ESTROGEN RECEPTOR REGULATION OF GLUCOSE AND FAT METABOLISM IN THE SKELETAL MUSCLE AND ADIPOSE TISSUE

BY

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ABSTRACT

Clinical studies suggest that postmenopausal women are at an increased risk for type 2 diabetes (T2D), and hormone replacement therapy can ameliorate this risk. Considerable clinical and experimental evidence exists demonstrating the ability of estrogen to modulate glucose metabolism in insulin responsive tissues such as the skeletal muscle and adipose tissue. Specifically, previous studies suggest estrogen receptor α (ERα) is involved in estrogen-mediated regulation of metabolism and is critical for the maintenance of whole body insulin action. However, very little is known regarding the mechanisms of action of ERα in insulin-responsive tissues.

In addition, clinical evidence demonstrates that many women gain weight following menopause. This increase in body weight is accompanied by an increase in abdominal adipose tissue, which greatly increases one's risk for T2D. These studies are supported by animal models of ovariectomy (OVX) in which removal of the ovaries results in increased total body weight and fat pad weight, which are ameliorated by estrogen treatment. However, the mechanism of estrogen's action remains unknown.

The purpose of our studies was to determine the effect of an obesity promoting high-fat diet (HFD) on skeletal muscle and adipose tissue estrogen receptor regulation and glucose metabolism in female rats with and without OVX. Furthermore we determined the effects of specific ERα activation on skeletal muscle glucose metabolism and adipose tissue triglyceride regulation.

We found that a HFD decreased whole body glucose intolerance, without decreasing insulin-stimulated skeletal muscle glucose uptake, as previously found in male

animal models. In female animal models, the HFD-induced decrease in whole body glucose tolerance likely occurred from alterations in the adipose tissue such as decreased glucose transporter 4 and ERα protein levels and increased activation of stress kinases.

Furthermore, specific activation of ERα increased glucose uptake and potentiated the insulin signaling pathway in skeletal muscle. In addition, specific ERα activation decreased body weight and fat pad weight, decreased proteins involved in lipogenesis, and increased proteins involved in lipolysis.

This information suggests novel roles of ERα in skeletal muscle glucose metabolism and adipocyte regulation and may help explain the metabolic differences between premenopausal and postmenopausal women.

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INTRODUCTION

INTRODUCTION

Obesity and metabolic syndrome.

Throughout recent history, epidemic rates of obesity emerged as a result of changes in our environment and lifestyle. The 2007-2008 National Health and Nutrition Examination Survey (NHANES) determined that obesity manifests 33.8% of the adult U.S. population (Flegal et al. 2010). The alarming obesity rate raises concern, as obesity greatly increases one's risk for type 2 diabetes (T2D), hypertension, and dyslipidemia, all of which belong to a constellation of metabolic abnormalities termed "metabolic syndrome" (Zimmet et al. 2005).

The origin of metabolic syndrome began almost 90 years ago when a physician noted that hyperglycemia and hypertension often occurred in parallel (Kylin 1923). The metabolic syndrome expanded its characteristics in 1979 when obesity, particularly upper body or central obesity, was also found to be associated with hyperglycemia and hypertension (Vague 1956). In 1988, Reaven brought to light the importance of understanding metabolic syndrome, or syndrome X, and spurred research focused on this cluster of metabolic abnormalities which increases one's risk of cardiovascular disease (Reaven 1988).

The criteria defining metabolic syndrome evolved over the past 20 years with reputable organizations such as the World Health Organization, the European Group for Studying Insulin Resistance, and the National Cholesterol Education Program all setting guidelines for diagnosing metabolic syndrome (NCEP 2001; Einhorn et al. 2003; Alberti et al. 2005). These guidelines include four common criteria: 1) hyperglycemia or T2D, 2) obesity, 3) dyslipidemia, and 4) hypertension. The differences in the definition of metabolic syndrome include putting importance on one of the criteria and detection method and definition of the criteria. The differences in criteria led to confusion in the clinical and research field and make comparisons among various research studies difficult. Therefore, these problems encouraged the International Diabetes Federation (IDF) to introduce a standardized definition of metabolic syndrome (Zimmet et al. 2005). In 2005, the IDF released a worldwide definition of metabolic syndrome which places an importance on central obesity. People are diagnosed with metabolic syndrome if they have central obesity and two of the following four criteria: 1) raised triglycerides, 2) reduced HDL-cholesterol, 3) raised blood pressure, and 4) raised fasting plasma glucose.

Central obesity prevailed as the essential component of metabolic syndrome as it independently increases one's risk for cardiovascular disease and the four criteria of metabolic syndrome (Fujioka et al. 1987; Despres et al. 1989; Seidell et al. 1990; Fujimoto et al. 1994; Kissebah and Krakower 1994; Boyko et al. 1995; Carey et al. 1997; Zimmet et al. 2001; Hu et al. 2004), and it is the number one risk factor for developing T2D (Kissebah and Peiris 1989; Chan et al. 1994; Despres 2006). Of the four criteria for metabolic syndrome, raised fasting plasma glucose, or T2D, places the greatest burden on

the U.S. According to the Centers for Disease Control and Prevention (CDC), T2D is the 7th leading cause of death in the U.S., and it is also one of the main causes of mortality and morbidity worldwide (Saltiel and Kahn 2001). In 2008, the CDC released a special press bulletin stating that the prevalence of diagnosed T2D in the U.S. increased by 14% from 2007 to 2008. The current diabetic population reached 24 million in 2008, which is nearly 8% of the U.S. population. In addition to these diagnosed cases of T2D, the number of people with undiagnosed T2D and those at risk for T2D totals an additional 57 million people. As such, research focused on the pathogenesis of obesity and T2D will give great insight to this widespread epidemic. Furthermore, the development of new treatment methods has the potential to greatly reduce mortality and morbidity on a global scale.

Diabetes.

The Merriam-Webster dictionary defines diabetes as "excretion of excessive amounts of urine". Diabetes as a whole encompasses various conditions which are divided into two sub-categories: mellitus and insipidus. Diabetes insipidus results from a deficiency in antidiuretic hormone or a resistance to antidiuretic hormone in the kidneys, both of which result in excessive urination. However, the term "diabetes" often refers to diabetes mellitus which results from a deficiency in insulin or a resistance to insulin. Diabetes mellitus includes two main sub-groups: type 1 diabetes (T1D) and T2D. T1D

accounts for 5-10% of all diabetes mellitus cases and results from a deficiency in insulin (CDC 2010). Insulin deficiency is thought to result from an autoimmune response which destroys the insulin-secreting β cells in the pancreas (Knip and Siljander 2008). Diagnosis of T1D commonly occurred in children, and therefore, was formerly called juvenile onset diabetes. T1D also requires treatment of exogenous insulin, and therefore, was formerly called insulin dependent diabetes (Cohen and Shaw 2007). However, the terms juvenile onset and insulin dependent diabetes are no longer used due to the inaccuracy of the terms. Today, adults are also diagnosed with T1D, and the alarming obesity rate in children has greatly increased the incidence of T2D in children (Klein et al. 2004). Furthermore, while those with T1D are insulin dependent, people with T2D may also become insulin dependent.

T2D accounts for 90-95% of all cases of diabetes mellitus and results from insulin resistance (CDC 2010). In the early stages of T2D, the pancreatic β cells produce insulin, but tissues are non-responsive to the insulin. As the pancreas tries to compensate by producing higher levels of insulin, β cell failure and insulin deficiency ultimately results (Weir and Bonner-Weir 2007). This insulin deficiency leads to a requirement of exogenous insulin to treat the disease (Prentki and Nolan 2006). Thus, as T2D was formerly known as non-insulin dependent diabetes, this term is no longer used. Diagnosis of T2D commonly occurred in adults, and therefore, was also called adult onset diabetes. However, as mentioned previously, the increase in childhood obesity and T2D makes this term invalid.

While T1D and T2D make up the most of the diabetic population, other forms of diabetes also exist. Gestational diabetes develops in 2-5% of all pregnancy and results in insulin resistance (CDC 2010). Gestational diabetes disappears after the pregnancy, although women who have gestational diabetes have up to a 70% greater risk for developing T2D later in life (Kim et al. 2002; Ben-Haroush et al. 2004). Other types of diabetes can result from specific genetic syndromes, surgery, drugs, malnutrition, infections, and other illnesses and account for 1-2% of all diagnosed cases of diabetes (CDC 2010).

Diagnosis of Diabetes.

The American Diabetes Association (ADA) set guidelines for diagnosing diabetes (ADA 2010a). Two commonly used tests to diagnose diabetes include the fasting plasma glucose (FPG) test and the oral glucose tolerance test (OGTT). The FPG determines one to be diabetic if fasting glucose levels are $\geq$ 126 mg/dL. Normal FPG levels are below 100 mg/dL, and individuals with FPG levels between 100 mg/dL and 125 mg/dL are considered pre-diabetic. When using the OGTT, patients with blood glucose levels $\geq$ 200 mg/dL two hours after consuming a glucose load are considered diabetic. Blood glucose levels should be < 140 mg/dL two hours after consuming a glucose load, and individuals with blood glucose levels between 140 mg/dL and 199 mg/dL are considered pre-diabetic. In January 2010 the ADA established an additional parameter for diagnosing

diabetes (ADA 2010b). Measuring glycated hemoglobin A1c (HbA1c) now serves as an additional means to diagnose diabetes. HbA1c indicates a person's average blood glucose over the past three months and is commonly used to assess how patients are managing their diabetes. A person without diabetes should have a HbA1c less than 5%. Under the new guidelines, a HbA1c between 5.7-6.4% indicates pre-diabetes and $\geq$ 6.5% indicates diabetes. The ADA recommends diabetics to keep their HbA1c below 7% (ADA 2010a).

Sex differences and T2D.

Differences exist between females and males with respect to the prevalence of T2D in human studies and inducing T2D in animal models. The prevalence of T2D is greater in males compared to females in developed nations such as Australia and Sweden (Welborn et al. 1989; Andersson et al. 1991). While a 2006 report from NHANES concluded that the prevalence of diagnosed T2D in the U.S. is similar by sex, the prevalence of undiagnosed diabetes and pre-diabetes is greater in males (Cowie et al. 2006). In addition, numerous human studies show greater insulin sensitivity in females (Nuutila et al. 1995; Donahue et al. 1997; Nilsson et al. 2000; Borissova et al. 2005; Vistisen et al. 2008; Hoeg et al. 2009; Karakelides et al. 2010).

In human studies, measuring whole body insulin sensitivity by the euglycemic-hyperinsulinemic clamp remains the gold standard (DeFronzo et al. 1979; Angioni et al.

2008). The euglycemic-hyperinsulinemic clamp involves the simultaneous infusion of glucose and insulin into the bloodstream with the purpose of maintaining constant blood glucose levels. During the process, the glucose infusion rate is measured. A higher glucose infusion rate (to keep the blood glucose levels constant) signifies greater glucose disposal into insulin-responsive tissues, and hence, greater insulin sensitivity. Numerous studies using the euglycemic-hyperinsulinemic clamp in healthy weight subjects demonstrate higher glucose infusion rates in pre-menopausal women than in age-matched men, which indicate greater insulin sensitivity in women (Nuutila et al. 1995; Nilsson et al. 2000; Borissova et al. 2005; Hoeg et al. 2009; Karakelides et al. 2010). The greater insulin sensitivity in women was present despite a greater number of total insulin receptors, high-affinity insulin receptors, and percent of receptors bound to insulin in males (Borissova et al. 2005). Some studies in these pre-menopausal women also demonstrate lower fasting glucose (Nilsson et al. 2000; Karakelides et al. 2010) and lower insulin secretion during an OGTT than men (Donahue et al. 1997), which suggests better glucose control in women. However, fasting insulin levels do not appear to be different between men and women (Nilsson et al. 2000). The increased whole body insulin sensitivity in women can be explained by greater glucose uptake into skeletal muscle (Nuutila et al. 1995; Hoeg et al. 2009). Nuutila et al. demonstrated a 47% greater rate of skeletal muscle glucose uptake in women during the euglycemic-hyperinsulinemic clamp, with rates of glucose uptake in cardiac muscle similar between men and women (Nuutila et al. 1995).

In addition to healthy weight women having greater insulin sensitivity than men, obese pre-menopausal women also demonstrate lower glucose infusion rates during the euglycemic-hyperinsulinemic clamp compared to obese men, indicating greater insulin sensitivity in obese women. In contrast to healthy weight subjects, no difference in fasting glucose is present between obese women and men (Vistisen et al. 2008).

In contrast to these studies showing greater insulin sensitivity in women, no difference in insulin sensitivity was present between healthy weight pre-menopausal women and men in dexamethasone-induced insulin resistance. Dexamethasone is a glucocorticoid analogue that induces insulin resistance by increasing plasma free fatty acids (Venkatesan et al. 1987; Guillaume-Gentil et al. 1993; Tappy et al. 1994) and decreasing skeletal muscle glucose uptake (Carter-Su and Okamoto 1985).

Numerous animal models exist to study T2D, with the high-fat feeding model being one of the most commonly used. The high-fat feeding model assess changes in adiposity, glucose metabolism, fatty acid regulation, and numerous other cellular mechanisms related to the pathogenesis and development of insulin resistance, glucose intolerance, and T2D. Most previous studies show that female rodents are less susceptible to T2D as a result of high-fat feeding than males (Corsetti et al. 2000; Coatmellec-Taglioni et al. 2002; Yakar et al. 2006; Hong et al. 2009). A 10 week HFD (50% kcal from fat) in male Sprague Dawley rats resulted in a significant increase in body weight, while the same HFD did not result in increased body weight in female animals (Coatmellec-Taglioni et al. 2002). An additional study showed that 20 weeks of high-fat feeding in mice (35% kcal from fat) resulted in a significant increase in body

weight and adipose tissue weight in both male and females, although the effect of the HFD was greater in males (Hong et al. 2009). However, not all studies show differences in susceptibility to T2D between male and female animals fed a high-fat diet (HFD) (Catala-Niell et al. 2008; Gomez-Perez et al. 2008). In contrast to the studies showing females rodents being less susceptible to high-fat feeding, 14 weeks of a HFD (30% kcal from fat) resulted in the same effect in female and male Wistar rats: the HFD increased body weight in 15 month old animals (Gomez-Perez et al. 2008), but it did not increase body weight in 2 month old animals (Catala-Niell et al. 2008). Variability in results from HFD studies stem from differences in assessed outcome variables, the duration and composition of the HFD, and the species/strain used in the study, to name a few.

While weight gain in response to a HFD may be a very important predictor for susceptibility to T2D, specific measurements of insulin resistance and glucose intolerance between female and male animals would better characterize these differences. In 2006, Yakar et al. found that a 10 week HFD (35% kcal from fat) significantly increased the body weight and percent body fat in both female and male mice (Yakar et al. 2006). However, the male animals also exhibited increased serum glucose and insulin levels, insulin resistance as measured by an insulin tolerance test, and impaired glucose tolerance as measured by a glucose tolerance test. Female animals did not display insulin resistance and impaired glucose tolerance. As adiposity is such an important predictor of T2D, the dimorphism between the sexes in susceptibility of HFD-induced T2D may be due to differences in body fat storage between males and females.

Sex differences in body fat distribution.

Differences in body fat storage exist between men and women. Men tend to store more of their fat in the abdominal area, and pre-menopausal women tend to store most of their fat in the gluteofemoral area (Vague 1956; Kvist et al. 1988; Lemieux et al. 2003). However, menopause brings about changes in fat storage in women. After menopause, the amount of total body fat and abdominal fat increases (Svendsen et al. 1995; Toth et al. 2000; Sites et al. 2002). As abdominal fat is the number one risk factor for developing T2D (Kissebah and Peiris 1989; Chan et al. 1994; Despres 2006), the difference in fat storage between pre-menopausal women and men may explain the protective effect women have over men against developing T2D.

In fact, women with predominately upper body obesity are more likely to have impaired glucose tolerance than women with predominately lower body obesity (Kissebah et al. 1982). In addition, decreased insulin sensitivity was highly associated with increased visceral adipose tissue in obese post-menopausal women. Obese post-menopausal women with greater amounts of visceral adipose tissue were more likely to have decreased insulin sensitivity (Brochu et al. 2000; Brochu et al. 2001). These studies suggest that the presence of abdominal fat in women diminishes the protective effect women have over men against developing T2D. However, a study comparing non-obese pre- and post-menopausal women demonstrated that the increase in abdominal fat after menopause did not result in increased fasting insulin and glucose levels or a lower

glucose infusion rate during the euglycemic-hyperinsulinemic clamp (Sites et al. 2002). Thus, while abdominal fat may indeed be the number one risk factor for developing T2D, reproductive hormones may also play a role in glucose regulation.

Insulin sensitivity during the menstrual cycle.

Numerous studies demonstrate changes in insulin sensitivity during the menstrual cycle in females (Jarrett and Graver 1968; Valdes and Elkind-Hirsch 1991; Widom et al. 1992; Escalante Pulido and Alpizar Salazar 1999), although additional studies demonstrate no differences (Yki-Jarvinen 1984; Toth et al. 1987; Diamond et al. 1993; Trout et al. 2007; Bingley et al. 2008). The intravenous glucose tolerance test (IVGTT) has been used to assess insulin sensitivity in various populations of women throughout their menstrual cycle. When conducting an IVGTT during the follicular and luteal phase in healthy, normal cycling women, insulin sensitivity was decreased during the luteal phase (Valdes and Elkind-Hirsch 1991; Escalante Pulido and Alpizar Salazar 1999) or showed no differences (Bingley et al. 2008). In addition, decreased insulin sensitivity during the luteal phase has also been shown in patients with type 1 diabetes (Widom et al. 1992), although another study demonstrated no difference in this population (Trout et al. 2007). When using the euglycemic-hyperinsulinemic clamp to assess insulin sensitivity, studies demonstrate no differences in insulin sensitivity throughout the menstrual cycle (Yki-Jarvinen 1984; Toth et al. 1987; Diamond et al. 1993). Although the discrepancies

among these results suggest that more studies are needed to assess changes in insulin sensitivity during the menstrual cycle, these studies also suggest that reproductive hormones may be involved in glucose regulation.

While the inconclusiveness of the previous studies may be due to the low sample size, other researchers have stratified their study population to decipher the reason why some women show differences in insulin sensitivity during their menstrual cycle and others do not. Gonzales-Ortiz et al. found that healthy women without a family history if T2D had decreased insulin sensitivity during the luteal phase, but healthy women with a family history of T2D did not (Gonzalez-Ortiz et al. 1998). Importantly, the changes in insulin sensitivity during the menstrual cycle has been attributed to changes in 17β-estradiol (E_2) levels, and not other reproductive hormones such as progesterone, testosterone, dihydrotestosterone, androstenedione, luteinizing hormone, follicular-stimulating hormone, or prolactin (Widom et al. 1992).

E_2 and glucose regulation in postmenopausal women.

Postmenopausal women incur a greater risk of T2D compared to premenopausal women (Lindheim et al. 1994; Lynch et al. 2002). Postmenopausal women have higher fasting blood glucose and insulin compared to age- and body mass index-matched premenopausal women (Lynch et al. 2002). Lindheim et al. also demonstrates decreased insulin sensitivity and glucose tolerance in healthy postmenopausal women compared to

premenopausal women (Lindheim et al. 1994). Additional studies show benefits of combined E_2/progestin hormone replacement therapy (HRT) (Andersson et al. 1997; Espeland et al. 1998; Kanaya et al. 2003; Margolis et al. 2004; Gower et al. 2006). The Heart and Estrogen/Progestin Replacement Study (HERS) and the Women's Health Initiative Hormone Trial (WHI) were two large-scale, national studies with over 2,500 and 16,000 subjects, respectively, which assessed the overall potential benefits and risks of HRT, including risk factors for T2D. The HERS found that the incidence of insulin resistance in healthy, postmenopausal women on HRT for one year was 35% less compared to postmenopausal women not on HRT (Kanaya et al. 2003). The WHI demonstrates that fasting glucose and insulin levels also decreased in postmenopausal women after one year of HRT (Margolis et al. 2004). (Lindheim et al. 1994; Lobo et al. 1994; Colacurci et al. 1998). In addition, a double blind study by Andersson et al. found that E_2 replacement for 3 months in postmenopausal women with T2D improved their glucose homeostasis versus women with T2D taking a placebo as measured by euglycemic-hyperinsulinemic clamp (Andersson et al. 1997). Of note, a recent study by Gower et al. randomized early postmenopausal women to HRT or placebo for two years. As menopause is closely associated with an increase in abdominal fat, this study assessed the independent effect of menopause on insulin sensitivity and found that HRT, even in the presence of increased abdominal fat, increased insulin sensitivity at the end of the two year study (Gower et al. 2006).

Studies demonstrating positive effects on glucose regulation with the use of combined HRT lead to questions as to whether E_2, progestin, or the combination of the

two hormones provides the benefits. Studies investigating this show that E_2 alone provides the greatest benefit (Cagnacci et al. 1992; Lindheim et al. 1994; Lobo et al. 1994; Cagnacci et al. 1997; Colacurci et al. 1998; Espeland et al. 1998), and this response may be attenuated with the combination of E_2 and progestin.

While E_2 replacement benefits postmenopausal women in terms of glucose regulation, the overall risks may outweigh the benefits. The WHI sought to determine the overall benefits and risks of HRT in postmenopausal women to serve as a guideline for clinical practice. The trial ended early due to the increased risk of severe health complications including breast cancer, thrombosis, and coronary heart disease in the HRT treated group (Nelson et al. 2002). Study investigators concluded that the overall risks of HRT exceeded the benefits. Therefore, understanding the mechanism by which E_2 may positively modulate glucose metabolism may provide important information for developing new treatment methods against T2D without the risks of HRT.

Ovariectomy in rodents models the postmenopausal state.

While clinical studies demonstrate the beneficial effect of E_2 on glucose metabolism, the molecular mechanisms of E_2 remain largely unknown. The use of rodent models has greatly contributed to the knowledge of cellular and molecular effects of E_2 on glucose metabolism. Ovariectomy (OVX) in rodents involves bi-lateral removal of the ovaries and models the postmenopausal state in humans.

Like postmenopausal women, OVX in rodents results in total body weight gain (Yakar et al. 2006; Nunez et al. 2007; Nunez et al. 2008; Hong et al. 2009) total body fat gain (Nunez et al. 2007), and a HFD combined with OVX further increases weight gain in female rodents (Yakar et al. 2006; Nunez et al. 2007; Nunez et al. 2008). The decreased susceptibility to T2D that females demonstrate over males also diminishes after rats undergo OVX (Hong et al. 2009) or become acyclic (Gomez-Perez et al. 2008).

OVX has also been shown to impair insulin sensitivity and glucose metabolism in animal models (Kumagai et al. 1993; Wagner et al. 1998; Yakar et al. 2006). Ten weeks post OVX in mice resulted in glucose intolerance as measured by GTT (Yakar et al. 2006). Kumagai et al. also found that rats ovariectomized for six months developed whole body insulin resistance and demonstrated decreased glucose uptake in skeletal muscle. E_2 replacement alone or in combination with progesterone ameliorated the insulin resistance, but progesterone alone had no effect (Kumagai et al. 1993). These studies demonstrate that OVX in rodents results in similar phenotypes and glucose metabolism seen in postmenopausal women and can serve as a model to study the cellular and molecular effects of E_2 on glucose metabolism.

Skeletal Muscle.

The skeletal muscle is highly metabolic and accounts for 75% of the glucose uptake that occurs in the body (Bjornholm and Zierath 2005). Therefore, the capability

for proper glucose disposal into the skeletal muscle serves an important metabolic function. While obesity leads to whole body insulin resistance, skeletal muscle insulin resistance often serves as an earlier indicator for the impending whole body insulin resistance and T2D.

The mammalian skeletal muscle is primarily made up of two different fiber types: slow-twitch and fast-twitch (Delp and Duan 1996). Slow-twitch fibers contain more mitochondria and myoglobin and have high oxidative capacity (Guyton and Hall 2006). Therefore, they are also called oxidative, red (due to the mitochondria-inducing red appearance), or type I fibers. Fast-twitch fibers contain fewer mitochondria and myoglobin and have high glycolytic capacity. Therefore, they are also referred to as glycolytic, white, or type II fibers. Variations exist within fast-twitch fibers. These fibers are further classified by their oxidative/glycolytic properties. Type IIa fibers are both oxidative and glycolytic and are considered intermediate fast-twitch fibers. Type IIb fibers are non-oxidative/glycolytic and are not typically expressed in humans (Spangenburg and Booth 2003). Type IId/x fibers contain oxidative and glycolytic properties that are between type IIa and type IIb fibers (Delp and Duan 1996). Certain rat muscles may primarily express either slow- or fast-twitch fibers and, therefore, provide a good model for studying fiber type-specific responses to a physiological state. For example, the soleus muscle in the hind limb consists predominantly of slow-twitch fibers (84% type I, 16% type IIa, 0% type IIb), and the extensor digitorum longus (EDL) muscle in the hind limb consists predominantly of fast-twitch fibers (3% type I, 57% type IIa, and 40% type IIb) (Ariano et al. 1973).

Slow- and fast-twitch fibers possess different insulin signaling characteristics. Slow-twitch fibers are more responsive to insulin as they exhibit a greater insulin binding capacity, increased activation of insulin signaling intermediates, and greater insulin-simulated glucose uptake compared to fast-twitch fibers (Bonen et al. 1981; James et al. 1985a; Ploug et al. 1987; Song et al. 1999). In contrast, exercise-induced glucose uptake may (James et al. 1985b) or may not (Ploug et al. 1987) be greater in slow-twitch fibers. Studies also suggest that glucose transporter 4 (GLUT4), the primary glucose transporter in skeletal muscle, levels are greater in slow-twitch fibers (type I), although GLUT4 may actually be greater in both type I and type IIa fibers compared to type IIb fibers (James et al. 1989; Henriksen et al. 1990). In addition, obese people have fewer type I fibers and greater type II fibers than lean people (Hickey et al. 1995). Therefore, estrogen's ability to alter glucose metabolism in the skeletal muscle may also depend on fiber type, and studying both slow- and fast-twitch muscle fibers is important.

Adipose tissue.

In mammals, two distinct types of adipose tissue exist. White adipose tissue is the primary site for storing lipids for energy, and brown adipose tissue specializes in thermogenesis as it generates heat through mitochondrial uncoupling of lipid oxidation. Adipocytes form from mesenchymal stem cells during cellular differentiation. While white adipose tissue derives from vascular cells, brown adipocytes arise from myogenic

precursors, thus giving each adipose tissue type its distinct properties and characteristics (reviewed in (Laharrague and Casteilla 2010). Excessive stores of white adipose tissue contribute to many disorders associated with metabolic syndrome, including T2D. Therefore, the focus on white adipose tissue deserves further attention.

White adipose tissue is present in the body in several subcutaneous and visceral areas. The tissue depots contain various cell types including adipocytes, preadipocytes, fibroblasts, endothelial cells, and multipotent stem cells which are able to differentiate into several cell types. Mature adipocytes account for one third of the cell population and can expand to accommodate lipid storage (reviewed in (Armani et al. 2010). In response to excess energy intake, expansion and accumulation of visceral fat occurs most notably and is linked to the development of metabolic disorders such as insulin resistance (Fox et al. 2007; Lee et al. 2010). Accumulation of subcutaneous adipose tissue occurs less in response to excess energy intake and is not as highly associated with metabolic disorders (Gillum 1987; Kissebah and Krakower 1994). In addition, Macotela et al. demonstrate increased insulin sensitivity in perigonadal adipose tissue (in the viscera) in female mice compared to male mice (Macotela et al. 2009), and this fat depot also has higher lipolytic capacity in females than in males (Pujol et al. 2003). This data suggests that estrogen's involvement in adipose tissue metabolic regulation may preferentially occur in the perigonadal depot.

In addition to white adipose tissue functioning as a storage site for lipids, it also functions as an endocrine organ by secreting hormones. These hormones are known as adipokines, and they regulate and integrate metabolic functions such as energy balance,

food intake and appetite, insulin sensitivity, blood pressure, and reproduction (Caprio et al. 2001). Dysfunctional secretion of adipokines and free fatty acids, combined with dysregulated disposal of glucose and lipids, contributes to the development of many metabolic disorders (Rosen and Spiegelman 2006; Lefterova and Lazar 2009). In addition, these alterations are associated with visceral fat accumulation (Berg and Scherer 2005; Wildman et al. 2008).

The function of the pancreas and insulin biosynthesis.

The pancreas performs both exocrine and endocrine functions, both of which pertain to nutrient utilization. The pancreas is made up of a variety of cells grouped into two major tissues: acini cells and islets of Langerhans. The acini cells perform the exocrine function of the pancreas by secreting digestive juices into duodenum. The cells in the islets of Langerhans perform the endocrine function and secrete hormones into the blood. The beta cells are the most prevalent cell in the islets, making up 60% of the total cells.

The beta cells synthesize and release insulin, which plays a key role in maintenance of whole body metabolism, and especially glucose metabolism. Insulin is a protein containing two polypeptide chains linked together by disulfide bonds. Translation of insulin mRNA in the beta cells first results in formation of pre-proinsulin, which is cleaved into proinsulin. C-peptide is then cleaved from proinsulin to form the

mature insulin. Intracellular storage vesicles contain insulin and C-peptide until its signaled release (Halban 1994). Glucose entry into the beta cells signals the release of insulin. Glucose enters the beta cells via diffusion through the glucose transporter (GLUT) 2. Increased production of ATP from the glucose inhibits the membrane ATP/K^+ pumps, resulting in increased intracellular K^+ levels. The increased K^+ level depolarizes the cell, resulting in opening of voltage-gated calcium channels. The increased intracellular calcium signals fusion of the insulin storage vesicles with the membrane which releases insulin into the blood stream (Guyton and Hall 2006).

Other cells reside in the islets of Langerhans in addition to beta cells, with the most prevalent being: 1) alpha cells which account for 25% of the cells in the islets and release glucagon; 2) delta cells which account for 10% of the cells in the islets and release somatostatin; and 3) the PP cells which account for 5% of the cells in the islets and secrete pancreatic poly peptide (Guyton and Hall 2006).

