11 β- HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN FELINE, EQUINE, AND OSSABAW SWINE ADIPOSE TISSUE

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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ABSTRACT

Enzymatic 11β- hydroxysteroid dehydrogenase type 1 (11β-HSD1) amplification of glucocorticoid concentrations in adipose tissue has been associated with obesity, diabetes, hypertension, dyslipidemia, and cardiovascular disease in humans and mice. Furthermore, it has been reported that mice that over-express 11βHSD1 activity in adipose tissue exhibit characteristics of visceral obesity and "metabolic syndrome". Analogous to the growing problem of obesity and related problems in humans, the incidence of obesity and related problems is also present in many animal species. Three different species were tested in order to determine the existence of the enzyme 11βHSD1 in adipose tissue across species and to quantitate the activity of 11βHSD1 within species across several adipose depots.

The first species tested was the feline. Six cats were each sampled from five different adipose depots: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP). Immuno-histochemistry was used to quantitate regional morphological differences and confirm the presence of 11βHSD1 in those tissues. There were significant differences ($P < 0.05$) in adipocyte diameter between adipose depots: SQI presented greater adipocyte diameter than FAL and OM, whereas RP presented greater adipocyte diameter than FAL, OM, and SQM. Additionally, cat 5 presented greater adipocyte diameters than cat 4 ($P < 0.05$).

Radiometric assay procedures were used to quantitate the amount of 11β-HSD1 activity. The adipose depot SQM presented higher 11β-HSD1 activity ($P < 0.05$) compared to SQI, RP, and OM. Cat 5 presented higher total 11β-HSD1 activity ($P <$

0.05) compared to cat 2 and cat 3. Adipocyte volume did not correlate to enzymatic activity.

The second species to be studied was the equine. Adipose tissue was collected from abdominal and subcutaneous depots from each of 23 horses. Immuno-histochemistry procedures confirmed the presence of the enzyme 11β-HSD1 and radiometric assay procedures were again used to quantitate the amount of 11β-HSD1 activity. There was no significant difference in 11β-HSD1 activity between subcutaneous and adipose tissue. Additionally, there was no correlation between 11β-HSD1 activity and body condition scores.

The third species to be studied was the porcine, specifically the Ossabaw swine. Adipose tissue was collected from abdominal and subcutaneous depots from each of eight pigs. Radiometric assay procedures of the amount of 11β-HSD1 activity revealed no significant difference in 11β-HSD1 activity between subcutaneous and adipose tissue.

In conclusion, these data provide a unique comparative perspective of the relationship between 11β-HSD1 activity and obesity.

CHAPTER I

INTRODUCTION

Obesity is a major human health concern of pandemic proportions that is particularly prevalent in adults and children in developed nations. In humans, obesity is associated with hypertension, hyperglycemia, insulin resistance, dyslipidemia, pro-inflammatory state, and pro-thrombotic state. Each of these scenarios is also descriptive of a complex referred to as the "metabolic syndrome", and as follows, obesity increases the relative risk of occurrence of the metabolic syndrome. Moreover, the metabolic syndrome also has strong morphological and metabolic similarities to Cushing's syndrome. Cushing's syndrome is characterized by excessive peripheral concentrations of cortisol, which exacerbates obesity. However, it is intriguing that in patients with metabolic syndrome, circulating concentrations of cortisol are at best only slightly elevated.

Several studies have now demonstrated the importance of peripheral tissue-specific enzymes that exert critical roles in the modulation of tissue specific steroid action. The focus of this review will be on the enzyme, 11β- hydroxysteroid dehydrogenase (11β-HSD), which is responsible for this tissue-specific metabolism of glucocorticoids, specifically catalyzing the interconversion of 11-hydroxy glucocorticoids (cortisol, corticosterone) and 11-keto forms (cortisone, 11-dehydrocorticosterone). The 11β-HSD1 amplification of glucocorticoids in adipose tissue has been associated with obesity, diabetes, hypertension, dyslipidemia, cardiovascular disease, and polycystic

ovarian syndrome in humans and mice. The studies presented herein focus on the objectives of:

1) locating the presence of 11β-HSD1 in fat tissues, and
2) quantitating the amount of enzymatic activity of 11β-HSD

in several adipose depots, in three different species, feline, equine, and Ossabaw swine. The hypothesis is that this comparative analysis, across tissue regions, within three different species will yield greater insight into the association between 11β-HSD1 activity and obesity.

CHAPTER II

LITERATURE REVIEW

Unprecedented in the history of civilization, the human population currently faces a pandemic of obesity among adults and children; a particularly pervasive problem in developed nations. Hedley et al. (2004) recently estimated that in the U.S., 2/3 of all adults are either overweight or obese. The cause of this problem can be attributed to many factors, but the root cause in developed nations has been attributed to sedentary lifestyle and bad nutritional habits. It has also been asserted that sedentary lifestyle and bad nutritional habits are a result of greater technological affluence, in light of the fact that in populations where technological progress is much slower, obesity is not a significant public health problem (Grundy, 2004). Certain advances in technology have greatly reduced the requirement of manual labor and concomitantly increased available food resources; a scenario that is ideal for obesity to occur. However, since 2005, obesity is being recognized as a problem in underdeveloped non-tech societies like China.

Undeniably, prevention and treatment of obesity are a major challenge for society. Programs have been implemented to start by changing the lifestyle of children, promoting more physical activity and better nutrition. It has been reported that children that have active physical lifestyles have better body weight control, lower blood pressure, improved psychological well-being, greater predisposition to increased physical activity in adulthood, increased life expectancy and decreased risk of cardiovascular diseases (Williams et al., 2002).

Obesity is caused by an energy imbalance whereby energy intake (diet) exceeds energy output (physical activity). In particular, it is believed that the consumption of fat (a energy-dense nutrient) is the major factor contributing to obesity. Typically, the body can regulate energy intake, however, this regulation is poor when energy as fat is consumed as a substantial portion of diets (Bray and Popkin, 1998).

Neel hypothesized in 1962 that it was discrepancies between the life-style of the Stone Age man vs. the modern man that led to obesity, diabetes, and hypertension. Genetic characteristics that were important to the survival of man in antiguity, such as low metabolic rate, sodium conservation, and rapid mobilization of insulin in response to a carbohydrate challenge, nowadays are considered a predisposition to obesity, diabetes and hypertension. This "obesity predisposed genotype" has been referred to as the "thrifty genotype" by Neel (1962).

Dynamically, the body stores excess calories as energy dense adipose tissue, which can be rapidly mobilized as a source of energy when there is lack of food. Adipose tissue has been described as a type of connective tissue comprised of lipid-filled cells surrounded by a matrix of collagen fibers, blood vessels, fibroblasts, and immune cells (Ahima and Flier, 2000). More recently, adipose tissue is considered more than an energy storage site, but rather a complex and highly active metabolic and endocrine organ (Ahima and Flier, 2000; Fruhbeck et al., 2001). Adipocytes actively secrete proteins that are involved in energy homeostasis and regulation of neuroendocrine, autonomic, and immune function. Sex steroids, peptide hormone precursors, pro-inflammatory cytokines, and adiponectin are some of the products secreted by adipose tissue (Flier and Spiegelman, 1996; Mohamed-Ali et al., 1998).

In conjunction with local regulation of fat stores, hormonal secretions from adipocytes function to inform the central nervous system (CNS) of its energy reserves via several receptors located in those tissues. Consequently, energy metabolism, neuroendocrine function, and immune function are collectively controlled by the interaction of hormones, the CNS, and adipose tissue (Kershaw and Flier, 2004). Several metabolic abnormalities can result from excesses as well as deficiencies in adipose tissue. Obesity is defined as an excess of adipose tissue, it is associated with hypertension, insulin resistance, dyslipidemia, and a pro-inflammatory state. These features also characterize what has been referred to as the "metabolic syndrome" in humans. Obesity, especially visceral obesity, increases the relative risk of metabolic syndrome (Grundy et al., 2004). Specific features of the metabolic syndrome are explained in greater detail in the paragraphs which follow. Finally, it is important to note that there are strong morphological and metabolic similarities between the metabolic syndrome and Cushing's syndrome; the latter condition of which is caused by an excess in circulating glucocorticoids and obesity (with or without the metabolic syndrome). Notably, obesity is characterized by not elevated plasma concentrations of cortisol (Walker and Seckl, 2001). This dichotomy between these two conditions may be due to local vs. peripheral causes of glucocorticoid production, secretion, and/or action (Jamieson et al., 1999).

Insulin resistance

Insulin resistance has been shown to be significantly correlated with obesity and high plasma concentrations of free fatty acids (FFA; Perseghin et al., 1997). The high concentrations of FFAs in plasma, associated with obesity, may be due to a greater amount of FFAs being released in association with an expansion in fat mass (Gordon,

1960; Jensen et al., 1989; Björntorp et al., 1969). Free fatty acids are released in increased amounts into the circulation in obese individuals (Heptulla et al., 2001). Free fatty acids release from adipocytes is initiated by the action of hormone sensitive lipase (HSL), which hydrolyzes stored triglyceride. The amount of FFA released is proportional to the amount of lipid in adipose tissue, more lipid present in adipose tissue will result in more FFA released. High insulin levels inhibit adipose tissue lipolysis, however, in individuals with obesity, insulin cannot reduce the amount of FFA released to normal levels (Grundy, 2004). Recent studies have shown that increased FFA concentrations induce insulin resistance in skeletal muscle by directly inhibiting insulin activation of glucose transport activity (Roden et al., 1996; Dresner et al., 1999). Dresner et al. (1999) concluded that insulin stimulation of GLUT 4 activity in skeletal muscle acts via increased insulin-stimulated phosphorylation of insulin receptor substrate (IRS)-1 that allows it to bind and activate phosphatidylinositol (PI) 3-kinase activity. This action results in activation of GLUT 4 activity in healthy individuals. Studies have demonstrated that increased plasma FFAs inhibit insulin activation of IRS-1 associated PI 3-kinase activity (Dresner et al., 1999). A similar mechanism is likely to happen in liver, where diacylglycerol activates a serine kinase cascade associated with PKC-ε, resulting in decreased tyrosine phosphorylation of IRS-2 which is a key mediator of insulin action in the liver (Previs et al., 2000; Samuel et al., 2004). Those studies provide evidence that it is not obesity per se that is inducing insulin resistance, but rather an accumulation of intracellular lipid metabolites like diacylglycerol (Petersen and Shulman, 2006). It is well known that insulin resistance is a risk factor for impaired glucose tolerance and type 2 diabetes. There are many factors that are involved in the development of insulin

resistance and the role of FFA is just one of those factors.

Hypertension

Studies in humans and animal models have shown that obesity is associated with increases in regional tissue blood flow, cardiac output, and arterial pressure (Messerli et al., 1981; Hall et al., 1993; Hall, 2000). Increased regional tissue blood flow has been attributed to a greater metabolic rate in the adipose tissue and thus greater local accumulation of vasodilator metabolites, which in turn results in a cascading effect on growth of the organs and tissues in response to their increased demands. Furthermore, because obesity is associated with increased blood flow, this factor, in conjunction with an expansion in extracellular tissue volume, results in greater cardiac output and thus greater work effort by the heart (Messerli et al., 1981; Hall et al., 1993). The increased work load eventually impairs cardiac systolic and diastolic function which precipitates tissue hypertrophy and remodeling (Carroll et al., 1997). Cardiac hypertrophy is also exacerbated by high sodium intake, which often occurs concurrently with high caloric intake (Carroll et al., 1998). Obesity hypertension, like other forms of hypertension, is related to abnormal kidney function which in this case is a result of the high demand to maintain sodium balance (Guyton, 1990; Hall, 1997). Sodium ions are controlled in a homeostatic process involving aldosterone which increases sodium ion absorption in the distal convoluted tubules. Aldosterone stimulates an increase in the re-absorption of sodium ions from the kidney tubules which causes an increase in the volume of water that is reabsorbed from the tubule. This increase in water re-absorption increases the volume of blood which ultimately raises the blood pressure. Moreover, another cause of obesity hypertension is higher sympathetic activity. An overactive sympathetic nervous system

leads to increased stress responses. High caloric intake also amplifies muscle sympathetic activity and increases plasma norepinephrine concentrations (Rumantir et al., 1999; Mansuo et al., 2000). Norepinephrine, in turn, causes vasoconstriction, an increased heart rate and a greater cardiac stroke volume output which results in increased blood pressure. Sympathetic activation increases blood pressure and causes sodium retention through the renal nerves. Obese humans also have an increase in renal sympathetic activity; renal nerves may play an important role in human obesity hypertension (Esler, 2000). Additionally, increased levels of fatty acids have also been known to contribute to hypertension by increasing sympathetic activity and/or by enhancing the vasoconstrictor response to sympathetic activation (Stepniakowski et al., 1995; Grekin et al., 1997). It is likely that the link between obesity and sympathetic activation is hyperleptinemia. Fasting plasma levels of leptin increase in proportion to adiposity (Zhang et al., 1994). Plasma leptin acts on the hypothalamus to regulate energy balance by decreasing appetite and increasing energy expenditure through sympathetic stimulation (Hall et al., 2001). Long-term infusion of leptin leads to a significantly increased arterial pressure and heart rate in rodents (Shek et al., 1998). A higher blood pressure is a risk factor for cardiovascular disease, as well as stroke, left ventricular hypertrophy, heart failure, and chronic renal failure (Chobanian et al., 2003).

