EFFECTS OF AGE AND ACUTE SUBMAXIMAL EXERCISE ON INFLAMMATORY CYTOKINES, CORTISOL, INSULIN, HEAT SHOCK PROTEINS AND SKELETAL MUSCLE MEDIATORS OF ENERGY HOMOESTASIS IN HORSES

By

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ABSTRACT OF THE DISSERTATION

Effects of age and acute submaximal exercise on inflammatory cytokines, cortisol, insulin, heat shock proteins and skeletal muscle mediators of energy homeostasis in horses

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There is a well documented decline in exercise capacity, immune function, insulin sensitivity and hypothalamic-pituitary-adrenal axis function in aged horses. Aerobic training can partially reverse age-related declines in performance measures, endocrine and immune function, and insulin sensitivity in horses. The mechanisms behind the adaptive response to exercise in horses require further research. Based on the comparative literature, several mediators of energy homeostasis and adaptation to exercise were identified for investigation in whole blood and skeletal muscle of horses.

Young and aged unconditioned Standardbred mares underwent an acute submaximal exercise test. Plasma cortisol, insulin and glucose concentrations were determined pre- and post-exercise. Whole blood and gluteus medius biopsies were analyzed for gene expression of inflammatory cytokines, *HSP70* and *HSP90* pre- and post-exercise. Skeletal muscle was also analyzed for protein content of HSP70, HSP90, AMPK, Akt and AS160 pre- and post-exercise.

Exercise increased plasma cortisol concentration in both young and aged mares, with the duration of the post-exercise rise in cortisol altered in aged horses. Although the magnitude of

the insulin response to exercise was not different between young and aged horses, plasma insulin was elevated sooner and declined earlier in aged mares. Subsequently, aged mares had a shorter duration of elevated plasma glucose concentration post-exercise than young mares. Exercise increased *IL-β* expression in whole blood of young and aged mares, with young mares having greater exercise-induced expression of *IL-β*. Cytokine expression was not altered in skeletal muscle, regardless of age or exercise.

Young and aged horses had increased *HSP70* expression in whole blood following exercise, with young horses exhibiting greater exercise-induced *HSP70* expression. *HSP90* expression in whole blood following exercise was increased only in young horses. *HSP70* and *HSP90* expression in skeletal muscle was increased following exercise in both young and aged horses, with age altering the timing of *HSP70* expression. There were no changes in skeletal muscle protein content of HSP70 and HSP90; or in activation or total protein concentration of AMPK, Akt and AS160, due to exercise or age.

In conclusion, mediators of energy homeostasis and the adaptive response to exercise are altered with age in horses.

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List of Abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
AUCc	Cortisol area under the curve
AIRg	Acute insulin response to glucose
Akt	Also known as Protein Kinase B (PKB)
AMPK	Adenosine monophosphate-activated protein kinase
AS160	Akt substrate of 160kDa
CV	Coefficient of variance
DI	Disposition index
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP	Heat shock protein
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IL-1	Interleukin 1
IL-6	Interleukin 6
MDA	Malondialdehyde
MLSS	Maximum blood lactate steady state
OBLA	Onset of blood lactate accumulation
PCV	Packed cell volume
RIA	Radioimmunoassay
SI	Insulin sensitivity
SG	Glucose effectiveness
TNF-α	Tumor necrosis factor alpha
V_{LA4}	Velocity at which blood lactate reaches 4 mmol/L
VO_{2max}	Maximal aerobic capacity

Chapter 1.

Introduction and Review of the Literature – The intersection of inflammation, insulin resistance and aging: Implications for the study of molecular signaling pathways in horses

Abstract

Inflammation-associated insulin resistance contributes to chronic disease in humans and other long-lived species, such as horses. Insulin resistance arises due to an imbalance among molecular signaling mediators in response to pro-inflammatory cytokines in the aged and obese. The mammalian heat shock protein response has received much attention as an avenue for attenuating inflammatory mediator signaling and for contributing to preservation and restoration of insulin signaling in metabolically important tissues. Data on heat shock proteins and inflammatory signaling mediators in untrained and aged horses are lacking, and horses represent an untapped resource for studying the mediator imbalance contributing to insulin resistance in a comparative model.

Introduction

Aging- and obesity-related loss of cellular function is associated with chronic, low-grade inflammation. Of particular interest, peripheral insulin resistance in aged or overweight individuals disrupts cellular ability to maintain homeostasis. Loss of homeostasis at the cellular level leads to loss of tissue function, further exacerbating the aging process. Inflammation, insulin resistance and aging represent a continuum that contributes to type 2 diabetes, cardiovascular disease and other disorders in humans and other mammals. While aging- and obesity-related loss of function and diseases have many factors, understanding the underlying imbalance of molecular signaling mediators in metabolically important tissues may present new avenues to address or preserve functionality of physiological systems. Specific mediators of insulin signaling and contributors to insulin resistance that warrant attention include members of the phosphoinositide 3-kinase (PI3K) and Akt signaling pathway, tumor necrosis factor-α (TNF-α), the serine-threonine kinases c-jun amino terminal kinase (JNK) and inhibitor of kappa B kinase (IKK) and heat shock proteins (HSPs).

Peripheral insulin resistance and disease

Glucose tolerance and the ability of metabolically important tissues of aged individuals to respond to insulin declines over time (DeFronzo, 1981; Narimiya et al., 1984; Chen et al., 1985; Hadden and Harris, 1987; Harris et al., 1987; Iozzo et al., 1999; Ford et al., 2002). Loss of insulin-mediated glucose uptake in the aged is primarily due to changes in body composition related to abdominal adiposity (Shimokata et al., 1991; Kohrt et al., 1993; Ferrannini et al., 1996), which contributes to increased insulin resistance in skeletal muscle through signaling defects downstream of the insulin receptor (Robert et al., 1982; Fink et al., 1983; Rowe et al., 1983; Chen et al., 1985; Jackson et al., 1988).

A major culprit in aging- and obesity-related peripheral insulin resistance is chronic inflammation. Over the last two decades, a growing body of evidence has linked obesity to chronic inflammation, with increased adiposity resulting in a consistent expression of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) from both macrophages and adipocytes (Kern et al., 2001; Wellen and Hotamisligil, 2005; Hotamisligil, 2006; Shoelson et al., 2006; Chung et al., 2008).

Insulin resistance is a well-known contributor to and marker of type 2 diabetes, and is predominantly triggered by environmental factors (Groop, 1999; Alberti et al., 2007). Over half of the adults in the United States studied in a cross-sectional survey from 1988-1994 were overweight or obese, with a strong correlation of type 2 diabetes mellitus occurring with increasing adiposity (Must et al., 1999). A population study conducted from 1999 – 2002 has shown that 9.3% of the United States population is afflicted with type 2 diabetes, with 6.5% diagnosed and 2.8% undiagnosed with the disease (Cowie et al., 2006). The prevalence of diagnosed type 2 diabetes increased by 1.3 % since a similar population survey was completed in 1994 (Lee et al., 2006; Ioannou et al., 2007). The study completed in 2002 also revealed that 52.1% of obese adolescents were insulin resistant (Lee et al., 2006). In overweight but otherwise healthy men and women, ranging in age from 30 to 75 years of age, individuals with excess visceral adipose tissue were found to be less insulin sensitive, while subjects with excess subcutaneous adipose tissue were more insulin sensitive (Amati et al., 2012).

Insulin resistance is not only a problem facing the United States, but is a health and economic threat to Latin America, with instances of diabetes expected to rapidly rise in the coming years (Barcelo, 2001; Barcelo et al., 2001; Barcelo and Rajpathak, 2001; Aschner, 2002; Barcelo et al., 2003; Barcelo et al., 2006; Barcelo et al., 2012). It is expected that diabetes will affect nearly 8%

of the global population by 2030, with the greatest increase of adults afflicted with diabetes occurring in developing countries (Alberti et al., 2007; Shaw et al., 2010).

Recently, the term metabolic syndrome has come to describe a general collection of risk factors for cardiovascular disease, including the characteristics of type 2 diabetes insulin resistance, obesity, hypertension and dyslipidemia (Kahn et al., 2005; Alberti and Zimmet, 2006; Alberti et al., 2006, 2007). The degree of insulin resistance correlates with age-related diseases such as hypertension, coronary heart disease, stroke and type 2 diabetes, with the most insulin resistant individuals having higher rates of disease, and the most insulin sensitive subjects leading lives free from disease (Yip et al., 1998; Facchini et al., 2001). Among 126 patients with hypertension, 50% were insulin resistant and had a 2-3 fold increase of abnormal risk factors for cardiovascular disease (Lima et al., 2009). In a genetically-induced, non-obese rodent model of insulin resistance, an increase of coronary risk factors was observed, such as increased blood pressure, increased plasma triglycerides and decreased plasma triglyceride clearance and decreased endothelium-dependent vascular relaxation (Abe et al., 1998). Additionally, wound healing is impaired in an induced diabetic state in rats (Bitar et al., 1999). Overall, the trends of increasing obesity and loss of insulin sensitivity, leading to chronic disease states, represent a grave health threat to the aged and obese.

Horses represent a naturally occurring model for study of insulin resistance as diagnosis and prevalence of the disorder, and the classification of equine metabolic syndrome, are on the rise (Geor and Frank, 2009; Waller et al., 2011a). Aging-related loss of function and insulin signaling are increasingly detrimental to the well-being of equine animals. Aging represents a growing challenge to the equine industry, with many horses over the age of 20 involved in competition or reproduction (Malinowski et al., 1997). Of particular concern, aged horses experience

disruptions in glucose homeostasis relating to insulin resistance (IR), which is implicated in loss of muscle mass, increased adiposity, declining immune function and laminitis (Frank et al., 2006).

Insulin resistance in horses has been studied in correlation with age and obesity. Aging and increased adiposity contribute to reduced insulin sensitivity and increased production of pro-inflammatory cytokines (Vick et al., 2007; Vick et al., 2008; Adams et al., 2009). Aging in horses is also associated with decline in aerobic capacity and exercise capability, declining immunity and chronic inflammation (McKeever and Malinowski, 1997; Horohov et al., 2010), and compromised thermoregulation in response to acute exercise (McKeever et al., 2010). Aerobic training can partially reverse age-related decline in performance measures, and insulin sensitivity in horses (Betros et al., 2002; Malinowski et al., 2002). However, the molecular mechanisms behind the adaptive response to exercise require further study for the benefit of all equine animals, and other long-lived species.

Exercise and diet are effective countermeasures to partially restore insulin sensitivity, through molecular mediators of insulin signaling, particularly in skeletal muscle, in the aged and insulin resistant (Ryan et al., 2011; Snel et al., 2012). For those unable to undertake necessary lifestyle changes to restore insulin sensitivity and combat age and inactivity, nutritional or pharmacological methods to reestablish the balance of molecular mediators in metabolically important tissues, such as skeletal muscle, may present new opportunities to preserve health and functionality. These means of restoring insulin sensitivity will be of benefit to horses that are unable to undertake strenuous activity due to age or injury. An understanding of proper insulin signaling and maintenance of cellular homeostasis, and the mechanisms causing

disruption of insulin signaling and cellular homeostasis that occurs during the inflamed state, is required before novel countermeasures can be pursued.

Mechanisms of insulin signaling leading to cellular uptake of glucose

Insulin-stimulated uptake of glucose occurs through translocation of glucose transporters (GLUTs) to the plasma membrane of cells (Cushman and Wardzala, 1980), with GLUT4 being the isoform most responsible for mediating glucose uptake in adipose and muscle tissue (Abel et al., 2001; Dugani and Klip, 2005; Ishiki and Klip, 2005; Thong et al., 2005). The signaling cascade leading to GLUT4 translocation to the plasma membrane of insulin-stimulated cells and uptake of glucose from the extracellular fluid relies on several regulated and interacting molecular mediators. In brief, the membrane bound insulin receptor autophosphorylates when stimulated by insulin, leading to tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). The signaling cascade continues through the PI3K pathway, which includes several important molecular mediators with multiple functions, to prompt GLUT4 translocation to the plasma membrane (Quon et al., 1995; Dugani and Klip, 2005; Ishiki and Klip, 2005; Thong et al., 2005).

One member of the PI3K pathway is Akt, which has many functions including direct stimulation of GLUT4 translocation to the cellular membrane (Quon et al., 1995; Dugani and Klip, 2005; Ishiki and Klip, 2005; Thong et al., 2005). Downstream from insulin-stimulated activation of PI3K, Akt is activated through phosphorylation and is responsible for stimulating GLUT4 translocation to the cell membrane, allowing glucose uptake into adipocytes (Kohn et al., 1996; Cong et al., 1997), and for directly inhibiting the serine/threonine kinase glycogen synthase kinase 3 (GSK-3) (Cross et al., 1995). Inhibition of Akt and subsequent inhibition of insulin signaling can occur due to activation of stress kinases by free fatty acids, as has been shown in endothelial cells (Wang et al., 2006b). Akt activation in response to insulin is suppressed in skeletal muscle of diabetic

patients (Krook et al., 1998), and acute pharmacological inhibition of Akt prevents GLUT4 translocation to the plasma membrane of adipocytes, impairing glucose uptake (Gonzalez and McGraw, 2006). Suppression of the insulin signaling pathway in human umbilical vein endothelial cells by use of inhibitory mutants of PI3K and Akt further supports their role as mediators of insulin signaling (Zeng et al., 2000).

Mechanisms of insulin resistance

Inhibition of insulin-induced GLUT4 translocation and glucose uptake is due to multiple factors leading to an imbalance of signaling molecules in metabolically important tissues. Skeletal muscle provides the greatest potential for glucose utilization, and is therefore the best target for restoring insulin sensitivity through nutritional, pharmacologic, or exercise treatments (Geiger and Gupte, 2011). However, understanding the role of cellular mediators of insulin sensitivity and inflammation of adipose tissue is also important. There is a strong correlation between whole-body glucose disposal and glucose transport into adipocytes (Ciaraldi et al., 1982). In type 2 diabetes, there is an early and continued insulin resistance in adipose tissue (Ost et al., 2010). In obese diabetic subjects, insulin stimulation of glucose uptake by adipocytes is reduced due to inactivation of the insulin receptor substrate and the impaired action of AKT (Ciaraldi et al., 2002; Ost et al., 2010). In aged rats, activation of IRS-1, Akt and GLUT4 translocation is impaired in white adipose tissue (Serrano et al., 2009). While whole-body insulin resistance may have roots in adipose tissue, with failure of insulin signaling in skeletal muscle occurring secondarily, in order to reverse the signaling defects prevalent in insulin resistance both tissues should undergo study to understand the basic mechanistic underpinnings of the disorder.

In premenopausal women TNF-α expression in adipose tissue increased with obesity, and a strong correlation was seen between TNF-α and insulin resistance. Reduction of body weight in

obese subjects decreased TNF-α and increased insulin sensitivity (Hotamisligil et al., 1995). In rodent models of obesity and diabetes, TNF-α expression increased in adipose tissue, while TNF-α protein increased both in adipose tissue and systemically (Hotamisligil et al., 1993). TNF-α knockout mice experienced improved insulin sensitivity in muscle and adipose tissue despite dietary or genetic induced obesity, as compared to controls (Uysal et al., 1997). Removal of TNF-α action in obese rats increased peripheral insulin sensitivity, as indicated by reduction of plasma glucose, insulin and FFA levels, leading the authors to conclude that TNF-α contributes to peripheral insulin resistance through inhibition of tyrosine kinase phosphorylation of IRS-1 in muscle and adipose tissue (Hotamisligil et al., 1993; Hotamisligil et al., 1994).

Secretion of pro-inflammatory cytokines from adipocytes and macrophages activate serine-threonine kinases, which modulate the insulin signaling pathway in metabolically important tissues such as liver, skeletal muscle and adipose tissue (Wellen and Hotamisligil, 2005; Shoelson et al., 2006). The serine-threonine kinases of importance are c-jun amino terminal kinase (JNK) and inhibitor of κ B kinase (IKK). For a complete review of the function and importance of JNK, see Bogoyevitch and Kobe, 2006. In brief, JNK is a member of the MAPK family, a group of Ser/Thr protein kinases which also includes GSK-3. JNK is a signaling mediator, especially of proinflammatory cytokines, and has roles in the stress/adaptive response and apoptosis. Different isoforms of JNK exist, and may perform different, sometimes tissue-specific, roles. JNK is known to both positively and negatively regulate transcription factors through phosphorylation, and has been determined to act on at least 50 substrates within the cytoplasm, mitochondria and nucleus (Bogoyevitch and Kobe, 2006).

