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INVESTIGATION OF ADIPOGENIC DIFFERENCES
BETWEEN BOVINE INTRAMUSCULAR AND
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ABSTRACT

INVESTIGATION OF ADIPOGENIC DIFFERENCES BETWEEN BOVINE INTRAMUSCULAR AND SUBCUTANEOUS PREADIPOCYTES

By

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Our objective was to examine signaling mechanisms most likely to explain the lower adipogenic capacity of bovine intramuscular (**IM**) preadipocytes when compared to bovine subcutaneous (**SC**) preadipocytes. We hypothesized that the lower adipogenesis of IM preadipocytes was caused by decreased glucocorticoid receptor expression (**GR**), sensitivity to glucocorticoids, peroxisome proliferator-activated receptor γ_2 (**PPARγ_2**) expression, and(or) PPARγ_2 ligand synthesis. Stromal-vascular cells, containing preadipocytes, were isolated from IM and SC adipose tissue of 3 Angus-cross steers. Immunoblot analysis detected GR immunoreactive bands of ~97, ~62, and ~48 kDa, which were equally expressed in IM and SC cells ($P > 0.50$). Intramuscular preadipocytes were less adipogenic than SC preadipocytes as determined by glycerol-3-phosphate dehydrogenase (**GPDH**) activity and oil red O staining ($P < 0.05$). Dexamethasone (**DEX**), a synthetic glucocorticoid, increased GPDH activity similarly in preadipocytes from both depots ($P < 0.05$). Dexamethasone increased the percentage of SC preadipocytes with lipid droplets ≥ 10 μm ($P = 0.002$), but had no effect on IM preadipocytes ($P > 0.27$). Immunoblot analysis revealed a PPARγ_2 immunoreactive band of ~53 kDa, which was expressed equally in IM and SC cells ($P = 0.39$). Conversely, IM cells secreted more of the presumptive PPAR ligand prostacyclin (**PGI_2**), than SC cells ($P = 0.046$). Because exposure of SC preadipocytes to an inhibitor of PGI_2 synthesis had no

effect on adipogenesis (P = 0.99), and exogenous $cPGI_2$ (PGI_2 analog) tended to enhance adipogenesis (P = 0.06), the greater secretion of PGI_2 by IM preadipocytes does not explain their lower adipogenesis. Exposing IM and SC preadipocytes to the cyclooxygenase (**COX**) inhibitor/PPARγ_2 ligand, ibuprofen (**IBU**) for 48 h or 12 d resulted in a treatment by depot interaction (P = 0.002). Ibuprofen exposure for 48 h enhanced DEX stimulation of GPDH activity only in IM cells (P = 0.009). Exposure to 100 μM and 500 μM IBU for 12 d enhanced DEX induction of differentiation in IM preadipocytes, whereas only 100 μM IBU enhanced DEX induction of differentiation in SC preadipocytes ($P \leq 0.05$). In the absence of DEX, exposure to IBU for 12 d maximally increased GPDH activity in IM preadipocytes by 12-fold, but only increased GPDH activity by 1.5-fold relative to control in SC preadipocytes ($P < 0.001$). Contrary to IBU, 500 μM aspirin (a COX inhibitor) did not affect GPDH activity either alone ($P > .37$), or combined with DEX ($P > 0.60$) in either cell population. Because IBU diminished adipogenic differences between IM and SC preadipocytes, it is suggested that these adipogenic differences may be partially related to differences in the endogenous activation of PPARγ_2. The use of selective PPARγ_2 agonists or antagonists offers potential to selectively alter adipogenesis in economically-important bovine adipose tissue depots.

I dedicate this dissertation to my wife, Catherine Mazak-Tumminia, whose support was essential for the completion of this project.
I am deeply indebted to you for keeping my hopes up when the end was not even seen as a possibility.
Gracias… de verdad que "somos mucho más que dos".

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADD1	Adipocyte determination and differentiation factor-1
ADRP	Adipocyte differentiation related protein
ALBP	Adipocyte lipid binding protein
ASP	Aspirin
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
$cPGI_2$	Carbaprostacyclin
C/EBP	CCAAT/Enhancer binding protein
CRF	Corticotropin releasing factor
COX	Cyclooxygenase
CREB	cAMP responsive element binding protein
DAPI	4', 6-Diamidino-2-phenylindole
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinase

FAS	Fatty acid synthase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GPDH	Glycerol-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
HCL	Hydrochloric acid
HPETE	Hydroperoxyeicosatetraenoic acids
IBU	Ibuprofen
IM	Intramuscular
JNK	c-Jun N-terminal kinases
LM	Longissimus muscle
LOX	Lipoxygenase
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MKP-1	MAPK phosphatase-1
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBFGC	National Bovine Functional Genomics Consortium
NDGA	Nordihydroguaracetic acid

NF-κβ	Nuclear factor kappa β
OM	Omental
ORO	Oil Red O
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol 3-kinase
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PR	Perirenal
Pref-1	Preadipocyte factor one
PVDF	Polyvinylidene fluoride
RXR	Retinoic X receptor
SAS	Statistical analysis systems
SC	Subcutaneous
SDS	Sodium dodecyl sulfate
SFM	Serum free media
SREBP-1c	Sterol responsive element-binding protein-1c
TBS	Tris buffer saline
TGZ	Troglitazone

TNF-α	Tumor necrosis factor alpha
TXA_2	Thromboxane A_2
USDA	United States Department of Agriculture

INTRODUCTION

Marbling, the adipose tissue embedded in the perimysial connective tissue matrix within skeletal muscle, is usually associated with beef palatability by consumers. Indeed, within a carcass maturity classification, marbling is the main determinant of USDA Quality Grade and producers are motivated to feed animals for long periods to attain premiums for highly marbled carcasses. Unfortunately, as producers fatten animals to meet these market demands, excessive subcutaneous (**SC**) fat results in significant economic losses. To selectively manipulate beef fat accretion, it is imperative to gain an understanding of the intrinsic differences between preadipocytes isolated from intramuscular (**IM**) and SC adipose tissues, and how these differences relate to the adipogenic capacity of the particular depots. The current understanding of preadipocyte differentiation suggests that observed adipogenic differences among bovine adipose tissues may be related to glucocorticoid receptor expression or activation. Alternatively, differences in peroxisome proliferator-activated receptor γ_2 (PPARγ_2) expression, or differences in the synthesis of PPARγ_2 activators may explain adipogenic differences among bovine adipose tissues. Identification of factors that selectively enhance adipogenesis of IM preadipocytes or selectively decrease adipogenesis of SC preadipocytes, would

create opportunities to develop practical methods to enhance beef quality. and(or) reduce waste.

CHAPTER I: REVIEW OF LITERATURE

Biological Importance of Adipose Tissues

Adipose tissues are essential for life because they function as energy reserves, insulation against cold, a source of metabolic water, hematopoietic tissue within the bone marrow (Klaus, 2001), and metabolic and appetite regulators crucial for the well being of the organism (Muoio et al., 1997; Houseknecht et al., 1998; Trayhurn et al., 2001; Frayn et al., 2003). Adipose tissues can be described as white adipose tissue and brown adipose tissue (Cinti, 2005), although these descriptors are the two extremes of a continuum (Pond, 1992, 1999). Whereas white adipose tissue accumulates energy as triacylglycerides after meals and releases energy when needed, brown adipose tissue's main function is thermogenesis through uncoupling oxidative phosphorylation (Cinti, 2005). At birth, most of the adipose tissues in ruminants can be considered brown adipose tissue, but the majority of these depots transform into white adipose tissue after birth (Ailhaud et al., 1992). The understanding of the function of adipose tissues has evolved dramatically in the last three decades, from being described as a "type of connective tissue that surrounds fat" (Allen et al., 1976), to now being recognized as integral and critical components of the endocrine system and essential in the organisms' normal physiology (Fruhbeck et al., 2001; Frayn et al., 2003).

During evolution, the development of adipose tissues was essential for homoeothermic animals to maintain their body temperature with a variable food supply (Klaus, 2001). In modern terrestrial mammals, adipose tissues have evolved to be encountered throughout the organism in more than twelve diverse (conspicuous and discrete) locations with very specialized functions (Pond, 1992). Generally, adipose tissues that primarily function in energy storage (i.e. perirenal (**PR**), subcutaneous (**SC**), inguinal) are located where they can expand rapidly without anatomical constraints during periods of high energy intake (Pond, 1999). Conspicuous adipose depots are composed of a nearly homogeneous adipocyte population that readily absorb lipids, are efficient in synthesizing *de novo* fatty acids, have high rates of lipolysis during fasting, and are good reporters of energy stores to the hypothalamus (Pond, 1999). Conversely, discrete adipose depots, like popliteal (found behind the knee), have an elaborate internal organization, a heterogeneous adipocyte population, and may function as metabolic regulators (Pond, 1992, 1999). For example, adipose tissues surrounding lymph nodes can be a source of paracrine substances and(or) essential fatty acids critical for proper function of the immune system (Pond, 1999; Klaus, 2001; Frayn et al., 2003; Pond, 2005). In addition, adipose tissues surrounding lymph nodes may provide fatty acids as nutritive fuel for immune cells (Frayn et al., 2003; Pond, 2005). Conditions of

malnourishment or obesity, where these specialized adipose depots are depleted of lipids or forced into lipid repositories, are related to malfunction of the immune system and susceptibility to infection (Pond, 1992, 1999). In the same way, other specialized adipose tissues infiltrate many organs like the pancreas, bone marrow, thymus and skeletal muscle (Klaus, 2001). Adipose tissue surrounding the perimysium within skeletal muscle could be described as one of these smaller, but specialized adipose depots.

Marbling

Marbling is the common name used to describe the adipose tissue embedded in the perimysial connective tissue matrix, and associated with blood capillary networks within skeletal muscle. The function of marbling, or intramuscular (**IM**) adipose tissue, remains unclear as it does not seem to confer any physiological advantage. In fact, adipose tissue development within muscle is rare in mammals and usually develops under extreme pathologies (Harper and Pethick, 2001). Independent of its physiological function, the accretion of IM fat is desirable in domestic cattle because it is positively associated with the palatability of beef, and is the main determinant of USDA Quality Grade within a carcass maturity classification (Kerth et al., 1999; Wheeler et al., 1999).

Marbling and Beef Palatability Traits

Palatability of beef results from the interaction of tenderness, juiciness, and flavor, parameters that are influenced by the degree of marbling (Nishimura et al., 1999; Owens and Gardner, 1999). Indeed, more than 90% of the steaks with a Slight degree of marbling, or better, are desirable in terms of tenderness, flavor, and overall palatability (Savell and Cross, 1988) and the lipid content of meat is very closely related with palatability (Owens and Gardner, 1999). The content of IM fat can improve the tenderness, juiciness and flavor of beef through various mechanisms.

Intramuscular fat is less dense and has a lower shear force than muscle. Therefore, as IM fat increases, the density of meat decreases, thereby improving meat tenderness (Savell and Cross, 1988). It has also been proposed that marbling compresses the connective tissue walls, decreasing their effective thickness and strength (Owens and Gardner, 1999). Marbling may also have a lubrication effect, increasing the sensation of tenderness when beef is consumed (Thompson, 2001). Intramuscular fat can increase beef juiciness by augmenting the water holding capacity of beef (Wheeler et al., 1999) and(or) reducing moisture loss during cooking by coating the meat (Owens and Gardner, 1999; Thompson, 2001). Intramuscular fat is also a reservoir of odoriferous compounds that are released upon cooking and potentially add to a favorable

eating experience (Savell and Cross, 1988). In fact, within a carcass maturity classification, steaks with a higher degree of marbling have been found more flavorful than steaks with lower marbling scores (Wheeler et al., 1999). It is the combination of these favorable palatability attributes of IM adipose tissue that makes its development essential for the beef industry.

The Economic Impact of Suboptimal Marbling Development to the Beef Industry

Although it is unknown if the lower adipogenic capacity of IM adipocytes is primarily determined by intrinsic characteristics of the IM adipocytes or the particular environment within IM adipose tissue, the deficient level of marbling development has great economic implications for the United States beef industry. Although beef cattle are currently fed high energy diets for extended periods of time in an attempt to assure a desirable degree of marbling, the National Beef Quality Audit-2000 found that insufficient development of marbling and the excess deposition of SC fat were among the top challenges of the industry (Smith et al., 2000a; McKenna et al., 2002). Indeed, beef quality as assessed by marbling score and USDA Quality Grade has not progressed since the early 1990s (Lorenzen et al., 1993; Boleman et al., 1998; McKenna et al., 2002). Furthermore, excessive development of adipose tissues other than IM is costly, as it is not unusual for 30% of the weight of a beef carcass to be waste adipose

tissue (Smith, 1995). It is estimated that for the United States beef industry, excessive fat deposition represents losses of $1.3 billion per year (Smith et al., 2000a). For this reason, it is in the best interest of the beef industry to better understand the developmental and constitutive differences among bovine adipose tissues in order to develop methods that optimize marbling development while minimizing adipose tissue accretion in other anatomical areas.

Unfortunately, three decades after the heterogeneity of bovine adipose tissues was proposed (Pothoven and Beitz, 1973), it is unknown if intrinsic differences among bovine preadipocytes and adipocytes from IM and SC adipose tissues are responsible for the marked morphologic and adipogenic differences. Nevertheless, a review of the research in lipid metabolism, adipose tissue physiology, and preadipocyte differentiation of other species (i.e. humans, rodents, swine) offers us insight into where developmental differences among bovine adipocytes may exist, and how they may be manipulated to enhance marbling development.

Adipogenic Potential of Intramuscular Adipose Tissue

Unfortunately, in parallel with the minor role of IM adipose tissue in energy storage, its capacity for lipid accretion is limited when compared to adipose depots like SC. Although the number of IM adipocytes account for

nearly 50% of the total fat cell population in the bovine body, IM fat accounts for only 10% of the total carcass fat (Allen, 1976). The mechanisms responsible for constraints in IM fat accretion are discussed below.

Fatty acid and triacylglyceride synthesis

Firstly, the capacity of IM adipose tissue to synthesize *de novo* fatty acids and triacylglycerides is restricted when compared to SC adipose tissue (Smith et al., 1998). Although IM and SC adipose tissues equally utilize glucose for *de novo* fatty acid synthesis (between 1 and 5 nM glucose/h/10^5 adipocytes), SC adipose tissue has a greater capacity to synthesize fatty acids from acetate, the main substrate for fatty acid synthesis in ruminants (Smith, 1995; Song et al., 2001). In SC adipocytes, acetate utilization in fatty acid synthesis produces up to 3,000 nM fatty acids/h/10^5 adipocytes, whereas IM adipose tissue is only capable of synthesizing between 1 and 300 nM fatty acids/h/10^5 adipocytes (Smith, 1995). In addition, when ruminants are fed high energy diets, the activity of adipogenic enzymes (i.e. ATP citrate lyase and NADP malate dehydrogenase) are increased in SC adipose tissue, but not in IM (Smith and Crouse, 1984). Furthermore, in adipose tissue explant studies, insulin is capable of increasing the rate of lipogenesis in SC adipose tissue but not in IM adipose tissue (Miller et al., 1991).

The low rate of triacylglyceride synthesis in IM adipose tissue can also be explained by the low activity of the enzyme phosphatidate phosphohydrolase

(Smith et al., 1998). Phosphatidate phosphohydrolase (also know as phosphatidic acid phosphatase) dephosphorylates diacylglycerol-3-phosphate (also knonw as L-phosphatidate or phosphatidic acid), converting it into D-1,2-diacylglycerol to which a third fatty acid is added resulting in a triacylglyceride. Phosphatidate phosphohydrolase is a rate limiting enzyme of triacylglyceride synthesis and its activity is influenced by nutrition and hormonal status to a greater extent than other enzymes involved in triacylglyceride synthesis (Coleman et al., 2000). Furthermore, there is a strong correlation between phosphatidate phosphohydrolase activity and triacylglyceride synthesis (Coleman et al., 2000). Phosphatidate phosphohydrolase activity in IM adipose tissue is 50% lower than the activity found in SC adipose tissue (Smith et al., 1998). Contrary to SC adipose tissue in which phosphatidate phosphohydrolase activity is drastically down regulated during starvation periods, this enzyme is not affected in IM adipose tissue by nutrient intake suggesting that it is constitutively expressed regardless of energy balance (Smith et al., 1998).

Phosphatidic acid, the substrate for phosphatidate phosphohydrolase is a common intermediate of triacylglyceride and phospholipid synthesis. The low activity of phosphatidate phosphohydrolase in IM adipose tissue suggests that the main function of the phosphatidic acid synthetic pathway in IM adipose tissue is the synthesis of phospholipids with structural or regulatory functions.

Uptake of dietary fatty acids

The low endogenous lipogenic capacity of IM adipose tissue may indicate that exogenous sources of fatty acids are more important than the utilization of substrates like acetate for de novo fatty acid synthesis in this adipose depot. Contrary to the differences in de novo fatty acid synthesis between IM and SC adipose tissue explants, there are no differences between the depots in the incorporation of palmitate into triacylglycerides (Lin et al., 1992). In fact, if the rate of palmitate esterification is expressed based on adipocyte volume, palmitate esterification is two times greater in IM adipocytes than SC adipocytes (Lin et al., 1992). However, blood flow to IM adipose tissue is less than that to abdominal depots in ruminants (Vernon, 1980). Furthermore, variations in the expression of lipoprotein lipase, the enzyme that facilitates the uptake of plasma fatty acids by adipocytes, are not related to adipose depot (Barber et al., 2000). Because typical ruminant diets are low in fatty acids, lipoprotein lipase may have a minor role in the supply of dietary fatty acids to adipocytes (Vernon, 1980; Bergen and Mersmann, 2005). Moreover, fatty acids of dietary origin accumulate preferentially in internal adipose tissue depots like PR and omental (**OM**), rather than IM (Barber et al., 2000; Eguinoa et al., 2003).

It may be that IM adipose tissue is involved in a regulatory role within the skeletal muscle, as its capacity to accrete lipid is significantly lower than SC

adipose tissue. If we also consider that starvation depresses triacylglyceride synthesis in bovine SC but not IM adipose tissue (Smith et al., 1998; Smith et al., 2000b) and IM lipid appears not to be mobilized in situations of acute energy need (Romans et al., 1974), an alternative role for IM adipose tissue, other than energy accumulation, is suggested.

Hypothetical function of intramuscular adipose tissue

Because IM adipose tissue is a potential source of arachidonic acid and its metabolites (eicosanoids), which are involved in signal transduction and gene regulation, IM adipocytes could be important in the regulation of skeletal muscle metabolism (Vernon and Houseknecht, 2000). In addition, IM adipose tissue could also serve as a paracrine source of leptin, a regulatory protein secreted from adipocytes (Houseknecht et al., 1998; Fruhbeck et al., 2001). Leptin has been shown to increase skeletal muscle fatty acid oxidation and decrease its triacylglyceride incorporation (Muoio et al., 1997; Frayn et al., 2003). Intramuscular adipocytes could also serve as a source of adiponectin and secretin, proteins that induce an increase in fatty acid oxidation and energy dissipation in skeletal muscle (Frayn et al., 2003). Because excessive lipid accumulation within skeletal muscle is related to severe myopathy, characterized by increased lymphocyte infiltration, fiber atrophy and fiber degeneration (Cortright et al., 1997), the secretion of regulatory molecules that

protect skeletal muscle from such metabolic aberrations could be an important function of IM adipose tissue.

Maturity of intramuscular adipocytes

Alternatively, the inferior adipogenic capacity of bovine IM adipose tissue could be merely related to differences in the maturity between IM adipocytes and adipocytes from highly adipogenic adipose depots. It has been determined that lipid synthesis and acetate oxidation to CO_2 increases with increasing bovine adipocyte size (Hood and Allen, 1978). In bovines, at a given chronological age, IM adipocytes are smaller than SC adipocytes and unable to synthesize fatty acids from dietary precursors as efficiently as SC adipocytes (Hood and Allen, 1978). Indeed, negative correlations have been found between adipocyte number per gram of IM adipose tissue and fatty acid synthesis, presumably because a large number of small adipocytes would contribute to IM adipose tissue mass albeit their low adipogenic activity (Smith et al., 1984). Furthermore, cattle breeds with low marbling potential like Santa Gertrudis, have more, but smaller adipocytes than cattle breeds with higher marbling potential (i.e. Angus) (Miller et al., 1991). Accordingly, Angus IM adipocytes showed higher lipogenic activity than Santa Gertrudis IM adipocytes (Miller et al., 1991). Of notice is the fact that Angus IM adipocytes presented an average diameter equivalent to Santa Gertrudis SC adipocytes (Miller et al., 1991). Cattle

breeds and animals within a breed, with higher marbling potential may have an IM adipocyte population that is more mature and able to utilize diet precursors for fatty acid synthesis more efficiently. However, within an animal, the lower maturity of IM adipocytes may preclude them from efficiently synthesizing triacylglycerides when compared to other depots.

Preadipocyte recruitment

Within cattle breeds, the number of IM adipocytes has been positively correlated to the amount of marbling (Hood and Allen, 1973; Smith et al., 1984). Rat retroperitoneal, epididimal, mesenteric and SC (inguinal) adipocytes secrete paracrine factors that recruit preadipocytes once a mass of 1.2 to 1.6 μg/adipocyte and(or) a size of 120 μm is reached (Faust et al., 1978; Ramsay et al., 1992). Considering the limited adipogenic capacity of IM adipocytes, an understanding of the paracrine factors that control preadipocyte recruitment and differentiation may reveal strategies to circumvent the need for IM adipocytes to reach a critical size before they can recruit more preadipocytes. Alternatively, treatments that could increase the adipogenic capacity of IM adipocytes may decrease the chronological age at which IM adipocytes reach a critical size needed to recruit preadipocytes, thus starting a differentiation cascade that results in highly marbled beef.

Adipogenesis: The Mechanism of Preadipocyte Differentiation

Commitment of mesodermal stem cells

Adipogenesis, the formation of adipose cells from mesoderm, is first detected late in fetal development as preparation for post-natal discontinuous food supply (Mandrup and Lane, 1997; Martin et al., 1998; Feve, 2005). The process of adipogenesis begins with the commitment of mesodermal stem cells to the adipocyte lineage (Figure 1-1), a process still poorly understood because established preadipocyte cell lines are already committed to the adipocyte lineage (Feve, 2005). However, the commitment of mesodermal cells to the adipocyte lineage may depend on the expression of a small number of regulatory genes (Konieczny and Emerson, 1984; Feve, 2005). Unipotent mesodermal cells committed to the adipogenic lineage are referred to as adipoblasts. Adipoblasts are characterized by the expression of early markers of the adipocyte phenotype, such as lipoprotein lipase, α-chain 2 of type VI collagen, and adipocyte differentiation related protein (**ADRP/adipophilin**) (Gaskins et al., 1989; Ailhaud et al., 1992; Mandrup and Lane, 1997). Adipoblast growth arrest at the G1/S boundary is critical for the cells to enter the preadipocyte state (Dani et al., 1989; Smas and Sul, 1995; Gregoire et al., 1998).

Preadipocyte factor one (**pref-1**) may initiate or maintain the inhibitory signals that keep the adipoblasts in the immature state (Gregoire et al., 1998).

Although the specific inhibitory mechanism(s) of pref-1 remain to be identified (Ailhaud, 2001; Feve, 2005), pref-1 may be a transducer of inhibitory signals from the extra cellular matrix into the cell interior, and(or) block the morphological changes (cytoskeleton remodeling) that are requisite for differentiation to proceed (Smas and Sul, 1995). Preadipocyte factor-1 also directly downregulates the expression of adipocyte lipid binding protein (**ALBP**; also know as aP2), which is needed for the selective uptake of long chain fatty acids that follows in the differentiation process (Smas and Sul, 1995; Gregoire et al., 1998).

After the downregulation of pref-1, the abundance of α-chain 2 of type VI collagen and lipoprotein lipase mRNAs increases in what has been named early differentiation (Dani et al., 1989; Dani et al., 1990). The extensive changes in the extracellular matrix that occur during this stage, such as an increase in the synthesis of collagen IV and a decrease in the synthesis of collagen III and I, may permit the cellular reorganization, movement, and reshaping needed to permit adipocyte specific gene expression (Sul et al., 1993).

The preadipocyte state is characterized by emerging of the expression of the nuclear receptors peroxisome proliferator activated receptor δ (**PPARδ**) and CCAAT/enhancer binding protein β and δ (**C/EBPβ and C/EBPδ**), along with the appearance of the prostacyclin (**PGI_2**) membrane receptor (Börglum et al., 1999;

Ailhaud, 2001). The expression of PPARδ is crucial for triggering preadipocyte clonal amplification, which can be induced by PPARδ activators like fatty acids and PGI_2 (Bastie et al., 1999; Jehl-Pietri et al., 2000; Bishop-Bailey and Wray, 2003).

Clonal amplification

Preadipocytes already expressing early differentiation markers, but without considerable triacylglyceride stores, then undergo at least one round of DNA replication (Ailhaud, 2001). This process is known as clonal amplification of committed cells and precedes the expression of late differentiation markers like ALBP, glycerol-3-phosphate dehydrogenase (**GPDH**), hormone-sensitive lipase, adipsin, and leptin (Négrel et al., 1989; Smas and Sul, 1995; Gregoire et al., 1998). This post-confluent mitosis and terminal differentiation may be initiated in part by increases in intracellular cAMP concentrations triggered by PGI_2 secretion (Négrel et al., 1989).

Prostacyclin exits the cell and binds to its specific cell-surface receptor, resulting in an activation of adenylate cyclase and an increase in intracellular cAMP (Négrel et al., 1989). The increase in intracellular cAMP is coupled with an increase in intracellular calcium (Ca^{+2}) concentration, which activates various kinases (i.e. calmodulin sensitive kinase II) that are involved in the control of DNA synthesis and the critical mitosis needed for terminal differentiation to

proceed (Gaillard et al., 1989; Négrel et al., 1989). High intracellular concentration of cAMP also downregulates the expression of Sp-1, a transcriptional repressor of CCAAT/enhancer binding protein α (**C/EBPα**), which is a transcription factor involved in preadipocyte terminal differentiation (Tang et al., 1999). In addition, activation of the transcription factor cAMP responsive element binding protein (**CREB**) is crucial during adipogenesis (Reusch et al., 2000). Furthermore, PGI_2 may act as a ligand for PPARδ, which is an inducer of clonal expansion (Hertz et al., 1996; Shao and Lazar, 1997; Wahle et al., 2003; Wise, 2003). However, PPARδ also induces peroxisome proliferator activated receptor γ_2 (**PPARγ_2**), an important regulator of differentiation that makes preadipocytes withdrawal from the clonal expansion phase (Chawla et al., 1994; Altiok et al., 1997; Morrison and Farmer, 1999; Wahle et al., 2003).

Growth arrest

After clonal expansion, preadipocytes enter into an irreversible growth arrest stage called G_D (Ntambi and Kim, 2000). The expression of PPARδ is involved in triggering clonal amplification, but at the same time PPARδ stimulates the expression of PPARγ_2, which upregulates the expression of the cyclin dependent kinase inhibitors p18 and p21, which induce cells to withdraw from the clonal expansion step (Morrison and Farmer, 1999). Furthermore, PPARγ_2 may contribute to the decrease of DNA binding and transcriptional

activity of the E2F-DP-1 complex that is implicated in the continuity of the proliferative stage (Altiok et al., 1997). Specifically, PPARγ_2 decreases the expression of protein phosphatase PP2Ac, which results in an increase in the phosphorylation state of the DP-1 transcription factor of the E2F-DP-1 complex and consequently, in a decrease in its DNA binding activity (Altiok et al., 1997). Peroxisome proliferator-activated receptor γ_2 also induces the expression of C/EBPα (Morrison and Farmer, 2000), which reciprocally stimulates PPARγ_2 expression, resulting in a positive feedback loop that maintains the expression of these transcription factors for the rest of the preadipocyte/adipocyte lifespan (Morrison and Farmer, 2000; Tamori et al., 2002; Fu et al., 2005). The upregulation of the C/EBPα in this stage is also crucial because the induction of C/EBPα has been related to an increase in the levels of the cyclin dependent kinase inhibitor, p21/SDI-1, which suppresses cell division (Sul et al., 1993; Smas and Sul, 1995; Gregoire et al., 1998). Furthermore, C/EBPα induces growth arrest through direct interaction with cell cycle protein E2F and cyclin dependent kinases 2 and 4 (Porse et al., 2001; Wang et al., 2001; Feve, 2005). In addition, PPARγ_2 and C/EBPα cooperatively develop insulin sensitivity in preadipocytes (Morrison and Farmer, 2000).

During this growth arrest stage the expression of late markers of adipogenic differentiation, like ALBP, acetyl-CoA carboxylase, fatty acid

synthase, glucose transporter 4, steroyl-CoA desaturase, and GPDH, among others, are first detected (Ailhaud et al., 1992; Ailhaud, 2001). Although C/EBPα induces fatty acid synthase and steroyl-CoA desaturase, and there is some redundancy between the genes induced by PPARγ_2 and C/EBPα (i.e. ALBP, GPDH, steroyl-CoA desaturase 1, and adipsin) (Soukas et al., 2001), PPARγ_2 is considered the master regulator of adipogenic gene expression (Schoonjans et al., 1996b; Tamori et al., 2002; Knouff and Auwerx, 2004; Rosen, 2005). Indeed, in PPARγ_2 -/- fibroblasts, induction of C/EBPα does not result in adipogenic conversion (Rosen et al., 2002; Rosen, 2005). This indicates that PPARγ_2 is absolutely required for adipogenesis, and C/EBPα plays a supplementary role by maintaining PPARγ_2 levels elevated in the cells. The crucial role of PPARγ_2 in the maturation of the preadipocytes is evidenced by its stimulation of adipogenic genes like lipoprotein lipase (Schoonjans et al., 1996a), ALBP (Tontonoz et al., 1994a; Tontonoz et al., 1994b), acyl-CoA synthase (Schoonjans et al., 1993; Schoonjans et al., 1995; Schoonjans et al., 1996b), fatty acid transporter protein (Schoonjans et al., 1996b), phosphoenol pyruvate carboxykinase (Tontonoz et al., 1995), steroyl-CoA desaturase 1 (Miller and Ntambi, 1996), malate dehydrogenase (Castelein et al., 1994), and GPDH (Soukas et al., 2001), all important for the development and maintenance of the mature adipocyte phenotype (Schoonjans et al., 1996b; Tamori et al., 2002).

Furthermore, out of 1,000 genes up-regulated during adipogenesis, 278 are down-regulated upon the repression of PPARγ2 expression using an antisense oligonucelotide (Perera et al., 2006). Negative regulators of adipogenesis like members of the GATA family of transcriptions factors (i.e. GATA-2 and GATA-3) suppress preadipocyte differentiation by interfering with PPARγ2 promoter activity (Tong et al., 2000). Furthermore, the Wnt family of secreted proteins, probably Wnt10b, potently represses adipogenesis by completely blocking the induction of PPARγ2 and C/EBPα (Ross et al., 2000; Longo et al., 2004).

Peroxisome proliferator-activated receptor γ2. Peroxisome proliferator-activated receptor γ is a member of the nuclear receptor super family that integrates the control of energy, lipid, and glucose homeostasis (Schoonjans et al., 1996b; Hamm et al., 1999; Chawla et al., 2001; Debril et al., 2001). Of the three isoforms of the PPAR subfamily (PPARα, PPARδ and PPARγ) (Brun et al., 1996a; Houseknecht et al., 2002), PPARγ is the most adipogenic (Forman et al., 1995; Brun et al., 1996a). Indeed, PPARγ knockout mouse embryos have been found to be completely devoid of adipose tissue (Barak et al., 1999).

From a single gene, various PPARγ mRNA variants are produced through alternative splicing. Of these mRNAs, PPARγ 1, 3, and 4 give rise to an identical protein, referred to as PPARγ1 (Fajas et al., 1998; Sundvold and Lien, 2001), while PPARγ2 mRNA produces a protein with 30 additional amino acids in the

N terminus (Kliewer et al., 1994). The actions of PPARγ are mediated by these two protein isoforms: PPARγ1, which is ubiquitously expressed, and the adipose tissue specific PPARγ2 (Chawla et al., 1994; Tontonoz et al., 1994a). However, only PPARγ2 is required during adipogenesis, as supported by the fact that exogenous delivery of PPARγ2 restores differentiation to PPARγ null preadipocytes, but PPARγ1 has no effect (Ren et al., 2002).