Metabolic functions of insulin and insulin signaling pathways.

After the release of insulin into the blood stream, insulin binds to membrane insulin receptors in insulin responsive tissues such as the liver, adipose tissue, and skeletal muscle. Insulin is most important for the regulation of glucose metabolism in these tissues. In the liver, insulin inhibits gluconeogenesis by inhibiting phosphoenolpyruvate carboxykinase (Barthel and Schmoll 2003). As mediated by

insulin, the skeletal muscle and adipose tissue are responsible for 75% (Bjornholm and Zierath 2005) and 10% (Klip et al. 1990), respectively, of the glucose disposal that occurs in the body. In this manner, insulin maintains circulating glucose levels between 80 and 100 mg/dL (Saltiel and Kahn 2001).

Insulin activates many cellular signaling cascades. Two of the main signaling pathways which result in increased glucose disposal into the skeletal muscle and adipose tissue include the insulin receptor substrate (IRS)/phosphatidylinositol 3 kinase (PI3K) and Cbl/Cbl associated protein (CAP) pathways, which will be detailed here.

The IRS/PI3K pathway begins with the binding of insulin to the insulin receptor (IR) which resides in the cell membrane (Figure 1). The IR is a dimeric tyrosine kinase receptor, and binding of insulin results in autophosphorylation on tyrosine residues (Taniguchi et al. 2006). Phosphorylation of the IR results in recruitment and phosphorylation of IRS proteins. There are six isoforms of the IRS proteins (IRS1-6) (Cai et al. 2003), and IRS-1 is the predominant isoform in skeletal muscle (Araki et al. 1994). All of the IRS proteins contain pleckstrin homology (PH) domains and phosphotyrosine binding domains which lead to recruitment of IRS to the IR. The IRS proteins contain approximately 20 tyrosine phosphorylation sites located at the C terminus and center of the protein (Gual et al. 2005) which are phosphorylated by the activated IR. Phosphorylated IRS proteins are capable of binding molecules containing Src-homology-2 domains, including PI3K. PI3K then catalyzes the formation of phosphatidylinositol triphosphate (PIP_3), which binds to and activates proteins containing the PH domain including phosphoinositide-dependent protein kinase-1 (PDK1).

Activation of PDK1 results in activation of Akt/PKB via phosphorylation on thronine 308 and serine 473 (Alessi et al. 1997; Sarbassov et al. 2005). Subsequently, Akt substrates of 160 kDa are activated, including TBC1D1 and TBC1D4 (also known as AS160) (Kane et al. 2002; Sano et al. 2003; Gonzalez and McGraw 2006; Taylor et al. 2008; Peck et al. 2009). While previous research only implicated TBC1D4 as a downstream target of Akt (hence the name Akt substrate 160 (AS160)), a newly discovered Akt substrate of 160 kDa (TBC1D1) was also found be activated downstream of Akt (Taylor et al. 2008; Peck et al. 2009). TBC1D1 and TBC1D4 are paralogs with sequences that are 47% identical (Roach et al. 2007). Both substrates contain Rab-GTPase-activating protein (GAP) domains (Miinea et al. 2005; Peck et al. 2009). Activation of TBC1D1 and TBC1D4 suppresses the GAP activity, which elevates the active GTP form of Rab proteins. The activated Rab proteins are involved in the cytoskeletal reorganization responsible for GLUT4 translocation to the plasma membrane (Sano et al. 2003; Sakamoto and Holman 2008; Zaid et al. 2008).

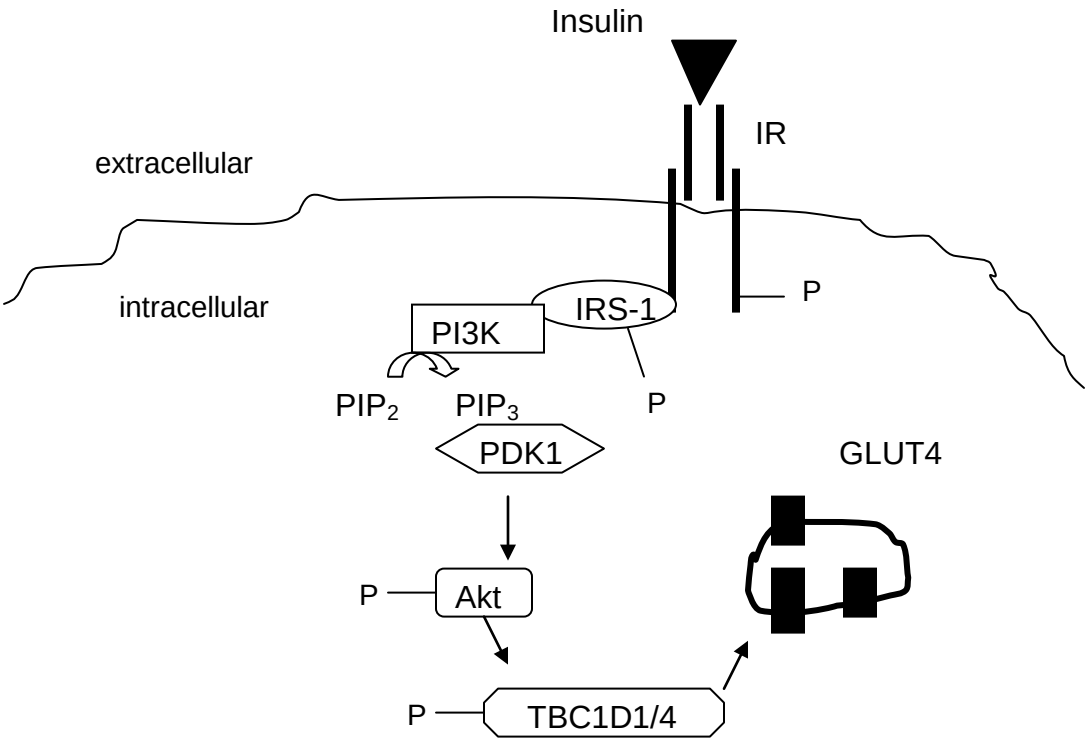

Figure 1: IRS/PI3K signaling cascade.

Increases in GLUT4 translocation to the membrane also result from activation of the Cbl-CAP insulin signaling pathway. In the Cbl-CAP pathway, insulin binds to the insulin receptor in the cell membrane which then recruits and activates the APS (adaptor with pleckstrin homology and Src homology-2 domains) protein (Ahmed et al. 2000; Hu and Hubbard 2005). APS subsequently phosphorylates and activates Cbl (Liu et al. 2002) which is accompanied by CAP (Ribon et al. 1998a; Ribon et al. 1998b; Lin et al. 2001). The activated Cbl-CAP complex ultimately leads to activation of TC10, which signals GLUT4 translocation to the cell membrane (Chiang et al. 2001; Chang et al. 2002). Some suggest that the Cbl-CAP insulin signaling pathway may be more specific to adipose tissue rather than skeletal muscle (JeBailey et al. 2004).

In addition to glucose metabolism, insulin also modulates fat and protein metabolism. In adipose tissue, insulin, via inhibition of the lipolytic enzyme lipase, inhibits lipolysis. Insulin also stimulates amino acid transport into cells, increases translation of mRNA, promotes protein synthesis and storage, and inhibits catabolism of proteins (Guyton and Hall 2006).

Insulin-independent GLUT4 translocation.

Insulin does not need to be present in order to signal GLUT4 translocation to the membrane to facilitate glucose uptake in the skeletal muscle. While muscle contraction and exercise (Lund et al. 1995; Kennedy et al. 1999), hypoxia (Mu et al. 2001; Wright et

al. 2005), and osmotic pressure (Chen et al. 1997) all stimulate glucose transport into muscle in the absence of insulin, muscle contraction and exercise has the greatest potential to improve glucose uptake in insulin resistant patients.

AMP-activated protein kinase (AMPK) is a heterotrimeric protein which contains a catalytic α subunit and two regulatory subunits (β and γ). AMPK possesses the ability to sense the energy status of a cell and plays a pivotal role in contraction mediated glucose transport (Kurth-Kraczek et al. 1999; Mu et al. 2001). When cellular stores of ATP decrease, AMPK is activated via phosphorylation on threonine 172 by kinases such as LKB-1 and CAMKKβ (Hurley et al. 2005; Kahn et al. 2005). AMPK activation can also occur by the AMP mimetic 5-aminoimidazole-4-carboxamide-riboside (AICAR) in resting muscle to stimulate glucose uptake (Merrill et al. 1997). The insulin independent AMPK signaling and the insulin dependent IRS/PI3K signaling converge at the level of TBC1D1/4 activation (Figure 2) (Thong et al. 2007; Taylor et al. 2008). Phosphorylation of AMPK on threonine 172 results in activation of TBC1D1/4 and subsequent GLUT4 translocation to the membrane. While both TBC1D1 and TBC1D4 are expressed in skeletal muscle, expression of TBC1D1 in adipose tissue is very low (Chavez et al. 2008; Taylor et al. 2008). In skeletal muscle, expression of TBC1D1 may be greater in mixed fiber type muscles such as the tibialis anterior compared to the soleus which is made up of primarily slow-twitch fibers (Taylor et al. 2008).

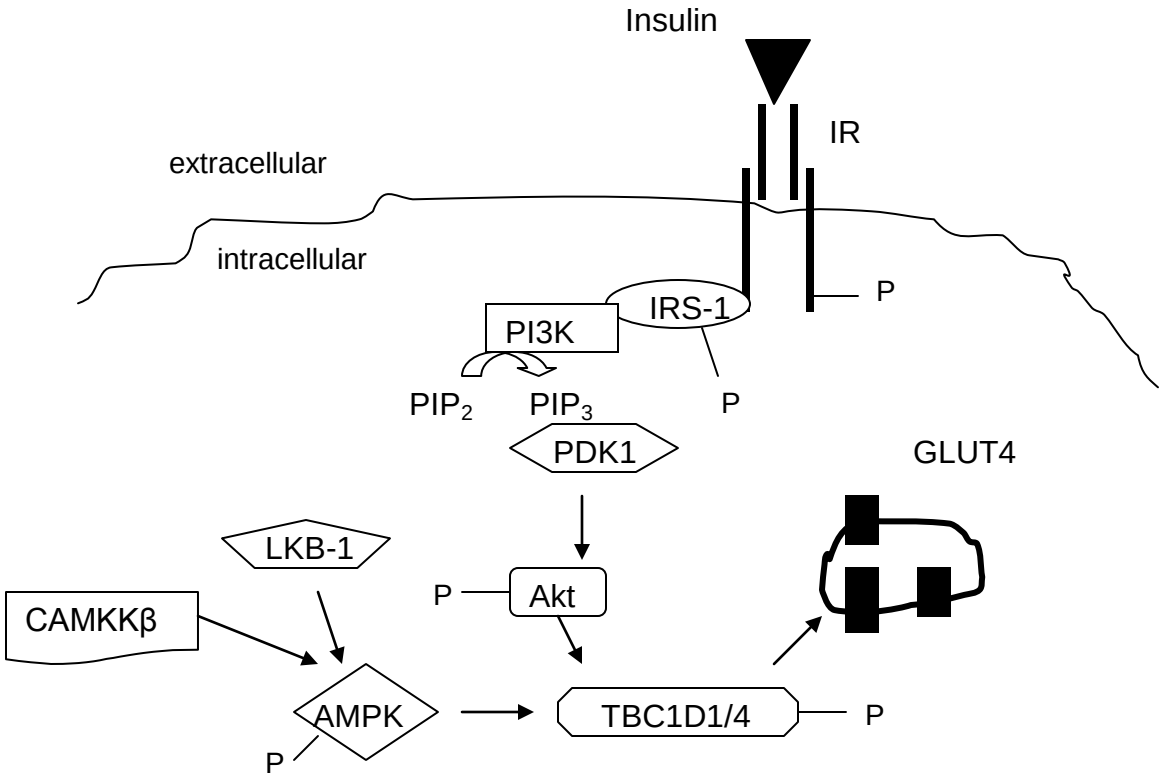

Figure 2: Convergence of the IRS/PI3K signaling cascade and insulin-independent AMPK signaling.

The energy sensing characteristic of AMPK also allows this protein to regulate fatty acid metabolism. AMPK inhibits the ability of acetyl CoA carboxylase (ACC) to form malonyl-CoA (Trumble et al. 1995; Winder et al. 1997). Decreased levels of malonyl-CoA allows for the transfer of long chain fatty acids into the mitochondria via carnitine palmitoyltransferase 1 (CPT1) (Ruderman et al. 1999). Decreasing intramuscular fatty acids stores via AMPK activation can improve glucose metabolism by increasing skeletal muscle insulin sensitivity (Merrill et al. 1997). Therefore, activation of AMPK may provide a two-fold benefit to improve glucose regulation by directly increasing glucose uptake via signaling GLUT4 translocation and by restoring insulin sensitivity via decreasing fatty acid stores.

Structure and function of GLUT4.

Glucose is a large, hydrophilic molecule which cannot penetrate the lipid bilayer of cells. Instead, glucose is transported across cell membranes via membrane associated glucose transporters. In skeletal muscle and adipose tissue, insulin stimulated glucose uptake occurs through GLUT4. GLUT4 is part of a 13 member GLUT family, whose transporters have 12 membrane-spanning helices and an intracellular amino- and carboxy- terminus (Joost and Thorens 2001; Scheepers et al. 2004). Glucose transport through GLUT4 occurs via ATP-independent facilitative diffusion (Joost and Thorens 2001).

When serum insulin concentrations are low, most GLUT4 molecules reside in intracellular storage vesicles, with 4-5% of GLUT4 proteins present at the plasma membrane (Klip et al. 1990). While the intracellular GLUT4 storage vesicles travel to the plasma membrane and undergo exocytosis during this time, the rate of endocytosis of GLUT4 proteins is greater than the rate of exocytosis (Satoh et al. 1993; Li et al. 2001). In the presence of insulin or activation of AMPK (described earlier), the GLUT4 storage vesicles translocate to the plasma membrane to facilitate glucose uptake (Bryant et al. 2002). This translocation of GLUT4 storage vesicles occurs via remodeling of the actin cytoskeleton (Guilherme et al. 2000). Further details regarding GLUT4 storage vesicle organization, translocation, and fusion with the plasma membrane remain controversial. However, GLUT4 likely co-localizes with vesicle-associated membrane protein (VAMP) 2 in intracellular storage vesicles (Malide et al. 1997). Exocytosis of GLUT4 storage vesicles is likely mediated by VAMP2 and soluble NSF-attachment protein (SNAP) 23 on the plasma membrane, as assisted by several accessory proteins including munc18c, syntaxin4-interacting protein (synip), and tomosyn (Widberg et al. 2003; Hodgkinson et al. 2005; Watson and Pessin 2006).

Other glucose transport proteins.

While GLUT4 is the primary regulator of glucose uptake in skeletal muscle and adipose tissue, GLUT1 is also present in the plasma membrane of these tissues and

facilitates glucose uptake. GLUT1 is not insulin responsive and remains in the plasma membrane to facilitate glucose uptake in the absence of insulin (Bell et al. 1990). GLUT1 also facilitates glucose uptake into many tissues in the body, especially erythrocytes and the brain. Neurons obtain glucose via GLUT3 transporters, and the liver, kidney, intestine, and pancreatic beta cells obtain glucose via GLUT2 (Joost and Thorens 2001). The kidneys and intestines also obtain glucose via sodium dependent glucose transporters (SGLT) (Bell et al. 1990). These symporters use the concentration gradient set up by Na^+/K^+-ATPase pumps and are, therefore, secondary active transporters. The SGLT facilitate Na^+ transport down its concentration gradient, which is coupled with glucose transport into the cell (Scheepers et al. 2004). SGLT are not present in skeletal muscle and adipose tissue (Asano et al. 2004).

Estrogen receptors.

The first estrogen receptor (ER) was discovered in the rat uterus in 1987 (Koike et al. 1987). Upon discovery of another ER in the rat prostate and ovary in 1996, the former ER was re-named ERα and the latter ER named ERβ (Kuiper et al. 1996). ERα and ERβ are products of two distinct genes (Menasce et al. 1993; Enmark et al. 1997). ERα is expressed primarily in the uterus, liver, kidney, and heart and ERβ in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Mueller and Korach 2001; Matthews and Gustafsson 2003). The ERs belong to the

nuclear receptor superfamily (Pettersson and Gustafsson 2001) which include the classical steroid hormones, orphan receptors, and adopted orphan receptors (reviewed in (Glass 2006)). The ERs belong to the classical steroid hormones which all contain a common domain structure. The C terminus contains a ligand binding domain which is responsible for ligand binding, receptor dimerization, and contains a ligand-dependent activation function. The DNA binding domain is located in the center and recognizes specific palindromic response elements on target genes. As the DNA binding domains in ERα and ERβ are highly conserved, ERα and ERβ bind estrogen response elements with similar affinity and specificity. However, physiologically, ERα and ERβ act differently depending on tissue, receptor level, and presence of ligand, co-activators, and co-repressors. The difference in function between ERα and ERβ may stem from a poorly conserved ligand-independent activation function located at the N terminus which lacks homology among the nuclear receptors (reviewed in (Matthews and Gustafsson 2003)).

The ERs may initiate cellular function via a genomic or non-genomic mechanism. The genomic, or classical, mechanism occurs following ligand binding to the ERs which typically reside in the nucleus, although current literature commonly detects the presence of ERα in the cytoplasm as well (reviewed in (Deroo and Korach 2006)). Upon activation of the ligand binding domain and receptor dimerization, the ERs bind to estrogen response elements on DNA to modulate gene transcription. The non-genomic mechanism of ER action has becoming increasingly important in studying non-reproductive physiological functions and disease states. This mechanism of ER action occurs following ligand binding to the ERs which reside in the cytoplasm or at the

membrane. The activated ERs then results in cellular responses such as increased levels of nitric oxide and calcium or activation of various signaling cascades and kinase activity (Kelly and Levin 2001; Nadal et al. 2001; Deroo and Korach 2006).

Non-reproductive functions of ERs.

While the importance of ERs in reproductive functions is well established, the ERs are becoming increasingly important in the study and development of cardiovascular diseases, brain degeneration, osteoporosis, and glucose metabolism. Estrogen, via the ERs, may protect against cardiovascular diseases by decreasing total cholesterol and LDL cholesterol levels in serum (Ohlsson et al. 2000) and also by increasing the vasodilator nitric oxide (Haynes et al. 2000). Activation of the ERs also promotes bone health by inhibiting the bone resorption function of nuclear factor-κB (NF-κB) (van den Wijngaard et al. 2000; Quaedackers et al. 2001). The anti-neurodegenerative effects of the ERs likely occur through the inhibition of apoptosis (Dubal et al. 1999; Maggi et al. 2000). Many of these non-reproductive functions of the ERs occur through the recently discovered non-genomic mechanisms previously mentioned. The ERs also play a role in glucose metabolism, with ERα and ERβ knockout (KO) mice providing much of the initial understanding of ER regulation of glucose control.

ER KO mice and glucose metabolism.

Glucose regulation in the body mostly occurs in the skeletal muscle, adipose tissue, and liver. ER KO mice suggest that the ERs may play a role in glucose regulation in all of these tissues, with the primary mediator being ERα. With respect to the liver, ERα KO mice show hepatic insulin resistance during the euglycemic-hyperinsulinemic clamp test. While hepatic glucose production decreases in wild-type (WT) mice, insulin is not able to decrease hepatic glucose production in ERα KO mice (Bryzgalova et al. 2006).

With respect to the adipose tissue, ERα KO mice have increased body weight and white adipose tissue weight compared to WT mice. ERα KO mice also have increased adipocyte size and number, although food intake does not differ (Heine et al. 2000). Similarly, aromatase KO mice, in which androgens cannot be converted to E_2, have increased body weight (Takeda et al. 2003) and adipose tissue weight (Fisher et al. 1998) compared to WT mice. In contrast, ERβ KO mice do not have increased adipose tissue weight or percent body fat compared to WT mice (Ohlsson et al. 2000). Therefore, E_2/ERα signaling appears to be an important regulator of body weight and adipocyte regulation.

In addition, ERα KO mice display a decrease in whole body glucose tolerance and insulin sensitivity and have decreased glucose uptake in the skeletal muscle (Heine et al. 2000; Bryzgalova et al. 2006; Ribas et al. 2009). ERα KO mice also have increased fasting glucose and insulin levels, which are not present in ERβ KO mice (Bryzgalova et

al. 2006). Aromatase KO mice also display a decrease in whole body glucose tolerance and increased insulin resistance, both of which can be ameliorated with E_2 replacement (Takeda et al. 2003). In addition, a case study of a human male lacking a functional ERα has impaired glucose tolerance and hyperinsulinemia (Smith et al. 1994). Likewise, humans that have aromatase deficiency are also hyperinsulinemic (Morishima et al. 1995). These data again suggest the importance of E_2/ERα signaling in whole body and skeletal muscle glucose regulation. As skeletal muscle is responsible for over 75% of the glucose regulation that occurs in the body (Bjornholm and Zierath 2005), understanding how E_2/ERα may be involved in skeletal muscle glucose metabolism could have a great impact on managing glucose regulation in the body.

Mechanisms of ER mediated glucose metabolism.

Recently, investigators utilized cell culture to examine the mechanism by which estrogen may play a role in glucose metabolism. Studies show that estrogen treatment in adipocytes increases insulin-stimulated glucose uptake and activation of the insulin signaling pathway more than insulin alone (Muraki et al. 2006; Nagira et al. 2006). Furthermore, Muraki et al. found that the beneficial effects of estrogen were abolished when adipocytes were co-treated with methylpiperidinopyrazole (MPP), a specific ERα inhibitor. The beneficial effects were restored with treatment of propylpyrazoletriol (PPT), a specific ERα activator (Muraki et al. 2006). These studies suggest that

activation of ERα can potentiate the insulin signaling pathway and glucose uptake in cultured adipocytes.

In skeletal muscle, E_2 may act on the insulin signaling pathway in a similar manner. Acute E_2 incubations (5 and 10 minutes) with skeletal muscle *in vitro* can increase activation of the insulin signaling pathway, including phosphorylation of Akt and its downstream targets, AS160 and TBC1D1 (Rogers et al. 2009). Furthermore, long-term E_2 treatment *in vivo* can improve whole body and skeletal muscle glucose metabolism in animals fed a high-fat diet (Riant et al. 2009). Therefore, activation of the estrogen receptors has the potential to positively modulate skeletal muscle glucose metabolism.

Additional studies suggest a second mechanism in which E_2 may play a role in glucose metabolism via regulation of GLUT4. GLUT4 is absolutely critical for glucose uptake in skeletal muscle. Regardless of the insulin signaling pathway's ability to function, low levels of GLUT4 can limit the rate of glucose uptake into the cell. NF-κB is a transcription factor that is activated by stimuli such as cellular stress, cytokines, and inflammation. The promoter region of GLUT4 contains a NF-κB binding site (Long and Pekala 1996b), and NF-κB represses GLUT4 transcription (Ruan et al. 2002). In a basal state, NF-κB is bound by the inhibitor of kappa B α (IκBα) in the cytosol and remains inactive. Upon activation of the stress kinase proteins, IκBα is phosphorylated, which signals its degradation by the proteosome. The free NF-κB is then activated and translocates to the nucleus where it functions as a transcription factor. Tumor necrosis factor α (TNF-α) is a cytokine that activates the NF-κB pathway and is highly expressed

in obese humans (Saghizadeh et al. 1996; Uysal et al. 1998). In addition, obese humans with T2DM have increased skeletal muscle NF-κB activation (Sriwijitkamol et al. 2006). Previous studies have also shown that rats on an obesity-promoting high-fat diet have decreased skeletal muscle GLUT4 protein (Kahn 1994; Han et al. 1995; Sevilla et al. 1997). Overall, these studies suggest that obesity increases the amount of TNF-α and leads to NF-κB activation, which is followed by a decrease in GLUT4 protein levels.

Estrogen's involvement in GLUT4 regulation comes in to play as new evidence points to NF-κB being regulated by ERα. While the ERs traditionally modulate gene expression by binding directly to DNA, studies also show that ERα modulates gene expression without binding directly to DNA, but by binding other transcription factors (Galien and Garcia 1997; Paech et al. 1997; Qin et al. 1999). Cell culture studies have shown that activated ERα can directly bind to NF-κB and decrease NF-κB–DNA binding (Stein and Yang 1995; Galien and Garcia 1997; Ray et al. 1997; Paimela et al. 2007). In the case of GLUT4, NF-κB is a negative transcription factor. With low levels of activated ERα, NF-κB may remain unchecked and decrease GLUT4 levels. In fact, females with polycyctic ovarian syndrome who have high androgen and low estrogen levels (and, therefore, have low ERα activation) have 35% less GLUT4 protein compared to control females (Rosenbaum et al. 1993). In addition, ERα KO mice show a decrease in GLUT4 mRNA levels (Barros et al. 2006b). Therefore, increased NF-κB activation via a high-fat diet and obesity combined with low ERα activation could decrease GLUT4 transcription, leading to a subsequent decrease in glucose uptake and insulin resistance.

This physiological condition (obesity and low E_2 levels) is present in most postmenopausal women, putting these women at a particular risk for insulin resistance.

GLUT4 transcriptional regulation.

GLUT4 protein levels change due to alterations in metabolism, hormones, and nutrition, and several transcription factors have been implicated in the regulation of GLUT4. GLUT4 mRNA and protein is decreased in the adipose tissue of obese humans and humans with T2D (Garvey et al. 1991; Pedersen et al. 1992; Shepherd and Kahn 1999). In skeletal muscle, GLUT4 is only decreased in morbidly obese humans (Shepherd and Kahn 1999). In animal models of obesity and T2D, discrepancies exist as to whether GLUT4 is decreased in the skeletal muscle and adipose tissue. In the *db/db* mouse model of obesity and T2D, in which the leptin receptor activity is deficient due to a point mutation, GLUT4 levels remain unchanged in both the adipose tissue and skeletal muscle (Friedman et al. 1992). In contrast, Zucker diabetic fatty rats display decreased GLUT4 in both the skeletal muscle and adipose tissue (Marette et al. 1993). However, non-genetic animal models of obesity and T2D may better compare to human studies of GLUT4 regulation.

The high-fat feeding model assesses changes in adiposity, glucose metabolism, fatty acid regulation, and numerous other cellular mechanisms related to the pathogenesis and development of insulin resistance, glucose intolerance, and T2D. Previous studies

show a decrease in GLUT4 protein in adipose tissue in rodents fed a high-fat diet (Leturque et al. 1991; Pedersen et al. 1991; Kahn 1994; Ikemoto et al. 1995). In skeletal muscle, a high-fat diet may (Kahn and Pedersen 1993; Han et al. 1995; Sevilla et al. 1997; Tremblay et al. 2001) or may not (Kusunoki et al. 1993; Rosholt et al. 1994; Zierath et al. 1997) decrease GLUT4 protein levels. Whether or not a high-fat diet decreases skeletal muscle GLUT4 may depend on the fatty acid composition of the diet. Diets high in arachidonic, stearic, and oleic acids may contribute to decreased GLUT4 expression (Tebbey et al. 1994; Long and Pekala 1996b). These fatty acids are omega-6 (arachidonic), saturated (stearic), and omega-9 (oleic) fatty acids that are very common in the western diet, as opposed to omega-3 fatty acids such α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) that are less common in the western diet. Although the mechanism by which a high-fat diet and/or obesity may contribute to decreased GLUT4 expression remains uncertain, tumor necrosis factor-α (TNF-α), nuclear factor-κB (NF-κB), and CCAAT/enhancer binding protein-α (C/EBP-α) may all work to suppress GLUT4 levels. The promoter region of GLUT4 contains a NF-κB binding site (Long and Pekala 1996a), and NF-κB represses GLUT4 transcription (Ruan et al. 2002). Fatty acids may induce activation of NF-κB to decrease GLUT4 (Jove et al. 2006), or TNF-α may activate NF-κB. TNF-α is a cytokine that activates the NF-κB pathway and is highly expressed in obese humans (Saghizadeh et al. 1996; Uysal et al. 1998). TNF-α can also repress GLUT4 gene expression through C/EBP-α in adipocytes (Liu and Matsumura 2006).

Insulin status can also alter GLUT4 protein levels. A decrease in insulin as induced by streptozotocin (Berger et al. 1989; Garvey et al. 1989; Sivitz et al. 1989; Kahn et al. 1991; Slieker et al. 1992; Napoli et al. 1995) or fasting (Berger et al. 1989; Sivitz et al. 1989; Charron and Kahn 1990) in animal models decreases GLUT4 in the adipose tissue, and re-feeding after a fast rapidly increases GLUT4 above pre-fasting levels (Berger et al. 1989; Sivitz et al. 1989; Charron and Kahn 1990). In skeletal muscle, numerous studies also support a streptozotocin-induced decrease in GLUT4 mRNA and protein levels (Bourey et al. 1990; Garvey et al. 1991; Kahn et al. 1991; Napoli et al. 1995; Munoz et al. 1996). However, this may only occur in primarily slow-twitch muscles, but not primarily fast-twitch muscles (Neufer et al. 1993). In contrast to adipose tissue, a three day fast increases skeletal muscle GLUT4 mRNA expression, although discrepancies exist as to which fiber type this occurs in. Fasting has been shown to increase GLUT4 mRNA in skeletal muscle made up of mixed and primarily slow-twitch fibers (Charron and Kahn 1990), although another report shows no change in primarily slow-twitch muscles, but an increase in primarily fast-twitch muscles (Neufer et al. 1993). Numerous transcription factors are activated or repressed upon decreased insulin levels and may contribute to these discrepancies. While the exact mechanisms of insulin-mediated GLUT4 transcriptional control remain unknown, sterol regulatory element binding protein-1 (SREBP-1) (Im et al. 2006), peroxisome proliferator activated receptor-α (PPAR-α) (Yechoor et al. 2002; Patti et al. 2003), olf-1/early B cell factor-1 (O/E-1) (Dowell and Cooke 2002), and nuclear factor-1 (NF-1) (Cooke and Lane 1999), and C/EBP-α (Kaestner et al. 1990) may all play a role.