Environmental factors influence the serine-threonine kinases, and contribute to aging- and obesity-related loss of function. Excess lipid accumulation in insulin-responsive tissue causes

increased deposition of lipid species such as diacylglycerol and ceramide, which contributes to insulin resistance through activation of JNK and IKK (Wellen and Hotamisligil, 2005; Hotamisligil, 2006; Shoelson et al., 2006; Watt et al., 2006; Hooper and Hooper, 2009). Constant activation of serine threonine kinases such as JNK and IKK by TNF-α results in serine phosphorylation of IRS-1 in insulin-responsive tissues, such as skeletal muscle, adipose tissue and liver, rendering it a poor substrate for insulin signaling by preventing tyrosine phosphorylation (Aguirre et al., 2000; Lee et al., 2003; Wellen and Hotamisligil, 2005; Hotamisligil, 2006). In aged rats, insulin receptor and IRS-1 phosphorylation, and activation of PI3K, was decreased in both skeletal muscle and liver (Carvalho et al., 1996; Zhu et al., 2005). In dietary and genetic models of obesity in mice, active JNK1 and JNK2 increased in liver, muscle and adipose tissue. However, only the absence of JNK1 protected against development of insulin resistance (Hirosumi et al., 2002). Recently, reduced phosphorylation of IRS-1 and Akt, and inhibitory phosphorylation of GSK-3, was found in adipocytes of a rodent model of surgically induced insulin resistance (Williams et al., 2012). Overall, the mechanisms underlying insulin resistance are at least in part due to increased pro-inflammatory cytokine production, causing increased activation of stress kinases, which interfere with the insulin signaling pathway.

Manipulation of insulin and inflammatory signaling pathways to restore insulin signaling

Experimental and pharmacological measures to restore insulin sensitivity and cellular functionality attempt to capitalize on the complex relationship between insulin signaling and inflammatory pathways in order to restore a signaling balance. Insulin treatment prevents TNF-α-induced apoptosis in human umbilical vein endothelial cells through activation of Akt, but this can be counterbalanced through the ability of TNF-α to deactivate Akt (Hermann et al., 2000). Pharmacological intervention to prevent obesity-related inflammation and insulin resistance in

high fat diet fed mice results in reduced pro-inflammatory cytokine production, decreased levels of active JNK in skeletal muscle and liver, and increased insulin-stimulated Akt signaling and glucose uptake in skeletal muscle (Vinolo et al., 2012). Silencing of the obesity-related gene NYGGF4 increases insulin-stimulated activation Akt and GLUT4 translocation to the cell membrane in myocytes in vitro (Zeng et al., 2012). Calorie restriction causes increased IRS-1, PI3K and Akt activation, leading to increased GLUT4 translocation to the membrane and glucose uptake in both fast- and slow-twitch skeletal muscle fibers in rats (Sharma et al., 2011). In mice, disruption of both JNK and IKK signaling pathways prevents obesity-related insulin resistance (Hirosumi et al., 2002; Arkan et al., 2005; Cai et al., 2005). Aerobic exercise training restores insulin sensitivity and oxidative capacity of human skeletal muscle (Short et al., 2003), through increased insulin-stimulated activity of Akt (Fujita et al., 2007). Experimental results have therefore illustrated that manipulation of the complex pathways involved in inflammation and insulin resistance through pharmacological agents, dietary changes and exercise can restore cellular functionality.

Endoplasmic reticulum stress and insulin resistance

The endoplasmic reticulum provides an essential role in proper cellular function through management of protein synthesis and folding. Evidence exists for a link between chronic endoplasmic reticulum (ER) stress and obesity-related insulin resistance through the actions of inflammatory mediators such as JNK (Ozcan et al., 2004; Hotamisligil, 2005; Nakatani et al., 2005; Ozawa et al., 2005; Wellen and Hotamisligil, 2005; Ozcan et al., 2006). Dietary and genetic induction of obesity in mice increases markers of ER stress and activation of JNK in liver and adipose tissue (Ozcan et al., 2004). In cultured hepatocytes, induction of ER stress causes increases in serine phosphorylation of IRS-1and reduced insulin-induced phosphorylation of Akt,

therefore inhibiting insulin action (Ozcan et al., 2004). Inhibition of JNK in cultured hepatocytes prevents the serine phosphorylation of IRS-1 that would have most likely occurred as a result of ER stress induction (Ozcan et al., 2004).

In response to acute ER stress, deployment of the unfolded protein response (UPR), allows the endoplasmic reticulum to adapt to the temporarily increased demands for protein folding, quality control and trafficking (Liu and Kaufman, 2003; Ron and Walter, 2007; Wang et al., 2009; Hotamisligil, 2010). The master regulator of the UPR is the chaperone protein GRP78, also known as BiP or HSPA5, which is a member of the HSP70 family of heat shock proteins (Liu and Kaufman, 2003; Ron and Walter, 2007; Wang et al., 2009; Hotamisligil, 2010).

In addition, oral administration of chemical chaperones stabilizes ER capacity and decreases JNK phosphorylation, while increasing IRS-1 and AKT activation and restoring insulin sensitivity, in liver and adipose tissue of obese and diabetic mice (Ozcan et al., 2006). In murine models of insulin resistance, overexpression of the molecular chaperone ORP150 protects hepatocytes from ER stress, and increases insulin sensitivity through tyrosine phosphorylation of IRS-1 and serine 473 phosphorylation of Akt (Nakatani et al., 2005; Ozawa et al., 2005). Failure of molecular chaperone function during chronic ER stress may therefore be an important factor in the development of insulin resistance.

Insulin signaling itself counteracts ER stress. Stimulation of the insulin receptor prevents apoptosis due to pharmacologically-induced ER stress in murine pancreatic β cells, while a graded reduction of the insulin receptor increased apoptosis (Srinivasan et al., 2005). Interference of the insulin signaling pathway exacerbates ER stress, as reduction of phosphorylated Akt increased apoptosis, while reduction of GSK-3 decreased apoptosis (Srinivasan et al., 2005). Failure of the endoplasmic reticulum in response to environmental

factors represents a specific and significant example of inflammatory-induced loss of cellular function and insulin sensitivity.

Heat shock proteins maintain cellular homeostasis and insulin signaling

As mentioned above, molecular chaperones play a critical role in proper cellular function, including insulin signaling, and may represent novel and effective pathways to restore insulin sensitivity. Constant challenges to homeostasis prompt adaptive responses in all organisms. Challenges and stressors may take many forms, but the adaptive response has similar properties regardless of the specific disturbance in homeostasis. The mechanism behind adaptation seems to rely on a highly conserved molecular response, which allows for cellular survivability in the face of environmental challenges and includes deployment of members of the heat shock protein family (Kilgore et al., 1998; Calderwood et al., 2009). Heat shock proteins (HSPs) were originally discovered in Drosophila in response to heat stress, hence the name (Morton et al., 2009). However, since their discovery, HSPs have been shown to be up-regulated in response to a variety of stressors.

Chaperone and stress response functions of HSPs

Newly synthesized proteins come under the care of molecular chaperones, which are responsible for performing post-translation modifications and translocation. During periods free from stress, members of the HSP family serve as these molecular chaperones, and are sometimes referred to as housekeeping proteins (Calderwood et al., 2009; Morton et al., 2009). They ensure the correct folding of newly synthesized proteins, thereby preventing accumulation of malformed polypeptides. Heat shock proteins also aide in polypeptide transportation through the cytoplasm, and act to refold denatured proteins (Calderwood et al., 2009; Morton et al.,

2009). While HSPs play an important role in the unchallenged cell, their most interesting activity occurs in response to homeostatic disturbances.

Generally speaking, HSPs maintain cellular survivability in the face of stress. “Stress proteins” and “cellular stress response” are commonly used to refer to HSPs and their expression (Locke, 1997). Various stimuli, including ischemia, protein degradation, hypoxia, acidosis, oxidative stress, increased extracellular calcium and energy substrate depletion, prompt the expression of HSPs (Madamanchi et al., 2001; Calderwood et al., 2009; Morton et al., 2009). Elevated levels of HSPs due to one stressor can provide cross-tolerance to additional disturbances of homeostasis (Locke, 1997; Calderwood et al., 2009; Morton et al., 2009). Therefore, adaptation in response to one challenge tends to increase functionality despite subsequent disturbances.

Transcription of HSP mRNA

The HSP response to extracellular stress depends on adequate translation of new stress proteins, and proper transcriptional control of HSP mRNA (**Figure 1-1**). Transcription of HSPs begins with trimerization of the transcription factor heat shock factor 1 (HSF-1) molecules in the cytoplasm, translocation to the nucleus where the homotrimer bindes to heat shock elements (HSE) – short nucleotide sequences located in the promoter region of the HSP-encoding gene – and hyperphosporylation of the HSF-1 trimer to activate transcription (Hensold et al., 1990; Price and Calderwood, 1991; Morimoto et al., 1992; Bruce et al., 1993; Cotto et al., 1996; Xia and Voellmy, 1997; Morton et al., 2009). In unstressed cells, it is thought that HSF1 is bound to constitutively expressed HSP70 and HSP90 molecules found in the cytoplasm. When homeostasis is disturbed, due to extracellular stress or unfolded proteins from the endoplasmic reticulum, HSP70 and HSP90 act to refold denatured proteins, and consequently unbind HSF1 molecules, freeing them to begin the transcription process (Morimoto et al., 1992; Morton et

al., 2009). The resulting production of HSPs will act to repair damaged proteins. When HSP expression reaches a level at which it is able to again bind to free HSF1, gene transcription is halted (Morimoto et al., 1992; Morton et al., 2009).

Figure 1-1

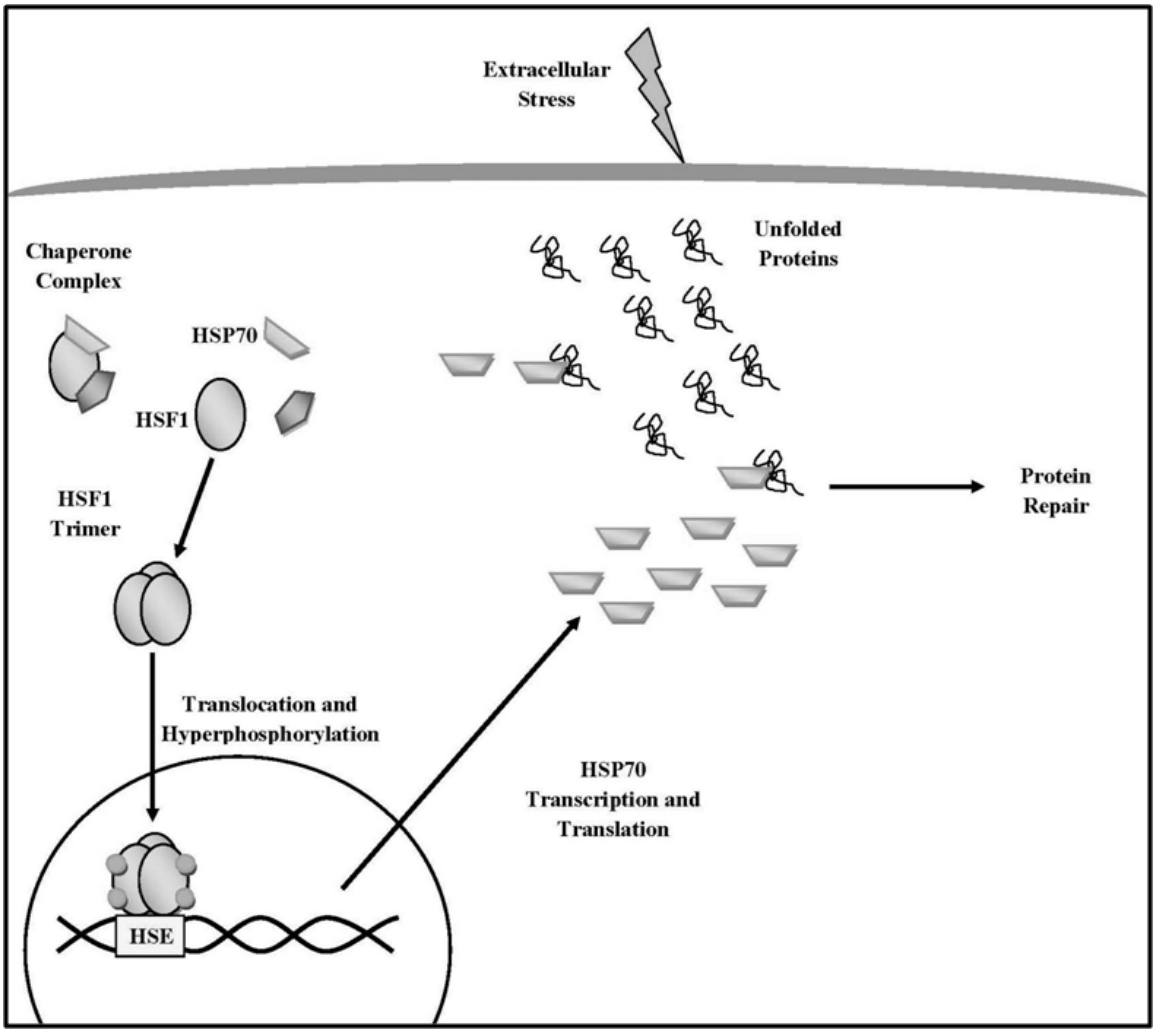


Figure 1-1. HSP70 response to extracellular stress. In response to denatured proteins created by extracellular stress, the chaperone complex consisting of HSF1, HSP70, and HSP90 disassociates. Free HSP70 then binds to unfolded proteins, marking them for repair. HSF1 forms homotrimers and translocates to the nucleus, where it binds to HSE and is hyperphosphorylated, activating *HSP70* transcription, leading to production of HSP70. The induced HSP70 then binds to any remaining unfolded/denatured proteins, contributing to restoration of homeostasis.

Cellular adaptation through the heat shock protein response

It is thought that HSPs promote recovery from stress by binding to misfolded and unfolded proteins, preventing their degradation by marking them for repair when the cellular environment becomes more favorable (Locke, 1997; Calderwood et al., 2009; Morton et al., 2009). Activation of HSPs by non-lethal disturbances of homeostasis results in stress tolerance and cytoprotection against otherwise subsequent lethal exposures to stress-induced molecular damage (Morimoto et al., 1992; Locke, 1997; Calderwood et al., 2009; Morton et al., 2009). This represents a crucial function, especially during periods of hyperphagia or oxidative stress. This mechanism is crucial to cellular survival in spite of further insults, and may be essential for the adaptive response to exercise.

Investigating the heat shock protein response to various homeostatic disturbances, including exercise, can have broad implications. The adaptive response to exercise, specifically the expression of HSPs in order to modulate repair and recovery, illuminates a non-pharmacological approach for improving the quality of life in the aged and unfit. In addition, targeting HSP regulation through nutritional or pharmacological intervention may supplement training regimens and increase quality of life for the aged, and generally enhance adaptive response to environmental stressors, such as challenges to the immune system.

Families of HSPs

Several varieties of heat shock proteins have previously been studied. All members of the HSP family perform housekeeping functions in most tissues, regardless of their specific type. HSPs are classified and named according to molecular mass, ranging in size from 8 – 90 kDa. The smallest member of the HSP family, ubiquitin, is 8 kDa in size, and will bind to damaged or denatured proteins and mark them for degredation (Morton et al., 2009).