Peroxisome proliferator-activated receptor γ2 forms heterodimers with the retinoic X receptor-α (**RXRα**), which, in the basal state, is bound to corepressor proteins (Knouff and Auwerx, 2004; Miard and Fajas, 2005). The transcriptional activity of PPARγ2 is increased by ligands (Brun et al., 1996a). Upon binding, these ligands cause PPARγ2 to change its conformation, which results in the release of corepressors and recruitment of coactivators (Knouff and Auwerx, 2004). Because the type of ligand determines which coactivators are recruited by the PPARγ2-RXRα heterodimer, and these coactivators determine the target genes of PPARγ2 (Yu et al., 1995; Debril et al., 2001; Houseknecht et al., 2002; Bishop-Bailey and Wray, 2003; Miard and Fajas, 2005), the type of ligand available is important in the regulation of adipogenesis.

Although many natural ligands for PPARγ2 have been identified (i.e. linoleic acid, linolenic acid, eicosapentaenoic acid, 15-deoxy-$\Delta^{12,14}$ –PGJ_2, PGI_2), the identification of the physiologically relevant ligand for this receptor has been

elusive (Forman et al., 1995; Houseknecht et al., 2002; Xie et al., 2006). It has been proposed that mature adipocytes secrete PGI_2, as a signal for preadipocyte recruitment (Négrel, 1999; Kim and Moustaid-Moussa, 2000; Ailhaud, 2001). As PGI_2 can activate PPARγ_2 (Hertz et al., 1996; Gregoire et al., 1998; Kim and Moustaid-Moussa, 2000; Wise, 2003; Feve, 2005), it has been proposed that PGI_2 synthesis may regulate adipose tissue development (Négrel et al., 1989; Gaillard et al., 1991; Darimont et al., 1994; Négrel, 1999; Kim and Moustaid-Moussa, 2000). Also, availability of PPARγ_2 ligands or ligand precursors in diets may be part of the connection between nutrition and adipose tissue development (Houseknecht et al., 2002).

In humans, PPARγ_2 mRNA expression is greater in SC preadipocytes than in visceral preadipocytes (Sewter et al., 2002). In addition, human PPARγ_2 mRNA expression is greater in subcutaneous adipose tissue (Lefebvre et al., 1998) than in visceral adipose tissue. This suggests that intrinsic differences in PPARγ_2 expression could be related to developmental differences between different adipose tissues. In fact, adipocyte determination and differentiation factor-1/sterol responsive element-binding protein-1c (**ADD1/SREBP-1c**), which enhances preadipocyte differentiation by inducing PPARγ_2 expression (Fajas et al., 1999) and controlling the synthesis of PPARγ_2 ligands (Kim et al., 1998), has a depot dependent pattern of expression (Gondret et al., 2001). This may be

related to different patterns of lipid accretion in distinct adipose depots. Although the expression of PPARγ_2 has been documented in bovine PR preadipocytes (Ohyama et al., 1998), its expression has not been reported for bovine preadipocytes from other depots.

Preadipocyte development and the mature adipocyte phenotype

With the activation of PPARγ_2, increases in de novo fatty acid synthesis, fatty acid uptake, and triacylglyceride synthesis result in the appearance of immature adipocytes. This stage is characterized by the appearance of very late markers of adipogenic differentiation, such as the expression of adrenergic receptors (α_2, β_1 and β_2), hormone sensitive lipase, and perilipin (Ailhaud, 2001). With continuous lipid accumulation, the phenotype of the mature adipocyte develops, characterized by the expression of leptin, angiotensinogen, adipsin, and other molecules in accordance with the endocrine role of the adipocyte (Kim and Moustaid-Moussa, 2000; Fruhbeck et al., 2001; Gregoire, 2001; Trayhurn et al., 2001).

Post-natal adipogenesis

After birth, the development of adipose tissue results from a combination of hypertrophy of mature fat cells and the recruitment, proliferation, and differentiation of preadipocytes as long as the organism is in a positive energy balance. Indeed, the recent discovery that adipose tissue is a source of

multipotent stem cells (Rodríguez et al., 2004) reveals the great capacity for energy storage of this organ.

Adipogenic Regulators of Preadipocyte Differentiation

There are a vast number of factors known to influence preadipocyte differentiation at various stages of the process. However, a more limited group of adipogenic inducers has been shown to selectively influence preadipocyte differentiation depending on the depot from which the cells were isolated. A description of those factors and reasoning on why they are good candidates as selective inducers of marbling development follows.

Glucocorticoids

Glucocorticoids are steroid hormones secreted by the adrenal cortex in response to adrenocorticotropic hormone (**ACTH**) secretion by the anterior pituitary. The anterior pituitary secretes ACTH in response to the secretion of corticotropin releasing factor (**CRF**) by the hypothalamus under conditions of physiological, physical, or psychosomatic stress. The most common natural glucocorticoids are cortisol and corticosterone. Glucocorticoids are transported in the blood by transcortin and then diffuse through the membrane of target cells spontaneously. Once in the cell, glucocorticoids bind to glucocorticoid receptors (**GR**). The glucocorticoid-GR complex then enters the nucleus where it binds DNA sequences of glucocorticoid inducible genes characterized by having

glucocorticoid response elements (**GRE**) with the base sequence TGTTCT (Bamberger et al., 1996).

With the refinement of in vitro techniques to cultivate preadipocytes, it has become clear that glucocorticoids are potent inducers of preadipocyte differentiation (Ramsay et al., 1989; Xu and Björntorp, 1990; Amri et al., 1991; Gaillard et al., 1991; Smas et al., 1999). Dexamethasone (**DEX**), a synthetic glucocorticoid, is part of the standard medium to trigger preadipocyte differentiation in cell culture systems. In fact, DEX exposure leads to an accumulation of adipocyte-specific mRNAs due to an acceleration of transcriptional activation of adipose-inducible genes (Gaskins et al., 1989). Furthermore, in some rodent models of obesity (i.e. ob/ob mouse), adrenalectomy prevents the development of obesity (Naeser, 1973).

Glucocorticoids derive their name from their role in glucose homeostasis, but are involved in the development, metabolism, and apoptosis of diverse cell types (Yudt and Cidlowski, 2002). The physiological responses to glucocorticoids differs between tissues and cell types (Yudt and Cidlowski, 2002). The tissue specific responsiveness to glucocorticoids depend on the presence of the hormone, existence and affinity of the GR, and ability of the cell to transduce the signal. In addition, the GR target genes must be active and not silenced by euchromatization (Bamberger et al., 1996). Because glucocorticoids

regulate the expression of the insulin receptor gene (McDonald and Goldfine, 1988), β adrenergic receptor gene (Hadcock and Malbon, 1988), and lipoprotein lipase activity (Xu and Björntorp, 1990), glucocorticoids can have a profound effect on adipose tissue distribution (Brönnegård et al., 1990). Glucocorticoid actions on adipose tissue are mediated through GR action, evidenced by the fact that GR antagonists like RU 38486 (also known as RU 486 or mifepristone) have been reported to inhibit DEX effects on adipogenic enzyme activity (Xu and Björntorp, 1990; Ottosson et al., 1995) and reverse obesity in fa/fa Zucker rats (Langley and York, 1990).

Glucocorticoid receptor isoforms. Although there is only one gene that codes for the GR, there are a diversity of GR isoforms. Through multiple promoters, alternative splicing, alternative translation initiation, and posttranslational modifications, various GR isoforms exist that fractionate into several bands upon electrophoresis (Yudt and Cidlowski, 2002).

Glucocorticoid receptor α (~97 kDa) is the isoform directly responsible for the regulation of gene expression exerted by glucocorticoids (Clark and Lasa, 2003). Upon ligand binding, GRα dissociates from various heat shock proteins and forms homodimers that migrate into the nucleus (Muller and Renkawitz, 1991; Bamberger et al., 1996). Inside the nucleus, GRα regulates transcription by directly binding glucocorticoid response elements in the regulatory region of

target genes and then, interacts with the basic transcription machinery to increase the rate of transcription of RNA polymerase II (Bamberger et al., 1996). Activated GRα can also induce rearrangement of the chromatin structure of particular promoter regions, allowing access of other transcription factors to previously silenced genes (Bamberger et al., 1996). By a similar mechanism GRα can bind negative glucocorticoid response elements, inhibiting the expression of particular genes (Muller and Renkawitz, 1991). Glucocorticoid receptor α can also negatively interact with diverse transcription factors by sequestering particular components needed for the transcription of their particular target genes (Bamberger et al., 1996). Indeed, the transcription of GRα is inhibited by activated GRα through this mechanism (Nobukuni et al., 1995). However, GRα expression can also be regulated by reducing GR mRNA stability or translatability (Dong et al., 1988; Bamberger et al., 1996), or diminishing the half-life of the GR protein (McIntyre and Samuels, 1985).

Glucocorticoid receptor ϱ has been identified in myeloma tumor cells (Krent et al., 1995). This isoform is missing a large portion of the ligand binding domain found in GRα, but it has been found to be involved in the upregulation of GRα mediated gene expression (Yudt and Cidlowski, 2002). In glucocorticoid resistant cells, the level of expression of this protein may represent up to 50% of the total GR protein (de Lange et al., 2001).

Another GR isoform named GRβ, is generated through alternative splicing of the final exon of GRα, which results in a protein that differs only in the carboxy terminus (Yudt and Cidlowski, 2002). Although GRβ is unable to bind glucocorticoids, it has been reported to inhibit the ligand activated GRα induction of glucocorticoid responsive genes in a concentration dependent manner (Bamberger et al., 1995). However, the in vivo relevance of GRβ as an inhibitor of GRα is still contested because transfection studies have indicated that proportions of 5:1 (GRβ:GRα) are required for a significant inhibition of GRα to occur, and this is higher than the ratio of these proteins at the tissue level (Yudt and Cidlowski, 2002). Although there is some evidence of nongenomic actions of glucocorticoids mediated by a membrane bound GR in neural and hepatic tissue (Borski, 2000), similar reports have not been published in adipose tissue. In conclusion, even though there is only one GR gene, the diversity of proteins derived from it could confer the cells with diverse glucocorticoid sensitivities.

Glucocorticoids and selective stimulation of adipose tissue accretion.

Glucocorticoid stimulation of adipose accretion varies among different adipose tissues in humans (Fried et al., 1993), pigs (Ramsay et al., 1989) and cattle (Brethour, 1972). Anatomical differences in adipose tissue accretion are seen in humans treated with glucocorticoids, where greater adipogenesis occurs

in abdominal adipose tissue (Rebuffé-Scrive et al., 1988; Fried et al., 1993; Boschmann, 2001). In swine, glucocorticoid stimulation of SC preadipocyte differentiation was greater in cells isolated from the shoulder than those from the ham, while PR preadipocytes were totally unresponsive to glucocorticoids (Ramsay et al., 1989). In cattle, glucocorticoid treatment was shown to selectively increase IM adipose development (Brethour, 1972) and a positive relationship has been found between plasma glucocorticoid concentration and the percentage of IM fat (Cramer and Shahied, 1974).

Among different tissues, glucocorticoid binding capacity is closely correlated with the magnitude of a response to glucocorticoids (Bamberger et al., 1996). Therefore, differences in GR expression among distinct adipose tissues (Rebuffé-Scrive et al., 1988) may help explain the particular pattern of adipose tissue accretion in cattle and humans undergoing glucocorticoid therapy. Indeed, the expression of GR has been shown to be variable among adipose tissues in various species. In the rat, different adipose tissues have GR of similar affinity, but epididimal adipose tissue showed greater glucocorticoid binding than PR, brown, and popliteal adipose tissues (Feldman and Loose, 1977). In humans, GR mRNA levels in female abdominal adipose tissue are 50% higher than in gluteal adipose tissue (Brönnegård et al., 1990). Furthermore, human OM adipose tissue has greater glucocorticoid binding than SC, independent of

gender (Pedersen et al., 1994). Although bovine preadipocytes are also considered a target for glucocorticoids, and glucocorticoids are commonly added to their differentiation media (Sato et al., 1996; Ailhaud, 2001; Brandebourg and Hu, 2005), to our knowledge no previous studies have documented GR expression in bovine preadipocytes. Likewise, we are unaware of any studies that have compared glucocorticoid induction of differentiation among bovine preadipocytes isolated from distinct adipose depots. Following are potential mechanisms by which glucocorticoids may enhance adipogenesis.

Downregulation of preadipocyte factor-1. In the early hours of differentiation the expression of more than 100 proteins is altered. One of them is pref-1, a transmembrane protein that initiates or maintains inhibitory signals to adipogenesis keeping the cells in a quiescent, non-differentiated state (Gregoire et al., 1998). Upon DEX exposure, the expression of pref-1 mRNA is reduced within 6 h without a concomitant increase in the expression of PPARγ mRNA (Smas et al., 1999). This rapid decrease in the expression of pref-1 indicates that it is not a result of adipocyte differentiation, but rather a direct consequence of glucocorticoid exposure (Smas et al., 1999). However, the mechanism by which pref-1 inhibits preadipocyte differentiation is not clear, as its specific inhibitory signal(s) is(are) unknown (Ailhaud, 2001; Feve, 2005).

Stimulation of the expression of CCAAT enhancer binding protein δ. Of the three members of the family of CCAAT enhancer binding proteins, C/EBPα, C/EBPβ and C/EBPδ, (Gregoire et al., 1998; Kim and Moustaid-Moussa, 2000), glucocorticoids directly induce the expression of C/EBPδ (Mandrup and Lane, 1997; Gregoire et al., 1998). Because of the role of C/EBPδ in induction of the clonal expansion phase discussed earlier, glucocorticoids may be important in this step of the differentiation process. However, glucocorticoids also contribute to the formation of C/EBPδ and C/EBPβ heterodimers, which stimulate the expression of PPARγ2 (Brun et al., 1996a; Gregoire et al., 1998) Peroxisome proliferator-activated receptor γ2 then causes the preadipocytes to exit the clonal expansion phase, implicating glucocorticoids with the induction of late differentiation.

Stimulation of lipoprotein lipase expression and activity. Glucocorticoids have a strong stimulatory effect on lipoprotein lipase activity and mRNA expression (Boschmann, 2001). In humans, these effects have been shown to be depot dependent as DEX administration causes a dramatic increase in lipoprotein mRNA, protein abundance and enzyme activity in OM adipose tissue, but only small increases in SC adipose tissue (Fried et al., 1993). In swine, hydrocortisone increased lipoprotein lipase activity and triacylglyceride synthesis in SC, but not PR adipose tissue (Ramsay et al., 1989).

Stimulation of adipocyte lipid binding protein expression. Adipocyte lipid binding protein, is a member of the fatty acid binding proteins that ameliorate the detergent effect of fatty acids, direct fatty acids to specific pathways, help establish fatty acid gradients between the interstitial space and the adipocyte cytoplasm, and are involved in the uptake of fatty acids by the adipocytes (Amri et al., 1991; Smith, 1995). In addition, ALBP is involved in the shuttling of fatty acids into triacylglyceride synthesizing organelles (Fruhbeck et al., 2001) and its expression is related to the induction of fatty acid and triacylglyceride synthesizing enzymes (Amri et al., 1991). The ALBP gene contains a GRE (Gaskins et al., 1989) and DEX has been shown to cause a dose dependent accumulation of ALBP mRNA (Amri et al., 1991). Glucocorticoid stimulation of ALBP expression may be crucial in adipocyte differentiation because ALBP has been implicated in the targeting of fatty acids to regulatory elements in the nucleus (Houseknecht et al., 2002). Consequently, glucocorticoids can influence the process of selective uptake of long chain fatty acids, differentiation, and adipocyte metabolism (Gaskins et al., 1989).

Influence on prostacyclin synthesis. Induction of differentiation by glucocorticoids may be due to an enhancement of PGI_2 synthesis and release in Ob 1771 preadipocytes (Gaillard et al., 1991). Glucocorticoids increase the activity of phospholipases, which are implicated in the enhancement of arachidonic acid

release from phospholipids in the cellular membrane (Gaillard et al., 1991). In Ob 1771 preadipocytes, this mobilization of arachidonic acid is coupled with an increase in the activity of cyclooxygenase (**COX**) activity and with the subsequent and exclusive increase in the activity of PGI_2 synthase (Négrel et al., 1989; Gaillard et al., 1991; Kim and Moustaid-Moussa, 2000). Accordingly, these events result in the preferential increase in PGI_2 production by Ob 1771 preadipocytes upon glucocorticoid exposure (Négrel et al., 1989; Gaillard et al., 1991; Kim and Moustaid-Moussa, 2000).

Glucocorticoids and arachidonic acid stimulate differentiation of Ob 1771 preadipocytes to a similar extent (Gaillard et al., 1989; Gaillard et al., 1991). In fact, the glucocorticoid enhancement of Ob1771 preadipocyte GPDH activity is diminished with concomitant exposure to arachidonic acid and completely abolished with the simultaneous exposure to PGI_2 (Gaillard et al., 1991). Additionally, aspirin, a COX inhibitor abolishes glucocorticoid enhancement of Ob1771 preadipocyte differentiation, and this effect is reversed by concomitant PGI_2 exposure (Gaillard et al., 1989; Négrel et al., 1989; Gaillard et al., 1991). Furthermore, when differentiating Ob1771 preadipocytes have been exposed to antibodies against PGI_2, adipogenesis has been abrogated (Catalioto et al., 1991).

As discussed earlier, PGI_2 induces increases in intracellular cAMP (Négrel et al., 1989), which activates kinases involved in the induction of the clonal

amplification essential for terminal differentiation (Gaillard et al., 1989; Négrel et al., 1989). Furthermore, PGI_2 may act as a PPAR ligand and activate PPARδ, which is an inducer of clonal expansion (Hertz et al., 1996; Shao and Lazar, 1997; Wahle et al., 2003; Wise, 2003) and PPARγ_2, the master regulator of adipocyte differentiation (Brun et al., 1996b; Bishop-Bailey and Wray, 2003; Feve, 2005). Because PGI_2 is considered a critical prostanoid in adipogenesis (Négrel, 1999), stimulation of PGI_2 production may be an important mechanism of glucocorticoid stimulation of preadipocyte differentiation (Gaillard et al., 1991; Martin et al., 1998).

The role of PGI_2 in adipogenesis has been previously challenged because the concentration of PGI_2 required to activate PPAR (Yu et al., 1995; Brun et al., 1996b; Hertz et al., 1996; Gupta et al., 2000) and(or) trigger preadipocyte differentiation is in the micromolar range (Négrel et al., 1989; Catalioto et al., 1991; Gaillard et al., 1991; Aubert et al., 1996; Aubert et al., 2000). In contrast, physiological concentrations have been measured in the nanomolar range (Funk, 2001; Bishop-Bailey and Wray, 2003). The adipogenic role of PGI_2 is further challenged by the results of experiments in which arachidonic acid has inhibited lipogenic gene expression, and supplementation of differentiation media with COX inhibitors reverses arachidonic acid prevention of adipogenesis (Casimir et al., 1996; Mater et al., 1998). However, the fact that various COX

inhibitors used to study the role of prostaglandins in preadipocyte differentiation have been reported to bind PPARγ (Lehmann et al., 1997) makes these results difficult to interpret.

Contrary to the finding that glucocorticoids stimulate PGI_2 secretion in Ob 1771 preadipocytes reported by Gaillard et al. (1991), others have found that glucocorticoids inhibit PGI_2 synthesis in non-adipose cultured cells (Blackwell et al., 1980; Hullin et al., 1989; Rosenstock et al., 1997). By abrogating the activity of COX, 3T3-L1 preadipocyte differentiation is accelerated (Yan et al., 2003). Consequently, COX products (i.e. PGI_2) appear to be negatively involved in adipogenesis. Recent evidence revealed that DEX exposure resulted in a decrease in the expression of COX, accompanied by a reduction in the secretion of prostaglandins and an enhancement of adipogenesis in 3T3-L1 preadipocytes (Xie et al., 2006). Glucocorticoids have been found to reduce prostaglandin synthesis through inhibition of phospholipase A_2 (**PLA_2**). This enzyme catalyzes the endogenous release of arachidonic acid, which is essential for the synthesis of prostaglandins (Heiko Mühla et al., 1992).

Based on this line of evidence it has been proposed that glucocorticoids may enhance preadipocyte differentiation by preventing arachidonic acid release and prostaglandin synthesis. However, COX inhibitors are not able to replicate glucocorticoid stimulation of differentiation (Casimir et al., 1996).

Nevertheless, when preadipocytes are exposed to high concentrations of COX inhibitors that activate PPARγ_2, an enhancement of differentiation has been reported (Lehmann et al., 1997). The evidence then suggests that COX inhibitors primarily induce preadipocyte differentiation through the activation of PPARγ_2, rather than by inhibiting prostaglandin synthesis.

Ligands of PPARγ_2

Although lipophilic molecules like linoleic acid, linolenic acid, eicosapentaenoic acid, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 are activators of PPARγ_2, the identity of the biologically relevant PPARγ_2 ligand(s) is(are) still unknown (Houseknecht et al., 2002; Xie et al., 2006). However, various synthetic PPARγ_2 ligands have been identified, of which the most studied are the thiazolidinediones. Supplementation with PPARγ_2 ligands can result in a depot-dependent enhancement of preadipocyte differentiation. Different PPARγ_2 ligands, by facilitating PPARγ_2 recruitment of different cofactors (Bishop-Bailey and Wray, 2003), ultimately can determine PPARγ_2 target genes (Knouff and Auwerx, 2004). Because site-specific differences in PPARγ_2 cofactor expression may exist in different adipose depots (Adams et al., 1997), there is a possibility that PPARγ_2 ligands could selectively enhance differentiation of preadipocytes in different adipose tissues. For instance, a PPARγ_2 ligand (indomethacin) has

been shown to enhance adipogenesis in bovine OM preadipocytes and not in SC preadipocytes (Wu et al., 2000).

Thiazolidinediones. The thiazolidinediones are a class of antidiabetic drugs with the ability of lowering plasma glucose by reducing insulin resistance in adipose tissues, skeletal muscle, and hepatic tissue (Hauner, 2002). In rodents and humans, thiazolidinediones are among the most potent inducers of preadipocyte differentiation (Adams et al., 1997; Tchkonia et al., 2002). This effect has been traditionally associated with the capability of these chemicals to activate PPARγ_2 (Kelly et al., 1999; Hauner, 2002; Houseknecht et al., 2002; Knouff and Auwerx, 2004). Indeed, the thiazolidinedione T-174 has been shown to induce the differentiation of bovine IM preadipocytes (Torii et al., 1998). Three thiazolidinediones, rosiglitazone, troglitazone (TGZ), and pioglitazone, are routinely used for the treatment of diabetes, and are the more studied of this class of compunds (Hauner, 2002).

1) Rosiglitazone. Although human SC and OM preadipocytes similarly express PPARγ isoforms, OM preadipocytes are unresponsive to the adipogenic stimulation of rosiglitazone (also know as BRL49653), while SC preadipocytes are responsive (Adams et al., 1997). In human SC preadipocytes, glucocorticoids and rosiglitazone synergistically induce adipogenic differentiation (Halvorsen et al., 2001). Conversely, in ovine preadipocytes rosiglitazone attenuates

differences in adipogenesis between SC and OM preadipocytes by enhancing OM preadipocyte differentiation to a greater extent than SC (Soret et al., 1999).

2) Troglitazone. In humans, the effects of TGZ on adipose tissue accretion and preadipocyte differentiation are depot dependent. Troglitazone caused a significant decrease in intra-abdominal adipose tissue mass, while total body fat was unchanged, suggesting that TGZ induced a redistribution of adipose tissue (Kelly et al., 1999). Furthermore, while no differences in the expression of PPARγ protein isoforms were found between human OM and SC preadipocytes, OM preadipocytes were less responsive to TGZ than SC preadipocytes (Adams et al., 1997). Conversely, bovine IM and SC preadipocytes were equally induced to differentiate by TGZ (Grant, 2005).

Although TGZ has been characterized to function as a PPARγ2 ligand (Kelly et al., 1999; Willi et al., 2002), recent evidence has revealed that TGZ also has non-genomic, PPARγ2 independent mechanisms of action (Takeda et al., 2001; Gardner et al., 2005) Specifically, TGZ has been found to activate phosphatidylinositol 3-kinase (**PI3K**), which subsequently activates various mitogen-activated protein kinases (**MAPK**) (Gardner et al., 2005). Of particular importance, PI3K activates extracellular signal-regulated kinase (**Erk**) that can activate MAPK phosphatase-1 (**MKP-1**) (Takeda et al., 2001). As an inhibitor of various MAPK (i.e. c-Jun N-terminal kinase (**JNK**), p18) (Clark and Lasa, 2003),

MKP-1 can trigger cell cycle withdrawal, an essential step for preadipocyte differentiation to proceed (Ailhaud, 2001). As inhibition of PI3K results in the prevention of 1246 and 3T3-L1 preadipocyte differentiation (Xia and Serrero, 1999), the rapid PPARγ_2 independent activation of PI3K by TGZ may be an important mechanism by which TGZ enhances preadipocyte differentiation.

Ibuprofen. Ibuprofen (**IBU**), a well established inhibitor of COX activity (Rome and Lands, 1975; Mitchell et al., 1993), has been characterized as a PPARγ ligand (Lehmann et al., 1997). Ibuprofen induces preadipocyte differentiation in 1246 preadipocytes (Ye and Serrero, 1998) and C3H10T1/2 clone 8 fibroblasts (Lehmann et al., 1997). However, at lower concentrations compatible with only COX inhibition (Rome and Lands, 1975), preadipocyte differentiation was not enhanced in C3H10T1/2 clone 8 fibroblasts (Lehmann et al., 1997). Another COX inhibitor (indomethacin) has also been shown to have adipogenic effects that are independent of COX inhibition (Knight et al., 1987).

Ibuprofen has been reported to induce the expression of adipophilin (Ye and Serrero, 1998). Because adipophilin enhances the uptake of long chain fatty acids and is involved in the intracellular storage of neutral lipids, its enhancement by IBU could be important in preadipocyte differentiation (Imamura et al., 2002). The adipophilin gene has been reported to have PPAR responsive elements (Targett-Adams et al., 2005) and IBU enhances the

transcription of adipophilin (Ye and Serrero, 1998). This strongly suggests that IBU enhances preadipocyte differentiation by functioning as a PPARγ_2 ligand (Lehmann et al., 1997).

Ibuprofen may induce preadipocyte differentiation through PPARγ_2 independent mechanisms. Ibuprofen has been shown to inhibit nuclear factor kappa β (**NF-$\kappa\beta$**) stimulation of gene transcription (Tegeder et al., 2001). One of the genes stimulated by NF-$\kappa\beta$ is tumor necrosis factor alpha (**TNFα**) (Clark and Lasa, 2003). Tumor necrosis factor α rapidly decreases PPARγ_2 mRNA and protein, and PPARγ_2 DNA binding activity (Gregoire et al., 1998). Tumor necrosis factor α also induces interleukin-6 expression in preadipocytes and adipocytes, which decreases lipoprotein lipase expression (Fried et al., 1998). In addition, TNFα is also linked to insulin resistance, inhibition of glucose intake and inhibition of preadipocyte differentiation (Fruhbeck et al., 2001).

Conclusion

The evidence presented supports the idea that adipose tissues are not inert or homogeneous organs. Bovine adipose tissues are no exception. Understanding the intrinsic differences between bovine adipose tissues would present a tremendous opportunity for manipulation of fat accretion in beef cattle.

The current understanding of the mechanisms of adipocyte development are mostly based on evidence gathered through experimentation using mouse cell lines. Because there are substantial differences in adipocyte metabolism even between similar species such as sheep and cattle (Smith, 1995), research is needed to establish if the mechanisms presented are valid for bovine preadipocytes. Furthermore, it is important to determine if intrinsic differences exist among preadipocytes isolated from different bovine adipose depots, and to understand how these differences may be related to the control of preadipocyte differentiation. This information may permit development of strategies to increase marbling (IM fat) and decrease SC fat in beef cattle. Consequently, we hypothesized that intrinsic differences exist among bovine preadipocytes isolated from distinct adipose tissues, and that these differences would be reflected by different capabilities to differentiate in culture. Furthermore, we predicted that adipogenesis may be differentially affected among depots by the supplementation of preadipocytes with various adipogenic factors.

The objectives of the subsequent studies were to determine if there were differences between IM and SC preadipocytes in: 1) expression of GR and response to glucocorticoids; 2) expression of PPARγ_2 and PGI_2 secretion; and 3) responsiveness to a PPARγ_2 activator (IBU).

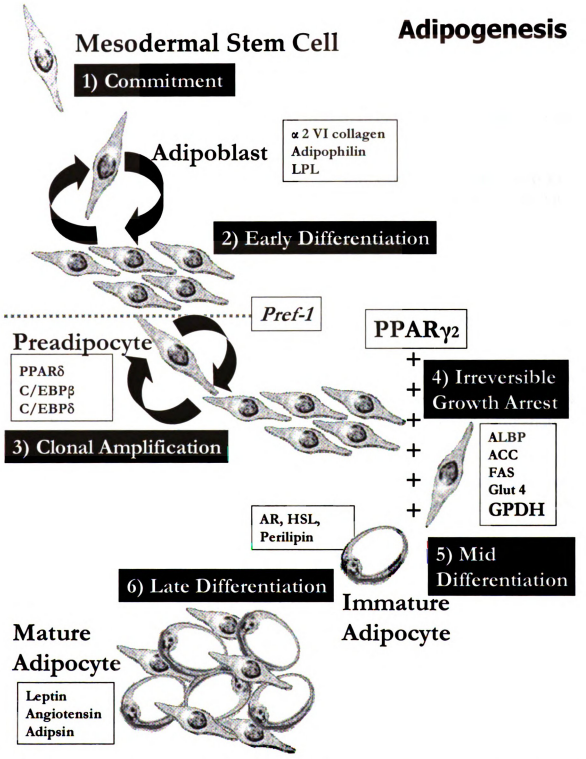

Figure 1-1: Simplified diagram of adipogenesis. Stages of differentiation are shown as black rectangles with white letters. Genes expressed in the different stages of adipogenesis are shown in white rectangles and black letters.

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CHAPTER II: BOVINE INTRAMUSCULAR, SUBCUTANEOUS, AND PERIRENAL PREADIPOCYTES EXPRESS SIMILAR GLUCOCORTICOID RECEPTOR ISOFORMS, BUT EXHIBIT DIFFERENT ADIPOGENIC CAPACITY

Abstract

The objectives of this study were to determine if differences exist in glucocorticoid receptor (GR) expression among bovine preadipocytes derived from intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue, and to evaluate the effects of dexamethasone (DEX) (a synthetic glucocorticoid) on adipogenesis of these cell populations. Preadipocytes isolated from IM, SC, and PR adipose tissues of two steers were propagated in culture and upon confluence were then exposed to 0 or 250 nM DEX for 48 h. Cell lysates were subjected to GR immunoblot analysis and immunoreactive protein bands of ~97, ~62, and ~48 kDa were detected. Relative to the β-actin immunoreactive band, the abundance of each GR immunoreactive band was similar among preadipocyte populations ($P > 0.50$). Dexamethasone exposure decreased the abundance of the ~97 and ~62 kDa GR immunoreactive bands in preadipocytes from the three depots ($P < 0.001$), but did not affect the expression of the ~48 kDa band ($P = 0.96$). Preadipocytes isolated from three steers were grown in culture, and upon confluence, were exposed to 0, 25, or 2500 nM DEX for 48 h. After an additional 10 d in differentiation media, the propensity to differentiate, was determined by glycerol-3-phosphate dehydrogenase (GPDH) specific activity

and oil red O staining. The propensity to differentiate was PR > SC > IM ($P < 0.05$). Compared with control, 2500 nM DEX increased GPDH activity in preadipocytes from all depots ($P < 0.05$). There was no interaction between adipose tissue depot and DEX concentration for GPDH activity, ($P = 0.99$). However, the percentage of PR preadipocytes with lipid droplets greater than 10 μm-diameter increased in response to DEX in a linear manner ($P < 0.02$), but only increased above control in SC cells exposed to 2500 nM DEX ($P = 0.002$). However, we failed to detect an increase in the percentage of IM preadipocytes with large (≥ 10 μm-diameter) lipid droplets upon DEX exposure ($P > 0.27$). These observations reflect an adipose tissue depot by DEX concentration interaction ($P = 0.03$). Relative differences in adipogenic capacity among preadipocytes isolated from IM, SC, and PR bovine adipose tissue were evident, although they express GR similarly. Dexamethasone enhanced adipogenic enzyme activity in all three depots, but did not significantly enhance morphological differentiation of IM preadipocytes.