In skeletal muscle, exercise-induced contraction of muscle fibers (Winder et al. 2006) and exercise training (Ploug et al. 1990; Wake et al. 1991) can also increase GLUT4 levels. Central to this regulation is myocyte enhancer factor 2 (MEF2). MEF2 is regulated by AMPK, a protein activated by muscle contraction (Knight et al. 2003; Ojuka 2004). AMPK also activates PPAR-γ coactivator 1 (PGC-1) (Ojuka 2004) and muscle GLUT4 enhancer factor (mGEF) (Knight et al. 2003). Together, MEF2, PGC-1, and mGEF work to increase GLUT4 as simulated by muscle contraction (Michael et al. 2001; Knight et al. 2003; Holmes et al. 2005). Kruppel-like Factor (KLF) 15 and myoD also work synergistically with MEF2 to upregulate GLUT4 gene expression (Czubryt et al. 2003).

Transgenic models used to study GLUT4.

Knocking out the activity of a specific gene can provide general information on the whole body impact of that gene. Surprisingly, GLUT4 knockout (KO) mice maintain normal plasma glucose levels, even following a GTT (Katz et al. 1995). However, these mice display hyperinsulinemia and insulin resistance in the fed state. As predicted, insulin-stimulated glucose transport into the skeletal muscle is abolished (Stenbit et al. 1996). Although GLUT4 KO mice do not display severe impairments of glucose metabolism, the importance of GLUT4 expression particularly presents itself in models of T2D. While a high-fat diet results in glucose intolerance in mice, a two-fold increase

in GLUT4 expression, via transgenic overexpression, protects against the high-fat diet-induced glucose intolerance (Ikemoto et al. 1995).

To better understand the role of GLUT4 in insulin-responsive tissues, mouse models have been developed which alter GLUT4 levels specifically in skeletal muscle and adipose tissue. Abel et al. developed an adipose-specific GLUT4 KO (Abel et al. 2001). These mice exhibit whole body insulin resistance and glucose intolerance, albeit no increase in body weight. GLUT4 expression is reduced by 70% in the adipose tissue, but unaltered in the skeletal muscle. Basal glucose transport in the adipose tissue is decreased by 40%, and insulin-stimulated glucose transport is decreased by 72%, accounting for much of the whole body insulin resistance and glucose intolerance. However, although no changes in GLUT4 levels occur in the skeletal muscle, these mice also display decreased glucose transport into the skeletal muscle. These authors suggest that cross-talk between adipose tissue and skeletal muscle may mediate this change due to secreted factors from the adipose tissue such as retinol binding protein 4. Overexpression of GLUT4 specifically in the adipose tissue increases basal and insulin-stimulated glucose transport into the adipose tissue (Shepherd et al. 1993), and these transgenic mice are partially protected against streptozotocin-induced diabetes (Tozzo et al. 1997). Therefore, GLUT4 levels in the adipose tissue play an important role in whole body glucose regulation.

Zisman et al. developed a skeletal muscle-specific GLUT4 KO (Zisman et al. 2000). These mice develop insulin resistance and glucose intolerance by eight weeks of age, and GLUT4 expression in the skeletal muscle is reduced by 95%. Non-insulin

stimulated glucose transport is decreased by 80% in the skeletal muscle, and insulin stimulation does not increase glucose transport. Secondary insulin resistance also develops in the adipose tissue, which is likely a result of glucose toxicity (Kim et al. 2001). Therefore, the presence of the insulin-responsive GLUT4 protein in skeletal muscle is important to ward off T2D and protect the body from glucose toxicity.

Research Goals.

Research focusing on the function of E_2 in skeletal muscle and adipose tissue glucose metabolism is gaining attention, and much more information has become available in the past few years. However, many gaps still exist in the literature. We assessed the outcome of a physiological model of insulin resistance combined with OVX on skeletal muscle and adipose tissue ER expression and glucose metabolism. Furthermore, we sought to determine the ability of activated ERα to modulate glucose uptake, insulin signaling, and GLUT4 in the skeletal muscle. Finally, as adipogenesis remains an important risk factor for T2D, we examined the extent to which activation of ERα may regulate fat storage and proteins involved in lipogenesis and lypolysis.

Chapter 2

Altered estrogen receptor expression in skeletal muscle and adipose tissue of female rats fed a high-fat diet

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ABSTRACT

Estrogen receptors (ERs) are expressed in adipose tissue and skeletal muscle, with potential implications for glucose metabolism and insulin signaling. Previous studies examining the role of ERs in glucose metabolism have primarily used knockout mouse models of ERα and ERβ and it is unknown whether ER expression is altered in response to an obesity-inducing high-fat diet (HFD). The purpose of the current study was to determine whether modulation of glucose metabolism in response to a HFD in intact and ovariectomized (OVX) female rats is associated with alterations in ER expression. Our results demonstrate that a 6 week HFD (60% calories from fat) in female rats induces whole body glucose intolerance with tissue specific effects isolated to the adipose tissue, and no observed differences in insulin-stimulated glucose uptake, GLUT4, or ERα protein expression levels in skeletal muscle. In chow-fed rats, OVX resulted in decreased ERα with a trend towards decreased GLUT4 expression in adipose tissue. Sham and OVX rats fed a HFD demonstrated a decrease in ERα and GLUT4 in adipose tissue. The HFD also increased activation of stress kinases (c-jun NH_2-terminal kinase and inhibitor of kappa B kinase β) in the sham rats and decreased expression of the protective heat shock protein 72 (HSP72) in both sham and OVX rats. Our findings suggest that decreased glucose metabolism and increased inflammation in adipose tissue with a HFD in female rats could stem from a significant decrease in ERα expression.

INTRODUCTION

Type 2 diabetes, one of the main causes of mortality and morbidity worldwide (Saltiel and Kahn 2001), is characterized by insulin resistance, glucose intolerance, and inflammation, and is closely associated with obesity. Clinical evidence suggests postmenopausal women have an increased risk of glucose intolerance and weight gain, and that this is accompanied by increased inflammation and decreased insulin sensitivity (Pfeilschifter et al. 2002; Sites et al. 2002; Carr 2003). Estrogen replacement therapy in postmenopausal women ameliorates the increased risk of type 2 diabetes (Andersson et al. 1997; Kanaya et al. 2003; Margolis et al. 2004), even in the presence of increased abdominal fat (Gower et al. 2006). While this beneficial effect of estrogen is evident, the molecular mechanisms of estrogen and its active metabolite, 17β-estradiol (E_2), in metabolic tissue remain unknown.

Estrogen exerts its effects through two nuclear receptors, estrogen receptor (ER) α and ERβ (Dahlman-Wright et al. 2006). ERα and ERβ are expressed in adipose tissue and skeletal muscle, with potential implications for glucose metabolism and insulin signaling. Previous studies demonstrate that ERα knockout mice are obese, insulin resistant, and exhibit glucose intolerance (Heine et al. 2000; Bryzgalova et al. 2006). A recent study by Ribas et al. (Ribas et al. 2009) further showed that ERα expression is critical for the maintenance of whole body insulin action and protection against tissue inflammation in response to high-fat feeding. These investigators suggest that ERα could play an important role in modulating inflammatory stress kinase proteins such as c-Jun NH_2-terminal kinase (JNK) (Ribas et al. 2009), known to interfere with insulin signaling

(Chung et al. 2008; Gupte et al. 2009a; Gupte et al. 2009b; Ribas et al. 2009). Despite this important new information, the role of ERs in the pathogenesis of insulin resistance and glucose intolerance is not clear. Previous studies examining the role of ERs in glucose metabolism have primarily used knockout mouse models of ERα and ERβ and it is unknown whether ER expression is altered in response to an obesity-inducing high-fat diet (HFD). As a result, the impact of a HFD on ER expression in adipose tissue and skeletal muscle, and thus the role of ERs in mediating the metabolic actions of estrogen, remains a fundamental question. Therefore, the purpose of the current study was to determine whether modulation of glucose metabolism in response to a HFD in intact and ovariectomized female rats is associated with alterations in ER expression.

MATERIALS AND METHODS

Materials. GLUT4 antibody (ab654) was purchased from Abcam (Cambridge, MA), ERα (MC-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), ERβ (#PA1-310B) was purchased from Affinity BioReagents (Rockford, IL), and HSP72 was purchased from Stressgen (Victoria, BC, Canada). Phospho-SAPK/JNK (T183/Y185), total SAPK/JNK, and IκBα were purchased from Cell Signaling (Beverly, MA). Goat anti-mouse HRP-conjugated secondary antibody was obtained from Bio-Rad (Hercules, CA) and donkey anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson (West Grove, PA). Enhanced chemiluminescence reagents were purchased from Fisher Scientific (Pittsburg, PA). [^{14}C]mannitol and 2-deoxy-[1,2-

^{3}H]glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from Sigma.

Experimental animals and treatment. Female Sprague Dawley rats (5 months old) were purchased from Charles River Laboratories (Wilmington, MA) and singly housed in a temperature-controlled (22 ± 2°C) room with 12-h light and dark cycles. Chow rats were fed ad libitum on a soy protein free diet (Harlan Teklad 2020X, Madison, WI, 10% calories from fat), whereas HF rats received a modified Kraegen diet (Storlien et al. 1986) of 60% calories from fat for 6 wk as previously used (Gupte et al. 2009a) which contains the following: 254 g/kg casein, 85 g/kg sucrose, 169 g/kg cornstarch, 11.7 g/kg vitamin mix, 1.3 g/kg choline chloride, 67 g/kg mineral mix, 51 g/kg bran, 3 g/kg methionine, 19 g/kg gelatin, 121 g/kg corn oil, 218 g/kg lard. A pre-set amount of food (in excess of what was needed) was administered to each animal. The remaining food was weighed two to three days later, prior to giving a new batch of food. At the start of the diet, animals underwent ovariectomy (OVX) or sham surgery under ketamine/atropine/xylazine anesthesia (60 mg/kg body wt ketamine, 0.4 mg/kg body wt atropine, 8 mg/kg body wt xylazine). Bilateral flank incisions were made under aseptic conditions. The ovaries were identified and either bilaterally removed via cauterization (OVX) or left intact (sham). Wounds were closed using sutures and wound clips. The following four groups were assessed (n= 5-6 rats/group): *1*) Chow Sham; *2*) Chow OVX; *3*) HF Sham; and *4*) HF OVX. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Intraperitoneal glucose tolerance test. An intraperitoneal glucose tolerance test (IPGTT) was performed during week 6 of the diet regimen. Overnight-fasted rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (2.5 mg/100 g body wt) and given a glucose load of 2 g/kg body wt in 0.9% saline. Tail blood samples were measured with a glucometer (Accu-Check) at time points 0, 15, 45, 60, 90, and 120 minutes after glucose injection. Serum insulin was measured via an ELISA according to the manufacturer's instructions (Alpco Diagnostics, 80-INSRT-E01; Salem, NH).

Tissue dissection. During week 7, overnight-fasted animals were anesthetized under ketamine/atropine/xylazine anesthesia (60 mg/kg body wt ketamine, 0.4 mg/kg body wt atropine, 8 mg/kg body wt xylazine). One soleus and one extensor digitorum longus (EDL) muscle was dissected from each animal, each split longitudinally into strips, and assessed for glucose transport. The remaining soleus and EDL muscle from each animal was frozen in liquid nitrogen for Western blot analysis. Gonadal fat was removed from the ovaries and uterine horns, weighed, and then frozen in liquid nitrogen. The uterus was also removed and weighed.

Measurement of glucose transport activity. Glucose transport was measured in soleus and EDL muscle strips as previously described (Gupte et al. 2009b). Muscles strips recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol, and a gas phase of 95% O_2-5% CO_2. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, the muscles were rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with or without insulin (2 mU/ml). After the rinse step, muscles were

incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2-[1,2-^{3}H]deoxyglucose (2-DG) (1.5 μCi/ml) and 36 mM [^{14}C]mannitol (0.2 μCi/ml), with or without insulin (2 mU/ml), with a gas phase of 95% O_2-5% CO_2 in a shaking incubator. The muscles were then blotted dry, clamp frozen in liquid nitrogen, and processed as described previously (Young et al. 1986; Geiger et al. 2006) for determination of intracellular 2-DG accumulation (^{3}H dpm) and extracellular space (^{14}C dpm) on a scintillation counter.

Serum estradiol measurement. Blood samples were collected at time of sacrifice and allowed to clot at room temperature for 30 minutes. Samples were spun at 17,500 x g for 20 minutes at 4°C. Serum estradiol levels were measured by Estradiol E2 Coat-a-Count Assay (Siemens Diagnostics, TKE21).

Western blotting. Muscles clamp frozen in liquid nitrogen were homogenized in a 12:1 (volume-to-weight) ratio of ice-cold buffer from Biosource (Invitrogen, Camarillo, CA) containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na3VO4; 20 mM Na4P2O7; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250μl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes at 4°C and then centrifuged for 20 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). Samples were prepared in 5X Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 5 minutes. Samples analyzed for GLUT4 protein were not boiled. Protein (30–75 μg) was separated on a SDS-PAGE (8.75–10%) gel followed by a wet transfer to a nitrocellulose membrane

for 60–90 minutes (200 mA). Total protein was visualized by Ponceau staining, and blots were normalized to the 45kDa band as previously described (Gupte et al. 2008). As the GLUT4 antibody only works with non-denatured protein, we chose to normalize all protein measurements to Ponceau staining, which does not require denaturing. Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated overnight with the appropriate primary antibodies. Antibodies were diluted in 1% nonfat dry milk in TBST or in 1% bovine serum albumin in TBST. Blots were incubated in a HRP-conjugated secondary antibody in 1% nonfat dry milk in TBST for 1 hour at room temperature and visualized by ECL. Bands were quantified using Image J densitometry. To serve as a positive control for ERα, uterine tissue was initially used to detect and quantify expression of the full length 66 kDa protein.

Statistical analysis. Results are presented as means $\pm$ SE. Statistical significance was set at $P < 0.05$ and determined by One-way or Two-way ANOVA and Student-Newman-Keuls post hoc test.

RESULTS

Effects of diet and OVX on food intake and body composition. Uterine weight is a commonly used bioassay to assess *in vivo* estrogen status. In the current study, OVX rats had significantly lower uterine weight compared to sham (0.415 $\pm$ 0.016 mg/g body weight vs. 1.618 $\pm$ 0.108 mg/g body weight; $P < 0.001$), confirming negligible estrogen

influence in OVX rats as a result of surgically removing the ovaries. To confirm the *in vivo* estrogen status, serum E_2 levels were also measured. OVX significantly decreased serum E_2 levels compared to sham treated animals (7.6 $\pm$ 0.9 pg/mL vs. 11.9 $\pm$ 1.8 pg/mL; $P<0.05$) which is consistent with previous reports in the literature (Haim et al. 2003; Imaoka et al. 2009). The 6 week HFD did not significantly alter uterine weight or serum E_2 levels in either OVX or sham treated animals, consistent with previously reported data (Bryzgalova et al. 2008; Akamine et al. 2010). Over the course of the 6 week diet regimen, female rats that underwent OVX demonstrated greater average daily food intake and increased body weight compared with sham rats, with no significant difference in food intake or weight gain as a result of the HFD in sham rats (Figures 3A and 3B). OVX animals fed a HFD demonstrated greater food intake and weight gain compared to all other groups. Both groups of HFD animals gained most of their weight in first 2-3 weeks of high-fat feeding. However, the body weight of the animals that underwent OVX and were fed a HFD increased at a greater rate during this period. After week 3, the OVX animals fed the HFD continued to gradually increase their body weight and the sham animals fed the HFD remained fairly constant. Assessment of fat mass, as measured by gonadal fat pad weight, revealed a different pattern from that observed for food intake and body weight. In spite of increased food intake and body weight as a result of OVX, no increase in gonadal fat was observed in this group (Figure 3C). The HFD resulted in a significant increase in gonadal fat pad weight in both sham- and OVX-treated rats.

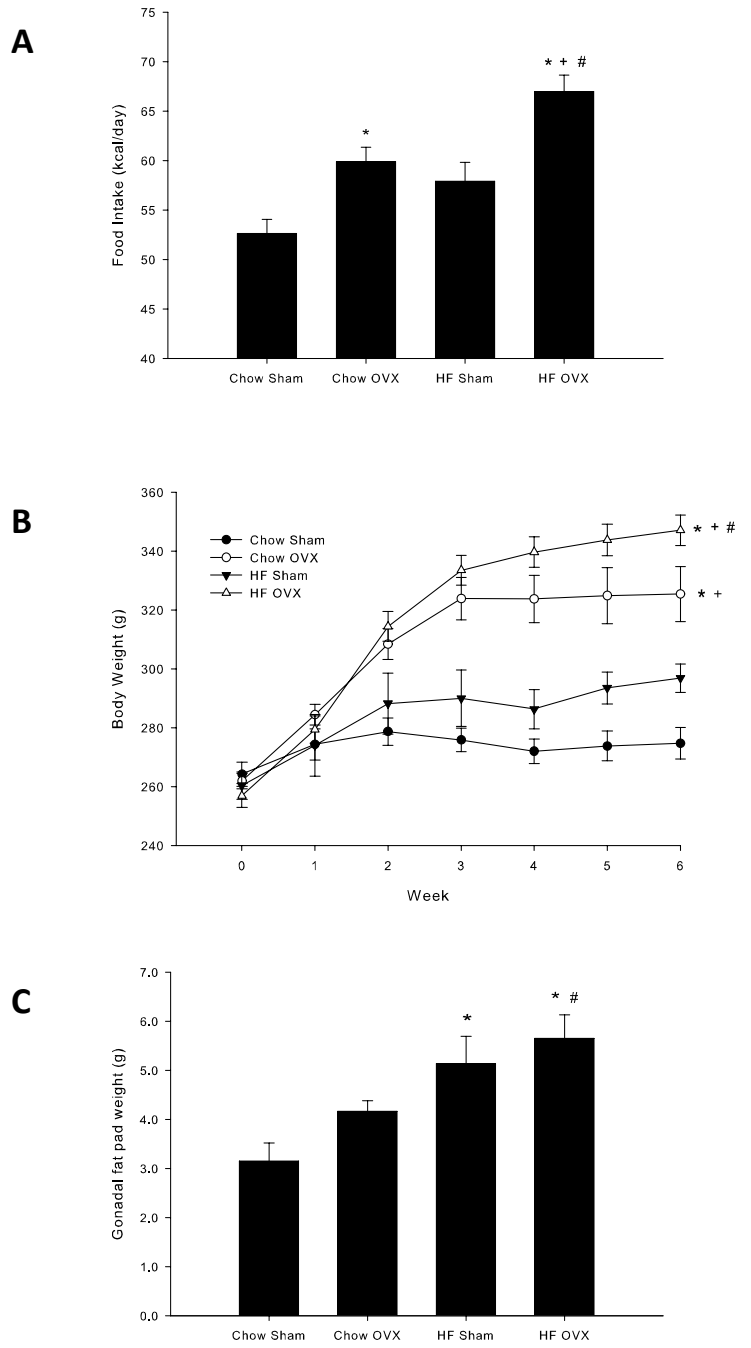

Figure 3. The combination of High Fat Diet (HFD) and ovariectomy (OVX) increases food intake, body weight, and gonadal fat weight. Average daily food intake was measured over the course of a 6 week chow or HFD (A), and body weight was measured weekly (B). At the end of the 6 week study, gonadal fat weight (C) was measured. Values are means $\pm$ SE for 5-6 rats per group. *p<0.05 vs. chow sham; +p<0.05 vs. HF sham; #p<0.05 vs. chow OVX.

Effects of diet and OVX on glucose tolerance. Fasting glucose and insulin levels did not differ across experimental groups at the end of the 6 week diet (Figures 4A and 4B, respectively). An intraperitoneal glucose tolerance test (IPGTT) was performed to assess whole body glucose clearance in response to a glucose challenge. The HFD resulted in a decrease in whole body glucose tolerance, as demonstrated by the inability of HFD rats to effectively clear glucose from their blood by the end of the 2 h test, compared to rats fed a chow diet (Figure 4C). While OVX rats fed a HFD had slightly lower glucose values throughout the test, these values were not significantly different from sham animals fed a HFD. Similarly, OVX did not significantly alter glucose clearance in chow-fed rats compared to sham controls. Serum insulin levels during the IPGTT did not differ among the groups (data not shown).

Effects of diet and OVX on insulin-stimulated skeletal muscle glucose uptake. To investigate the effects of diet and OVX on skeletal muscle glucose uptake, we performed 2-DG uptake assays on the predominately slow-twitch soleus or the predominately fast-twitch EDL muscles. Insulin-stimulated glucose uptake increased above basal in all groups examined (Figures 5A and 5B, respectively). However, no differences in basal- or insulin-stimulated skeletal muscle glucose uptake were observed across treatment groups in either the soleus or EDL muscles.

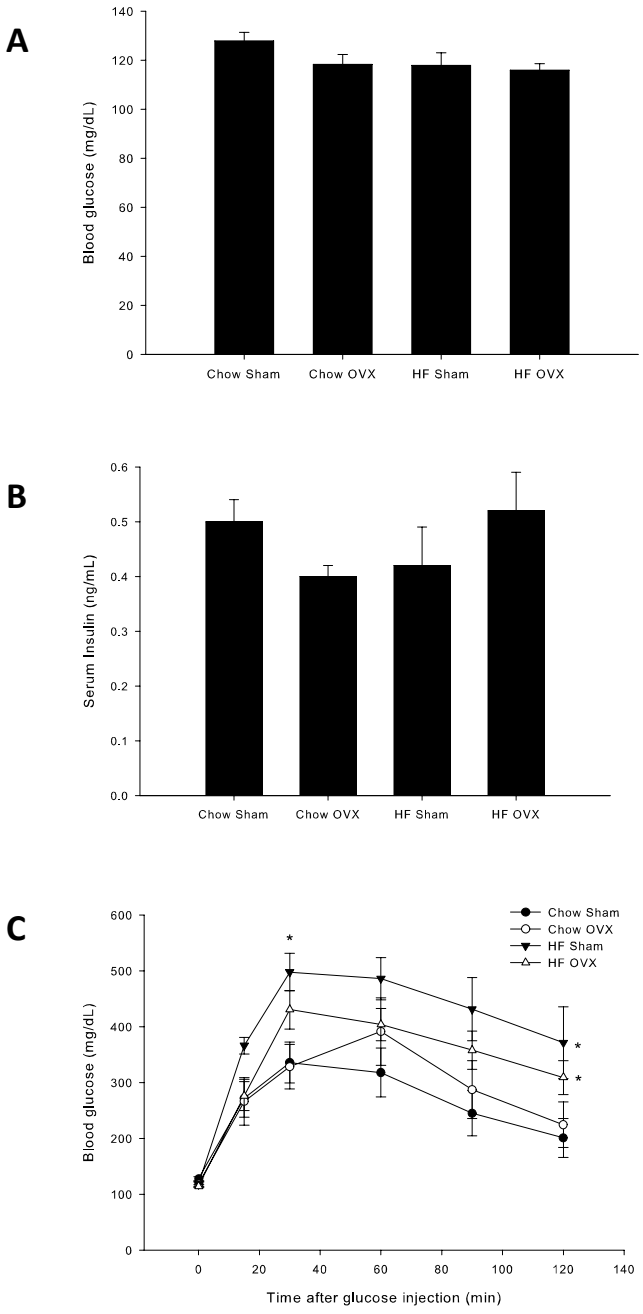

Figure 4. A six week HFD decreases glucose tolerance in female rats. At the end of the 6 week diet, rats were fasted overnight and fasting blood glucose (A) and fasting serum insulin (B) were measured. An intraperitoneal glucose tolerance test (IPGTT) was then performed (C). Rats were injected with a glucose load of 2 g/kg body wt i.p. Blood glucose was measured at time 0, 15, 30, 60, 90, and 120 min after injection using a glucometer. Values are means $\pm$ SE for 5-6 rats per group. Serum insulin values are means $\pm$ SE for 2-6 rats per group. *P<0.05 vs. chow sham.

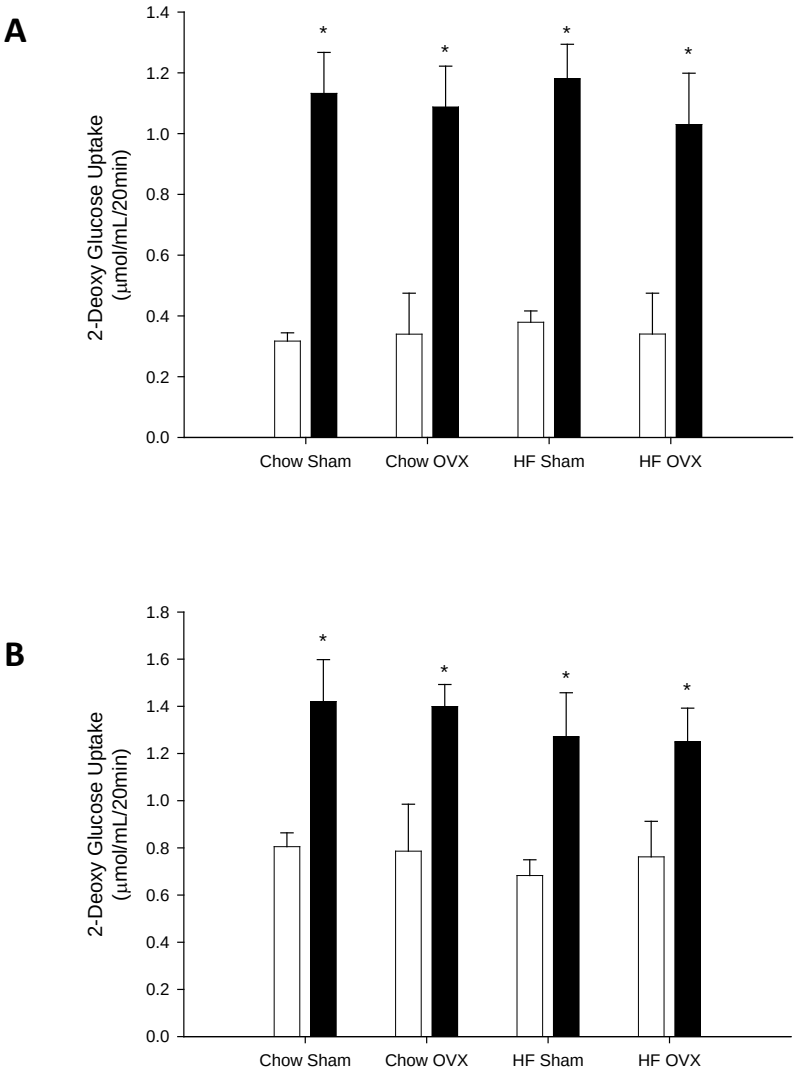

Figure 5. Insulin-stimulated skeletal muscle glucose transport was not altered by a HFD or OVX. Insulin-stimulated glucose transport was measured in soleus (A) and EDL (B) muscles. Muscles were incubated in the absence of insulin (open bars) or in the presence of insulin (2 mU/ml, solid bars), along with 2-[1,2-3H]deoxyglucose and [14C]mannitol. Values are means $\pm$ SE for 5-6 rats per group. *P<0.05 insulin vs. basal.

Effects of diet and OVX on ERα, ERβ, and GLUT4 protein levels. The effects of high-fat feeding on ER protein expression in metabolic tissue has not been previously examined in non-transgenic animal models. ERα and ERβ are both prevalent in skeletal muscle and adipose tissue, with ERα expression shown to be more highly expressed than ERβ in insulin-sensitive tissue (Ribas et al. 2009). Neither OVX nor the HFD had an effect on ERα expression in the soleus or EDL muscles (Figures 6A and 6B). However, ERα expression was significantly decreased in adipose tissue in response to OVX and the HFD (Figure 6C). In OVX rats fed a HFD, the decrease in ERα was not greater than with either intervention alone. In contrast with ERα expression, there was an effect of OVX and diet on ERβ expression in skeletal muscle, but these effects were isolated to the soleus muscle. In this muscle, OVX and a HFD resulted in significant decreases in ERβ expression compared to sham controls (Figure 6D). The combination of a HFD with OVX did not result in a greater decrease in ERβ expression in soleus muscle and no changes with OVX or diet were observed in the EDL muscle (Figure 6E). In the adipose tissue, ERβ expression was unchanged by OVX or a HFD (Figure 6F).

The effect of a HFD on GLUT4 protein expression is equivocal with some studies demonstrating a decrease or no change in GLUT4 protein expression as a result of high-fat feeding (Kahn and Pedersen 1993; Kusunoki et al. 1993; Rosholt et al. 1994; Han et al. 1995; Sevilla et al. 1997; Zierath et al. 1997; Tremblay et al. 2001). In female rats subject to OVX or sham surgery, a 6 week HFD had no effect on GLUT4 protein expression in either the soleus or EDL muscle (Figures 7A and 7B). However, the HFD dramatically reduced GLUT4 protein expression in adipose tissue in both sham- and

OVX- treated rats (65% and 52%, respectively, Figure 7C). OVX in chow-fed rats resulted in lower GLUT4 levels in adipose tissue compared to sham-treated chow rats, although these differences were not statistically significant (P = 0.07).

