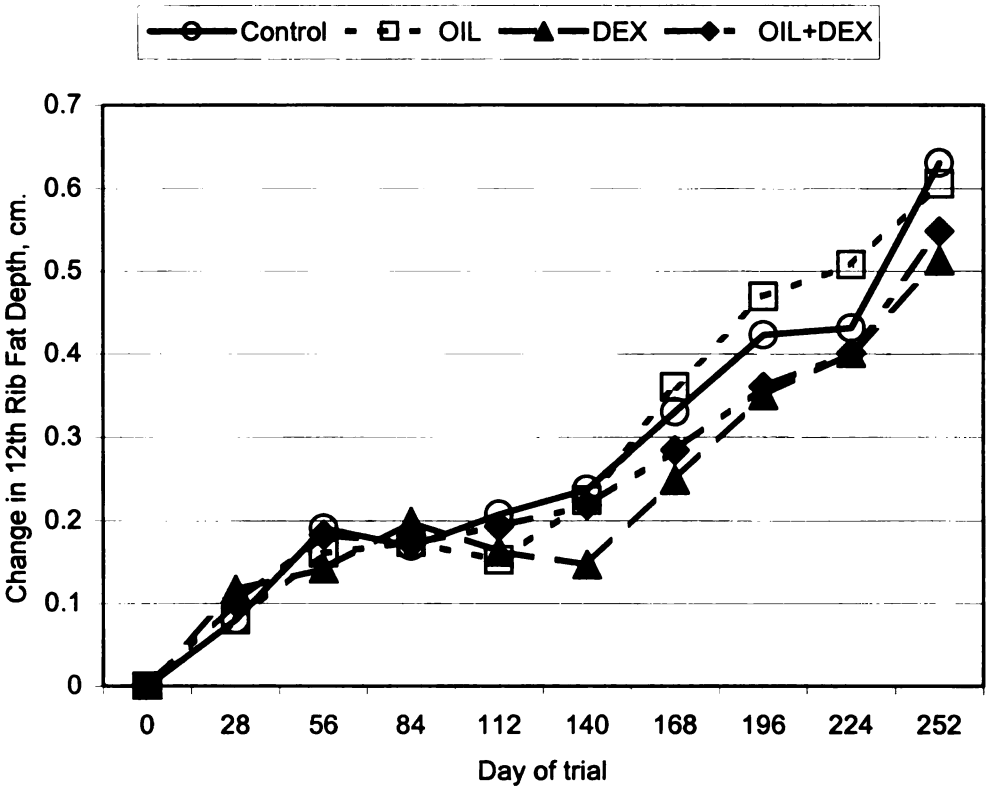
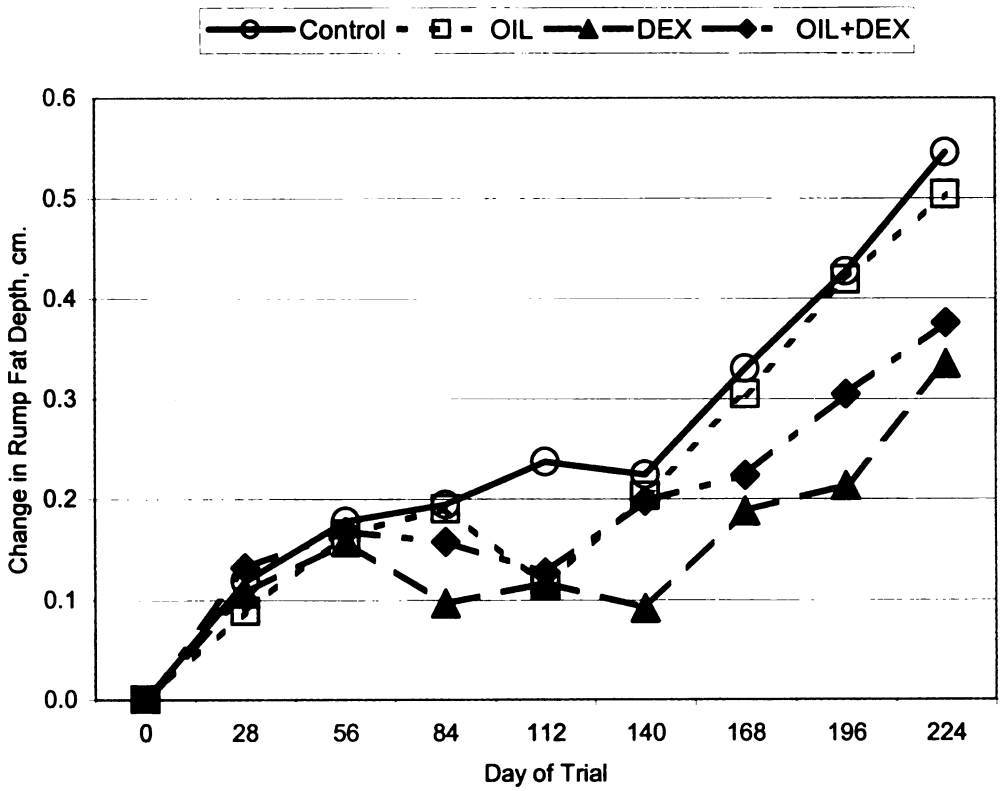


Figure 2-3. Ultrasound readings of subcutaneous rump fat taken at 28 d increments for steers that were control, received supplemental sunflower oil in the diet (OIL), received treatments of dexamethasone (DEX), or received supplemental sunflower oil in the diet and received treatments of dexamethasone (OIL+DEX).