HSP70 is the most highly conserved and studied of the heat shock proteins. The cognate isoform of HSP70 is sometime referred to as HSP73, while the inducible form is referred to HSP72. Members of the HSP70 family perform a wide array of functions, including roles in cell signaling pathways, mRNA stabilization and degradation and apoptosis. It goes without saying that HSP70 is an important molecular chaperone in unstressed cells and maintainer of correct protein folding, translocation, repair and degradation following perturbations of homeostasis. HSP70 expression has been shown to increase following pre-conditioning stressors, and after both acute exercise and exercise training (Garramone et al., 1994; Lepore et al., 2000; Liu et al., 2000; Khassaf et al., 2001; Morton et al., 2006).

HSP90 is a chaperone of substrate proteins, such as protein kinases, transcription factors and steroid hormone receptors (Morton et al., 2009). HSP90 constitutively binds to steroid hormone receptors, such as the glucocorticoid receptor, until such a time as the receptor complexes with its client hormone. The hormone-receptor complex then disassociates from its chaperone HSP, and enters the nucleus to initiate transcription. When transcription is terminated, the steroid receptor once again binds to its chaperone, which preserves its structure and position within the cell until the next hormonal signal arrives (Pratt, 1993). Interestingly, impaired recovery from heat shock has been shown to result from HSP90 inhibition, suggesting that interactions among the different members of the HSP family are required for proper cellular recovery and protection from stress (Duncan, 2005). It is worth recalling that HSP70 and HSP90 are involved in auto-regulation of the HSP response through binding of HSF1, and that by inhibiting the response of HSP90 one may reduce HSF1 action on the genome.

Exercise and the HSP response

Exercise has been shown to up-regulate HSP content in tissues collected from various mammal species, however there are limited and sometimes conflicting data concerning HSP response in human skeletal muscle (Morton et al., 2009). Slow myosin containing fibers appear to have higher abundance of HSPs, with elevated protein levels occurring as early as one day following acute exercise and lasting up to a week post exercise (Morton et al., 2006; Tupling et al., 2007; Gupte et al., 2008; Geiger and Gupte, 2011). However, there is no reported difference in basal levels of HSPs among different muscle fiber types, and HSPs are elevated in response to exercise in all fiber types (Tupling et al., 2007). Still, the type of exercise performed and site of sample collection may influence patterns of HSP expression and protein abundance, and studies investigating differential expression of HSPs should be performed with this in mind. Furthermore, some studies focus only on changes in mRNA transcription, which may not fully reflect changes in protein levels after exercise. However, as HSP protein abundance is transcriptionally controlled results of mRNA studies are reported here, with *HSP72* reflecting results quantifying mRNA, while HSP72 will refer to findings from studies which quantified protein.

Rodent studies have shown that acute bouts of exercise have increased HSP72 content in both liver (Salo et al., 1991) and skeletal muscle (Locke et al., 1990; Salo et al., 1991; Skidmore et al., 1995; Hernando and Manso, 1997). In untrained human subjects, following lengthening contractions of the biceps brachii, HSP27 and HSP72 expression was found to have increased 2- and 10-fold, respectively, 48 hours after exercise (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003). Studies involving non-damaging exercise protocols, such as running at the anaerobic threshold for 30-60 minutes, resulted in a 4- to 6-fold increase in *HSP72* levels,

however protein levels were not elevated 2, 3, 8, or 24 hours following exercise (Puntschart et al., 1996; Walsh et al., 2001). Mature male rats subjected to both heat shock and eccentric exercise had lower creatine kinase and mononuclear cell infiltration, and higher HSP72 in skeletal muscle as compared to animals that underwent eccentric exercise alone, indicating that HSP72 may protect muscle cells from damage and facilitate muscle remodeling (Touchberry et al., 2012). The intensity and type of exercise, in the presence or absence of other stressors, may therefore be a determining factor in changes in HSP protein expression.

Adaptations due to training decrease the homeostatic imbalance resulting from acute stress, therefore reducing the overall stress response. Trained muscles have an increased and more rapid HSP response than sedentary muscles to acute exercise in both rodents and humans (Fehrenbach et al., 2000; Gonzalez et al., 2000). In comparison to skeletal muscle of unconditioned men, trained males had higher levels of αB-crystallin and HSP72 (Morton et al., 2008). Trained individuals do not show a stress response to customary exercise, indicating that the adaptive response increases the threshold of homeostatic disruption required to elicit the stress response (Morton et al., 2008). In rats subjected to a single bout of high intensity exercise, HSP72 expression was increased at 6 and 24 hours post-exercise, and had returned to resting levels after two days (Ogata et al., 2009). In the same study, rats undergoing eight weeks of high intensity exercise had a prolonged elevation of HSP72 protein, without an accompanying increase in *HSP72* transcription, for up to two weeks after the cessation of training (Ogata et al., 2009).

Resistance training also increases HSP accumulation. In rats undergoing 4.5 weeks of electrically stimulated stretch-shortening contractions to simulate heavy resistance training, HSP72 and HSP27 abundance increased in both young and aged animals post-training (Murlasits et al.,

2006). Resistance training also increased HSP72 and HSP27 protein accumulation in aged animals, but not to levels comparable to those seen in young animals (Murlasits et al., 2006). Interestingly, increased protein expression was not due to increased *HSP72* transcription in response to training in either the young or aged animals (Murlasits et al., 2006).

The HSP response in equine skeletal muscle

Data on HSP expression in horses is limited. To the author's knowledge, the first record of HSP expression in the horse was part of a larger characterization of the heat stress response in the lymphocytes of various livestock species, which demonstrated that induction of HSP72 does occur in equine tissue (Guerriero and Raynes, 1990). The limited other work investigating HSPs in equine tissue has been carried out in Finnish Standardbreds.

One of the few studies concerning HSP expression in exercising horses investigated whether moderate intensity training, without changes in volume or intensity, changes the expression of HSP72 (Poso et al., 2002). Ten horses, aged 4 -14 years, were exercised three days per week for three months. Horses performed three submaximal 60 minute exercise tests: an initial test after three months of training, a second test two weeks later, and a final test after an additional three weeks. Results indicated that training increased transcription of *HSP72*, albeit only temporarily as mRNA levels returned to baseline within one day, in response to an exercise challenge. It was also observed that peak *HSP72* transcription correlated with peak blood lactate concentration (Poso et al., 2002).

In a second study, 8 trotters, aged 6 – 9 years, were subjected to a modified standard exercise test in order to determine the effect of one bout of moderate exercise on HSP expression (Kinnunen et al., 2005). Muscle biopsies were taken before exercise and four hours post-exercise, and were analyzed for HSP70, HSP90 and HSF1 expression. The bout of acute exercise

did not induce HSF-1 DNA binding activity, and HSF-1 protein expression was not affected by the acute exercise. Expression of HSP72 and HSP90 was not altered at 4 hours post-exercise, however, markers of oxidative stress were elevated (Kinnunen et al., 2005). Analysis of tissue only at four hours post-exercise may account for the lack of any increase in HSP expression, as actual protein expression may not be elevated until several hours into recovery. Also, these were trained animals which most likely had high levels of constitutively expressed HSPs that rapidly attenuated the disturbance in homeostasis caused by exercise, therefore precluding the need for increased HSP translation.

In a third Finnish study, six trained standardbreds, aged 5 – 13 years, were used to study the affect of α-lipoic acid, a known antioxidant, supplementation on HSP72 expression (Kinnunen et al., 2009). Animals underwent a standardized exercise test in order to determine the speed at which V_{La4} occurred for each horse. Based on this data, during the performance test to determine HSP expression, the speed was kept below the anaerobic threshold. Performance tests were completed before and after 5 weeks of antioxidant supplementation. Muscle biopsies were taken at the time of each exercise test; specifically at rest, and at 6, 24 and 48 hours post-exercise. Each horse served as its own control (Kinnunen et al., 2009). Supplementation did not change the expression of constitutive HSP70 at any time point. However, at 24 hours post-exercise HSP72 expression was elevated in response to treatment as compared to control samples. At 48 hours post-exercise there was no difference in HSP72 expression between control and supplemented samples. HSP90 expression was not altered by either acute exercise or antioxidant supplementation (Kinnunen et al., 2009). Although lipoic acid supplementation appeared to transiently increase HSP72 expression, the pattern of HSP expression in untrained and aged horses in response to acute exercise and exercise training remains uninvestigated, and supplementation may have an even more beneficial effect in these groups of animals. This lack

of characterization of the equine HSP response hinders complete exploration of the factors contributing to insulin resistance and age-related decline of function in horses. Only after a more complete understanding of HSPs in metabolically important equine tissues is gained can an accurate assessment of the effectiveness of exercise training, and nutritional or pharmacological treatments, on HSP expression be carried out.

Evidence for a role of HSPs in insulin signaling

Recently there has been increased interest in the role of HSPs in the natural defense and adapative mechanisms that may preserve cellular function despite several pathologies, including cancer, neurodegeneration, cardiovascular disease, senescence and type 2 diabetes (Geiger and Gupte, 2011). As skeletal muscle is capable of up to 75% of glucose uptake mediated by insulin (Geiger and Gupte, 2011), the possibility of inducing the HSP response in order to maintain skeletal muscle functionality represents a tempting target for pharmacological and non-pharmacological intervention aimed at improving health and quality of life through restoration of insulin signaling. Slow-twitch muscle fibers appear to be more sensitive to insulin in rodents (Bonen et al., 1981; James et al., 1986; Song et al., 1999), and have a greater role in whole body glucose uptake in humans (Hickey et al., 1995a; Hickey et al., 1995b; Zierath et al., 1996; Nyholm et al., 1997). As mentioned above, HSP72 appears to have a greater role in type I as compared to type II fibers (Morton et al., 2006; Tupling et al., 2007; Gupte et al., 2008; Geiger and Gupte, 2011), therefore harnessing or enhancing the HSP response in these fiber types may substantially preserve or restore insulin sensitivity and functionality in the aged and obese. The growing body of evidence has led to the hypothesis that increased HSP expression, whether by exercise, nutrition, or pharmacological means, reduces the inflammatory pathways that inhibit insulin signaling, thereby restoring insulin sensitivity(McCarty, 2006; Geiger and Gupte, 2011).

HSP function itself may increase energy demand through increased ATP utilization, serving to maintain mitochondrial function and reduce oxidative stress despite a hypercaloric environment (Geiger and Gupte, 2011).

A cyclical model of insulin resistance involving heat shock proteins has been put forward whereby inflammation causes insulin resistance and impairs insulin signaling, which reduces expression of HSPs, allowing damaged proteins to accumulate while simultaneously removing the anti-inflammatory actions of HSPs, leading to loss of cellular and tissue functionality, contributing to greater inflammation and insulin resistance (Hooper, 2007; Hooper and Hooper, 2009). Low heat shock factor-1, *HSP* levels and HSP expression have been observed in insulin-sensitive tissue of diabetic humans and rats (Bruce et al., 2003; Atalay et al., 2004; Hooper and Hooper, 2009). A sedentary lifestyle, coupled with high caloric intake and elevated level of stress hormones, such as glucocorticoids, impairs insulin signaling, reducing HSP function, and allowing for unmodulated inflammation and insulin resistance (Hooper and Hooper, 2009). This exacerbates the loss of HSP chaperone function, leading to loss of mitochondrial and endoplasmic reticulum function, and increased apoptosis (Hooper, 2007; Hooper and Hooper, 2009). Expression of HSPs prevent activation of inflammatory kinases (Gabai et al., 1997; Li et al., 2008; Hooper and Hooper, 2009) and ameliorate the effects of pro-inflammatory transcription factors (Stice and Knowlton, 2008; Hooper and Hooper, 2009). Reduced basal abundance of *HSP72,* and reduced *HSP72* expression in response to a euglycemic-hyperinsulinemic clamp in skeletal muscle of patients with type 2 diabetes has been demonstrated (Kurucz et al., 2002; Bruce et al., 2003). These studies also demonstrated that levels of *HSP72* correlate with insulin sensitivity in skeletal muscle (Kurucz et al., 2002; Bruce et al., 2003).

Age-related decline in functionality correlates with the failure of cellular and molecular mechanisms of repair and upkeep. As aging occurs, the ability to activate the transcriptional pathways leading to HSP expression is greatly reduced in neural tissue, skeletal and cardiac muscle and the liver. This allows for the aggregation of malformed proteins and reduced cellular function, the hallmark of aging (Calderwood et al., 2009). The decline of HSP activity may be related to reduced HSF1/HSE binding ability, which diminishes transcription of *HSP72*, as has been demonstrated in rat hepatocytes (Heydari et al., 2000). Reduced expression of HSP72 in skeletal muscle has been correlated with individuals with type 2 diabetes (Kurucz et al., 2002; Bruce et al., 2003). In obese, insulin-resistant humans, skeletal muscle HSP72 induction is reduced as well (Chung et al., 2008). In diabetic patients, HSP72 expression in peripheral blood mononuclear cells was decreased by 75% (Burkart et al., 2008). In diet-induced insulin resistance in male rats, myocardial expression of HSP72 was reduced (Ooie et al., 2005). Evidence from aged, insulin-resistant and STZ-induced diabetic rats further supports the link between reduced HSP expression and loss of insulin sensitivity (Atalay et al., 2004; Gupte et al., 2008). In skeletal muscle of rats, glucose uptake and insulin signaling, measured by activation of IRS-1, Akt and Akt substrate of 160kDa (AS160), decreased with age; activation of JNK and GSK-3 increased with age; and expression HSP72 and activation of HSP27 decreased with age (Gupte et al., 2008). However, heat shock protein function can be restored to some degree through exercise training despite insulin resistance. In STZ-induced diabetic rats, it was found that endurance training up-regulates HSP72 expression (Atalay et al., 2004).

Adaptation through the HSP response restores insulin signaling and cellular functionality. Elevating core body temperature to 41.5°C for 15 minutes increases HSP72 expression in skeletal muscle, liver and adipocytes in rodents. This response was blunted in animals on a high-fat diet, but this inhibition can be reversed by repeated heat treatments (Chung et al., 2008).

Heat treatment can increase glucose uptake and up-regulate the insulin signaling pathway in skeletal muscle of both aged rodents and rodents fed a high fat diet (Geiger and Gupte, 2011).

Chronic inflammation in the aged – referred to as inflammaging – in humans and animals, has been characterized by increased pro-inflammatory cytokines, such as TNF-α and IL-6 (Salvioli et al., 2006; Horohov et al., 2010), and reactive oxidative species may underpin the increase in pro-inflammatory cytokines found during aging (Horohov et al., 2010). Abundant reactive oxidative species and decreased antioxidant defenses may contribute to insulin resistance through activation of inflammatory pathways, such as JNK and IKK. Heat treatment has been found to subsequently increase HSP72 and decrease JNK and IKK (Geiger and Gupte, 2011). Exercise training could accomplish the same up-regulation of HSPs in the aged, obese and insulin-resistant, hence counteracting chronic inflammation and the increased presence of reactive oxidative species.

Reduced expression of HSP in aged and insulin-resistant individuals may be caused by inhibition of HSF1, due to chronic inflammation (Geiger and Gupte, 2011). HSP70 over-expression in rats prevents elevated levels of TNF-α and IL-6 in response to an LPS challenge, perhaps through modulation of the IKK/NF-$_{K}$β pathway (Dokladny et al., 2010). Overall, HSP up-regulation can reduce some age-related pathologies, such as decline in muscle function, insulin resistance, damage caused by oxidative stress and chronic inflammation (Escobedo et al., 2004; Broome et al., 2006; Chung et al., 2008; Stice and Knowlton, 2008).

Interactions among HSF1, HSPs and inflammatory mediators

As alluded to above, HSPs, especially members of the HSP70 family, interact with mediators of inflammation. The heat shock response adds a new facet to insulin signaling and insulin resistance (**Figure 1-2**).

Interest in the role of HSPs in restoration of insulin signaling began with a study performed over ten years ago. Increasing the body temperature of patients with type 2 diabetes through hot tub therapy (37.8°C to 41.0°C for 30 minutes per day, 6 days a week, for three weeks) lowered body weight, fasting glucose and glycosylated hemoglobin (Hooper, 1999). At the time the mechanism behind these changes was unknown. In the years since, investigations have revealed multiple ways in which mediators of inflammation and the insulin signaling pathway and heat shock response interact.