Dyslipidemia

Dyslipidemia has been shown to be strongly associated with obesity in humans. The characteristics of dyslipidemia include increased triglycerides, increased small LDL particles, decreased HDL cholesterol, and increased total apolipoprotein B-100 (Grundy, 1995). High levels of plasma FFA resulting from an excess in adipose tissue, is the

primary cause of fatty liver disease. It is likely that the liver takes up the FFAs that were not utilized by skeletal muscle or other tissues (Grundy, 2000). Consequently, the liver oxidizes the FFAs or re-esterifies them into triglycerides which are then incorporated into triglyceride-rich lipoproteins. Ultimately, the triglyceride-rich lipoproteins will be secreted into the blood stream and undergoes lipolysis by adipose tissue lipoprotein lipase. The resulting fatty acids then enter the adipocytes where they are re-esterified into triglycerides (Grundy, 2000). In obesity, the influx of fatty acids exceeds the liver's capacity to dispose of them, resulting in an accumulation of liver triglycerides. In order to re-distribute the excess of fatty acids to peripheral adipose and skeletal muscle tissue the triglycerides are incorporated into very low-density lipoprotein (VLDL); consequently, elevated VLDL is a hallmark of obesity (Grundy et al., 1979). The accumulation of triglycerides in the liver demands an increase in lipoprotein particles to carry triglycerides into the circulation (Boren et al., 1991). Apolipoprotein B-100 (apo B) is one of the carriers of triglycerides, it is generally partitioned between low-density lipoprotein (LDL) and VLDL. Therefore, obese individuals tend to have high levels of apo B. Additionally, high serum triglycerides reduce high-density lipoprotein (HDL) by replacing the HDL with VLDL triglycerides. Moreover, individuals with obesity-induced fatty liver disease have increased syntheses of hepatic lipase, which degrades HDL, thus exacerbating the decline in HDL (Grundy, 2004).

Pro-inflammatory state

A pro-inflammatory state is defined as a lipid-induced injury that initiates invasion of macrophages followed by proliferation of smooth muscle cells (Grundy, 2000). These processes have classic characteristics of chronic inflammation.

Inflammatory responses have been associated with the onset, development, and evolution of atherosclerotic lesions (Maseri, 1997; Pasceri et al., 1999). A nonspecific but sensitive marker of acute inflammatory response is increased serum levels of C-reactive protein. Shah (2000) affirmed that elevated plasma concentrations of the acute-phase reactant, C-reactive protein, is a risk factor for atherosclerosis and coronary heart disease. Obese persons and particularly those with the metabolic syndrome also have elevated serum levels of C-reactive protein (Visser et al., 1999; Ridker, 2003). This finding has lead to the suggestion that obesity is a pro-inflammatory condition; however, a mechanistic connection has not been definitively established.

Multiple factors and hormones have now been reported to be secreted from adipocytes and other cells in the adipose tissue. Some of the adipose tissue secreted proteins are described in more detail hereafter.

Leptin

Leptin is a 16-kDa polypeptide containing 167 amino acids with structural homology cytokines. Typically the peripheral concentration of leptin is proportional to the adipose tissue mass and nutritional status of the individual as a whole. Some studies report that leptin is secreted in higher quantities from subcutaneous than visceral adipose tissue (Fain et al., 2004; Wajchenberg, 2000). Leptin is regulated by several factors, its secretion is increased by insulin, glucocorticoids, TNFα, estrogens, and CCAAT/enhancer-binding protein-α and decreased by β3-adrenergic activity, androgens, free fatty acids, GH, and peroxisome proliferator-activated receptor-γ agonists (Margetic et al., 2002). The site of leptin's action is likely ubiquitous since its receptors have been found on all tissues tested thus far (Bjorbaek and Kahn, 2004). The specific effects of

leptin on energy intake and expenditure are mediated via hypothalamic pathways, while other effects are mediated via direct action on peripheral tissues (Bjorbaek and Kahn, 2004). Leptin's primary purpose is believed to function as a metabolic signal of energy reserves (Flier, 1998). During caloric restriction, leptin levels decrease thus resulting in increased appetite and decreased energy expenditure. The opposite situation occurs when the individual has ingested enough calories; the level of leptin increases, thus decreasing appetite and increasing energy expenditure. In some forms of obesity, the leptin feedback loop has been disrupted suggesting a condition referred to as "leptin resistance" (Bjorbaek and Kahn, 2004; Flier, 2004). Leptin has several other important endocrine effects such as regulation of immune function, reproduction, hematopoiesis, angiogenesis, and bone development (Margetic et al., 2002; Lord et al., 1998). Leptin prevents suppression of the immune system in case of malnutrition, which causes suppression of immune system in leptin deficiency (Lord et al., 1998). In addition, leptin regulates neuroendocrine function and traditional endocrine systems.

Tumor Necrosis Factor-α

Tumor Necrosis Factor-α (TNFα) is a 26-kDa transmembrane protein that is cleaved into a 17-kDa biologically active protein classified as a pro-inflammatory cytokine. Ruan and Lodish (2003) described TNFα as an endotoxin-induced factor that causes necrosis of tumors. Also, some studies have implicated TNFα in obesity and insulin resistance (Ruan and Lodish, 2003; Hotamisligil, 2003; Hotamisligil et al., 1993). Several studies have provided evidence that expression of TNFα is higher in subcutaneous than visceral adipose tissue in humans (Fain et al., 2004; Wajchenberg, 2000). Adiposity and insulin resistance have been positively correlated to increased

expression of TNFα by adipose tissue in obese humans and rodents (Ruan and Lodish, 2003; Hotamisligil, 2003; Hotamisligil et al., 1993; Fernadez-Real and Ricart, 2003). Insulin resistance is induced by chronic exposure to TNFα *in vivo* and *in vitro* (Ruan and Lodish, 2003). Moreover, insulin sensitivity improves with treatments that neutralize soluble TNFα receptors in rodents with obesity but not in humans (Ruan and Lodish, 2003). Also, insulin resistance and circulating FFA improve significantly with deletion of the TNFα target gene or its receptors (Uysal et al., 1997). One of the mechanisms of actions of TNFα is through its influence on gene expression in tissues like adipose tissue and liver (Ruan et al., 2002). In adipose tissue, TNFα changes the expression of several adipocyte-secreted factors, suppresses genes for transcription factors involved in lipogenesis and adipogenesis, and represses genes involved in storage and uptake of glucose and FFA (Ruan et al., 2002). In liver, TNFα increases expression of genes involved in *de novo* synthesis of fatty acids and cholesterol and suppresses expression genes involved in glucose metabolism and uptake (Ruan et al., 2002). Other mechanisms of action of TNFα include its ability to decrease the impact of insulin signaling by activation of serine kinases (Hotamisligil, 2003). Serine kinases increase phosphorylation of insulin receptors, which make them a poor substrate for insulin receptor kinases and increase their degradation (Hotamisligil, 2003). Also, TNFα increases serum levels of FFA which induce insulin resistance in multiple tissues (Ruan et al., 2002).

Adiponectin

Adiponectin is a 30-kDa polypeptide that is highly and specifically expressed in differentiated adipocytes and circulates at high levels in the bloodstream (Chandran et al., 2003). There are two receptors for adiponectin, AdipoR1 and AdipoR2. In muscle,

AdipoR1 is expressed, which is a low-affinity receptor for full-length adiponectin and high-affinity receptor for globular adiponectin (Yamauchi et al., 2003). Expression of adiponectin is higher in subcutaneous than visceral adipose tissue (Fain et al., 2004). In liver, AdioR2 is expressed, which is an intermediate-affinity receptor for both globular and full-length adiponectin (Yamauchi et al., 2003). Studies have established an inverse association between adiponectin and insulin resistance (Chandran et al., 2003; Diez and Iglesias, 2003). Hotta et al. (2001) found that before the onset of obesity and insulin resistance in nonhuman primates, adiponectin levels decrease, leading to the hypotheses that hypoadiponectinemia contributes to the pathogenesis of these conditions. The cause of low adiponectin levels with insulin resistance is either obesity or lipodystrophy (Chandran et al., 2003; Diez and Iglesias, 2003). In cases where improvement in insulin sensitivity occurs, such as after weight reduction or treatment with insulin-sensitizing drugs, the level of adiponectin increases (Chandran et al., 2003; Diez and Iglesias, 2003). Moreover, obesity and insulin resistance are associated with several polymorphisms in the adiponectin gene (Chandran et al., 2003; Diez and Iglesias, 2003). Studies in mice that are adiponectin-deficient have revealed that these mice prematurely develop diet induced glucose intolerance and insulin resistance (Kubota et al., 2002; Maeda et al., 2002). Conversely, mice overexpressing adiponectin exhibit improved glucose tolerance, insulin sensitivity, and serum FFA (Maeda et al., 2002; Combs, 2004). The effects of adiponectin in the liver include enhanced insulin sensitivity, decreased influx of FFA, increased fatty acid oxidation, and reduced hepatic glucose output (Chandran et al., 2003; Diez and Iglesias, 2003). The effects of adiponectin in the muscle are fatty acid oxidation and increased glucose use (Chandran et al., 2003; Diez and Iglesias, 2003). Furthermore,

in the vascular wall adiponectin inhibits monocyte adhesion by decreasing expression of adhesion molecules and inhibits macrophage transformation to foam cells by inhibiting expression of migrating smooth muscle cells in response to growth factors (Chandran et al., 2003; Diez and Iglesias, 2003). Collectively, these studies have provided evidence that adiponectin's metabolic effects are anti-diabetic, anti-inflammatory, and anti-atherogenic.

Resistin

Resistin is a ca. 12-kDa polypeptide that belongs to a family called resistin-like molecules. Resistin was initially identified in 2001 when obese rodents were found to have elevated serum levels of resistin (Steppan et al., 2001). Expression of resistin in visceral adipose tissues is 15 times greater than that found in subcutaneous adipose tissue in rodents (Banerjee and Lazar, 2003). Banerjee and Lazar (2003) suggested that resistin was a possible link between obesity and insulin resistance because of its effects on insulin action. Cultured adipocytes treated with recombinant resistin responded with insulin-stimulated glucose uptake while anti-resistin antibodies prevented this effect (Steppan et al., 2001). Moreover, under euglycemic hyperinsulinemic conditions, infusion of resistin caused hepatic insulin resistance (Rajala et al., 2003).

In humans, resistin is expressed at very low levels in adipocytes and human and murine resistin share 68% homology (Banerjee and Lazar, 2003). Several studies have failed to provide a consistent link between resistin expression in adipose tissue or circulating resistin levels and insulin resistance (Banerjee and Lazar, 2003).

Interleukin-6

Interleukin-6 (IL-6) is another cytokine and it circulates in multiple glycosylated

forms ranging from 22 to 27-kDa in size. Also, IL-6 is associated with obesity and insulin resistance (Fernadez-Real and Ricart, 2003). There is a positive correlation between adipose tissue expression and circulating levels of IL-6 with obesity and insulin resistance (Fernadez-Real and Ricart, 2003). Visceral adipose tissue has been reported to express and secrete 2 to 3 times more IL-6 than subcutaneous adipose tissues (Fain et al., 2004; Wajchenberg, 2000). Moreover, plasma levels of IL-6 have been used to predict the development of cardiovascular diseases and type 2 diabetes (Fernadez-Real and Ricart, 2003). Peripheral administration of IL-6 leads to hyperglycemia, insulin resistance and hyperlipidemia in humans and rodents (Fernadez-Real and Ricart, 2003). In addition, IL-6 reduces expression of insulin receptor signaling components and induces suppressor of cytokines signaling 3, a negative regulator of both leptin and insulin signaling (Senn et al., 2003). Furthermore, IL-6 decreases adiponectin secretion and inhibits adipogenesis (Fernadez-Real and Ricart, 2003). These findings provide evidence that IL-6 is likely to contributes to the development of insulin resistance in obese individuals.

Macrophages and monocyte chemoattractant protein-1

Macrophages and monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is expressed and secreted by adipose tissue, it recruits monocytes to sites of inflammation (Wellen and Hotamisligil, 2003). Increased adipose tissue infiltration by macrophages is correlated to obesity (Wellen and Hotamisligil, 2003; Weisberg et al., 2003; Xu et al., 2003). Tumor necrosis factor-α, IL-6, and other inflammatory factors that contribute to insulin resistance are secreted by activated macrophages (Wellen and Hotamisligil, 2003). Obese rodents have high circulating concentrations of MCP-1 and elevated tissue expression of MCP-1, suggesting that infiltration of macrophages

mediated by MCP-1 may contribute to the metabolic abnormalities related to obesity and insulin resistance (Takahashi et al., 2003; Sartipy and Loskutoff, 2003). It has been suggested that MCP-1 directly contributes to adipose tissue insulin resistance because MCP-1 decreased insulin-stimulated glucose uptake and insulin-induced receptor tyrosine phosphorylation in cultured adipocytes (Sartipy and Loskutoff, 2003; Gerhardt et al., 2001). Furthermore, peripheral administration of MCP-1 increases circulating monocytes, promotes accumulation of monocytes in collateral arteries, and increases neointimal (new or thickened layer of arterial intima) formation in mice (Takahashi et al., 2003; van Royen et al., 2003). Collectively, these studies provide evidence that MCP-1 is playing a role in the development of atherosclerosis.

Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 is one of several proteins of the hemostasis and fibrinolytic system that are secreted by adipocytes (Mertens and Van Gaal, 2002). The primary inhibitor of fibrinolysis is PAI-1, which is a member of the serine protease inhibitor family (Mertens and Van Gaal, 2002). Visceral adipose tissue expression and secretion of PAI-1 is greater than subcutaneous adipose tissue expression and secretion (Fain et al., 2004; Wajchenberg, 2000). Atherogenesis and angiogenesis are other biological processes in which PAI-1 is involved (Mertens and Van Gaal, 2002). Obese and insulin resistant individuals have elevated plasma levels of PAI-1, also there is a positive correlation between high levels of PAI-1 with features of the metabolic syndrome, risk for cardiovascular disease and type 2 diabetes (Mertens and Van Gaal, 2002; Juhan-Vague et al., 2003). Mice with target deletion in PAI-1 exhibited reduced weight gain on a high fat diet, increased energy expenditure, improved glucose tolerance,

and enhanced insulin sensitivity (Ma et al., 2004). Therefore, it is likely that there is a connection between PAI-1, obesity, insulin resistance, and cardiovascular disease.