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CHAPTER III

DIFFERENTIAL GENE EXPRESSION OF SUBCUTANEOUS AND INTRAMUSCULAR ADIPOSE TISSUE FROM BEEF STEERS TREATED WITH DEXAMETHASONE

ABSTRACT

The objective of this study was to investigate the effects of dexamethasone on subcutaneous and intramuscular adipose tissue based on differences in expressed genes. Four Angus steers (585 ± 27 kg) of similar origin were assigned to two groups and received either a single injection of saline (**CON**) or of 0.1 mg dexamethasone/kg of body weight (**DEX**) 24 h before harvest. Tissue samples of subcutaneous and intramuscular adipose were obtained postmortem and immediately flash-frozen in liquid nitrogen for subsequent RNA isolations. Analysis of differential gene expression was carried out by using the isolated RNA to probe bovine cDNA microarrays. The presence of glucocorticoid receptor mRNA in each tissue was also examined using Northern blot hybridization. The microarray analysis revealed that DEX up-regulated ($P < 0.01$) expression of urokinase receptor mRNA in both adipose tissue depots, suggesting induction of the mitotic cell cycle and preadipose cell proliferation. In addition, intramuscular adipose tissue from both treatment groups exhibited significantly greater expression of genes involved in cellular proliferation (MDM2; c-myc; hypoxanthine phosphoribosyltransferase; alpha-tubulin) than tissue collected from the

subcutaneous depot. Intramuscular adipose tissue was also observed to have greater expression ($P < 0.05$) of four genes involved in cellular apoptosis (Caspase-4, serine protease inhibitor p19, bax-alpha, and TRAF5) than in subcutaneous tissue. Northern blot analysis confirmed expression of the glucocorticoid receptor (**GR**) in both types of adipose tissue; however, expression was significantly lower ($P < 0.01$) in intramuscular than in subcutaneous adipose depots. Administration of DEX lowered GR mRNA abundance in both adipose depots ($P < 0.05$), suggesting negative feedback of the steroid on expression of its receptor. The results of this study suggest that subcutaneous adipose tissue is different from intramuscular adipose tissue at the level of expressed genes, and that subcutaneous adipose depots may be more sensitive to the lipolytic effects of glucocorticoids due to greater abundance of GR than intramuscular depots. Further studies are needed for characterization of the differences between the two depots to determine the reasons behind the differential gene expression that is observed.

Introduction

Evidence has accumulated to indicate that intramuscular adipocytes represent a cell population different from more extensively investigated subcutaneous adipocytes (Lin et al., 1992). In examining the differences between adipose tissue depots on a cellular level it was observed that intramuscular adipocytes had a smaller cell diameter and cell volume than subcutaneous adipocytes (May et al., 1994). Adipocytes from subcutaneous depots are larger and more spherical in shape and contain greater accumulation of triacylglycerides than adipocytes from intramuscular depots. This is likely due, in part, to a greater number of adipocytes being more mature at a given age of the animal in subcutaneous versus intramuscular fat. Terminal cellular differentiation is a process in which cells begin to arrest the proliferative cycle and take on characteristics of a specific mature cell type. The greater accumulation of triacylglycerides exhibited in subcutaneous adipocytes may be the result of a greater number of differentiated adipocytes in that depot. Glucocorticoid, or its synthetic analog dexamethasone, is routinely used in adipocyte cell cultures to induce adipocyte differentiation (Martin et al., 1998).

Because of the apparent biological differences of adipocytes among depot locations and the influence of glucocorticoid hormones on adipocyte differentiation, the purpose of this study was to identify unique

aspects of intramuscular and subcutaneous adipose on a molecular level as well as to characterize a response to dexamethasone treatment within the two depots by surveying differences in expressed gene profiles.

Materials and Methods

Four Angus steers of similar origin (585 ± 27 kg) were allotted by ultrasound intramuscular fat percentage to one of two treatments. Steers received either an injection of 0.1 mg dexamethasone/kg body weight (**DEX**) (Azium®, Schering Corp, Kenilworth, NJ; 2 mg dexamethasone/mL) or an equivalent volume of physiological saline (**CON**). A single injection was administered intramuscularly in the neck 24 h before harvest.

Sample collection. Steers were harvested at the Michigan State University Meats Laboratory. Boneless rib steaks approximately 5 to 8 cm thick were obtained from between the 10^{th} and 12^{th} rib immediately after exsanguination by cutting directly through the hide. Sections of subcutaneous fat were sliced into approximately one gram pieces. The portion of longissimus dorsi muscle was dissected and tissue snips of intramuscular adipose tissue were removed. Care was taken to obtain sample free from visible muscle fibers. The tissue snips were snap frozen in liquid nitrogen, collected into 50 mL conical polypropylene tubes and placed on dry ice. Each of the two sample types (subcutaneous and intramuscular adipose tissue) was collected within 30 min of exsanguination. Upon the completion of sample collections, samples were stored at -80°C.