Hormone and neuroendocrine signaling may influence expression of HSPs in response to various stimuli. Insulin itself will increase HSP expression through inhibition of GSK-3 by Akt. Disruptions of the insulin signaling pathway will activate GSK-3, which phophorylates serine residues on HSF-1, thereby preventing its tanslocation to the nucleus (Chu et al., 1998; Hooper and Hooper, 2009). Genetic disruption of IGF-1/insulin signaling in mice will disrupt transcription of *HSP72* in many tissues (Swindell, 2009; Swindell et al., 2009). Signaling through adrenergic receptors induced HSP72 expression in brown adipose tissue of mice (Matz et al., 1996). Restraint stress is capable of inducing *HSP72* expression in the adrenal cortex (Blake et al., 1993) and aorta through sympathetic nervous system and hypothalamic-pituitary-adrenal axis signaling, respectively (Udelsman et al., 1991). *HSP72* transcription in the adrenal cortex is dependent on ACTH (Blake et al., 1991b), and suppression or perturbation of hypothalamic-pituitary-adrenal axis signaling will decrease *HSP72* (Udelsman et al., 1994) and HSP72 and HSP27(Gordon et al., 1994). Overall, there appears to be complex endocrine/neuroendocrine signaling interactions that influence the heat shock protein response, and the interaction among HSPs and mediators of inflammation and insulin signaling.

It has been shown *in vitro* that HSF1 has multiple serine residues, which are excellent targets for phosphorylation, and activation of transcription is dependent upon proper phosphorylation (Holmberg et al., 2001; Guettouche et al., 2005). It is indicated that HSF-1 can be deactivated by JNK, thereby reducing HSP production (Park and Liu, 2001; Hooper and Hooper, 2009; Geiger and Gupte, 2011). Prevention of HSF-1 hyperphosphorylation and subsequent binding to HSE can occur due to phosphorylation by JNK, through binding at a small recognition domain, known as the D domain (Dai et al., 2000). However, due to the presence of multiple isoforms of JNK, and their sometimes tissue-specific roles, the relationship between HSF-1 and JNK requires further study. Serine residue phosphorylation of HSF-1 by MAPK/ERK family members and GSK-3 will inhibit transcription of HSP72 *in vitro* (Knauf et al., 1996; Chu et al., 1998) and *in vivo* (Chu et al., 1996; Knauf et al., 1996), which represents a means of repressing HSP over-production during times of cellular growth and protein synthesis. Failure to inhibit GSK-3, such as during insulin resistance will prevent HSP expression, however (Hooper, 2007; Geiger and Gupte, 2011). Mitogen activated protein kinase-activated protein kinase 2 (MK2), a mediator of proinflammatory cytokine signaling and production, phosphorylates the ser-121 residue of HSF-1, effectively deactivating it *in vitro* (Wang et al., 2006a). Overall, inhibition of HSP72 transcription and disruption of insulin signaling, contributing to loss of homeostasis and cellular functionality, occur through constant inflammatory signaling and through the actions of JNK.

Increasing the levels of HSP70 protein in skeletal muscle may prove important to restoring insulin sensitivity in the face of chronic inflammation. Members of the HSP70 family provide a crucial check on the activation of JNK. Activation of JNK and development of insulin resistance in fat-fed mice was prevented by overexpression of HSP72 and weekly heat shock treatments. Furthermore, inhibition of GSK-3 increased HSF-1 and HSP72 levels, decreased JNK activation and improved insulin signaling in ob/ob mice (Chung et al., 2008). In a murine model of

genetically-induced diabetes, induction and overexpression of HSP72 prevented TNF-α-induced phosphorylation of JNK and ER stress in pancreatic β-cells, preserving their function and survival (Kondo et al., 2012). Non-lethal heat treatment increased HSP72 expression in response to subsequent heat stress or other stressful stimuli, and inhibited the activation of JNK *in vitro* (Gabai et al., 1997). Heat treatment of aged rats improved insulin-stimulated glucose uptake and inhibited JNK activation through induction of HSP72 in skeletal muscle (Gupte et al., 2010).

Figure 1-2

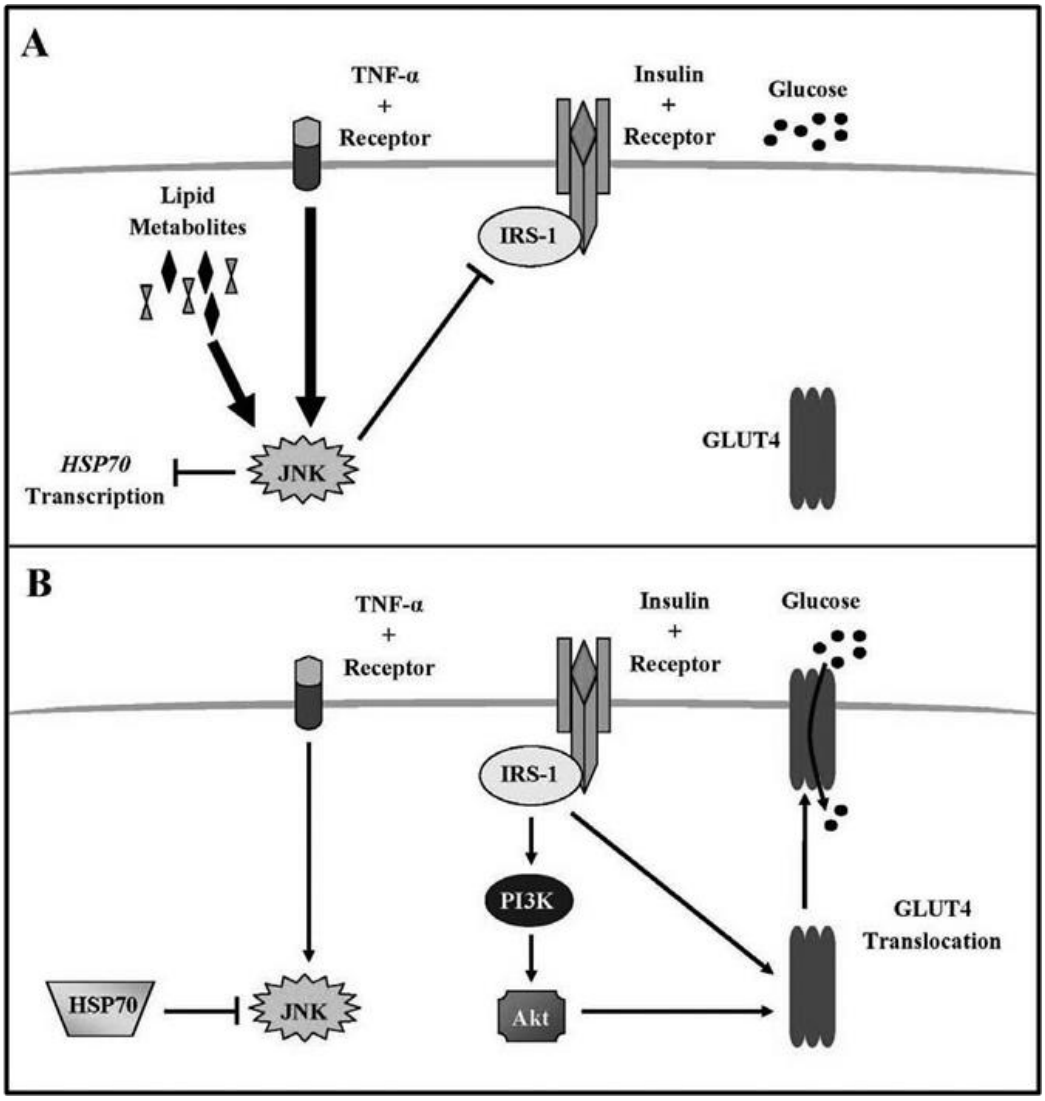


Figure 1-2. Imbalance among inflammatory signaling mediators and HSP70 contributing to insulin resistance and the restoration of insulin signaling through decreased active JNK. (A) Inflammatory cytokines, such as TNF-α, and increased intercellular deposition of lipid metabolites, such as ceramide and diacylglycerol, cause increased activation of JNK. JNK phosphorylates IRS-1, preventing its activation by insulin, thereby preventing GLUT4 translocation to the cellular membrane. JNK also prevents activation of the HSF1 homotrimer, preventing transcription of *HSP70* and subsequent production of HSP70. Thus, damaged and unfolded proteins aggregate, contributing to loss of cellular function. (B) Restoration of HSP70, removal of lipid metabolites and decreased TNF-α signaling through exercise and dietary modification will decrease activation of JNK. Without interference from JNK, IRS-1 is activated by insulin, allowing activation of the PI3K pathway and subsequent translocation of GLUT4 to the cellular membrane.

Reduction of HSP72, concurrent with increased phosphorylation of JNK, has been demonstrated in the skeletal muscle of obese, insulin resistant humans in comparison to healthy subjects (Chung et al., 2008). Increasing body temperature of mice 41.5°C for 15 minutes induced a transient increase in skeletal muscle HSP72, but the HSP72 response to heat treatment was absent in mice fed a high fat diet (Chung et al., 2008). Feeding mice a high fat diet increased JNK phosphorylation in skeletal muscle, as well as causing insulin resistance and elevated fasting glucose and insulin levels. These outcomes were attenuated in mice fed a high fat diet that were subjected to heat treatment once per week for 16 weeks (Chung et al., 2008). Feeding a high fat diet induced mild fasting hyperglycemia and significant fasting hyperinsulinemia in mice, but these effects were absent in transgenic mice overexpressing HSP72 in cardiac and skeletal muscle (Chung et al., 2008). Additionally, the high fat diet induced significant phosphorylation of JNK, and inhibition of AKT in wild type mice, while in HSP72-overexpressing mice the high fat diet did not result in elevated JNK activation or inhibition of AKT (Chung et al., 2008).

In a series of experiments on the relationship between HSP72 and JNK, Park and colleagues (2001) concluded that HSP72 modulates stress-activated signaling through direct inhibition of JNK (Park et al., 2001). An acute heat treatment of 43°C for 20 minutes reduced JNK1 activity following subsequent stress. Also, constitutive overexpression of HSP72 inhibited JNK1 activation through binding. Inhibition of JNK-dependent apoptosis was prevented by HSP72 expression. Conversely, the lack of HSP72 expression in response to mild heat shock led to JNK activation and apoptosis (Park et al., 2001). Male rats fed a high fat diet and subjected to weekly heat treatment (41°C for 20 minutes) in order to induce HSPs experienced improved glucose tolerance and insulin-stimulated glucose transport, and reduced JNK and IKK activation in skeletal muscle when compared to rats fed the high fat diet alone (Gupte et al., 2009b). It

appears as if preservation of the balance between HSP70 and JNK is required for maintenance of homeostasis and insulin signaling.

Induction of the HSP response through pharmacological or nutritional means to counteract chronic inflammation

Hydroxylamine derivatives possibly activate HSP72 through membrane-associate stress signaling and by prolonging the binding of HSF-1 to HSE within DNA (Kurthy et al., 2002; Hargitai et al., 2003; Torok et al., 2003; Kieran et al., 2004; Vigh et al., 2007). Acute administration of the hydroxylamine derivative BGP-15, in the presence of cotreatment with heat, increased both HSF-1 and HSP72 *in vitro* (Chung et al., 2008). Treatment of leptin –deficient (ob/ob) mice with BGP-15 for 15 days resulted in increased HSP72 and prevented phosphorylation of JNK in skeletal muscle, reduced fasting levels of glucose and insulin and increased glucose disposal rate during a hyperinsulinemic euglycemic clamp (Chung et al., 2008).

The antioxidant lipoic acid, when fed to rats on a high fat diet, increased HSP72 levels and decreased JNK activation in skeletal muscle, but supplementation had no effect on chow-fed rats (Gupte et al., 2009a). Lipoic acid treatment of muscle cells in vitro induced expression of HSPs, concurrent with prevention of TNF-α stimulated activation of JNK and IKK (Gupte et al., 2009a). In elderly subjects, daily supplementation of a fermented papaya extract for three months increased serum HSP72 levels and reduced circulating TNF-α and IL-6 (Marotta et al., 2007). Nutritional intervention may therefore be an avenue through which stimulation of HSPs can prevent dietary and inflammatory-induced insulin resistance.

Summary and conclusions

Insulin resistance, as a factor in type 2 diabetes and the metabolic syndrome, creates a grave health and economic threat to the aging global population. The insulin signaling pathway, etiology of insulin resistance and the interplay among heat shock proteins and inflammatory mediators, represents a complex system of interacting molecules in metabolically important tissues. Heat shock proteins are crucial to maintaining cellular homeostasis and for the adaptation to stress. Horses represent an untapped resource to study the modalities of insulin resistance, and to investigate potential countermeasures to restore insulin sensitivity through harnessing the heat shock response.

No studies to date have investigated the relationship between HSPs and insulin resistance in horses. Understanding HSPs and molecular mediators of inflammation and insulin signaling in aged and untrained equine animals will open the door to developing training and nutritional strategies to restore functionality to the aging equine population, and provide insight into human metabolic disease. Unlike rodents, which are typically sacrificed at various ages during the course of an experiment, horses can be sampled serially along their lifespan. This would allow a unique model to study the role of HSPs and molecules involved in inflammation and insulin signaling under various dietary and environmental conditions, as well as providing opportunities to study the interactive effects of aging and exercise on these processes. The horse is an excellent model of exercise physiology as control of cardiovascular function, via the sympathetic and parasympathetic divisions of the autonomic nervous system, and thermoregulation, via evaporative cooling, are similar to human physiology. Like humans, the population of aged horses is expanding, with similar age- and obesity-related health challenges. Profiling the heat shock protein response and the role of mediators of insulin-signaling and

inflammation in aged and untrained horses will give valuable insight to the adaptive response to exercise. As the equine population increases in age and declines in functionality, potential increases of HSPs through training regimens, nutraceutical supplementation, or pharmacological intervention may reverse age-and obesity-related disorders and disease.

Using equids as a model for heat shock protein response and insulin resistance creates an opportunity for study in highly trainable animals in order to ask several basic questions. First, is there an additive effect of exercise, heat treatment and nutritional or pharmacological intervention on expression of HSPs and restoration of insulin sensitivity in mammals? What are the mechanistic interactions between HSPs, stress kinases and insulin-signaling intermediates in exercising animals, and how does this compare to findings in humans and other models? If increased expression of HSPs can benefit the aged and insulin resistant, how can we most effectively up-regulate their expression? Heat shock proteins are crucial for an effective adaptive response to disturbances of homeostasis in mammalian tissues, and may present a therapeutic avenue for treatment of age- and obesity-related disease, such as insulin resistance in humans and horses.

Chapter 2.

Effects of age and submaximal exercise on physiological markers of stress and inflammatory cytokines in Standardbred mares

Abstract

We hypothesized that the cortisol response to acute exercise, markers of oxidative stress and the expression of inflammatory cytokines are altered by aging and in response to acute submaximal exercise. Young (n=6; 5.5 ± 2.8 years) and aged (n=6; 22.6 ± 2.25 years) unconditioned Standardbred mares underwent an acute submaximal exercise test which consisted of running at the velocity at which blood lactate reaches 4 mmol/L until fatigue. Blood samples were collected and analyzed for plasma cortisol and malondialdehyde concentrations, and for cytokine gene expression pre- and post-exercise. Gluteus medius biopsies were obtained for analysis of cytokine gene expression in skeletal muscle pre- and at 0, 4, 24 and 48 hours post-exercise. Plasma cortisol concentration was measured via radioimmunoassay. Plasma malondialdehyde concentration was determined via TBARS assay. Blood and skeletal muscle cytokine expression was determined via RT-PCR. Data were analyzed for main effects using a two-way ANOVA for repeated measures to evaluate the differences due to age and exercise. Post-hoc comparisons of means were conducted using Student-Neuman-Keuls for pair wise multiple comparisons where appropriate. The null hypothesis was rejected when $P \leq 0.05$. Acute submaximal exercise increased plasma cortisol concentration in both young and aged mares, and the duration of the post-exercise rise in cortisol was altered in aged horses. Plasma malondialdehyde concentration, and expression of *TNF-α* and *IL-6* were unchanged in blood and muscle regardless of age or exercise. Exercise increased *IL-1β* expression in whole blood of young and aged mares, with young mares having greater exercise-induced expression at 2 ($P<0.001$) and 4 ($P=0.019$) hours post-exercise. There was no change in *IL-1β* expression in skeletal muscle, regardless of age or exercise. The age-related changes in cortisol and *IL-1β* expression following acute submaximal exercise can have implications for energy homeostasis and the adaption to such disturbances at a cellular and whole animal level.