Introduction

Glucocorticoid induction of adipose tissue development has anatomical specificity. In swine, glucocorticoid stimulation of subcutaneous (**SC**) preadipocyte differentiation was greater in cells isolated from the shoulder than those from the ham (Ramsay et al., 1989). Additionally, preferential accretion of

abdominal adipose tissue has been seen in humans treated with glucocorticoids (Rebuffé-Scrive et al., 1988; Fried et al., 1993; Boschmann, 2001). In cattle, glucocorticoid treatment was shown to increase intramuscular (**IM**) adipose tissue development (Brethour, 1972).

In humans and rats, the differing abundance of glucocorticoid receptors (**GR**) among adipose tissue depots is associated with the differential development of adipose depots following glucocorticoid supplementation (Feldman and Loose, 1977; Brönnegård et al., 1990; Rebuffé-Scrive et al., 1990).

Although preadipocytes are also considered a target for glucocorticoids, and glucocorticoids are commonly added to the differentiation media for cultured preadipocytes (Sato et al., 1996; Ailhaud, 2001; Brandebourg and Hu, 2005), to our knowledge, no previous studies have documented GR expression in bovine preadipocytes. If differences in GR expression exist between bovine preadipocytes from different adipose tissues, it could present a possibility to selectively alter adipose tissue accretion. Because IM adipose tissue accretion is positively associated with beef palatability and is the main determinant of beef quality within a carcass maturity classification, any treatment that could selectively enhance IM adipose tissue development, or selectively reduce SC fat, would potentially benefit the beef industry.

Therefore, the objectives of this study were to compare GR expression among bovine IM, SC, and perirenal (**PR**) preadipocytes, and to compare the effects of a glucocorticoid on the differentiation of these distinct cell populations. We hypothesized that bovine IM, SC, and PR preadipocytes would present differences in GR expression, and differences in the adipogenic response to glucocorticoids.

Materials and Methods

Isolation of bovine preadipocytes

Preadipocytes from IM, SC, and PR adipose tissues were isolated using a modification of a protocol previously described (Forest et al., 1987). Briefly, adipose tissue samples were collected immediately after exsanguination of one Angus steer and two Angus x Simmental steers (13.5 mo. old, 558 to 563 kg). Procedures were approved by the Michigan State University Committee on Animal Use and Care (AUF No. 10/03-130-00). Portions of the *longissimus* muscle (**LM**) between the 12th and 13th ribs and adipose tissue surrounding the kidneys were removed and immediately placed in sterile, ice-cold PBS (pH 7.2) and transported to the laboratory. Under sterile conditions, SC adipose tissue was separated from the LM and visible fibrous connective tissue surrounding SC adipose tissue was removed. Intramuscular adipose tissue was carefully excised from LM. For each adipose tissue depot, samples were minced and

approximately 3 g were aliquoted into 50 mL conical tubes and digested in 6 mL Dulbecco's Modified Eagle's Medium (**DMEM**; Invitrogen Corp., Carlsbad, CA) supplemented with 12 mg of collagenase (from *Clostridium histolyticum*, Type II, >125 collagen digestion units/mg solid and 0.5 to 5.0 furylacryloyl-Leu-Gly-Pro-Ala hydrolyzing units/mg solid) and 2% BSA. Samples were incubated in a 37°C water bath, and were inverted at 0, 5, 10, and 15 min. After 15 min, samples were transferred to an incubator (Lab-Line Instruments Inc. Melrose Park, IL) and further digested with shaking (230 rpm) for 45 min at 37°C. The digested tissue was then sequentially filtered through 1000, 500, and 53 μm nylon mesh and the filtrates were centrifuged for 10 min at 800 × *g*. Resulting supernatants were collected and centrifuged again for 10 min at 800 × *g*. The pellets were resuspended in growth medium (DMEM [5.5 mM glucose] supplemented with antibiotic-antimycotic (Final concentration: 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B), 0.05 mg/mL gentamicin, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate, 1,000 μM octanoate, and 10% fetal bovine serum [**FBS**]). Unless otherwise stated, all reagents were of tissue culture grade and were purchased from Sigma (St. Louis, MO).

The resulting suspension of preadipocytes was seeded at 0.05 g equivalent per cm^2 in 35 mm-diameter cell culture wells (Corning Inc., Corning, NY).

Alternatively, cells were suspended in freezing medium (base medium supplemented with a final concentration of 20% FBS and 10% dimethyl sulfoxide) and aliquoted into 1.8 mL cryogenic vials. Cells were placed at -20°C for two hours, and overnight at -80°C, before storage in liquid nitrogen for later use.

Analysis of glucocorticoid receptor expression

Cell culture. Primary preadipocytes from the IM, SC, and PR adipose tissue of two steers were propagated in culture and in their 4th passage, preadipocytes were seeded at a density of 3,600 cells/cm^2 in 35 mm-diameter cell culture wells. Cells were allowed to proliferate to confluence (5 d) in growth medium, while incubated in a humidified atmosphere (37°C, 95% air and 5% CO^2). Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and the preadipocytes were exposed to modified growth medium (1% FBS) containing 0 or 250 nM dexamethasone (**DEX**) for 48 h. Identical procedures were followed in four individual trials.

Immunoblot analysis. After 48 h of exposure to 0 or 250 nM DEX, preadipocyte monolayers were washed twice with ice-cold PBS and subsequently solubilized by the addition of hot (95°C) electrophoresis sample buffer (62 mM Tris-HCL (pH 6.8), 2% SDS, and 10% glycerol). Cell lysates from two wells per treatment were pooled and immediately stored at -20°C. Protein

concentrations were determined using the bicinchoninic acid assay (**BCA**, Pierce Biotechnology Inc., Rockford, IL). Prior to electrophoresis, protein samples were diluted to equal concentrations (0.33 μg/μL) by the addition of electrophoresis sample buffer supplemented with β-mercaptoethanol (5%) and bromophenol blue (0.01 %). Samples were then boiled for 3 min and 20 μg of protein per sample were subjected to SDS-PAGE using 7.5% (37.5:1 acrylamide/bis acrylamide) (Bio-Rad Laboratories, Hercules, CA) separating mini-gels, with 4% (37.5:1 acrylamide/bis acrylamide) stacking gels. After PAGE, proteins were transferred to polyvinylidine difluoride membranes at 4°C for 2 h at 100 V in a buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol using a using a Bio Rad Mini-Trans-Blot electrophoretic transfer cell. Lanes with the molecular weight standards were cut from the membrane and stained with amido black. The remaining membranes were then cut just above the 45 kDa band to allow for individual β-actin and GR antibody labeling. Nonspecific antibody binding was prevented by incubating the membranes for 1 h in blocking solution (Tris buffered saline [198.2 mM Tris, 1.3 M NaCl, 26.8 mM KCl; pH 7.2] containing 0.1% Tween-20 [Bio-Rad Laboratories, Hercules, CA] and 5% non-fat dry milk). The membranes were then incubated overnight at 4°C in blocking solution containing 1 μg/mL of a polyclonal (rabbit) anti-GR antibody (PA1-511A, Affinity BioReagents, Inc., Golden, CO). After the primary antibody incubation,

membranes were washed three times with blocking solution and incubated for 1 h in blocking solution containing 1:1000 (vol/vol) of an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (A3683, Sigma, Inc.). Membranes were washed three times with blocking solution and three times with TBS (0.1% Tween-20), and immunoreactive bands were detected upon the addition of 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (Bio-Rad Laboratories, Hercules, CA). Utilizing a similar procedure, the appropriate membrane sections were incubated for 1 h in blocking solution containing 0.16 μg/mL of a monoclonal anti-β-actin antibody (ab6276, Ab Cam, Inc., Cambridge, UK) and subsequently incubated for 1 h in blocking solution containing 1:1000 (vol/vol) of an alkaline phosphatase conjugated goat anti-mouse IgG antibody (A3562, Sigma, Inc.). Images of immunoblots were acquired using a Fluor-S Multilmager (Bio-Rad Laboratories) and analyzed with Discovery Series Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Abundance of GR immunoreactive bands was normalized based on β-actin immunoreactive band intensity to account for loading differences.

Analysis of the effects of glucocorticoids on bovine preadipocyte differentiation

Cell culture. Primary preadipocytes from the IM, SC, and PR adipose tissue of three steers were propagated in culture and secondary cultures were seeded at a density of 4,600 cells/cm^2 in 35 mm-diameter cell culture wells. Cells

were allowed to proliferate to confluence (4 d) in growth medium. Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and differentiation treatments were applied. Differentiation medium was supplemented with 280 nM bovine insulin and 5 μL/mL bovine serum lipids (Ex-Cyte; Serologicals Corp., Norcross, GA). Preadipocytes were exposed to 0, 25, or 2500 nM DEX for 48 h. Previously, DEX optimized bovine clonal SC preadipocyte differentiation at a concentration of 2500 nM (not shown). Each treatment was applied to two wells of a 6-well plate, in two replicate plates for each of the three steers. After 48 h, treatment media were replaced with basic differentiation medium supplemented with 280 nM bovine insulin and 5 μL/mL bovine serum lipids and fresh medium was provided every 2 d for 12 d.

Glycerol-3-phosphate dehydrogenase activity. Cell differentiation was quantified biochemically by measuring glycerol-3-phosphate dehydrogenase (**GPDH**) enzyme activity using a modification of a method previously described (Adams et al., 1997). Cells were washed twice with ice-cold PBS. Contents of two wells per treatment were harvested and combined and in a total volume of 200 μL of ice-cold Tris (5 mM, pH 7.4) containing 1 mM EDTA and 50 μM dithiothreitol (extraction buffer). Each sample was transferred into prechilled 1.5 mL microcentrifuge tubes, and then disrupted by sonification three times at

40 W (3 s bursts with 1 min cooling on ice between bursts) using a Sonifier-Cell Disrupter 350 (Branson Sonic Power Co., Danbury, CT). Samples were centrifuged at 16,000 × g for 15 min at 2°C. Solutions containing 50 μL of the resulting supernatant and 150 μL of assay buffer were assayed for GPDH activity in duplicate, within 30 min of isolation. The final concentration of the assay mixture was: 100 mM triethanolamine-HCL (pH 7.4), 2.5 mM EDTA, 50 μM dithiothreitol, 0.8 mM dihydroxyacetone phosphate, and 0.317 mM NADH. Each reaction was initiated by the addition of assay buffer to the supernatant in one well of a 96-well plate (Immulon 1B; Fisher Scientific, Hampton, NH). Fifty μL of extraction buffer served as the reagent blank. To obtain the reaction rate, the ΔA340 was recorded at 15 s intervals for 6 min at 30°C using a spectrophotometer (Versamax Tunable Microplate Reader, Molecular Devices, Sunnyvale, CA). All reactions measured were linear for at least 200 s and the Vmax used to calculate GPDH activity was obtained from the linear range. Enzyme activity was expressed as nanomoles of NADH oxidized • min^{-1} • mg $protein^{-1}$. Protein concentrations of soluble cell lysates were determined by BCA protein assay (Pierce Biotechnology Inc., Rockford, IL).

Analysis of morphological differentiation. Cell differentiation was morphologically assessed by counting the number of cells containing lipid droplets stained with oil red O (**ORO**). The ORO solution was prepared using a

protocol previously described (Ramírez-Zacarías et al., 1992). Cells were fixed by addition of 3.7% formaldehyde (Mallinckrodt Baker Inc., Phillipsburg, NJ) in PBS for 4 min. After fixation, cells were washed twice with PBS and incubated at room temperature with ORO solution for 1 h. Residual ORO solution was aspirated and the cells were washed twice with distilled water (15 min incubation/wash). Cell nuclei were stained by adding 1 mL giemsa solution (1g Giemsa, 66 mL glycerol, 66 mL methanol) to each well for 1 h, after which the cells were washed twice in distilled water, and stored dry at 4°C. Cells were visualized within 8 h of staining. Digital photographs were taken using a Nikon CoolPix 5000 digital camera (Nikon Inc., Melville, NY) fitted to a Zeiss inverted microscope (Carl Zeiss Inc., Thornwood, NY). Five fields of view, selected a priori, were photographed for each treatment replicate. Total cells were counted, and the percentage of ORO positive cells and differentiated cells were determined. Differentiated cells were defined as having one or more lipid droplet(s) with a diameter of 10 μm or larger, determined using an electronically generated ruler.

Statistical analysis

Data were analyzed using the Mixed Model procedure of SAS (SAS, Cary, NC). For the GPDH and immunoblot data, pooled cells from two wells of a six-well plate were considered the experimental unit, while for morphological

differentiation a single well was the experimental unit. For the immunoblot data, means were calculated using the fixed effects of DEX, depot, and DEX × depot, with steer included as a random variable. To satisfy the conditions of normality and homogeneity of variance, GPDH data were $\log_e$ transformed. For GPDH and morphological differentiation data, means were calculated using the fixed effects of DEX, depot, and DEX × depot, with steer and steer × replication included as random variables. When the main effects were significant ($P < 0.05$), mean differences were analyzed utilizing Tukey's multiple comparisons. Correlation analysis was perform utilizing the CORR procedure of SAS where the variables analyzed were GPDH activity, percentage of differentiated cells, and percentage of ORO positive cells.

Results

Glucocorticoid receptor (GR) expression

The immunoblot analysis of protein isolates from IM, SC, and PR bovine preadipocytes revealed three GR immunoreactive bands of ~97, ~66, and ~48 kDa (Figure 2-1). No differences in the abundance of these immunoreactive bands across the three depots were found ($P > 0.50$). The relative expression of the ~97 kDa and ~48 kDa bands were approximately 2-fold more abundant than the ~66 kDa band in these cells. Dexamethasone exposure reduced the level of the ~97 kDa isoform by 36.5 ± 8.9%, and reduced ~66 kDa isoform expression by

72.5 ± 23.7% in preadipocytes from all depots ($P < 0.001$). However, the level of expression of the ~48 kDa immunoreactive band was not affected by DEX treatment ($P = 0.96$).

DEX effects on adipogenic enzyme activity

Supplementation of differentiation media with 25 nM DEX increased GPDH activity in preadipocytes from all depots ($P < 0.001$), and there was no interaction ($P = 0.99$) between DEX concentration and depot of origin of the preadipocytes (Figure 2-2). However, 2500 nM DEX did not significantly increase GPDH activity over 25 nM DEX levels ($P = 0.45$). Intrinsic differences existed in the propensity of the preadipocytes from different depots to undergo adipogenic differentiation. Independent of treatment, PR preadipocytes were the most adipogenic, followed by SC preadipocytes with GPDH activities 37.5% lower, while IM preadipocytes had GPDH activities 74.5% lower than PR preadipocytes ($P < 0.001$).

Morphological assessment of lipid accumulation

The percentage of PR preadipocytes with lipid droplets greater than 10 μm-diameter increased in response to DEX in a linear manner ($P < 0.02$), but only increased above control in SC cells exposed to 2500 nM DEX ($P = 0.002$; Figure 2-3). However, we failed to detect an increase in the percentage of IM preadipocytes with large (≥ 10 μm-diameter) lipid droplets upon DEX exposure

($P > 0.27$). These observations reflect an adipose tissue depot by DEX concentration interaction ($P = 0.03$). The percentage of preadipocytes with lipid droplets smaller than 10 μm-diameter averaged 21.3% and was not influenced by depot ($P = 0.18$) or DEX exposure ($P = 0.84$; Figure 2-4). Photomicrographs representing average fields of view are shown in Figure 2-5.

To evaluate our criterias for adipogenic differentiation, we compared percentage of preadipocytes with lipid droplets ≥ 10 μM-diameter with biochemical differentiation data (GPDH activity). Correlation analysis revealed linear relationships ($r = 0.95$, $P < 0.001$) between the percentage of morphological differentiated cells and GPDH activity in the three depots (Figure 2-6). Independently, the correlation between the percentage of morphological differentiated cells and GPDH activity was: IM preadipocytes ($r = 0.97$, $P < 0.001$); SC preadipocytes ($r = .90$, $P < 0.001$); and PR preadipocytes ($r = 0.98$, $P < 0.001$). The percentage of of cells containing lipid droplets smaller than 10 μM-diameter was not correlated ($P = 0.19$) with GPDH activity in SC preadipocytes. However, the percentage of cells containing lipid droplets smaller than 10 μM-diameter was negatively correlated with GPDH activity in IM preadipocytes ($r = -0.54$, $P = 0.02$), and tended to be negatively correlated with GPDH activity in PR preadipocytes ($r = -0.44$, $P = 0.07$).

Discussion

To our knowledge, this is the first study that documents the expression of GR in cultured bovine preadipocytes. Immunoblot analysis of protein homogenates resulted in distinct GR immunoreactive bands of ~97, ~66, and ~48 kDa, which were similar among preadipocytes isolated from different bovine adipose tissues. Although only one GR gene has been identified, multiple promoters, alternative splicing, alternative translation initiation, and posttranslational modifications result in production of various isoforms that fractionate into several bands upon electrophoresis (Brönnegård et al., 1995; Yudt and Cidlowski, 2002). The ~97 kDa isoform is the best characterized GRα isoform, and presumably the primary mediator of glucocorticoid action.

We found that upon DEX exposure the ~97 kDa GR immunoreactive band was downregulated, which is similar to previous results in human adipocytes (Brönnegård et al., 1995). Upon glucocorticoid binding, GRα itself can inhibit GRα expression by repressing the transcription of the GR gene (Nobukuni et al., 1995) and by reducing GRα mRNA stability or translatability (Bamberger et al., 1996). The ability of DEX to downregulate the ~97 kDa GR immunoreactive band detected in our study suggests that cultured bovine preadipocytes express a functional GRα.

The function of the ~66 kDa putative GR isoform is uncertain. Although the abundance of the ~66 kDa immunoreactive band was not different among preadipocytes isolated from different adipose depots, its abundance was also decreased by DEX exposure, suggesting a possible role in glucocorticoid signaling.

The specific identity of the ~48 kDa putative isoform is not known. However, this immunoreactive band may be functionally equivalent to a splice variant named GRρ which has been identified in myeloma tumor cells (Yudt and Cidlowski, 2002). In some cells, the level of expression of this protein may represent 10 to 50% of the total GR proteins (de Lange et al., 2001). In our preadipocytes, the relative expression of the ~48 kDa immunoreactive band was 40% of the total GR immunoreactive bands. The GRρ isoform has been documented to be constituted of only 676 amino acids, compared to 777 amino acids in GRα. Although GRρ lacks a ligand binding domain (Yudt and Cidlowski, 2002), it may be involved in the upregulation of GRα mediated gene expression (de Lange et al., 2001).

Bovine preadipocyte differentiation, as measured by GPDH activity, was induced by DEX, independent of the adipose tissue depot from which the cells were isolated. The responsiveness to glucocorticoids is closely related to the abundance of GR in many tissues (i.e. hepatic, lymphatic) (Rosseau et al., 1972;

Bamberger et al., 1996). The proportionally comparable induction of differentiation among IM, SC, and PR preadipocytes by DEX exposure is consistent with our observation that cultured bovine preadipocytes express similar quantities of GR.

We hypothesized that glucocorticoids would preferentially enhance adipogenic differentiation in IM preadipocytes. However, the inferior GPDH activity of IM preadipocytes was not selectively improved by DEX exposure. This implies that differences in adipogenic capacity among these cell populations may be due to signaling pathways unrelated to GR. Although DEX increased GPDH activity in IM preadipocytes, we failed to detect an increase in the percentage of IM preadipocytes with large (≥ 10 μm-diameter) lipid droplets upon DEX exposure. The lower adipogenic capacity of IM preadipocytes may suggest that cell cultures isolated from IM adipose tissue may contain a lower proportion of adipogenic cells. Nevertheless, under similar culture conditions (i.e. supplementation with bovine serum lipids) clonal analysis of cells isolated from IM and SC adipose tissue revealed no differences in the percentages of colonies that were adipogenic, although the number of cells accumulating lipid within a colony was higher in SC cells (Grant, 2005).

Almost all mammalian cells accumulate minuscule lipid droplets that mainly serve as cholesterol ester reservoirs used in the synthesis and

maintenance of membranes (Murphy and Vance, 1999; Wolins et al., 2005). Conversely, energy storage is primarily found in adipocytes that package triacylglycerides in large (10 to 100 μm in diameter) lipid droplets (Wolins et al., 2005). Other cell types, have a limited capacity to store triacylglycerides and seldom accumulate large lipid droplets (Wolins et al., 2005). We used the presence of lipid droplets ≥ 10 μm-diameter as a morphological indicator of preadipocyte differentiation. The percentage of cells with lipid droplets ≥ 10 μm-diameter was highly correlated with GPDH activity among the preadipocytes of the three depots, validating our criterias for determining adipogenic differentiation. In fact, the percentage of cells with lipid droplets < 10 μm-diameter was not correlated with GPDH activity in SC preadipocytes, and was negatively correlated with GPDH activity in PR and IM preadipocytes.

Glucocorticoid influence on preadipocyte differentiation has been well documented in various species (Ramsay et al., 1989; Xu and Björntorp, 1990) and cell lines (Gaillard et al., 1991; Smas et al., 1999). Interestingly, porcine PR preadipocytes have been reported as unresponsive to glucocorticoid exposure, while SC preadipocytes isolated from the shoulder and ham regions exhibited dose-dependent increases in GPDH activity in response to hydrocortisone (Ramsay et al., 1989). In our study, bovine PR preadipocytes were responsive to DEX and exhibited the highest propensity to differentiate.

Our results do not reveal the mechanism of action of DEX stimulation of bovine IM adipose tissue development observed by Brethour (1972). However, it is important to acknowledge that glucocorticoids have dramatic effects on whole body nutrient metabolism and an increase in IM fat accretion may be the indirect result of glucocorticoids creating a state of insulin resistance in SC adipose tissue and(or) other organs (Rebuffé-Scrive et al., 1988; Corah et al., 1995; Cortright et al., 1997), which could increase substrate availability for IM preadipocytes, and as a consequence, augment IM fat development.

Dexamethasone equally promoted adipogenic enzyme activity in IM, SC, and PR bovine preadipocytes, and consequently did not abolish their adipogenic differences. It is then suggested that adipogenic differences among these cells are controlled by factors unrelated to GR expression or activation. Peroxisome proliferator-activated receptor γ_2 (**PPARγ_2**) is considered the master regulator of adipogenesis (Schoonjans et al., 1996; Knouff and Auwerx, 2004). Therefore, differences in adipogenic capacity between bovine preadipocytes could be related to differences in PPARγ_2 expression. However, the activity of PPARγ_2 is regulated by the availability of lipophilic molecules that could be different between preadipocyte populations. Consequently, adipogenic differences among bovine preadipocytes isolated from distinct adipose depots may result

from intrinsic differences in the endogenous activation of PPARγ_2 among distinct bovine preadipocyte populations.

We conclude that cultured bovine preadipocytes from IM, Sc, and PR adipose tissue express similar quantities of GR immunoreactive bands. Dexamethasone exposure resulted in downregulation of ~97 and ~62 kDa GR immunoreactive bands in preadipocytes from all depots, suggesting similarity in at least one aspect of GR function. Bovine preadipocytes exhibited different propensities to biochemically differentiate (GPDH: PR > SC > IM), but DEX induced proportionally similar increases in GPDH activity in preadipocytes from all depots. In contrast with SC and PR preadipocytes, we failed to detect an increase in the proportion of IM preadipocytes with lipid droplets ≥ 10 μm upon DEX expossure. Because differences among the preadipocyte populations may be related to signaling pathways unrelated to GR, future studies aiming to understand the observed differences among preadipocytes from different depots should focus on studying the expression of other regulators of adipogenesis and(or) differences in secretion of adipogenic molecules.

Implications

Cultured preadipocytes isolated from bovine intramuscular adipose tissue have a limited ability to accumulate lipid when compared to perirenal and subcutaneous preadipocytes. Because glucocorticoid receptor is equally

expressed in intramuscular, subcutaneous, and perirenal preadipocytes, and dexamethasone increased adipogenic enzymatic activity in equal proportions in these cell populations, the observed differences in adipogenic capacity among these cell populations appears to be unrelated to glucocorticoid receptor function. Experiments aiming to compare the expression of other proteins known to be involved in the regulation of preadipocyte differentiation may help decipher what is unique about intramuscular preadipocytes and facilitate the discovery of methods to selectively increase intramuscular lipid accretion in cattle.

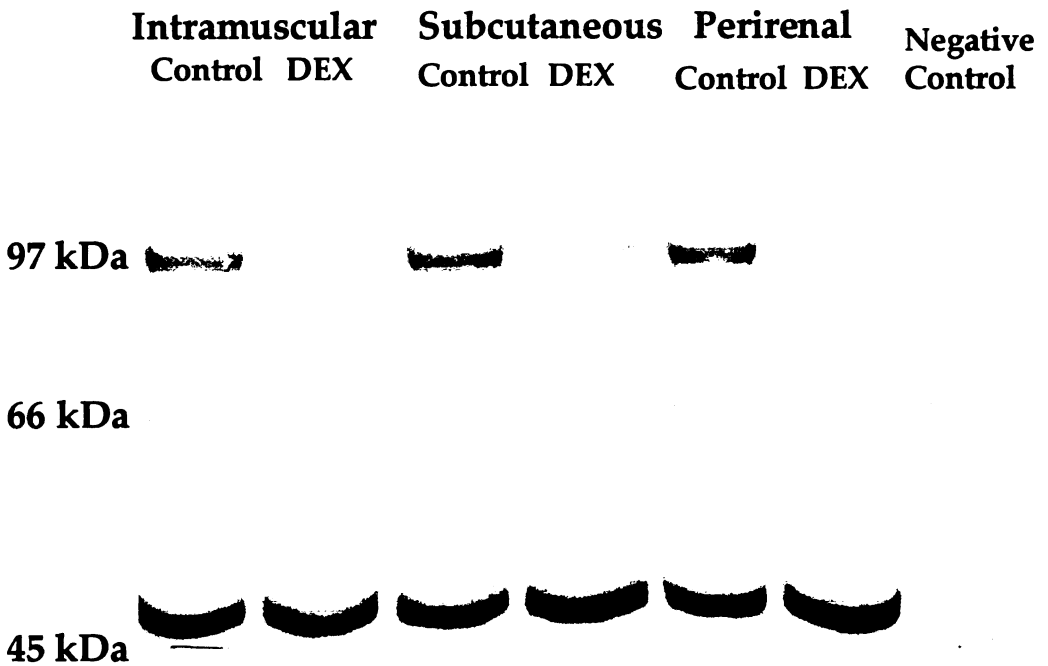


Figure 2-1. Glucocorticoid receptor immunoblot of bovine intramuscular, subcutaneous, and perirenal preadipocytes. Cells were grown to confluence and then exposed to 0 or 250 nM dexamethasone (DEX) for 48 h. Twenty micrograms of protein per sample were separated by gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Membranes were subsequently incubated overnight with a polyclonal antibody raised against the glucocorticoid receptor, which detected major immunoreactive bands of ~97, ~66, and ~48 kDA. Positions of molecular weight standards are indicated to the left.

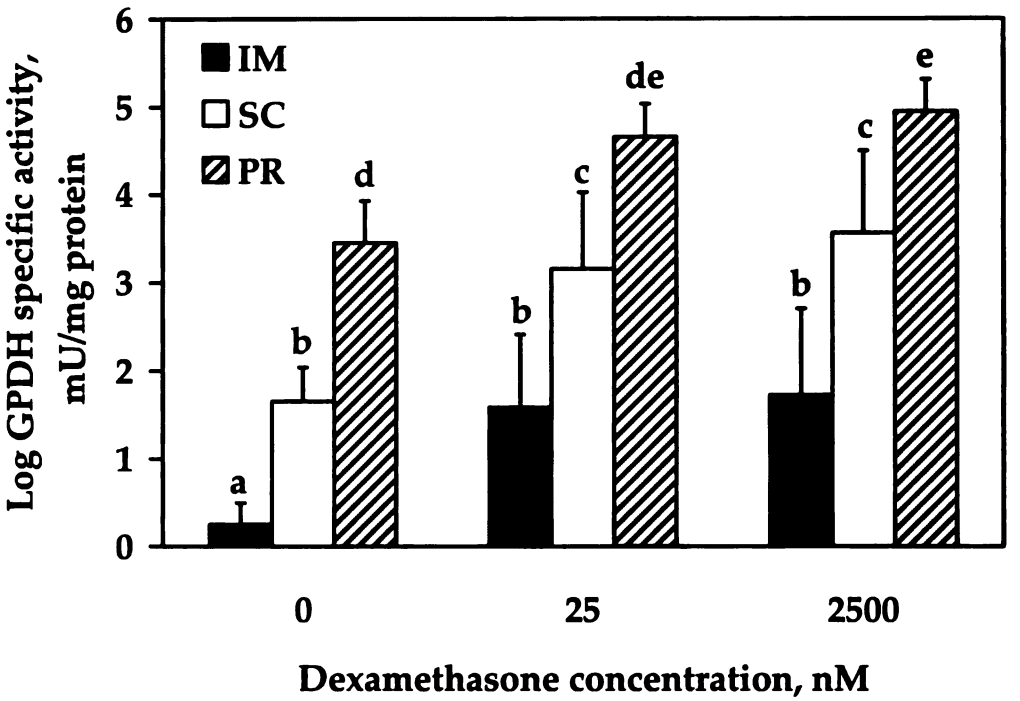


Figure 2-2. Effect of dexamethasone (DEX) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine preadipocytes isolated from bovine intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue of three steers. Bovine preadipocytes were grown to confluence and subsequently exposed to DEX for 48 h, and differentiation media for 10 additional days. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Bars represent means ± SEM. Means with different superscripts differ ($P < 0.05$).

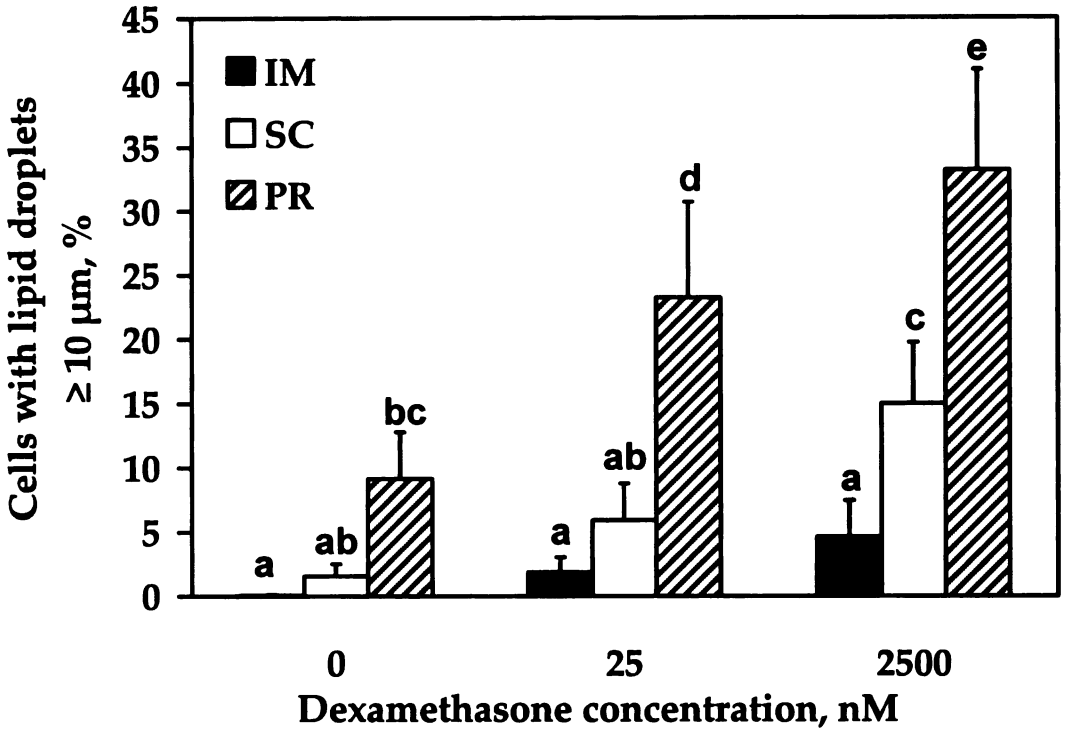


Figure 2-3. Effect of dexamethasone (DEX) concentration on the percentage of differentiated preadipocytes (cells with a lipid droplet ≥ 10 µm). Bovine preadipocytes isolated from intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue of three steers were grown to confluence and subsequently exposed to the indicated DEX concentrations for 48 h and differentiation media for 10 additional days. The percentage of differentiated preadipocytes was determined by microscopy 12 d after addition of treatments. Bars represent means ± SEM. There was an interaction ($P = 0.03$) between DEX concentration and depot. Means with different superscripts differ ($P < 0.05$).