Extraction of total RNA. Total RNA was extracted using TRIzol® reagent (Life Technologies, Gaithersburg, MD) by the following

procedures. Tissue samples were homogenized in 1 mL of TRIzol® reagent per 50 to 100 mg of tissue. Homogenized samples were incubated for 5 min at 30°C after which 0.2 mL of chloroform was added per 1 mL of TRIzol®. The sample was mixed, incubated for a second time at 30°C for 3 min and centrifuged at 12,000 x *g* for 10 min. Following centrifugation, the colorless, aqueous phase containing RNA was removed and the RNA precipitated using isopropyl alcohol at 0.5 mL per 1 mL of TRIzol®. Samples were then incubated at 30°C for 10 min and centrifuged at 12,000 x *g* for 10 min. After the RNA was precipitated, it was washed with 75% ethanol using 1 mL of 75% ethanol per 1mL of original TRIzol® reagent and centrifuged at 12,000 x *g* for 10 min. The 75% ethanol wash was then repeated. After extraction, RNA was dried and redissolved in RNase-free water by passing the solution through a pipette and incubating for 10 min at 55°C. One-tenth the sample volume of 3 M sodium acetate and 2.5 times the sample volume of 100% ethanol were then added and mixed with sample for precipitation. The dissolved sample was then stored at -80°C for overnight precipitation of RNA. Samples were then recovered and dissolved in RNAse-free water. Concentration and purity of RNA was determined by analyzing a 1/10 dilution of each sample using a DU-650 spectrophotometer (Beckman, Schaumberg, IL). After determination of concentration, integrity of RNA samples was checked by ethidium bromide staining and visualization of

gel electrophoresed 28S and 18S rRNA bands using procedures outlined by Weber et al. (2001).

RNA analysis. Microarray analysis of differential gene expression among adipose tissue samples was carried out in the Michigan State University Center for Animal Functional Genomics.

Samples of RNA from the same location and treatment group were pooled for analysis to obtain the necessary 20 μg for microarray analysis. Microarray analysis was performed in a loop design (Kerr and Churchill, 2001) to examine differences between subcutaneous and intramuscular adipose tissue samples with and without dexamethasone treatment. The design included four microarray slides that provided for each sample to be labeled with both Cy3 (green) and Cy5 (red) fluorescent dyes (Amersham Pharmacia Biotech, Piscataway, NJ) once during the design. The four arrays that comprised the design were prepared to demonstrate four different comparisons of adipose tissue sample: (1) subcutaneous, dexamethasone-treated (Cy5) versus subcutaneous, control (Cy3) (2) intramuscular, dexamethasone-treated (Cy3) versus intramuscular, control (Cy5) (3) subcutaneous, dexamethasone-treated (Cy3) and intramuscular, dexamethasone-treated (Cy5) (4) subcutaneous, control (Cy5) versus intramuscular,control (Cy3).

Synthesis of cDNA was carried out using the Atlas Glass Fluorescent Labeling Kit (Clonetech Laboratories, Palo Alto, CA). Fluorescent dye labeling and cDNA purification were carried out according

to the manufacturer's instructions. Samples were then used to interrogate cDNA microarrays containing 1056 unique bovine ESTs derived from a bovine total leukocyte library (Burton et al., 2001; Yao et al., 2001) and targeted genes involved in transcription, cell signaling, cell migration, and apoptosis (Coussens and Nobis, 2002). Probe was allowed to hybridize to a microarray slide for 18 h using a GeneTAC Hybridization Station (Genomic Solutions, Ann Arbor, MI). The hybridized slide was scanned using a GeneTAC LS 4 and GeneTAC Integrator 3.3 software (Genomic Solutions, Ann Arbor, MI) was used to obtain spot ratios from the scanned microarray slide.

Northern hybridization. Northern blot analysis was carried out using procedures outlined by Weber et al. (2001). Samples of RNA for Northern blot analysis of GR mRNA abundance were pooled as described above for microarray analysis, providing four different treatment groups. Each of the four samples were loaded in duplicate across a total of eight lanes (10 μg per lane) and electrophoresed in a 1.2% agarose denaturing gel. Separated RNA was then transferred to a nylon membrane by use of a Turbo Blotter apparatus (Schleicher and Schuell Bioscience, Inc. USA, Keene, NH) according to the manufacturer's instructions. RNA was UV crosslinked on the membrane. The membrane was then pre-hybridized at 42°C for 4 h with a solution containing 50% formamide, 5X Denhardt's, 6X SSC, 0.1% SDS, 0.05 M phospate buffer, 1.0 mM EDTA, and 0.15 mg per mL yeast tRNA. Samples of mRNA were hybridized in the same solution

at the same temperature for 18 h with a ^{32}P labeled probe for the glucocorticoid receptor 9-α subunit. The GR cDNA probe used in this study was obtained from Dr. J. L. Burton (Michigan State University, East Lansing, MI). The cDNA was developed by PCR amplification using a primer pair designed from sequence of human GR exon 9-α (Hollenberg et al., 1985; Encio and Detera-Wadleigh, 1991) and Holstein genomic DNA as the template. The 1281 bp PCR amplification product was cloned and sequenced as described by Weber et al., (2001) and was 88% homologous to corresponding cDNA sequence of exon 9-α in the human GR cDNA (Hollenberg et al., 1985). Following probe hybridization, the membrane was washed at room temperature with 2X SSC, 0.1% SDS for 15 min and three times with 0.1X SSC, 0.1% SDS at 65°C. The membrane was then exposed to BioMax MS film (Fisher Scientific, Pittsburgh, PA) for 8 d at -80°C. The membrane was then stripped of radioactive probe and rehybridized as described above with a ^{32}P labeled probe for β-actin. The β-actin cDNA was obtained from Dr. L. Kedes (Stanford University School of Medicine; Palto Alto, CA) and is described by Ponte et al. (1984) and donated for this study by Dr. J. L. Burton. The membrane was exposed to film at -80°C for 2 d. Quantitative analysis of mRNA abundance was measured using a GS-710 calibrated imaging densitometer and multi-analyst software (Bio-Rad Laboratories, Hercules, CA) and GR expression was shown as a ratio to beta actin expression.