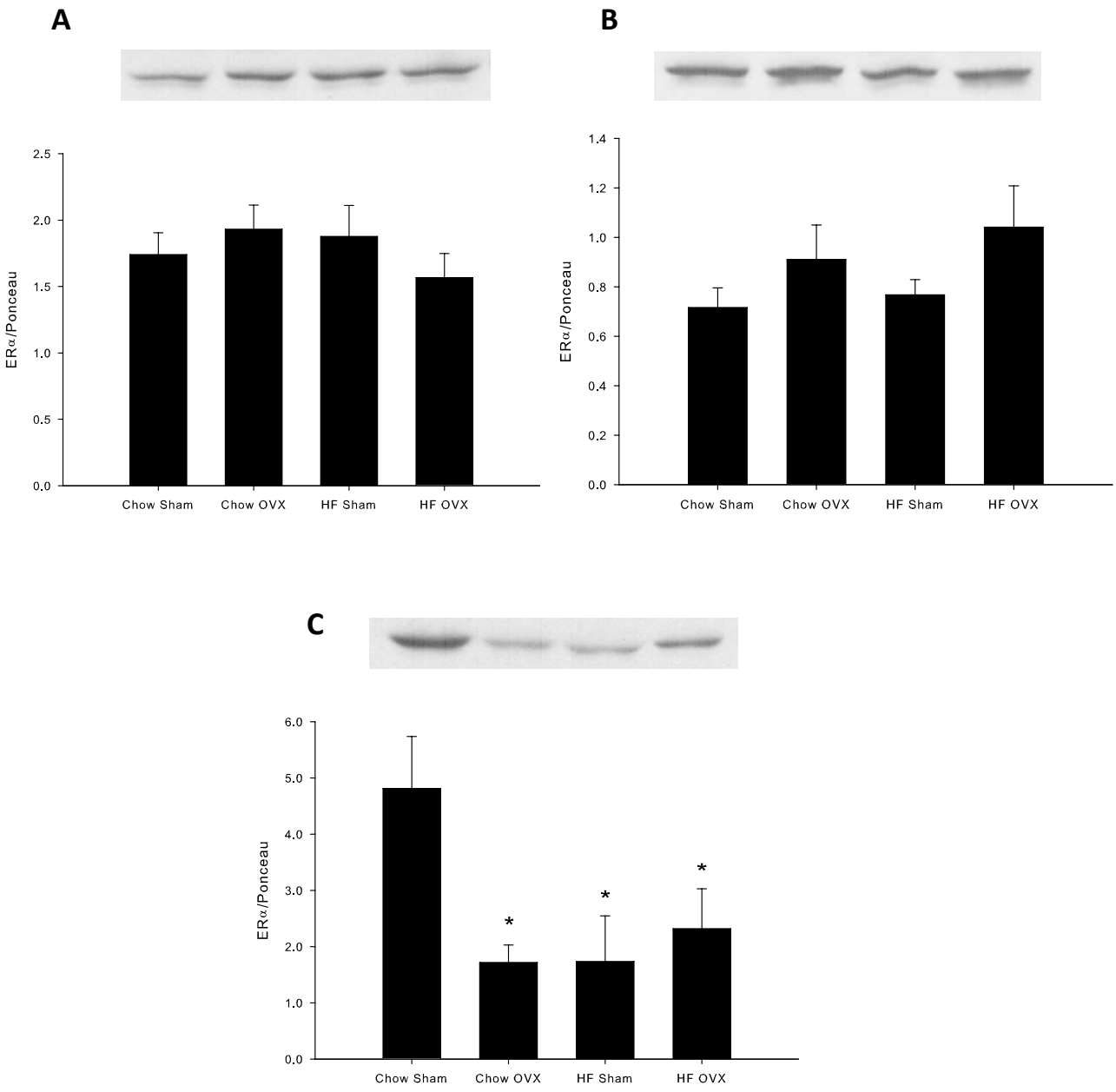

Figure 6.

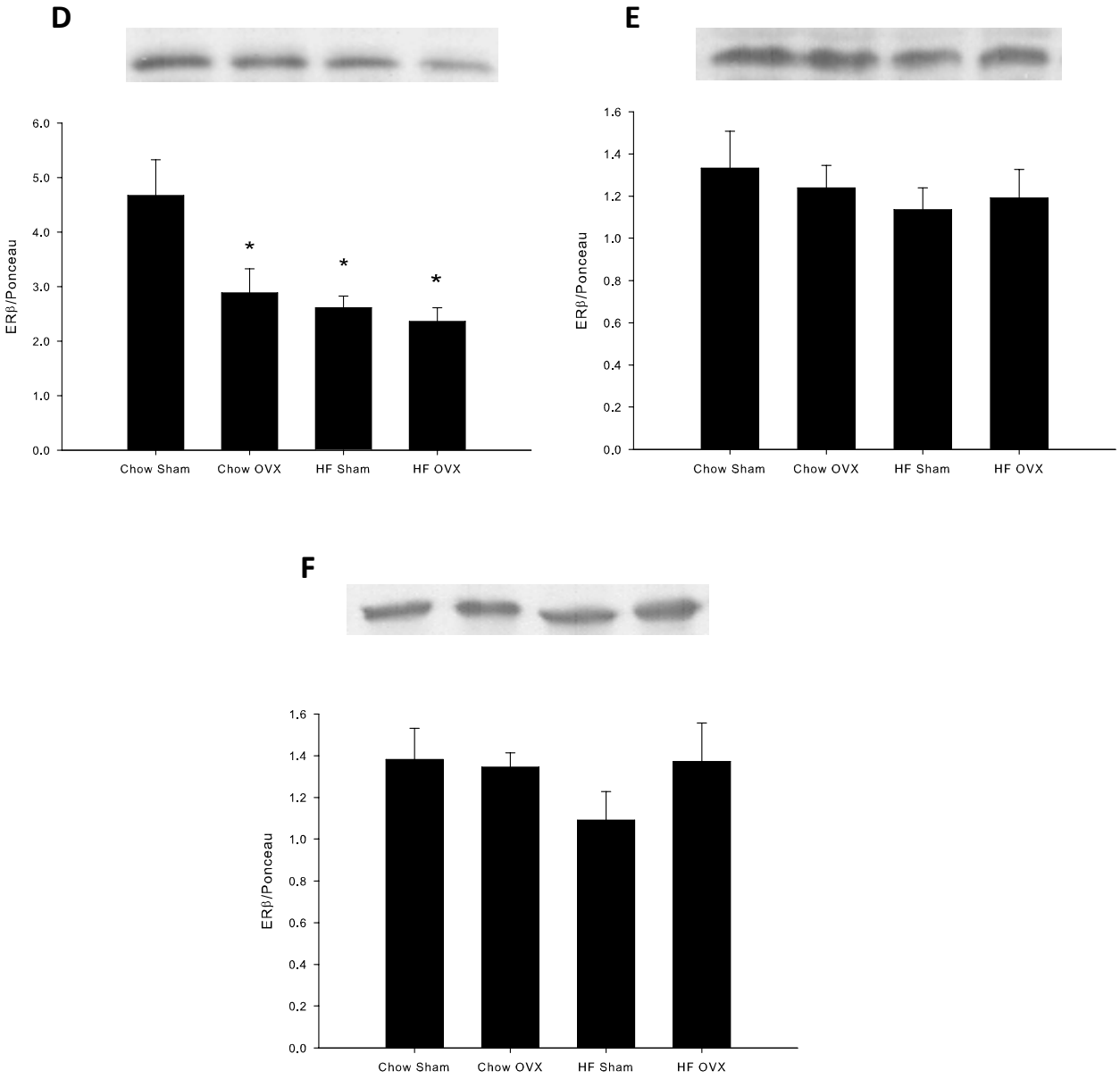

Figure 6. HFD and OVX decrease ERα in adipose tissue and ERβ in soleus muscle. ERα (A-C) and ERβ (D-F) protein levels were measured in the soleus (A, D) and EDL (B, E) muscles and adipose tissue (C, F) by Western blot analysis. Protein levels were normalized to total protein measured by Ponceau staining. Values are means ± SE for 5-6 samples per group. *P<0.05 vs. chow sham.

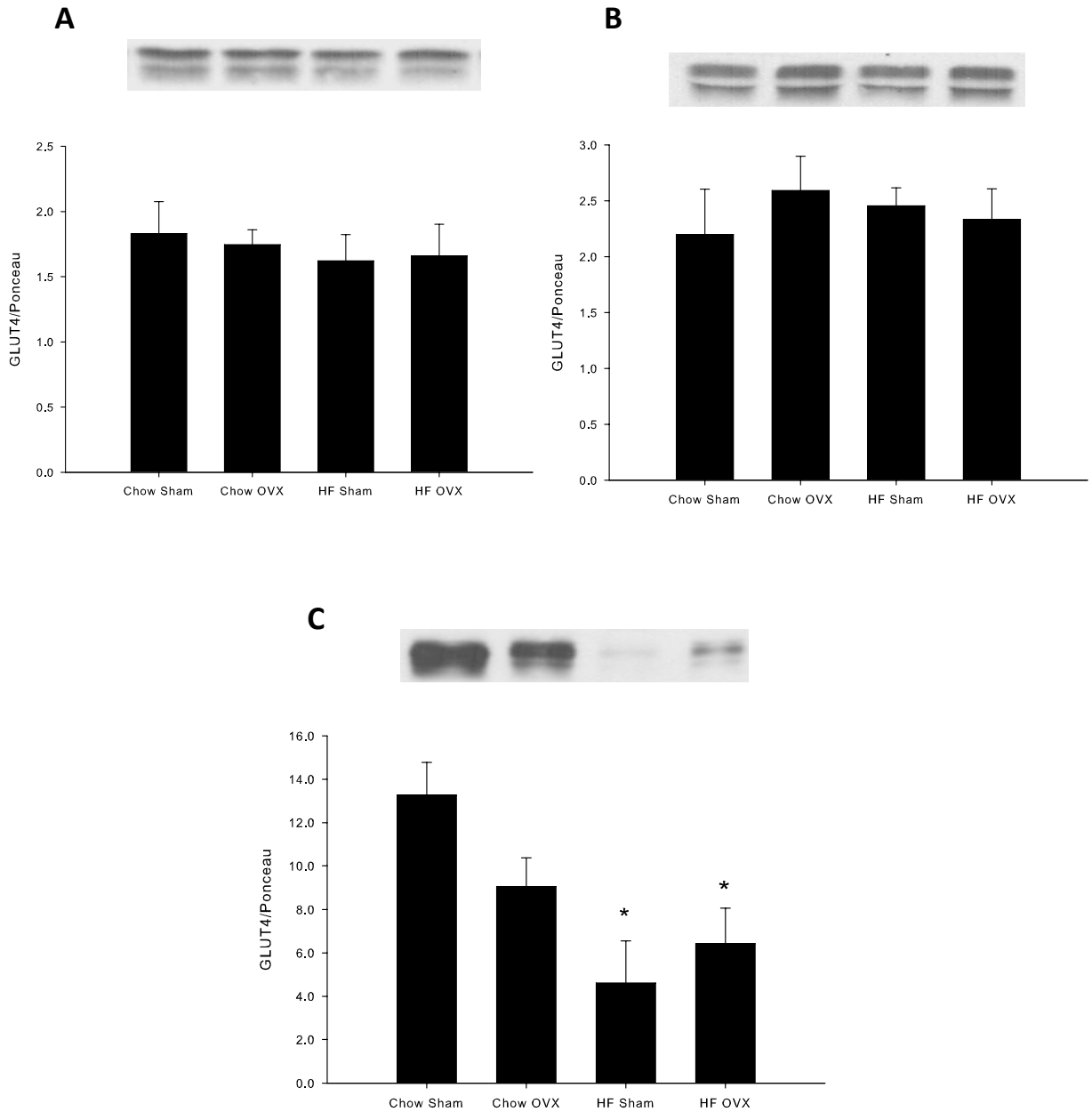

Figure 7. HFD and OVX decrease GLUT4 protein levels in adipose tissue. Protein levels were measured in soleus muscle (A), EDL muscle (B), and adipose tissue (C) by Western blot analysis. Protein levels were normalized to total protein measured by Ponceau staining. Values are means $\pm$ SE for 5-6 muscles per group. *P<0.05 vs. chow sham.

Effects of diet and OVX on stress kinases and HSP72 protein levels. Activation of the stress kinases c-Jun NH_2-terminal kinase (JNK) and inhibitor of kappa B kinase β (IKKβ) were assessed via Western blot analysis. JNK activation was assessed by measuring changes in JNK protein phosphorylation and IKKβ by protein levels of IκBα, the downstream protein targeted for degradation by IKKβ. JNK phosphorylation was increased as a result of the HFD in adipose tissue (Figure 8A), but no change in JNK phosphorylation occurred in either soleus or EDL muscle in response to diet (data not shown). JNK phosphorylation with OVX treatment alone or in combination with a HFD was not different than chow-fed sham animals in adipose tissue or skeletal muscle. Activation of IKKβ was also increased with the HFD in adipose tissue, as indicated by decreased expression of IκBα (Figure 8B). No changes in adipose tissue IκBα protein expression occurred with OVX in either chow or high fat-fed rats. In addition, no changes were observed in IκBα expression in either the soleus or EDL muscle as a result of diet or OVX (data not shown). 6 weeks of a HFD dramatically decreased protein levels of heat shock protein 72 (HSP72) in the adipose tissue of both sham-treated and OVX rats (Figure 8C). OVX alone had no effect on protein levels of HSP72 in adipose tissue. Neither the HFD nor OVX resulted in alterations in HSP72 protein expression in the soleus or EDL muscles (data not shown).

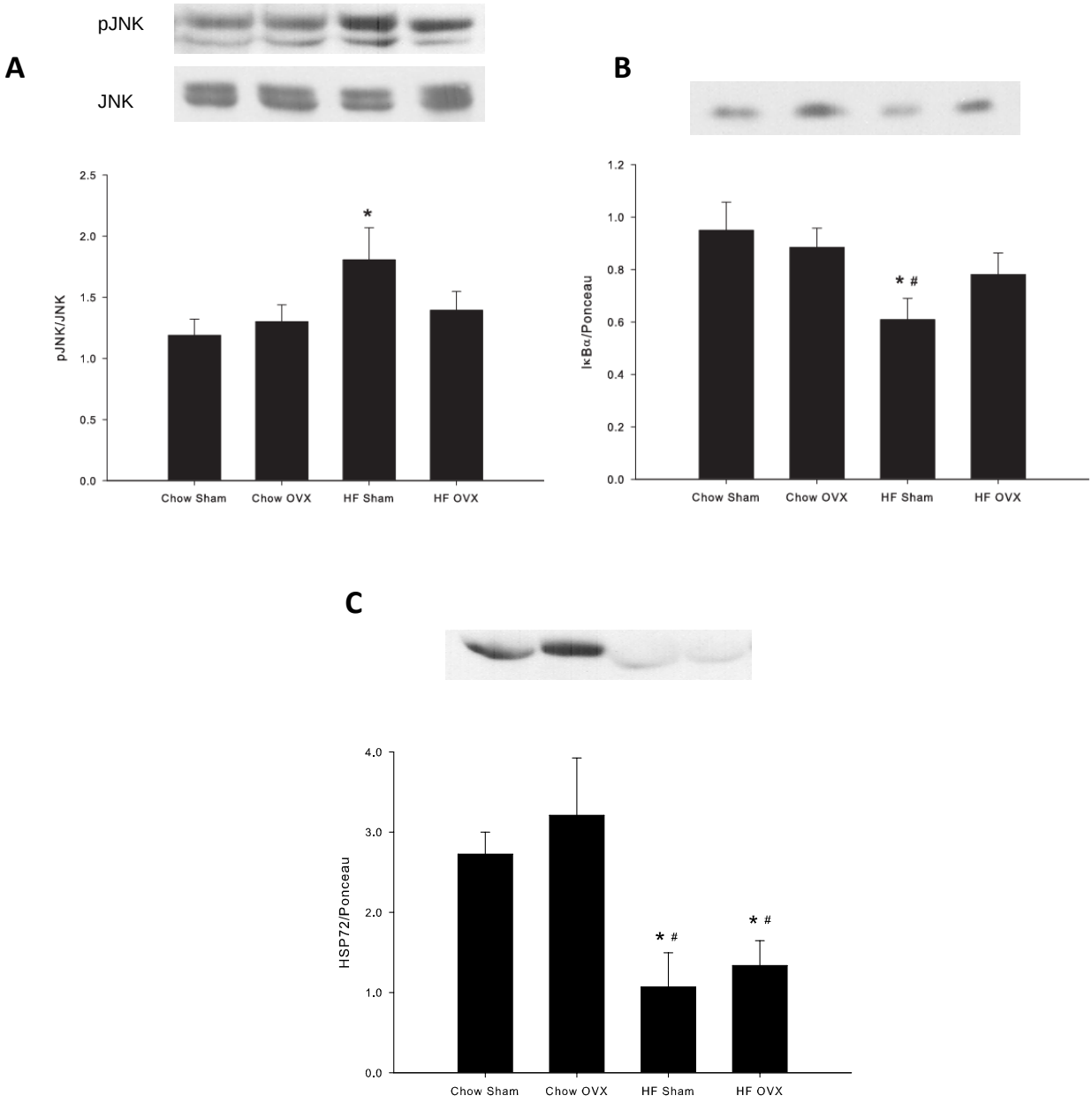

Figure 8. HFD increases stress kinase activation and decreases HSP72 expression in adipose tissue. pJNK/total JNK (A), IκBα (B), and HSP72 (C) protein levels were measured by Western blot analysis. Both the 46 kDa and 54 kDa bands were quantified for pJNK and JNK. Non-phosphorylated protein levels were normalized to total protein measured by Ponceau staining. Values are means $\pm$ SE for 5-6 samples per group. *P<0.05 vs. chow sham; #P<0.05 vs. chow OVX.

DISCUSSION

The purpose of the current study was to examine the effects of a HFD on adipose tissue and skeletal muscle glucose metabolism in female rats with and without OVX, and to determine whether modulation of glucose metabolism in response to a HFD could be attributed to alterations in ER expression. While a short-term HFD in female rats induced whole body glucose intolerance, tissue specific effects were isolated to the adipose tissue with no observed differences in insulin-stimulated glucose uptake, GLUT4, or ERα protein expression levels in skeletal muscle. GLUT4 protein decreased dramatically in adipose tissue of OVX and sham treated rats as a result of a HFD, as did expression of ERα, the ER isoform previously shown to positively mediate glucose metabolism (Barros et al. 2006b; Muraki et al. 2006; Barros et al. 2009). Increased stress kinase activation and decreased HSP72 expression in adipose tissue in response to a HFD further demonstrates the impact of high-fat feeding on this tissue. These new findings highlight the differential effects of high-fat feeding in female compared to male rats, with previous studies demonstrating a significant decrease in skeletal muscle glucose metabolism in response to a HFD in male rats (Pedersen et al. 1991; Han et al. 1995; Zierath et al. 1997; Tremblay et al. 2001; Gupte et al. 2009a). In addition, our findings suggest a high-fat diet induced loss of ERα in adipose tissue may be a contributing factor in the pathogenesis of glucose intolerance in female rats.

Ribas et al. (Ribas et al. 2009) recently showed that female ERα knockout mice have decreased whole body glucose tolerance compared to wild-type mice, suggesting that the absence of ERα results in decreased glucose metabolism. While this data

indicates that ERα is critical for the maintenance of whole body insulin action, the effect of a HFD on ER expression in insulin responsive tissue was unknown. Our findings reveal that ERα expression was decreased with a HFD only in the adipose tissue, which also displayed decreased GLUT4 protein and likely reflects lower glucose utilization in this tissue. OVX animals fed a chow diet demonstrated decreased ERα without corresponding changes in GLUT4, whole body glucose tolerance, or markers of inflammation. While the role of ERα in mediating glucose metabolism cannot be firmly established from this data, these findings suggest ERα-mediated effects may be dependent on additional changes induced by the HFD (stress kinase activation and HSP expression changes). In contrast, in the insulin-responsive skeletal muscle tissue, ERα expression was unchanged as was glucose uptake and GLUT4 protein expression levels. It is still possible that in insulin-resistant skeletal muscle (such as that from male rats fed a HFD), alterations in ERα expression could occur and contribute to changes in glucose metabolism. Other data support our findings of an adipose tissue specific effect of the HFD in female rats. For example, Riant et al. (Riant et al. 2009) demonstrated that the combination of a HFD and OVX resulted in decreased glucose utilization in adipose tissue with no changes in soleus or EDL muscles in female mice (these investigators did not assess ER expression changes). Our findings of decreased ERα in adipose tissue in the current study support the idea that ERα is the primary functioning ER in adipose tissue (Barros et al. 2009). In turn, ERβ has been suggested as the primary functioning ER in skeletal muscle (Barros et al. 2009), which is coincident with our findings of decreased ERβ in the soleus muscle in response to a HFD. Decreased ERβ, the ER

isoform suggested to have a suppressive role on GLUT4 expression (Barros et al. 2006b), could result in protection from HFD-induced insulin resistance in skeletal muscle. The effects of estrogen on skeletal muscle likely depend on the balance between the two receptors and future studies are needed to determine the regulatory roles of ERs in skeletal muscle.

Barros et al. (Barros et al. 2006b; Barros et al. 2009) have previously shown that ERs modulate GLUT4 expression in adipose tissue and skeletal muscle. Although the potential mechanism has yet to be demonstrated in skeletal muscle, ERα could modulate GLUT4 expression through specificity protein 1 and nuclear factor-kappa B. Ribas et al. did not find a decrease in skeletal muscle GLUT4 expression in ERα knockout mice despite insulin resistance and decreased glucose uptake in these mice (Ribas et al. 2009). As these investigators point out, GLUT4 expression is regulated by redundant transcriptional pathways and ERα is likely only one of these pathways. However, our findings, and others (Barros et al. 2009) seem to suggest that ERα modulation of GLUT4 occurs primarily in the adipose tissue and future studies will be needed to assess transcriptional control of GLUT4 by ERα in adipose tissue.

Estrogen has the potential to regulate fat storage and triacylglyceride accumulation by altering transcription of lipogenic proteins such as SREBP-1 and its downstream targets, ACC, and FAS (D'Eon et al. 2005; Bryzgalova et al. 2006; Paquette et al. 2007; Jiang et al. 2009; Chen et al. 2010). The effects of estrogen on lipogenic pathways have primarily been assessed in response to estrogen treatment or replacement. For example, Phrakonkham et al. (Phrakonkham et al. 2008) demonstrated that estrogen

treatment increased FAS expression in cultured adipocytes. However, other studies have demonstrated opposite effects, with estrogen treatment in mice shown to decrease ACC and FAS mRNA in adipose tissue (D'Eon et al. 2005; Bryzgalova et al. 2008). As has been previously shown, physiological estrogen levels may positively modulate glucose metabolism while high or low estrogen levels have a different effect (Muraki et al. 2006; Nagira et al. 2006). More studies are needed to assess the role of estrogen and ER expression in modulating lipogenic pathways in cycling, OVX and estrogen- treated animals.

Increased lipid intermediates and oxidative stress in insulin-responsive tissues can result in activation of stress kinases (Yuan et al. 2001; Hirosumi et al. 2002; Ropelle et al. 2006; Gupte et al. 2009a; Gupte et al. 2009b). We (Gupte et al. 2009a; Gupte et al. 2009b) and others (Chung et al. 2008; Ribas et al. 2009) have previously shown that increased stress kinase activation and decreased HSP expression contribute to decreased insulin signaling and glucose uptake in skeletal muscle. Further evidence suggests that ERα may be involved in stress kinase activation and HSP expression. Ribas et al. demonstrate increased activation of JNK in skeletal muscle and adipose tissue of ERα knockout mice (Ribas et al. 2009). When challenged with a HFD, ERα knockout mice display greater JNK activation and decreased HSP72 expression in adipose tissue compared to high-fat fed wild-type mice (Ribas et al. 2009). These data suggest that ERα may contribute to glucose regulation by positively modulating stress kinase activation and HSP expression. Evidence of inflammation, increased stress kinase activation (increased pJNK and decreased IκBα), and decreased HSP72 expression in adipose tissue

was observed in the present study, although these changes did not always correlate with changes in ERα levels. With OVX alone, ERα protein was decreased in adipose tissue without changes in inflammation observed with a HFD (increased stress kinases and decreased HSP72). As OVX alone did not result in increased adiposity or glucose intolerance, it is possible the combination of decreased ERα and increased inflammation, as observed with the HFD, is critical for glucose intolerance. These results indicate the complex interplay of diet, hormones and inflammation in insulin-responsive tissue requiring further investigation.

While this study focuses on the effect of a HFD and OVX on adipose tissue and skeletal muscle glucose metabolism, the liver is also an important regulator of glucose metabolism. In ERα knockout mice, modest hepatic insulin resistance is present as demonstrated by elevated hepatic glucose production during insulin stimulation and decreased insulin receptor substrate-PI 3-kinase p85 association compared to wild type mice (Ribas et al. 2009). Plausibly, ERα knockouts could have impaired signal transducer and activator of transcription 3 (STAT3) function. Estrogen treatment upregulates STAT3, which suppresses key enzymes in glucose homeostatis, including the gluconeogenic genes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Gao et al. 2006; Lundholm et al. 2008; Ramadoss et al. 2009). Future studies are needed to assess the effects of OVX and a HFD on ER protein expression in the liver and their role in regulating hapatocyte substrate metabolism.

While previous studies demonstrate the importance of the ERs in regulating glucose metabolism, the impact of a HFD on ER expression in skeletal muscle and

adipose tissue was unknown. Findings from the present study indicate a short-term HFD in female rats induced whole body glucose intolerance, along with decreased ERα □and GLUT4 in adipose tissue. In contrast with previous findings using male rodents, a short-term HFD did not decrease skeletal muscle glucose uptake in female rats. In addition, decreased ERβ expression was observed in the soleus muscle with no changes in skeletal muscle ERα expression. Future studies are needed to determine the tissue specific regulation of ERs and how altered ER expression and/or function may contribute to increased susceptibility to type 2 diabetes.

Chapter 3

In vivo stimulation of estrogen receptor α increases insulin-stimulated skeletal muscle glucose uptake

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Abstract

Previous studies suggest estrogen receptor α (ERα) is involved in estrogen-mediated regulation of glucose metabolism and is critical for maintenance of whole body insulin action. Despite this, the effect of direct ERα modulation in insulin-responsive tissues is unknown. The purpose of the current study was to determine the impact of ERα activation, using the ER subtype-selective ligand propylpyrazoletriyl (PPT), on skeletal muscle glucose uptake. 2 month-old female Sprague Dawley rats, ovariectomized for 1 week, were given subcutaneous injections of PPT (10 mg/kg), estradiol benzoate (EB; 20 μg/kg), the ERβ agonist diarylpropionitrile (DPN, 10 mg/kg), or vehicle every 24 hours for 3 days. On the fourth day, insulin-stimulated skeletal muscle glucose uptake was measured *in vitro* and insulin signaling intermediates were assessed via Western blotting. Activation of ERα with PPT resulted in increased insulin-stimulated glucose uptake into the slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles, activation of insulin signaling intermediates (as measured by pAkt and PAS) and phosphorylation of AMPK. GLUT4 protein was increased only in the EDL muscle. Rats treated with EB or DPN for 3 days did not show an increase in insulin-stimulated skeletal muscle glucose uptake compared to vehicle-treated animals. These new findings reveal that direct activation of ERα positively mediates glucose uptake and insulin action in skeletal muscle. Evidence that estrogens and ERα stimulate glucose uptake has important implications for understanding mechanisms of glucose homeostasis, particularly in postmenopausal women.

Introduction

Numerous clinical and basic studies demonstrate that estrogens contribute to glucose homeostasis (Louet et al. 2004). The beneficial effects of estrogens on insulin action and glucose homeostasis are supported by studies showing insulin sensitivity is higher in premenopausal women compared with age-matched men (Nuutila et al. 1995; Donahue et al. 1997). Following menopause, a significant decline in insulin sensitivity occurs along with a corresponding increase in fat mass (Lindheim et al. 1994; Carr 2003; Alonso et al. 2006; Moreno et al. 2010). Ovariectomy has also been shown to impair insulin sensitivity and glucose metabolism in animal models (Kumagai et al. 1993; Wagner et al. 1998). In addition, estrogen replacement can ameliorate the increased risk for type 2 diabetes in postmenopausal women and improve whole body (Lindheim et al. 1994; Margolis et al. 2004; Alonso et al. 2006; Riant et al. 2009; Moreno et al. 2010) and skeletal muscle glucose metabolism (Riant et al. 2009; Moreno et al. 2010).

The physiological actions of estrogens are mediated by two receptors, estrogen receptor (ER) α and ERβ. Both ERα and ERβ are expressed in a variety of tissues, with ERα more highly expressed in insulin-sensitive tissue (Ribas et al. 2009). Increased adiposity occurs in humans and mice as a result of decreased ERα activation (Smith et al. 1994; Heine et al. 2000), and mice with global knockout of ERα exhibit impaired glucose tolerance and skeletal muscle insulin resistance (Heine et al. 2000; Bryzgalova et al. 2006; Riant et al. 2009). Based on this evidence, the beneficial effects of estrogens on glucose metabolism are thought to be mediated by ERα. However, while there is strong clinical evidence demonstrating a relationship between ERα expression levels and the

incidence of insulin resistance and increased adiposity, the ability of ERα to positively mediate insulin action and increase glucose uptake *in vivo* is unknown.

The purpose of the current study was to determine the impact of *in vivo* ERα activation on skeletal muscle glucose uptake and insulin action. Skeletal muscle accounts for 75% of glucose regulation in the body (DeFronzo et al. 1985) and, as a result, has a significant impact on whole body glucose homeostasis. In the current study, we utilized estradiol benzoate (EB) and the compound propylpyrazoletriyl (PPT), a potent ERα agonist. PPT is capable of binding with high affinity and 400-fold preference to ERα, and exhibits almost no binding to ERβ (Stauffer et al. 2000). For comparison, the ERβ agonist diarylpropionitrile (DPN), which binds to ERβ at a 70-fold higher affinity than ERα (Meyers et al. 2001), was also used. Our results demonstrate that activation of ERα with PPT increases insulin-stimulated glucose uptake and insulin signaling in skeletal muscle. These findings provide new insight into the role of estrogen receptors in mediating glucose uptake, a finding with important implications for postmenopausal women at increased risk for type 2 diabetes.

Methods

Ethical approval

The authors have read "Reporting ethical matters in the Journal of Physiology: standards and advice." (Drummond 2009), and our experiments comply with the policies and regulations of *The Journal of Physiology* and the UK regulations on animal

experimentation. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Materials

GLUT4 antibody (ab654) and tubulin (ab7291) were purchased from Abcam (Cambridge, MA). pAkt (S473), total Akt, phospho-(ser-thr) Akt substrate (PAS), pERα (S118)□, pAMPK (T172), and total AMPK were purchased from Cell Signaling (Beverly, MA). pAS160 (T642) and total AS160 were purchased from Millipore (Billerica, MA). ERα (MC-20) was purchased from Santa Cruz (Santa Cruz, CA) and ERβ (PA1-310B) was purchased from Thermo Fisher Scientific (Rockford, IL). Donkey anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson (West Grove, PA) and goat anti-mouse HRP-conjugated secondary antibody was purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were purchased from Fisher Scientific (Pittsburg, PA). [^{14}C]mannitol and 2-deoxy-[1,2-^{3}H]glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). Estradiol benzoate (E9000; EB) was purchased from Sigma. Propylpyrazoletriyl (PPT) and diarylpropionitrile (DPN) were purchased from Tocris Bioscience (Ellisville, MO). All other reagents were obtained from Sigma.