Proteins of the renin angiotensin system

Adipose tissue produces proteins that are part of the renin angiotensin system (RAS), such as renin, angiotensinogen, angiotensin I, angiotensin II, angiotensin receptors type 1 and 2, angiotensin-converting enzyme (ACE), and other proteases capable of producing angiotensin II (Engeli et al., 2003; Goossens et al., 2003). Visceral adipose tissue expression of angiotensinogen, ACE, and angiotensin I receptors is greater than that of subcutaneous tissues (Engeli et al., 2003; Goossens et al., 2003). Many of the effects of the RAS, all of which contributes to blood pressure regulation, are mediated by angiotensin II including increasing vascular tone, aldosterone secretion from the adrenal gland, and sodium and water reabsorption from the kidney (Kershaw and Flier, 2004). Transgenic mice overexpressing angiotensinogen in adipose tissue exhibit high blood pressure and increased adipose tissue mass, while mice with targeted gene deletion of angiotensinogen exhibit low blood pressure and adipose tissue mass (Massiera et al., 2001a; Massiera et al., 2001b). In addition, angiotensin II promotes lipogenesis and indirectly stimulates prostaglandin synthesis leading to stimulation of adipocyte growth and differentiation (Engeli et al., 2003). Also, angiotensin II alters blood flow and sympathetic nervous system activity by binding receptors on adipocytes as well as stromo-vascular cells and nerve terminals (Engeli et al., 2003). Moreover, angiotensin II increases glycogenolysis and hepatic gluconeogenesis, decreases insulin-dependent glucose uptake, inhibits lipolysis, and promotes lipogenesis (Engeli et al., 2003). Adipose tissue RAS is involved in many of the metabolic syndrome features, especially

hypertension.

Other factor that plays a critical role in obesity and metabolic syndrome is the metabolism of glucocorticoids. Glucocorticoids are involved in several metabolic processes including insulin sensitivity, glucose homeostasis, lipid metabolism, and adipogenesis (Friedman et al., 1996). Plasma glucocorticoid levels are generally regulated via the activity of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates circulating glucocorticoid levels by a short-term neuroendocrine feedback circuit. In acute stress situations there is a brief increase in HPA activity, which interacts with elevated epinephrine, glucagon, and sympathetic neural activity to elevate blood glucose concentrations, ensuring adequate substrate for brain and muscle.

The excess of glucocorticoids promotes visceral obesity, insulin resistance, dyslipidemia, and hypertension (Friedman et al., 1996). Several studies have suggested a correlation between obesity and increased urinary free cortisol excretion (Strain et al., 1980; Marin et al., 1992; Pasquali et al., 1993). Moreover, some studies affirmed that obese men and women have total cortisol production rate somewhat elevated (Andrew et al., 1998; Fraser et al., 1999; Stewart and Krozowski, 1999). However, plasma cortisol levels are not usually elevated in most individuals with metabolic syndrome and in some individuals cortisol levels are reduced in obesity without metabolic syndrome (Walker and Seckl, 2001).

The enzyme 11 β-hydroxysteroid dehydrogenase (11 β-HSD) plays an important role in peripheral metabolism of cortisol. 11 β-HSD catalyses the interconversion of hormonally inactive 11 β-ketoglucocorticoid metabolites (cortisone in humans and 11 β-dehydrocorticosterone in mice) and hormonally active 11 β-hydroxylated metabolites

(cortisol in humans and corticosterone in mice; Amelung et al., 1953). Monder and White (1993) described 11 β-HSD activity in a broad range of cells and tissues. Later on, two isozymes were characterized, 11 β-HSD type 1 and 11 β-HSD type 2 (White et al., 1997; Stewart and Krozowski, 1999).

11 β-HSD type 2 converts active cortisol into the inactive form cortisone via a high-affinity (low Km), nicotinamide adenine dinucleotide (NAD)-dependent process (Albiston et al., 1994; Brown et al., 1996). 11 β-HSD2 is expressed in sweat glands, distal nephron, salivary glands, and other tissues where aldosterone induces its classical effects on sodium excretion (Smith et al., 1996; Hirasawa et al., 1997). The main function of 11 β-HSD2 is to protect the mineralocorticoid receptor (MR) from binding cortisol (Edwards et al., 1988; Funder et al., 1988). Inhibition or disruption of the 11 β-HSD 2 gene causes the syndrome of apparent mineralocorticoid excess, in which glucocorticoids occupy renal MR leading to sodium retention, hypertension, and hypokalemia (Kotelevtsev et al., 1999; Mune et al., 1995; Dave-Sharma et al., 1998).

11 β-HSD type 1 can convert cortisone into cortisol and vice-versa, it is a much-lower affinity, nicotinamide adenine dinucleotide phosphate (NADP(H))-dependent process (Low et al., 1994). 11 β-HSD1 is widely distributed and it is predominantly 11-ketoreductase which converts cortisone into cortisol (Low et al., 1994; Jamieson et al., 1995). The predominant 11-ketoreductase activity of 11 β-HSD1 may be due to the fact that 11 β-HSD1 is co-localized with hexose-6-phosphate dehydrogenase, which is a NADPH-generating enzyme (Ozols, 1995; Draper et al., 2003). The tissues that express 11 β-HSD1 in humans and rodents includes liver, adipose tissue, lung, skeletal muscle, cardiac tissue, vascular smooth muscle, anterior pituitary gland, brain and adrenal cortex

(Moisan et al., 1990; Tannin et al., 1991; Napolitano et al., 1998; Christy et al., 2003). Remarkably, those locations present higher expression of glucocorticoid receptors than mineralocorticoid receptors, except heart and hippocampus that primarily express MR with a high-affinity for glucocorticoids (Whorwood et al., 1991).

Adipose tissue 11 β-HSD1 plays a role in regulation of metabolism and differentiation of preadipocytes into adipocytes (Gaillard et al., 1991). Mice overexpressing 11 βHSD1 presented much higher local concentrations of corticosterone in mesenteric adipose tissue (Masuzaki et al., 2001). Moreover, transgenic mice had serum corticosterone concentrations similar to their non-transgenic littermates (Masuzaki et al., 2001). Also, transgenic mice that over express 11 βHSD1 exhibit body weights 25-35% higher than their non-transgenic littermates when receiving high fat diets, and most of the additional weight was restricted to mesenteric fat (Masuzaki et al., 2001). Furthermore, 11 βHSD1 overexpressing mice developed all the major features of the metabolic syndrome: they were markedly glucose intolerant and insulin resistant. Additionally, they exhibit dyslipidemia with elevated FFA and triglyceride levels (Masuzaki et al., 2001).

Intriguing is the fact that mice overexpressing 11 βHSD1 had elevated expression of angiotensinogen mRNA in adipose tissue, which is normally expressed at low levels. The hypertension that is present in those mice may be therefore due to the glucocorticoid-regulated transcript of angiotensinogen (Masuzaki et al., 2001). An excess of glucocorticoids in adipose tissue decreases the expression of insulin-sensitizing factor adiponectin and increases expression of TNFα, which causes insulin resistance (Fasshauer et al., 2002; Viengchareun et al., 2002). However, adipose mRNA-encoding

resistin is reduced possibly due to the excess of glucocorticoids (Viengchareun et al., 2002).

Another study utilized knockout (KO) mice for 11 βHSD1, which originated from two different genetic backgrounds; one an obesity-resistant strain and the other an obesity-prone strain. Mice were then fed a high fat diet and the 11 βHSD1 KO obesity-prone strain gained significantly less weight than controls, probably due to an enhanced metabolic rate (Morton et al., 2002). Also, the 11 βHSD1 KO obesity-prone mice were found to be insulin sensitized and resistant to hyperglycemia, such as occurs with high-fat feeding in white mice (Morton et al., 2002). 11 βHSD1 KO mice also exhibited increased adiponectin mRNA, while TNFα and resistin were reduced. Therefore, 11 βHSD1 KO mice had improved glucose tolerance, increased insulin sensitivity, and reduced intra-tissue glucocorticoid levels (Harris et al., 2001).

Some studies have found differences in 11 βHSD1 activity among fat depots, and among species. In humans, 11 βHSD1 activity measured in adipose stromal cells and cells from the visceral tissue were found to be higher than subcutaneous tissues (Bujalska et al., 1997). Conversely, 11 βHSD1 mRNA expression and activity were higher in peripheral than visceral depots in non-obese strains of mice (Morton et al., 2003). These facts provide evidence that elevated glucocorticoid reactivation (or interconversion) correlates with increased differentiation potential of this depot. In agreement with this premise, humans perhaps have higher activity in visceral adipose because this depot has more glucocorticoid receptors and/or has greater capacity for glucocorticoid-induced differentiation of immature to mature adipocytes (Masuzaki et al., 2001). Interestingly, in mice while increased subcutaneous adipose 11 βHSD1 is associated with insulin

resistance in obesity, there is no correlation with visceral adiposity or hypertension. Mice and men differ in those aspects, although 11 βHSD1 was measured only in cultured cells (Tomlinson et al., 2002).

Obesity can differ distinctly depending on the species studied, and each species responds in a different way to excess accumulations of adipose tissue. The following is a brief review of obesity issues in several species.

SWINE OBESITY

Swine and humans share several similarities, such as omnivorous habits, propensity to sedentary habits and obesity, lipoprotein metabolism, and most importantly, similar cardiovascular systems (Douglas, 1972; Mahley et al., 1975; Lee, 1986; Bell and Gerrity, 1992). Considering these similarities, pigs are frequently used in cardiovascular and obesity research. Several unique strains of pigs are typically used and will be reviewed here.

Ossabaw pigs are descendants of Iberian swine that were abandoned on Ossabaw Island, off the coast of Georgia, approximately 500 years ago. Those animals had to adapt to the availability of food on the island. They developed the capacity to drink sea water and survive long periods of starvation accumulating fat when food was available. When those pigs are fed high fat diets in captivity they become obese, insulin resistant, glucose intolerant, hyper-triglyceridemic, and hyper-cholesterolemic (Martin et al. 1973; Wangsness et al., 1977; Etherton and Kris-Etherton, 1980; Wangsness et al., 1980). It is likely that Ossabaw pigs have the thrifty genotype, especially because they haven't had any genetic selection efforts applied by humans for over 500 years.

Recent studies provided evidence that Ossabaw pigs fed high-fat diets had an increase in fat 2 times greater than the increase in body weight, while Yucatan pigs had an almost identical increase in fat and body weight (Witczak et al., 2005). Also, female Ossabaw pigs exhibit a preferential increase in fat mass rather than in lean mass. There were no differences in fat accumulation between subcutaneous and intra-abdominal depots (Dyson et al., 2006). When comparing subcutaneous fat thickness between Ossabaw females versus Yucatan females, Ossabaw exhibited a greater efficiency of adipogenesis (Dyson et al., 2006). In addition, Ossabaw female pigs that were fed a high-fat diet developed insulin resistance in a period of 9 weeks (Dyson et al., 2006). In contrast, Yucatan pigs fed a high-fat diet had increased body weight but did not develop insulin resistance, not even in a 20 week study (Boullion et al., 2003; Witczak et al., 2005).

Interestingly, swine have a greater tolerance for glucose than humans. It is suggested that swine have greater insulin secretory reserve when compared with humans (Larsen and Rolin, 2004). Moreover, female Ossabaw pigs, fed a high-fat diet exhibited an enormous LDL: HDL ratio, higher than any previously reported for swine (Dyson et al., 2006; Dixon et al., 2002; Witczak and Sturek, 2004). Female Ossabaw pigs are predisposed to coronary artery disease, the high LDL: HDL ratio is a strong predictor of coronary artery disease (Dixon et al., 2002). The anatomical and physiological similarities between swine and humans likely make them the best animal model for human physiology. Finally, perhaps Ossabaw pigs are an ideal animal model for humans because they are capable of developing the features of the metabolic syndrome.

FELINE OBESITY

Obesity in household cats has become a relevant problem in the last decade. Obesity in cats increases the risk of lameness, diabetes mellitus, dermatopathy, urinary tract disease, and neoplasia (Rand and Appleton, 2001; Appleton et al., 2001). A study conducted on 8000 adult cats revealed that the prevalence of obesity was greatest in neutered males and middle-aged cats (Lund et al., 2005). There are similarities in the development of obesity and insulin resistance between humans and cats. First, there are many similarities in the life-style of household cats and humans from developed nations; both have unrestricted availability of high caloric density diets with restricted exercise. Thus, cats also have a great imbalance in the energy intake/expenditure equation. Hoenig et al. (2006a) reported that obese cats are markedly insulin resistant, as determined from the use of the euglycemic and hyperinsulinemic clamp models. It was found that there was a decrease in insulin sensitivity and glucose effectiveness within an increase in body mass index (Hoenig et al., 2006a). Insulin secretion in diabetic cats is low and unstable because prolonged insulin resistance results in reduced secretory capacity (Hoenig et al., 2000).

Furthermore, like humans obese cats exhibit high plasma levels of leptin and appear to be resistant to leptin effects (Backus et al., 2000; Hoenig and Ferguson, 2003; Yildiz and Haznedaroglu, 2006). Another similarity between cats and humans is that obese cats exhibit high concentrations of FFA, triglycerides, and cholesterol (Wilkins et al., 2004). However, it is interesting that obese and/or diabetic cats are not predisposed to atherogenesis, coronary artery disease, or high blood pressure (Sennello et al., 2003). Moreover, a recent study reported that obese cats exhibited low fat activity and plasma

levels of lipoprotein lipase (Hoenig et al., 2006b). The fact that the cats in the study had long-standing obesity and high levels of TNFα might have resulted in low lipoprotein lipase activity (Hoenig et al., 2006b). On the other hand, obese cats exhibit higher lipoprotein lipase activity in muscle when compared to lean cats (Hoenig et al., 2006b). This finding suggests an increase in lipid deposition in muscle, which has been associated with insulin resistance (Wilkins et al., 2004; Perseghin et al., 1999; Boden et al., 2001).