Introduction

Aging represents a growing challenge to the equine industry, with many horses over the age of 20 involved in competition or reproduction (Malinowski et al., 1997). Aging in horses is associated with decline in aerobic capacity and exercise capability, altered immune function and chronic inflammation (McKeever and Malinowski, 1997; Horohov et al., 1999; Horohov et al., 2010; Walker et al., 2010), and compromised thermoregulation in response to acute exercise (McKeever et al., 2010). Endocrine function of horses is also altered with age. Plasma cortisol concentrations and the diurnal levels of cortisol are altered in aged horses (Horohov et al., 2002), and the exercise-induced increase of plasma cortisol is blunted in aged horses (Horohov et al., 1999; Malinowski et al., 2006). Functionality of the pituitary and adrenal glands is also altered in aged horses (Liburt et al., 2013). Taken together, these findings indicate that the hypothalamic-pituitary-adrenal axis is altered with aging in horses, with implications for energy homeostasis, immune function, exercise capacity, and adaptation to disturbances in homeostasis.

Of particular concern, aged horses experience disruptions in glucose homeostasis relating to insulin resistance (IR), which is implicated in loss of muscle mass, increased adiposity, declining immune function and laminitis (Frank et al., 2006). Loss of insulin sensitivity in horses has also been studied in correlation with age and obesity. Aging and increased adiposity contribute to reduced insulin sensitivity and increased production of pro-inflammatory cytokines in horses (Malinowski et al., 2002; Vick et al., 2007; Vick et al., 2008; Adams et al., 2009). Aerobic training can partially reverse age-related decline in performance measures, endocrine and immune function, and insulin sensitivity in horses (Betros et al., 2002; Malinowski et al., 2002;

Malinowski et al., 2006). However, the molecular mechanisms behind the adaptive response to exercise conditioning in horses require further research.

The objective of this study was to determine the physiological response to a single bout of submaximal exercise in young and aged horses. Prior studies of aged horses have used strenuous high intensity exercise, and have not investigated the effects of a single bout of submaximal exercise. Understanding the effects of submaximal exercise will help to elucidate the adaptive mechanisms at play during exercise condition. Horses were first assessed for aerobic capacity, and then underwent an acute running exercise session at the velocity at which blood lactate reaches 4 mmol/L (V_{LA4}), which is historically associated with a level of sustained exertion without fatigue-related injuries in horses and humans (Bourgela et al., 1991). We hypothesized that the cortisol response to acute exercise, markers of oxidative stress and the expression of inflammatory cytokines are altered by aging and in response to acute submaximal exercise.

Materials and Methods

Animals

Twelve unconditioned Standardbred mares were separated into a young group (n=6; average age = 5.5 ± 2.8 years) and an aged group (n=6; average age = 22.6 ± 2.25 years). Overall, Standardbreds have low genetic diversity and high inbreeding coefficients (McCue et al., 2012; Petersen et al., 2013), therefore a random sampling of young and aged mares was deemed to be an appropriate model for this experiment. Mares were housed in two-acre drylots and given *ad libitum* hay, water, and mineral blocks. Feed was removed before exercise and sample collection. All mares were previously acclimated to the equine exercise physiology laboratory and procedures. None of the mares exhibited phenotypic signs of pituitary pars intermedia

dysfunction or equine metabolic syndrome, such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). All horses were of similar body weight and body condition. This experiment took place during the early spring, but mares did not exhibit any signs of estrus, indicating that the study took place within the seasonal anestrous period. All methods and procedures used in this experiment were approved by the Rutgers University Institutional Animal Care Review Board.

Exercise Tests – Incremental exercise and acute submaximal exercise tests

Each mare's maximal aerobic capacity (VO_{2max}) and velocity at which blood lactate reaches 4 mmol/L (V_{LA4}) was determined by completing an incremental exercise test on a Sato-I high - speed treadmill. The treadmill started at 4 m/s for 1 min, increased to 6 m/s, and subsequently increased 1 m/s every 60 seconds until fatigue. An indirect open-flow calorimeter measured VO_{2max}, which was defined as the point when there was no further increase in VO_2 despite increases in speed.

After completion of the incremental exercise test, each horse completed an acute submaximal exercise test which consisted of running at the velocity corresponding with V_{LA4} until fatigue. Every testing day three horses completed the exercise tests and three horses served as standing controls. Four weeks after the acute submaximal exercise tests horses were crossed-over; where previously exercised mares became standing controls and previously standing control mares completed the acute submaximal exercise tests.

Sample Collection

Thirty minutes before the first exercise test a catheter was inserted into each mare's jugular vein using local lidocaine anesthesia. Whole blood was collected from the catheters with EDTA

and lithium heparin tubes for plasma analysis before exercise at -30 min and -1 min, every 5 min during exercise, directly after exercise, and 30 min, 60 min, 2 hr, 4 hr, 24 hr, and 48 hr post exercise. Whole blood was collected via venipuncture with PAXgene™ blood RNA tubes for mRNA analysis at -30 min, and 0 min, 2 hr, 4 hr, 24 hr, and 48 hr post exercise, and were stored at -80 °C until analyzed. Blood samples were also collected at the same time points for standing control mares. All EDTA and lithium heparin samples were kept on ice and centrifuged at 3000 x g for 10 minutes at 4°C. Plasma samples were immediately measured for lactate, and were stored at -80 °C for further analysis. Plasma from EDTA tubes was used for Cortisol Radioimmunoassay (RIA) and Thiobarbituric Acid Reactive Substances (TBARS) assay. Plasma collected in lithium heparin tubes was used for Insulin RIA.

Additionally, rectal temperatures were determined at -1 min pre-exercise and at 0 min post-exercise in all mares. Rectal temperature measurements were taken at the same time points in standing control mares.

Percutaneous skeletal muscle biopsies of the gluteus medius were obtained in a time-matched manner from exercised and standing control horses via Bergström biopsy needle at a site one-third the distance along a line running from the tuber coxae to the root of the tail (Lindholm and Piehl, 1974), 30 minutes prior to the bout of acute exercise, immediately following exercise, and at 4, 24 and 48 hours into recovery. Biopsies were obtained from a depth of 8 cm. Samples were immersed in RNALater (Qiagen, Valencia, CA) and stored first at 4 °C for 24 hours and then at -80 °C until analysis for gene expression via RT-PCR.

Assays and sample measurement

Packed cell volume (PCV) and plasma total protein concentration were measured in duplicate using the microhematocrit technique and refractometry, respectively. Plasma lactate

concentration was measured in duplicate via enzyme-electrode interface (ABL 800 Flex, Radiometer America, Westlake, OH).

Plasma cortisol was measured via RIA using a commercially available kit (ImmuChem Cortisol, MP Biomeicals, Solon, OH) previously validated in horses(Freestone et al., 1991). Samples were counted for 1 min in a gamma counter (Packard Instrument Co, Meridan, CT). Within assay coefficient of variation for cortisol was 5.35%.

Plasma malondialdehyde (MDA) was determined through lipid peroxidation quantification via measurement of TBARS using a commercially available kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI). Lipoprotein fractions were acid precipitated from plasma samples and samples were loaded in duplicate on a 96-well plate. Absorbance was measured at 540 nm and MDA values were calculated using the formula: MDA (μM) = [(Corrected Absorbance – y-intercept)/Slope], where corrected absorbance was the absorbance value of the standard subtracted from all sample absorbance values, and the y-intercept and slope were obtained from the MDA standard curve.

Cytokine quantification

Quantification of *TNF-α*, *IL-1β* and *IL-6* expression in whole blood and gluteus medius skeletal muscle was completed in duplicate via RT-PCR as previously described (Breathnach et al., 2006; Adams et al., 2009; Liburt et al., 2012). Whole blood total RNA was isolated using spin columns (PAXgene Blood RNA Kit IVD, Qiagen, Valencia, CA) according to manufacturer's instructions.

Skeletal muscle biopsies were homogenized in a cell lysis buffer (RNA-Stat60 Reagent, Tel-Test INC., Friendsweed, TX). Sterile zirconium oxide beads were used to disrupt the tissue in a mixing mill instrument (Retsch MM301 Mixing Mill, Clifton, NJ). Total RNA was isolated from the

samples using chloroform extractions, isopropanol precipitation and ethanol wash, according to manufacturer's instructions. Sampling of skeletal muscle concurrently with peripheral blood could potentially lend clues to patterns for cytokine expression on a whole-animal scale.

Quantitative and qualitative parameters of the RNA preparations were assessed using a BioTek Epoch (Thermo Fisher Scientific, Wilmington, DE) spectrophotometer. In all cases, optical density (OD) $_{260/280}$ ratios were greater than 1.0 and RNA yields were greater than 29 ng/µl. RNA (1 µg) was reverse transcribed into cDNA in an 80 µl reaction containing a master mix of 16 µl of avian myeloblastosis virus (AMV) buffer (5x), 16 µl of $MgCl_2$, 4µl of dNTP, 1 µl of RNAasin, 1 µl oligo deoxythymine (dt) and 0.5 µl of AMV reverse transcriptase (Promega, Madison, WI). Cytokine-specific cDNA was then amplified and quantified by real-time PCR (ABI Systems 7900 Fast Real-Time PCR System, Foster City, CA) using primers based on sequences for equine cytokines and *β-glucuronidase* (β-gus) (Breathnach et al., 2006). Intron-spanning primers and 6-carboxyfluorescein (FAM)-labelled probes for equine cytokines and *β-gus*, provided as Assays-on-Demand kits (ABI, Foster City, CA), were added to 10 µl reactions in 384-well plates containing the 5 µl Bioline SensiFAST™ Probe Hi-ROX Mix (Bioline USA Inc, Taunton, MA), 0.5 µl of primer-probe, and 4.5 µl of cDNA. The following PCR conditions were employed: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected using β-gus as an internal control for each sample (Breathnach et al., 2006). Relative differences in cytokine mRNA expression resulting from exercise were determined by relative quantification (RQ). RQ provides accurate comparison between the initial levels of target cDNA in a sample without requiring that the exact copy number be determined. The RQ is calculated using the

equation $RQ=2^{-\Delta\Delta Ct}$ where ΔΔCt = ΔCt (sample) – ΔCt (Calibrator), and ΔCt = Ct gene of interest – Ct housekeeping gene (Livak and Schmittgen, 2001). The pre-exercise samples for each group of horses (young and aged) were averaged and the group average used as the calibrator for subsequent calculation of RQ and reflects the change in cytokine gene expression post-exercise relative to the calibrator for its respective group. Negative controls included template negative samples and genomic DNA controls. In this methodology, hematocrit was not standardized and white blood cells were not counted. Whole blood contained, by definition, all red and white blood cells present, and expression was normalized to the housekeeping gene to account for variations between samples.

Statistical analysis

Data were analyzed for main effects using a two-way ANOVA for repeated measures to evaluate the differences between young and aged mares and in response to exercise for physiological parameters, plasma cortisol and MDA concentrations, and cytokine expression in whole blood and skeletal muscle (SigmaStat 3.1, Systat Software, San Jose, CA). Outliers were identified via Grubb's test and were excluded from statistical analysis when $P<0.001$. Post-hoc comparisons of means were conducted using Student-Neuman-Keuls for pair wise multiple comparisons, where appropriate. Linear regression analysis was used to determine correlations between factors, where appropriate. The null hypothesis was rejected when $P\leq0.05$.

Results

Body condition and incremental exercise test

Prior to the start of the study, young and aged mares were assessed for body weight, and an incremental exercise test to fatigue was performed to determine maximal oxygen uptake

(VO_{2max}) and velocity at which blood lactate reaches 4 mmol/L (V_{LA4}). There was no significant difference in body weight between age groups (*P* >0.05) (**Table 2-1**). While not significantly different (P=0.055), aged mares displayed lower maximal oxygen uptake (VO_{2max}) and young mares had a higher V_{LA4} than aged mares (*P*=0.003) (**Table 2-1**).

Table 2-1

	Young (n=6)	**Aged (n=6)**
Body Weight (Kg)	459 ± 13	478 ± 21
VO_{2max} (ml/Kg/min)	129 ± 6	111 ± 7
V_{LA4} (m/s)	7 ± 0.4[a]	5 ± 0.4[b]
Different superscripts (a, b) denote differences between age groups.		

Table 2-1. Mean ± s.e. body weight and results of incremental exercise tests.

Physiological response to acute submaximal exercise

Figure 2-1 displays physiological responses to acute submaximal exercise. On days of the submaximal exercise tests, young mares exercised for an average of 1024 ± 133.52 seconds while aged mares exercised for an average of 872 ± 64.61 seconds, however there was no significant difference in running time between groups (*P*>0.05). Both young and aged mares had elevated PCV (*P*<0.001) and plasma total protein (*P*<0.001) at 0 minutes post exercise, but values returned resting levels by 30 minutes post-exercise. There was no effect of age on either PCV or plasma total protein in exercised or control horses (*P*>0.05). Rectal temperature was not changed in young or aged standing control horses (*P*>0.05), but was significantly elevated in response to exercise (*P*<0.001). There was no difference in rectal temperature between exercised young or exercised aged horses (*P*>0.05).

Displayed in **Figure 2-2** are plasma lactate concentrations in response to submaximal exercise. Both young and aged mares had plasma lactate concentrations above 4 mmol/L at completion of the acute submaximal exercise test. Plasma lactate concentration peaked in both young (n=6)

and aged (n=6) mares at the 0 minutes post-exercise (P<0.001). Plasma lactate concentration remained elevated in young and aged mares at 30 (young, P<0.001; aged, P<0.001) and 60 (young, P=0.043; aged P=0.001) minutes post-exercise. Young mares had a higher peak lactate concentration at 0 minutes post-exercise than aged mares (P<0.001). Plasma lactate concentrations at 30 minutes post-exercise were also significantly different between aged groups (P=0.023). There was no change in plasma lactate in either young or aged control horses (P>0.05).

Taken together, these data indicate that a prolonged bout of submaximal exercise was sufficient to cause body temperature changes and fluid shifts in both groups of mares, and that the exercise protocol was a significant physiological challenge.

Plasma cortisol and MDA

RIA analysis of plasma showed that cortisol was elevated at 0 minutes post-exercise (young P=0.01; aged P=0.009), and peaked at 30 minutes post-exercise in both young and aged mares (P<0.001). However, there was no difference between age groups at 30 minutes post-exercise (P>0.05). Cortisol remained elevated in young mares at 60 minutes (P<0.001) and 2 hours (P=0.007) post-exercise, but not in aged mares (**Figure 2-3**).

Exercise increased cortisol area under the curve (AUCc) as compared to standing controls in young mares (P=0.003) and aged mares (P=0.023). In response to exercise, young mares had a mean 65.35% greater AUCc than aged mares (P=0.019). There was no difference in AUCc between young and aged standing controls (P>0.05).

Plasma MDA concentration was determined via TBARS assay. Neither age nor exercise significantly altered plasma MDA concentrations (P>0.05) (**Figure 2-4**).

Figure 2-1. Physiological response to acute submaximal exercise. Mean ± s.e. packed cell volume (PCV) (2-1.A), plasma total protein (2-1.B) and rectal temperature (2-1.C) in young (n=6) and aged (n=6), exercised and standing control mares. PCV and plasma total protein data points represented are those collected from before exercise or standing control treatments (-30 and -1 min), and at 0, 30, 60 min, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Rectal temperature was measured before exercise or control (-1 min) and at 0 min post-exercise or standing control.