Statistical analysis. Data from each array were normalized using the LOESS procedure of SAS (SAS Inst. Inc., Cary, NC) as described by Cleveland and Grosse (1991). Normalization was assessed by constructing *M-A* scatter plots before and after normalization (Figure 3-11) in which log intensity ratios $M = \log(Cy3/Cy5) = \log Cy3 - \log Cy5$ were plotted against mean log intensities $A = (\log Cy3 + \log Cy5)/2$ for each array spot as described by Yang et al., (2002). The resulting normalized log intensities were then analyzed using the Mixed Model procedure of SAS. The model included fixed effects of dye, treatment, adipose tissue depot, and treatment × tissue interaction as independent variables. The level of probability at which main effects and interactions were considered significant was $P < 0.05$ and $P < 0.10$, respectively.

Results and Discussion

Northern blot analysis. Northern blot analysis was performed to examine glucocorticoid receptor (α-isoform) mRNA abundance between the two adipose tissue depots and in response to dexamethasone administration. Samples of RNA were hybridized with a ^{32}P-labeled probe specific for the 9-α subunit of the bovine glucocorticoid receptor. Subcutaneous adipose exhibited higher abundance of GRα mRNA message ($P < 0.01$) (Figures 3-1, 3-2) for GR than did intramuscular adipose, even after adjustment for loading inefficiency through β-actin normalization (Table 3-1). Additionally, it was observed that in both adipose tissue depots, dexamethasone acutely down-regulated expression of GRα mRNA ($P < 0.05$). These results are in agreement with Preisler et al. (2000) who showed that bovine GRα proteins were down-regulated in white blood cells in response to high glucocorticoid concentrations at parturition. The northern blot analysis confirmed the expression of GR in bovine adipose tissue as well as a response in gene expression to dexamethasone treatment. Northern blot analysis for expression of β-actin revealed additional bands within the intramuscualr adipose samples that appeared to result from expression of α-actin. This suggests that intramuscular adipose samples were most likely contaminated with a small amount of muscle tissue from dissection.

cDNA microarray analysis. A number of genes were significantly differentially expressed due to treatment with dexamethasone or location of adipose tissue. The cDNA microarray analysis revealed genes that changed expression in adipose tissue due to treatment with dexamethasone. The most biologically significant gene that showed differential expression upon stimulation by the glucocorticoid was the urokinase receptor ($P<0.01$) (Figure 3-3). Expression of the urokinase receptor has been associated with angiogenic function in epithelial cells (Mignatti et al., 1991) and its expression appears to be induced by increases in fibroblast growth factor (Mignatti et al., 1991; Pepper et al., 1993). However, more recent work indicates that the urokinase receptor and its associated cellular proteins also play a role in cell cycle control and proliferation, and that expression of the urokinase receptor increases as the cell cycle progresses (Zavizion et al., 1998). Glucocorticoid stimulation increased urokinase receptor expression in both adipose depots suggesting an increase in cell cycle activity of preadipose cells. Assuming that the glucocorticoid was able to withdraw preadipocytes from the proliferative cycle for differentiation, this increase in cell cycle activity may be the result of the mitotic clonal expansion that precedes terminal differentiation (Gregoire et al., 1998).

In addition to the urokinase receptor, several genes that are involved in cell adhesion were differentially expressed in response to glucocorticoid treatment. The glycoproteins P-selectin, CD6, CD28, and

GLYCAM-1 expression were all affected by dexamethasone administration. The findings of Burton et al. (1995) and Burton and Kerhli (1995) for similar cell adhesion molecule expression leukocytes showed a pronounced down regulation by dexamethasone. In the current study, CD6 was down-regulated with DEX ($P < 0.05$), however expression of CD28 and P-selectin were increased (both $P < 0.05$). The increase in expression of P-selectin may be partially explained by the work of Xia et al. (1998) who found P-selectin expression in cells is decreased by most anti-inflammatory drugs, with the exception of glucocorticoids. This does not, however, explain the apparent up-regulation of P-selectin expression. It was also noted that GLYCAM-1, a gene that codes for a mucin-like glycoprotein was differentially expressed between adipose tissue depot and appeared to be differentially regulated by dexamethasone administration. However biological function of this gene and its gene product does not appear to be determined. An explanation of the differential expression of genes that code for cell adhesion molecules in adipose tissue is not apparent. However, because the products of these genes are glycoproteins that are involved in cell membrane composition, it is possible that the differential expression may be the result of changes in cell size or number that were evoked by the dexamethasone treatment.