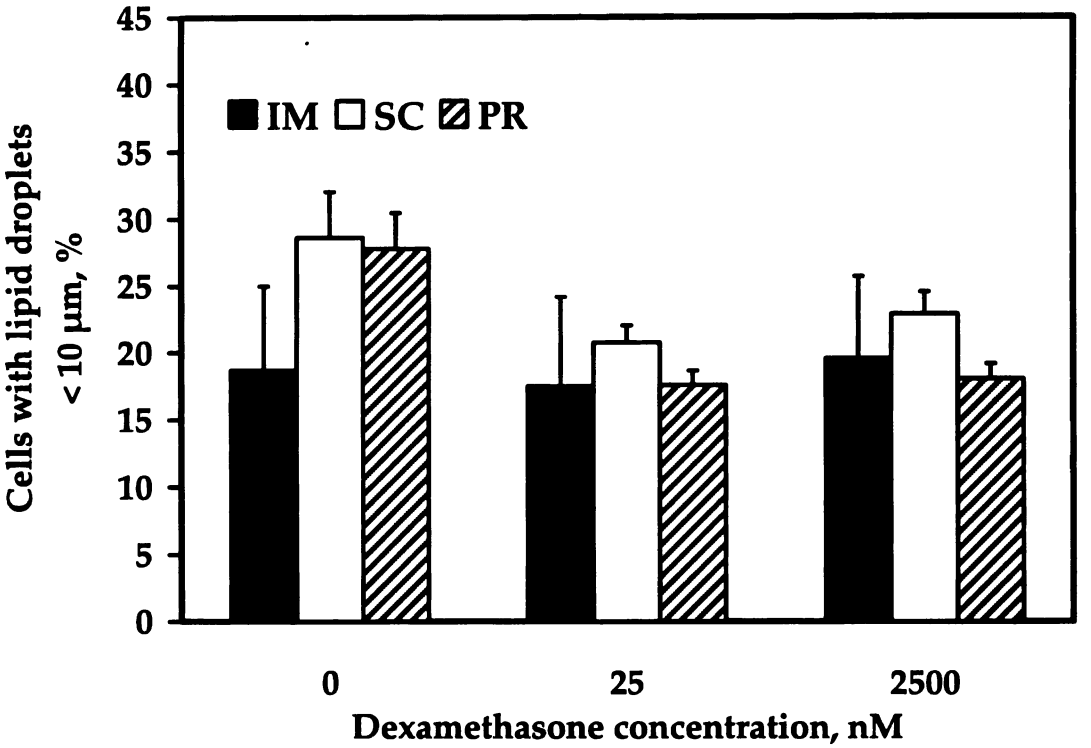
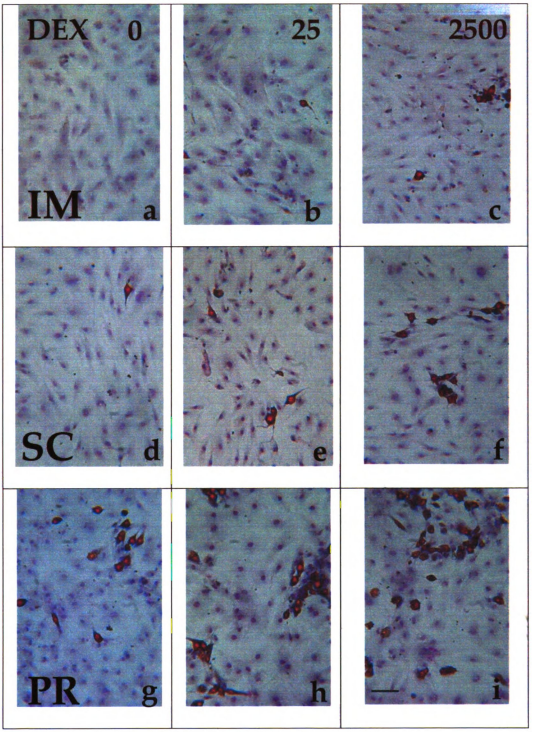


Figure 2-4. Effect of dexamethasone (DEX) concentration on the percentage of preadipocytes with a lipid droplet < 10 µm. Bovine preadipocytes isolated from intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue of three steers were grown to confluence and subsequently exposed to the indicated DEX concentrations for 48 h and differentiation media for 10 additional days. Preadipocyte lipid droplets were evaluated by microscopy 12 d after addition of treatments. Bars represent means ± SEM. Means did not differ ($P > 0.20$).

Figure 2-5. Effect of dexamethasone (DEX) concentration on morphological differentiation of bovine preadipocytes. Bovine preadipocytes isolated from intramuscular (IM; a, b, c), subcutaneous (SC; d, e, f), and perirenal (PR; g, h, i) adipose tissue of three steers were grown to confluence and subsequently exposed to 0 (a, d, g), 25 (b, e, h) or 2500 (c, f, i) nM DEX for 48 h and differentiation media for 10 additional days. Photomicrographs were taken 12 d after addition of treatments. Lipid droplets in cells were stained with oil red O and cell nuclei were counterstained with giemsa. Photomicrographs shown represent average fields of view. Bar = 100 μM (Panel i).

Figure 2-5. Effects of dexamethasone on morphological differentiation of bovine preadipocytes



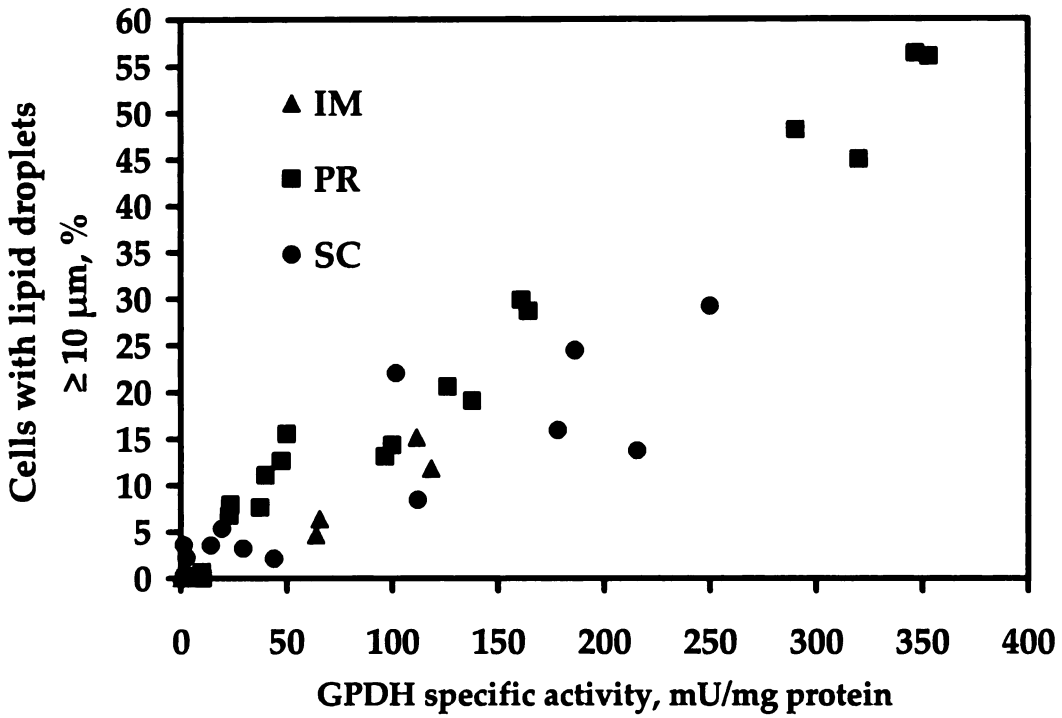


Figure 2-6. Relationship between the specific glycerol-3-phosphate dehydrogenase (GPDH) activity and percentage of cells with a lipid droplet ≥ 10 μm ($r = 0.95$, $P < 0.001$) in preadipocytes isolated from intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissues.

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CHAPTER III: DIFFERENCES IN ADIPOGENESIS BETWEEN BOVINE INTRAMUSCULAR AND SUBCUTANEOUS PREADIPOCYTES ARE NOT RELATED TO EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA TWO OR SECRETION OF PROSTACYCLIN

Abstract

The objectives of these experiments were to determine if intramuscular (IM) and subcutaneous (SC) bovine preadipocytes differ in expression of peroxisome proliferator activated receptor γ_2 (PPARγ_2) or in secretion of prostacyclin (PGI_2), a presumptive endogenous PPAR activator. Preadipocytes isolated from IM and SC adipose tissues of three steers were propagated in culture and upon confluence were exposed to 0 or 25 nM dexamethasone (DEX) for 48 h. After exposure to differentiation media for an additional 10 d, cell lysates were subjected to PPARγ_2 immunoblot analysis, which revealed an immunoreactive band of ~53 kDa. There was no interaction between DEX treatment and preadipocyte depot ($P = 0.90$) in the relative expression of PPARγ_2. Expression of PPARγ_2 was also equivalent between IM and SC preadipocytes ($P = 0.39$), and DEX did not affect PPARγ_2 abundance ($P = 0.98$). Heterogeneous preadipocytes isolated from a steer and clonal preadipocytes derived from a second steer were grown to confluence and exposed to 0 or 25 nM DEX for 48 h. Media were collected every 12 h for 48 h and assayed for the stable PGI_2 derivatives 6-keto-prostaglandin $F_{1\alpha}$ (6k-$PGF_{1\alpha}$) and 2,3-dinor-6-keto-

prostaglandin $F_{1\alpha}$ (2,3d-6k-$PGF_{1\alpha}$). After 12 d in differentiation media, glycerol-3-phosphate dehydrogenase (GPDH) analysis was performed. Intramuscular preadipocytes secreted more PGI_2 derivatives than SC preadipocytes (P = 0.046) and DEX decreased secretion of the PGI_2 derivatives equally in cells from both depots (P = 0.001). Although 25 nM DEX increased GPDH activity in both preadipocyte populations (P < 0.001), IM preadipocytes were less adipogenic than SC preadipocytes (P < 0.001). Exposure of clonal SC preadipocytes to 1 μM $cPGI_2$ tended (P = 0.06) to enhance differentiation over control conditions, and tended (P = 0.09) to increase the adipogenic enhancement stimulated by DEX. However, supplementation with 0.01 or 0.1 μM $cPGI_2$ did not increase differentiation (P > 0.65). Although ibuprofen (IBU), an inhibitor of PGI_2 synthesis, did not affect adipogenesis in clonal SC preadipocytes (P = 0.99), 100 μM IBU enhanced (P = 0.01) adipogenesis in the presence of DEX while an intermediate concentration (50 μM) tended (P = 0.08) to enhance DEX effects. We conclude that adipogenic differences between IM and SC bovine preadipocytes are not explained by differences in $PPAR\gamma_2$ expression or PGI_2 secretion.

Introduction

Bovine preadipocytes isolated from intramuscular (**IM**) and subcutaneous (**SC**) adipose tissue have different propensities to accumulate lipid (Chapter II).

This likely results from differences in the expression or activation of proteins involved in the regulation of adipogenesis. Peroxisome proliferator activated receptor gamma two (**PPARγ_2**), a ligand activated transcription factor, is considered the master regulator of adipocyte differentiation and lipid accretion (Schoonjans et al., 1996; Knouff and Auwerx, 2004). In humans, PPARγ_2 mRNA expression is greater in SC preadipocytes than in visceral preadipocytes (Sewter et al., 2002). Although the expression of PPARγ_2 has been documented in bovine perirenal preadipocytes (Ohyama et al., 1998), its expression in bovine preadipocytes from economically important adipose depots has not been reported.

Stimulation of adipogenesis by PPARγ_2 depends on its activation by ligands, such as those derived from arachidonic acid (Bishop-Bailey and Wray, 2003; Knouff and Auwerx, 2004). One of these derivatives, prostacyclin (PGI_2), is considered a PPAR activator (Aubert et al., 1996; Hertz et al., 1996; Wise, 2003) and an inducer of mouse (Ob1771) preadipocyte differentiation (Négrel et al., 1989; Négrel, 1999).

We hypothesized that SC bovine preadipocytes are more adipogenic than IM preadipocytes because SC preadipocytes express more PPARγ_2 and secrete more PGI_2. Therefore, the objectives of this study were to compare PPARγ_2 expression and PGI_2 secretion between IM and SC bovine preadipocytes.

Materials and Methods

Isolation of bovine preadipocytes

Preadipocytes from IM and SC adipose tissue were isolated using a modification of a protocol previously published (Forest et al., 1987) and described in Chapter II.

Preadipocyte cloning

Preadipocytes from IM and SC adipose tissue of one steer were seeded at 0.05 g per cm^2 in 35 mm-diameter cell culture wells (Corning Inc.) and grown to 70% confluence. Cells were then trypsinized (0.5 g/L trypsin and 0.02 g/L ethylenediaminetetraacetic acid (**EDTA**) in PBS [pH 7.2]) and seeded at 5 cells/cm^2 in 100 mm-diameter culture plates or at one cell/6.2 mm-diameter well in 96-well plates. Cells were then incubated in growth medium for 10 d. Individual colonies were isolated utilizing cloning-rings in 100 mm-diameter culture plates, or directly from 6.2 mm-diameter wells. Each clone was individually transferred into 16 mm-diameter culture wells. All surviving clones were then grown and sequentially transferred into 35 mm-diameter culture plates, then into 100 mm-diameter culture plates (1,800 cells/cm^2) after reaching 70% confluence. Cells were cryopreserved after a sub-sample of each clone (IM n = 48; SC n = 64) was seeded at 10,000 cells/cm^2 in 16 mm-diameter cell culture wells for evaluation of their adipogenic capacity. Clones were

allowed to proliferate to confluence in growth medium, which was replaced every 2 d. After reaching confluence, cells were washed twice with PBS and exposed to differentiation medium: DMEM (1% FBS) supplemented with 1.74 nM insulin, 20 mM glucose and 10 mM acetate. Differentiation medium was supplemented with 250 nM dexamethasone (**DEX**) for 48 h. After 48 h, DEX treated medium was replaced with fresh differentiation medium, and subsequently fresh medium was provided every 2 d for 10 d. Adipogenic clones were identified morphologically as those containing at least two cells stained with oil red O. The oil red O solution was prepared and used following a protocol previously described (Ramírez-Zacarías et al., 1992). An individual adipogenic clone from each adipose depot (IM and SC) was thawed at the time of the experiments.

General procedures

Cell culture. Preadipocytes from IM and SC adipose tissue were seeded at a density of 4,160 cells/cm^2 in 35 mm-diameter cell culture wells. Cells were allowed to proliferate to confluence (4 d) in a humidified atmosphere (37°C, 95% air and 5% CO_2) while incubated in growth medium (DMEM [5.5 mM glucose] supplemented with antibiotic-antimycotic (Final concentration: 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B), 0.05 mg/mL gentamicin, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate,

1,000 μM octanoate, and 10% fetal bovine serum [**FBS**]). Unless otherwise stated, all reagents were of tissue culture grade and were purchased from Sigma (St. Louis, MO). Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and differentiation treatments were applied. Differentiation medium was DMEM (5.5 mM glucose), supplemented with antibiotic-antimycotic (Final concentration: 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B), 0.05 mg/mL gentamicyn, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate, 280 nM bovine insulin and 5 μL/mL bovine serum lipids (Ex-Cyte; Serologicals Corp., Norcross, GA). Unless otherwise specified, DEX was included in the differentiation media for the initial 48 h, after which, cells were washed twice with PBS and fresh treatment media were provided every 2 d for an additional 10 d.

Glycerol-3-phosphate dehydrogenase activity. Cell differentiation was quantified biochemically by measuring glycerol-3-phosphate dehydrogenase (**GPDH**) enzyme activity using a modification of a method previously published (Adams et al., 1997) and described in Chapter II.

Experiment 1: Analysis of PPARγ2 expression

This experiment was conducted to compare the expression of PPARγ2 between bovine IM and SC preadipocytes. Primary preadipocytes from the IM

and SC adipose tissue of three steers were propagated in culture and secondary cultures were grown to confluence and exposed to differentiation medium supplemented with 0 or 25 nM DEX for 48 h. Fresh differentiation medium was provided for an additional 10 d. Each treatment was applied to two 35 mm-diameter wells of a 6-well plate, in two replicate plates for each of the three steers.

Immunoblot Analysis. Cell monolayers were washed twice with ice-cold PBS and subsequently solubilized by the addition of hot (95°C) electrophoresis sample buffer (62 mM Tris-HCL (pH 6.8), 2% SDS, and 10% glycerol). Cell lysates from two wells per treatment were pooled and immediately stored at -20°C. Protein concentrations were determined using the bicinchoninic acid assay (**BCA**, Pierce Biotechnology Inc., Rockford, IL). Prior to electrophoresis, protein samples were thawed and diluted to equal protein concentrations (0.83 μg/μL) by the addition of electrophoresis sample buffer supplemented with β-mercaptoethanol (5%) and bromophenol blue (0.01%). Samples were then boiled for 3 min and 50 μg of protein per sample were subjected to SDS-PAGE using 12.5% (37.5:1 acrylamide/bis acrylamide) (Bio-Rad Laboratories, Hercules, CA) separating mini-gels (0.75 mm thick), with 4% (37.5:1 acrylamide/bis acrylamide) stacking gels. After SDS-PAGE, proteins were transferred to polyvinylidine difluoride membranes at 4°C for 2 h at 100 V in a buffer

containing 25 mM Tris, 193 mM glycine, and 15% methanol, using a Bio Rad Mini-Trans-Blot electrophoretic transfer cell. Pre-stained molecular weight standards were used. The membranes were cut above the 45 kDa band to allow for individual PPARγ_2 and β-actin immunolabeling. Nonspecific antibody binding was prevented by incubating the membranes for 1 h in blocking solution (Tris buffered saline [198.2 mM Tris, 1.3 M NaCl, 26.8 mM KCl; pH 7.2] containing 0.1% Tween-20 [Bio-Rad Laboratories] and 5% non-fat dry milk). The membranes were then incubated for 2 h at room temperature in blocking solution containing 1 μg/mL of a polyclonal (rabbit) anti-PPARγ_2 antibody (PA1-824, Affinity BioReagents, Inc., Golden, CO). After the primary antibody incubation, membranes were washed three times with blocking solution and incubated for 1 h in blocking solution containing 1:1000 (vol/vol) of an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (A3683, Sigma, Inc.). Membranes were then washed three times with blocking solution and three times with TBS (0.1% Tween-20), and immunoreactive bands were detected upon the addition of 5-bromo-4-chloro-3-indoyl phosphate/nitroblue-tetrazolium (Bio-Rad Laboratories). Utilizing a similar procedure, the appropriate membrane sections were incubated for 1 h in blocking solution containing 0.16 μg/mL of a monoclonal anti-β-actin antibody (ab6276, Ab Cam, Inc., Cambridge, UK) and subsequently incubated for 1 h in blocking solution

containing 1:1000 (vol/vol) of an alkaline phosphatase conjugated goat anti-mouse IgG antibody (A3562, Sigma, Inc.). Images of immunoblots were acquired using a Fluor-S Multilmager (Bio-Rad Laboratories) and analyzed with Discovery Series Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Abundance of PPARγ_2 immunoreactive bands was normalized based on β-actin immunoreactive band intensity to account for loading differences.

Experiment 2. Evaluation of PGI_2 secretion and GPDH activity.

The purpose of this experiment was to compare the secretion of PGI_2 between bovine IM and SC preadipocytes. Secondary IM and SC preadipocytes from one steer, in addition to clonal preadipocytes (6^{th} passage) derived from IM and SC adipose tissue of a second steer were utilized. Cells were exposed to differentiation medium supplemented with 0 or 25 nM DEX for 48 h. Each treatment was applied to two 35 mm-diameter wells of a 6-well plate, in two replicate plates for each of the two steers. After 12 d in differentiation medium, GPDH analysis was performed. Because equivalent results were obtained from heterogeneous and clonal preadipocytes, and there was no interaction between cell type (heterogeneous or clonal preadipocytes), DEX treatment, depot and(or) time ($P > 0.15$), the results obtained from heterogeneous and clonal preadipocytes were pooled.

Measurements of PGI_2. Because PGI_2 has a half life of only 2 to 3 min in buffer, PGI_2 secretion was quantified indirectly by the measurement of its non-enzymatic hydration products 6-keto-prostaglandin $F_{1\alpha}$ (6k-$PGF_{1\alpha}$) and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (2,3d-6k-$PGF_{1\alpha}$) with an enzyme immunoassay kit (900-025, Assay Designs, Inc., Ann Arbor, MI). Samples of 250 μL of differentiation media supplemented with 0 or 25 nM DEX were collected at 12, 24, 36, and 48 h and immediately frozen at -80°C until assayed. Within one week of sample collection, media samples were thawed and 100 μL per sample were incubated in each of duplicate wells in a 96-well assay plate for 2 h with a polyclonal (sheep) anti-6-keto-$PGF_{1\alpha}$ antibody and alkaline phosphatase-conjugated 6k-$PGF_{1\alpha}$. The assay plate was placed in a plate shaker (Lab-Line Instruments Inc.) at 500 rpm during incubation. The wells were then emptied and washed 3 times (300 μL wash solution per well). Wells were then incubated for 45 min without shaking, with 200 μL of alkaline phosphatase substrate (p-nitrophenyl phosphate). Finally, 50 μL of stop solution were added to each well and the optical density was immediately read at 405 nm in a spectrophotometer (Versamax Tunable Microplate Reader, Molecular Devices), and the concentration of 6k-$PGF_{1\alpha}$ and 2,3d-6k-$PGF_{1\alpha}$ was quantified by using a standard curve developed by the serial dilution of 2,3d-6k-$PGF_{1\alpha}$. The standard curve concentrations utilized were 7.81, 31.25, 125, 500, and 2000 pg/mL. Nonspecific

binding and background optical density were calculated and used to correct the spectrophotometer readings. The concentration of PGI_2 derivatives were determined by interpolation from the standard curve utilizing a 4 parameter logistic curve fitting program (TableCurve 2D, Statistical Solutions, Saugus, MA). Prostacyclin derivatives were not detected in media (0 or 25 nM DEX) before exposure to preadipocytes.

Experiment 3: Evaluation of the effects of carbaprostacyclin on preadipocyte differentiation

This experiment aimed to evaluate the effects of carbaprostacyclin (**cPGI$_2$**), at concentrations previously shown to induce adipogenic differentiation in Ob1771 mouse preadipocytes (Catalioto et al., 1991), on the adipogenic differentiation of bovine SC preadipocytes. Clonal SC preadipocytes (6^{th} passage) were grown to confluence and exposed to unsupplemented differentiation media (control), or differentiation media supplemented with 1 μM cPGI$_2$, or 25 nM DEX in combination with 0, 0.01, 0.1, or 1.0 μM cPGI$_2$ for 48 h. Carbaprostacyclin was diluted in ethanol (5 mg/mL), and treatments not containing cPGI$_2$ were supplemented with equivalent concentrations of ethanol. For this and all subsequent experiments, treatment media were applied for 48 h. Treatment media were then replaced with fresh differentiation medium, and fresh medium was provided every 2 d for 10 d. Each treatment was applied to

two wells of a 6-well plate, in two replicate plates. After 12 d in differentiation media GPDH analysis was performed.

Experiment 4: Evaluation of the effects of ibuprofen in preadipocyte differentiation

This experiment was designed to evaluate the effect of ibuprofen (**IBU**), a cyclooxygenase (**COX**) inhibitor, on clonal SC preadipocyte adipogenesis. Cyclooxygenase is the enzyme that catalyzes the rate-limiting reaction during the biosynthesis of PGI_2 and other prostanoids (Funk, 2001). Clonal SC preadipocytes (6^{th} passage) were grown to confluence and exposed to unsupplemented differentiation media (control), or differentiation media supplemented with 100 μM IBU, or 25 nM DEX in combination with 0, 10, 50, or 100 μM IBU for 48 h.

Statistical analysis

Data were analyzed using the Mixed Model procedure of SAS (SAS, Cary, NC). In all experiments, pooled samples from two 35 mm-diameter wells of a 6-well-plate were considered the experimental unit. When main effects were significant ($P < 0.05$), differences between means were evaluated utilizing Tukey's multiple comparison test. In Exp. 1, means were calculated using the fixed effects of DEX, depot, and DEX × depot. In Exp. 2, PGI_2 data were analyzed with repeated measures, where means were calculated using the fixed effects of depot, treatment (0 or 25 nM DEX), time, and their interactions. Means

for GPDH data were calculated using the fixed effects of DEX, depot, and DEX × depot. In Exp. 3, means for GPDH data were calculated using the fixed effects of DEX, and cPGI$_2$. In Exp. 4, means for GPDH data were calculated using the fixed effects of DEX and IBU. In Exp. 2, 3, and 4, GPDH data, and in Exp. 2, PGI$_2$ data, were $\log_e$ transformed to satisfy the conditions of normality and homogeneity of variance. In Exp 1. and 2, steer and steer by replication were included as random variables, while in Exp. 3 and 4 replication was included as a random variable.

Results and Discussion

The objectives of these studies were to characterize differences in PPARγ_2 expression and PGI$_2$ secretion between preadipocytes isolated from economically important adipose depots (i.e. IM and SC), and evaluate their relation to the differences in adipogenic capacity exhibited by these cell populations.

Experiment 1

We hypothesized that differences in adipogenic capacity between bovine preadipocytes were related to differences in PPARγ_2 expression. Immunoblot analysis revealed a PPARγ_2 immunoreactive band of ~53 kDa in control and DEX treated IM and SC preadipocytes (Figure 3-1). No interaction (P = 0.90) between DEX treatment (0 or 25 nM) and depot (IM or SC) existed for PPARγ_2

protein expression. Expression of PPARγ2 was not different between IM and SC bovine preadipocytes (P = 0.39) and DEX treatment had no effect on the abundance of this protein (P = 0.98).

Human SC preadipocytes express more PPARγ2 mRNA than omental (**OM**) preadipocytes (Sewter et al., 2002). Conversely, no differences in the expression of PPARγ2 protein have been detected between human SC and OM preadipocytes (Adams et al., 1997). Although they exhibit similar patterns of PPARγ2 protein expression, human OM preadipocytes are less adipogenic, and have been shown to be less sensitive to a PPARγ2 agonist (rosiglitazone) than SC preadipocytes (Adams et al., 1997). The expression of PPARγ2 has been previously reported in bovine perirenal preadipocytes (Ohyama et al., 1998), but our study is the first to establish that PPARγ2 protein expression is equivalent in bovine IM and SC preadipocytes.

Differences in adipogenic capacity between bovine SC and OM preadipocytes were abolished when a PPARγ2 ligand (indomethacin) was added to differentiation media (Wu et al., 2000). This suggests that adipogenic differences between preadipocytes from different adipose depots may be related to differences in PPARγ2 ligand synthesis rather than PPARγ2 expression. The activity of PPARγ2 may be regulated by the availability of lipophilic molecules

like PGI_2 (Hertz et al., 1996; Bishop-Bailey and Wray, 2003), a potent adipogenic inducer (Négrel et al., 1989; Négrel, 1999).

Preadipocyte differentiation has been shown to be dependent on the presence of arachidonic acid (Gaillard et al., 1989). To date, PGI_2 is the only adipogenic arachidonic acid metabolite demonstrated to be produced by preadipocytes (Aubert et al., 1996; Négrel, 1999; Ailhaud, 2001). Cyclooxygenase catalyzes the rate-limiting reaction in the biosynthesis of PGI_2 (Figure 3-2). Exposing Ob 1771 preadipocytes to COX inhibitors abrogates adipogenic differentiation. Because the abrogation of Ob 1771 preadipocyte adipogenesis caused by COX inhibitors is prevented by concomitant exposure to $cPGI_2$ (Négrel et al., 1989), it has been suggested that Ob 1771 preadipocyte adipogenesis may be regulated through PGI_2 secretion (Gaillard et al., 1991).

Experiment 2

We hypothesized that bovine IM preadipocytes were less adipogenic than SC preadipocytes because they secreted lower levels of PGI_2. However, it was found that IM preadipocytes secreted greater amounts of PGI_2 ($P = 0.046$) (Figure 3-3). There were no 2- or 3-way interactions among DEX, depot, and time for PGI_2 secretion ($P > 0.26$). The concentration of PGI_2 in differentiation media increased with time, up to 36 h, independent of DEX treatment or depot ($P <$

0.001), and exposure to DEX decreased PGI_2 secretion in IM and SC preadipocytes ($P = 0.002$).

In contrast to our results, DEX was shown to increase PGI_2 secretion in Ob 1771 preadipocytes (Gaillard et al., 1991). However, consistent with our results, DEX has previously been shown to decrease PGI_2 secretion in non-adipose cultured cells (Blackwell et al., 1980; Hullin et al., 1989; Rosenstock et al., 1997), and to reduce prostaglandin synthesis through inhibition of phospholipase A_2 (Figure 3-2), the enzyme that catalyzes the endogenous release of arachidonic acid, the precursor of prostaglandins (Heiko Mühla et al., 1992).

Although DEX decreased PGI_2 secretion, DEX also stimulated GPDH activity in IM and SC preadipocytes as expected ($P < 0.001$) (Figure 3-4). There was no interaction ($P = 0.64$) between DEX concentration and adipose tissue depot. However, IM preadipocytes exhibited less propensity to differentiate than SC ($P < 0.001$).

Experiment 3

Because IM preadipocytes were least adipogenic than SC preadipocytes while secreting higher levels of PGI_2, we proceeded to investigate if PGI_2 may have an inhibitory role in the adipogenesis of bovine preadipocytes. Arachidonic acid, the precursor of PGI_2 synthesis, has been reported to inhibit lipogenic gene expression (Mater et al., 1998). The arachidonic acid mediated

abrogation of adipogenesis is reversed by inhibitors of COX (Casimir et al., 1996; Mater et al., 1998; Petersen et al., 2003). Because COX is the rate limiting enzyme in the synthesis of prostaglandins, these experiments suggest that arachidonic acid anti-adipogenic effects are mediated through prostaglandins (Figure 3-2). Therefore, we evaluated the effects of micromolar concentrations of $cPGI_2$ in the adipogenic differentiation of bovine clonal SC preadipocytes. As expected, DEX enhanced preadipocyte adipogenesis ($P = 0.04$) (Figure 3-5). Exposure to 1 μM $cPGI_2$ tended ($P = 0.06$) to enhance differentiation over control conditions, and tended ($P = 0.09$) to increase the adipogenic enhancement stimulated by DEX. However, supplementation with 0.01 or 0.1 μM $cPGI_2$ did not increase differentiation ($P > 0.65$), even though these concentrations are more than 25 times higher than our previous measurements of PGI_2 secretion (3.52 nM).