Experimental animals and treatment

2 month-old female Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in a temperature-controlled (22 ± 2°C) room with 12-h light and dark cycles and given free access to food and water. Animals underwent bilateral ovariectomy (OVX) under ketamine/atropine/xylazine anesthesia (intraperitoneal injection of 60 mg/kg body wt ketamine, 0.4 mg/kg body wt atropine, 8 mg/kg body wt xylazine). Bilateral flank incisions were made under aseptic conditions. The ovaries were identified and bilaterally removed via cauterization. In a subset of 6 animals, incisions were made but the ovaries were left intact for evaluation of endogenous estradiol levels at sacrifice. Wounds were closed using sutures and wound clips. One week following surgery, OVX animals received subcutaneous injections once every 24 hours for 3 days (N = 6 animals per group) of EB (20 μg/kg body wt) dissolved in 90% corn oil/10% ethanol, PPT (10 mg/kg body wt) dissolved in DMSO, or DPN (10 mg/kg body wt) dissolved in DMSO. This dose of PPT and DPN has previously been used in *in vivo* rodent studies (Harris et al. 2002; Lee et al. 2005). The dose of EB was chosen to produce physiological levels of serum estradiol (Hurn and Macrae 2000; Haim et al. 2003). EB is commonly used in research studies and is a conjugate-salt form of 17β-estradiol. Like all conjugate molecules, the benzoate moiety dissociates from the 17β-estradiol moiety when dissolved in solution. Thus, the solution of EB injected into the animals contains free 17β-estradiol which binds to the ERs. Vehicle treatments were 90% corn oil/10% ethanol or DMSO, as appropriate. No differences in uterine weight or glucose uptake were observed between the two vehicle treatments, and therefore these measurements were combined in the results section. Rats were fasted 10 hours prior to

muscle incubation and glucose transport experiments. 24 hours following the final injection, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (2.5 mg/100 g body wt) for removal of the soleus and extensor digitorum longus (EDL) muscles. The uterus was also removed and weighed. Rats were sacrificed by cervical dislocation.

Muscle incubation

The soleus and EDL muscles were dissected and each split longitudinally into two strips to allow for adequate diffusion of substrates, as described previously (Henriksen and Holloszy 1991; Gupte et al. 2008). Two muscle strips per rat were assessed for glucose transport and two strips for Western blot analysis. Muscle strips designated for Western blot analysis recovered from the dissection for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol, and a gas phase of 95% O2-5% CO_2 (recovery medium). The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, one muscle strip was transferred to recovery medium containing 2 mU/mL insulin, and the other muscle strip was left without insulin (basal) in recovery medium for 30 min and then clamp frozen in liquid nitrogen.

Measurement of glucose transport activity

Glucose transport was measured in soleus and EDL muscle strips. Muscle strips were incubated after dissection in recovery medium for 60 min at 35°C and then rinsed for 30 min at 29°C in 2 mL of oxygenated KHB containing 40 mM mannitol, with or without insulin (2 mU/mL). After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2-[1,2-^{3}H]deoxyglucose (2-DG) (1.5 μCi/mL) and 36 mM [^{14}C]mannitol (0.2 μCi/mL), with or without insulin (2 mU/mL), with a gas phase of 95% O_2-5% CO_2 in a shaking incubator. The muscles were then lightly blotted, clamp frozen in liquid nitrogen, and processed as described previously (Young et al. 1986; Geiger et al. 2006) for determination of intracellular 2-DG accumulation (^{3}H dpm) and extracellular space (^{14}C dpm) on a scintillation counter.

Serum estradiol measurement

Blood samples were collected at time of sacrifice and allowed to clot at room temperature for 30 minutes. Samples were spun at 17,500 x g for 20 minutes at 4°C. Serum estradiol levels were measured by Estradiol E2 Coat-a-Count Assay (Siemens Diagnostics, TKE21).

Western blotting

Muscles incubated with and without insulin and clamp frozen in liquid nitrogen were homogenized in a 12:1 (volume-to-weight) ratio of ice-cold buffer from Biosource

(Invitrogen, Camarillo, CA) containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na_3VO_4; 20 mM $Na_4P_2O_7$7; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250µl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes at 4°C and then centrifuged for 20 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). Samples were prepared in 5X Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 5 minutes. Samples analyzed for GLUT4 protein were not boiled. Protein (30–100 µg) was separated on a SDS-PAGE (7.5–10%) gel followed by a wet transfer to a nitrocellulose membrane for 60–90 minutes (200 mA). Total protein was visualized by Ponceau staining, and GLUT4 blots were normalized to the 45kDa band. Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated overnight with the appropriate primary antibodies. Antibodies were diluted in 5% nonfat dry milk in TBST or in 1% bovine serum albumin in TBST. Blots were incubated in a HRP-conjugated secondary antibody in 1% nonfat dry milk in TBST for 1 hour at room temperature and visualized by ECL. Western blots first probed for phosphorylated proteins were stripped and probed for total protein expression for normalization, and non-phosphorylated proteins were stripped and probed for tubulin expression for normalization. Blots were stripped for 20 minutes at 55°C in buffer containing 62.5mM Tris-HCl, 2% SDS and 100mM 2-mercaptoethanol. Blots were then rinsed three times in TBST for 15 minutes each, blocked in 5% milk in TBST for 1 hour, and incubated in the appropriate primary

antibody overnight. The GLUT4 antibody only works with non-denatured protein, and the tubulin antibody requires denaturation. Therefore, Western blots that were ran with non-denatured samples and probed for GLUT4 were not able to be stripped and re-probed for tubulin for normalization. Therefore, Ponceau staining, which does not require denaturing, was used for normalizing as previously described (Gupte et al. 2008). We identified the 56 kDa ERβ protein by using the rat hypothalamus as a positive control (Figure 9). This tissue has previously been used as a positive control (Kalbe et al. 2007). Bands were quantified using Image J densitometry.

Statistical analysis

Results are presented as means $\pm$ SE, and statistical significance was set at $P < 0.05$. Symbols on the uterine weight, pERα, total ERα, pAMPK, and GLUT4 graphs represent differences determined by one-way ANOVA. Symbols on the glucose transport, pAkt, and PAS-160 graphs represent differences determined by a Student-Newman Keuls post hoc test following a significant interaction as determined by two-way ANOVA.

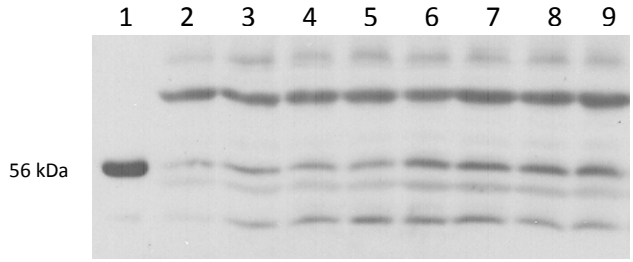

Figure 9: ERα protein identified by the hypothalamus. Female Sprague Dawley rat hypothalamus and soleus muscles were homogenized and total protein was measured as stated in the materials and methods. ERα protein was measured by Western blot analysis. Lane 1 represents the hypothalamus (3 μg protein) and lanes 2-9 represents soleus muscle samples (100 μg protein).

Results

In vivo effects of estrogen and PPT treatment

In order to control for endogenous estrogen levels, all animals underwent OVX for 1 week. At sacrifice, serum estadiol levels were significantly decreased as a result of OVX (11.9 $\pm$ 1.8 pg/mL vs. 6.8 $\pm$ 0.4 pg/mL for intact and OVX animals, respectively; $p<0.01$). Serum estradiol levels were significantly greater in EB treated animals (14.5 $\pm$ 2.0 pg/mL) compared to vehicle treated animals (6.8 $\pm$ 0.4 pg/mL; $p<0.001$). Estradiol levels for EB treated animals were within physiological values for cycling rodents (Hurn and Macrae 2000; Haim et al. 2003).

Administration of estrogen and PPT has been shown to increase uterine weight by activation of ERα (Harris et al. 2002; Frasor et al. 2003; Stygar et al. 2007). In this manner, uterine weight can serve as a bioassay for the *in vivo* effects of estrogen and PPT. In OVX female rats, administration of both EB and PPT for 3 days resulted in a significant and similar increase in uterine weight compared to vehicle treated controls (Figure 8), which suggests that ERα is being activated to the same extent in the uterus. Consistent with previous findings (Harris et al. 2002; Frasor et al. 2003), a higher dose of PPT than EB was needed to increase uterine weight. In contrast, administration of the ERβ agonist DPN for 3 days had no effect on uterine weight (Figure 10) as previously shown (Frasor et al. 2003).

Prior to treatment with EB, PPT, or DPN, body weight was not different among groups. After treatment, body weight did not change in animals treated with EB (vehicle

179.2 ± 3.2 g vs. EB 172.2 ± 2.8 g) or DPN (vehicle 184.8 ± 3.6 g vs. DPN 181.5 ± 2.9 g) but decreased in animals treated with PPT (vehicle 183.3 ± 2.0 g vs. PPT 169.3 ± 1.8 g; $p<0.001$).

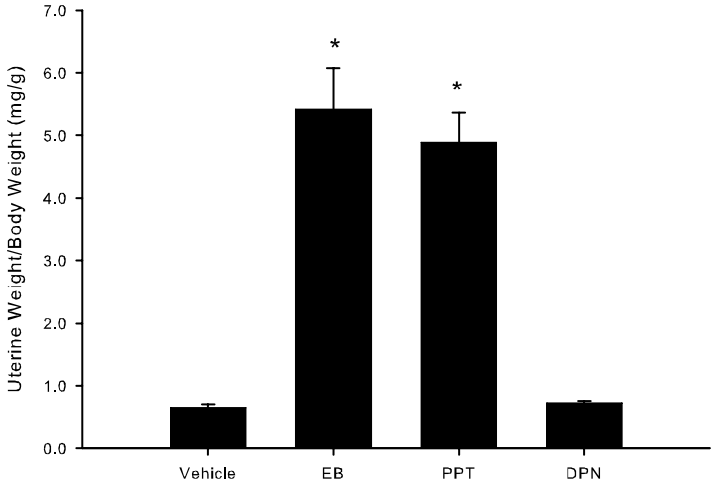

Figure 10: Administration of EB and PPT activates ERα as indicated by an increase in uterine weight. Female Sprague Dawley rats were give subcutaneous injections of EB (20 μg/kg), PPT (10 mg/kg), DPN (10mg/kg), or vehicle for 3 days. Uterine weight was measured at time of sacrifice. Symbol represents differences determined by one-way ANOVA. *$p<0.001$ vs. vehicle; N = 6/group

PPT increases insulin-stimulated glucose transport in soleus and EDL muscle

To determine the impact of ERα stimulation on skeletal muscle glucose uptake, we performed *in vitro* 2-DG uptake assays on the predominately slow-twitch soleus or the predominately fast-twitch EDL muscles following EB, PPT, or DPN administration. Insulin-stimulated glucose uptake was significantly increased above basal in all treatment groups in both the soleus and EDL muscles (Figure 11A and 11B, respectively). Three day treatment with EB did not augment insulin-stimulated glucose uptake compared to that observed with vehicle alone. However, specifically activating ERα with PPT resulted in a greater increase in insulin-stimulated glucose transport in both the soleus and EDL muscles compared to vehicle treated controls. This observed increase in the soleus and EDL muscles was 108% and 55%, respectively, greater than insulin-stimulated glucose transport in rats treated with vehicle. Treatment with the ERβ agonist DPN had no effect on insulin-stimulated skeletal muscle glucose uptake.

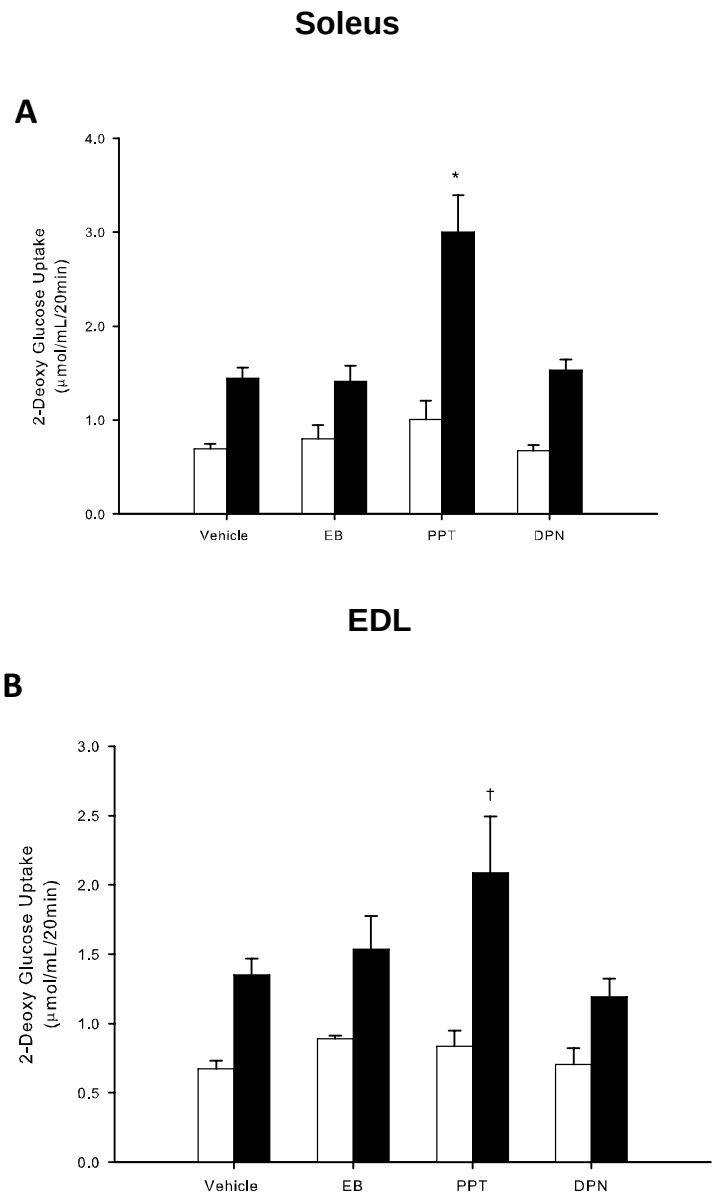

Figure 11: Skeletal muscle glucose transport in female rats treated with vehicle, EB, PPT, and DPN. Female Sprague Dawley rats were give subcutaneous injections of EB (20 µg/kg), PPT (10 mg/kg), DPN (10mg/kg) or vehicle for 3 days. Insulin-stimulated glucose transport was measured in soleus (A) and EDL (B) muscles as described in the materials and methods. Briefly, muscles were incubated in the absence of insulin (open bars) or in the presence of insulin (2 mU/ml, solid bars), along with 2-[1,2-3H]deoxyglucose. *$p<0.001$ and †$p<0.05$ vs. insulin-stimulated vehicle indicates a significant interaction as determined by two-way ANOVA. The horizontal line indicates a significant main effect of insulin relative to basal across all treatment groups, #$p<0.05$; N = 6/group.

PPT activates ERα in skeletal muscle

Given the significant effect of PPT on skeletal muscle glucose uptake, we next investigated the cell signaling pathways altered by PPT in skeletal muscle. Phosphorylation of ERα, a measure of protein activation (Weigel 1996; Joel et al. 1998), significantly increased in soleus and EDL muscles (Figures 12C and 12D, respectively) following 3 days of PPT administration compared to vehicle-treated controls. Total ERα also decreased following PPT treatment (Figures 12C and 12D). No change in ERβ occurred in soleus or EDL muscles as a result of PPT treatment (Figures 12C and 12D), highlighting the specificity of PPT for ERα in skeletal muscle.

PPT increases insulin-stimulated phosphorylation of Akt and AMPK in soleus and EDL muscle

Akt is a protein kinase in the insulin/IRS-1/PI3K signaling cascade, and activation of this protein is crucial for insulin-stimulated glucose transport. Our results demonstrate that activation of Akt in response to insulin stimulation, as measured by phosphorylation of Akt on serine 473, was increased in the soleus and EDL muscles from rats treated with PPT (Figures 13A and 13B). An additional pathway for signaling GLUT4 translocation to the membrane and increasing glucose transport, independent of insulin, is by phosphorylation of AMP-activated protein kinase (AMPK). PPT treatment resulted in increased phosphorylation of AMPK in both the soleus and EDL muscles (Figures 13C and 13D, respectively).

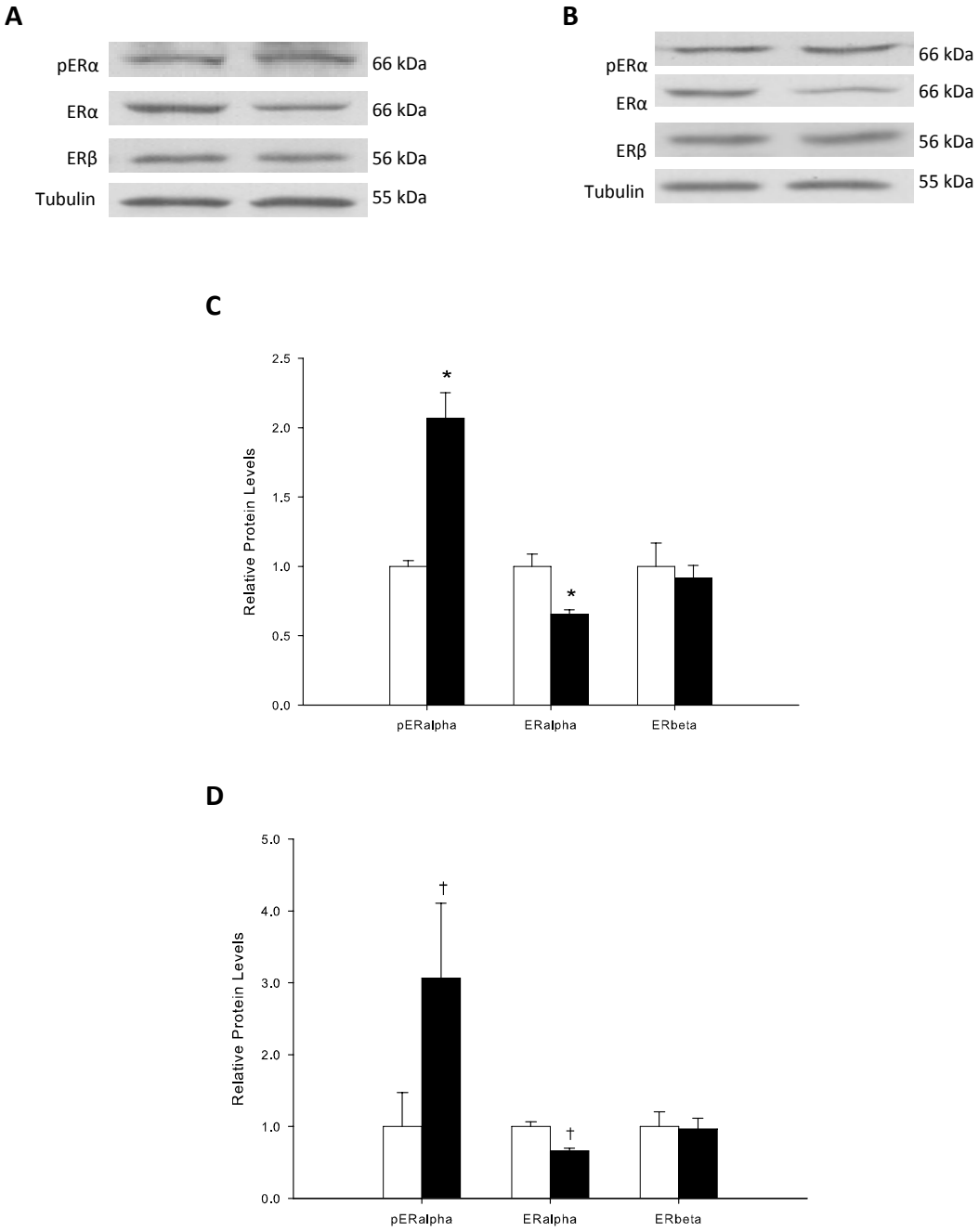

Figure 12: PPT activates ERα in skeletal muscle. Female Sprague Dawley rats were given subcutaneous injections of PPT (10 mg/kg) or vehicle for 3 days. Western blot analysis measured phosphorylation of ERα normalized to total ERα and total ERα and total ERβ normalized to tubulin in the soleus (C) and EDL (D) for vehicle (open bars) and PPT-treated (closed bars) animals. Representative blots are shown for the soleus (A) and EDL (B). *$p<0.05$ and †$p<0.01$ vs. vehicle as determined by one-way ANOVA; N = 6/group

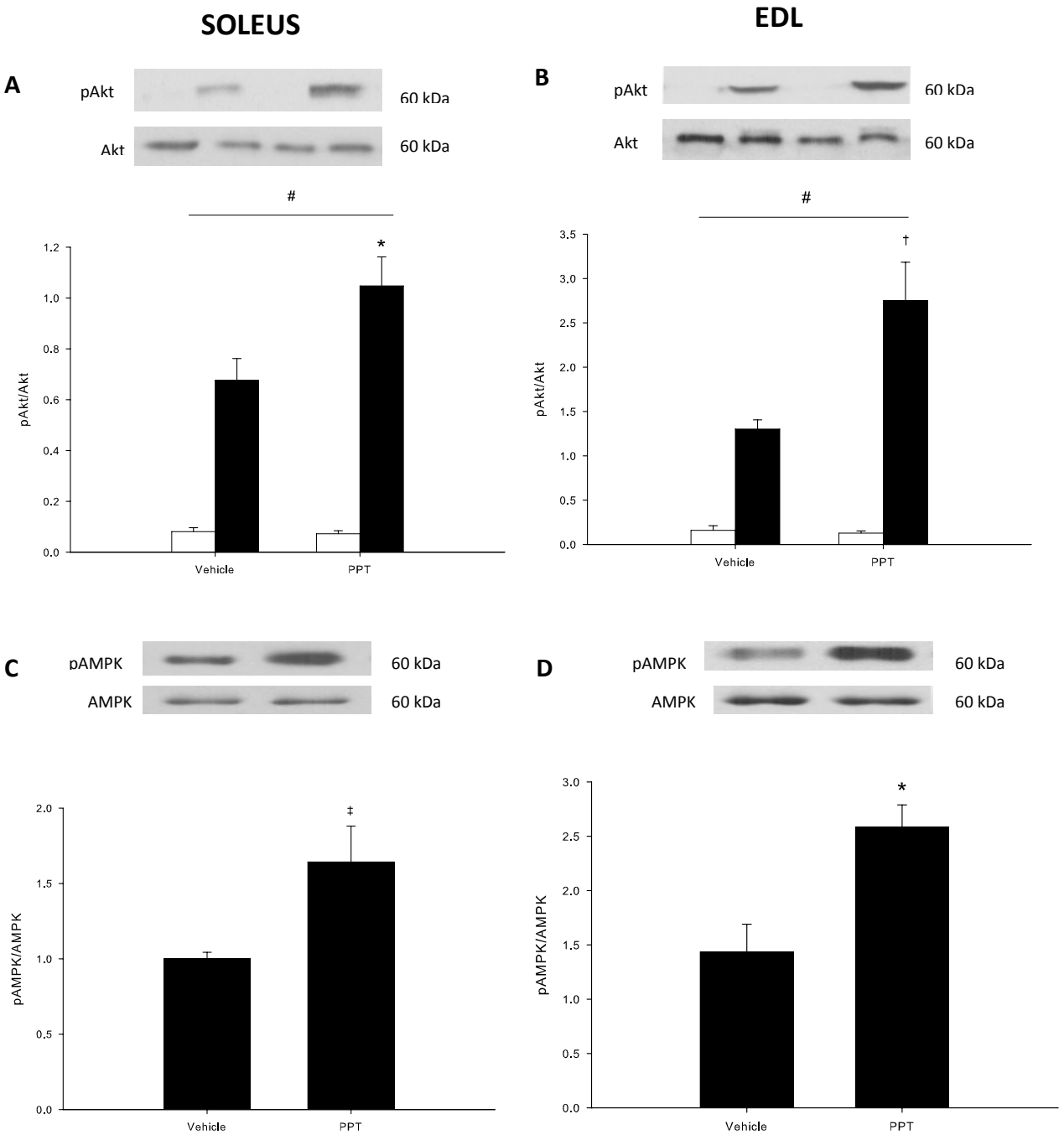

Figure 13: *In vivo* activation of ERα via PPT increases insulin-stimulated phosphorylation of Akt and AMPK in the soleus and EDL. Female Sprague Dawley rats were treated with PPT as stated in the materials and methods. Activation of Akt was measured by Western blot analysis of pAkt normalized to Akt in the absence of insulin (open bars) or in the presence of insulin (2 mU/ml, solid bars) in the soleus (A) and EDL (B). *p<0.01, †p<0.001 on (A) and (B) vs. insulin-stimulated vehicle indicates a significant interaction as determined by two-way ANOVA. The horizontal line indicates a significant main effect of insulin relative to basal, #p<0.05. Activation of AMPK was measured by Western blot analysis of pAMPK normalized to total AMPK in the soleus (C) and EDL (D). Symbols on (C) and (D) represent differences determined by one-way ANOVA. *p<0.01, ‡p<0.05 vs. vehicle; N = 6/group.

PPT increases insulin-stimulated phosphorylation of PAS-160 in soleus and EDL muscle

Activation of Akt and AMPK results in downstream activation of Akt substrates with a molecular weight of 160 kDa, including AS160. Detection of activated Akt substrates can collectively be identified by a phospho-Akt substrate (PAS) antibody. Activation of Akt substrates with a molecular weight of 160 kDa (PAS-160) in response to insulin stimulation was increased in the soleus and EDL muscles from rats treated with PPT (Figures 14A and 14B). We measured specific activation of AS160 by phosphorylation of threonine 642 and found no additional increase in insulin-stimulated activation in the soleus and EDL muscles from rats treated with PPT compared to rats treated with vehicle (Figures 14C and 14D).

PPT increases GLUT4 protein in the EDL muscle

Insulin and AMPK both signal translocation of GLUT4 to the cell membrane where GLUT4 then transports glucose into the cell. Rats treated with PPT demonstrated increased GLUT4 protein levels in EDL muscles (Figure 15B). In contrast, GLUT4 protein was unaltered in response to PPT in soleus muscles (Figure 15A). GLUT4 levels were not altered as a result of EB or DPN treatment (Figure 15C-15F).

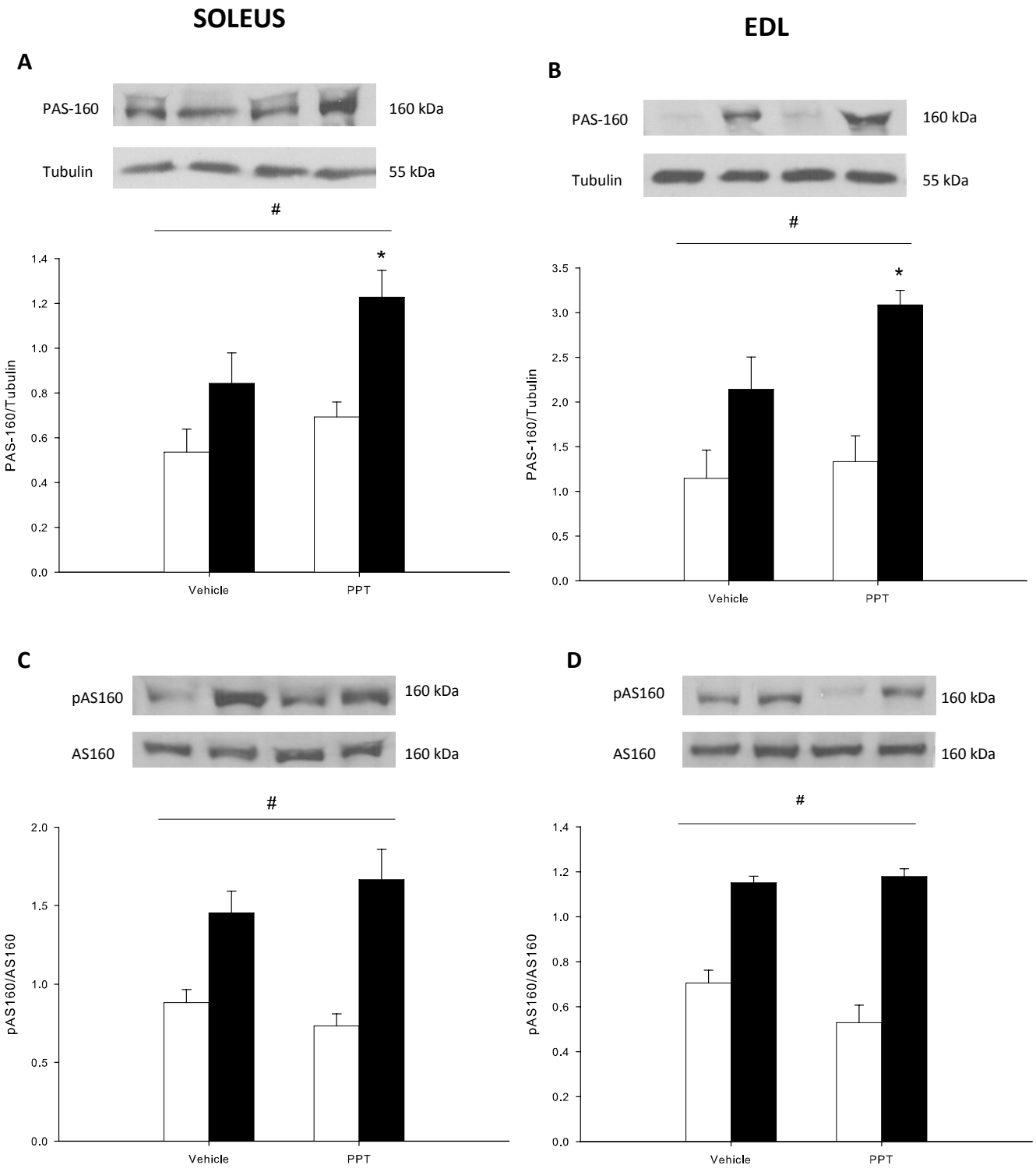

Figure 14: *In vivo* activation of ER*α* via PPT increases insulin-stimulated phosphorylation of PAS-160 in the soleus and EDL. Female Sprague Dawley rats were treated with PPT as stated in the materials and methods. Downstream activation of Akt and AMPK was measured by Western blot analysis of PAS-160 normalized to tubulin (A, B) and pAS160 normalized to total AS160 (C, D) in the absence of insulin (open bars) or in the presence of insulin (2 mU/ml, solid bars) in the soleus (A, C) and EDL (B, D). *$p<0.05$ vs. insulin-stimulated vehicle indicates a significant interaction as determined by two-way ANOVA. The horizontal lines indicates a significant effect of insulin relative to basal, #$p<0.05$; N = 6/group.