Cats are carnivores and they require high-protein and prefer high-fat diets. Frank et al. (2001) reported that high-protein diets decrease insulin requirements and improved glucose control in diabetic cats. The mechanism involved in this process is not fully understood. Other studies reported that a high-protein diet is beneficial for weight loss and leads to greater fat loss with preservation of lean body mass (Szabo et al., 2000; Laflamme and Hannah, 2005). Yet another study reported that cats on a high-protein diet had high energy expenditure, suggesting a long-term beneficial effect of protein on weight gain (Hoenig et al., 2006c). Also, this study reported that weight loss led to complete restoration of insulin sensitivity.

In cats, Schipper et al. (2004) confirmed the presence of 11 β-HSD2 in the kidney by RT-PCR, and also had found the presence of 11 β-HSD1 in the liver. Even though they found 11 β-HSD1 in the kidney they hypothesized that dehydrogenase activity was the predominant reaction because the velocity of the dehydrogenase reaction is greater than the reductase reaction. In human kidney only 11 β-HSD2 activity was found (Albiston et al., 1994; Kataoka et al., 2002). In feline liver 11 β-HSD1 presence was detected and confirmed by RT-PCR with predominantly reductase activity (Schipper et al., 2004). Schipper et al. (2004) also found that feline 11 β-HSD2 has 90% of homology

with the human 11 β-HSD2.

EQUINE OBESITY

Obesity and related problems are dramatically affecting the horse industry. Obesity is a risk factor for the development of certain health abnormalities in horses, such as equine metabolic syndrome, laminitis, and infertility (Jeffcott et al., 1986; Pass et al., 1998). Compared to horses, ponies have a tendency to get obese and to develop laminitis (Freestone et al., 1992; Alford et al. 2001). Some studies have associated insulin resistance with predisposition to laminitis in ponies (Coffman and Colles, 1983; Jeffcott et al., 1986; Treiber et al., 2005a; Treiber et al., 2005b). In humans, insulin resistance is associated with altered insulin signaling, which is involved in reduced glucose availability to insulin-sensitivity cells, vasoconstriction, endothelial damage, and the inflammatory response (DeFronzo and Ferrannini, 1991; Fonseca et al., 2004). Those factors combined with "trigger factors", such as exotoxins, endotoxins, and amines, will promote hoof separation (Bailey et al., 2004).

Matrix metalloproteinases (MMPs) are proteases (exotoxins) released by disturbed microflora and active collagens, MMP-2 and -9 are particularly implicated in the pathogenesis of laminitis (Mungall et al., 2001; Pollitt, 2004). Studies have been reported suggesting that insulin suppresses MMP-9 and glucose stimulates MMP-9 in humans (Aljada et al., 2004; Dandona et al., 2003). Therefore, the combination of insulin resistance and glucose intolerance may be involved in the disruption of the laminae by activation of MMP-9. In addition, the vascular changes observed in cardiovascular disease and diabetes in humans may be similar in the horse being the vascular component

of laminitis (Hinckley et al., 1996; Hood et al., 1978; Robinson et al., 1976).

Moreover, some studies have demonstrated that high concentrations of circulating insulin can affect reproductive activity by modification of GnRH-mediated LH release and also by directly affecting ovarian activity (Bruning et al., 2000; Diamanti and Bergiele, 2001; Mao et al., 2001). Sessions et al. (2004) suggested that high concentrations of circulating insulin modified follicular development and luteal function of mares' ovaries.

CHAPTER III

DEVELOPMENT OF AN 11 β- HYDROXYSTEROID DEHYDROGENASE ACTIVITY ASSAY FOR ADIPOSE TISSUE

Following the discovery of the enzyme, 11 β HSD, by Amelung and colleagues in 1953, several methods have been used to quantitate 11 β HSD activity in various tissues. Table 1 provides a summary review of the unique and diverse methodological approaches that have been employed to quantitate 11 β HSD activity in various tissues in the literature heretofore.

Slight et al. (1993), with their assessment of 11 β HSD activity in cultured heart and peripheral vascular tissues set in motion a "race" of efforts to measure 11 β HSD activity. These initial efforts were then quickly followed by the work of Low et al. (1994) who examined 11 β HSD 1 activity in intact mammalian COS-7 cells and by Stewart et al. (1994), who characterized 11 β HSD activity in human renal tissue; finding only that dehydrogenase activity was the primary pathway believed to exist in those tissues. Hundertmark et al. (1995) then reported the presence of 11 β HSD1 reductase in fetal lung cultures and confirmed the relationship between 11 β HSD 1 activity to glucocorticoid induced surfactant phosphatidylcholine syntheses. In 1995, Jamieson and coworkers also examined 11 β HSD activity in rats hepatocytes and reported that the hepatocytes predominantly expressed reductase activity. Thereafter, Rajan et al. (1996) examined 11 β HSD activity in a primary culture of hippocampal cells and also determined that predominantly 11 β reductase activity was present.

More recently, it was asserted that 11-ketoreductase activity was relatively unstable in tissue homogenates, despite efforts to stabilize it with the addition of a plentiful supply of the co-factor NADPH. It is now known that this pathway predominantly exists only *in vivo* in those tissues (Draper et al., 2003). Contemporaneously, other investigators chose to focus on measuring dehydrogenase activity because this pathway has been asserted as the preferential reaction pathway when 11 β HSD is liberated from its intracellular environment. In 2001, Rask and coworkers became the first to measure 11 β HSD1 activity in human adipose tissue and this was followed shortly thereafter by Tomlinson and coworkers (2002) who measured 11 β HSD 1 reductase activity in adipose tissue, adipocytes, and pre-adipocytes in humans. Morton and coworkers (2004) tested the effect of high fat diets in different strains of mice and found 11 β HSD1 dehydrogenase activity in adipose, liver, and muscle tissue. Again, Table 1 provides a summary of the methods described in these aforementioned studies.

Our laboratory utilized a combination of these methodologies to establish a robust and repeatable procedure for quantitating 11 β HSD1 activity. Our first step was to mince adipose tissue rather than homogenize it to minimize the possibility that 11 β HSD 1 reductase activity was relatively unstable in tissue homogenates. We minced the tissues to be studied, first with scissors, followed by the use of scalpel blade scraping to disrupt the cell walls. Once 100 mg of minced tissue was acquired, it was then incubated in borosilicate glass tubes with either 20 nM or 2.5 nM of unlabeled cortisone or 11-dehydrocorticosterone (Sigma Chemical Co., UK), plus ^{3}H-cortisone (Amersham International, USA) or ^{3}H-11-dehydrocorticosterone, respectively. It should be noted that aliquots of ^{3}H-11-dehydrocorticosterone were prepared fresh prior to each assay by

derivatizing ^{3}H-corticosterone as previously described (Yang and Yu, 1994). Known quantities of ^{3}H-11-dehydrocorticosterone were then incubated in the presence of the minced – scraped tissues with 0.1 mM, 0.2 mM, or 1 mM of NADPH (Alexis Biochemicals, San Diego, CA), depending on the species and tissues being tested. One ml of Krebs Ringer Buffer (pH 7.4) was then added to the mixture, which was then incubated at 37^{o}C for 30 min, 1 hr, or 24 hrs, again depending on the species and tissues tested. The incubation time points, concentrations of substrate, and cofactor were ultimately optimized to achieve linearity within assay for each tissue type and species.

Following the incubations, the incubation media was extracted utilizing a C_{18} solid phase extraction column (J. T. Baker, USA) as described by others (Slight et al., 1993). Briefly, the columns were activated by washing the columns with 2 ml of methanol followed by 2 ml of distilled water, without drying the column following the addition of the water. The incubation media was then applied to the C_{18} solid phase extraction columns and washed with 2 ml of 1:4 methanol/water followed by 2 ml of methanol, the latter fraction of which contained the steroids. This latter methanol fraction (which contained the steroids) was evaporated to dryness under nitrogen, thus concentrating the steroids into the very bottom of the tubes. Acetone (200 µl) was then added to the dried extracts to wash all steroids to the bottom of the tube and to transfer the extract to TLC plates.

The steroids were then separated by TLC using a mobile phase consisting of ethanol and chloroform (8:92). Unlabeled steroid control lanes were ran in parallel with the sample extracts for quantative control over isolation, identification, and the spatial aspect of each steroid under UV light. The spots containing the intra-converted steroids

were then identified (based on the control steroids) and scrapped from the surface of the TLC plates. Thereafter the steroids within the scrapings were extracted from the powder by spinning the samples for 15 min at 1500 rpm with 1 ml of isopropanol. The isopropanol / ^{3}H steroid mixture was then decanted into liquid scintillation counting vials and dried under nitrogen. After drying was complete, 6 ml of liquid scintillation cocktail was added (Ultima Gold, PerkinElmer) and the samples were counted. Enzyme activity was calculated and expressed as a percentage of conversion of one steroid to its complement form.

The results of these procedures are described in the following chapters.

Table 1. Details of the methods used to measure 11 β HSD activity.

	Tissue	^{3}H- Form	Cofactor	Buffer	Extraction	Incubation
Slight et al.(1993)	Heart and Vascular cells - cell sonicate	Cortisol or Corticosterone	0.5 mM/L $NADP^{+}$	_	C_{18} solid phase extraction	1 h- 37^{o} C
Low et al.(1994)	Mammalian COS-7 cells – cell homogenate	11-dehydro-corticosterone or corticosterone	1 mM $NADP^{+}$	Krebs Ringer Buffer , pH 7.4	Ethyl acetate	10 min- 37^{o} C
Stewart et al.(1994	Kidney homogenate	Cortisol	100 µM/L NAD or $NADP^{+}$	Phosphate Buffer pH 7.6	Dichloro-methane	30 min- 37^{o} C
Hundertmark et al. (1995)	Fetal Lung culture - homogenate	Corticosterone	0.3 mM/L $NADP^{+}$	Tris-HCl Buffer pH 9.0	Ethyl ether	1 h- 37^{o} C
Jamieson et al. (1995)	Hepatocytes cell homogenates	11-dehydro-corticosterone or corticosterone	200 µM $NADP^{+}$ or NADPH	Krebs Ringer Buffer, pH 7.4	Ethyl acetate	10 to 60min-37^{o} C
Rajan et al.(1996)	Cultured hippocampal cells homogenates	11-dehydro-corticosterone or corticosterone	400 µM $NADP^{+}$ or NADPH	_	Ethyl acetate	1 h - 37^{o} C
Rask et al.(2001)	Adipose tissue homogenate	Cortisol	2 mM $NADP^{+}$	Krebs Ringer Buffer, pH 7.4	_	3, 6, 20 and 30 h - 37^{o} C
Tomlinson et al. (2002)	Preadipocytes and adipocytes - intact cells	Cortisone	_	_	Dichloro-methane	16 h- 37^{o} C
Morton et al. (2006)	Adipose, liver and muscle - homogenates	Corticosterone	400 µM $NADP^{+}$	_	Ethyl acetate	10min,1h and 6h - 37^{o} C

CHAPTER IV

11 β - HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN FELINE ADIPOSE TISSUE

Abstract

The role of 11 β-Hydroxysteroid dehydrogenase (11 β-HSD) type 1 is to amplify intracellular glucocorticoid action in adipose tissue. Transgenic mice that overexpress 11βHSD1 in adipose tissue exhibit visceral obesity and the hallmarks of metabolic syndrome. In contrast, mice lacking 11βHSD1 activity in adipose tissues exhibit a protective metabolic phenotype; thus further implicating 11βHSD1 in obesity and the metabolic syndrome.

Within the last decade, obesity has become a pervasive problem in humans, and household pets have been caught up in this struggle to avoid obesity. Industry experts now agree that obesity has become the most common nutritional disorder in cats. Feline obesity further exacerbates health problems such as, diabetes mellitus and skin problems. The objective of this study was to determine if 11βHSD1 was present in feline adipose tissue, and if present to quantitate the 11βHSD1 activity in multiple adipose depots and correlate the activity to a morphological characterization of those tissues. The hypothesis of this study was that, like humans, cats exhibit regional tissue (adipose) differences in 11βHSD1 activity.

Samples (adipose tissue) were collected from six intact male cats with healthy body weights (10.13 ± 1.33), each sampled from five different adipose depots: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP). Slides of the fat samples were fixed, sectioned, and then subjected to immuno-histochemistry procedures to detect the presence of 11βHSD1. Morphological (adipocyte size) and immuno-histochemical determinations were then made. In addition, aliquots of the same samples were subjected to radiometric assay procedures to reaffirm the presence and radiometrically quantitate the activity of 11βHSD1 in the adipose depots. Data were analyzed using ANOVA using a threshold value of $P < 0.05$ to denote significance.

The adipose depot SQM had greater 11β-HSD1 activity ($P < 0.05$) compared to SQI, RP, and OM. Cat 5 had greater total 11β-HSD1 activity ($P < 0.05$) compared to cat 2 or cat 3. There were significant differences ($P < 0.05$) in adipocyte diameter between adipose depots; SQI had greater adipocyte diameters than FAL or OM, whereas RP had greater adipocyte diameters than FAL, OM, or SQM. Additionally, cat 5 had greater adipocyte diameters than cat 4 ($P < 0.05$). These data provide evidence that 11βHSD1 activity is present in cat adipose tissue, but these data fail to provide evidence of a correlation between adipocyte size and 11βHSD1 activity.

Introduction

Obesity in household cats has increased dramatically within the last decade and has become the most common nutritional disorder in cats (Donoghue and Scarlett, 1998). Furthermore, obesity exacerbates diabetes mellitus and skin problems in cats and also

increases cat's risk of urinary tract disease, and some neoplastic conditions. (Rand and Appleton, 2001; Appleton et al., 2001). Middle-aged neutered male cats are particularly susceptible, and tend to become obese more frequently that the rest of the adult cat population (Scarlett et al., 1994).