Prostacyclin (1 μM) may have tended to enhanced differentiation acting as a $PPAR\gamma_2$ ligand. Contrary to our experiment in which we utilized a lipid supplement (5 μL/mL), previous work performed with Ob 1771 preadipocytes (Gaillard et al., 1989; Négrel et al., 1989; Gaillard et al., 1991) has utilized serum free media supplemented only with arachidonic acid as a source of fatty acid. We speculate that in the absence of a variety of fatty acids, $cPGI_2$ may induce adipogenesis more dramatically than when preadipocytes are exposed to a variety of lipids and fatty acids that serve as $PPAR\gamma_2$ ligands or $PPAR\gamma_2$ ligand

precursors. Although PGI_2 may play a role in stimulating bovine preadipocyte adipogenesis, lower secretion of PGI_2 does not explain the greater propensity of SC preadipocytes to differentiate, when compared with IM preadipocytes.

Experiment 4

Intramuscular preadipocytes were less adipogenic than SC preadipocytes while secreting higher levels of PGI_2. Furthermore, micromolar concentrations of $cPGI_2$ enhanced clonal SC preadipocyte adipogenesis. To examine the role of endogenous PGI_2 in clonal SC preadipocyte adipogenesis, we exposed the preadipocytes to IBU, a COX inibitor. We found that 10 μM IBU did not affect DEX enhancement of differentiation ($P = 0.99$) (Figure 3-6). In contrast, we found that 100 μM IBU enhanced ($P = 0.01$) DEX induction of preadipocyte differentiation, while an intermediate concentration (50 μM) tended ($P = 0.08$) to enhance DEX effects. However, exposing the cells to 100 μM IBU without DEX did not have an effect on differentiation when compared to control conditions ($P = 0.99$). In agreement with our study, Lehmann et al. (1997) found that, in the absence of DEX, 100 μM IBU failed to promote lipogeneis in C3H10T1/2 murine fibroblasts, even though cells were exposed to IBU for 9 d. High concentrations of IBU (≥ 100 μM) have been shown to activate $PPAR\gamma_2$, suggesting that IBU may stimulate adipogenesis through a COX independent mechanism.

Because IBU has been previously shown to inhibit COX activity at a concentration of 10 μM (Rome and Lands, 1975; Mitchell et al., 1993; Neupert et al., 1997), but this concentration did not affect adipogenesis in our study, the adipogenic role of endogenous PGI_2 seems unlikely. Previously, the role of endogenous PGI_2 has been questioned because the concentration of PGI_2 required to activate PPAR *in vitro* (Yu et al., 1995; Brun et al., 1996; Hertz et al., 1996; Gupta et al., 2000) and trigger Ob 1771 preadipocyte differentiation is in the micromolar range (Négrel et al., 1989; Catalioto et al., 1991; Gaillard et al., 1991; Aubert et al., 1996; Aubert et al., 2000), which is significantly higher than that measured in Ob 1771 mouse preadipocytes (Catalioto et al., 1991), primary cultures of rat preadipocytes (Shillabeer et al., 1998), and bovine endothelial cells (Rosenstock et al., 1997). Therefore, we hypothesized that other arachidonic acid metabolites could be adipogenic regulators responsible for differences between IM and SC preadipocytes. Indeed, arachidonic acid derivative products of lipoxygenase (**LOX**) enzymatic action, like 8[s]-HETE (Figure 3-2), are PPAR activators and inducers of 3T3-L1 preadipocyte adipogenesis (Yu et al., 1995). In addition, exposing mouse preadipocytes to nordihydroguaracetic acid (**NDGA**), a LOX inhibitor, reduces their adipogenesis (Shillabeer et al., 1998; Madsen et al., 2003). However, we found that adding NDGA (10 or 20 μM) to differentiation media had no effect ($P > 0.95$) on the differentiation of bovine clonal SC

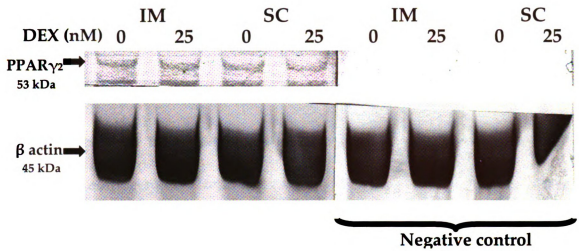

Figure 3-1. Western immunoblot of PPARγ2 from cultured bovine preadipocytes. Preadipocytes isolated from intramuscular (IM) and subcutaneous (SC) adipose tissue were grown to confluence and then exposed to 0 or 25 nM dexamethasone (DEX) for 48 h. Twelve days after addition of treatments, total protein was collected and 50 μg of protein per sample were separated by gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Membranes were cut above the 45 kDa band and the appropriate portions were incubated with an antibody raised against peroxisome proliferator-activated receptor γ2 (PPARγ2) or β-actin, which resulted in immunoreactive bands of ~53kDa (PPARγ2) and ~45kDa (β-actin). Membrane sections used as negative control were not incubated with the antibody raised against PPARγ2, but all other procedures were identical among all membrane sections. Positions of molecular weight standars are indicated to the left.

preadipocytes (Appendix D). Concentrations of 40 μM or higher were toxic to the preadipocytes.

In conclusion, IM preadipocytes are less adipogenic than SC preadipocytes even though they similarly expressed PPARγ2, an important regulator of adipogenesis. Intramuscular preadipocytes secreted higher levels of PGI_2 than SC preadipocytes, and supplementing SC preadipocytes with $cPGI_2$ and(or) IBU, an inhibitor of PGI_2 synthesis, did not have an effect on SC preadipocyte adipogenesis. Therefore, differences in PGI_2 secretion do not explain the greater adipogenic capacity of bovine SC preadipocytes. We propose that adipogenic differences between IM and SC preadipocytes are a result of differences in the secretion of a biologically relevant PPARγ2 ligand that is not PGI_2.

Implications

Adipogenic differences between intramuscular and subcutaneous preadipocytes are not explained by differences in the expression of peroxisome proliferator-activated receptor gamma two (PPARγ2), an important regulator of adipogenesis, or by differences in the secretion of a presumptive PPAR activator, prostacyclin. Consequently, adipogenic differences between intramuscular and subcutaneous preadipocytes may be a result of differences in the synthesis of a biologically relevant PPARγ2 ligand that is not prostacyclin.

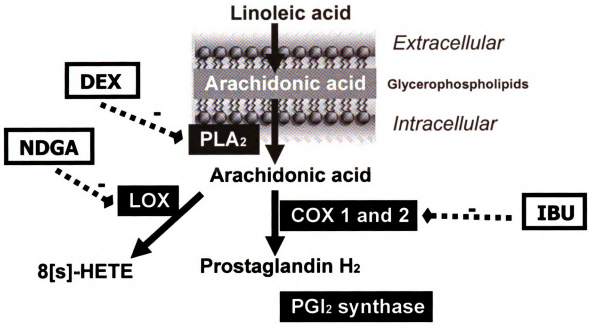

Figure 3-2. Simplified diagram of the arachidonic acid metabolism cascade. Arachidonic acid is derived from linoleic acid, an essential fatty acid which must be obtained from the diet. Arachidonic acid is normally found esterified to glycerophospholipids found in cellular membranes. Activation of phospholipase A_2 (PLA_2) results in the release of arachidonic acid and its subsequent metabolism by various enzymes like cyclooxygenase 1 and 2 (COX), lipoxygenase (LOX), and cytochrome P450 (not shown). Arachidonic acid metabolism through COX results in the production of prostaglandin H_2 which is the precursor of prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, TXA_2, and PGI_2). Arachidonic acid metabolism through LOX results in the production of hydroperoxyeicosatetraenoic (HPETE) acids which are precursors of 8-[s]-HETE and other leukotrienes and lipoxins. Dexamethasone (DEX) inhibits PLA_2 activity, ibuprofen (IBU) is an inhibitor of COX activity, and nordihydroguaracetic acid (NDGA) is a LOX inhibitor.

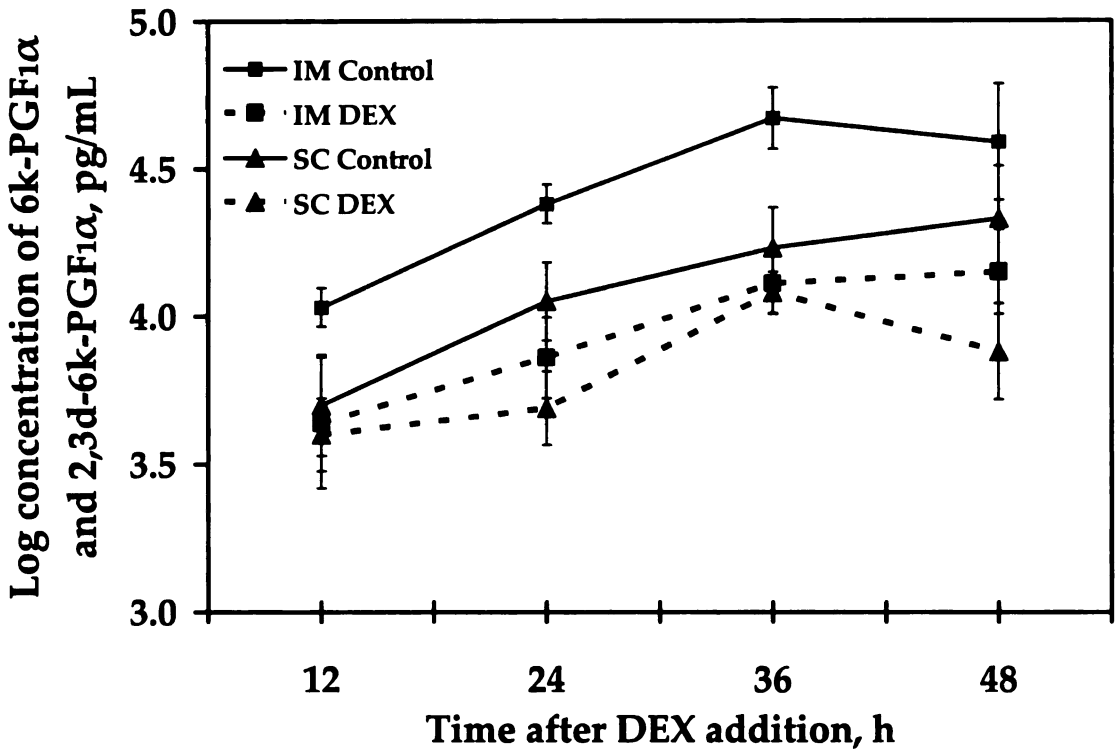

Figure 3-3. Effect of dexamethasone (DEX) on the combined secretion of the prostacyclin (PGI_2) derivatives 6k-$PGF_{1\alpha}$ and 2,3d-6k-$PGF_{1\alpha}$ from bovine heterogeneous and clonal preadipocytes isolated from intramuscular (IM) and subcutaneous (SC) adipose tissue. Preadipocytes were grown to confluence and exposed to 0 nM (control) or 25 nM DEX. Points represent PGI_2 derivative concentration as means ± SEM (Depot effect, $P = 0.046$; DEX effect, $P = 0.002$; Time effect, $P < 0.001$).

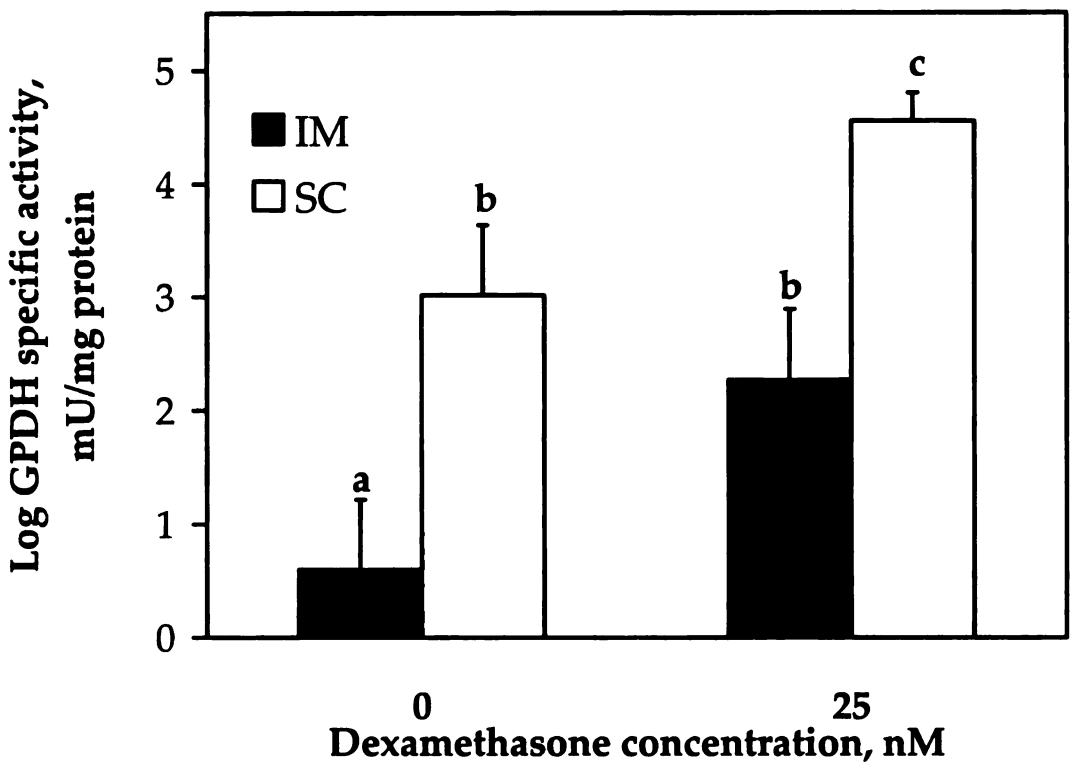

Figure 3-4. Effect of dexamethasone (DEX) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous and clonal preadipocytes isolated from bovine intramuscular (IM) and subcutaneous (SC) adipose tissue of two steers. Bovine preadipocytes were grown to confluence and exposed to DEX for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d addition of treatments. Means with different superscripts differ ($P < 0.05$).

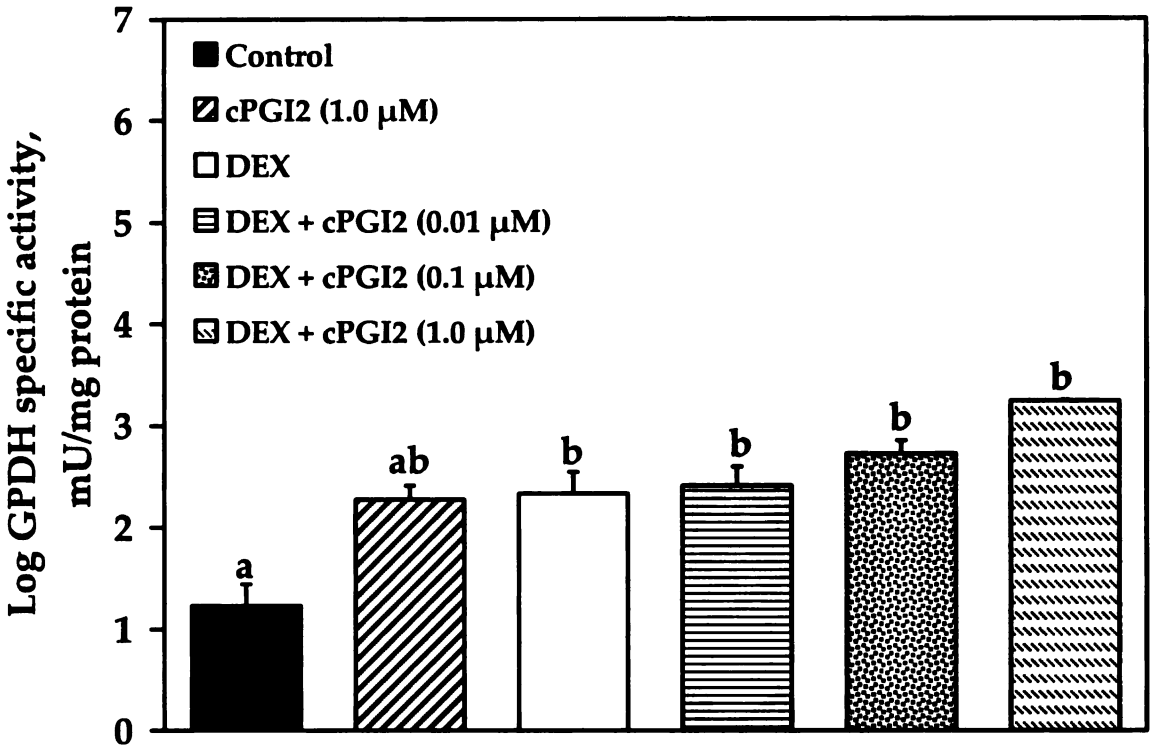

Figure 3-5. Effect of dexamethasone (DEX) and carbaprostacyclin ($cPGI_2$) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine clonal subcutaneous preadipocytes. Preadipocytes were grown to confluence and exposed to 25 nM DEX and denoted concentrations of $cPGI_2$ for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

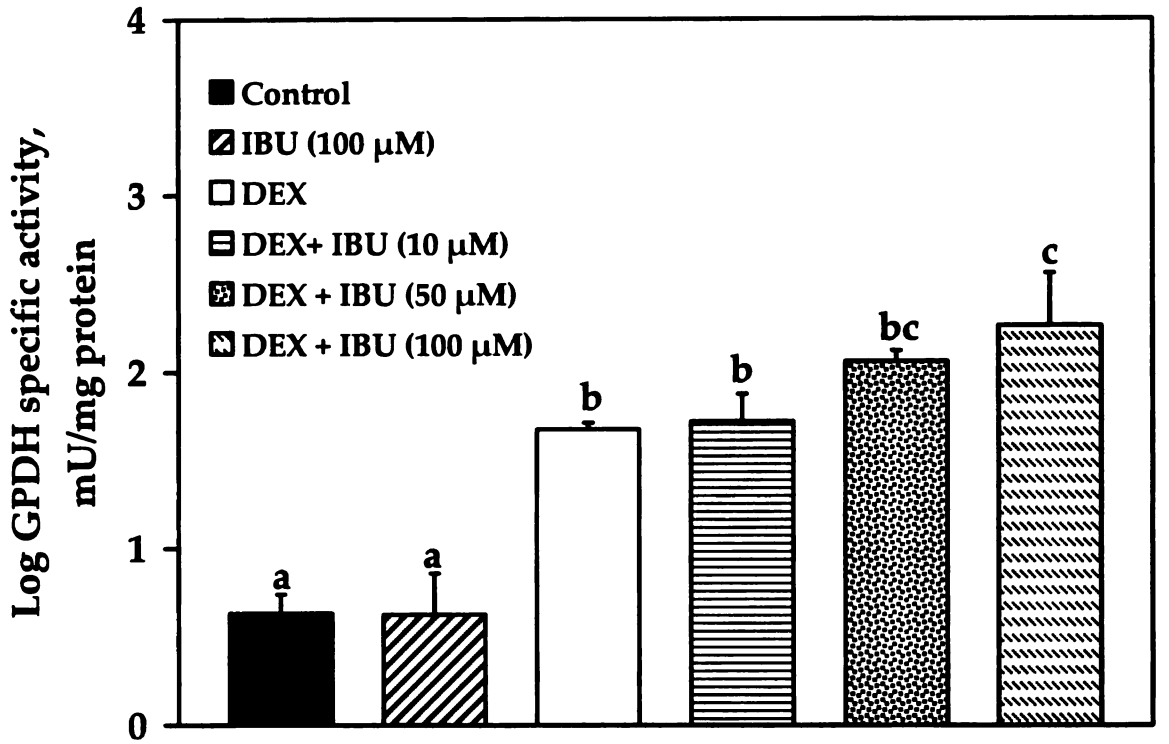

Figure 3-6. Effect of dexamethasone (DEX) and ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in clonal bovine subcutaneous preadipocytes. Preadipocytes were grown to confluence and exposed to 0 nM (control) or 25 nM DEX and the denoted concentrations of IBU for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

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CHAPTER IV: IBUPROFEN PREFERENTIALLY ENHANCES ADIPOGENESIS IN BOVINE INTRAMUSCULAR PREADIPOCYTES WHEN COMPARED TO SUBCUTANEOUS PREADIPOCYTES

Abstract

Our objectives were to determine if adipogenic differences between bovine intramuscular (IM) and subcutaneous (SC) preadipocytes could be reduced by an activator of peroxisome proliferator-activated receptor γ_2 (PPARγ_2). In Exp. 1, clonal SC preadipocytes were exposed to control media or 25 nM dexamethasone (DEX), 100 μM ibuprofen (IBU), 40 μM troglitazone (TGZ), or their combinations for 48 h. Ibuprofen did not enhance clonal SC preadipocyte GPDH activity ($P = 0.99$). Although TGZ enhanced GPDH activity over control ($P < 0.001$), concomitant exposure to IBU and TGZ diminished TGZ stimulation of differentiation ($P < 0.001$). Dexamethasone increased GPDH activity ($P < 0.001$), and this effect was enhanced by the concomitant exposure to IBU ($P = 0.01$). Conversely, DEX and TGZ did not have additive effects ($P = 0.17$). Subsequent experiments demonstrated an interaction between IBU treatment and depot ($P < 0.001$). In Exp. 2, bovine clonal and heterogeneous IM and SC preadipocytes were exposed to 0 or 25 nM DEX and IBU at 0, 250, 500, 1000, or 2000 μM for 48 h. Exposure to IBU did not enhance DEX stimulation of GPDH activity in SC preadipocytes ($P > 0.54$). Conversely, exposure to 1000 or 2000 μM IBU enhanced DEX stimulation of GPDH activity in IM preadipocytes

($P = 0.01$). In Exp. 3, heterogeneous IM and SC preadipocytes were exposed to 0 or 25 nM DEX and IBU at 0, 10, 100, 500, or 1000 μM for 12 d. Exposure to 100 μM and 500 μM IBU enhanced DEX stimulation of differentiation in IM preadipocytes, while only 100 μM IBU enhanced DEX induction of differentiation in SC ($P \leq 0.05$). In Exp. 4, heterogenous IM and SC preadipocytes were exposed to 0 or 25 nM DEX for 48 h. In addition, cells were exposed to IBU at 0, 10, 100, 500, or 1000 μM, or aspirin (ASP) at 0 or 500 μM for 12 d. Exposure to 10, 100, and 500 μM IBU enhanced GPDH activity in SC preadipocytes ($P < 0.001$), while 500 μM IBU enhanced GPDH activity in IM preadipocytes ($P < 0.004$). However, the maximum induction of GPDH activity by 500 μM IBU was much greater in IM than SC preadipocytes (12-fold vs. 1.7-fold over control, respectively). Contrary to the effect of IBU, 500 μM ASP did not affect GPDH activity either alone ($P > 0.37$), or combined with DEX ($P > 0.60$) in either cell population. Because IBU diminished adipogenic differences between IM and SC preadipocytes, we conclude that their adipogenic differences are partially related to differences in the endogenous activation of PPARγ_2.

Introduction

Bovine intramuscular (**IM**) and subcutaneous (**SC**) preadipocytes differ in adipogenic capacity (Chapter II and III) while equally expressing peroxisome

proliferator activated receptor γ2 (**PPARγ2**) (Chapter III), a key regulator of adipogenesis. Because PPARγ2 function depends on its activation by ligands (Bishop-Bailey and Wray, 2003), adipogenic differences between IM and SC preadipocytes may be related to differences in the synthesis of biologically relevant PPARγ2 ligands, other than prostacyclin (**PGI2**) (Chapter III). Supplementation with an exogenous PPARγ2 ligand, troglitazone (**TGZ**), equally stimulated differentiation of bovine IM and SC preadipocytes (Grant, 2005). Conversely, supplementation with the PPARγ2 ligand indomethacin preferentially induced adipogenesis in bovine omental (**OM**) preadipocytes when compared to SC preadipocytes, which resulted in the elimination of their adipogenic differences (Wu et al., 2000). Furthermore, although human SC and OM preadipocytes similarly express PPARγ2, rosiglitazone, another PPARγ2 ligand, exclusively stimulates adipogenesis in SC preadipocytes (Adams et al., 1997). Consequently, specific PPARγ2 ligands may selectively enhance adipogenesis of bovine IM preadipocytes, and thereby, selectively stimulate IM fat (marbling) development.

Ibuprofen (**IBU**), a cyclooxygenase (**COX**) inhibitor that also is a PPARγ2 ligand (Lehmann et al., 1997), induces adipogenesis in murine preadipocytes (Ye and Serrero, 1998). Ibuprofen has also been shown to marginally increase bovine SC preadipocyte differentiation (Chapter III). Because bovine IM

preadipocytes may have a limited adipogenic capacity as a result of limited synthesis of biologically relevant PPARγ_2 ligands, we hypothesized that IBU, a PPARγ_2 ligand, would preferentially enhance adipogenesis of bovine IM preadipocytes. The objectives of these experiments were to evaluate the effects of IBU on adipogenesis of IM and SC preadipocytes.

Materials and Methods

Isolation and cloning of bovine preadipocytes

Preadipocytes from IM, and SC adipose tissue were isolated using a modification of a protocol previously published (Forest et al., 1987) and described in Chapter II. Preadipocytes from IM and SC adipose tissue of one steer were cloned as described in Chapter III.

General Procedures

Cell culture. Preadipocytes from IM and SC adipose tissue were seeded at a density of 4,160 cells/cm^2 in 35 mm-diameter cell culture wells. Cells were allowed to proliferate to confluence (4 d) in a humidified atmosphere (37°C, 95% air and 5% CO_2) while incubated in growth medium (DMEM [5.5 mM glucose] supplemented with antibiotic-antimycotic (Final concentration: 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B), 0.05 mg/mL gentamicin, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate, 1,000 μM octanoate, and 10% fetal bovine serum [**FBS**]). Unless otherwise

stated, all reagents were of tissue culture grade and were purchased from Sigma (St. Louis, MO). Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and differentiation treatments were applied. Differentiation medium was DMEM (5.5 mM glucose), supplemented with antibiotic-antimycotic (Final concentration: 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and 0.25 μg/mL amphotericin B), 0.05 mg/mL gentamicyn, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate, 280 nM bovine insulin and 5 μL/mL bovine serum lipids (Ex-Cyte; Serologicals Corp., Norcross, GA). After 48 h of DEX exposure, plates were washed twice with PBS and differentiation medium was replaced with fresh media every 2 d for 10 d.

Glycerol-3-phosphate dehydrogenase activity. Cell differentiation was quantified biochemically by measuring glycerol-3-phosphate dehydrogenase (**GPDH**) enzyme activity using a modification of a method previously published (Adams et al., 1997) and described in Chapter II.

Experiment 1: Effects of ibuprofen and troglitazone on bovine preadipocyte adipogenesis

The objective of this experiment was to compare the adipogenic effects of IBU with the effects of troglitazone (**TGZ**), a well studied thiazolidinedione with potent adipogenic properties, partially mediated by PPARγ_2 activation (Takeda et al., 2001; Hauner, 2002; Gardner et al., 2005). Clonal bovine SC preadipocytes

(6th passage) were grown to confluence and exposed to unsupplemented differentiation medium (control), or differentiation medium supplemented with 25 nM DEX, 100 μM IBU, 40 μM TGZ, or their combinations for 48 h. Troglitazone has been previously shown to optimize bovine preadipocyte differentiation at a concentration of 40 μM (Grant, 2005). Troglitazone was solubilized (2.5 mg/mL) in ethanol, therefore treatments not containing TGZ were supplemented with equivalent concentrations of ethanol. After 48 h, treatment media were replaced with fresh differentiation medium, and fresh medium was subsequently provided every 2 d for 10 d. Each treatment was applied to two 35 mm-diameter wells of a 6-well plate, in two replicate plates.

Experiment 2: Effects of 48 h of ibuprofen exposure on the adipogenesis of bovine IM and SC preadipocytes

The objective of this experiment was to compare the effects of IBU exposure for 48 h on adipogenesis in bovine IM and SC preadipocytes. Clonal (6th passage) and heterogenous (2nd passage) IM and SC preadipocytes were grown to confluence and exposed to unsupplemented differentiation medium (control) or differentiation medium supplemented with 25 nM DEX and IBU at 0, 250, 500, 1000, or 2000 μM for 48 h. After 48 h, treatment media were replaced with fresh differentiation medium and fresh medium was subsequently provided, every 2 d for 10 d. In this and subsequent experiments each treatment

was applied to two 35 mm-diameter wells of a 6-well plate, in two replicate plates for each of two steers. Adipogenesis was quantified by measuring GPDH activity. Because there was no interaction between cell type (heterogeneous or clonal preadipocytes) and treatment (DEX and IBU) ($P = 0.62$), the results obtained from heterogeneous and clonal preadipocytes were pooled.

Experiment 3: Effects of exposure to ibuprofen for 12 d on the adipogenesis of bovine IM and SC preadipocytes

The objective of this experiment was to compare the effects of IBU exposure for 12 d on adipogenesis in bovine IM and SC heterogeneous preadipocytes (2nd passage). Intramuscular and SC preadipocytes were grown to confluence and exposed to unsupplemented differentiation medium (control) or differentiation medium supplemented with 25 nM DEX for the initial 48 h and IBU at 0, 10, 100, 500, or 1000 μM for 12 d. Fresh treatment media were provided every 2 d for the 12 d period.

Experiment 4: Effects of ibuprofen, aspirin, and indomethacin administration for 12 d in the adipogenesis of bovine IM and SC preadipocytes

The objective of this experiment was to compare the effects of IBU, aspirin (**ASP**), and indomethacin (**IND**) exposure for 12 d on bovine IM and SC heterogeneous preadipocytes (2nd passage) . Ibuprofen, ASP, and IND are well

established COX inhibitors (Neupert et al., 1997; Tegeder et al., 2001). While IBU and IND are PPARγ_2 activators, ASP is not (Lehmann et al., 1997). Alternatively, only IBU and ASP are inhibitors of neural factor kappa beta (**NF-κβ**) (Tegeder et al., 2001). Neural factor-κβ is involved in the stimulation of the expression of tumor-necrosis factor alpha (**TNF-α**), a negative regulator of adipogenesis (Ntambi and Kim, 2000). Heterogenous IM and SC preadipocytes were grown to confluence and exposed to unsupplemented differentiation medium (control) or differentiation medium supplemented with 25 nM DEX for the initial 48 h. In addition, preadipocytes were exposed for 12 d to IBU at 0, 10, 100, 500, or 1000 μM, or ASP at 0 or 500 μM, or IND at 0 or 500 μM. Fresh treatment media were provided every 2 d for the 12 d period.

Statistical analysis

Data were analyzed using the Mixed Model procedure of SAS (SAS, Cary, NC). In all experiments, pooled cells from two wells of a 6-well plate were considered the experimental unit. When main effects were significant ($P < 0.05$), differences between means were evaluated utilizing Tukey's multiple comparison test. In Exp. 1, data means were calculated using the fixed effects of DEX, IBU and TGZ. In Exp. 2 and 3, data means were calculated using the fixed effects of DEX, IBU, depot, and their interactions, with steer and steer × replication included as random variables. In Exp. 4, data means were calculated

using the fixed effects of DEX, IBU, ASP, depot, and their interactions, with steer and steer × replication included as random variables. To satisfy the conditions of normality and homogeneity of variance, GPDH data were $\log_e$ transformed in Exp. 1, and GPDH data were square root transformed in Exp. 2.

Results

The primary objective of these experiments was to determine if IBU preferentially enhanced adipogenesis in bovine intramuscular preadipocytes when compared to subcutaneous preadipocytes. Furthermore, we compared the adipogenic effects of IBU to those of ASP, another well established COX inhibitor which, contrary to IBU, does not activate PPARγ_2.

Experiment 1

Addition of 100 μM IBU to differentiation media for 48 h did not enhance clonal SC preadipocyte GPDH activity over control levels ($P = 0.99$; Figure 4-1). Conversely, TGZ enhanced GPDH activity over control ($P < 0.001$). However, concomitant exposure of IBU and TGZ diminished TGZ stimulation of differentiation ($P < 0.001$). As expected, DEX (25 nM) increased GPDH activity ($P < 0.001$), and this effect was enhanced by the concomitant exposure to IBU ($P = 0.01$). Conversely, DEX and TGZ did not have additive effects ($P = 0.17$). The combination of DEX, IBU and TGZ resulted in higher GPDH activity than

obtained with TGZ alone (P = 0.02), but similar to GPDH activity of cells treated with DEX and TGZ.