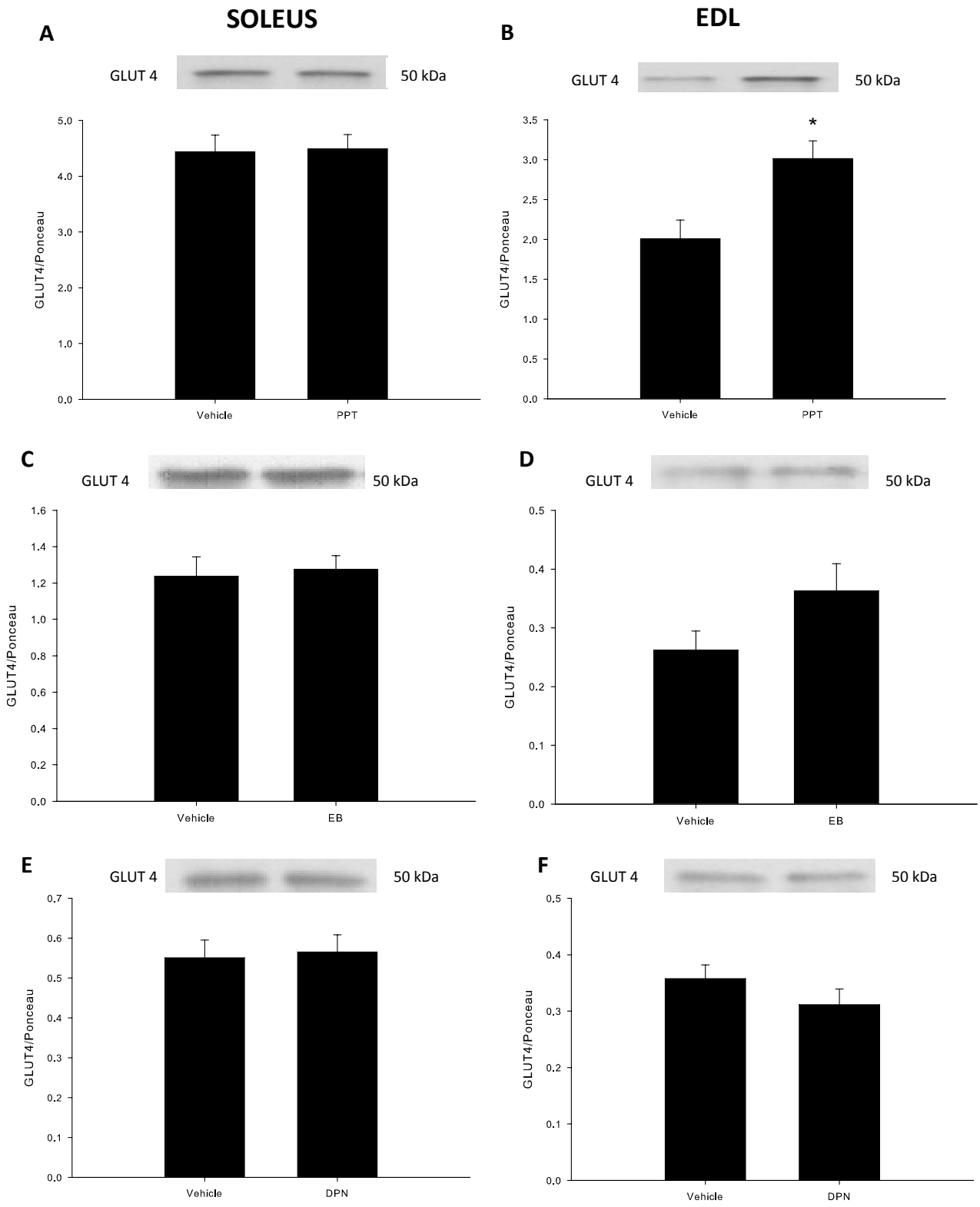

Figure 15: *In vivo* activation of ERα via PPT increases GLUT4 protein in the EDL muscle. Female Sprague Dawley rats were treated with PPT, EB, or DPN as stated in the materials and methods. GLUT4 was measured by Western blot analysis and normalized to total protein as measured by Ponceau staining in animals treated with PPT (A and B), EB (C and D), and DPN (E and F). Symbol represents differences determined by one-way ANOVA. *$p<0.05$ vs. vehicle; N = 6/group

Discussion

The purpose of the current study was to determine the effect of direct ERα modulation on skeletal muscle glucose uptake. While studies utilizing ERα-deficient mice demonstrate impaired glucose tolerance in the absence of ERα (Heine et al. 2000; Bryzgalova et al. 2006; Riant et al. 2009), *in vivo* activation of ERα in control female rodents had not previously been tested. Three day treatment with PPT, a specific agonist of ERα resulted in increased glucose uptake and activation of Akt, PAS-160, and AMPK in both soleus and EDL muscles. In addition, activation of ERα resulted in increased GLUT4 protein in the EDL muscle. This new evidence of the ability of short-term modulation of ERα to increase glucose uptake has important implications for understanding the regulation of glucose uptake, particularly in postmenopausal women.

In a previous study by Ribas et al. (Ribas et al. 2009), ERα knockout mice demonstrated impaired glucose tolerance and reduced insulin sensitivity in liver and skeletal muscle while on a normal chow diet. The decrease in glucose disposal rate in the knockout mice was attributed primarily to impaired insulin action in skeletal muscle. This is in contrast to a study by Bryzgalova et al. (Bryzgalova et al. 2006) that attributed deceased glucose tolerance in ERα knockout mice primarily to the liver. Our current findings demonstrating a dramatic increase in insulin-stimulated skeletal muscle glucose uptake in control animals treated with the ERα agonist PPT support the idea that ERα plays an important role in skeletal muscle insulin sensitivity. Using ER-subtype specific ligands is an alternative and complementary approach to using knockout animals. It is

encouraging that both methods demonstrate an important role for ERα in mediating skeletal muscle insulin sensitivity.

To our knowledge, only one previous study has looked at the effect of PPT on glucose uptake in skeletal muscle. In this study, ob/ob mice were treated with PPT for 7 days prior to measurement of insulin-stimulated glucose uptake (Lundholm et al. 2008). In contrast with the current study, there was no increase in soleus or EDL muscle glucose uptake in ob/ob mice treated with PPT. This difference can most likely be attributed to the lower dose of PPT used in the previous study (1mg/kg body weight vs. 10 mg/kg body weight). Further, the use of hyperglycemic and hyperinsulinemic ob/ob mice in the previous study is markedly different from the control OVX rats used in the current study. Additional experiments will be needed to determine the ability of a higher dose of PPT to improve insulin sensitivity in insulin resistant or type 2 diabetes animal models.

Estrogen and PPT have been shown to potentiate the insulin signaling pathway and increase glucose transport in adipocytes in culture (Muraki et al. 2006; Nagira et al. 2006) and estrogen has been shown to increase phosphorylation of Akt (Vasconsuelo et al. 2008) and AMPK (D'Eon et al. 2008) in C2C12 muscle cells. In the first study to test the effects of estrogen on skeletal muscle *in vitro,* acute incubations (5 and 10 min) with estrogen increased phosphorylation of Akt, AMPK and TBC1D1/4 in soleus muscle (Rogers et al. 2009). However, incubation in estrogen for 10 minutes did not increase insulin-stimulated glucose transport. Our findings show that 3 days of estrogen treatment *in vivo* also had no effect on insulin-stimulated glucose uptake in soleus or EDL muscles. These findings are the first to show that both in isolated rodent skeletal muscle and

following *in vivo* administration, acute estrogen treatment did not produce a measurable increase in skeletal muscle glucose uptake. This may be due to the specific expression pattern and activation of ERs in skeletal muscle.

Previous studies indicate that estrogens and estrogen receptor modulators will produce a distinct phenotype in cells that express predominately ERα compared to those expressing predominantly ERβ (Kian Tee et al. 2004). A number of studies suggest that ERα is more highly expressed in insulin sensitive tissues (Deroo and Korach 2006; Heldring et al. 2007), and this expression pattern was also recently demonstrated in mouse skeletal muscle (Ribas et al. 2009; Baltgalvis et al.). In contrast, Barros et al. (Barros et al. 2009) suggest that ERβ expression predominates in skeletal muscle, although these investigators primarily focused on nuclear ER expression and the results were not quantified. ERα and ERβ are known to demonstrate a complex inter-regulatory relationship that varies with the target tissue. For example, activation of ERα can oppose the action of ERβ and act as a negative regulator in glucose metabolism (Matthews and Gustafsson 2003; Barros et al. 2006a; Barros et al. 2006b). As estrogen activates both ERs, the lack of an effect of estrogen on skeletal muscle glucose uptake in the present study could be due to ERβ activation off-setting any stimulation of ERα via estrogen. As DPN administration did not decrease insulin-stimulated glucose uptake in the present study, stimulation of both ERα and ERβ by estrogen could prevent direct activation of ERα due to the unique active conformation formed between estrogen and these two receptors. In addition to the interregulatory actions of the ERs on each other, estrogen and estrogen receptor modulators exert distinct tissue-specific effects by recruiting different

coregulatory proteins to ERs (Shang and Brown 2002; Kian Tee et al. 2004). This difference in coregulatory protein recruitment can result in a modulator having agonist or antagonist properties. The specific coregulatory proteins involved in ERα activation by PPT and estrogen in skeletal muscle have yet to be identified. Previous studies have shown that long-term estrogen treatment can improve whole body and skeletal muscle glucose metabolism in animals fed a high-fat diet or as a result of aging (Riant et al. 2009; Moreno et al. 2010). Aging or metabolic disease could alter the ratio of ERα to ERβ in skeletal muscle and change the tissue response to estrogen and estrogen receptor modulators. Future studies are needed to determine the impact of insulin resistance, and subsequent chronic estrogen treatment, on ER expression and activation patterns in skeletal muscle.

In the current study, PPT increased ERα phosphorylation in skeletal muscle relative to total ERα, while total ERα expression was decreased compared to vehicle-treated controls. Phosphorylation of ERα may occur in the cytoplasm or the nucleus and is important for receptor dimerization and DNA binding (Arnold et al. 1995a; Arnold et al. 1995b). However, the extent to which phosphorylation of ERα contributes to the involvement of ERα in cell signaling cascades is unknown, and little is known regarding the effect of PPT on phosphorylation of ERα. To our knowledge, this is the first report of ERα activation and expression characterized in skeletal muscle as a result of PPT treatment. Studies thus far have mainly focused on estrogen's phosphorylation of ERα. Phosphorylation on tyrosine residues likely represents basal phosphorylation of ERα in the absence of estrogen (Migliaccio et al. 1986; Arnold et al. 1995a), and activation of

ERα results in phosphorylation of serine residues (Washburn et al. 1991; Weigel 1996). Specifically, Joel et al. reports that estrogen treatment in MCF-7 cells results in increased phosphorylation on Ser118 (Joel et al. 1998). In addition, mutation of Ser118 resulted in a 40% reduction in transactivation activity in response to estrogen (Le Goff et al. 1994). We chose to assess Ser118 due to a more recent report showing increased phosphorylation of ERα on Ser118 in the soleus muscle due to resveratrol treatment, which resulted in enhanced glucose uptake (Deng et al. 2008).

In contrast to the previously mentioned studies on ERα phosphorylation, the present study used PPT as an ERα agonist. In MCF-7 cells, Joel et al. demonstrated an increase in estrogen-stimulated pERα on Ser118 relative to total ERα, with no decrease in total ERα (Joel et al. 1998). In contrast, while our study in rat skeletal muscle demonstrates an increase in PPT-stimulated pERα on Ser118 relative to total ERα, we also saw a decrease in total ERα. In fact, more recent reports demonstrate that phosphorylation of ERα on Ser118 leads to protein degradation of ERα (reviewed in (Murphy et al. 2011)). Ultimately, our study demonstrates that of the total ERα present, more ERα is phosphorylated on Ser118 in the PPT treated animals than in the vehicle treated animals. A recent study by Baltgalvis et al. (Baltgalvis et al. 2010) demonstrated that one week of OVX resulted in an increase in ERα gene and protein expression compared to sham-treated animals. Two days of estradiol treatment in OVX female mice in this same study resulted in a decrease in ERα levels, similar to our results with PPT. In addition, we have recently shown that ER protein levels are altered in the skeletal muscle and adipose tissue in response to OVX and a high-fat diet (Gorres et al. 2011a). The

specificity of PPT for ERα in skeletal muscle is further demonstrated by the lack of effect of PPT on ERβ expression. In addition to regulation of ERα by phosphorylation, localization of ERα proteins (nuclear, cytosolic, membrane-associated) could also play an important role in activation and regulation in response to PPT and should be pursued in future studies.

PPT also results in direct activation of the insulin signaling pathway as shown by increased phosphorylation of Akt. To our knowledge, this is the first evidence of the effects of PPT on insulin signaling. In support of our findings, stimulation of the insulin signaling pathway with resveretrol in C2C12 myotubes was shown to be dependent on ERα activation (Deng et al. 2008). Phosphorylation of AS160 on threonine 642 is commonly used to assess activation of AS160 and, hence, activation of the insulin signaling pathway downstream of Akt. Our results indicate that rats treated with PPT have increased insulin-stimulated phospho-Akt substrate (PAS), but not as a result of a significant increase in pAS160 on threonine 642. The PAS immunoreactivity at 160 kDa includes both AS160 and a paralog of AS160, TBC1D1. As a result, an additional site of phosphorylation on AS160 or the TBC1D1 protein could be activated with PPT and result in increased glucose transport.

Specific activation of ERα *in vivo* with PPT results in increased pAMPK in soleus and EDL muscles. In support of this finding, ERα-KO mice demonstrate decreased pAMPK in skeletal muscle (Ribas et al. 2009). Furthermore, skeletal muscle simulated with estrogen *in vivo* and *in vitro* can increase AMPK activation (D'Eon et al. 2008; Riant et al. 2009; Rogers et al. 2009), with a recent study showing estrogen-induced AMPK

activation is mediated by ERα (Rogers et al. 2009). Together, these findings suggest that ERα acts as a positive modulator of AMPK activation. AMPK activation can result in increased basal glucose transport, an effect that was not observed in the present study. The amount of AMPK phosphorylation may have been insufficient to alter basal glucose uptake in the present study. AMPK can phosphorylate both AS160 (Treebak et al. 2006; Chen et al. 2008) and TBC1D1 (Geraghty et al. 2007; Pehmoller et al. 2009), although with phospho-specific sites distinct from those activated by Akt, to stimulate an increase in glucose uptake. This potential for differential regulation has important implications for the regulation of glucose uptake and will need to be further explored in the context of ERα activation.

The ERs have been shown to be involved in modulation of GLUT4 transcription, with ERα acting as a positive modulator and ERβ acting as a negative modulator (Barros et al. 2006a; Barros et al. 2006b; Barros et al. 2009). Barros et al. (Barros et al. 2006a) have proposed a mechanism by which ERα could bind to nuclear factor-kappa B (NF-κB), a transcription factor with the potential to repress GLUT4 expression. The binding of ERα to NF-κB could inhibit this transcription factor's repression of GLUT4 and thereby increase GLUT4 expression. Without ERα present, GLUT4 is decreased in the gastrocnemius muscle of male ERα knock-out (ERα-KO) mice (Barros et al. 2006b). However, a more recent report shows that female ERα-KO mice do not have decreased GLUT4 in the quadriceps or soleus muscle (Ribas et al. 2009). In the current study, activation of ERα resulted in increased GLUT4 in the EDL (fast-twitch) but not in the soleus (slow-twitch), which suggests that the ability of ERα to regulate GLUT4 may be

fiber type specific. As we measured total GLUT4 protein levels, we do not know if the increase in GLUT4 in the EDL contributed to the increase in insulin-stimulated glucose uptake. It is possible that the increase in total GLUT4 did not contribute to an increase in GLUT4 at the membrane or in glucose transport. If so, then this would explain the disparity between the soleus and EDL GLUT4 and glucose transport data. However, as GLUT4 is an important protein for insulin-stimulated glucose uptake, it is important to understand factors which modulate GLUT4, and the ability to modulate GLUT4 by direct ERα activation has not previously been shown. While numerous transcriptional pathways regulate GLUT4 (Murgia et al. 2009), acute ERα activation may be an additional mechanism for modulating GLUT4.

A recent study reported that 8 weeks of OVX increased circulating glycerol, NEFA, and glucose, and estrogen treatment reversed this (Wohlers and Spangenburg 2010). While long-term OVX and estrogen treatment had significant systemic metabolic effects, it is unlikely that one week of OVX or 3 days of PPT treatment resulted in significant changes in plasma glucose, insulin, or lipids, although we did not measure these factors in the current study. We think the PPT effects demonstrated in this study are a result of acute changes in signaling and protein expression in the skeletal muscle, although future studies will be needed to confirm a lack of systemic effect on plasma glucose, insulin, and lipid levels. In addition, the decrease in body weight with PPT treatment suggests that ERα activation may affect body weight and metabolism. These factors may also play an important role in increasing skeletal muscle glucose uptake and insulin signaling and will be addressed in future studies.

In summary, our study demonstrates that the ERα agonist PPT results in increased insulin-stimulated glucose uptake into the skeletal muscle via potentiating the insulin signaling pathway, activating AMPK, and increasing GLUT4 protein. Specific activation of ERα may provide an additional means by which drug treatments can be developed that have a positive impact on glucose metabolism. Future studies are needed to determine the long term effects of estrogens, insulin resistance and type 2 diabetes on ER expression and activation in skeletal muscle.

Author Contributions

B.K.G and P.C.G designed the experiments, analyzed and interpreted data, and wrote the paper. B.K.G, G.L.B, J.K.M, and P.C.G collected the data. All authors approved the final version of the manuscript. These experiments were carried out at the University of Kansas Medical Center.

Chapter 4

In vivo stimulation of estrogen receptor α modulates proteins involved in adipocyte regulation in female rats

Brittany K. Gorres, Gregory L. Bomhoff, Paige C. Geiger

Submitted.

ABSTRACT

Clinical evidence demonstrates that many women gain weight following menopause. This increase in body weight is accompanied by an increase in abdominal adipose tissue. These studies are supported by animal models of ovariectomy (OVX) in which removal of the ovaries results in increased total body weight and fat pad weight. Previous animal studies demonstrate that estrogen treatment following OVX can decrease triacylglyceride accumulation via decreasing expression of proteins in the lipogenic pathway. However, as estrogen may exert its effects via various receptors, the mechanism of estrogen's action is unknown. The purpose of the current study was to determine the effects of *in vivo* estrogen receptor α (ERα) activation on body weight regulation, *de novo* fatty acid synthesis, and lipolysis. Ovariectomized 3 month-old female Sprague Dawley rats were given subcutaneous injections of propylpyrazoletriyl (PPT; 10 mg/kg) or vehicle (DMSO) every 24 hours for 3 days. *In vivo a*ctivation of ERα with PPT resulted in decreased body weight and adipose tissue weight and decreased proteins involved in *de novo* fatty acid synthesis such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Furthermore, ERα activation increased proteins involved in lipolysis and fatty acid uptake in the adipose tissue such as adipose triglyceride lipase (ATGL), perilipin, and lipoprotein lipase (LPL). Serum triglycerides, glycerol, and non-esterfied fatty acids were unchanged. These findings provide new insight into the role of ERα in modulating adipogenesis. Modulation of ERα may represent a powerful tool in the prevention of obesity, particularly in postmenopausal women at increased risk for the disease.

INTRODUCTION

Ovariectomy (OVX) involves removal of the ovaries in rodents, and this models the post-menopausal state. OVX results in increased body weight and adipose tissue weight gain (Yakar et al. 2006; Nunez et al. 2007; Nunez et al. 2008; Hong et al. 2009). Estrogen is an important regulator of metabolism, and previous animal studies demonstrate that estrogen treatment following OVX can decrease adiposity and triacylglyceride accumulation via decreasing expression of proteins in the lipogenic pathway (D'Eon et al. 2005). However, as estrogen may exert its effects via various receptors, the mechanisms of estrogen's action remain unknown. Estrogen primarily mediates its effects by binding to its receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). The function of these ERs in adipocyte regulation and lipid metabolism has not been studied with specific ER activation.

ERα knock-out (KO) mice have increased body weight, adipose tissue weight, and adipocyte size and number compared to wild-type (WT) mice (Heine et al. 2000). Similarly, aromatase KO mice, in which androgens cannot be converted to estrogen, have increased body weight (Takeda et al. 2003) and adipose tissue weight (Fisher et al. 1998) compared to WT mice. In contrast, ERβ KO mice do not have increased adipose tissue weight or percent body fat compared to WT mice (Ohlsson et al. 2000). Therefore, estrogen/ERα signaling appears to be an important regulator of body weight and adipocyte regulation. However, the KO models display the deleterious long-term effects of estrogen/ERα deficiency. Studies have not determined if acute ERα activation could provide beneficial effects on adipocyte and body weight regulation. We have previously

shown that acute ERα activation positively modulates skeletal muscle glucose metabolism (Gorres et al. 2011b), and favorable effects may also occur in the adipose tissue.

The purpose of the current study was to determine the effects of *in vivo* ERα activation on body weight regulation, *de novo* fatty acid synthesis, and lipolysis. We administered propylpyrazoletriyl (PPT), a potent ERα agonist, to OVX female rats for three days. Our results demonstrate that *in vivo* activation of ERα decreases body weight and adipose tissue weight, as well as proteins involved in *de novo* fatty acid synthesis. Furthermore, ERα activation increased proteins involved in lipolysis in the adipose tissue. These findings provide new insight into the role of ERα in modulating adipogenesis. Modulation of ERα may represent a powerful tool in the prevention of obesity, particularly in postmenopausal women at increased risk for the disease.

MATERIALS AND METHODS

Materials. Fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), adipose triglyceride lipase (ATGL), and pERα (S118) antibodies were purchased from Cell Signaling (Beverly, MA). ERα (MC-20), perilipin (67164), and lipoprotein lipase (32885) were purchased from Santa Cruz (Santa Cruz, CA). ERβ (PA1-310B) was purchased from Thermo Fisher Scientific (Rockford, IL) and tubulin (ab7291) was purchased from Abcam (Cambridge, MA). Donkey anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson (West Grove, PA), and goat anti-mouse HRP-conjugated secondary antibody was purchased from Bio-Rad (Hercules, CA).

Enhanced chemiluminescence reagents were purchased from Fisher Scientific (Pittsburg, PA). Propylpyrazoletriyl (PPT) was purchased from Tocris Bioscience (Ellisville, MO). All other reagents were obtained from Sigma.

Experimental animals and treatment. 3 month-old female Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in a temperature-controlled (22 ± 2°C) room with 12-h light and dark cycles and given free access to food and water. Animals underwent bilateral ovariectomy (OVX) under ketamine/atropine/xylazine anesthesia (intraperitoneal injection of 60 mg/kg body wt ketamine, 0.4 mg/kg body wt atropine, 8 mg/kg body wt xylazine). Bilateral flank incisions were made under aseptic conditions. The ovaries were identified and bilaterally removed via cauterization. Wounds were closed using sutures and wound clips. One week following surgery, OVX animals received subcutaneous injections once every 24 hours for 3 days (N = 6 animals per group) of PPT (10 mg/kg body wt) dissolved in DMSO or DMSO. This dose of PPT has previously been used in *in vivo* rodent studies (Harris et al. 2002; Lee et al. 2005). Body weight was measured daily prior to injections. Rats were fasted 10 hours prior to sacrifice. 24 hours following the final injection, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (2.5 mg/100 g body wt). Tail blood was used to determine glucose levels using a glucometer (Accu-Check) prior to tissue dissection. Periuterine white adipose tissue was removed, weighed, and frozen in liquid nitrogen. The right soleus and extensor digitorum longus (EDL) muscles were also removed and weighed.

Serum insulin, triglyceride (TG), glycerol, and non-esterfied fatty acid (NEFA) measurement. Blood samples were collected from the heart at time of sacrifice. Samples were placed on ice for at least 30 minutes, then spun at 3,000 rpm for 60 minutes at 4°C. The top serum portion was removed for measurement of insulin (Alpco Diagnostics, 80-INSRT-E01; Salem, NH), triglyceride (Cayman Chemical, 10010303; Ann Arbor, MI), glycerol (Sigma, F6428; St. Louis, MO), and NEFA (Wako Diagnostics; Richmond, VA) according to the manufacturer's instructions.

Western blotting. Adipose tissue was homogenized in a 2:1 (volume-to-weight) ratio of ice-cold buffer from Biosource (Invitrogen, Camarillo, CA) containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na3VO4; 20 mM Na4P2O7; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250µl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes at 4°C and then centrifuged for 20 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). Samples were prepared in 5X Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 5 minutes. Protein (100 µg) was separated on a SDS-PAGE (7.5–10%) gel followed by a wet transfer to a nitrocellulose membrane for 60–90 minutes (200 mA). Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated overnight with the appropriate primary antibodies. Blots were incubated in a HRP-conjugated secondary antibody in 1% nonfat dry milk in TBST for 1 hour at room temperature and visualized by ECL. Western blots were stripped and

probed for tubulin expression for normalization. Bands were quantified using Image J densitometry.

Statistical analysis. Results are presented as means $\pm$ SE. Statistical significance was set at $P<0.05$ and determined by a one-way repeated measures ANOVA or one-way ANOVA and Student-Newman-Keuls post hoc test.

RESULTS

PPT decreases body weight and adipose tissue weight. Body weight was measured on days 1-3, prior to administering the PPT or vehicle, and on day 4, prior to sacrifice. Compared to the vehicle treated group, body weight decreased in the animals treated with PPT over time, as measured by a one-way repeated measures ANOVA (#$p<0.001$; Figure 16A). The decrease in body weight with PPT treatment paralleled a decrease in periuterine adipose tissue weight in the PPT treated group (Figure 16B). Skeletal muscle weight did not change in the soleus (Figure 16C) or extensor digitorum longus (Figure 16D) muscles with PPT treatment.

Effects of PPT on fasting blood glucose and serum insulin. Body weight and adipose tissue weight are predictors of fasting blood glucose and serum insulin levels. Therefore, we measured fasting blood glucose and serum insulin levels in the vehicle and PPT treated animals but found that these values were not different between groups (Figures 17A and 17B).