The problems associated with obesity are far ranging. Adipose tissue is not only a passive reservoir for energy storage, it is considered a complex and highly active metabolic and endocrine organ (Ahima and Flier, 2000; Fruhbeck et al., 2001). It secrets proteins actively involved in energy homeostasis and regulation of neuroendocrine, autonomic and immune function. Sex steroids, peptide hormone precursors, pro-inflammatory cytokines, and adiponectin are some of the other products secreted by adipose tissue (Flier and Spiegelman, 1996; Mohamed-Ali et al., 1998). Consequently, several metabolic abnormalities can result from excess or a deficiency of adipose tissue.

Obesity in humans is associated with hypertension, hyperglycemia, insulin resistance, dyslipidemia, and the pro-inflammatory state (Grundy et al., 2004). These conditions are not only hallmarks of the metabolic syndrome, but moreover, obesity increases the relative risk of these metabolic syndrome features (Grundy et al., 2004). Treatment of individuals with metabolic syndrome are not so straight forward, because there are confounding morphological and metabolic similarities to Cushing's syndrome in many of the affected individuals (Walker and Seckl, 2001). Cushing's syndrome is characterized by excessive peripheral concentrations of cortisol, which leads to the occurrence of secondary obesity. An interesting distinction between individuals with Cushing's syndrome versus those with metabolic syndrome is that in individuals with the

metabolic syndrome, cortisol levels are modestly, if at all, elevated (Walker and Seckl, 2001).

Most recently, the perception that circulating concentrations corticosteroids were the predominant determinant of corticosteroid action has changed. It has now been found that tissue-specific enzymes exert a far greater influence in certain tissues than could be attributed to peripheral concentrations of corticosteroids (Stulnig and Waldhäusl, 2004; Seckl and Walker, 2001). The enzyme that is responsible for this tissue-specific reaction to glucocorticoids is 11β- hydroxysteroid dehydrogenase, which catalyzes the interconversion of 11-hydroxy glucocorticoids (cortisol, corticosterone) and 11-keto forms (cortisone, 11-dehydrocorticosterone) (Amelung et al. 1953). 11β-HSD1 was found to be highly expressed in adipose tissue in humans and mice, particularly localized in the visceral adipose tissues (Stulnig and Waldhäusl, 2004; Seckl and Walker, 2001). Furthermore, it is critical to realize that the amplification of glucocorticoids concentrations in adipose tissue by 11β-HSD1 does not seem to effect systemic concentrations of glucocorticoids (Stulnig and Waldhäusl, 2004; Seckl and Walker, 2001).

The relative importance of 11β-HSD1 amplification of glucocorticoids in adipose tissue lies in the fact that 11β-HSD1 has now been directly associated with or identified as a causal factor of obesity, diabetes, hypertension, dyslipidemia, cardiovascular disease, and polycystic ovarian syndrome in humans and mice (Stulnig and Waldhäusl, 2004; Seckl and Walker, 2001). Polymorphisms in the 11β-HSD1 gene has also been linked to the predisposition to obesity (Draper et al., 2002; Gelernter-Yaniv et al., 2003). Recently, Walker and coworkers (1995) reported that pharmacological inhibition of 11β-HSD1 in humans increased insulin sensitivity. Furthermore, transgenic mice that over-express

11β-HSD1 in adipocytes had elevated local glucocorticoid concentrations in adipose tissues but normal serum glucocorticoids (Masuzaki et al., 2001) and presented features of the metabolic syndrome (hypertension, dyslipidemia, insulin resistance, and hepatic steatosis) and visceral obesity (Masuzaki et al., 2001). In contrast, 11β-HSD1 knockout mice were reported to exhibit favorable metabolic phenotypes characterized by improved insulin sensitivity and lipid profiles (Morton et al., 2001).

Recently, Schipper and coworkers (2004) confirmed the presence of 11β-HSD1 and 11β-HSD2 in feline kidney and 11β-HSD1 in feline liver. Because 11β-HSD1 is involved with obesity and metabolic syndrome in humans and mice is hypothesized to be involved in obesity and insulin resistance in cats.

The goal of this study was to determine if 11β-HSD1 activity exists in feline adipose tissue and if so, compare 11β-HSD1 activity from different adipose deposition sites. A final goal of this study was determine if there exists a correlation between adipocyte volume and 11β-HSD1 activity in the different depots.

Material and Methods

Animals

Samples were collected from six purpose-bred intact male cats during anesthesia or after euthanasia. Mean age of the cats was 2.46 ± 0.6 years and mean body weight was 10.13 ± 1.33 kg. Tissue samples were collected from each cat from two subcutaneous and three abdominal sites identified as: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP). After tissue collection the samples were frozen in liquid nitrogen and stored at - 80 °C until assayed.

11β-HSD1 enzyme activity

Several assay optimization efforts were performed to ensure robust and repeatable linearity within and across assays utilizing the feline tissues. Briefly, the tissue was minced first with scissors and after with a scalpel blade to disrupt the cells wall. Then, 100 mg of minced adipose tissue was added to borosilicate glass tubes containing 20 nM of unlabeled 11-dehydrocorticosterone (Sigma Chemical Co., UK), and ^{3}H- 11-dehydrocorticosterone. Prior to each assay, ^{3}H- 11-dehydrocorticosterone was prepared fresh from ^{3}H- corticosterone (65.0 Ci/mmol, Amersham Biosciences UK Limited) as previously described (Yang and Yu, 1994). To this mixture, 0.1 mM NADPH (Sigma, UK) was added along with 900 µL of Krebs-Ringer buffer (pH 7.4) to bring the total media volume to 1 ml. The mixture was then incubated at 37^{o}C for 30 min.

Following the incubation, the incubation media was extracted utilizing a C_{18} solid phase extraction column (J. T. Baker, USA) as described by others (Slight et al., 1993). Briefly, the columns were activated by washing the columns with 2 ml of methanol followed by 2 ml of distilled water, without drying the column following the addition of the water. The incubation media was then applied to the C_{18} solid phase extraction columns and washed with 2 ml of 1:4 methanol/water followed by 2 ml of methanol, the latter fraction of which contained steroids. This latter methanol fraction (which contained the steroids) was evaporated to dryness under nitrogen, thus concentrating steroids into the very bottom of the tubes. Acetone (200 µl) was then added to the dried extracts to wash all steroids to the bottom of the tube and to transfer the extract to TLC plates.

The steroids were then separated by TLC using a mobile phase consisting of ethanol and chloroform (8:92). Unlabeled steroid control lanes were ran in parallel with

the sample extracts for quantative control over isolation, identification, and the spatial aspect of each steroid under UV light. The spots containing the intra-converted steroids were then identified (based on the control steroids) and scrapped from the surface of the TLC plates. Thereafter the steroids within the scrapings were extracted from the powder by spinning the samples for 15 min at 1,500 rpm with 1 ml of isopropanol. The isopropanol / ^{3}H steroid mixture was then decanted into liquid scintillation counting vials and dried under nitrogen. After drying was complete, 6 ml of liquid scintillation cocktail was added (Ultima Gold, Perkin Elmer) and the samples were counted. Enzyme activity was calculated and expressed as a percentage of conversion of one steroid to its complement form.

Determination of adipocyte volume

Portions of the tissue samples, from each cat and adipose depot, were embedded in paraffin, sectioned, processed, and stained with hematoxylin and eosin. Regions of representatively sized cells were photographed at X200 magnification. The total number of cells in a 0.5 x 0.5 mm area was counted and incomplete cells were counted only if more than half of the cell was inside the designated area. The average volume of adipocytes was calculated using the formula: diameter = 1.1 x 2x [area/ cell number x π)]^{½} . Volume was calculated as: volume = (diameter x π^{3})/6 (Ashwell et al., 1976; Zhang et al., 2002).

Immuno-histochemistry

Samples of adipose tissue were embedding in paraffin and sections were cut at 4 microns and placed onto slides. Slides were microwaved briefly in order to fix the tissue to the slide. Thereafter, the slides were deparaffinized in Dako Target Retrieval solution at 90-100^{o}C for 30 min using a steamer. Endogenous peroxidase was avoided by fixing and embedding slides with solutions containing 3% H_2O_2. Background staining was minimized by incubating samples with avidin and biotin blocking solutions prior to antibody reaction. A non-serum protein blocking solution was applied to minimize non-specific protein binding. Thereafter, slides were incubated overnight at 4^{o}C with primary rabbit polyclonal anti-rat 11β-HSD1, 1:100 dilution. The next day the slides were incubated with a biotinylated anti-rabbit secondary antibody and then with peroxidase-labeled streptavidin. To enhance visualization of primary antibody staining, diaminobenzine and diaminobenzine enhancer was added. The slides were then counterstained with hematoxylin, dehydrated, and cover-slipped with permanent mounting media. A positive and negative control sample was prepared along with the samples for reference perspective.

Statistical analyses

All data were expressed as means ± standard error. Data were analyzed using ANOVA and PROC GLM procedures of SAS (SAS Inst. Inc., Cary, NC) a significance threshold set at $P < 0.05$.

Results

Immuno-histochemistry

All adipose depot samples were found to stain positively for 11β-HSD1. The staining was most intense in the nucleus of the adipocytes and macrophages present in the sections (Figure 1 and 2).

Adipocyte volume

There were significant differences ($P < 0.05$) in adipocyte diameter between adipose depots, SQI had greater adipocyte diameters than FAL or OM, whereas RP had greater adipocyte diameters than FAL, OM, or SQM (Table 2). In addition, cat 5 was found to have greater overall adipocyte diameters than cat 4 ($P < 0.05$).

11β-HSD1 enzyme activity

11β-HSD1 activity was present in all adipose tissue samples. 11β-HSD1 activity was NADPH concentration dependent in converting 11-dehydrocorticosterone into corticosterone. The adipose depot SQM had greater 11β-HSD1 activity ($P < 0.05$) compared to SQI, RP, or OM (Figure 3 and Table 3). Also, cat 5 had greater total 11β-HSD1 activity ($P < 0.05$) than cat 2 or cat 3 (Table 4). Adipocyte volume did not correlate significantly with enzymatic activity.

Discussion

These data provide immuno-histochemical and radiometric evidence of the presence of 11β-HSD1 in feline adipose tissue. This corroborates the discovery of 11β-

HSD1 mRNA expression in human adipose tissue and rat white adipose tissue (Bujalska et al., 1997; Napolitano et al., 1998). We found that all feline adipose tissue samples tested were positive for 11β-HSD1 by immuno-histochemical staining and functionally expressed 11β-HSD1 enzymatic activity. A notable, and yet expected finding was that the 11β-HSD1 enzymatic activity was NADPH concentration dependent in converting 11-dehydrocorticosterone into corticosterone. As a perspective, 3T3 cell lines, that have been used as model tissue culture systems for adipose tissue expresses only 11β-HSD1 and the reaction in those intact cells is exclusively reductase (Napolitano et al., 1998).

We anticipated in this study that we would find that different adipose depots would have different 11β-HSD1 enzymatic activity. This hypothesis was based on the observations of Bujalska et al. (1999) who reported that cultures of omental human stromal cells have greater 11β-HSD1 reductase activity than subcutaneous stromal cells. In contrast, Morton and coworkers (2003) reported that peripheral adipose 11β-HSD1 expression and activity was higher than visceral adipose expression and activity in non-obese strains of mice. These reports lead us to hypothesize that increased glucocorticoid reactivation may correlate with a specific adipose depot. In agreement with this perception, it has been reported that humans perhaps have greatest 11β-HSD1 activity in visceral adipose because this depot has more glucocorticoid receptors and/or has greater capacity for glucocorticoid-induced differentiation of immature to mature adipocytes (Masuzaki et al., 2001). An interesting observation in mice is the fact that while increased subcutaneous adipose 11 βHSD1 is associated with insulin resistance in obesity, there is no correlation with visceral adiposity or hypertension. Mice and men differ in those

aspects, although it should be noted that 11 βHSD1 activity was measured only in cell cultures from these two species and not from fresh biopsies (Tomlinson et al., 2002).

In our study we found that the SQM adipose depot had significantly more 11 βHSD1 activity than OM, SQI or RP, but not different from FAL. The reason for this difference in activity between adipose depots in cats is still unknown. Notable however is the fact that Hoenig and Rand (2005) reported that abdominal fat is the primary site of fat accumulation in cats. More recently, Hoeing and coworkers (2007) reported that the distribution of abdominal fat in obese cats is balanced between subcutaneous and intra-abdominal depots.

Implications

Confirmation of 11 βHSD1 existence and reductase activity in feline adipose tissue provides some evidence that 11 βHSD1 activity may be involved in obesity and related health problems in cats. We found differences in 11 βHSD1 activity between adipose depots and between cats. However in contrast to other reports correlating 11 βHSD1 with obesity and related health problems, we failed to find evidence to support this hypothesis in cats.