Differential gene expression was also observed between adipose tissue depots for a number of genes. MDM2, a cellular protein that is associated with the p53 tumor suppressor protein, had significantly greater

expression in intramuscular adipose (Figure 3-6). The p53 tumor suppressor protein functions in the halting of cellular proliferation (Mulligan and Jacks, 1998). The MDM2 protein acts as an oncogene by binding to and inactivating p53 (Lundgren et al., 1997). Thus, the greater expression of MDM2 in intramuscular adipose suggests that a larger percentage of cells within that depot are still experiencing proliferation. Furthermore, intramuscular adipose exhibited more abundant mRNA messages for hypoxanthine phophoribosyltransferase (Figure 3-4), a rate-limiting enzyme in purine synthesis. This suggests greater levels of nucleotide synthesis and thus higher proliferative activity of cells in intramuscular adipose. Additional evidence of a greater percentage of preadipose cells in the intramuscular depot is shown by the greater expression ($P < 0.05$) of alpha-tubulin in intramuscular versus subcutaneous adipose (Figure 3-5). Alpha-tubulin is a protein involved in the cytoskeletal composition of cells. The morphological changes in cell shape that accompany terminal adipocyte differentiation are marked by a decrease in such structural proteins as tubulin and actin (Spiegelman and Farmer, 1982).

Four different genes involved in cellular apoptosis were more highly expressed in intramuscular adipose ($P < 0.05$). Caspase-4, a protein involved in the fas-mediated pro-apoptotis pathway (Kamada et al., 1997; Martin and Panja, 2002), pro-apoptosis regulator bax-alpha, and the serine protease inhibitor p19 exhibited greater expression in intramuscular tissue (Figures 3-10, 3-7, and 3-9, respectively). Additionally, mRNA for

TRAF5, a factor associated with tumor necrosis factor-α (TNF-α) that serves to mediate pro-death effects of this potent cytokine in signaling pathways (Bradley and Pober, 2001), was more abundant in intramuscular adipose (Figure 3-8).

Implications

The results of this study point to fundamental differences between intramuscular and subcutaneous adipose on a molecular (gene expression) level. However, differential gene expression in adipose tissue due to depot location opens the possibility for independent manipulation of adipose tissue depots.

Additionally, bovine adipose tissue expressed abundant glucocorticoid receptor-alpha, especially in subcutaneous adipose tissue. This suggests that glucocorticoids may provide a useful way to regulate differential fat accretion in specific adipose depots.

Table 3-1. Densitometer quantifications of northern blots for GR9α and β-actin

Sample	Lane	GR9α density	β-actin density	Adj. GR9α density[a]
1-Subcutaneous, control	1	27.742	9.921	2.796
1-Subcutaneous, dex-treated	2	6.305	6.405	0.984
1-Intramuscular, control	3	7.261	8.565	0.848
1-Intramuscular, dex-treated	4	4.852	9.563	0.507
2-Subcutaneous, control	5	24.206	11.342	2.134
2-Subcutaneous, dex-treated	6	14.873	8.766	1.697
2-Intramuscular, control	7	9.041	8.712	1.037
2-Intramuscular, dex-treated	8	5.150	8.946	0.576

[a]GR9α density divided by β-actin density

Figure 3-1. Northern blots: Glucocorticoid receptor 9-alpha subunit mRNA (top); beta-actin (bottom) for subcutaneous (SQ) or intramuscular (IM) adipose tissue treated with saline (C) or dexamethasone (D).

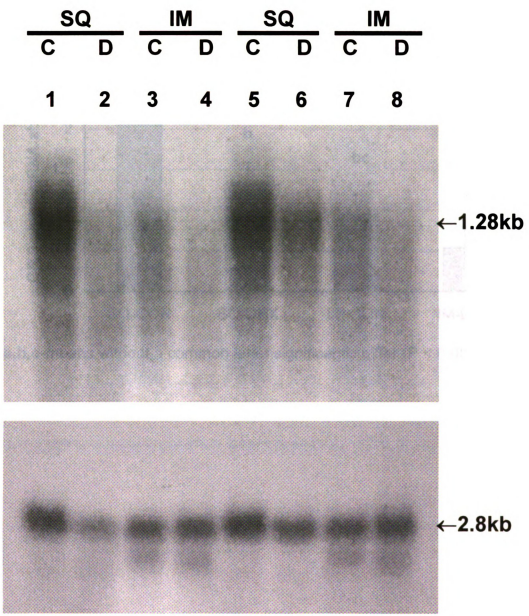
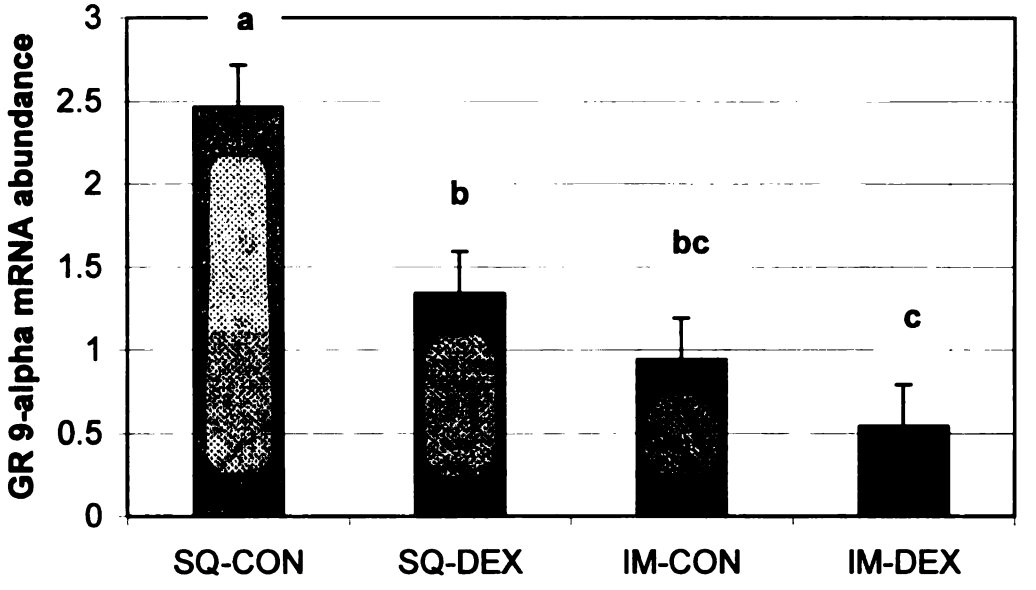
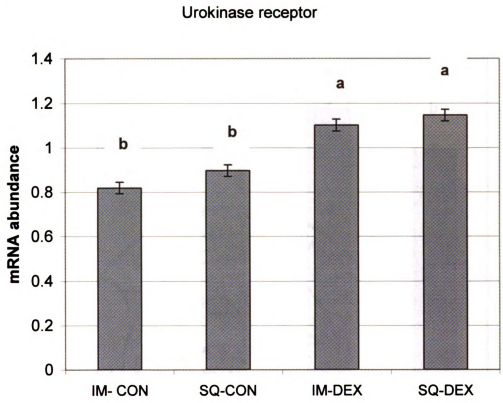