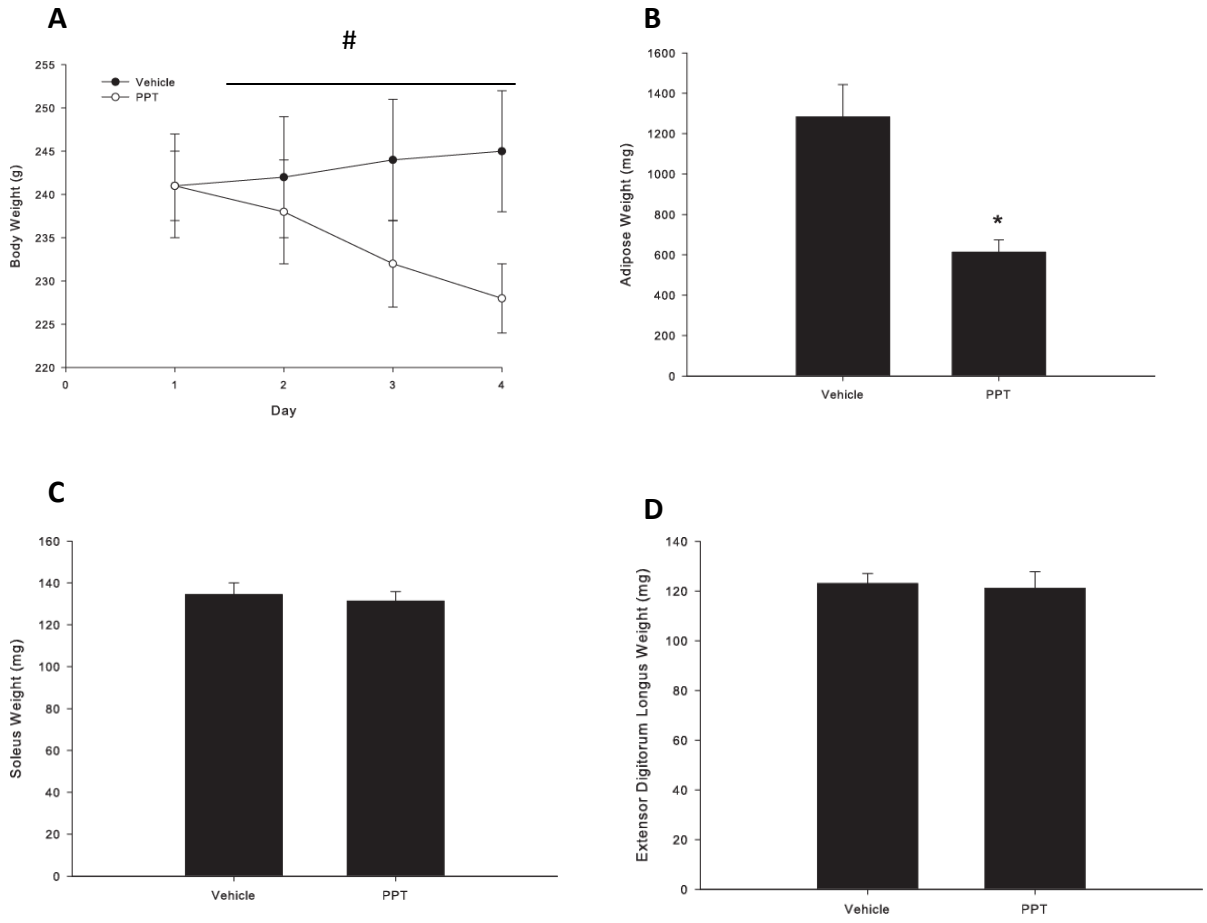

Figure 16: PPT decreases body weight and adipose tissue weight. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Body weight (A) was measured prior to injections on days 1-3 and prior to sacrifice on day 4. Body weight decreased in animals treated with PPT over time, as measured by a one-way repeated measures ANOVA (#$p<0.001$). Periuterine adipose tissue (B), soleus (C), and EDL (D) were measured at time of sacrifice. *$P<0.01$; N = 6 animals per group

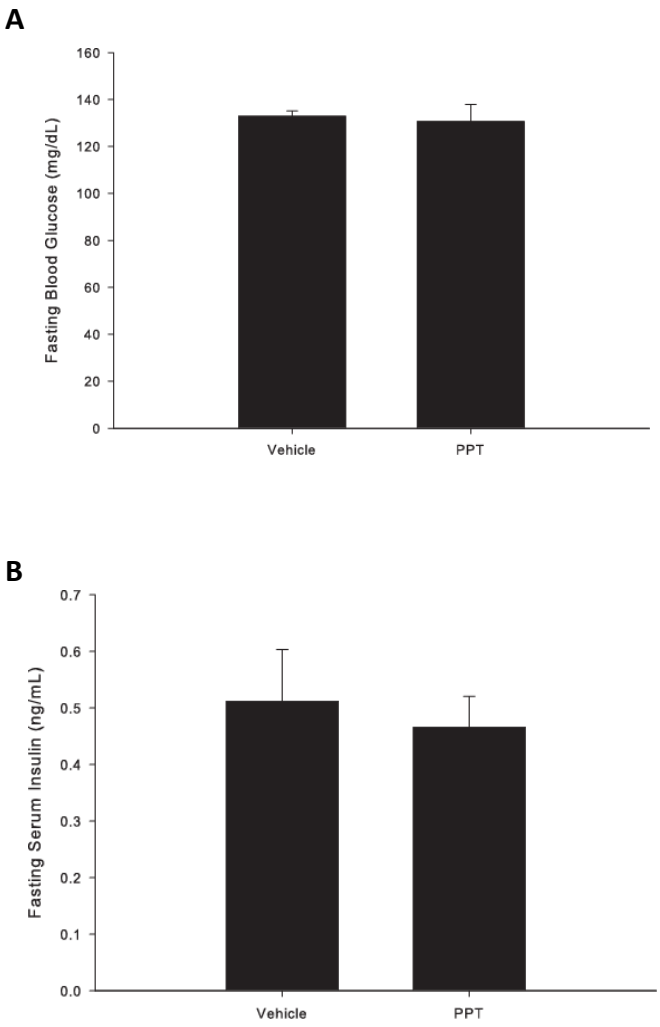

Figure 17: No change in fasting blood glucose or fasting serum insulin levels. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Blood was taken at time of sacrifice. N = 6 animals per group

PPT activates ERα in the adipose tissue. Phosphorylation of ERα is a measure of protein activation (Weigel 1996; Joel et al. 1998; Deng et al. 2008). PPT significantly increased pERα in the adipose tissue (Figure 18A) following 3 days of PPT administration compared to vehicle-treated controls. Total ERα decreased following PPT treatment (Figure 18B), and no change in ERβ occurred (Figure 18C), highlighting the specificity of PPT for ERα in the adipose tissue.

PPT decreases proteins involved in *de novo* lipid synthesis in the adipose tissue. The decrease in adipose tissue weight in the PPT treated group may indicate that lipogenesis and/or lipolysis are altered in the adipose tissue. ACC and FAS are proteins involved in *de novo* lipid synthesis. In accordance with the decrease in adipose tissue weight, PPT treatment significantly decreased ACC and FAS protein levels in the adipose tissue (Figures 19A and 19B).

PPT increases proteins involved in lipolysis and fatty acid uptake in the adipose tissue. While the decrease in factors involved in *de novo* lipid synthesis likely contributes to the decrease in adipose tissue weight with PPT treatment, we also measured proteins involved in the regulation of lipolysis, such as ATGL and perilipin. PPT treatment significantly increased ATGL and perilipin protein levels in the adipose tissue (Figures 20A and 20B). The potential for fatty acid uptake into the adipose tissue also increased with PPT treatment, as evident by the increase in LPL (Figure 20C).

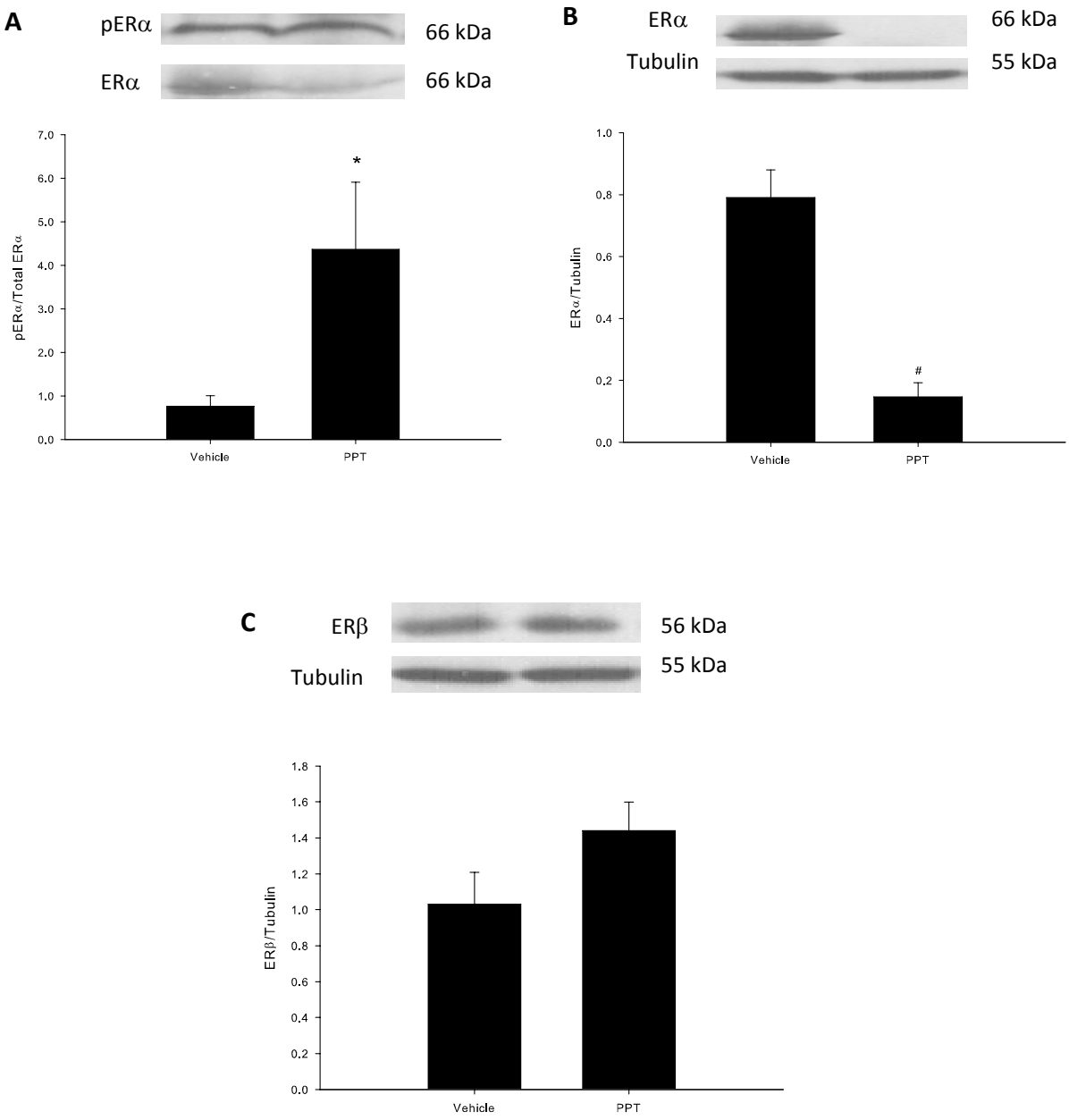

Figure 18: PPT activates ERα in the adipose tissue. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, the rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Periuterine adipose tissue was removed at time of sacrifice. Phosphorylation of ERα was measured by Western blot analysis and normalized to total ERα (A). Total ERα (B) and total ERβ (C) were also measured and normalized to tubulin. *P<0.05, #P<0.001; N = 6 animals per group

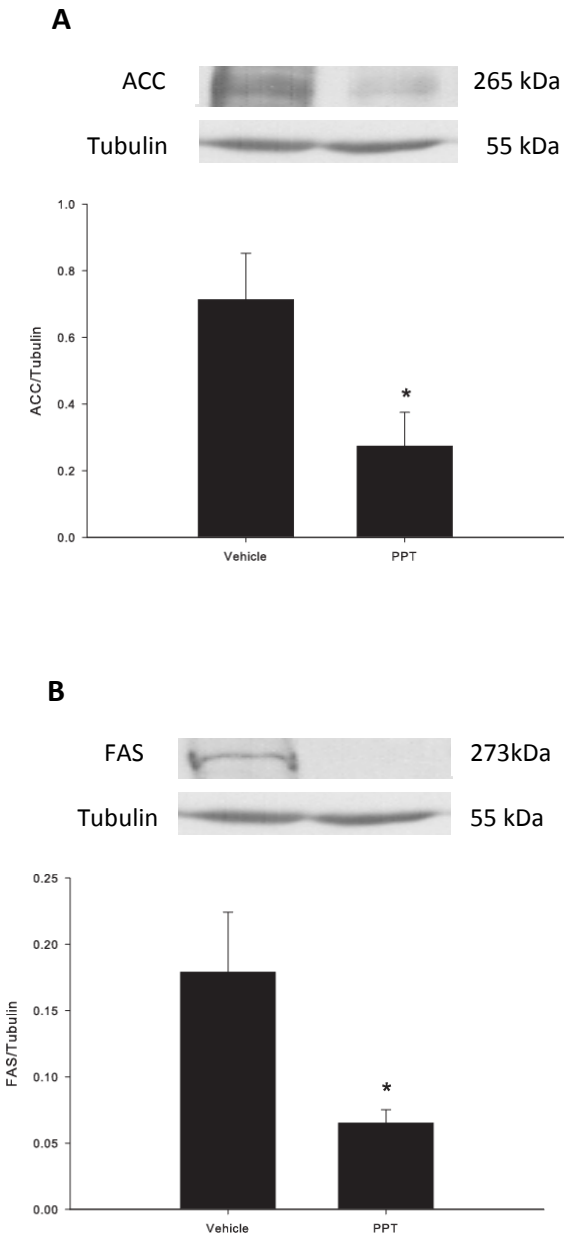

Figure 19: PPT decreases proteins involved in *de novo* fatty acid synthesis. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, the rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Periuterine adipose tissue was removed at time of sacrifice. ACC (A) and FAS (B) protein levels were determined by Western blot analysis and normalized to tubulin. *P<0.05; N = 6 animals per group

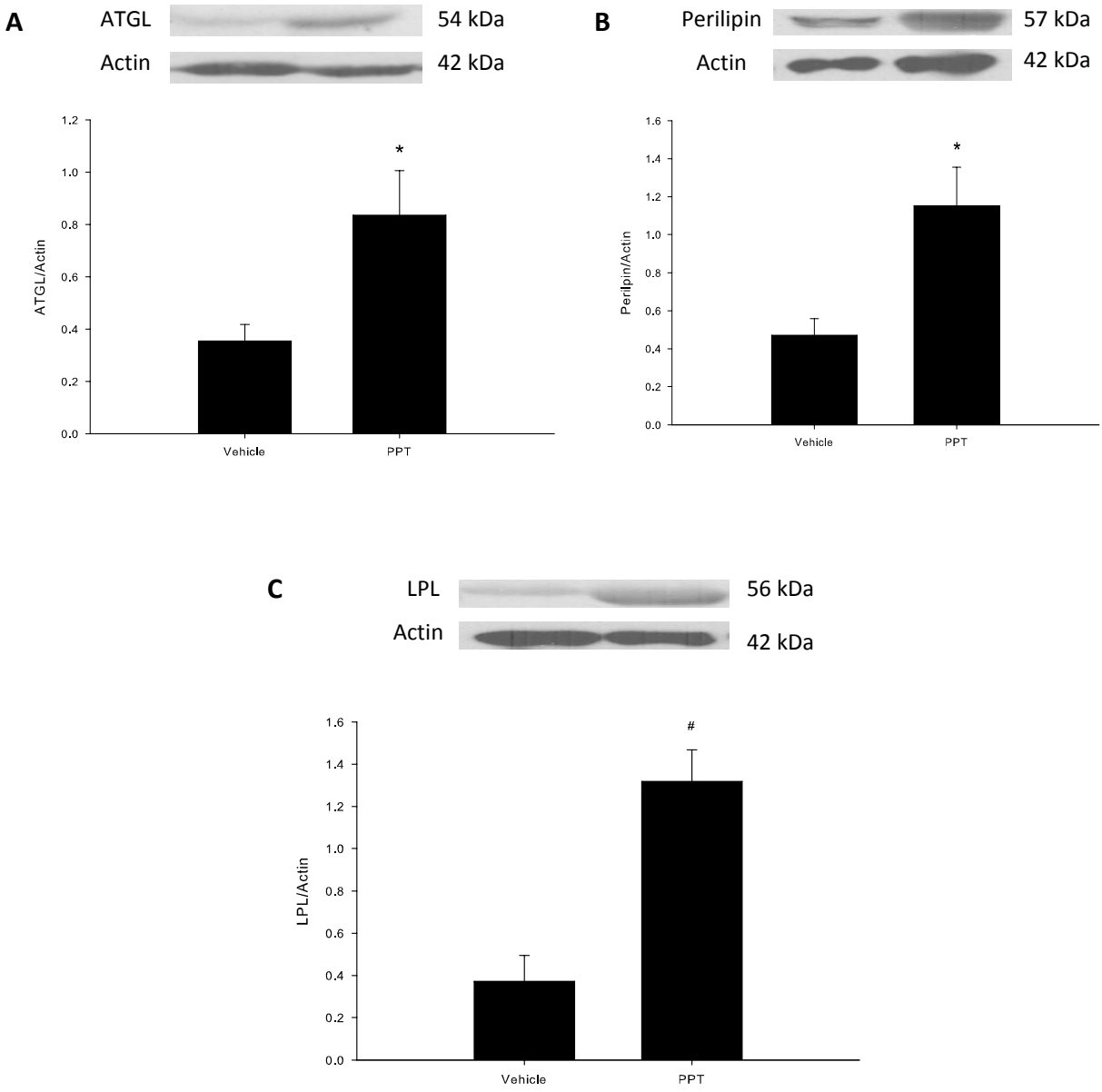

Figure 20: PPT increases proteins involved in lipolysis and fatty acid uptake in the adipose tissue. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Periuterine adipose tissue was removed at time of sacrifice. ATGL (A), perilipin (B), and LPL (C) protein levels were determined by Western blot analysis and normalized to actin. *P<0.05, #P<0.001; N = 6 animals per group

Effects of PPT on serum TG, glycerol, and NEFA. As lipolysis in the adipose tissue increased in animals treated with PPT, we measured serum TG, glycerol, and NEFA levels, which are also indicators of lipolysis. Although there was a trend towards decreased serum TG levels with PPT treatment (P=0.08), there were no significant differences in serum TG, glycerol, or NEFA levels (Figure 21) between groups.

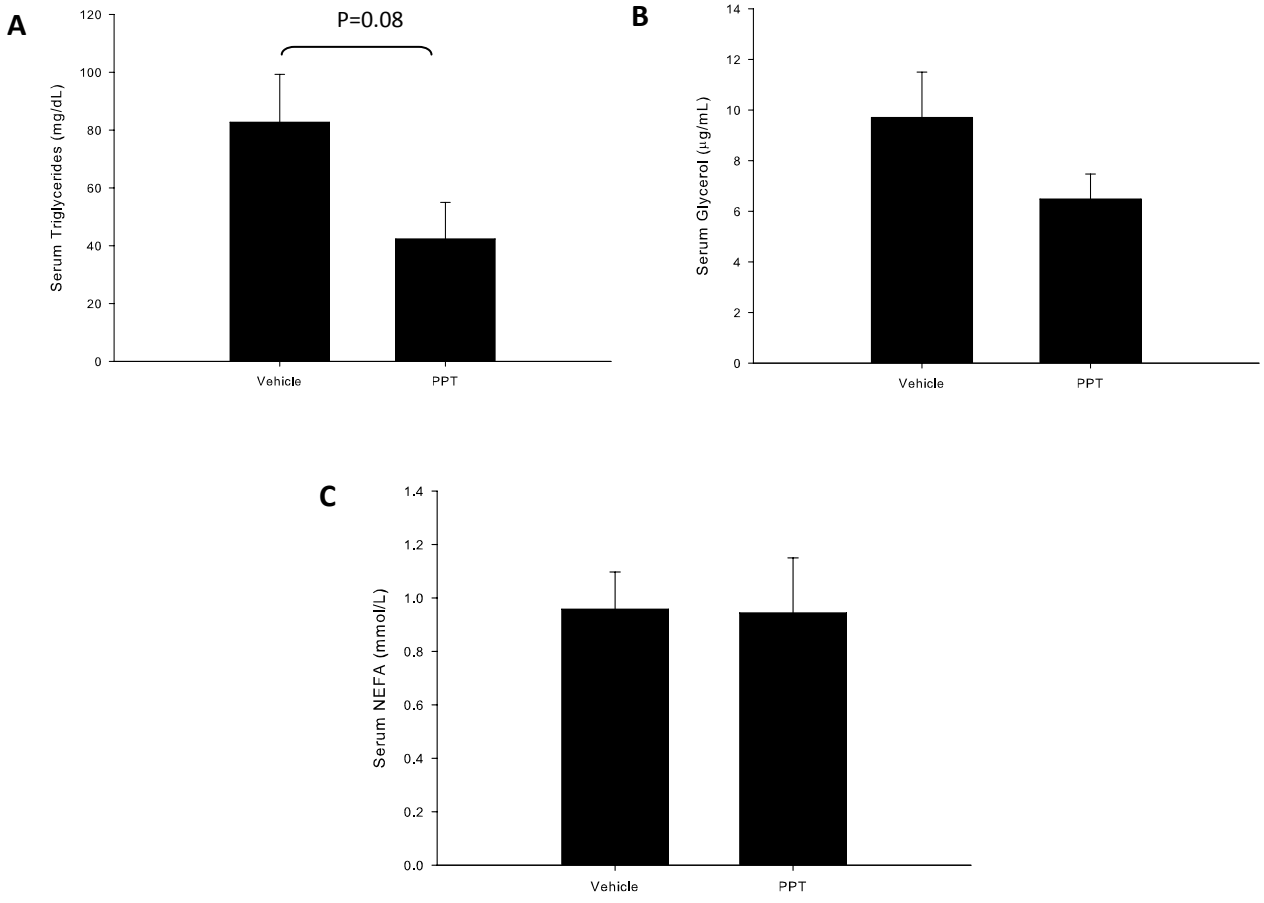

Figure 21: Effect of PPT on serum TG, glycerol, and NEFA. Female Sprague Dawley rats underwent OVX surgery. Seven days post surgery, rats were given PPT injections (10mg/kg) or vehicle (DMSO) every 24 hours for 3 days. Blood was taken at time of sacrifice, and serum TG (A), glycerol (B), and NEFA (C) were measured according to the manufactures' instructions. N = 6 animals per group

DISCUSSION

The purpose of the current study was to determine the effect of direct ERα modulation on body weight and fatty acid metabolism in the adipose tissue. While previous studies demonstrate the ability of estrogen to modulate adiposity and be protective against high-fat diet-induced obesity (D'Eon et al. 2005; Bryzgalova et al. 2008; Priego et al. 2008; Wohlers and Spangenburg 2010), the signaling pathway by with estrogen acts was previously unknown. We show for the first time that modulation of adipogenesis and lipolysis occurs with specific ERα activation. Three day treatment with PPT, a specific agonist of ERα resulted in decreased body and adipose tissue weight. In addition, proteins involved in *de novo* lipid synthesis, such as ACC and FAS decreased, while proteins involved in lipolysis, such as ATGL and perilipin, increased. This new evidence demonstrates the ability of short-term activation of ERα to modulate fatty acid metabolism in the adipose tissue.

Previous studies show that estrogen can modulate body weight and adipose tissue weight. In female rats, body weight and adipose tissue weight increased in response to high-fat feeding for ten months, and one month of estrogen treatment following this diet regime significantly decreased total body weight and adipose tissue weight (Bryzgalova et al. 2008). Estrogen can also modulate adiposity in the absence of a HFD, as estrogen treatment for 40 days in female mice decreased adipose tissue weight and adipocyte size (D'Eon et al. 2005). These beneficial effects of estrogen likely occurred through activation of ERα, as our data show that specific ERα activation results in decreased body weight and adipose tissue weight.

Sex differences exist between males and females with respect to regulation of adiposity and fuel partitioning, and the high-fat feeding model has been used to study these differences. Priego et al. demonstrate that the response to overfeeding via a high-fat diet (HFD) is sex-dependent (Priego et al. 2008). Female rats fed a HFD for six months were better protected against the HFD by being able to modulate genes involved in fatty acid metabolism to protect against energy overload and decrease lipogenesis. Specifically, the female rats were able to up-regulate LPL and decrease ACC and FAS in the adipose tissue.

ACC and FAS are proteins involved in *de novo* lipid synthesis. ACC catalyzes the formation of malonyl CoA from acetyl-CoA, and FAS catalyzes the formation of free fatty acids from malonyl CoA (Wakil et al. 1983; Kim 1997). Our data show that specific ERα activation decreases ACC and FAS protein levels, thus contributing to the decreased adipose tissue weight. This in agreement with previous studies showing that estrogen decreases FAS and ACC in the adipose tissue (D'Eon et al. 2005; Bryzgalova et al. 2008). This new information on ERα's role in fatty acid synthesis provides a means by which estrogen may contribute to the sex differences in adipocyte regulation.

LPL promotes fatty acid uptake into the adipose tissue, and its regulation by estrogen remains controversial. D'Eon et al. report that estrogen treatment for 40 days in mice decreased LPL gene expression in the adipose tissue, contributing to a decrease in fatty acid uptake (D'Eon et al. 2005). However, this treatment method increased LPL and proteins involved in lipolysis and energy dissipation in the skeletal muscle, suggesting that estrogen also has the ability to promote the use of lipids as fuel. In fact, Priego et al.

(Priego et al. 2008) report that LPL protein increased in the adipose tissue of female rats in response to a HFD. In contrast, LPL protein levels in males did not increase in the adipose tissue when fed a HFD. These sex differences in LPL regulation suggest that estrogen may be involved in LPL modulation, with estrogen being able to increase LPL in times of need. Our data show that specific ERα activation increased LPL protein levels in the adipose tissue. This increased ability for fatty acid uptake was mirrored by an increased capability for the fatty acids to undergo lipolysis.

Lipolysis of triglycerides provides energy from fat stores and involves many steps and regulatory proteins. ATGL performs the first step in lipolysis by catalyzing the formation of diacylglycerol from triacylglycerol (Jenkins et al. 2004; Villena et al. 2004; Zimmermann et al. 2004), which releases a fatty acid for subsequent β-oxidation. This is also the rate limiting step in cyclic AMP-dependent protein kinase (PKA)-stimulated lipolysis (Kershaw et al. 2006; Miyoshi et al. 2006). Perilipin plays a role in basal lipolysis and PKA-stimulated lipolysis. While perilipin inhibits lipolysis in a basal state (Martinez-Botas et al. 2000; Tansey et al. 2001), perilipin promotes PKA-stimulated lipolysis (Sztalryd et al. 2003; Zhang et al. 2003; Miyoshi et al. 2006). A previous study reports that estrogen treatment in mice enhanced epinephrine-stimulated lipolysis by increasing levels of perilipin protein (D'Eon et al. 2005). Furthermore, Wohlers et al. report that OVX mice have significantly lower perilipin protein levels compared to mice with intact ovaries and endogenous estrogen levels, and estrogen treatment in the OVX mice restored the protein levels (Wohlers and Spangenburg 2010). Our data are in

agreement with these studies and suggest that the estrogen-mediated increase in perilipin occur through activation of ERα.

Although this study demonstrates that lipolysis increased with ERα activation, no changes in serum TG, glycerol, or NEFA occurred. This is in agreement with human studies showing that serum TG levels remained unchanged after estrogen or combined estrogen/progestin replacement therapy. However, regulation of serum NEFA with estrogen treatment remains controversial. While Wohlers et al. report that the decrease in adipose tissue weight with estrogen treatment in OVX mice corresponds with a decrease in serum glycerol and NEFA levels (Wohlers and Spangenburg 2010), D'Eon et al. report that the decrease in adipose tissue weight with estrogen treatment in OVX mice corresponds with an increase in serum NEFA levels (D'Eon et al. 2005). In addition, estrogen-mediated glycerol release from the adipose tissue was bi-modally regulated: estrogen treatment resulted in lower glycerol release under basal conditions and higher glycerol release following lipolytic stimulation (D'Eon et al. 2005). We show no change in serum glycerol or NEFA levels with acute, specific ERα activation. Therefore, while activation of ERα decreases adipose tissue weight and promotes lipolysis in the adipose tissue, more studies are needed to ascertain how these changes correspond to alterations in circulating NEFA and glycerol levels.

We previously showed that acute activation of ERα positively modulates skeletal muscle glucose metabolism (Gorres et al. 2011b). Similarly, this study demonstrates that many changes also occur in the adipose tissue. While we saw a trend towards decreased serum TG levels (Figure 6), longer treatment may be needed to see whole body effects of

ERα activation. A previous study investigated the long-term effect of PPT treatment in ob/ob mice (Lundholm et al. 2008). PPT treatment for 30 days decreased fasting blood glucose and improved glucose tolerance, as measured by an intraparitoneal glucose tolerance test. However, body weight did not change after PPT treatment. Hepatic lipid levels were also measured. While estrogen treatment decreased total lipids, TG, and cholesterol, these changes did not occur with PPT treatment.

In conclusion, our data demonstrate that certain beneficial effects of estrogen on adipocyte regulation occur through activation of ERα. Specifically, activation of ERα decreased proteins involved in *de novo* fatty acid synthesis and increased proteins involved in lipolysis in the adipose tissue. This resulted in decreased body weight and adipose tissue weight. These data provide insight to the previous studies showing sex differences in fat storage and the response to high-fat feeding. Furthermore, modulation of ERα may represent a powerful tool in the prevention of obesity, particularly in postmenopausal women at increased risk for the disease.

Chapter 5

SUMMARY OF RESULTS AND DISCUSSION

Summary of Results.

Our studies examined the role of activated ERs in skeletal muscle glucose metabolism and adipocyte regulation. The principle finding and conclusions are as follows:

1. A short-term HFD in female rats resulted in whole body glucose intolerance and altered ER expression in the adipose tissue and skeletal muscle. Most effects occurred in the adipose tissue including decreased ERα and GLUT4 protein levels and increased stress kinase activation. In the skeletal muscle, no changes in insulin-stimulated glucose uptake occurred, as does in male rats. Skeletal muscle changes were limited to a decrease ERβ protein in response to OVX and a HFD.
2. Specific activation of ERα increases insulin-stimulated skeletal muscle glucose uptake via potentiating the insulin signaling pathway, increasing GLUT4 protein, and increasing activation of AMPK. Activation of ERβ or ERα and ERβ together did not increase skeletal muscle glucose transport. These findings suggest that the previously established benefits of E_2 on glucose metabolism likely occur through activation of ERα.
3. Specific activation of ERα decreases proteins involved in *de novo* lipid synthesis, periuterine adipose tissue, and body weight and increases proteins involved in lipolysis in adipose tissue. This decrease in adipose tissue weight in the abdominal region may also provide benefit to the improved skeletal muscle glucose regulation with ERα activation.

Discussion.

Estrogen versus selective estrogen receptor modulators.

Our findings display the beneficial role of specific ERα activation on skeletal muscle glucose uptake and adipocyte regulation. Yet clinical studies in humans (Andersson et al. 1997; Espeland et al. 1998; Kanaya et al. 2003; Margolis et al. 2004; Gower et al. 2006) and animal models (Bryzgalova et al. 2008; Riant et al. 2009) demonstrate that E_2 (which activates both ERα and ERβ) also protects against glucose intolerance. In these animal models, E_2 was protective against HFD-induced insulin resistance. Bryzgalova et al. (2008) demonstrated that 9 months of high-fat feeding in female mice resulted in the common outcomes including increased body weight, increased abdominal adipose weight, increased fasting glucose and insulin levels, and decreased whole body glucose tolerance. However, following 9 months of high-fat feeding, 1 month of E_2 treatment in conjunction with the high-fat feeding ameliorated the outcomes of the HFD. Riant et al. (2009) found similar results. As measured by the euglycemic-hyperinsulinemic clamp, whole body insulin resistance developed in OVX rats fed a HFD for 4 weeks. However, OVX animals fed the HFD and treated with E_2 for 4 weeks displayed protection against insulin resistance.