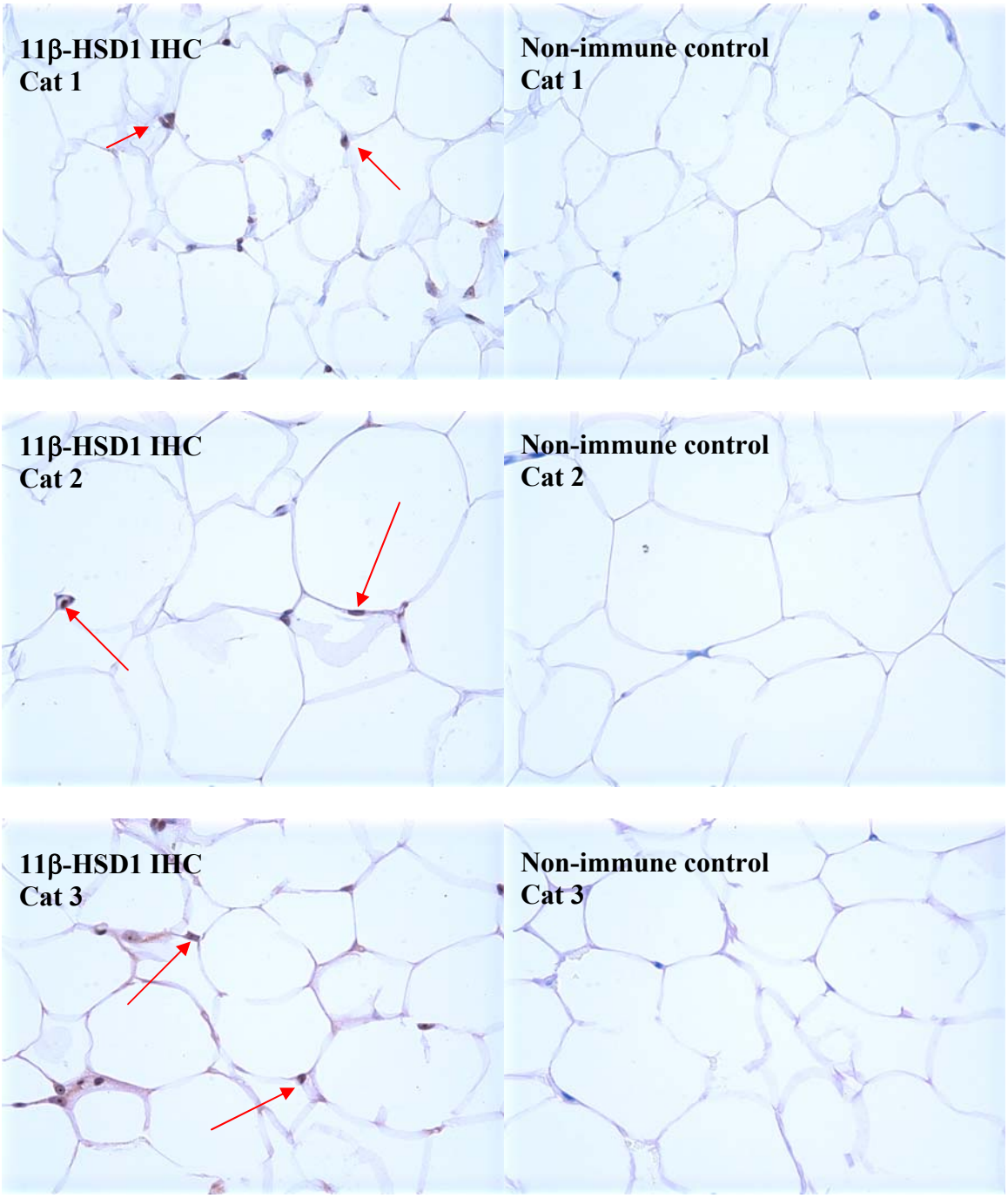

Figure 1. Sections of feline adipose tissue submitted to 11 βHSD1 immuno-histochemistry detection procedures (Cat 1, 2, and 3).

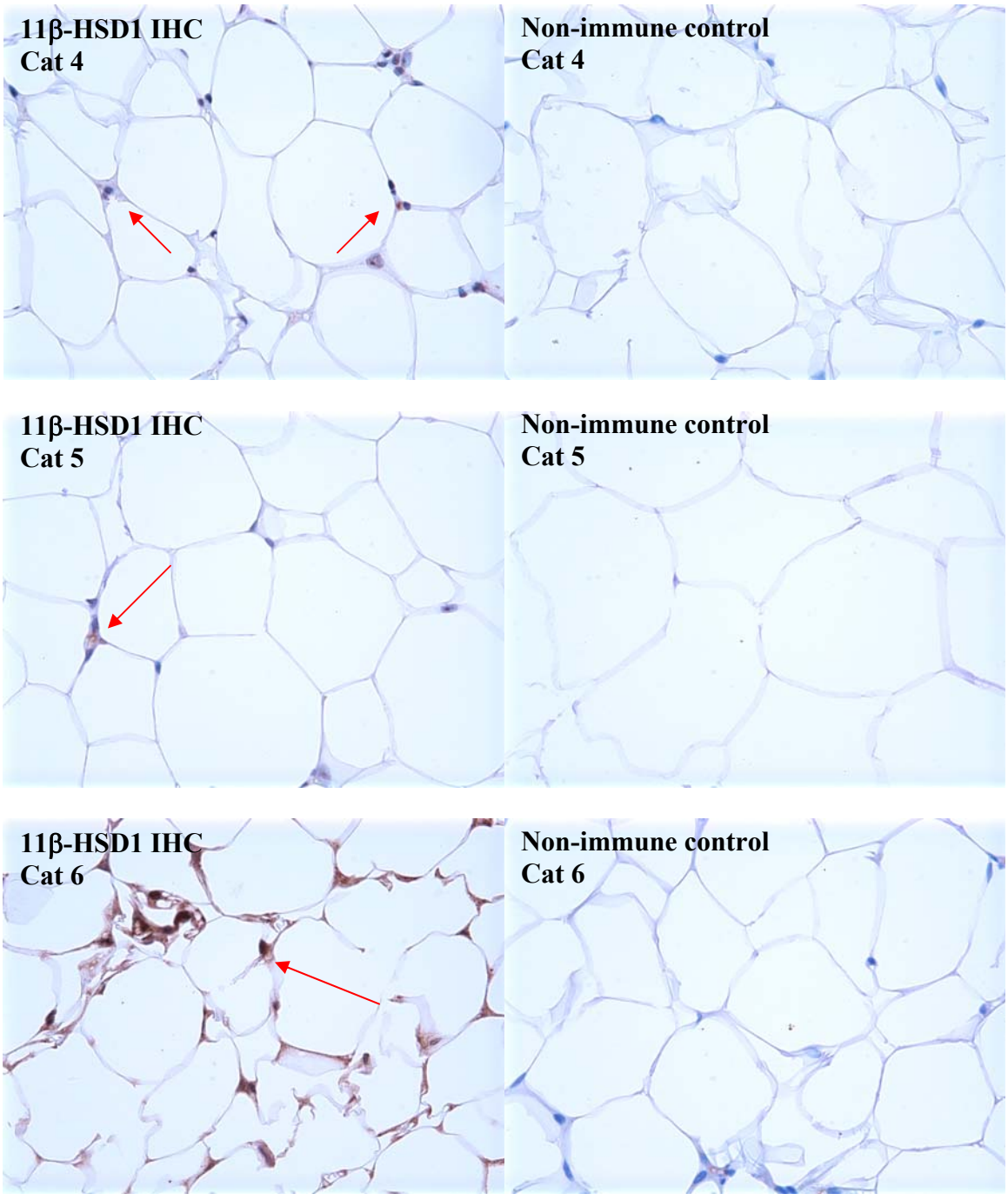

Figure 2. Sections of feline adipose tissue submitted to 11 βHSD1 immuno-histochemistry detection procedures (Cat 4, 5, and 6).

Table 2. Adipocyte diameter in different adipose depots. Adipose depots are identified as: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP).

	FAL	**OM**	**SQM**	**SQI**	**RP**
Adipocyte diameter (μm)	$101.63^{a} \pm 4.6$	$101.64^{a} \pm 9.0$	$98.96^{ab} \pm 5.6$	$91.37^{bc} \pm 10.8$	$85.35^{c} \pm 4.4$

Data are in means. Significant differences are indicated by *, $P < 0.05$.

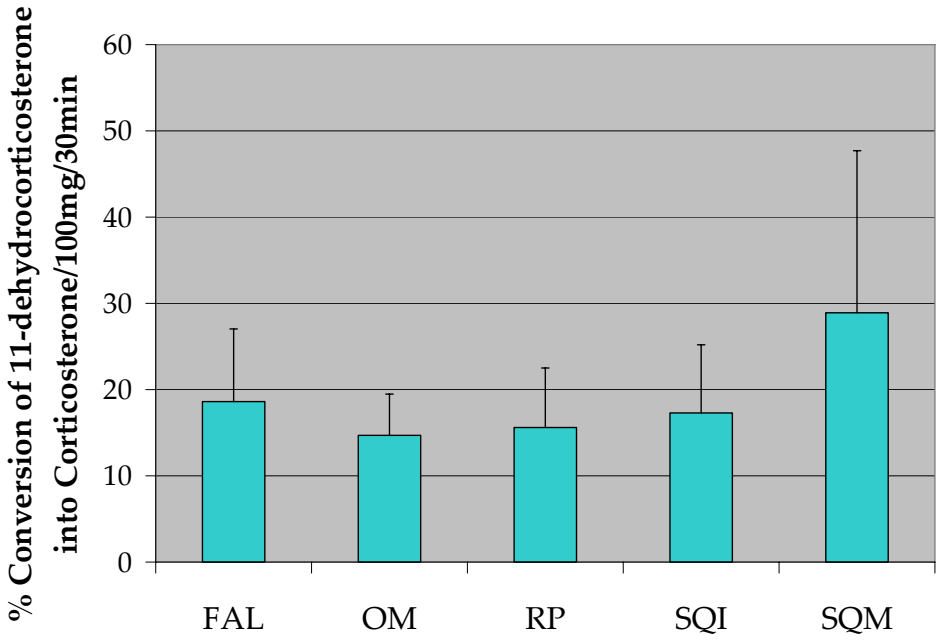

Figure 3. 11β-HSD1 enzymatic activity in feline adipose depots. Adipose depots are identified as: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP).

Table 3. 11β- HSD1 activity in different adipose depots. Adipose depots are identified as: subcutaneous midline (SQM), subcutaneous inguinal (SQI), omental (OM), falciform (FAL), and retro-peritoneal (RP).

	SQM	**FAL**	**SQI**	**RP**	**OM**
11β-HSD1 activity (% conversion)	$28.92^{a} \pm 18.8$	$18.68^{ab} \pm 8.4$	$17.32^{b} \pm 7.9$	$15.66^{b} \pm 6.9$	$14.73^{b} \pm 4.7$

Data are in means. Significant differences are indicated by letters, $P < 0.05$.

Table 4. Differences between cats in total 11β- HSD1 activity.

	1	**2**	**3**	**4**	**5**	**6**
11β- HSD1 activity (% conversion)	23.02^{ab}	13.19^{cb}	9.83^{c}	20.95^{abc}	27.28^{a}	20.10^{abc}

Data are in means. Significant differences are indicated by letters, $P < 0.05$.

CHAPTER V

11 β - HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN EQUINE ADIPOSE TISSUE

Abstract

In the horse industry there is an ever increasing incidence of obesity and in horses, this problem is significant because it predisposes the affected animal to greater risk for laminitis, infertility, and equine metabolic syndrome. In humans and mice, tissue content of 11βHSD1 enzymatic activity is correlated with obesity, diabetes, hypertension, dyslipidemia, and cardiovascular disease. Moreover, mice treated so as to over-express 11βHSD1 activity in adipose tissue exhibit characteristics of visceral obesity and metabolic syndrome. Our objective was to determine if 11βHSD1 activity exists in horse adipose tissue and, if present, to quantitate the amount of activity in abdominal vs. subcutaneous fat depots. Our hypothesis was that 11βHSD1 is present in horse adipose tissue and that its activity is greater in abdominal vs. subcutaneous fat depots. Samples of abdominal (retro-peritoneal) and subcutaneous (base of the tail) adipose tissue were collected from 23 horses. Horse body condition scores were categorized as obese (7 to 9), subtle overweight (6), normal (4 to 5), or thin (1 to 3). Fat samples from each adipose depot were submitted for immuno-histochemistry procedures to detect the presence of 11βHSD1 in abdominal and subcutaneous adipose tissue. In addition, radiometric assay procedures were used to reaffirm the presence of 11βHSD1 activity and to quantitate it in abdominal and subcutaneous adipose tissue locations. Data were analyzed by PROC

GLM and PROC MIXED procedures of SAS and a threshold for significance was set at $P < 0.05$. We found no difference in 11βHSD1 activity between subcutaneous and abdominal adipose tissue nor did its activity correlate with body condition scores. These data provide evidence that 11βHSD1 activity is present in horse subcutaneous and abdominal adipose tissue, but these data fail to provide evidence that these tissues differ in their enzymatic activity or that body condition score and 11βHSD1 activity are correlated as reported to occur in humans. It should be noted however that these observations do not rule out a potential role of 11β-HSD1 in obesity and related problems in horses.

Introduction

While the world's population of adults and children in developed nations face a pandemic occurrence of obesity, populations of companion animals are equally at risk. In the horse industry there is an ever increasing incidence of obesity, which is a particularly significant problem, because it predisposes affected horses to greater risk for laminitis, infertility, insulin resistance, and equine metabolic syndrome (Jeffcott et al., 1986; Pass et al., 1998).

Within the past decade, the function of fat tissue (adipose tissue) has undergone a transformation from what was once considered to be a static amorphous lipid storage site to what is now know to be a complex and highly dynamic metabolic and endocrine organ capable of secreting hormones and factors that are involved in energy homeostasis and regulation of neuroendocrine, autonomic, and immune function (Ahima and Flier, 2000; Fruhbeck et al., 2001). Sex steroids, glucocorticoids, peptide hormone precursors, pro-

inflammatory cytokines, and adiponectin are a few of the products secreted by adipose tissue (Flier and Spiegelman, 1996; Mohamed-Ali et al., 1998).