Figure 3-2. Northern blot densitomer quantifications for glucocorticoid receptor-alpha mRNA abundance in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



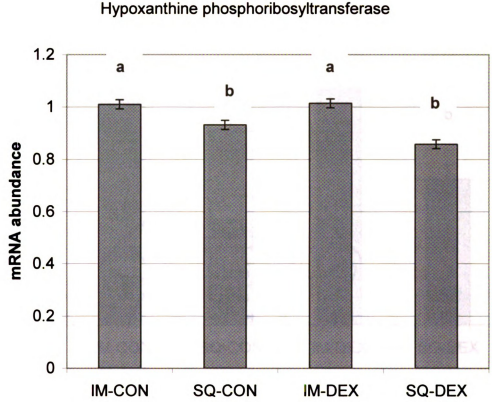
a,b,c-means without a common letter significantly differ ($P < 0.05$).

Figure 3-3. Expression of mRNA for urokinase receptor in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



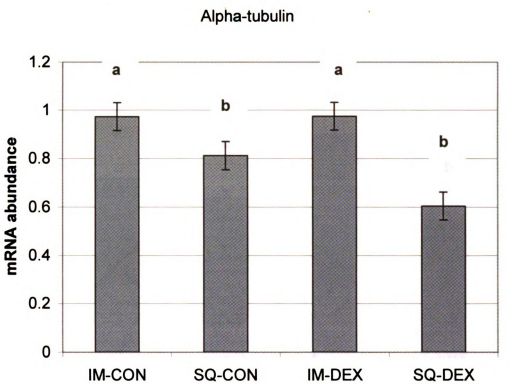
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-4. Expression of mRNA for hypoxanthine phosphoribosyltransferase in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



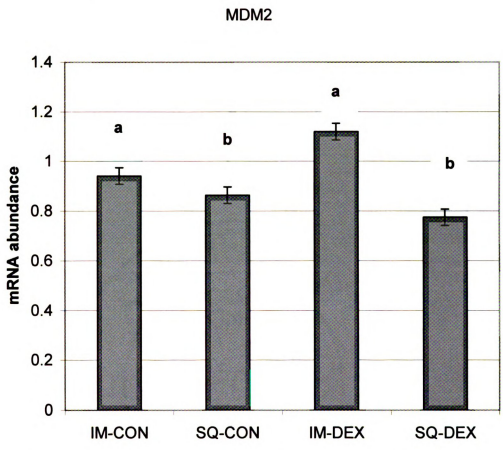
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-5. Expression of mRNA for alpha tubulin in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



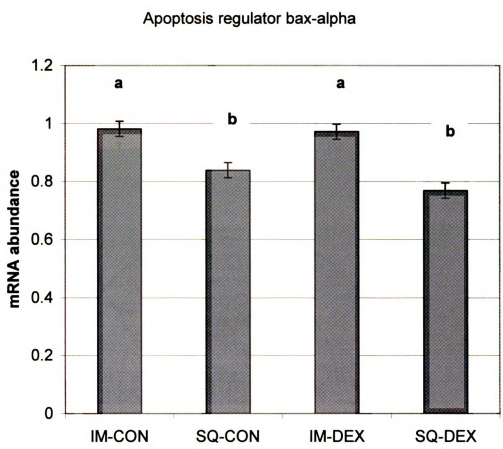
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-6. Expression of mRNA for MDM2 in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