Experiment 2

Subcutaneous preadipocytes were more adipogenic than IM preadipocytes (P < 0.01), and DEX enhanced GPDH activity in both preadipocyte populations (P < 0.001; Figure 4-2). There was an interaction between IBU treatment and depot (P = 0.002). Exposure to IBU for 48 h did not enhance DEX stimulation of GPDH activity in SC preadipocytes (P > 0.54). Conversely, exposure to 1000 or 2000 μM IBU enhanced DEX stimulation of GPDH activity in IM preadipocytes (P = 0.01).

Experiment 3

Long term (12 d) exposure of bovine preadipocytes to IBU also resulted in a treatment by depot interaction (P < 0.001) (Figure 4-3). Exposure to 100 μM IBU enhanced (P = 0.03) DEX induction of differentiation in IM preadipocytes, whereas 500 μM IBU tended (P = 0.05) to enhanced DEX induction of differentiation in IM preadipocytes. In SC preadipocytes, 100 μM IBU enhanced DEX induction of differentiation (P < 0.001), but 500 μM IBU did not (P = 0.99). Increasing the concentration of IBU to 1000 μM reduced (P < 0.001) DEX stimulation of GPDH in SC, but not IM preadipocytes (P = 0.99).

Experiment 4: Effects of exposure to ibuprofen, aspirin, and indomethacin for 12 d on the adipogenesis of bovine IM and SC preadipocytes

In the absence of DEX, exposure of bovine preadipocytes to IBU for 12 d resulted in a treatment by depot interaction ($P < 0.001$) (Figure 4-4). Exposure to 10, 100, and 500 μM IBU enhanced GPDH activity in SC preadipocytes ($P < 0.001$), whereas 500 μM IBU enhanced GPDH activity ($P < 0.004$) in IM preadipocytes. However, at 1000 μM IBU the activity of GPDH was higher than control only in IM cells ($P = 0.003$). As expected, under control conditions SC preadipocytes had higher GPDH activity than IM preadipocytes ($P < 0.001$). However, the maximum induction of GPDH activity by IBU (500 μM) was much greater in IM than SC preadipocytes (12-fold vs. 1.7-fold over control, respectively) (Figure 4-4; Appendix E). Based on the observed morphological changes (vacuoles, protuberances, and frequent stress fibers) caused by 1000 μM IBU in IM and SC preadipocytes, we suggest that 1000 μM IBU was harmful to the cells, resulting in a lower GPDH activity than at 500 μM IBU (not shown).

Contrary to the effects of IBU, 500 μM ASP did not enhance GPDH activity either alone ($P > 0.37$) or in combination with DEX ($P > 0.60$) in either cell population (Figure 4-5). Indomethacin (500 μM) was toxic to the cells.

Discussion

We have demonstrated that IBU has the ability to preferentially stimulate adipogenic differentiation in bovine IM preadipocytes when compared with SC preadipocytes. Previously, human (Adams et al., 1997) and bovine (Wu et al., 2000) SC and OM preadipocytes have been shown to exhibit differential responses to supplementation with PPARγ_2 ligands other than IBU. Collectively, these data suggest that adipogenic differences between IM and SC preadipocytes may be, at least in part, related to differences in the ability of cells from these depots to synthesize unique PPARγ_2 ligand(s).

Although 1000 μM IBU for 48 h enhanced DEX-induced adipogenesis in IM preadipocytes, IBU failed to enhance SC preadipocyte adipogenesis. Because 1,000 μM IBU is nearly 500 times the IBU concentration required to inhibit COX (Tegeder et al., 2001), we attributed the minor enhancement of adipogenesis to the PPARγ_2 activating capabilities of IBU.

Previous studies have found that long term (9 d) exposure to IBU enhanced adipogenic differentiation in 1246 murine preadipocytes (Ye and Serrero, 1998) and C3H10T1/2 clone 8 murine fibroblasts (Lehmann et al., 1997). Therefore, we proceeded to evaluate if longer periods of exposure to IBU could amplify the differential effects of IBU, and further reduce or abolish adipogenic differences between IM and SC preadipocytes. Exposure to IBU for 12 d

resulted in subtle stimulation of DEX-induced adipogenesis in both IM and SC preadipocytes, although IM cells were responsive to a broader range of IBU concentrations. In the absence of DEX, IBU at 10 μM enhanced GPDH activity in SC preadipocytes, but not in IM preadipocytes. Furthermore, 100 and 500 μM IBU enhanced GPDH activity by approximately 1.7-fold in SC preadipocytes. Conversely, 500 μM IBU, a concentration at which IBU effectively activates PPARγ2 (Lehmann et al., 1997), enhanced GPDH activity 12-fold in IM preadipocytes. The minor enhancement of adipogenesis in SC preadipocytes by 10 to 500 μM IBU suggests that IBU action in these cells may be independent of PPARγ2 activation. In contrast, the dramatic increase in IM adipogenesis elicited by 500 μM IBU suggests that IBU may effectively activate PPARγ2, emulating a level of PPARγ2 activation more near that obtained by natural PPARγ2 ligand(s) in SC preadipocytes.

Ibuprofen is a well established inhibitor of COX activity (Rome and Lands, 1975; Mitchell et al., 1993), and may thereby enhance preadipocyte differentiation. Cyclooxygenase is the enzyme that catalyzes the rate-limiting reaction during the biosynthesis of prostanoids (Funk, 2001). Prostanoids have been associated positively (Négrel et al., 1989; Négrel, 1999) and negatively (Mater et al., 1998; Petersen et al., 2003; Yan et al., 2003; Xie et al., 2006) with adipogenesis. However, in this and previous studies (Chapter III) we have

found that bovine IM preadipocyte differentiation is not affected by IBU exposure at concentrations compatible with COX inhibition (≤ 10 μM IBU) (Rome and Lands, 1975), but below those required for PPARγ2 activation (Lehmann et al., 1997). Alternatively, IBU may enhance preadipocyte adipogenesis by indirectly inhibiting the expression of an anti-adipogenic protein, TNF-α. Tumor necrosis factor-α inhibits preadipocyte adipogenesis (Fruhbeck et al., 2001), by decreasing the expression of PPARγ2 (Gregoire et al., 1998) and lipoprotein lipase (Fried et al., 1998), and by inducing insulin resistance (Fruhbeck et al., 2001). Ibuprofen has been shown to inhibit NF-κβ stimulation of gene transcription (Tegeder et al., 2001), and TNF-α is one of the genes stimulated by NF-κβ (Clark and Lasa, 2003). Aspirin has also been reported to inhibit NF-κβ actions (Tegeder et al., 2001), but does not stimulate preadipocyte differentiation (Gaillard et al., 1989; Catalioto et al., 1991) or activate PPARγ2 (Lehmann et al., 1997). We found that 500 μM ASP did not affect GPDH activity in either preadipocyte population. These data suggests that IBU may induce bovine IM preadipocyte adipogenesis independently of COX inhibition and NF-κβ signaling, acting as a PPARγ2 ligand.

Previous research in our laboratory demonstrated that the potent PPARγ2 ligand TGZ, equally increased adipogenesis in bovine IM and SC preadipocytes (Grant, 2005). We initially compared the effects of IBU and TGZ on

adipogenesis of clonal SC preadipocytes. In contrast to TGZ, IBU incubation for 48 h did not enhance the GPDH activity of bovine SC clonal preadipocytes without concomitant exposure to DEX. Furthermore, IBU suppressed TGZ stimulation of GPDH activity when DEX was not included in the differentiation media. These observations indicate that IBU may stimulate bovine preadipocyte differentiation through a different mechanism than TGZ.

Although TGZ is a member of the thiazolidinedione family of antidiabetic drugs characterized as PPARγ_2 ligands (Kelly et al., 1999; Willi et al., 2002), recent evidence has revealed that TGZ also has non-genomic, PPARγ_2 independent mechanisms of action (Gardner et al., 2005) Specifically, TGZ has been found to activate phosphatidylinositol 3-kinase (**PI3K**), which subsequently activates various mitogen-activated protein kinases (**MAPK**) (Gardner et al., 2005). Of particular importance, PI3K activates extracellular signal-regulated kinase (**Erk**) that in turn can activate MAPK phosphatase-1 (**MKP-1**) (Takeda et al., 2001). As an inhibitor of various MAPK (i.e. c-Jun N-terminal kinase (**JNK**), p18) (Clark and Lasa, 2003), MKP-1 can trigger cell cycle withdrawal, an essential step for the progression of preadipocyte differentiation (Ailhaud, 2001). Inhibition of PI3K prevents differentiation of 1246 and 3T3-L1 preadipocytes (Xia and Serrero, 1999). The rapid, PPARγ_2 independent activation of PI3K by TGZ may be a crucial difference between IBU and TGZ

mechanisms of action. Interestingly, glucocorticoids have also been reported to activate MKP-1 (Clark and Lasa, 2003), offering a common pathway between DEX and TGZ that may partially explain the absence of an additive adipogenic effect between them under our conditions. However, TGZ also serves as a high affinity PPARγ_2 ligand (Hauner, 2002; Houseknecht et al., 2002), which may explain the greater stimulatory effect of TGZ when compared to DEX.

Ibuprofen could function as a conditional antagonist of TGZ. Indomethacin, a PPARγ_2 activator (Lehmann et al., 1997) that stimulates preadipocyte differentiation (Knight et al., 1987), can also act as a PPARγ_2 conditional antagonist in the presence of rosiglitazone, which is a PPARγ_2 ligand of higher activity (Bishop-Bailey and Warner, 2003). Because IBU has been described as a low activity PPARγ_2 ligand (Lehmann et al., 1997), in the absence of a high activity ligand, IBU could act as a PPARγ_2 activator, explaining its enhancement of preadipocyte differentiation observed in our study. However, in the presence of TGZ, a high activity PPARγ_2 ligand (Hauner, 2002; Houseknecht et al., 2002), IBU could act as a conditional antagonist, resulting in the observed suppression of TGZ enhancement of preadipocyte differentiation.

Various PPARγ_2 ligands have the ability to induce PPARγ_2 to recruit different coactivators (i.e. PPARγ co-activator 1α [**PGC1α**]), and these

coactivators determine PPARγ_2 target genes (Knouff and Auwerx, 2004). Thus, IBU may preferentially enhance adipogenesis of bovine IM preadipocytes by recruiting PPARγ_2 coactivators that may be differentially expressed in IM preadipocytes.

In conclusion, IBU preferentially increases adipogenesis in bovine IM preadipocytes compared to SC preadipocytes. It is suggested that adipogenic differences between IM and SC preadipocytes may be related to differences in the endogenous activation of PPARγ_2.

Implications

Bovine intramuscular and subcutaneous preadipocytes differ in their ability to accumulate lipid. Ibuprofen preferentially increased adipogenesis in intramuscular preadipocytes, under conditions that it likely functions as an activator of peroxisome proliferator-activated receptor γ_2 (PPARγ_2). Therefore, the observed differences in adipogenic capacity among intramuscular and subcutaneous preadipocytes may be related to differences in the endogenous activation of PPARγ_2. The preferential enhancement of adipogenesis in intramuscular preadipocytes by ibuprofen may present a mechanism to increase intramuscular lipid accretion in cattle with little or no increase in subcutaneous fat accretion.

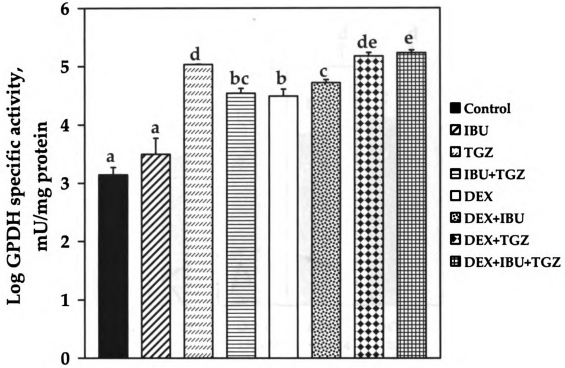

Figure 4-1. Effect of dexamethasone (DEX), ibuprofen (IBU), and troglitazone (TGZ) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine clonal subcutaneous preadipocytes. Preadipocytes were grown to confluence and exposed to differentiation medium (control) or differentiation medium supplemented with 25 nM DEX, 100 μM IBU, 40 μM TGZ, or their combinations for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

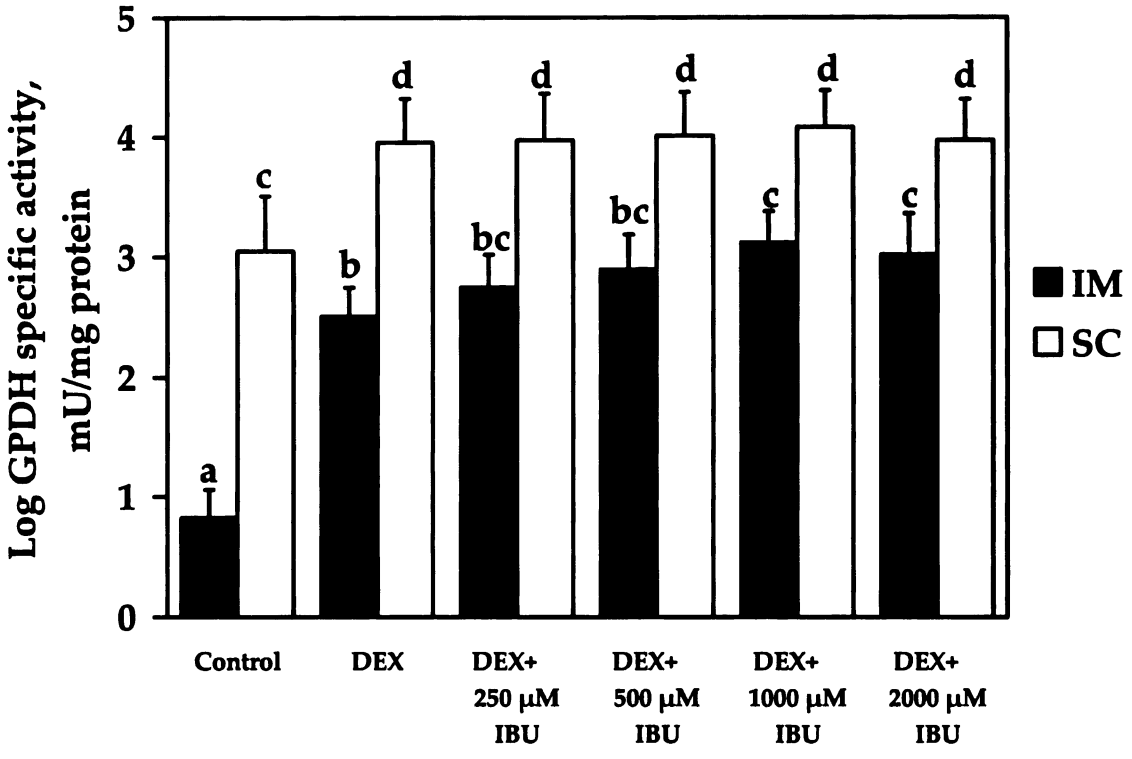

Figure 4-2. Effect of dexamethasone (DEX) and ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine clonal and heterogeneous preadipocytes isolated from intramuscular (IM) and subcutaneous (SC) adipose tissue. Preadipocytes were grown to confluence and exposed to differentiation medium (control) or differentiation medium supplemented with 25 nM DEX and IBU at 0, 250, 500, 1000, or 2000 μM for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

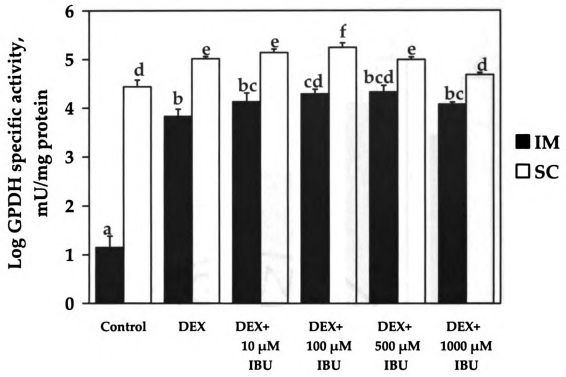

Figure 4-3. Effect of dexamethasone (DEX) and ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous intramuscular (IM) and subcutaneous (SC) preadipocytes. Preadipocytes were grown to confluence and exposed to differentiation medium (control) or differentiation medium supplemented with 25 nM DEX for 48 h, and IBU at 0, 10, 100, 500, or 1000 μM for 12d. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

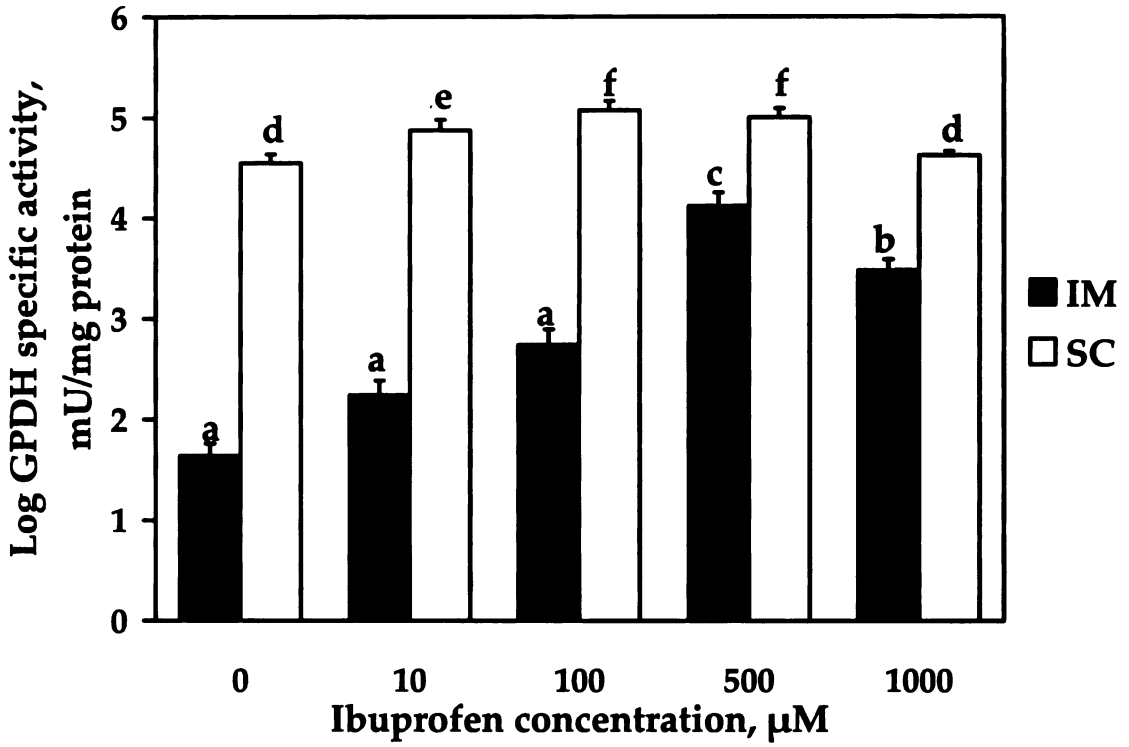

Figure 4-4. Effect of ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous intramuscular (IM) and subcutaneous (SC) preadipocytes. Preadipocytes were grown to confluence and exposed to differentiation media supplemented with IBU at 0 (control), 10, 100, 500, or 1000 μM for 12d. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

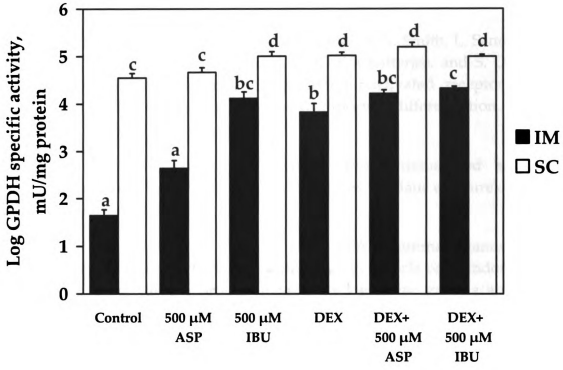

Figure 4-5. Effect of aspirin (ASP), ibuprofen (IBU), and dexamethasone (DEX) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous intramuscular (IM) and subcutaneous (SC) preadipocytes. Preadipocytes were grown to confluence and exposed to differentiation medium (control), or differentiation medium supplemented with 0 or 25 nM DEX for 48 h and ASP at 0 or 500 μM, or IBU at 0 or 500 μM for 12d. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

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CHAPTER V: INTERPRETIVE SUMMARY

Because intramuscular (**IM**) fat (marbling) is the main determinant of USDA Quality Grade within a carcass maturity classification, beef producers are motivated to feed cattle high energy diets for long periods to attain premiums for highly marbled carcasses. Unfortunately, as cattle are fed for long periods, excessive subcutaneous (**SC**) fat develops, and results in economic losses for the U.S. beef industry. Therefore, the beef industry would benefit from knowing how to selectively stimulate marbling development with reliability, or selectively reduce SC adipose tissue accretion. To selectively manipulate beef fat accretion, it is necessary to understand the intrinsic differences between IM and SC preadipocytes, and comprehend how these differences relate to adipogenesis. Accordingly, the objectives of the experiments contained in this dissertation were to determine if the lower adipogenic capacity of bovine IM preadipocytes when compared to bovine SC preadipocytes was related to differences in: 1) glucocorticoid receptor (**GR**) expression and response to glucocorticoids; 2) peroxisome proliferator-activated receptor (**PPARγ_2**) expression and prostacyclin (**PGI_2**) secretion, or alternatively; 3) if the adipogenic capacity of IM preadipocytes could be preferentially enhanced by ibuprofen (**IBU**), a PPARγ_2 activator.

Glucocorticoid induction of adipose tissue development has been shown to have anatomical specificity *in vivo*. Therefore, in the first set of experiments we aimed to determine if there were differences in GR expression among bovine IM, SC, and perirenal (**PR**) preadipocytes. Moreover, we also compared the effects of dexamethasone (**DEX**), a synthetic glucocorticoid, on adipogenesis among bovine IM, SC, and PR preadipocytes.

Preadipocytes from the three depots similarly expressed the ~97 kDa immunoreactive band consistent with the size of GR isoform α, presumably the GR isoform responsible for inducing glucocorticoid effects. Accordingly, DEX equally induced adipogenic differentiation in IM, SC, and PR preadipocytes, as determined by the stimulation of glycerol-3-phosphate dehydrogenase (**GPDH**) activity. The proportionally comparable induction of differentiation among IM, SC, and PR preadipocytes by DEX exposure is consistent with our observation that cultured bovine preadipocytes express similar quantities of GR. Although DEX increased the percentage of SC and PR preadipocytes with large lipid droplets (10 ≥ μm-diameter), we did not detect an effect of DEX in the percentage of IM preadipocytes with large lipid droplets. Independent of DEX exposure, the propensity for adipogenic differentiation was PR > SC > IM, which suggests that adipogenic differences among these cell populations are due to signaling pathways unrelated to GR.

Peroxisome proliferator-activated receptor γ_2 has been characterized as the master regulator of adipogenesis and accordingly, a pleiotropic regulator of the adipocyte phenotype. Therefore, we determined if there were differences in PPARγ_2 expression between bovine IM and SC preadipocytes. Moreover, we compared the secretion of PGI_2, a PPAR activator and potent adipogenic molecule, between bovine IM and SC preadipocytes.

Preadipocytes from IM and SC adipose tissue expressed PPARγ_2 equally, although they exhibited different adipogenic phenotypes. Because PPARγ_2 is a marker of mid differentiation, this may reflect an uncoupling between mid differentiation and the mature adipocyte phenotype. Perhaps, the mechanisms that initiate preadipocyte differentiation are different from those that regulate the extent of lipid accumulation. For instance, PPARγ_2 may have different roles in distinct phases of adipogenesis, and these different roles may be subject to dissimilar regulation in IM and SC preadipocytes. Alternatively, because PPARγ_2 activity is regulated by the availability of ligands, differences in the synthesis of PPARγ_2 ligands between IM and SC preadipocytes may help explain their adipogenic differences.

Fatty acids and metabolites of arachidonic acid (i.e. prostaglandins) have been characterized as PPARγ_2 activators. Of the metabolites of arachidonic acid detected in cultured preadipocytes, PGI_2 is the only has been characterized as a

potent adipogenic molecule and a PPAR activator. Therefore, we proceeded to test the hypothesis that adipogenic differences between IM and SC preadipocytes could be related to differences in the secretion of PGI_2. Unexpectedly, IM preadipocytes secreted more PGI_2 than SC preadipocytes. Therefore, we speculated that perhaps PGI_2 and(or) other prostaglandins may actually be a negative regulator of adipogenesis in our conditions. Nevertheless, a PGI_2 analog, carbaprostacyclin, tended to enhanced adipogenesis of clonal SC preadipocytes, as previously reported for the Ob 1771 mouse preadipocyte cell line. Moreover, IBU (10 μM), a well established inhibitor of prostaglandin synthesis, did not affect adipogenesis of clonal SC preadipocytes. Supplementation with $cPGI_2$ at hyperphysiological concentrations may be able to substitute for the endogenous $PPAR\gamma_2$ ligand, and consequently trigger preadipocyte differentiation. However, in bovine preadipocytes, endogenous PGI_2 secretion might have a role unrelated to adipogenesis.

While using IBU as an inhibitor of prostaglandin synthesis, it was revealed that high concentrations (100 μM) of IBU enhanced the DEX stimulation of adipogenesis in clonal SC preadipocytes. Review of current literature unveiled that 100 μM IBU can activate $PPAR\gamma_2$. Therefore, our objective for the third set of experiments was to compare the adipogenic effects of IBU between bovine IM and SC preadipocytes.

Firstly, we compared the adipogenic effects of IBU with those induced by troglitazone (**TGZ**), a potent PPARγ_2 activator, by incubating bovine clonal SC preadipocytes with these substances for 48 h. We found that although TGZ enhanced GPDH activity over control, in the absence of DEX, concomitant exposure of IBU and TGZ diminished TGZ stimulation of differentiation. This observation indicated that IBU, being a weak PPARγ_2 activator, may act as a conditional PPARγ_2 antagonist in the presence of TGZ. These further supported the hypothesis that IBU adipogenic induction is mediated through a PPARγ_2 mechanism. However, IBU and TGZ presented different modes of action. For instance, contrary to TGZ, exposure of clonal SC preadipocytes to IBU for 48 h did not enhance preadipocyte differentiation without concomitant exposure to DEX. Indeed, previous research has revealed that although TGZ has important PPARγ_2 mediated effects, it also has non-genomic actions that could explain the different adipogenic actions between TGZ and IBU.

Various PPARγ_2 ligands have the ability to induce PPARγ_2 to recruit different coactivators, and these coactivators determine PPARγ_2 target genes. We speculated that PPARγ_2 coactivators are differently expressed between IM and SC preadipocytes and that IBU could selectively induce IM preadipocyte differentiation. We found that IBU exposure for 48 h selectively enhanced DEX induction of IM preadipocyte differentiation. However, the effect was relatively

small, and SC preadipocytes were still considerably more adipogenic than IM cells. Conceivably, activation of PPARγ2 for only 48 h may not be enough time to greatly enhance adipogenesis in IM preadipocytes. We then hypothesized that continuous exposure (12 d) to IBU could be able to diminish the adipogenic differences between IM and SC preadipocytes by stimulating adipogenesis more effectively in IM preadipocytes.

Exposure to 100 μM IBU (12 d) enhanced DEX induction of differentiation in both IM and SC preadipocytes. Conversely, 500 μM IBU enhanced DEX induction of differentiation in IM, but not in SC preadipocytes. Furthermore, in the absence of DEX, exposure to 10 and 100 μM IBU (12 d) enhanced GPDH activity in SC, but not IM preadipocytes. Conversely, the maximum induction of GPDH activity by IBU (500 μM) was much greater in IM than SC preadipocytes (12-fold vs. 1.7-fold over control, respectively). It is important to note that IBU at 10 μM inhibits cyclooxygenase (**COX**) activity but does not activate PPARγ2. Furthermore, 100 μM IBU has been described as a weak PPARγ2 ligand. The adipogenic induction by 10 μM IBU and comparable but modest effects of 100 and 500 μM IBU suggest that IBU enhancement of SC preadipocyte adipogenesis might be independent of PPARγ2 activation. Conversely, in IM preadipocytes, 10 or 100 μM IBU did not enhance adipogenesis, but 500 μM IBU, a concentration at which IBU effectively activates

PPARγ2, enhanced GPDH activity 12-fold over control. These data suggest that adipogenic differences between IM and SC preadipocytes might be partially explained by differences in the synthesis of ligands for PPARγ2. Ibuprofen, acting as a PPARγ2 ligand, may partially compensate for this difference. Alternatively, both cell populations may secrete PPARγ2 ligands equivalently, but IM cells may express a different set of coactivators. It is conceivable, that IBU may be able to recruit the particular set of coactivators expressed by IM preadipocytes.

The possibility exists that IBU enhancement of preadipocyte differentiation could be partially independent of PPARγ2 activation. Indeed, IBU is a well established inhibitor of COX activity, and has been reported to inhibit the actions of neural factor kappa beta (**NF-κβ**). However, another well established COX inhibitor, aspirin (**ASP**; 500 μM), that has also been reported to inhibit the actions of (**NF-κβ**), did not enhance GPDH activity either alone or in combination with DEX in both cell populations. These results further support that IBU adipogenic stimulation is dependent upon PPARγ2 activation.

To better explain the observed differences in adipogenesis between IM and SC preadipocytes, it would be important to determine if IBU effects are unique. If similar results are obtained with other PPARγ2 activators like indomethacin or rosiglitazone, the hypothesis that the observed adipogenic

differences are related, at least in part, to the synthesis of the physiological PPARγ_2 ligand would be supported. However, if the selective enhancement of IM preadipocyte differentiation is a characteristic unique to IBU, it would suggest that IBU may be able to allow PPARγ_2 to recruit the coactivators available within IM preadipocytes.

Alternatively, IBU may downregulate potential PPARγ_2 corepressors expressed by IM preadipocytes, in addition to function as a PPARγ_2 ligand. The function of these corepressors may be to assure that IM preadipocytes abide by their proposed regulatory role and do not become large energy reservoirs. Therefore, experiments that compare the expression of PPARγ_2 coactivators and(or) corepressors between IM and SC preadipocytes would certainly help clarify the understanding of our observations. Moreover, studies that evaluate how PPARγ_2 interactions with other proteins change upon IBU exposure would be valuable.

While IBU mechanism(s) of selectively inducing IM preadipocyte differentiation still needs to be better delineated, I believe that pilot studies, whose aim is to evaluate IBU effects on marbling development, are worth pursuing. Ibuprofen has been used to treat bovine mastitis and to improve fertilization rate during artificial insemination. The actual dosage that may successfully enhance IM adipose tissue development needs to be determined

empirically, but concentrations used in the mentioned studies may offer a reasonable starting point. In addition, because IBU is a widely utilized drug for the treatment of a variety of human ailments, its usage by the beef industry certainly would be more acceptable to consumers than the use of TGZ and other PPAR ligands that have been associated with severe hepatotoxicity in diabetic patients.

We have demonstrated that bovine IM and SC preadipocytes have intrinsic adipogenic differences. We propose that these adipogenic differences are partially caused by differences in endogenous PPARγ_2 activation. Even though we cannot rule out an alternative mechanism(s) of action for IBU, we suggest that IBU compensates for a lower secretion of appropriate PPARγ_2 ligands in IM preadipocytes. Future studies should focus in determining if IBU effects are unique, or shared by other PPARγ_2 activators and on better deciphering IBU mechanism(s) of action. Because supplementation of cattle with IBU may offer a strategy to selectively increase marbling with reliability, *in vivo* studies to determine appropriate IBU dosage to influence marbling development, as well as practical modes of delivery, are also worth pursuing.