Our data demonstrate that this beneficial effect of E_2 likely occurs through activation of ERα. However, our data showed no beneficial effect of E_2 on glucose

uptake. This may be due to the fact that our model is markedly different than the high-fat feeding models which do demonstrate a beneficial effect of E_2 on glucose regulation. High-fat feeding may alter the protein levels of the ERs in the body. This is important because E_2 activates both ERα and ERβ, and while ERα may have a beneficial effect on glucose metabolism, ERβ can oppose the action of ERα and act as a negative regulator in glucose metabolism (Matthews and Gustafsson 2003; Barros et al. 2006a; Barros et al. 2006b). Prior to our HFD study, the effect of a HFD on ER protein levels was unknown. We showed that ERβ expression was significantly decreased in the soleus muscle in response to the HFD. This may allow E_2 to primarily activate ERα and provide the beneficial effects on glucose regulation. Therefore, the ratio of ERα to ERβ may be an important predictor of the ultimate function of E_2 on glucose metabolism.

Models used to study E_2 and glucose regulation.

Various models are used to study E_2 and glucose regulation. Using animals with intact ovaries provides a model of endogenous, cycling hormone levels. Yet, most common in the literature, researchers use OVX animals and administer E_2 via injection or a time-release pellet. While this method allows the researchers to control the amount of E_2 given to each animal, serum E_2 levels still fluctuate. Injections of E_2 result in a spike of serum E_2, which usually peak at 1 hour post injection (Medlock et al. 1991; Zoubina et al. 2001), and the E_2 fully subsides by 48 hours (Haim et al. 2003). Time-release E_2

pellets may be a better choice for delivering a constant dose of E_2 for a longer period of time. These pellets usually produce serum E_2 levels that peak at around 6 hours, and then remain steady for the remainder of the treatment (Medlock et al. 1991). However, these pellets do not provide the physiological cycling of serum E_2 levels. A benefit to giving back E_2 is that researchers can show the independent effect of E_2 on the desired outcome. However, altering the E_2 level, via OVX and/or E_2 replacement may also affect other sex hormones, which is generally not measured or taken into account. In conclusion, the outcomes of a study may only pertain to the situation which is modeled. For example, any effect of E_2 replacement in an OVX animal may not be the same as what occurs in an endogenous, cycling animal. Furthermore, the rat estrus cycle is 4 days (Westwood 2008), and the human reproductive cycle is 28 days. Therefore, the response to endogenous, cycling ovarian function may also differ among species.

The age of the animal being studied is also important to consider. OVX is commonly used to model the post-menopausal state. However, this procedure is typically performed on 2-3 month old rats, which represents early adolescence. Certainly, using rats older than 10 months (when the rat's reproductive cycle stops) represents the best physiological animal model. However, using older rats comes with its own set of challenges and confounding factors. Aging itself is a risk factor for insulin resistance (DeFronzo 1981; Bolinder et al. 1982; Jackson 1990). In fact, a decline in insulin sensitivity begins in the third or fourth decade of one's life, and it continues to decline over one's lifespan (DeFronzo 1981). While the exact mechanism of aging's impact on insulin resistance is still being explored, oxidative stress and mitochondrial dysfunction

are likely contributors (Short et al. 2005; Asghar and Lokhandwala 2006). In order to assess the independent effect of E_2 on glucose metabolism without the factors associated with aging, younger animals are commonly used in research studies.

The fact that ageing is a risk factor for insulin resistance questions whether the decline in insulin sensitivity in females is in fact due to E_2 loss or if it is due to the increase in oxidative stress and mitochondrial dysfunction. While the decline in insulin sensitivity occurs in men and women, the decrease in insulin sensitivity is more pronounced in post-menopausal women compared to age-matched men (Borissova et al. 2005). This suggests that menopause in women further impacts the age-associated decline in insulin sensitivity.

Adipokines and insulin resistance.

Adipokines are cytokines secreted from the adipose tissue that can act as an autocrine, paracrine, or endocrine hormone. Increasingly, numerous reports demonstrate that adipokines play an important role in insulin resistance via mediating cross-talk between adipose tissue and skeletal muscle. While our studies did not focus on this aspect, our data show that PPT impacts both skeletal muscle and adipose tissue. Therefore, we must consider the possibility that adipokines are also being modulated and may contribute to the changes in skeletal muscle insulin sensitivity.

Leptin and adiponectin are two adipokines which improve muscle insulin sensitivity (Yamauchi et al. 2001; Singh et al. 2003; Yaspelkis et al. 2004). These

proteins increase fatty acid oxidation and decrease lipid content in the skeletal muscle to contribute to the increased insulin sensitivity (Muoio et al. 1997; Minokoshi et al. 2002; Steinberg et al. 2002; Tomas et al. 2002; Yamauchi et al. 2002). Serum adiponectin levels are inversely correlated with body mass index (BMI) (Arita et al. 1999), which signifies that overweight and obese people have lower adiponectin levels and, hence, decreased muscle insulin sensitivity. In fact, people with T2D have lower serum adiponectin levels compared to BMI-matched controls (Hotta et al. 2000).

Retinol binding protein 4 (RBP4) is an adipokine which contributes to decreased insulin sensitivity, and obesity and T2D results in increased serum RBP4 levels (Basualdo et al. 1997; Abahusain et al. 1999; Yang et al. 2005). Mice fed a HFD also have increased serum RPB4 levels (Yang et al. 2005). Yang et al. (2005) demonstrate that the skeletal muscle insulin resistance present in mice with adipose tissue-specific GLUT4 KO is due to an increase in RBP4 levels. Moreover, genetic deletion of RBP4 enhances insulin sensitivity (Yang et al. 2005). Thus, RBP4 is an important mediator of adipose tissue to skeletal muscle cross talk, with the ability to decrease insulin sensitivity.

Very few studies have looked at the possibility of adipokines contributing the gender dimorphisms in insulin sensitivity and if E_2 may modulate the adipokines. Serum adiponectin levels are lower in men than women and in male rats compared to female rats (Nishizawa et al. 2002; Gomez-Perez et al. 2008), which may contribute to the fact that females are more insulin sensitive than males. However, there are no differences in adiponectin levels between pre- and post-menopausal women or OVX and intact mice (Nishizawa et al. 2002; Nunez et al. 2008), but studies show that E_2 treatment decreases

serum adiponectin levels in mice (Bryzgalova et al. 2008; Riant et al. 2009). Therefore, the role of E_2 in modulating adiponectin levels and the potential for this to play a role in insulin sensitivity remains unknown.

In our studies, treating rats for three days with PPT decreased adipogenesis and enhanced skeletal muscle insulin sensitivity. While our data show that the PPT increased cell signaling intermediates in the skeletal muscle (e.g. pAkt and pAMPK), we also showed that the PPT has an effect on adipocyte regulation. Therefore, modulation of leptin, adiponectin, and RBP4 may also be contributing factors to the observed changes in the skeletal muscle. Future studies are needed to investigate this relationship in detail.

Chapter 6

FUTURE DIRECTIONS

Much research in the E_2/glucose regulation field has focused on the regulation of GLUT4 by the ERs. While we have shown that *in vivo* activation of ERα via PPT increased GLUT4 protein levels in the EDL muscle, exploring the mechanism by which ERα acts to increase GLUT4 is an area that needs future exploration.

The protein of interest which may link ERα and GLUT4 is NF-κB. NF-κB is a transcription factor activated by stimuli such as cellular stress, cytokines, and inflammation. The promoter region of GLUT4 contains a NF-κB binding site (Long and Pekala 1996b), and NF-κB represses GLUT4 transcription (Ruan et al. 2002). NF-κB may be regulated by ERα, as activated ERα can directly bind to NF-κB and decrease NF-κB–DNA binding (Stein and Yang 1995; Galien and Garcia 1997; Ray et al. 1997; Paimela et al. 2007). This decrease in NF-κB–DNA binding may contribute to the increase in GLUT4 with ERα activation. However, controversy still exists in the field. In male ERα KO mice, GLUT4 mRNA and protein is thought to be decreased in the gastrocnemius muscle (slow- and fast-twitch fibers) (Barros et al. 2006b). Yet, in female ERα KO mice, GLUT4 mRNA and protein is unchanged in the quadriceps (slow- and fast-twitch fibers) and soleus (primarily fast-twitch fibers) muscles (Ribas et al. 2009). We too see differences in GLUT4 protein levels based on the muscle and fiber-type examined. While GLUT4 increased in the EDL (primarily fast-twitch fibers) with ERα activation, no change in GLUT4 was present in the soleus muscle (primarily slow-twitch fibers). Known differences in glucose regulation exist between the fiber types. For example, GLUT4 protein level is greater in slow-twitch muscles compared to fast-twitch muscles (Henriksen et al. 1990), and the regulation of GLUT4 by ERα may also be

different depending on fiber type. In addition, stress kinase proteins modulate glucose metabolism, and the level of stress kinases differ between fiber type, with fast-twitch muscles having greater stress kinase levels compared to slow-twitch muscles (Gupte et al. 2008). As stress kinase levels are higher in fast-twitch muscles, the ability of ERα to interact with NF-κB and alter GLUT4 levels may be greater in fast-twitch fibers. Also, the lower amount of GLUT4 in fast-twitch muscles may allow GLUT4 to have room to be up-regulated. Future studies are needed to determine the extent to which NF-κB and ERα interact to modulate GLUT4 levels.

Localization of the ERs in skeletal muscle.

While numerous studies show the presence of ERα and ERβ in skeletal muscle of various species including humans, mice, and rats (Kalbe et al. 2007), little is known about the specific localization of these receptors in skeletal muscle. Knowing the localization of these proteins may predict the function of the receptors. For example, while nuclear localization may suggest a role in gene regulation, membrane and cytoplasmic localization may suggest a role in cell signaling events. To our knowledge, we have shown for the first time staining of ERα and ERβ in rat skeletal muscle. ERα was localized throughout the cell, including the cytoplasm, nucleus, and membrane, in both the soleus and EDL (Figure 22A), and ERβ was localized to the nucleus in the soleus and throughout the cell in the EDL (Figure 22B). Other studies show the presence of ERs in

various locations of skeletal muscle, with differences being attributed to species studied and antibodies used. Immunohistochemical analyses in pig skeletal muscle using various ERα and ERβ antibodies show the presence of ERα either in the nucleus or undetected, albeit the presence of ERα shown via mRNA and Western blot; and ERβ was shown to have either a nuclear localization or both a nuclear and cytoplasmic localization depending on the source of the ERβ antibody (Kalbe et al. 2007). In humans, staining for ERα and ERβ reveals the presence of the proteins in the nucleus (Wiik et al. 2009), yet ERα has also been undetected (Wiik et al. 2003). In mice, ERα and ERβ was shown to be present in the nucleus (Barros et al. 2006b). While determining the localization of the ERs in skeletal muscle is a new endeavor, clearly the source of the antibody plays a role in detection. The location of the ERs in skeletal muscle may also differ by species.

Localizing the ERs in the skeletal muscle may provide a useful tool for determining ERα–NF-κB interaction. In an inactive state, NF-κB is bound by IκBα at a specific epitope on the p65 subunit of NF-κB. To activate NF-κB, IκBα is phosphorylated, which signals its degradation by the proteosome. The p65 epitope is now exposed, and a specific p65 antibody can detect this epitope, hence, detecting activated NF-κB (Kaltschmidt et al. 1994a; Kaltschmidt et al. 1994b; Kaltschmidt et al. 1995). Co-immunostaining of ERα and activated p65 may serve as a method to determine ERα–NF-κB interaction, both quantitatively and qualitatively (location in the cell).

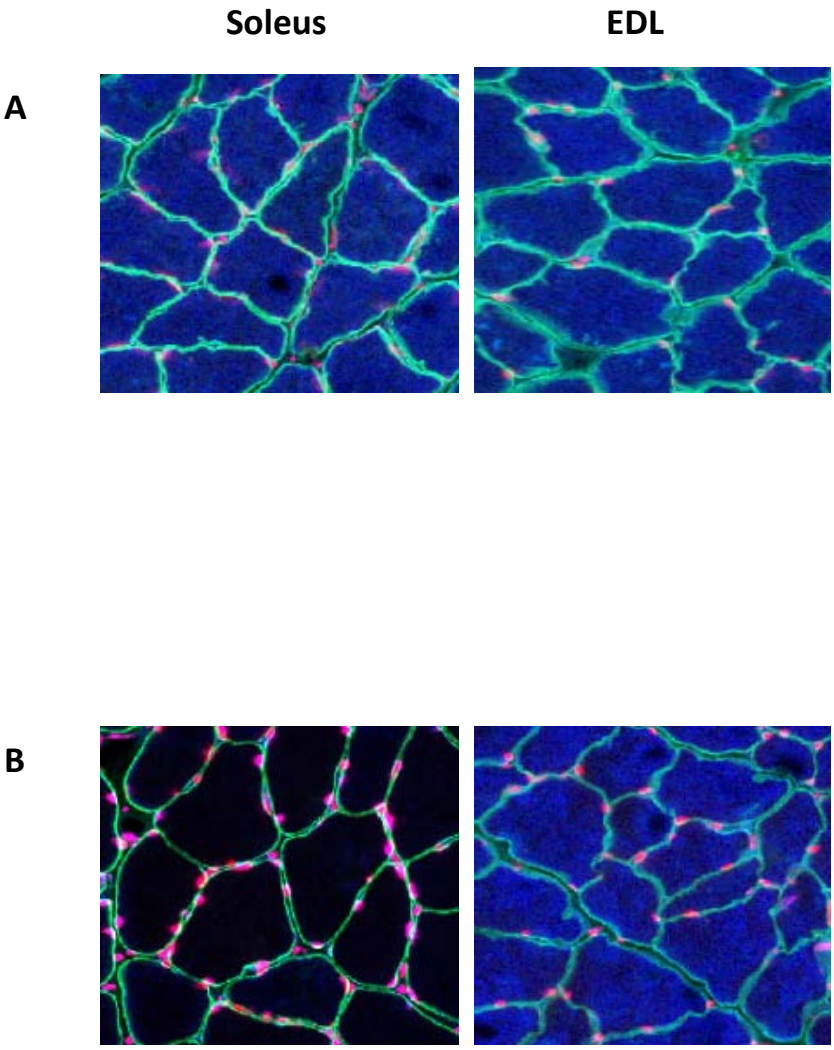

Figure 22: Immunohistochemistry of ERα and ERβ in female Sprague Dawley rats. The soleus and EDL muscles were removed from female Sprague Dawley rats an frozen in isopentane. 10 micron cross-sections were cut. ERα is localized throughout the cell, including the cytoplasm, nucleus, and membrane, in both the soleus and EDL (A). ERβ is localized to the nucleus in the soleus and throughout the cell in the EDL (B). ERα and ERβ are shown in blue, dystrophin (membrane) is shown in green, and propidium iodide (nucleus; red) is shown merged with ERα/β (pink).

Phosphorylation of NF-κB p65 signals activation.

Numerous studies report the ability of the p65 subunit of NF-κB to be phosphorylated. p65 is phosphorylated at numerous residues, but we chose to focus on serine phosphorylation as this has been suggested to be important for its transcription activity (Hayden and Ghosh 2004). Specifically, measuring phosphorylation of p65 on serine residues may indicate transcriptional activity and an association to GLUT4 regulation. Phosphorylation of p65 can occur in the cytoplasm or in the nucleus in response to stimuli. TNF-α is a known stimulus for p65 phosphorylation on various serine residues (reviewed in (Viatour et al. 2005)). TNF-α is a cytokine that is highly expressed in obese humans (Saghizadeh et al. 1996; Uysal et al. 1998), and obese humans with T2D have increased skeletal muscle NF-κB activation (Sriwijitkamol et al. 2006). Therefore, we chose to focus on serine residues which are phosphorylated via TNF-α stimulation. Numerous research studies show that TNF-α-stimulated Ser536 phosphorylation is required for nuclear translocation and enhances p65 transactivation potential (Sakurai et al. 1999; Jiang et al. 2003; O'Mahony et al. 2004). Specifically, Zhong et al. demonstrated enhanced binding of p65 to DNA with Ser536 phosphorylation (Zhong et al. 1998). Furthermore, inhibitory peptides corresponding to amino acids 525-536 of p65 inhibit TNF-α-induced NF-κB activity (Takada et al. 2004).

To explore the association between ERα activation and p65 regulation, we measured phosphorylation of p65 on Ser536 in the EDL muscles of rats treated with PPT. Rats treated with PPT showed increased GLUT4 protein in the EDL. However, activation of p65 (as measured by phosphorylation of Ser536) did not decrease (Figure

23A). A decrease in p65 phosphorylation would indicate less activation of p65, and p65 is a negative modulator of GLUT4 transcription. While we suspected that the p65 phosphorylation may result from TNF-α stimulation (due to its up-regulation in obesity and T2D), no current evidence has established this association. Therefore, we also measured phosphorylation of p65 on Ser468, which is not induced by TNF-α stimulation (Williams et al. 2008). No differences in p65 Ser468 were measured between vehicle and PPT treated animals in the EDL muscle (Figure 23B).

While we did not see any changes in p65 phosphorylation on Ser536 and Ser468, we cannot conclude whether or not p65 is altered with PPT treatment. Numerous other phosphorylation sites may be modified as a result of ERα activation. Particularly, numerous reports also suggest that Ser276 and Ser529 play an important role in regulating p65 transcriptional activity (reviewed in (Viatour et al. 2005)). In addition, we measured p65 phosphorylation 24 hours following the final PPT treatment. p65 phosphorylation may occur in a time-dependent fashion to regulate GLUT4 levels, and optimal phosphorylation may occur prior to 24 hours. Finally, measuring phosphorylation of p65 may not be the optimal technique to detect p65 activation. While the literature supports the fact that p65 is phosphorylated upon activation, the standard technique of electrophoretic mobility shift assay (EMSA) would likely provide the best evidence for p65 activation.

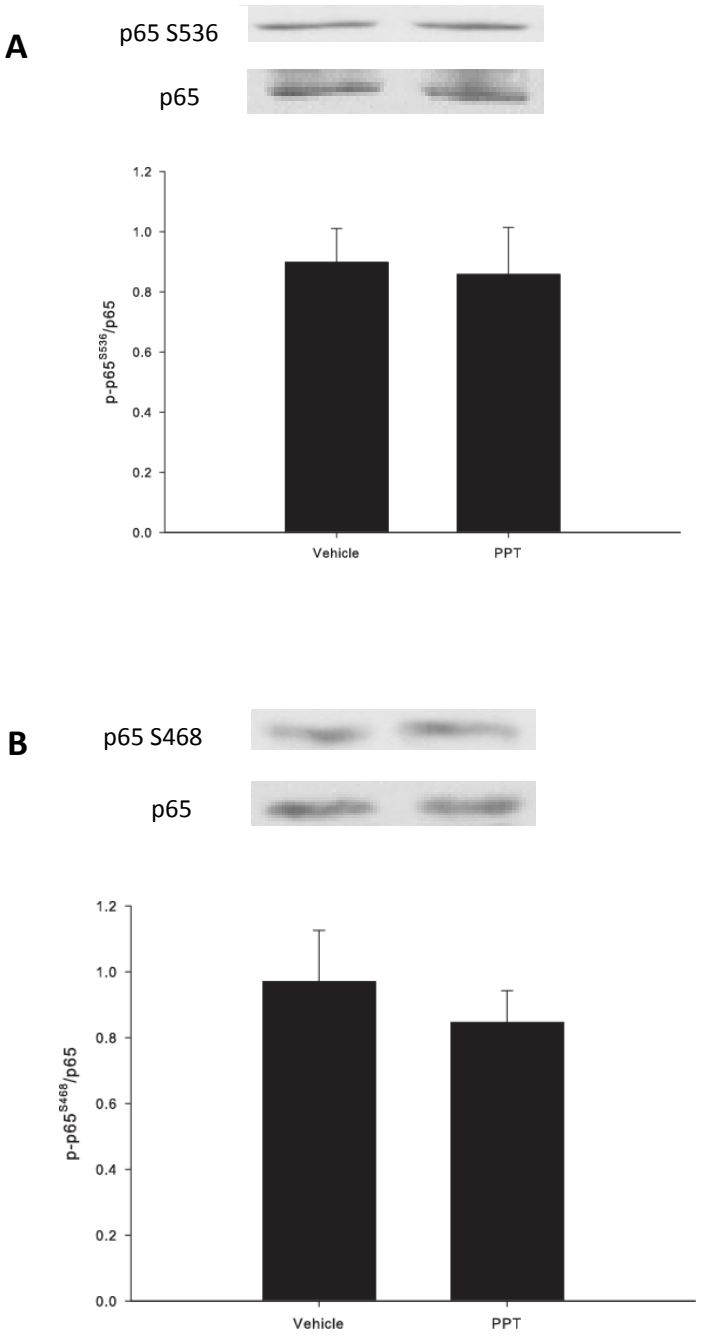

Figure 23: No change in p-p65 in the EDL muscle from in PPT-treated rats. Female Sprague Dawley rats were given subcutaneous injections of PPT (10 mg/kg) or vehicle for 3 days. Phosphorylation of p-65 was measured in the EDL muscle by Western blot analysis on residues Ser536 (A) and Ser468 (B) and normalized to total p65. Values are means ± SE for 6 samples per group.

Using L6 cells to study ERα–NF-κB interaction.

While evidence exists for the possibility of ERα to interact with NF-κB and regulate GLUT4 (see introduction), the details involving the interaction remain in its infancy. To better guide researchers, a cell culture model may provide a better starting point. A cell culture model would allow researchers to quickly manipulate the cells and make initial measurements regarding ERα–NF-κB interaction and GLUT4 regulation. L6 cells are a common skeletal muscle cell line originally isolated from rat thigh muscle. This cell line may provide a useful mean to study ERα, NF-κB, and GLUT4 interaction via treating the cells with various substances such as estrogen, specific estrogen receptor agonists/antagonists, NF-κB activators, and siRNA to name a few. In fact, treating L6 cells will TNF-α (a cytokine up-regulated in obesity and T2D) results in decreased GLUT4 protein (Figure 24). Exploring how this decrease in GLUT4 protein is altered in the presence of additional substances may provide better evidence for ERα–NF-κB interaction and GLUT4 regulation.

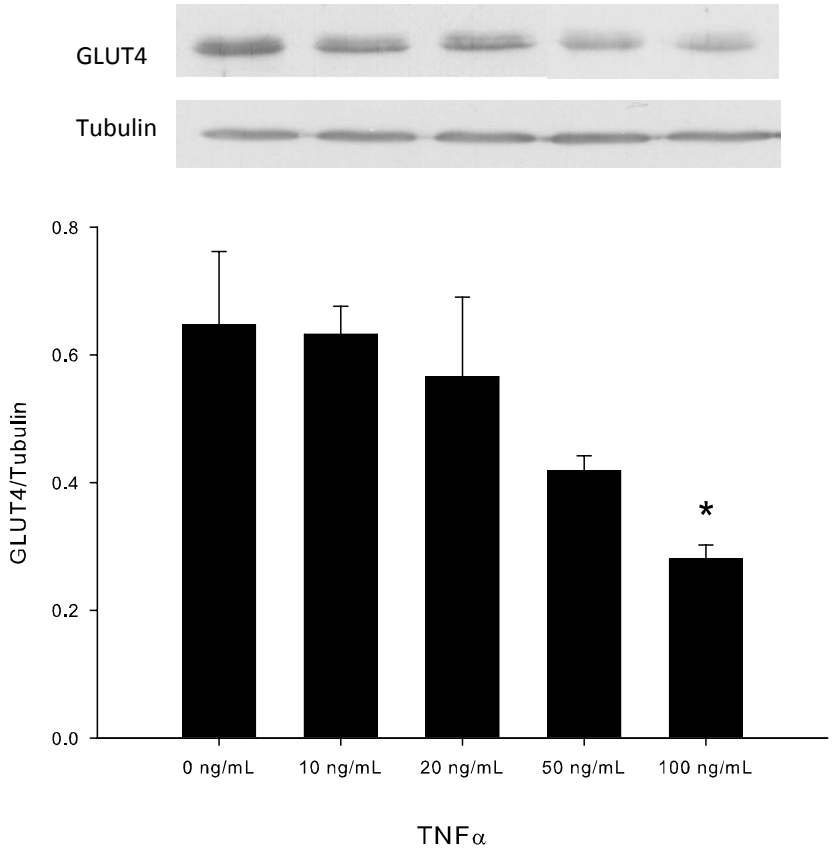

Figure 24: TNFα treatment decreases GLUT4 protein in L6 cells. L6 cells were treated with various concentrations of TNFα for 48 hours. GLUT4 protein levels were measured by Western blot analysis and normalized to tubulin. Values are means ± SE for 3-6 samples per group; *P=0.066 vs. 0 (ng/mL).

Additional model for studying estrogen and metabolic regulation.

We have additional data suggesting that alternative models could be used to study the interaction between estrogen and metabolic regulation. NADH cytochrome b(5) oxidoreductase (Ncb5or) is a flavoheme reductase involved in desaturation of fatty acids (Larade et al. 2008). Ncb5or is widely expressed in various tissues and is located in the endoplasmic reticulum (Xie et al. 2004; Zhu et al. 2004). The desaturation of fatty acids is an important step in triglyceride synthesis and fat storage. Therefore, loss of Ncb5or results in lipoatrophy due to the inability to synthesize triglycerides and store fatty acids (Larade et al. 2008). As a means of compensation, the fatty acids are metabolized in the endoplasmic reticulum, which results in overloading and endoplasmic reticulum stress-induced lipotoxicity (Zhang et al. 2010). The absence of Ncb5or also results in insulin-deficient diabetes, resulting from a loss of pancreatic β cell function (Xie et al. 2004). When challenged with a high-fat diet (HFD), Ncb5or KO mice become diabetic at an even earlier age as metabolizing the increased amounts of fatty acids poses an extreme challenge (Zhu et al. unpublished data). Interestingly, female Ncb5or KO mice become diabetic at a much later age, suggesting that the female sex hormones may provide protective benefits. In fact, OVX in female Ncb5or KO mice advances the age of onset of diabetes compared to intact animals (Figure 25). The mechanism of how estrogen may provide beneficial effects in protecting against the fatty acid overload remains unknown.

However, estrogen is known to promote mitochondrial biogenesis and increase mitochondrial function. When challenged with a HFD, female rats demonstrated higher

mitochondrial oxygen consumption and cytochrome c oxidase activity, and a better capacity to counteract the oxidative stress-induced insulin resistance by increasing expression of UCP3 in the skeletal muscle (Gomez-Perez et al. 2008). The mechanism by which estrogen increases mitochondrial function has been explored on a genomic level. Estrogen increases nuclear respiratory factor-1 (NRF-1) and up-regulates mitochondrial biogenesis via activation of ERα (Mattingly et al. 2008). NRF-1 is a transcription factor that, along with PGC-1α, increases transcription of proteins involved in mitochondrial function, particularly Tfam (mtDNA maintenance factor) and TFB (mitochondrial transcription factor B) (Scarpulla 2006). Tfam and TFB work to promote mitochondrial DNA transcription and mitochondrial biogenesis via acting as transcription factors (reviewed in (Klinge 2008). An increase in mitochondrial biogenesis due to estrogen may better support fatty acid oxidation and decrease oxidative stress in female Ncb5or KO mice, providing a longer protection against the lipotoxicity observed in the male Ncb5or KO mice. However, this theory remains speculative and more research is need. Using Ncb5or KO mice may provide an additional model for studying the role of estrogen in metabolic regulation.

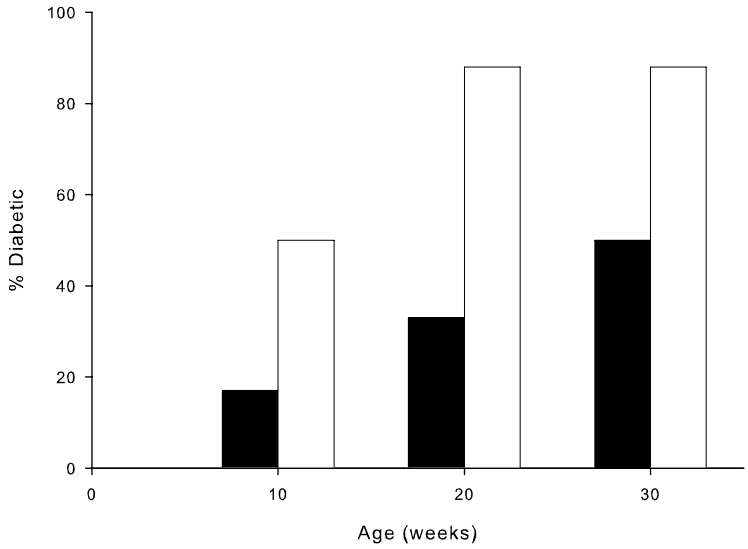

Figure 25: OVX in female Ncb5or KO mice advances the age of onset of diabetes. Ovaries were removed from female Ncb5or KO mice (OVX) or left intact (sham) at 4 weeks of age. Blood glucose was monitored every 2 days. Diabetes was defined as a blood glucose level > 200 mg/dL. Sham animals are shown in closed bars (N=6), and OVX animals are shown in open bars (N=8).

The impact of ERs on glucose metabolism, adipogenesis, and T2D.

In sum, the data presented here constitute novel information regarding estrogen's (specifically ERα) involvement in glucose metabolism and adipocyte regulation. The signaling pathways and proteins regulated by ERα provide pertinent information for understanding skeletal muscle glucose metabolism, lipolysis, and lipogenesis. Furthermore, these data are new information to help explain the molecular differences between males and females with regard to fat storage and risk for insulin resistance and T2D.

Whether or not activation of ERα can prevent and/or treat T2D remains an important question. While we and others provide evidence suggesting ERα plays a role in insulin resistance, many other pathologies and physiological pathways contribute to T2D. The extent to which ERα, and activation of ERα, contributes to insulin resistance has yet to be determined. However, if this pathway would be pursued for potential drug treatments, tissue specific drug analogs are needed, as traditional estrogen therapy has already proven to produce greater health risks than the benefits obtained for decreasing the risk of insulin resistance. Moreover, as drug development involves immense time, cost, and risk, exercise remains an established means by which T2D, and many of its consequences, can be reversed.

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