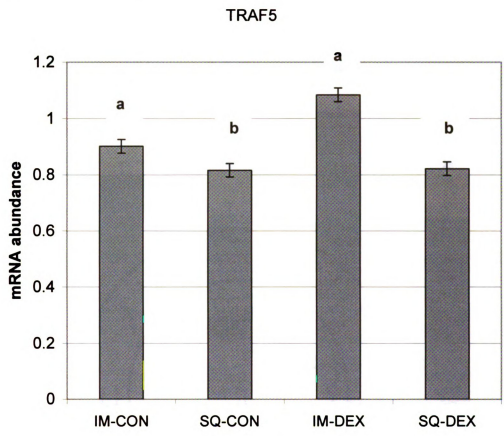
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-7. Expression of mRNA for Apoptosis regulator bax-alpha in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



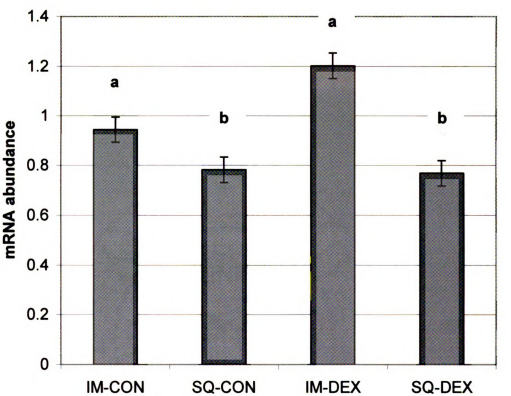
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-8. Expression of mRNA for TRAF5 in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



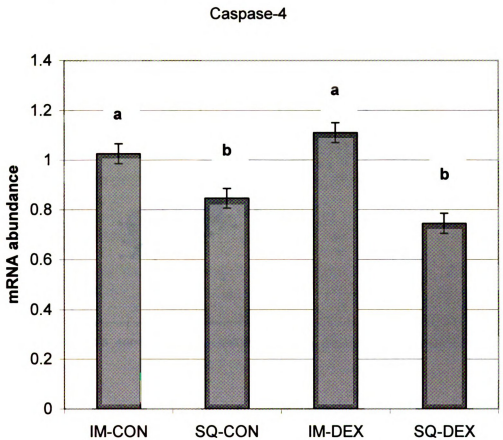
a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-9. Expression of mRNA for serine protease inhibitor p19 in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



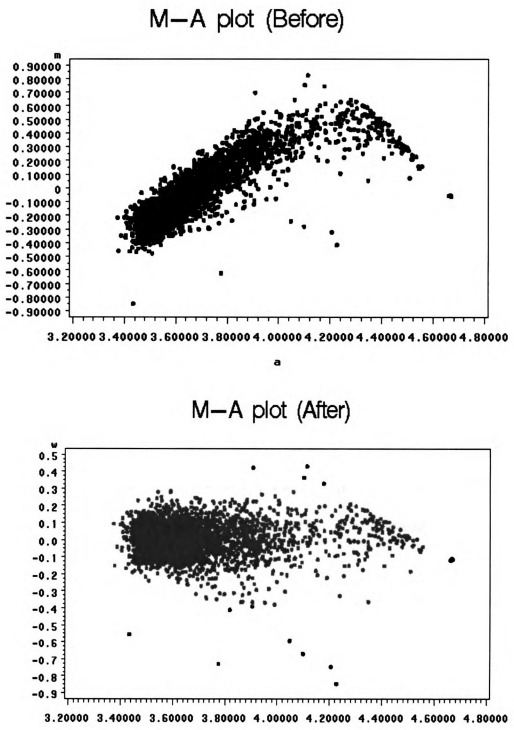
a,**b**- means without a common letter significantly differ ($P < 0.05$).

Figure 3-10. Expression of mRNA for Caspase-4 in intramuscular (IM) and subcutaneous (SQ) adipose tissue from steers treated with saline (CON) or dexamethasone (DEX)



a,b- means without a common letter significantly differ ($P < 0.05$).

Figure 3-11. M-A scatterplot of data from array comparing intramuscular, control tissue vs. intramuscular, dexamethasone-treated tissue before and after normalization



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CHAPTER IV

INTERPRETIVE SUMMARY

Adipose tissue of beef cattle, its rate of development, and its cost of accretion, form a cornerstone of profitability in the current beef industry. Accumulation of subcutaneous fat has historically been used as an indicator of quality in live cattle and as a tool to determine a market end point for fed slaughter cattle. However, actual correlation of quality to subcutaneous fat levels has been shown to be inconsistent at best, to highly ineffective at worst. In addition, the development of subcutaneous fat requires a greater input of dietary energy than lean growth and thus comes at the expense of decreased feed efficiency. From a carcass standpoint, increases in subcutaneous adipose require greater trim and result in a lower percentage of carcass weight that goes into retail product, decreasing revenues. In contrast, increased intramuscular adipose in beef carcasses is desirable, and it is the amount of visible intramuscular fat or marbling that is the primary determinant of quality grade in beef. Because cattle in the U.S. do not have sufficient marbling to meet consumer demand in the current industry, decreased revenues are the result. For these reasons, the focus of research and production has centered around the goal of decreasing subcutaneous fat while concurrently increasing intramuscular fat. The realization of this challenge has been made more difficult by the lack of response of intramuscular fat

to manipulation through management, nutrition, or physiology. Despite the difficulty of influencing intramuscular fat deposition, studies have shown that intramuscular and subcutaneous adipose tissue exhibit unique characteristics when compared on a cellular level. Therefore, the objectives of the current studies were to evoke and characterize differential responses between two adipose tissue depots. The purpose of the objectives was one of discovery and meant to provide a point of origin for further investigations on differential adipose tissue accretion.