Figure 2-1

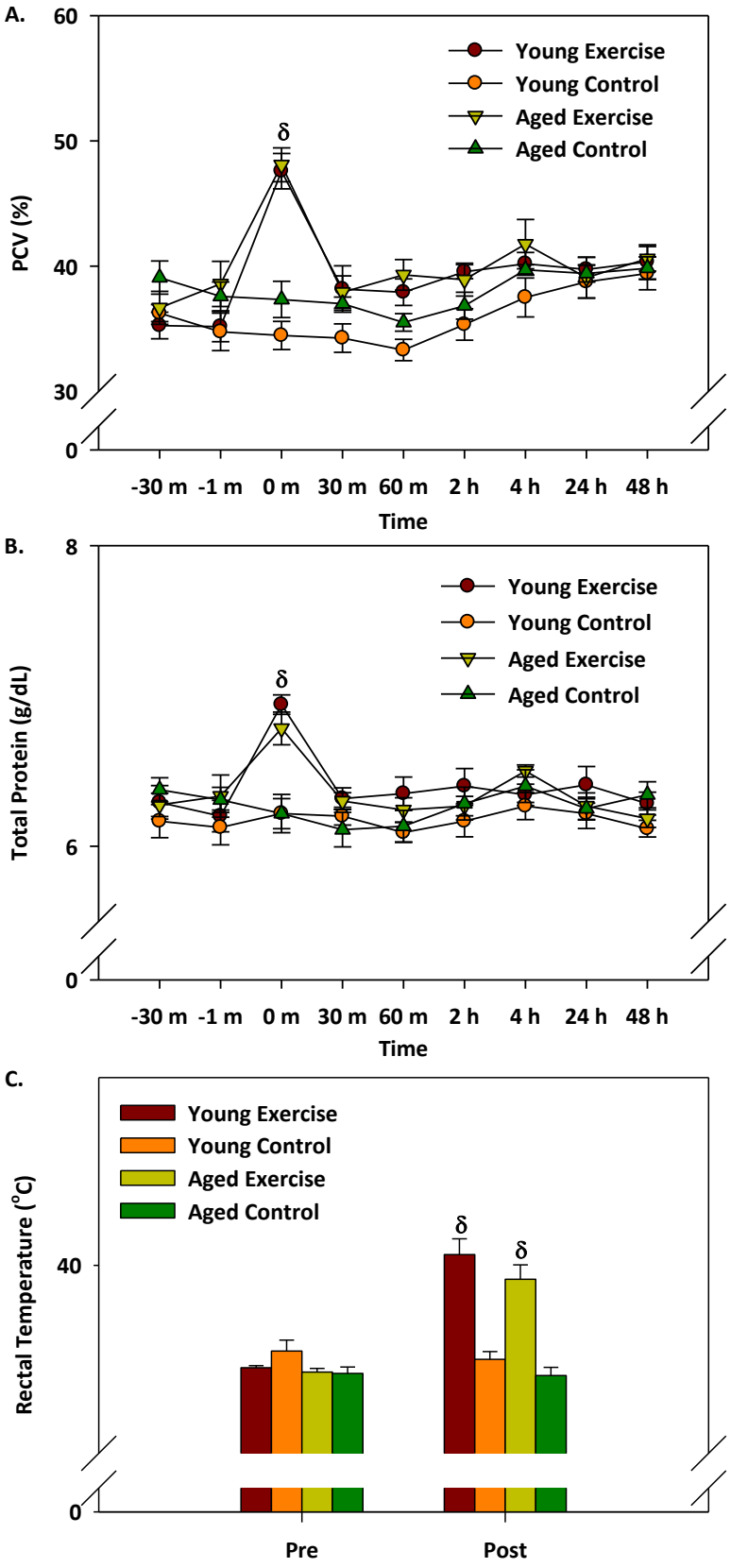

A.
PCV (%)
60
50
40
30
0
δ
Young Exercise
Young Control
Aged Exercise
Aged Control
-30 m
-1 m
0 m
30 m
60 m
2 h
4 h
24 h
48 h
Time
B.
Total Protein (g/dL)
8
6
0
δ
Young Exercise
Young Control
Aged Exercise
Aged Control
-30 m
-1 m
0 m
30 m
60 m
2 h
4 h
24 h
48 h
Time
C.
Rectal Temperature (°C)
40
0
Young Exercise
Young Control
Aged Exercise
Aged Control
δ
δ
Pre
Post

Figure 2-2

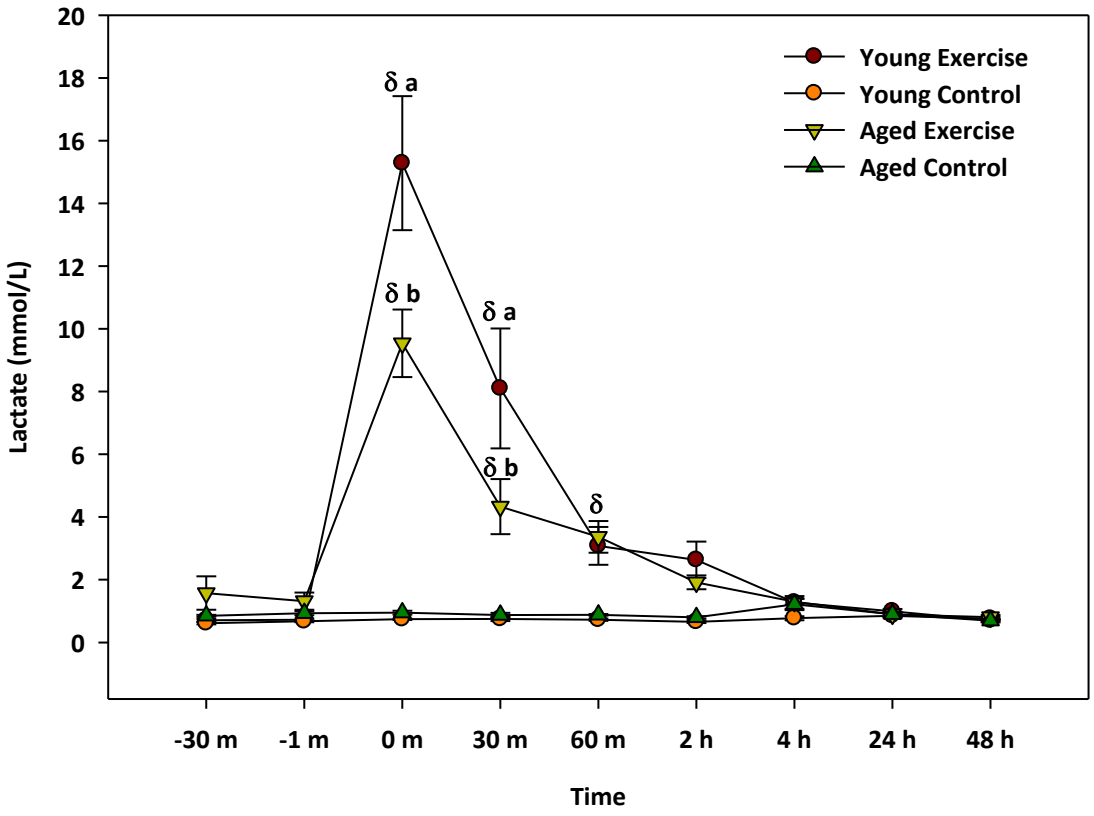


Figure 2-2. Mean ± s.e plasma lactate concentrations in response to acute submaximal exercise. Plasma lactate concentrations in young (n=6) and aged (n=6 mares in response to acute submaximal exercise or standing control. Data points represented are those collected from before exercise or control treatments (-30 and -1 min), and at 0, 30, 60 min, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 2-3

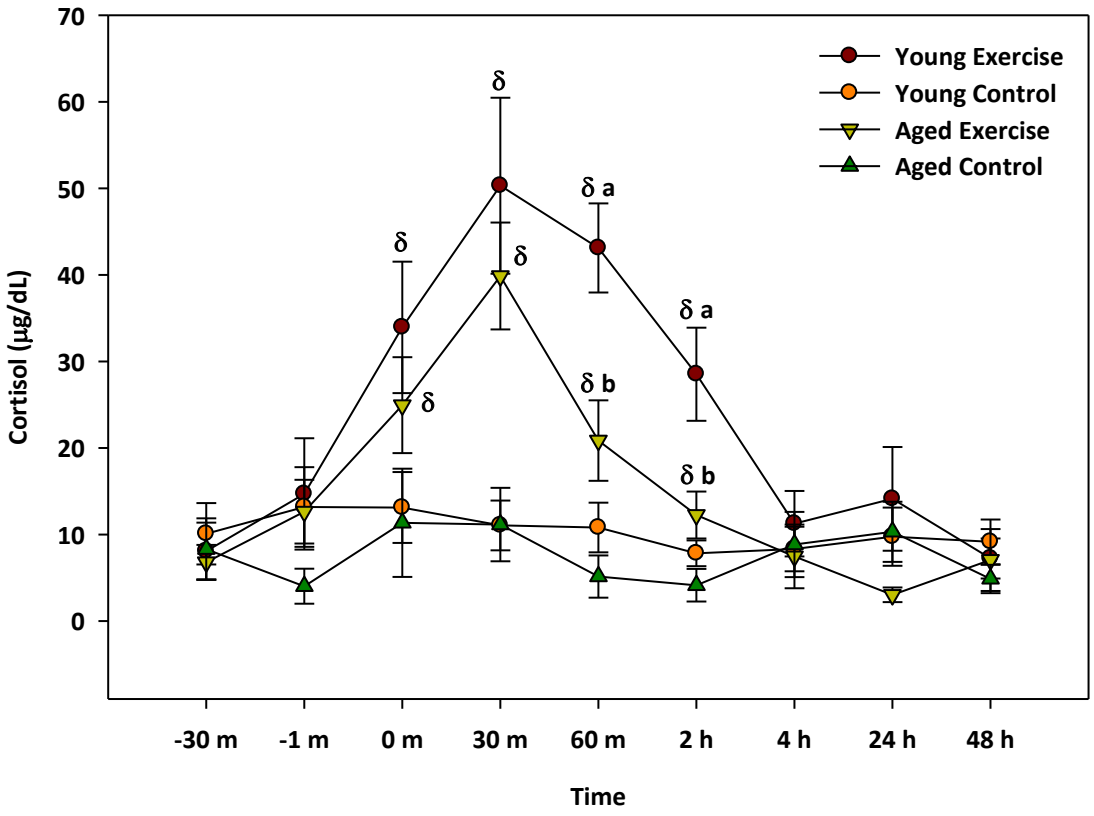


Figure 2-3. Mean ± s.e. plasma cortisol concentrations in response to acute submaximal exercise. Plasma cortisol concentrations in young (n=6) and aged (n=6 mares in response to acute submaximal exercise or standing control. Data points represented are those collected from before exercise or control treatments (-30 and -1 min), and at 0, 30, 60 min, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 2-4

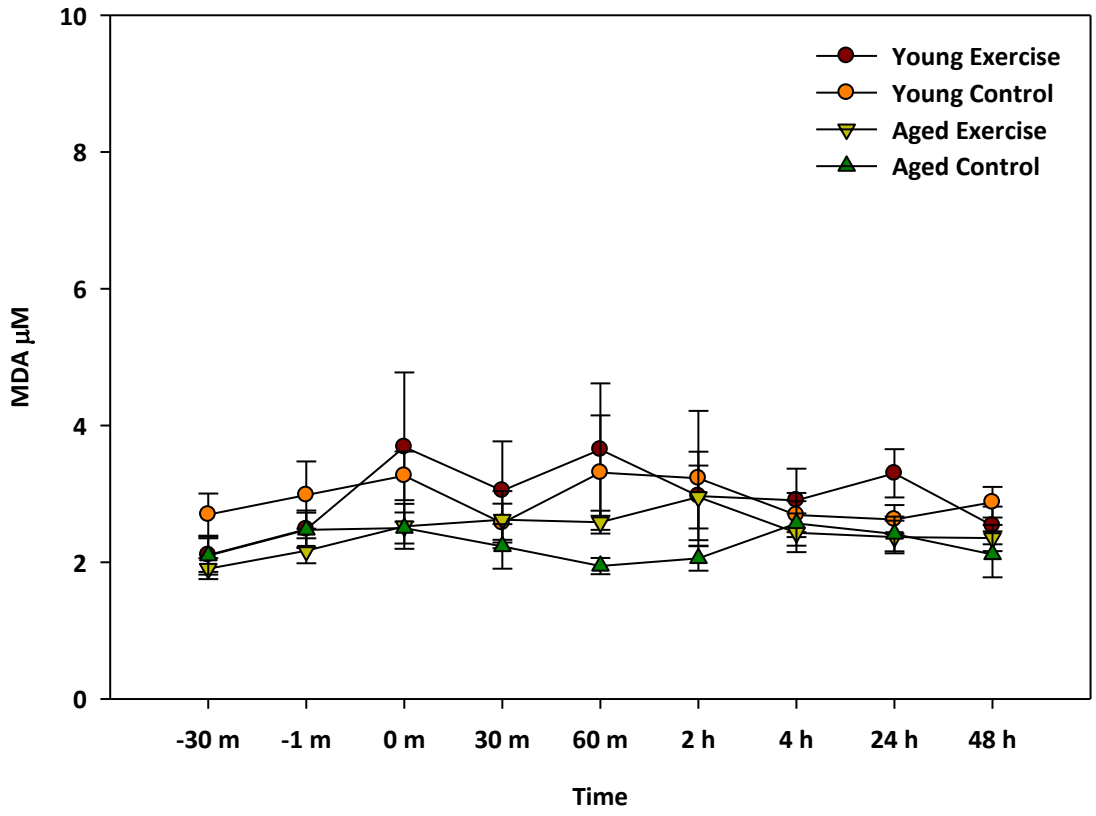


Figure 2-4. Mean ± s.e plasma malondialdehyde concentrations in response to acute submaximal exercise. Plasma malondialdehyde concentrations in young (n=6) and aged (n=6 mares in response to acute submaximal exercise or standing control. Data points represented are those collected from before exercise or control treatments (-30 and -1 min), and at 0, 30, 60 min, 2, 4, 24 and 48 post-exercise or standing control.

Whole blood cytokine expression

Cytokine expression in whole blood in response to acute submaximal exercise is shown in **Figure 2-5**. There was no change in whole blood expression of *TNF-α* in young or aged horses at any time post-exercise ($P>0.05$). Additionally, there was no difference within or between young and aged standing controls for expression of *TNF-α* ($P>0.05$).

Exercise increased *IL-1β* expression in whole blood of young and aged horses at 2 hours (young, $P<0.001$; aged, $P=0.007$) and 4 hours (young, $P=0.014$; aged, $P=0.019$) post-exercise. In young horses, *IL-1β* expression increased 5.5- and 4.2-fold at 2 and 4 hours post-exercise, respectively, as compared to rest. In aged horses, *IL-1β* expression increased 2.6- and 2.3-fold at 2 and 4 hours post-exercise, respectively. Young exercised horses had greater *IL-1β* expression than aged horses at 2 hours ($P<0.001$) and 4 hours ($P=0.019$) post-exercise. There was a 2.2- and 1.9-fold difference in *IL-1β* expression between young and aged horses at 2 and 4 hours post-exercise, respectively. There were no differences within or between young and aged standing controls ($P>0.05$).

There were no changes of *IL-6* expression due to age or acute exercise ($P>0.05$), and there were no changes of *IL-6* expression in young or aged standing controls ($P>0.05$).

Skeletal muscle cytokine expression

Neither age nor acute submaximal exercise altered expression of *TNF-α*, *IL-1β* or *IL-6* in gluteus medius skeletal muscle in any samples collected during the present study ($P>0.05$) (**Figure 2-6**).

Regression analysis

Linear regression analysis revealed no correlation between plasma cortisol concentration and *IL-1β* expression following exercise in either aged ($R=0.05$; $P>0.05$) or young mares ($R=0.02$;

P>0.05). Additionally, no correlation was found between plasma lactate concentration and *IL-1β* expression following exercise in either aged (R=0.04; *P*>0.05) or young horses (R=0.12; *P*>0.05). Finally, there was a significant correlation between plasma lactate and plasma cortisol concentrations following exercise in both aged (R=0.45; *P*<0.001) and young mares (R=0.5; *P*<0.001).