APPENDICES

APPENDIX A: CLONAL EFFICIENCY AND ADIPOGENIC CAPACITY OF CELLS ISOLATED FROM BOVINE INTRAMUSCULAR, SUBCUTANEOUS, AND PERIRENAL ADIPOSE TISSUE

Abstract

The objectives of this study were to evaluate clonal efficiency, proportion of adipogenic clones, and adipogenic capacity, among cells derived from intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue. Adipose tissues were isolated from an Angus steer (556 kg, 13.5 mo. old), and 1 g equivalent was seeded at 0.05 g / cm^2 in growth media. Four to seven days after seeding, cells were serially diluted and seeded in six, 96-well plates: 4 plates for control, one plate with 1 ng/mL of fibroblast growth factor (FGF) and one plate with 10 ng/mL FGF. Clonal efficiency was evaluated 8 to 13 d later. There was an interaction between depot and treatment ($P < 0.001$). Perirenal cells did not respond to FGF treatment ($P = 1.00$). In control or 1 ng/mL FGF, intramuscular (IM) and subcutaneous cells presented equivalent clonal efficiencies ($P = 1.00$). Exposure to 1 ng/mL FGF increased clonal efficiency exclusively in SC cells ($P = .003$), but 10 ng/mL FGF did not ($P > 0.74$). In IM cells, 10 ng/mL FGF decreased clonal efficiency ($P < 0.05$). Surviving clones from each depot (IM n = 48; SC n = 64; PR n = 11) were seeded at 10,000 cells/cm^2. At confluence the cells were exposed to growth media supplemented with 1% FBS, 10 ng/mL insulin, 20 mM glucose, and 10 mM acetate with or without 1 ng/mL

FGF. Cells were also exposed to 250 nM dexamethasone (DEX) for 48 h. Proportion of adipogenic clones was determined by oil red O staining. There was no FGF treatment effect ($P = 0.25$) or treatment by depot interaction ($P = 0.60$). There was no difference in the proportion of adipogenic clones between SC and PR cells (49%) ($P = 0.60$), but both were higher than the proportion of IM adipogenic clones (12.5%) ($P < 0.002$). Clonal adipogenic cells isolated from IM, SC, and PR adipose tissue were seeded at 4,600 cells/cm^2 and upon confluence, were exposed to 0, 25, or 2500 nM DEX for 48 h. After 12 d, glycerol-3-phosphate dehydrogenase (GPDH) enzymatic analysis was performed. There was an interaction between treatment and depot ($P < 0.001$). Independent of DEX treatment, IM cells were less adipogenic that SC or PR ($P < 0.003$). Although SC cells were more adipogenic than PR at 0 ($P < 0.001$) and 25 nM DEX ($P < 0.001$), when exposed to 2,500 nM DEX these differences disappeared ($P = 0.33$). Dexamethasone increased GPDH activity in a dose dependent manner in IM and PR cells ($P < 0.004$). In contrast, in SC cells, the activity of GPDH was increased by exposure to 25 nM DEX ($P = 0.04$), but 2,500 nM DEX did not enhance differentiation further ($P = 0.37$). Differences in clonal efficiency and adipogenic capacity among cells isolated from distinct bovine adipose tissues are evident in culture.

Introduction

Adipogenic differences exist between bovine preadipocytes isolated from distinct adipose depots (Chapter II, III, and IV; Ohyama et al., 1998; Wu et al., 2000). Because adipose tissue is comprised of many different cells (i.e. pericytes, fibroblasts, preadipocytes, etc.) (Ailhaud, 2001), it is not known if adipogenic differences are a result of different proportions of adipogenic cells in the different adipose depots. Knowing the proportion of non-adipogenic to adipogenic cells is needed to determine if there are differences in the adipogenic potential of preadipocytes from different adipose depots. By studying the differentiation of cell colonies derived from a single adipogenic cell (clones), comparisons of adipogenesis between cells isolated from different adipose depots would not have the confounding factor of different proportions of adipogenic cells being responsible of observed differences.

The growth of clonal cells also presents a challenge because the ability of cells to survive without the support of the surrounding heterogeneous cell population may be impaired. Consequently, evaluation of the ability of growth factors to improve survival of clonal cells is important when developing such a model. Fibroblast growth factor (**FGF**) has been utilized in the cloning of bovine preadipocytes (Aso et al., 1995), but in other studies, FGF has induced differentiation in ovine preadipocytes (Broad and Ham, 1983). Because

proliferation and differentiation are mutually exclusive, the usefulness of FGF in bovine preadipocyte cloning success is uncertain.

Therefore, the objectives of the study were to evaluate the clonal efficiency of cells derived from bovine intramuscular (**IM**), subcutaneous (**SC**), and perirenal (**PR**) adipose tissue and to study the influence of FGF in clonal efficiency. Furthermore, we aimed to determine if there were differences in the proportion of adipogenic clones, and(or) if there were differences in adipogenic enzyme activity among clones isolated from IM, SC, and PR adipose tissue, that were previously characterized as adipogenic.

Materials and Methods

Isolation and cloning of bovine preadipocytes

Preadipocytes from IM, SC, and PR adipose tissue were isolated using a modification of a protocol previously published (Forest et al., 1987) and described in Chapter II. Preadipocytes from IM, SC, and PR adipose tissue of one steer were cloned as described in Chapter III.

Determination of clonal efficiency

Four to seven days after the seeding, cells were trypsinized, counted, serially diluted to 5 cells per mL and seeded in 6 mm-diameter wells of 96-well plates at a proportion of 200 μL per well, and allowed to grow while incubated in a humidified atmosphere (37°C, 95% air and 5% CO_2). Therefore, the seeding

was expected to place one cell per well on average. Six, 96-well plates per depot were utilized: 4 plates with growth media (control), one plate with growth media supplemented with 1 ng/mL of FGF and one plate with growth media supplemented with 10 ng/mL FGF. Within each depot and treatment, the proportion of wells that had a single proliferative colony was considered the clonal efficiency and was evaluated 8 to 13 days after the initial seeding. Wells with more than one colony were not considered in the clonal efficiency analysis.

Determination of the proportion of adipogenic clones

Surviving clones from each depot (IM n = 48; SC n = 64; PR n = 11) were isolated and seeded at 10,000 cells/cm^2 in 35 mm-diameter cell culture wells. Cells were allowed to proliferate to confluence in growth media. Growth media was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and the preadipocytes were exposed to growth media (1% FBS) supplemented with 1.74 nM insulin, 20 mM glucose and 10 mM acetate with or without 1 ng/mL FGF. Preadipocytes were also exposed to 250 nM dexamethasone (**DEX**) for 48 h. After 12 d in culture, cell differentiation was morphologically assessed by determining the number of clones containing at least two cells stained with oil red O (**ORO**). The ORO solution was prepared using a protocol previously described (Ramírez-Zacarías et al., 1992). Cells were fixed by addition of 3.7% formaldehyde (Mallinckrodt Baker Inc., Phillipsburg,

NJ) in PBS for 4 min. After fixation, cells were washed twice with PBS and incubated at room temperature with ORO solution for 1 h. Residual ORO solution was aspirated and the cells were washed twice with distilled water (15 min incubation/wash). Cell nuclei were stained by adding 1 mL giemsa solution (1g Giemsa, 66 mL glycerol, 66 mL methanol) to each well for 1 h, after which the cells were washed twice in distilled water, and stored dry at 4°C. Cells were visualized within 8 h of staining.

Determination of differences in adipogenic capacity

Cell culture. Clonal preadipocytes isolated from IM, PR, and SC adipose tissue of a steer were proliferated in culture and in their 6th passage were seeded at a density of 4,600 cells/cm^2 in 35 mm-diameter cell culture wells, and allowed to proliferate to confluence (4 d) in growth medium. Growth medium was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and experimental differentiation treatments applied. Differentiation medium was DMEM (5.5 mM glucose), supplemented with 1% antibiotic-antimycotic, 0.1% gentamicyn, 33 μM biotin, 17 μM pantothenate, 200 μM ascorbate, 280 nM bovine insulin and 5 μL/mL bovine serum lipids (Ex-Cyte; Serologicals Corp., Norcross, GA). Preadipocytes were exposed to 0, 25, or 2500 nM DEX for 48 h. Each treatment was applied to two wells of a 6-well plate, in two replicates for each adipose depot. After 48 h, treatment media were

replaced with basic differentiation medium supplemented with 280 nM bovine insulin and 5 μL/mL bovine serum lipids and fresh medium was provided every 2 d for 12 d.

Glycerol-3-phosphate dehydrogenase activity. Cell differentiation was quantified biochemically by measuring glycerol-3-phosphate dehydrogenase (**GPDH**) enzyme activity using a modification of a method previously published (Adams et al., 1997) and described in Chapter II.

Statistical analysis

Data were analyzed using the Mixed Model procedure of SAS (SAS, Cary, NC). For the evaluation of morphological differentiation and clonal efficiency, a single well was the experimental unit, while for GPDH data, pooled cells from two wells of a six-well plate were considered the experimental unit. For the clonal efficiency data, means were calculated using the fixed effect of depot. For morphological differentiation data, means were calculated using the fixed effects of depot, FGF and FGF × depot. To satisfy the conditions of normality and homogeneity of variance, GPDH data were $\log_e$ transformed. For GPDH data, means were calculated using the fixed effects of DEX, depot, and DEX × depot with replication included as a random variable. When the main effects were significant ($P < 0.05$), mean differences were analyzed utilizing Tukey's multiple comparison test.

Results

Clonal efficiency

The obtained clonal efficiencies showed an interaction between adipose depot (IM, SC, and PR) and treatment (0, 1, or 10 ng/mL FGF ($P < 0.001$) (Figure A-1). Perirenal cells, showed the lowest clonal efficiency (13.8%) among the three depots ($P < 0.001$), and their clonal efficiency was not affected by FGF treatment ($P = 0.99$). Under control conditions or when exposed to 1 ng/mL FGF, IM and SC cells presented similar clonal efficiencies ($P = 0.99$). Compared to control, exposure to 1 ng/mL FGF increased clonal efficiency from 49.7 to 69.8%, exclusively in SC cells ($P = .003$), however, when exposed to 10 ng/mL FGF, SC clonal efficiency (59%) was not different from control or 1 ng/mL FGF ($P > 0.74$). Although exposure to 1 ng/mL FGF did not enhance IM clonal efficiency (56.8%) compared to control ($P = 0.18$), 10 ng/mL decreased clonal efficiency to 33.7% ($P < 0.05$).

Proportion of adipogenic clones

Adipose depot (IM, SC, PR) of origin affected the proportion of adipogenic clones ($P < 0.001$) (Figure A-2). However, there was no effect of treatment (0 or 1 ng/ml FGF) ($P = 0.25$) or a treatment by depot interaction ($P = 0.60$) in the proportion of adipogenic clones. Although there was no difference in the proportion of adipogenic clones between SC and PR cells (49%) ($P = 0.60$),

both showed a higher proportion of adipogenic clones that IM cells (12.5%) ($P < 0.002$).

Adipogenic capacity

There was an interaction between treatment (0, 25, and 2500 nM DEX) and depot (IM, SC, and PR) ($P < 0.001$) for adipogenic capacity of the clones (Figure A-3). Although SC clones were more adipogenic than PR at 0 or 25 nM DEX ($P < 0.001$), when exposed to 2,500 nM DEX these differences disappeared ($P = 0.33$) (Figure 3). In IM and PR clones GPDH activity increased in response to DEX in a dose-dependent manner ($P < 0.004$). Conversely, in SC clones GPDH activity was increased at 25 nM DEX ($P = 0.04$), but 2,500 nM DEX did not further enhance GPDH activity ($P = 0.37$). Independent of treatment, IM clones were less adipogenic than SC or PR clones ($P< 0.003$).

Discussion

Our results support the hypothesis that intrinsic differences exist between cells isolated from IM, SC, and PR adipose tissues. The study reveled that the ability of clones to survive in culture varies among cells isolated from these adipose depots, and resulted in a lower number of clones obtained from the PR depot. The usefulness of FGF to enhance clonal efficiency may only be granted in SC cells. Furthermore, it was determined that in cells isolated from the IM adipose tissue, a lower proportion of clones were able to accumulate lipid when

compared to clones isolated from SC and PR adipose depots. In addition, an IM adipogenic clone showed lower adipogenic enzyme activity than SC and PR adipogenic clones.

Our data shows that adipogenic differences exist between cells isolated from different bovine adipose tissues. It has been determined that hyperplasia in PR adipose tissue is completed earlier than in IM and SC adipose depots (Hood and Allen, 1973; Cianzio et al., 1985). As a result, clonal efficiency would be expected to be higher in late developing adipose tissues like SC and IM when compared to early developing adipose tissue, like PR, where the population of proliferative/progenitor cells may have been diminished in a nearly mature animal. Indeed, differences in clonal efficiencies have been previously reported. In the rat, clones from PR adipose tissue have higher clonal efficiencies (34%) than epididimal cells (29.3%), and in both tissues, clonal efficiencies decreased with age of the donor (Kirkland et al., 1990).

Alternatively, our results may reflect the differences in adipose tissue growth in different anatomical locations. In rats, it has been determined that the growth of internal adipose depots depend more on hypertrophy than hyperplasia, while surface adipose depots rely more on hyperplasia for their growth (DiGirolamo et al., 1998). In general terms, the clonal efficiency results may not only reflect differences in the stage of development of IM, SC, and PR

adipose tissue, but also intrinsic differences in the growth patterns of the different adipose depots.

Contrary to our observed differences in the proportion of adipogenic clones among cells isolated from different bovine adipose depots, in humans, independent of adipose depot (subcutaneous, mesenteric, and omental), more than 90% of the isolated clones accumulated lipid when compared after the clones were maintained in differentiation media for up to 60 days (Tchkonia et al., 2002). However, when clones were compared at 15 days in differentiation media, the proportion of adipogenic clones were 85% for SC, 75% for mesenteric, and 55% for omental (Tchkonia et al., 2002). Consequently, because we observed the cells for only 12 days, it could be speculated that our results may reflect that bovine preadipocytes from different depots differ in the time required for differentiation to proceed. This assertion is supported by our observations that PPARγ_2, a transcription factor expressed almost exclusively in adipose cells (Bishop-Bailey and Wray, 2003), is equally expressed among heterogeneous preadipocyte cultures from IM, SC, and PR adipose tissue (Chapter III).

Even though FGF has been previously used in cloning media for bovine IM preadipocytes (Aso et al., 1995), in retrospect, according to our results this treatment may not have been needed or may even have hampered the cloning

procedure. Although FGF has been described as a mitogen for preadipocytes (Yamasaki et al., 1999), other studies have found that FGF is adipogenic (Broad and Ham, 1983; Gabrielsson et al., 2002). Because different FGF concentrations may have different effects on cells, and 22 different FGF isoforms have been identified (Gabrielsson et al., 2002), the effects of FGF in clonal efficiency and adipogenesis are uncertain.

The lesser propensity of bovine IM adipose tissue to develop *in vivo* may result from a smaller proportion of adipogenic cells and(or) a lower adipogenic enzymatic activity of the cells contained within IM adipose tissue. Various studies have determined that IM adipose tissue has a lower capacity to synthesize lipids (Smith and Crouse, 1984; Smith et al., 1984; Miller et al., 1991). The capacity of SC adipose tissue to synthesize fatty acids from acetate (the main substrate for fatty acid synthesis in ruminants) is greater that in IM adipose tissue (Smith, 1995). In addition, when ruminants are exposed to high energy diets the activities of enzymes involved in adipose tissue metabolism (i.e. ATP citrate lyase and NADP malate dehydrogenase) are increased in SC adipose tissue but not in IM adipose tissue (Smith and Crouse, 1984). The resistance of IM adipose tissue to accumulate lipid may be a result of a lower proportion of adipogenic cells in this adipose depot, and(or) that these cells have an intrinsic constraint for lipid accumulation.

In conclusion, cells isolated from bovine IM, SC, and PR adipose tissue present distinct characteristics in terms of clonal efficiency, proportion of adipogenic clones, and adipogenic enzyme activity of adipogenic clones. The evidence presented suggests that intrinsic differences among cells isolated from different adipose depots, may be responsible for observed developmental differences of distinct adipose depots in the bovine.

Implications

The utilization of adipogenic cells derived from a single progenitor cell (clones) isolated form different adipose depots can help in discerning intrinsic differences between preadipocytes from distinct anatomical locations. During the cloning procedure it should be expected that the number of clones obtained from adipose depots would be smaller in adipose tissues that develop earlier in the bovine's life (i.e. perirenal), as the population of proliferative cells may have diminished with time, when compared to intramuscular and subcutaneous adipose tissue. In our culture conditions, the utilization of fibroblast growth factor to enhance clonal efficiency is not recommended. The study also suggests that the reduce capacity of IM adipose tissue to develop in the bovine compared to other depots may result from a lower proportion of adipogenic cells, combined with the possibility that intramuscular adipogenic cells have a lower adipogenic capacity than subcutaneous and perirenal cells.

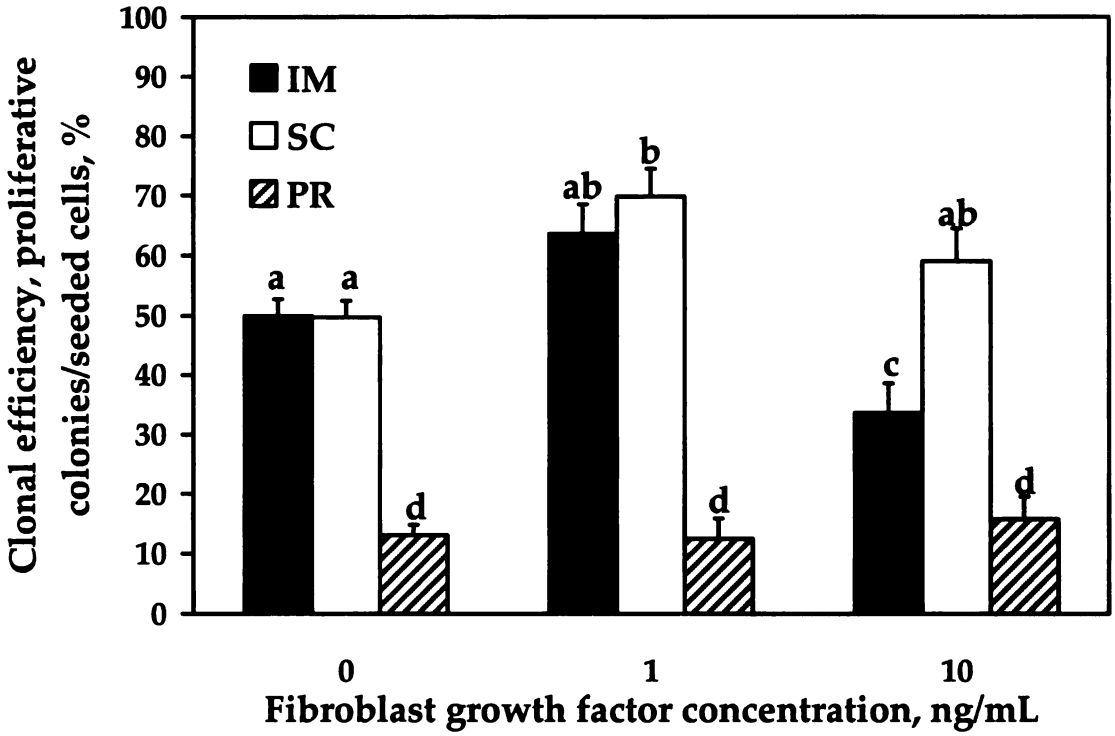

Figure A-1. Differences in clonal efficiency among cells isolated from different adipose depots. Cells isolated from bovine intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue of a steer were cloned by serial dilution. Cells were incubated with growth media supplemented with 0 (control), 1, or 10 ng/mL fibroblast growth factor (FGF). Clonal efficiency was determined as the proportion of proliferative colonies to the total number of cells seeded. Means with different superscripts differ ($P < 0.05$).

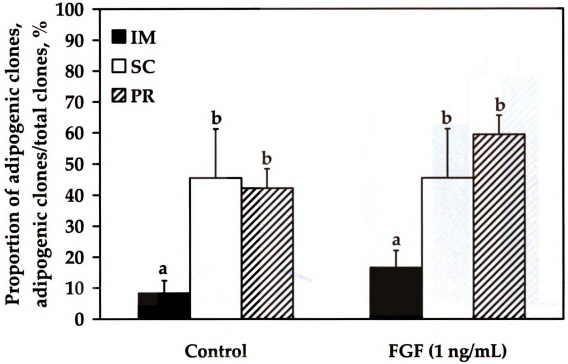

Figure A-2. Differences in the proportions of isolated clones that were adipogenic. Cells isolated from bovine intramuscular (IM; n = 12), subcutaneous (SC; n = 64), and perirenal (PR; n = 11) adipose tissue of a steer were cloned, grown to confluence, and subsequently exposed to differentiation media supplemented with 0 (control) or 1 ng/mL fibroblast growth factor (FGF) for 12 d. The percentage of clonal colonies containing at least two cells with lipid droplets were determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

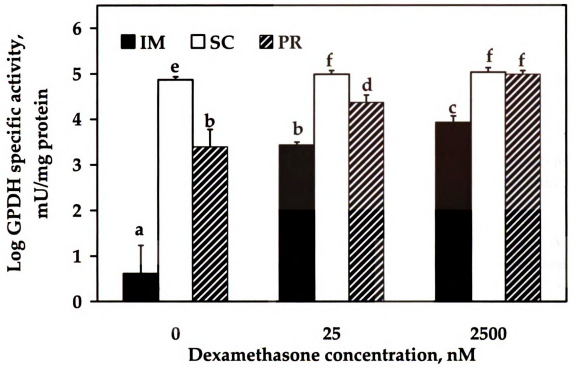

Figure A-3. Effect of dexamethasone (DEX) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in clonal adipogenic cells isolated from bovine intramuscular (IM), subcutaneous (SC), and perirenal (PR) adipose tissue of a steer. Cells were grown to confluence and subsequently exposed to DEX for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

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APPENDIX B: INFLUENCE OF DEXAMETHASONE ON THE GENE EXPRESSION OF BOVINE INTRAMUSCULAR PREADIPOCYTES

Abstract

The objective of this study was to evaluate the changes in gene expression induced by dexamethasone (DEX) (a synthetic glucocorticoid) in bovine intramuscular (IM) preadipocytes utilizing microarray technology. Intramuscular preadipocytes from a steer were proliferated in culture and at the 6^{th} passage were seeded at a density of 1,300 cells/cm^2 in six, 100 mm-diameter cell culture wells per treatment. Preadipocytes were propagated in culture and upon confluence were then exposed to 0 or 250 nM DEX for 48 h. After 48 h, total RNA (10 μg) from control and DEX treated bovine intramuscular preadipocytes were extracted and used as templates in reverse transcription reactions. After first-strand cDNA synthesis, cDNAs from control and DEX treated preadipocytes were differentially labeled using *N*-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes. The National Bovine Functional Genomics Consortium (NBFGC) microarray library was utilized in this study to interrogate 18,263 unique transcripts. Utilizing the "dye swap" experimental design, samples were exposed to hybridization for 18 h. After microarray data normalization, it was determined that compared to control values, 48 h DEX treatment of bovine IM preadipocytes caused the differential expression (P <

0.05) of 583 genes. Of the differentially expressed genes, 31% (184) were found to have a known function while 68% (399) of the genes' functions remain undefined. Dexamethasone treatment caused the differential expression of many genes known to be involved in cell cycle regulation, metabolism, transcription, immunity and apoptosis. Genes involved in cell cycle regulation, carbohydrate, and lipid metabolism accounted for 16% of the differentially expressed genes. Among the genes found to be differentially expressed were those coding for enzymes involved in fatty acid metabolism including acetyl-CoA carboxylase and steroyl-CoA desaturase, fatty acid binding protein, and the adipogenic protein angiotensinogen. It can be concluded that DEX had a positive effect on the adipogenic gene expression of bovine IM preadipocytes.

Introduction

Understanding the biological process underlying intramuscular (**IM**) adipose tissue accretion in bovines is critical to improve beef production efficiency and meat quality. In particular, the characterization of genes whose expression is altered during IM preadipocyte adipogenesis would help us identify potential targets to selectively increase IM adipose (marbling) development and(or) molecular predictors of marbling development.

Microarray technology offers the prospect to obtain a comprehensive appraisal of the transcriptional profile of bovine IM adipogenesis. Although

various studies have utilized microarray analysis to characterize the transcriptional profile of adipogenesis in human (Urs et al., 2004) and mouse (Soukas et al., 2001) preadipocytes, we are unaware of any study that has utilized microarray technology to characterize the transcriptional profile of differentiating bovine preadipocytes.

Even though glucocorticoids are commonly added to the differentiation cocktail for cultured preadipocytes (Sato et al., 1996; Ailhaud, 2001; Brandebourg and Hu, 2005), a comprehensive analysis of glucocorticoid induced changes in gene expression has not been reported for cultured bovine preadipocytes.

Therefore, we hypothesized that glucocorticoids would stimulate the expression of genes involved in the adipogenesis of these IM preadipocytes. The objective of the study was to utilize cDNA microarrays to discern the alterations in the transcriptional profile of bovine IM preadipocytes upon glucocorticoid exposure.

Materials and Methods

Isolation of bovine preadipocytes

Preadipocytes from IM adipose tissue were isolated using a modification of a protocol previously published (Forest et al., 1987) and described in Chapter II.

Cell culture

Intramuscular preadipocytes were proliferated in culture and at the sixth passage were seeded at a density of 1,300 cells/cm^2 in 100 mm-diameter cell culture plates. Six cell cultures plates were exposed to each treatment. Cells were allowed to proliferate to confluence (6 d) in growth media, while incubated in a humidified atmosphere (37°C, 95% air and 5% CO_2). Growth media was replaced every 2 d. After reaching confluence, plates were washed twice with PBS and the preadipocytes were exposed to modified growth medium (1% FBS) containing 0 or 250 nM dexamethasone (**DEX**) for 48 h.

RNA extraction

After 48 h, RNA extraction was performed using a modification of the Tri Reagent-RNA/DNA/protein isolation protocol (Molecular Research Center Inc, Cincinnati, OH). Briefly, confluent preadipocytes were washed 3 times with 37°C PBS, detach from the plate with trypsin (0.5 g/L trypsin and 0.02 g/L ethylenediaminetetraacetic acid (**EDTA**) in PBS [pH 7.2]), and cells from the six culture plates per treatment were collected and combined in 50 mL centrifuge tubes. The preadipocytes were then pelletized upon centrifugation for 5 min at 3000 × *g*. The supernatant was discarded and 2 mL Tri Reagent added to the pellets. The preadipocyte homogenate was mixed and stored at room temperature for 5 min after which it was supplemented with 200 μL

bromochloropropane and vortexed vigorously for 15 s. Homogenates were centrifuged (11,000 × *g*) for 15 min at 4°C, after which the supernatants were transferred to new vials, mixed with 1.0 mL isopropanol, and centrifuged, (11,000 × *g*) for 8 min at 4°C. Resulting RNA pellets were washed by addition of 2 mL 75% ethanol in diethyl pyrocarbonate treated distilled water, vortexed briefly, and centrifuged at 8,000 × *g* for 5 min at 4°C. After the pellet dry, it was dissolved in 50 μL of DEPC treated distilled water. To achieve complete solubilization, the samples were incubated in a water bath for 15 min at ~57°C. The RNA extracts were stored at –80°C until use. Concentration and purity of RNA was determined by analyzing a 1/100 dilution of each sample using a spectrophotometer (Model DU-650, Beckman, Schaumberg, IL). Upon gel electrophoresis the integrity of the RNA samples was evaluated by evaluation of the 18S and 28S rRNA bands after ethidium bromide staining. Quality and integrity of RNA was further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Preparation of labeled cDNA

Complimentary DNA synthesis was performed following a procedure previously published (Coussens et al., 2002). Briefly, total RNA (10 μg) from control and DEX treated bovine intramuscular preadipocytes were used as templates in reverse transcription reactions, utilizing SuperScript III (Invitrogen

Corp., Carlsbad, CA) and oligo (dT)15 as a primer. After first-strand synthesis, cDNAs from control and DEX treated preadipocytes were differentially labeled using *N*-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes (Amersham Pharmacia, Ltd., Piscataway, NJ). The "dye swap" design was used to compensate for potential dye bias. Labeled cDNAs were purified to remove unincorporated dyes using cDNA labeling purification modules (Invitrogen Corp.). Differentially labeled samples were then combined and concentrated to 10 μL by using Microcon 30 spin concentrators (Millipore Corp., Bedford, MA).

Microarray hybridization

Microarray hybridization was performed for 18 h after addition of 100 μL of SlideHyb-3 (Ambion Inc., Alameda, CA) to the concentrated Cy3–Cy5-labeled probe cDNAs. The procedure was performed in a commercial microarray hybridization station, utilizing a step-down hybridization protocol (GeneTAC; Genomics Solutions Inc., Ann Arbor, MI). The National Bovine Functional Genomics Consortium (**NBFGC**) microarray library was utilized in this study, and has been previously described in detail (Suchyta et al., 2003). In brief, the NBFGC microarray contains 18,263 unique transcripts, 96 *Bos taurus* ß-actin spots, 96 *Bos taurus* GAPDH spots, 120 lambda Q spots, 241 negative spots (3x SSC), and 384 blank spots. Accordingly, the number of total spots on the 20 x 20 patch configuration is 19,200 (400 spots in each patch). Information for each

NBFGC microarray clone can be found at the NBFGC web site (http://nbfgc.msu.edu). After hybridization, cDNA microarrays were washed, rinsed once in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) and once in double-distilled water. Lastly, microarrays were dried by centrifugation in a cushioned 50 mL conical centrifuge tube. Microarrays were then scanned using a GeneTAC LS IV microarray scanner. GeneTAC LS analyzer software (Genomic Solutions Inc.) was used to determine total spot intensities for both dyes.

Microarray data analysis

Two NBFGC microarrays were used to interrogate 18,263 genes against the RNA isolated from control and DEX treated preadipocytes using the "dye swap" experimental design to account for variation due to dye. Array-specific data normalization was then performed using the LOESS procedure of SAS (SAS, Cary, NC). For each microarray, accomplishment of data normalization was evaluated by plotting log intensity ratio M (log Cy3 – logCy5), versus the mean log intensities *A* ([logCy3 + log Cy5]/2). The normalized data was back-transformed before statistical analyses. To correct for background dye intensities, the median value of the negative spots was determined and subtracted from sample spot intensity values. The natural log of the resulting values was then calculated and submitted to a Student's T-test using Excel (Microsoft, Corp., Redmond, WA). Fold change ratios were calculated using the

average intensity value of treated vs. control for each gene. Resulting P-values were used to determine level of significance values for genes of interest. Genes having P-values less than 0.05 were considered significant and were evaluated for known function using the NBFGC gene library search.

Results and Discussion

When compared to control values, 48 h DEX treatment of bovine IM preadipocytes caused the differential expression ($P < 0.05$) of 583 genes from a total of 18,263 within the NBFGC library. Of the differentially expressed genes, 31% (184) were found to have a known function while 68% (399) of the genes' functions remain undefined.

Ontological clustering of differentially expressed genes with known functions uncovered several important findings. Although many of the genes with a known function proved difficult to categorize and were thus depicted as "other", DEX treatment did cause the differential expression of many genes known to be involved in cell cycle regulation, metabolism, transcription, immunity and apoptosis (Figure B-1). Of most interest within this study, genes involved in cell cycle regulation, carbohydrate, and lipid metabolism accounted for 16% of the differentially expressed genes (Table B-1). Selected genes from this list will be discussed in detail.

Within *in vitro* preadipocyte cell studies, DEX is often used to stimulate adipose differentiation, which is partially controlled through growth arrest and exit from the cell cycle (Gregoire et al., 1998; Soukas et al., 2001). Our study confirms these actions of DEX as genes such as Zinc finger protein ZPR1, Cdt1/DNA replication factor, CDK-activating kinase assembly factor MAT1, and MAPK 6, all of which have roles in stimulating or maintaining mitogenesis, were downregulated by DEX ($P < 0.03$). Because growth arrest is a crucial requirement for adipocyte differentiation (Gregoire et al., 1998), DEX seems to induce differentiation, at least partially, by making preadipocytes withdraw form the cell cycle.