Glucocorticoid production by adipocytes is of particular interest because of the relationship between the corticosteroids and obesity. In humans and mice, there is a strong morphological and metabolic similarity between the Cushing's syndrome, which is caused by an excess of circulating glucocorticoids, and obesity, yet with obesity peripheral concentrations of cortisol are not necessarily elevated (Walker and Seckl, 2001). This latter paradox may be explained by increased local intracellular glucocorticoid reactivation (Jamieson et al., 1999). The enzyme 11 β-hydroxysteroid dehydrogenase (11 β-HSD) plays a critical role in peripheral metabolism of cortisol. The enzyme 11 β-Hydroxysteroid dehydrogenase (11 β-HSD) type 1 amplifies intracellular glucocorticoid by catalyzing the activation of inert cortisone into cortisol (Amelung et al., 1953). Transgenic mice that over-express 11β-HSD1 in adipocytes had elevated local glucocorticoid concentrations in adipose tissues but normal serum glucocorticoids (Masuzaki et al., 2001) and those mice also presented features of the metabolic syndrome (hypertension, dyslipidemia, insulin resistance, and hepatic steatosis) and visceral obesity (Masuzaki et al., 2001). In contrast, 11β-HSD1 knockout mice were reported to exhibit favorable metabolic phenotypes characterized by improved insulin sensitivity and lipid profiles (Morton et al. 2001).

Based on these observations, our hypothesis was that 11 βHSD1 enzymatic activity could be found in equine adipose tissue and that the enzymatic activity of 11 βHSD1 was dependent on the regional differences in expression; notably those regions

most susceptible to fat accumulation. We also sought to determine if a correlation existed between body condition score and 11β-HSD1 activity in the adipose depots.

Material and Methods

Animals

Samples of abdominal and subcutaneous adipose tissue were collected from 23 horses after euthanasia. The samples were always collected from the same location from each horse. More specifically, subcutaneous fat was obtained from the fat pad located just cranial to the base of the tail, and intra-abdominal fat was acquired from the lining of the ventral aspect of the abdominal body wall at the level of intercostal space 12. Horses were from different breeds, both sexes, and had ages ranging from 4 to 28 years. Body condition (range 1 to 9 = obese) were assessed according to the procedure described by Henneke et al. (1983). Horses that scored: 9 to 7 = obese (O), scored 6 = subtle overweight (SO), scored 5 to 4 = normal (N), or scored 3 to 1 = thin (T). Immediately following the collection of the adipose samples they were directly frozen in liquid nitrogen and stored at -80 °C until assayed.

Immuno-histochemistry

Portions of each sample of adipose tissue was embedding in paraffin and sections were cut at 4 microns and placed on glass slides. Slides were microwaved briefly in order to fix the tissue on the slide. Thereafter, the slides were deparaffinized in Dako Target Retrieval solution at 90 to 100^{o}C for 30 min using a steamer. Endogenous peroxidase activity was minimized by incorporating 3% H_2O_2 during the embedding process.

Background staining was minimized by pre-incubating the samples with avidin and biotin blocking solution. A non-serum protein blocking solution was applied to minimize the occurrence of non-specific protein binding. Thereafter, the slides are incubated overnight at 4^{o}C with primary rabbit polyclonal anti-rat 11β-HSD1 1:100 dilution. The next day the slides were incubated with a biotinylated anti-rabbit secondary antibody and then peroxidase-labeled streptavidin was applied. To enhance visualization of primary antibody staining, diaminobenzine and diaminobenzine enhancer were applied. Slides were then counterstained with hematoxylin, dehydrated, and cover-slipped with permanent mounting media. Both positive and negative control slides were processed concurrent with the samples for reference perspective.

11β-HSD1 enzyme activity

Several assay optimization efforts were performed to ensure robust and repeatable linearity within and across assays utilizing the feline tissues. Briefly, the tissue was minced first with scissors and after with a scalpel blade to disrupt the cells wall. Then, 100 mg of minced adipose tissue was added to borosilicate glass tubes containing 2.5 nM of unlabeled cortisone (Sigma Chemical Co., UK), and ^{3}H- cortisone (40 Ci/mmol, Amersham Biosciences UK Limited) as previously described (Yang and Yu, 1994). To this mixture, 1 mM NADPH (Sigma, UK) was added along with 900 µL of Krebs-Ringer buffer (pH 7.4) to bring the total media volume to 1 ml. The mixture was then incubated at 37^{o}C for 24 hours.

Following the incubation, the incubation media was extracted utilizing a C_{18} solid phase extraction column (J. T. Baker, USA) as described by others (Slight et al., 1993).

Briefly, the columns were activated by washing the columns with 2 ml of methanol followed by 2 ml of distilled water, without drying the column following the addition of the water. The incubation media was then applied to the C_{18} solid phase extraction columns and washed with 2 ml of 1:4 methanol/water followed by 2 ml of methanol, the latter fraction of which contained steroids. This latter methanol fraction (which contained the steroids) was evaporated to dryness under nitrogen, thus concentrating steroids into the very bottom of the tubes. Acetone (200 µl) was then added to the dried extracts to wash all steroids to the bottom of the tube and to transfer the extract to TLC plates.

The steroids were then separated by TLC using a mobile phase consisting of ethanol and chloroform (8:92). Unlabeled steroid control lanes were ran in parallel with the sample extracts for quantative control over isolation, identification, and the spatial aspect of each steroid under UV light. The spots containing the intra-converted steroids were then identified (based on the control steroids) and scrapped from the surface of the TLC plates. Thereafter the steroids within the scrapings were extracted from the powder by spinning the samples for 15 min at 1,500 rpm with 1 ml of isopropanol. The isopropanol / ^{3}H steroid mixture was then decanted into liquid scintillation counting vials and dried under nitrogen. After drying was complete, 6 ml of liquid scintillation cocktail was added (Ultima Gold, Perkin Elmer) and the samples were counted. Enzyme activity was calculated and expressed as a percentage of conversion of one steroid to its complement form.

Statistical analyses

Data were analyzed by PROC GLM and PROC MIXED procedures of SAS (SAS Inst. Inc., Cary, NC) with a significance threshold set at $P < 0.05$.

Results

Immuno-histochemistry

All equine adipose tissue samples exhibited positive staining for 11β-HSD1. The staining was most intense in the nucleus of the adipocytes and in macrophages present in the sections (Figure 4).

11β-HSD1 enzyme activity

11β-HSD1 activity was present in all adipose tissue samples (Figure 4), however, there was no significant difference in 11β-HSD1 activity among the different depots sampled (Figure 5). Finally, there was also no correlation between 11β-HSD1 activity and body condition categories of the horse (Figure 6).

Discussion

These data provide evidence that 11β-HSD1 enzymatic activity is present in horse adipose tissue, as confirmed by both immuno-histochemistry and radiometric assay procedures used to quantitate activity of 11βHSD1 in those tissues. The 11β-HSD1 enzyme activity that was found was NADPH dependent in converting cortisone into cortisol. This corroborates the notion that the preponderance of 11β-HSD1 activity is believed to be dictated by the co-localization with hexose-6-phosphate dehydrogenase,

which is a NADPH-generating enzyme (Ozols, 1995; Draper et al., 2003). Previously, Bujalska et al. (1999) reported that fresh isolates from adipocytes have mainly 11β-HSD1 reductase activity in humans.

Paulmyer-Lacroix et al. (2002) reported that obese humans exhibited the greatest amount of 11β-HSD1 gene expression in subcutaneous and visceral adipose tissue when compared to lean subjects. Increased 11β-HSD1 gene expression has been associated with increased 11β-HSD1 enzymatic activity (Stewart and Krozowski, 1999; Tomlinson et al., 2000). However, Tomlinson et al. (2002) reported that adipose tissue, adipocytes and preadipocytes did not exhibit differences in 11β-HSD1 mRNA levels between omental and subcutaneous depots.

In this study, we found that 11β-HSD1 activity did not differ between subcutaneous and visceral adipose depots, nor did it differ with body condition score, age or gender of the horse. These data should be interpreted with caution, because even though no differences in 11β-HSD1 activity level were found it does not rule out a potential role of 11β-HSD1 in obesity and related problems in the equine.

Implications

This study provides evidence that 1β-HSD1 reductase activity exists in subcutaneous and visceral adipose tissue in horses, but the 1β-HSD1 level of activity did not differ, regardless of the tissue depot studied, body condition score, age or gender of the horse.

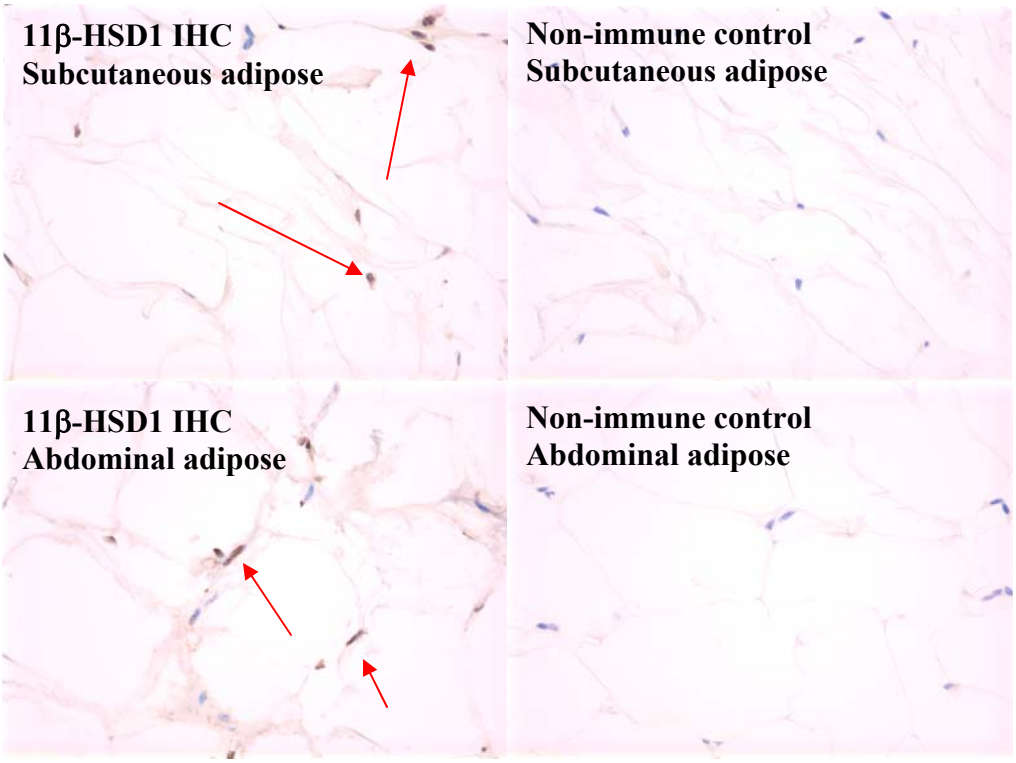

Figure 4. Positive and negative staining for 11β-HSD1 in equine adipose tissue.

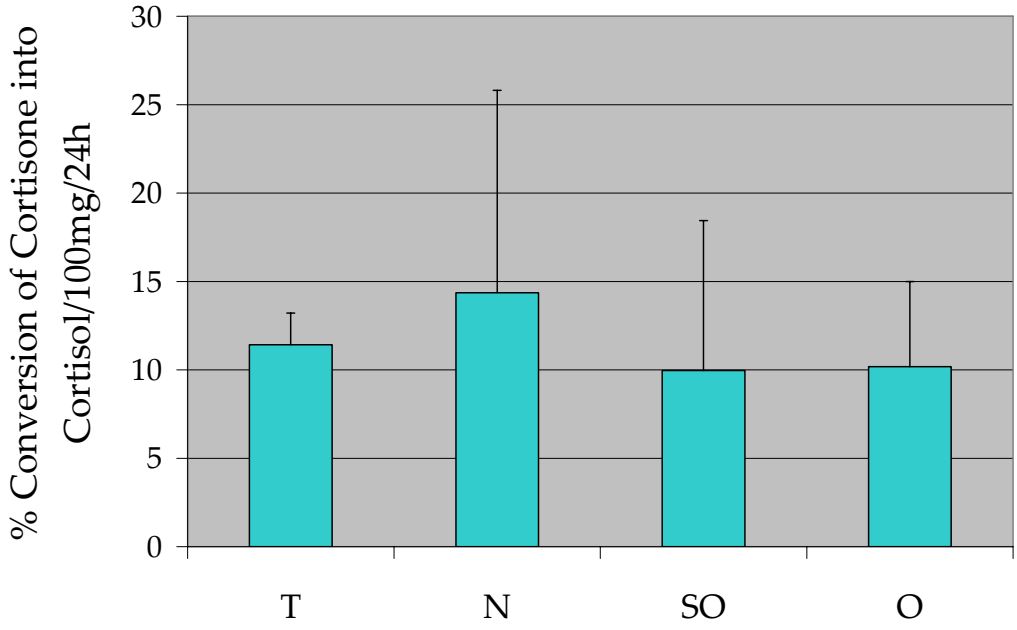

Figure 5. 11β-HSD activity in subcutaneous and abdominal adipose tissue from horses with different body condition scores. Horses that scored: 9 to 7 = obese (O), scored 6 = subtle overweight (SO), scored 5 to 4 = normal (N), or scored 3 to 1 = thin (T).