In the initial experiment fat deposition of early weaned beef steers was examined in response to administration of dexamethasone therapy and supplementation of sunflower oil in the diet. Previous work had pointed to a differential response of intramuscular and subcutaneous adipose to glucocorticoid exposure. Past research also indicated the effects of glucocorticoids may be mediated through an increase in arachadonic acid metabolites, namely prostacyclins. Arachadonic acid is synthesized from the precursor linoleic acid, which is found in high concentrations in sunflower oil. Thus the treatments were chosen to form a 2 x 2 factorial study.

Ultrasound readings were taken on steers throughout the study to monitor fat deposition during the course of the trial. The results of the ultrasound showed that dexamethasone did not elicit a significant response in intramuscular tissue. Dexamethasone did lower subcutaneous fat over the trial but not significantly. Over the duration of

the experiment, the supplementation of sunflower oil did show an increase in intramuscular fat but again, it was not to a significant degree. Despite the positive results of the ultrasound, significant differences did not exist in final carcass characteristics for quality or yield. Additionally, it was determined that the fatty acid profile of steaks from the steer carcasses was not significantly affected by treatment.

Although the treatments appeared to stimulate a response in adipose tissue, the results of the end measurements of the study were inconclusive. This may have been confounded by an extended finishing period for the steers (138 d) after the cessation of treatment. Overall, the results of the study point to dexamethasone and sunflower oil as possible tools for the manipulation of adipose tissue in fed beef cattle. However, the time, duration, and dosage of dexamethasone for optimum response require further investigation for determination.

In the second experiment, intramuscular and subcutaneous adipose tissue samples were obtained from four fed beef steers at slaughter. Steers were administered a single injection of either saline or dexamethasone 24 h before harvest. Messenger RNA was extracted from tissue samples and differential gene expression between treatment groups and between adipose depot location was determined by cDNA microarray analysis and Northern blot hybridization. One gene that was differentially expressed upon stimulation of the glucocorticoid was the urokinase receptor. The urokinase receptor and its associated cellular proteins play

a role in cell cycle control and proliferation and expression of the urokinase receptor increases as the cell cycle progresses. Glucocorticoid stimulation increased urokinase receptor expression in both adipose depots suggesting an increase in cell cycle activity of preadipose cells. If the glucocorticoid was able to withdraw preadipocytes from the proliferative cycle for differentiation, this increase in cell cycle activity may be the result of the mitotic clonal expansion that precedes terminal differentiation.

Differential gene expression was also observed between adipose tissue depots for a number of genes. MDM2, a cellular protein that acts as an oncogene by binding to and inactivating p53 tumor suppressor gene exhibited greater expression in intramuscular adipose suggesting a larger percentage of cells within that depot are still experiencing proliferation. Furthermore, intramuscular adipose exhibited more abundant mRNA messages for hypoxanthine phophoribosyltransferase, a rate-limiting enzyme in purine synthesis, further indicating proliferative activity of cells. Additional evidence of a higher percentage of preadipose cells in the intramuscular depot is shown by the differential expression of alpha-tubulin. The significantly higher expression of structural proteins such as alpha-tubulin in intramuscular tissue, suggests a less mature, preadipose cell type.

In addition there were four genes that were differentially expressed between depots that are associated with cellular apoptosis. These

included pro-apoptosis regulator bax-alpha, serine protease inhibitor p19, Caspase-4, and TRAF5. Each of these genes had a significantly greater expression in intramuscular adipose tissue suggesting the cells within that depot are undergoing a greater rate of apoptosis.

Northern hybridization of mRNA with a radioactive probe specific for the 9-α subunit of the glucocorticoid receptor (**GR**), confirmed its expression in both adipose locations. Additionally, it was shown that GR expression was greater in subcutaneous adipose, and that dexamethasone treatment reduced expression of GR in both types of adipose. The results show that not only was dexamethasone successful in evoking a response in adipose tissue from both depots, but also that the differential level of GR expression may provide a means for separate manipulation of intramuscular and subcutaneous adipose depots.

The results of the study point to glucocorticoids as a potential endocrine regulator in adipose that has the capability to elicit a response that is unique to adipose depot location. Furthermore the identification of the urokinase receptor gene as a possible target of glucocorticoid-induced response provides a clue in the elucidation of the pathways involved in dexamethasone-mediated differentiation. Identification of the differential expression of MDM2 between intramuscular and subcutaneous adipose, gives a potential subject for further study of biological differences between the two tissues. Additionally, with the differential gene expression taken as a whole, it provides a new perspective on the two different adipose

tissue depots as unique cell populations. With intramuscular adipose tissue representing a group of cells that have greater rates of proliferation and apoptosis, these cells appear to have a greater turnover of cells. In contrast, cells from subcutaneous tissue appear to represent a more stable cell population in which lipid metabolism is a more primary function of the cells. Further investigations into the exact function of specific genes in the molecular pathways of adipocyte growth and metabolism will be required before any practical uses of their differential expression can be realized in adipose tissue development. However, it does provide a new aspect for research in this area of production as well as a greater understanding of the biological function of the different adipose tissue depots.


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