During preadipocyte differentiation DEX has been shown to cause an increase in triacylglyceride synthesis in bovine IM preadipocytes (Sato et al., 1996) and in rodent preadipocyte cell lines (Gaillard et al., 1991). Also, glucocorticoid treatment has resulted in an increase in the expression of lipoprotein lipase, an enzyme involved in the uptake of plasma fatty acids, in human adipose tissue (Fried et al., 1993). In our study, genes critical to fatty acid synthesis and accumulation within adipocytes were upregulated by DEX treatment. Acetyl-CoA carboxylase 1, which catalyzes the rate-limiting reaction in the biogenesis of long-chain fatty acids, was upregulated by DEX ($P = 0.01$). An up-regulation of Acetyl-CoA carboxylase 1 would help explain the increase

in lipid droplet accumulation seen in bovine IM preadipocytes following exposure to DEX (Sato et al., 1996). In addition, acetyl-CoA carboxylase 1 is a key regulator of lipogenesis in the bovine, and interestingly, it has been reported that animals that have a greater tendency to fatten have higher activity of this enzyme in their adipose tissues (Allen et al., 1976). Another enzyme involved in fatty acid metabolism upregulated by DEX within our study was stearoyl-CoA desaturase ($P = 0.01$). This enzyme converts sterate, palmitate, and myristate into their respective unsaturated fatty acids (Sturdivant et al., 1992). The high concentration of oleate (unsaturated fatty acid of sterate) in bovine adipose tissue reflects the importance of stearoyl-CoA desaturase in ruminant lipid metabolism (Lin et al., 1992). It is interesting to note that cattle breeds with greater propensities to deposit IM fat (i.e. Waygu) exhibit greater stearoyl-CoA desaturase activity in their adipose tissues (Sturdivant et al., 1992; Wang et al., 2005). Additionally, a greater expression of stearoyl-CoA desaturase contributes positively to beef flavor. This is caused by a decrease in the melting point of IM fat as a result of a greater unsaturated/saturated fatty acid ratio (Nishimura et al., 1999). Because unsaturated fatty acids can serve as ligands for peroxisome proliferator activated receptor γ_2 (PPARγ_2), a crucial regulator of adipogenesis (Bishop-Bailey and Wray, 2003), it can be speculated that DEX may increase IM

fat development at least partially, by increasing the availability of PPARγ_2 ligands, as a result of steroyl-CoA desaturase induction.

The expression of fatty acid binding protein (**FABP**) was also increased by DEX (P = 0.04), similar to previous microarray studies in rodent preadipocyte cell lines (Soukas et al., 2001). Fatty acid binding protein constitutes 6% of total cytosolic protein in murine mature adipocytes and is involved in extracellular fatty acid uptake (Amri et al., 1991). Also, FABP is involved in intracellular fatty acid trafficking, targeting of fatty acids towards organelles involved in triacylglyceride synthesis and fatty acid oxidation (Frühbeck et al., 2001). In murine preadipocyte cell lines, DEX acts directly through the glucocorticoid receptor to induce FABP, causing an increase in FABP mRNA within hours of treatment (Amri et al., 1991). Furthermore, the emergence of FABP is closely related to induction of fatty acid and triacylglycerol synthesizing enzymes (Frühbeck et al., 2001). The up-regulation of this gene suggests that DEX is involved in the maturation of preadipocytes by stimulating the biochemical machinery necessary for intracellular lipid accretion. Interestingly, the FABP gene is more highly expressed in cattle breeds with a high propensity to accumulate IM adipose tissue (i.e. Waygu) (Wang et al., 2005).

Lipid accumulation within preadipocytes can result from fatty acid uptake or *de novo* synthesis. Within ruminants, it has been suggested that both

glucose and acetate may be utilized as fatty acid precursors (Smith and Crouse., 1984). Our study suggests that as preadipocytes differentiate after DEX induction, their capability for glucose uptake and resulting triacylglyceride synthesis may increase. It was discovered that glucose-transporter 3 (GLUT-3) was upregulated by DEX ($P = 0.01$), along with allograft inflammatory factor-1 ($P = 0.04$), a gene known to be associated with both glucose/insulin regulation and inhibition of cell proliferation. It therefore appears that DEX could have the capacity to enhance glucose uptake and use in bovine IM preadipocytes.

Angiotensinogen is another gene important to adipose development that was upregulated by DEX in our study ($P = 0.007$). Glucocorticoids have been previously implicated in an upregulation of angiotensinogen gene expression (Kim and Moustaid-Moussa, 2000). There appears to be a positive relationship between angiotensinogen mRNA level and rate of adipocyte growth (Kim and Moustaid-Moussa, 2000). Angiotensin II, a the physiologically active derivative of angiotensinogen cleavage, is implicated in adipose tissue development by stimulating the production and release of prostacyclin by mature adipocytes (Gregoire et al., 1998), which, in turn, may stimulate preadipocyte recruitment and differentiation (Martin et al., 1998). In addition, angiotensin II may regulate adipose tissue blood supply, and as a consequence substrate availability for

lipid accretion. Angiotensin II also promotes triacylglyceride storage and stimulates the secretion of leptin (Kim and Moustaid-Moussa, 2000).

It can be concluded that DEX treatment of IM bovine preadipocytes decreased the expression of genes involved in cell proliferation, consequently directing the cells toward terminal differentiation. In addition, important genes involved in lipid synthesis and uptake were up-regulated by DEX, as well as genes involved in carbohydrate metabolism. Dexamethasone also increased the expression of angiotensinogen, a key molecule involved in adipocyte development and function. Thus, DEX treatment resulted in a gene expression pattern consistent with the terminally differentiated phenotype in bovine IM preadipocytes. Future studies comparing DEX effects on gene expression of preadipocytes isolated from different bovine adipose depots would increase our understanding of the biological processes that make bovine adipose depots unique.

Summary

Dexamethasone exposure resulted in a comprehensive alteration in the gene expression profile of bovine preadipocytes isolated from intramuscular adipose tissue. Importantly, many genes involved in fatty acid uptake, metabolism, and modification were up-regulated by dexamethasone. Because the expression of an important adipogenic protein, angiotensinogen, was also

up-regulated by dexamethasone, an adipogenic role for this glucocorticoid is supported. Future studies comparing the gene expression profile of bovine preadipocytes isolated from distinct adipose depots would aid in elucidation of adipose depot differences in bovine preadipocyte adipogenesis.

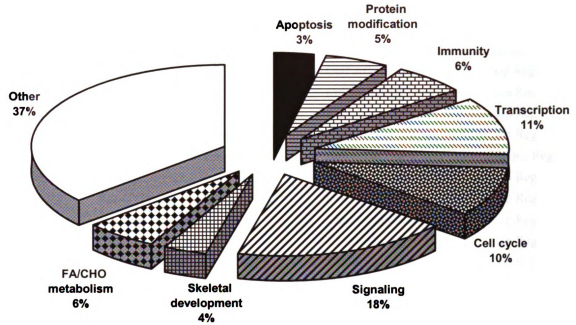

Figure B-1. Ontological clustering of genes with known function that upon microarray analysis were determined to be differentially expressed ($P < 0.05$) in bovine intramuscular preadipocytes upon dexamethasone exposure (250 nM, 48 h).

Table B-1. Selected genes related to preadipocyte adipogenesis that upon cDNA microarray analysis were determined to be differentially expressed ($P < 0.05$) in bovine intramuscular preadipocytes upon dexamethasone treatment.

Gene ID	Gene name[a]	Function	DEX effect
AW656833A	**Angiotensinogen**	Cell differentiation	Up Reg
BE482150	MAP kinase kinase 2	Cell signaling	Up Reg
BE665388	**Acetyl-CoA Carboxylase 1**	Fatty acid synthesis	Up Reg
BE665830	**Stearoyl-CoA desaturase**	Fatty acid synthesis	Up Reg
BF774305	Rab6 GTPase-activating protein	Cell cycle	Down Reg
BE757609	Arfaptin	Cell signaling	Up Reg
AW657014	Ras-GTPase-activating protein 1	Cell signaling	Up Reg
BE665566	Triacylglycerol lipase	Lipid catabolism	Up Reg
AW668928	**Glut-3**	Glucose transport	Up Reg
AW655845	Retinoic acid receptor alpha	Cell differentiation	Up Reg
AW308502	**Zinc finger protein ZPR1**	Cell proliferation	Down Reg
AW298838	**CDK-activating kinase assembly factor MAT1**	Cell proliferation	Down Reg
BE684394	P150 (regulatory protein of PI3K)	Cell signaling	Down Reg
BF706948	**MAPK 6**	Cell signaling	Down Reg
BF230835	ARP2/3 complex 16 kDa subunit	Cytoskeleton organization	Down Reg
BE237542	Rho-GTPase-activating protein 1	Cytoskeleton organization	Down Reg
BE483745	Collagen alpha 1(XIV) chain precursor	ECM reorganization	Down Reg
BF077525	Mouse Ten-m/Odz	Inhibitor cell proliferation	Up Reg
BE665570	**Cdt1/DNA replication factor**	DNA replication	Down Reg
BF073071	Tyrosine Phosphatase	Cell signaling	Down Reg
AW659446	Mitotic spindle assem. chkpt protein MAD2B	Cell cycle	Up Reg
AW657666	LDL receptor	Lipid metabolism	Down Reg
AW658776	IP_3 receptor type 1	Cell signaling	Up Reg
BE668078	Aldose reductase	Carbohydrate metabolism	Up Reg
AW652540	Hematopoietic PBX-interacting protein	Cell differentiation	Up Reg
AW652741	**Allograft inflammatory factor-1**	Inhibitor cell proliferation	Up Reg
BE808535	Phosphatidylserine-specific phospholipase A1	Cell signaling	Down Reg
BE749837	**Fatty acid binding protein**	Lipid metabolism	Up Reg

[a]Genes in bold are discussed in the text.

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APPENDIX C: IMMUNOCYTOCHEMICAL DETECTION OF GLUCOCORTICOID RECEPTOR IN CLONAL BOVINE PREADIPOCYTES ISOLATED FROM INTRAMUSCULAR, SUBCUTANEOUS, AND PERIRENAL ADIPOSE TISSUE

Objectives

To detect the expression of the glucocorticoid receptor on bovine clonal preadipocytes isolated from intramuscular (**IM**), subcutaneous (**SC**), and perirenal (**PR**) adipose tissue, and to evaluate the subcellular localization of the glucocorticoid receptor in these cells.

Materials and Methods

Isolation and cloning of bovine preadipocytes

Preadipocytes from IM, SC, and PR adipose tissue were isolated using a modification of a protocol previously published (Forest et al., 1987) and described in Chapter II. Preadipocytes from IM, SC, and PR adipose tissue of one steer were cloned as described in Chapter III.

Immunocytochemical detection of glucocorticoid receptor

Clonal preadipocyte cultures from IM, SC, and PR adipose tissue in the 6th passage were seeded on glass cover slips at approximately 13,000 cells/cm^2 and allowed to attach overnight while exposed to serum free media (**SFM**). Non-attached cells were then removed by three gentle washes with SFM. Attached cells were then incubated in SFM supplemented with 0 or 250 nM dexamethasone (**DEX**) for 2 h in a humidified atmosphere (37°C, 95% air and 5%

CO_2). Immunolabeling was performed utilizing a modification of a protocol previously described (Brönnegård et al., 1995). Briefly, after the incubation, cells were fixed with 1% formalin in PBS (30 min). Intrinsic fluorescence was quenched with 0.1 M glycine in PBS (5 min), and cells were permeabilized with 0.05% Triton X-100 in PBS (30 min). To minimize non-specific antibody binding, cells were then incubated with 5% sheep serum in PBS for 30 min. Incubation with 1 μg/mL of a polyclonal (rabbit) anti-glucocorticoid receptor (**GR**) antibody (PA1-511A, Affinity BioReagents, Inc., Golden, CO) or control rabbit IgG at a concentration of 1 μg/mL, was performed overnight at 4°C. Cells were then washed five times, for 5 min each, with PBS (1% sheep serum). The washes were followed with a 2 h incubation with a secondary antibody, fluorescein isothiocyanate (**FITC**) conjugated, affinity purified, goat anti-rabbit IgG. Five washes (5 min each) with PBS (1% sheep serum) followed, after which glass cover slips were mounted exposed to 4', 6-diamidino-2-phenylindole (**DAPI**), and mounted on glass microscope slides and sealed with nail polish. Fluorescence stained cells were then visualized and photographed with a camera (Leica Micosystems Digital Imaging, Cambridge, United Kingdom) attached to a fluorescence sensitive microscope (Leica Microskopie, Wetzlar, Germany).

Results

During immunocytochemistry analysis, GR was predominantly and consistently observed in the nuclei of both control preadipocytes, and those exposed to 250 nM DEX (Figure C-1, Panels A and C). Only faint cytoplasmic staining, above background, was visualized and this observation was consistent among cells isolated from the different adipose tissues. Cells incubated with irrelevant rabbit IgG, as a negative control, only presented a very faint, non specific labeling (Figure C-1, Panel E).

Discussion

It was found that GR immunostaining was predominant in the nuclei of preadipocytes isolated from the three depots independent of ligand. Previous reports have also shown that independent of ligand presence, GR immunolabeling results in intense nuclear staining surrounded by cytoplasmic staining (Brönnegård et al., 1990; Sackey et al., 1996; Oakley et al., 1997; Yudt and Cidlowski, 2001). Perhaps, removing control cells from exposure to FBS only 24 h before the immunocytochemical staining was not sufficient to eliminate the effects of glucocorticoids on GR localization. However, we have obtained similar nuclear staining of preadipocytes following 12 d exposure to serum-free media devoid of glucocorticoids. Alternatively, because according to imunoblot analysis (Chapter II) the used antibody in this study detected various

GR isoforms, GR immunostaining in the nucleus and cytoplasm of bovine preadipocytes may reflect distinct subcellular locations of the different isoforms (Yudt and Cidlowski, 2001, 2002).

Figure C-1. Glucocorticoid receptor (GR) immunostaining of bovine preadipocytes, after being exposed to 0 (A) or 250 nM dexamethasone (DEX) for 2 h (C). Incubation with an antibody against GR revealed positive staining relative to incubation with control rabbit IgG (E). Cells with DNA labeled with 4', 6-diamidino-2-phenylindole (DAPI) are shown in panels B, D, and F. Bar = 100 μM.

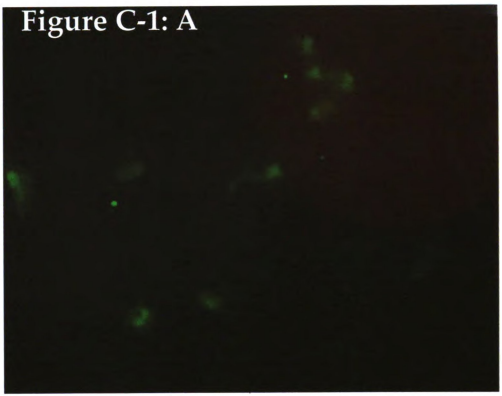
Figure C-1: A

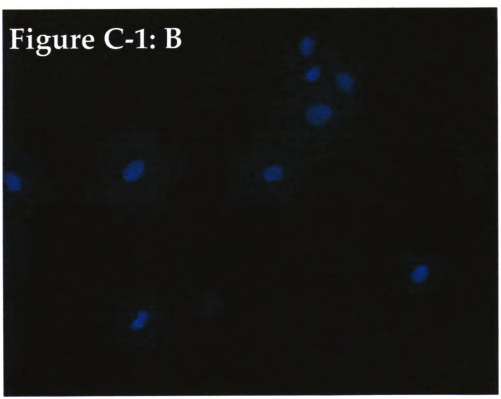
Figure C-1: B

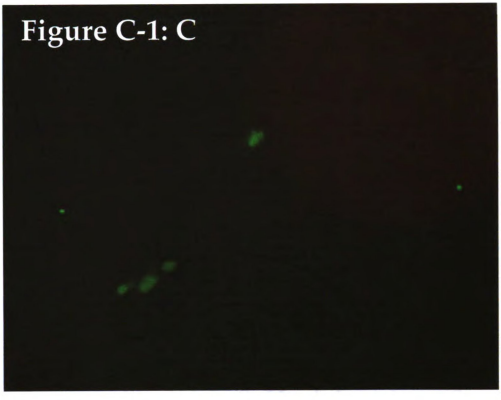
Figure C-1: C

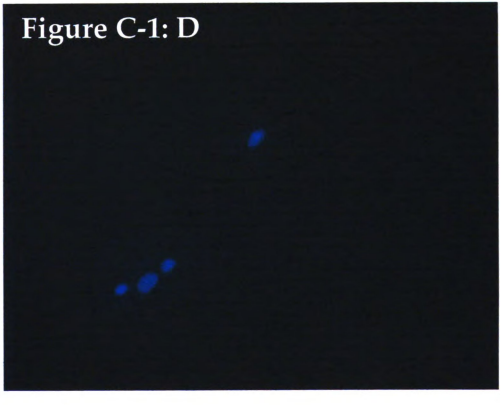
Figure C-1: D

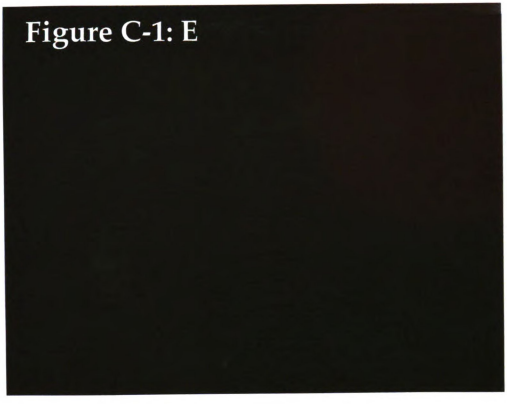
Figure C-1: E

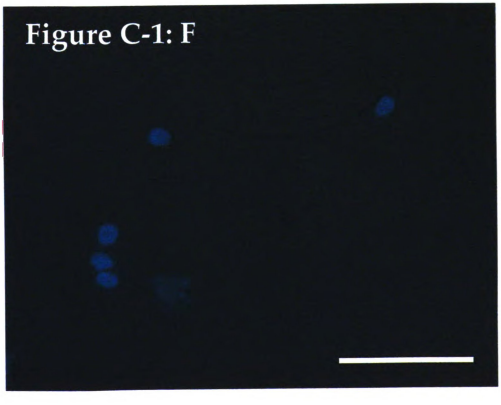
Figure C-1: F

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APPENDIX D: EFFECT OF NORDIHYDROGUAIARETIC ACID ON THE ACTIVITY OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE IN BOVINE SUBCUTANEOUS PREADIPOCYTES

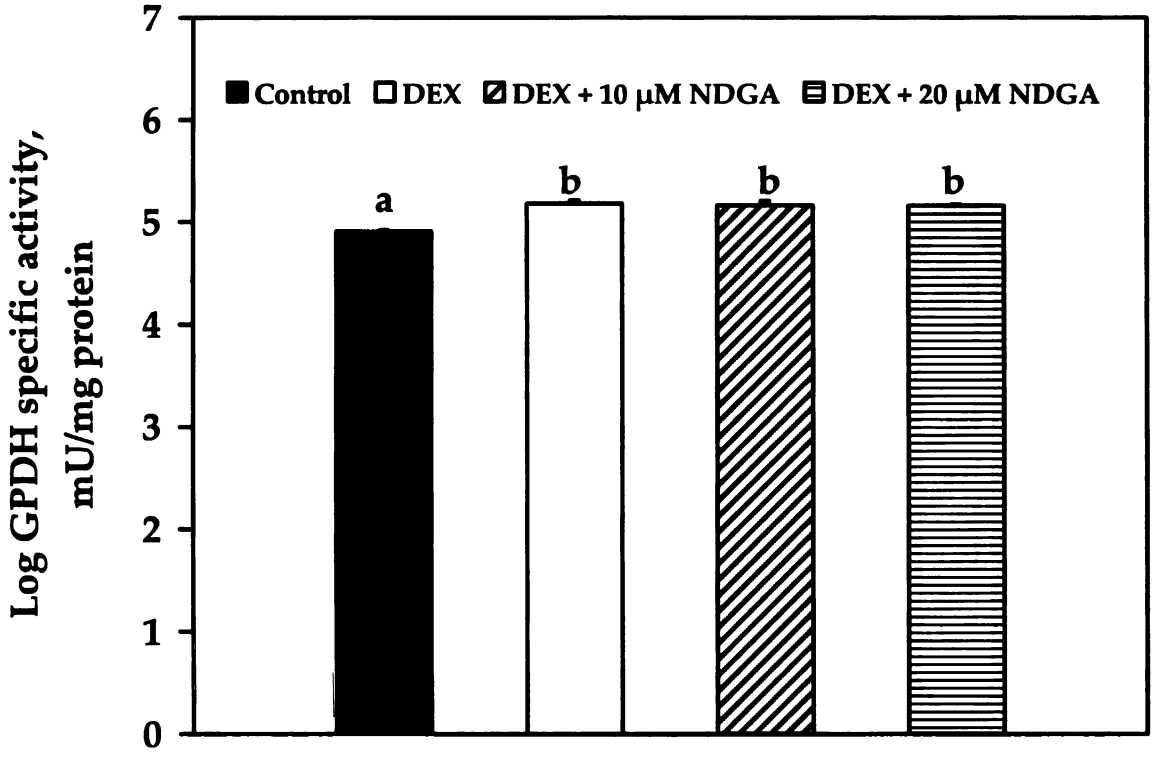

Figure D-1. Effect of 25 nM dexamethasone (DEX) and nordihydroguaiaretic acid (NDGA) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine clonal subcutaneous preadipocytes. Preadipocytes were grown to confluence and exposed to 0 nM (control) or 25 nM DEX and 0, 10 or 20 μM NDGA for 48 h. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

APPENDIX E: EFFECT OF IBUPROFEN ON THE ACTIVITY OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE IN BOVINE HETEROGENEOUS INTRAMUSCULAR AND SUBCUTANEOUS PREADIPOCYTES

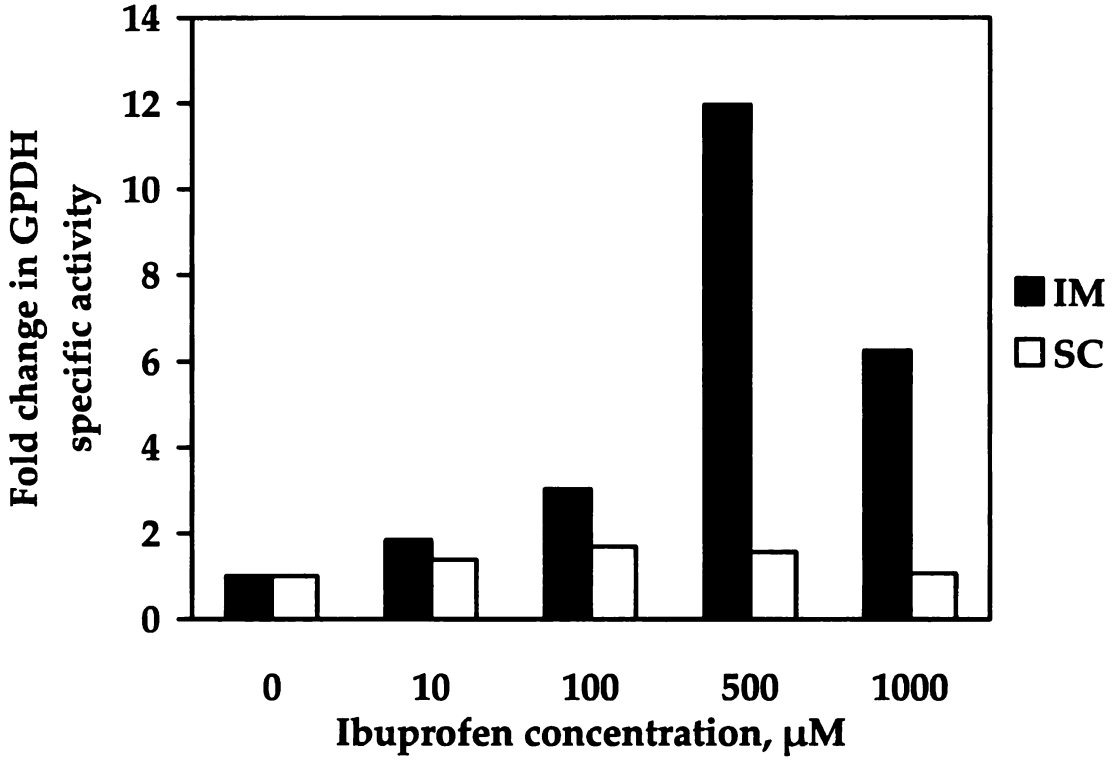

Figure E-1. Effect of ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous intramuscular (IM) and subcutaneous (SC) preadipocytes (fold change compared to control). Preadipocytes were grown to confluence and exposed to differentiation media supplemented with IBU at 0 (control), 10, 100, 500, or 1000 μM for 12 d. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

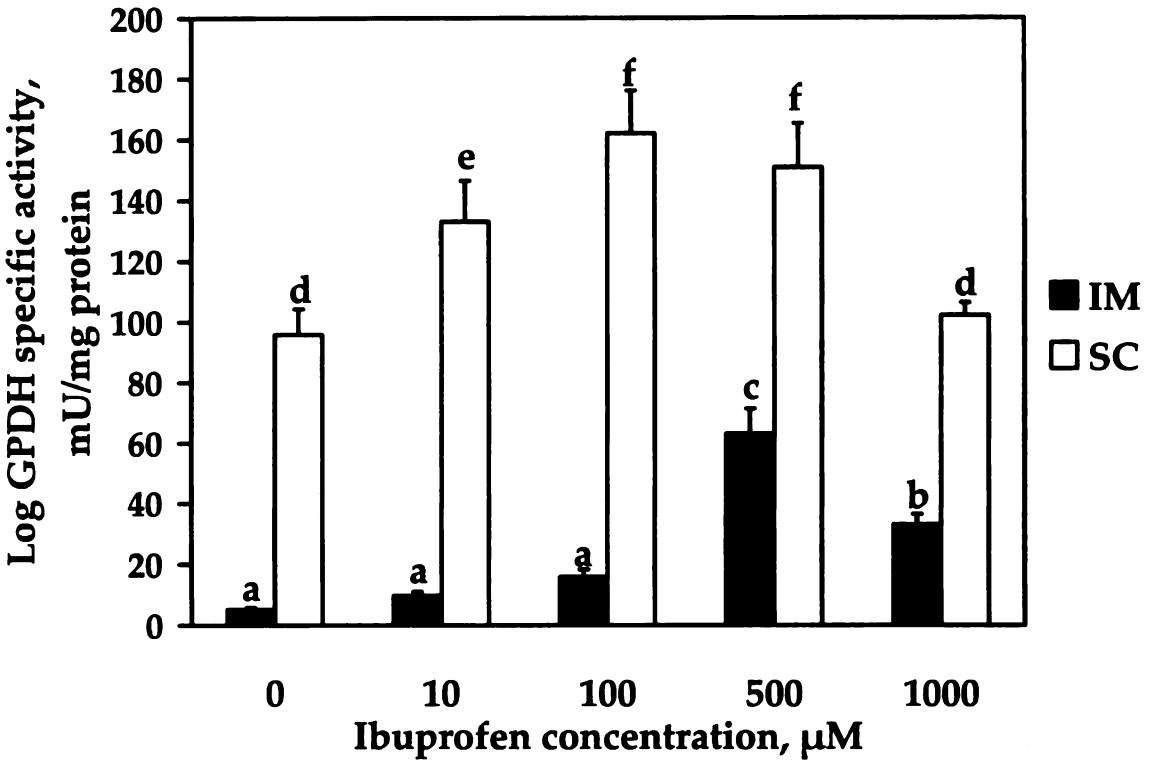

Figure E-2. Effect of ibuprofen (IBU) on the activity of glycerol-3-phosphate dehydrogenase (GPDH) in bovine heterogeneous intramuscular (IM) and subcutaneous (SC) preadipocytes. Preadipocytes were grown to confluence and exposed to differentiation media supplemented with IBU at 0 (control), 10, 100, 500 or 1000 μM for 12 d. Glycerol-3-phosphate dehydrogenase activity was determined 12 d after addition of treatments. Means with different superscripts differ ($P < 0.05$).

APPENDIX F: CELLS UTILIZED WITHIN THIS DISSERTATION

Chapter II: Bovine intramuscular, subcutaneous, and perirenal preadipocytes express similar glucocorticoid receptor isoforms, but exhibit different adipogenic capacity

GR Immunoblot analysis: Heterogeneous preadipocytes from steers 3 and 4 (4^{th} passage)

GPDH analysis: Heterogeneous preadipocytes from steers 3 and 4, and 5 (2^{nd} passage)

ORO analysis: Heterogeneous preadipocytes from steers 3 and 4, and 5 (2^{nd} passage)

Chapter III: Differences in adipogenesis between bovine intramuscular and subcutaneous preadipocytes are not related to expression of PPARγ_2 or secretion of PGI_2

PPARγ_2 Immunoblot analysis: Heterogeneous preadipocytes from steers 3 and 4, and 5 (2^{nd} passage)

PGI_2 Enzyme immuno assay and GPDH activity: Heterogeneous preadipocytes from steer 5 (2nd passage), and clones IM_3-C_{32}-P_6 and SC_3-C_{31}-P_6 (6th passage, steer 3)

Evaluation of the effects of carbaprostacyclin on preadipocyte differentiation: GPDH analysis: Clone SC_3-C_{31}-P_6 (6th passage, steer 3)

Evaluation of the effects of ibuprofen on preadipocyte differentiation: GPDH analysis: Clone SC_3-C_{31}-P_6 (6th passage, steer 3)

Chapter IV: Ibuprofen preferentially enhances adipogenesis in bovine intramuscular preadipocytes when compared to subcutaneous preadipocytes.

Effects of ibuprofen and troglitazone on bovine preadipocytes adipogenesis: GPDH analysis: Clone SC_3-C_{31}-P_6 (6th passage, steer 3)

Effects of 48 h of ibuprofen exposure on the adipogenesis of bovine IM and SC preadipocytes. Heterogeneous preadipocytes from steers 3 and 5 (2nd passage), and clones IM_3-C_{32}-P_6 and SC_3-C_{31}-P_6 (6th passage, steer 3)

Effects of exposure to ibuprofen for 12 d on the adipogenesis of bovine IM and SC preadipocytes. Heterogeneous preadipocytes from steers 3 and 5 (2nd passage)

Effects of ibuprofen, aspirin, and indomethacin administration for 12 d in the adipogenesis of bovine IM and SC preadipocytes. Heterogeneous preadipocytes from steers 3 and 5 (2^{nd} passage)

Appendix A: Clonal efficiency and adipogenic capacity of cells isolated from bovine intramuscular, subcutaneous, and perirenal adipose tissue

Clonal efficiency: Primary stromal-vascular cells from steer 3

Determination of proportion of adipogenic clones: Surviving clones from steer 3 (4^{th} passage)

Determination of differences in adipogenic capacity: Clones IM_3-C_{32}-P_6, SC_3-C_{31}-P_6, and PR_3-C_2-P_6 (6^{th} passage, steer 3)

Appendix B: Influence of dexamethasone on the gene expression of bovine intramuscular preadipocytes

Microarray analysis: Heterogeneous IM preadipocytes (6^{th} passage, steer 3)

Appendix C: Immunocytochemical detection of glucocorticoid receptor in clonal bovine preadipocytes isolated from intramuscular, subcutaneous, and perirenal adipose tissue

Immunocytochemical analysis: Clones IM_3-C_{32}-P_6, SC_3-C_{31}-P_6, and PR_3-C_2-P_6 (6^{th} passage, steer 3)

APPENDIX D: Effect of nordihydroguaiaretic acid on the activity of glycerol-3-phosphate dehydrogenase in bovine subcutaneous preadipocytes

GPDH analysis: Clone SC_3-C_{31}-P_6 (6^{th} passage, steer 3)