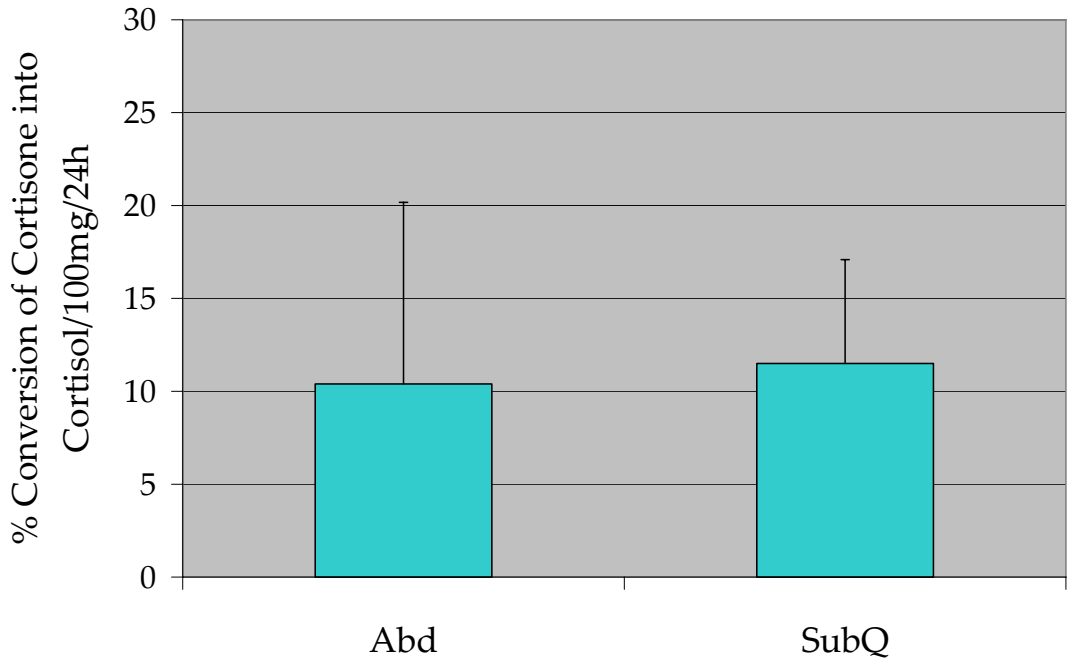

Figure 6. 11β HSD1 activity in horse abdominal (Abd) and subcutaneous (SubQ)adipose tissue.

CHAPTER VI

11 β - HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN ADIPOSE TISSUE OF OSSABAW PIGS

Abstract

11 β - hydroxysteroid dehydrogenase type 1 (11 βHSD1) plays a critical role in amplifying levels of cortisol in adipose tissues; and cortisol is known to be involved in visceral obesity and metabolic syndrome. Therefore, 11 βHSD1 has been considered a promising target to ameliorate some of the aspect of the metabolic syndrome. In the current study, Ossabaw pigs were utilized because of their metabolic similarities to humans, and when fed high fat diets become obese, insulin resistant and glucose intolerant, hypertriglyceridemic, and hypercholesterolemic. Our objective was to quantitate the amount of 11βHSD1 activity in mesenteric vs. subcutaneous adipose tissue from Ossabaw pigs. Our hypothesis was that 11βHSD1 activity would be more abundant in mesenteric vs. subcutaneous adipose tissue. Thus, mesenteric and subcutaneous adipose tissue samples were collected from eight Ossabaw female pigs, and radiometric assay procedures were used to quantitate activity of 11βHSD1 in those tissues. We found that 11βHSD1 activity did not differ between subcutaneous and mesenteric adipose tissues, but this does not rule out a potential role of 11β-HSD1 in obesity and related problems in Ossabaw pigs.

Introduction

Obesity is a major human health concern of pandemic proportions that is particularly prevalent in adults and children in developed nations. In humans, obesity is associated with hypertension, hyperglycemia, insulin resistance, dyslipidemia, and a pro-inflammatory state. Each of these scenarios is also descriptive of a complex referred to as the "metabolic syndrome", and as follows, obesity increases the relative risk of the occurrence of the metabolic syndrome. Moreover, the metabolic syndrome also shares strong morphological and metabolic similarities to Cushing's syndrome. Cushing's syndrome is characterized by excessive peripheral concentrations of cortisol, which exacerbates obesity. However, it is intriguing that in patients with metabolic syndrome, peripheral concentrations of cortisol are at best only slightly elevated.

Several studies have now begun to focus more on the role of peripheral tissue-specific enzymes on localized tissue response; in particular 11β- hydroxysteroid dehydrogenase activity (11β-HSD; Amelung et al., 1953). The uniqueness of 11β-HSD is that it is a cellular component of certain cells (ex. adipocytes), which functions to metabolize glucocorticoids by catalyzing the interconversion of 11-hydroxy glucocorticoids (cortisol, corticosterone) and 11-keto forms (cortisone, 11-dehydrocorticosterone). In effect 11β-HSD1 amplifies the action of glucocorticoids in adipose tissues and its activity level has been positively correlated with incidence of obesity, diabetes, hypertension, dyslipidemia, cardiovascular disease, and polycystic ovarian syndrome in humans and mice (Stulnig and Waldhäusl, 2004; Seckl and Walker, 2001). Polymorphisms in the 11β-HSD1 gene have also been associated with predisposition to obesity (Draper et al., 2002; Gelernter-Yaniv et al., 2003). Recently,

Walker and coworkers (1995) reported that pharmacological inhibition of 11β-HSD1 in humans increased insulin sensitivity. Furthermore, transgenic mice that over-express 11β-HSD1 in adipocytes had elevated local glucocorticoid concentrations in adipose tissues but normal serum glucocorticoids (Masuzaki et al., 2001) and those mice also presented features of the metabolic syndrome (hypertension, dyslipidemia, insulin resistance, and hepatic steatosis) and visceral obesity (Masuzaki et al., 2001). In contrast, 11β-HSD1 knockout mice were reported to exhibit favorable metabolic phenotypes characterized by improved insulin sensitivity and lipid profiles (Morton et al., 2001). Most recently, swine models for human health have come into vogue, as swine share several similarities with humans, such as omnivorous habits, propensity to sedentary habits and obesity, lipoprotein metabolism, and a similar cardiovascular system (Douglas, 1972; Mahley et al., 1975; Lee, 1986; Bell and Gerrity, 1992). In particular, Ossabaw pigs have come of interest because these pigs, when fed high fat diets in captivity become obese, insulin resistant and glucose intolerant, hypertriglyceridemic, and hypercholesterolemic (Martin et al. 1973; Wangsness et al., 1977; Etherton and Kris-Etherton, 1980; Wangsness et al., 1980).

The goal of this study was to determine if there 11β-HSD1 activity exists in Ossabaw pig adipose tissue and if so, to determine if 11β-HSD1 activity differs between subcutaneous and mesenteric adipose depots. Because pigs Ossabaw exhibit characteristics typical of the metabolic syndrome in humans we expected to find differences in 11β-HSD1 activity between subcutaneous and mesenteric adipose depots, similar to what has been reported in humans.

Materials and Methods

Animals

Samples of mesenteric and subcutaneous adipose tissue were collected from eight Ossabaw pigs after euthanasia. The Ossabaw pigs were females that ranged in age from 1.5 to 2 years of age. Immediately following adipose tissue collection, the samples were directly frozen in liquid nitrogen and stored at - 80 °C until assayed.

11β-HSD1 enzyme activity

Several assay optimization efforts were performed to ensure robust and repeatable linearity within and across assays utilizing the porcine tissues. Briefly, the tissue was minced first with scissors and after with a scalpel blade to disrupt the cells' walls. Then, 200 mg of minced adipose tissue was added to borosilicate glass tubes containing 2.5 nM of unlabeled cortisone (Sigma Chemical Co., UK), and ^{3}H- cortisone. (40 Ci/mmol, Amersham Biosciences UK Limited). To this mixture, 0.2 mM NADPH (Sigma, UK) was added along with 800 µL of Krebs-Ringer buffer (pH 7.4) to bring the total media volume to 1 ml. The mixture was then incubated at 37^{o}C for 1 hour.

Following the incubation, the incubation media was extracted utilizing a C_{18} solid phase extraction column (J. T. Baker, USA) as described by others (Slight et al., 1993). Briefly, the columns were activated by washing the columns with 2 ml of methanol followed by 2 ml of distilled water, without drying the column following the addition of the water. The incubation media was then applied to the C_{18} solid phase extraction columns and washed with 2 ml of 1:4 methanol/water followed by 2 ml of methanol, the latter fraction of which contained steroids. This latter methanol fraction (which contained

steroids) was evaporated to dryness under nitrogen, thus concentrating steroids into the very bottom of the tubes. Acetone (200 ul) was then added to the dried extracts to wash all steroids to the bottom of the tube and to transfer the extract to TLC plates.

The steroids were then separated by TLC using a mobile phase consisting of ethanol and chloroform (8:92). Unlabeled steroid control lanes were ran in parallel with the sample extracts for quantative control over isolation, identification, and the spatial aspect of each steroid under UV light. The spots containing the intra-converted steroids were then identified (based on the control steroids) and scrapped from the surface of the TLC plates. Thereafter the steroids within the scrapings were extracted from the powder by spinning the samples for 15 min at 1,500 rpm with 1 ml of isopropanol. The isopropanol / ^{3}H steroid mixture was then decanted into liquid scintillation counting vials and dried under nitrogen. After drying was complete, 6 ml of liquid scintillation cocktail was added (Ultima Gold, Perkin Elmer) and the samples were counted. Enzyme activity was calculated and expressed as a percentage of conversion of one steroid to its complement form.

Statistical analyses

Data were analyzed by Proc GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with a significance threshold set at $P < 0.05$.

Results

Despite the fact that 11β-HSD1 activity was detectable in the subcutaneous and mesenteric adipose tissue of Ossabaw pigs, there was no significant difference in enzyme activity levels between these two adipose depots.

Discussion

11 β-HSD1 activity was detectable in the subcutaneous and mesenteric adipose tissue of Ossabaw pigs, however in contrast to an earlier report utilizing Yucatan pigs (Slight et al., 2003), there was no significant difference in 11 β-HSD1 activity between mesenteric and subcutaneous adipose tissue depots. The 11 β-HSD1 enzyme activity that was found was NADPH dependent in converting cortisone into cortisol. This corroborates the notion that the preponderance of 11 β-HSD1 activity is believed to be dictated by the co-localization with hexose-6-phosphate dehydrogenase, which is a NADPH-generating enzyme (Ozols, 1995; Draper et al., 2003). Previously, Bujalska and coworkers reported that fresh isolates from adipocytes have mainly 11 β-HSD1 reductase activity.

In humans, the effect of glucocorticoids on adipocyte differentiation is most notable clinically in individuals with Cushing's syndrome. These affected individuals exhibit pronounced central or abdominal obesity which is also one of the characteristics of the metabolic syndrome (Rebuffe-Scrive et al., 1988; Mayo-Smith et al., 1989). It has also been asserted that the prevalence of abdominal obesity in Cushing's syndrome individuals is associated with a high level of glucocorticoid receptor mRNA expression in the abdominal adipose tissue (Masuzaki et al., 2001). While contrasting the human, mice

that exhibit high levels of 11 βHSD1 in the subcutaneous adipose tissues also exhibit insulin resistance with obesity and there is no correlation between 11 βHSD1 activity with visceral adiposity or hypertension. Mice and men differ on those aspects, although these data should be interpreted with caution, as 11 βHSD1 was measured in cell cultures and not fresh biopsies (Tomlinson et al., 2002).

In 1999, Bujalska et al., reported that more 11β-HSD1 activity was resident in omental adipose than in subcutaneous adipose. Morton et al., (2003) reported that 11β-HSD1 expression and activity was greater in peripheral adipose depots than visceral adipose depots in non-obese strains of mice. Our data provide evidence that 11β-HSD1 activity does not differ between mesenteric versus subcutaneous adipose depots in Ossabaw pigs.

Implications

This study provides evidence that 1β-HSD1 reductase activity exists in mesenteric and subcutaneous adipose tissue of Ossabaw pigs, but the level of activity did not differ between the two depots. These data should be interpreted with caution, because even though no differences in activity level existed it does not rule out a potential role of 11β-HSD1 in obesity and related problems in Ossabaw pigs.

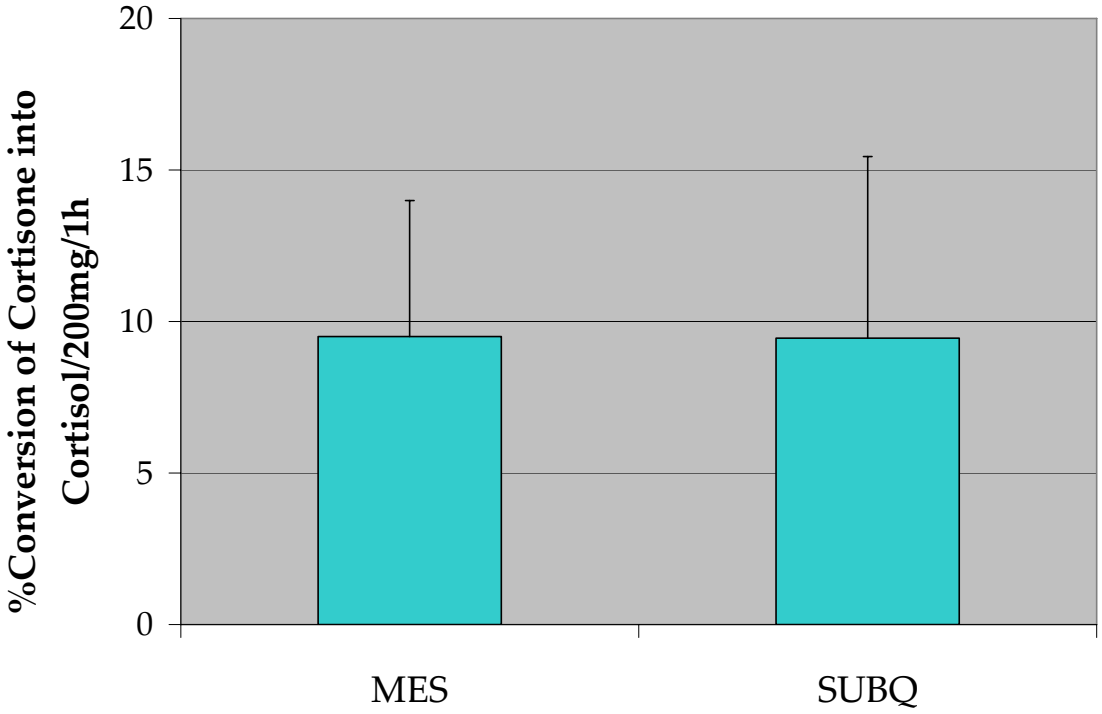

Figure 7. 11β HSD1 activity in Ossabaw pigs; mesenteric (MES) and subcutaneous adipose tissue (SUBQ).

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