Figure 2-5. Cytokine expression in whole blood in response to acute submaximal exercise. Mean ± s.e. relative quantity values for expression of *TNF-α* (2-5.A), *IL-1β* (2-5.B) and *IL-6* (2-5.C) in whole blood of young (n=6) and aged (n=6) mares in response to acute submaximal exercise or standing control. Data points represented are those collected from horses before exercise or control treatments (-30 and -1 min), and at 0, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 2-5

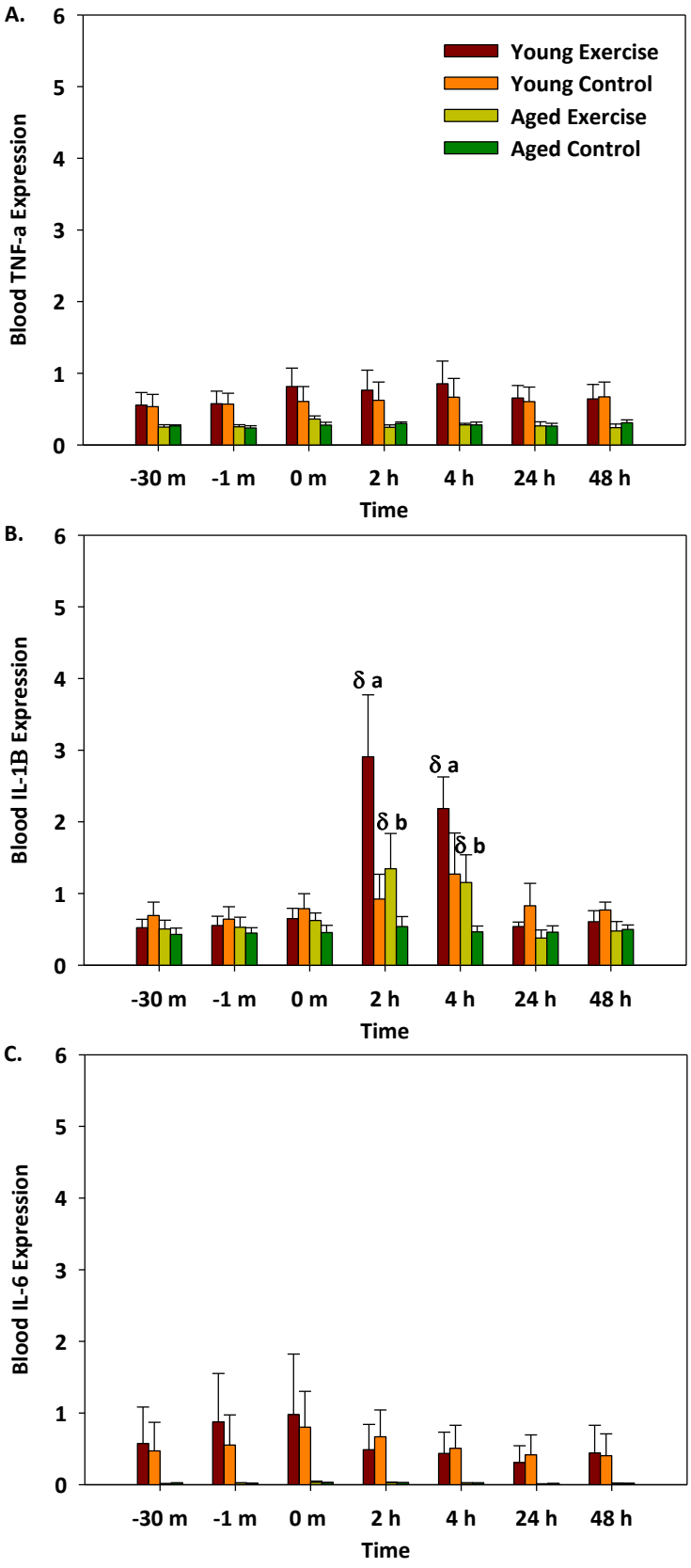
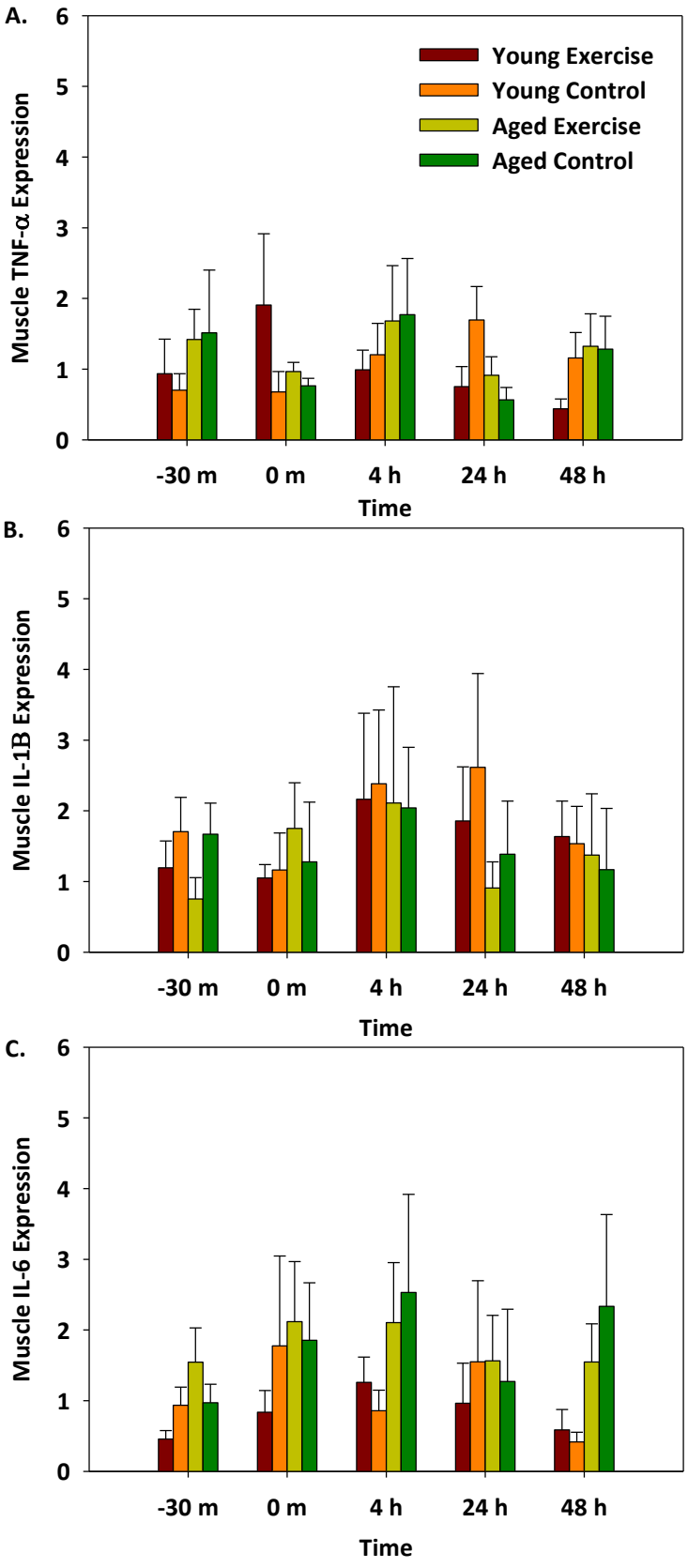

A.
Blood TNF-a Expression
Young Exercise
Young Control
Aged Exercise
Aged Control
0
1
2
3
4
5
6
-30 m
-1 m
0 m
2 h
4 h
24 h
48 h
Time
B.
Blood IL-1B Expression
δ a
δ b
δ a
δ b
0
1
2
3
4
5
6
-30 m
-1 m
0 m
2 h
4 h
24 h
48 h
Time
C.
Blood IL-6 Expression
0
1
2
3
4
5
6
-30 m
-1 m
0 m
2 h
4 h
24 h
48 h
Time

Figure 2-6. Cytokine expression in skeletal muscle in response to acute submaximal exercise. Mean ± s.e. relative quantity values for expression of *TNF-α* (2-6.A), *IL-1β* (2-6.B) and *IL-6* (2-6.C) in gluteus medius of young (n=6) and aged (n=6) mares in response to acute submaximal exercise or standing control. Data points represented are those collected from horses before exercise or control treatments (-30 min), and at 0, 4, 24 and 48 post-exercise or standing control.

Figure 2-6


A.
Muscle TNF-α Expression
Young Exercise
Young Control
Aged Exercise
Aged Control
0
1
2
3
4
5
6
-30 m
0 m
4 h
24 h
48 h
Time
B.
Muscle IL-1B Expression
0
1
2
3
4
5
6
-30 m
0 m
4 h
24 h
48 h
Time
C.
Muscle IL-6 Expression
0
1
2
3
4
5
6
-30 m
0 m
4 h
24 h
48 h
Time

Discussion

This study tested the effects of an acute bout of submaximal exercise consisting of running at V_{LA4} on the physiological response to exercise in young and aged horses. Observations for variables such as packed cell volume, plasma total protein and rectal temperature indicate that both young and aged mares underwent a significant physiological challenge. Exercise-induced splenic contraction in horses rapidly increases PCV of circulating blood in proportion to work load until complete mobilization of red blood cells occurs (Persson, 1967; Persson et al., 1973). Both PCV and plasma total protein have been used previously as a method of assessing fluid shifts in response to acute exercise in horses (McKeever et al., 1993b, a). Taken together, increases in PCV, plasma total protein and rectal temperature indicate that both young and aged horses in the current study underwent a comparable physiological challenge during exercise to fatigue at V_{LA4}.

Although all horses exercised at V_{LA4}, plasma lactate concentrations were above 4 mmol/L in both young and aged horses at the end of exercise. V_{LA4} is historically associated with a level of sustained exertion without fatigue-related injuries in horses and humans (Bourgela et al., 1991). A common concept is that a higher V_{LA4} indicates a greater level of fitness and higher exercise capacity in horses (Castejon et al., 1994). Metabolically speaking, V_{LA4} represents the maximum work intensity at which ATP is produced aerobically and blood lactate concentrations are stable because lactate is released from contracting muscles at the same rate it is utilized by other tissues (Poso et al., 2008). V_{LA4} is frequently determined on the track or through use of a high-speed treadmill, and is commonly referred to as the anaerobic threshold, the onset of blood lactate accumulation (OBLA), or maximal lactate steady state (MLSS) (Castejon et al., 1994; Gerard et al., 2014; Votion, 2014). Anaerobic threshold is commonly used to evaluate level of

fitness and aerobic capacity, and is used to indicate the maximum workload a trainer can employ while avoiding fatigue-related injuries in exercising horses. Training at V_{LA4} was shown to increase exercise tolerance in Standardbreds (Gottlieb-Vedi et al., 1995), and aged horses have been shown to have reduced V_{LA4} and exercise capacity (McKeever and Malinowski, 1997). The age-associated difference in post-exercise V_{LA4} reported here may be associated with changes in muscle fiber type and lactate dehydrogenase with advanced age. Muscle respiratory capacity determines the workload at which blood lactate begins to accumulate (Ivy et al., 1980), with lactate concentration and total lactate dehydrogenase higher in fast twitch, oxidative muscle fibers (Tesch et al., 1978). Fast twitch, oxidative muscle fibers and lactate dehydrogenase decrease with age in rats (Singh and Kanungo, 1968) and man (Larsson et al., 1978). Aged horses also experience a loss of oxidative fiber types (Lehnhard et al., 2004; Kim et al., 2005). Therefore, the lower plasma lactate concentrations in aged horses at 0 and 30 minutes post-exercise reported here may be due to lower activity of lactate dehydrogenase. Although the use of V_{LA4} as a determinant of anaerobic threshold and maximal lactate steady state may be controversial in horses (Bourgela et al., 1991; Valette et al., 1993; Persson, 1997; Lindner, 2010), it remains useful as a marker of fitness and as a level of physiological challenge.

Plasma cortisol concentrations in both young and aged mares increased due to exercise, with both groups having peak plasma cortisol concentrations at 30 minutes following acute exercise. It has previously been reported in horses that peak plasma cortisol concentrations are dependent on exercise intensity (Linden et al., 1991; Nagata et al., 1999), and that plasma cortisol concentrations peak at 30 minutes post-exercise in horses (Malinowski et al., 2006; Gordon et al., 2007). Young horses in this study had a prolonged elevation of plasma cortisol until 2 hours post-exercise, while plasma cortisol had returned to values similar to resting concentrations by 60 minutes post-exercise in aged mares. This supports previous findings that

HPA axis functionality, especially as it relates to adrenal release of cortisol following exercise, is impaired in aged horses (Horohov et al., 1999; Malinowski et al., 2006; Liburt et al., 2013). While an acute bout of submaximal exercise was enough to prompt a cortisol response in both young and aged horses, the duration of the cortisol response was affected by age. This differential cortisol response can have implications on energy substrate homeostasis, and on molecular mechanisms for adaptation to disturbances in homeostasis at a cellular level.

Plasma MDA is used as a biomarker for oxidative stress in humans and rodents (Alessio and Goldfarb, 1988; Alessio et al., 1988; Nielsen et al., 1997; Powers et al., 2002; Rodriguez et al., 2003). Exercise training in horses has been shown to alter MDA concentrations, indicating an adaptive response and altered oxidative stress dynamics (Chiaradiaa et al., 1998; Avellini et al., 1999; White et al., 2001; Marlin et al., 2002). However, neither age nor the acute submaximal exercise test reported here altered plasma MDA concentrations in Standardbred mares. The adaptive response to exercise in horses as relates to antioxidant status is most likely exercise type and intensity dependent.

Cytokines are molecular messengers that modulate the immune and endocrine response to physiological challenges, such as exercise (Moyna et al., 1996; LaManca et al., 1999; Pedersen and Hoffman-Goetz, 2000). Previous work has demonstrated an exercise-induced change in cytokine expression in horses (Streltsova et al., 2006; Donovan et al., 2007; Liburt et al., 2010a; Liburt et al., 2010b; Horohov et al., 2012).

Changes in TNF-α expression following acute exercise in whole blood and skeletal muscle of horses were observed following incremental exercise tests to fatigue (Streltsova et al., 2006; Liburt et al., 2010a; Liburt et al., 2010b). Exercise intensity appears to influence *TNF-α* expression, as high intensity exercise increases gene expression (Donovan et al., 2007) but lower

intensity acute exercise did not alter expression of *TNF-α (Horohov et al., 2012)*. *TNF-α* expression in blood and muscle was not altered by either age or acute exercise in the present study. *TNF-α* is considered a general marker of inflammation (Pedersen and Hoffman-Goetz, 2000; Suzuki et al., 2002), and is involved in the response to muscle damage and impaired skeletal muscle glucose uptake (Kimura et al., 2001; Steensberg et al., 2003).

IL-1β is considered a pro-inflammatory cytokine with a role in the response to acute exercise (Ostrowski et al., 1998; Ostrowski et al., 1999; Moldoveanu et al., 2001). Previous work in horses demonstrated an increase in *IL-1β* expression in whole blood following intense exercise (Donovan et al., 2007; Liburt et al., 2010a; Horohov et al., 2012). It is reported here that both young and aged horses had increased *IL-1β* expression in blood at 2 and 4 hours post-exercise. Peak *IL-1β* expression occurred at 2 hours following exercise in both young and aged horses, supporting earlier findings (Liburt et al., 2010a; Horohov et al., 2012). There was an age-related difference in *IL-1β* expression, with young mares having greater gene expression at 2 and 4 hours post-exercise. Acute changes in IL-1β are associated with tissue repair in humans (Dennis et al., 2004), therefore age-related differences in *IL-1β* following an acute exercise challenge may indicate impaired tissue repair and adaptation in aged horses.

In response to acute running exercise, expression of *IL-6* in horses appears to be exercise intensity dependent. Following incremental exercise tests to fatigue, no change in *IL-6* expression in whole blood was observed (Streltsova et al., 2006; Liburt et al., 2010b), however *IL-6* expression in skeletal muscle was seen to increase following acute exercise (Liburt et al., 2010a). Following intense running exercise on a high-speed treadmill, IL-6 expression in blood was seen to increase following exercise, and remain elevated until 6 hours post-exercise (Donovan et al., 2007). However, these findings were in horses that had undergone an 8 week

training period (Donovan et al., 2007). Increased IL-6 expression was also seen in horses following initial race training (Horohov et al., 2012); supporting the belief that *IL-6* contributes to an anti-inflammatory state in response to exercise conditioning. It is reported here that neither age nor acute submaximal exercise prompted a change in *IL-6* expression in blood or muscle of unfit Standardbred mares. It can be inferred that the exercise challenge employed in this study did not cause metabolic or physiological changes requiring inflammatory modulation by IL-6 in either the blood or skeletal muscle.

Inflammatory cytokine action is modulated by cortisol, which is generally considered to be anti-inflammatory. Production of several inflammatory cytokines, including TNF-α, IL-1, and IL-6, is suppressed by cortisol (Derijk and Sternberg, 1994; Chrousos, 1995; Wilder, 1995). The rise in cortisol seen in both young and aged mares following acute submaximal exercise may therefore be responsible for the lack of TNF-α and IL-6 expression seen here.

Aging has been associated with chronic inflammation in horses (Horohov et al., 1999; Horohov et al., 2010) other species (DeFronzo, 1981; Narimiya et al., 1984; Chen et al., 1985; Hadden and Harris, 1987; Harris et al., 1987; Iozzo et al., 1999; Ford et al., 2002), with implications for insulin sensitivity, and endocrine and immune function. However, chronic inflammation in the aged has been associated primarily with adipose tissue and obesity in horses (Vick et al., 2007; Vick et al., 2008; Adams et al., 2009) and other species (Shimokata et al., 1991; Kohrt et al., 1993; Hotamisligil et al., 1995; Ferrannini et al., 1996; Kern et al., 2001; Wellen and Hotamisligil, 2005; Hotamisligil, 2006; Shoelson et al., 2006; Chung et al., 2008). The horses used in this study were not considered obese, and had no clinical characteristics of endocrine dysfunction. There is no evidence here to suggest that aging alone would contribute to chronic inflammation in clinically healthy, unfit Standardbred mares.

In conclusion, acute submaximal exercise was a significant physiological challenge, highlighting the differential response of cortisol and IL-1β to acute disturbances in homeostasis in young and aged mares. These findings have implications for understanding the molecular adaptations to exercise conditioning in horses, and the role of aging in declining exercise performance.

Chapter 3.

HSP70 and HSP90 gene expression and protein content in whole blood and skeletal muscle in Standardbred mares

Abstract

Heat shock proteins (HSPs) are important mediators of the cellular response to disturbances in homeostasis. HSPs decline with age, but have been shown to increase following acute exercise and exercise conditioning. Little work has been done to investigate HSPs in horses. We hypothesized that *HSP70* and *HSP90* expression in whole blood and skeletal muscle, and HSP70 and HSP90 protein concentrations in skeletal muscle are altered by age and in response to acute submaximal exercise in horses. Young (n=6; 5.5 ± 2.8 years) and aged (n=6; 22.6 ± 2.25 years) unconditioned Standardbred mares underwent an acute submaximal exercise test which consisted of running at the velocity at which blood lactate reaches 4 mmol/L until fatigue. Whole blood and gluteus medius biopsy samples were collected and analyzed for *HSP70* and *HSP90* expression via RT-PCR. Muscle biopsy samples were analyzed for HSP70 and HSP90 protein content via western immunoblotting. Data were analyzed for main effects using a two-way ANOVA for repeated measures. Post-hoc comparisons of means were conducted using Student-Neuman-Keuls for pair wise multiple comparisons. The null hypothesis was rejected when $P \leq 0.05$. Both young and aged horses had increased *HSP70* expression in whole blood following acute exercise, with young horses exhibiting 3-fold greater *HSP70* expression than aged mares at 2 hours post-exercise. *HSP90* expression in whole blood following exercise was increased only in young horses. Both young and aged horses had increased *HSP90* expression in skeletal muscle following exercise, but there was no difference due to age. However, the timing of *HSP70* expression was different between young and aged horse. There were no changes in HSP70 and HSP90 protein content in skeletal muscle due to acute submaximal exercise or age. In conclusion, the magnitude and timing of the HSP expression following acute submaximal exercise is altered by age in horses. Quantification of HSP expression in whole blood may be a useful biomarker, with implications for cellular adaptation and survival in aged horses.

Introduction

Aging in horses is associated with a decline in capacity for strenuous exercise and compromised thermoregulation, altered immune function and chronic inflammation, and decline in hypothalamic-pituitary-adrenal axis functionality (McKeever and Malinowski, 1997; Horohov et al., 1999; Horohov et al., 2002; Malinowski et al., 2006; Horohov et al., 2010; McKeever et al., 2010; Liburt et al., 2013). Overall, aged horses have reduced ability to maintain homeostasis and adapt to physiological challenges. This is of concern because 15 percent of horses in the United States are over the age of 20 and many continue to be used for competition and reproduction (Malinowski et al., 1997). Aerobic training has been shown to partially reverse age-related declines in endocrine and immune function, and to increase exercise capacity in aged horses (Betros et al., 2002; Malinowski et al., 2002; Malinowski et al., 2006). Molecular mechanisms behind the adaptive response to exercise in horses remain to be fully elucidated, however.

Heat shock proteins (HSPs) are involved in the cellular response to disturbances in homeostasis (Kilgore et al., 1998), and are believed to be important for adaptation and survivability in response to repeated exposure to stressors, including exercise (Garramone et al., 1994; Lepore et al., 2000; Maglara et al., 2003; McArdle et al., 2004b). Although active at a cellular level, HSPs are crucial to adaptation to stress on a whole organism scale (Moseley, 1997). Heat shock protein 70 (HSP70) is highly conserved and has multiple roles in cellular function (Samali and Orrenius, 1998; Laroia et al., 1999; Gabai and Sherman, 2002; Goldberg, 2003). HSP70 has roles as a molecular chaperone and in cellular survival, where it ensures correct protein folding and translocation, while facilitating degradation of unstable proteins and preventing their aggregation (Kiang and Tsokos, 1998; Kregel, 2002). HSP70 is the most inducible and abundant member of the HSP family (Katschinski, 2004).

Heat shock protein 90 (HSP90) is also a molecular chaperone that ensures proper folding and activation of substrate proteins, such as protein kinases, transcription factors and steroid hormone receptors (Welch, 1992; Morton et al., 2009). HSP90 also prevents aggregation of unstable proteins by marking them for repair (Freeman and Morimoto, 1996; Morton et al., 2009). HSP70 and HSP90 are believed to regulate their own expression through a multi-chaperone complex with the transcription factor Heat Shock Factor 1 (HSF1) (Morimoto et al., 1992; Morton et al., 2009).

A blunted HSP response may contribute to the loss of cellular functionality seen in aging. Heat shock protein production following either heat stress or exercise is reduced in white blood cells and tissue from aged rodents and humans (Deguchi et al., 1988; Kregel and Moseley, 1996; Jurivich et al., 1997; Rao et al., 1999; Njemini et al., 2002; Vasilaki et al., 2002; Njemini et al., 2003; Marotta et al., 2007; Gupte et al., 2008). It is believed that reduced HSP production associated with aging is due to transcription inhibition (Heydari et al., 2000; Calderwood et al., 2009). The age-related decline of HSPs has been shown to be reversible, especially in fast-twitch oxidative muscles, by exercise conditioning in rats (Naito et al., 2001a)

Few studies have investigated HSPs in horses (Poso et al., 2002; Kinnunen et al., 2005; Kinnunen et al., 2009), with no studies to date investigating the HSP response to acute exercise in aged and unfit horses. We hypothesized that *HSP70* and *HSP90* expression in whole blood and skeletal muscle, and HSP70 and HSP90 protein concentrations in skeletal muscle are altered by aging and in response to acute submaximal exercise in horses.

Materials and methods

Animals

Twelve unconditioned Standardbred mares were separated into an aged group (n=6; average age = 22.6 ± 2.25 years) and a young group (n=6; average age = 5.5 ± 2.8 years). Standardbreds have low genetic diversity and high inbreeding coefficients (McCue et al., 2012; Petersen et al., 2013), therefore a random sampling of young and aged mares was deemed to be an appropriate model for this experiment. Mares were housed in two-acre drylots and given *ad libitum* hay, water, and mineral blocks. Feed was removed before exercise and sample collection. All mares were previously acclimated to the equine exercise physiology laboratory and procedures. None of the mares exhibited phenotypic signs of pituitary pars intermedia dysfunction or equine metabolic syndrome, such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). All horses were of similar body weight and body condition. This experiment took place during the early spring, but mares did not exhibit any signs of estrus, indicating that the study took place within the seasonal anestrous period. All methods and procedures used in this experiment were approved by the Rutgers University Institutional Animal Care Review Board.

A*cute submaximal exercise tests and sample collection*

The exercise test in this study employed a cross-over design in which mares were randomly assigned to Exercise (E) or Control (C) groups, and were then crossed-over so that each horse served as its own standing control. Prior to undergoing an acute bout of submaximal exercise, all mares first underwent an incremental exercise test on a Sato-I high-speed treadmill to determine the velocity at which blood lactate reaches 4 mmol/L (V_{LA4}). After completion of the incremental exercise test, each horse completed an acute submaximal exercise test which consisted of running at the velocity corresponding with V_{LA4} until fatigue. A short bout of

exercise, such as a graded exercise test, has been shown to not be significant enough to alter HSP levels in skeletal muscle (Locke et al., 1994; Naito et al., 2001b), indicating a prolonged bout of submaximal exercise may be more beneficial in studying the effects of acute exercise on heat shock proteins in skeletal muscle (Naito et al., 2001b; Morton et al., 2006).

Every testing day three horses completed the exercise tests and three horses served as standing controls. Four weeks after the acute submaximal exercise tests the horses were crossed-over so that the previously exercised mares became standing controls and the previously standing control mares completed the acute submaximal exercise tests.

Whole blood was collected via venipuncture with PAXgene™ blood RNA tubes (Qiagen, Valencia, CA) for mRNA analysis at -30 min, and 0 min, 2 hr, 4 hr, 24 hr, and 48 hr post exercise. Blood samples were also collected at the same time points from the standing control mares. Blood for mRNA analysis was stored at -80 °C until analysis.

Percutaneous skeletal muscle biopsies of the gluteus medius were obtained in a time-matched manner from exercised and standing control horses via Bergström biopsy needle at a site one-third the distance along a line running from the tuber coxae to the root of the tail (Lindholm and Piehl, 1974), 30 minutes prior to the bout of acute exercise, immediately following exercise, and at 4, 24 and 48 hours into recovery. Biopsies were obtained from a depth of 8 cm. Samples were immediately frozen in liquid nitrogen and stored at -80° C until analyzed via western immunoblotting for protein concentration, or were immersed in RNALater (Qiagen, Valencia, CA) and stored first at 4 °C for 24 hours and then at -80 °C until analysis for gene expression via RT-PCR.

HSP mRNA quantification

Quantification of *HSP70* and *HSP90* was completed in duplicate via RT-PCR as previously described (Breathnach et al., 2006; Adams et al., 2009; Liburt et al., 2012). Whole blood total RNA was isolated using spin columns (PAXgene Blood RNA Kit IVD, Qiagen, Valencia, CA) according to manufacturer's instructions.

Skeletal muscle biopsies were homogenized in a cell lysis buffer (RNA-Stat60 Reagent, Tel-Test INC., Friendsweed, TX). Sterile zirconium oxide beads were used to disrupt the tissue in a mixing mill instrument (Retsch MM301 Mixing Mill, Clifton, NJ). Total RNA was isolated from the samples using chloroform extractions, isopropanol precipitation and ethanol wash, according to manufacturer's instructions.

Quantitative and qualitative parameters of the RNA preparations were assessed using a BioTek Epoch (Thermo Fisher Scientific, Wilmington, DE) spectrophotometer. In all cases, optical density (OD) $_{260/280}$ ratios were greater than 1.0 and RNA yields were greater than 29 ng/µl. RNA (1 µg) was reverse transcribed into cDNA in an 80 µl reaction containing a master mix of 16 µl of avian myeloblastosis virus (AMV) buffer (5x), 16 µl of MgCl2, 4µl of dNTP, 1 µl of RNAasin, 1 µl oligo deoxythymine (dt) and 0.5 µl of AMV reverse transcriptase (Promega, Madison, WI). HSP70- and HSP90-specific cDNA was then amplified and quantified by real-time PCR (ABI Systems 7900 Fast Real-Time PCR System, Foster City, CA) using primers based on sequences for equine *HSP70*, *HSP90* and *β-glucuronidase* (*β-gus*) (Breathnach et al., 2006). Intron-spanning primers and 6-carboxyfluorescein (FAM)-labelled probes for *HSP90* and *β-gus*, provided as Assays-on-Demand, and probes for *HSP70* provided as Assays-by-Design kits (ABI, Foster City, CA), were added to 10 µl reactions in 384-well plates containing the 5 µl Bioline SensiFAST™ Probe Hi-ROX Mix (Bioline USA Inc, Taunton, MA), 0.5 µl of primer-probe, and 4.5 µl of cDNA. In

order to prevent genomic DNA contamination in RNA samples from blood and skeletal muscle, samples were treated with DNase (Ambion DNA-free kit, Life Technologies, Grand Island, NY) prior to amplification of *HSP70*. The following PCR conditions were employed: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected using *β-gus* as an internal control for each sample (Breathnach et al., 2006). Relative differences in cytokine mRNA expression resulting from exercise were determined by relative quantification (RQ). RQ provides accurate comparison between the initial levels of target cDNA in a sample without requiring that the exact copy number be determined. The RQ is calculated using the equation $RQ=2^{-\Delta\Delta Ct}$ where ΔΔCt = ΔCt (sample) – ΔCt (Calibrator), and ΔCt = Ct gene of interest – Ct housekeeping gene (Livak and Schmittgen, 2001). The -30 exercise and control samples for each group of horses (young and aged) were averaged and the group average used as the calibrator for subsequent calculation of RQ and reflects the change in cytokine gene expression post-expression relative to the calibrator for its respective group. Negative controls included template negative samples and genomic DNA controls. In this methodology, hematocrit was not standardized and white blood cells were not counted. Whole blood contained, by definition, all red and white blood cells present, and expression was normalized to the housekeeping gene to account for variations between samples. Sampling of skeletal muscle concurrently with peripheral blood could potentially lend clues to patterns for *HSP70* and *HSP90* expression on a whole-animal scale.

Western immunoblotting

Skeletal muscle samples were suspended in lysis buffer (1% Triton-X 100, 50 mM HEPES, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 0.1% SDS, supplemented with 0.1 mM PMSF, 1 µg/ml each of aprotinin, leupeptin, and trypsin inhibitor, 10 mM NaF and 2 mM NaO) and homogenized via mechanical disruption (BeadBug Microtube Homogenizer, Benchmark, Edison, NJ). Samples were incubated at 4 °C for one hour with gentle agitation then centrifuged 10,000xg for 10 min. The resulting supernatants were assayed for protein with the BioRad Protein Assay (BioRad, Hercules, Ca). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 10% gels. Proteins were transferred to PVDF (0.45 mM; Millipore, Bedford, MA) membranes. Equal sample loading and successful protein transfer was verified by Ponceau-S stain (G-Biosciences, St. Louis, MO). Membranes were blocked for 1 h at room temperature in Tris–buffered saline+0.05% Tween-20 (v/v) (TBS-T) and 5% non-fat dried milk (w/v), and incubated with primary antisera at 4°C overnight with gentle agitation.

Primary antibodies for HSP70 (Abcam, Cambridge, MA) and HSP90 (Santa Cruz Biotechnology, Dallas, TX) predicted to work in equine samples were used to detect proteins of interest. A polyclonal primary antibody for GAPDH (Imgenex, San Diego, CA) was used to confirm equal loading and for signal quantification. Membranes were then washed in TBS-T and incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibodies. Peroxidase activity was detected with ECL Prime (GE, Pittsburgh, PA). Band intensity for proteins of interest was quantified via densitometry (FluorChem, ProteinSimple, Santa Clara, CA), with values made relative to a standard sample run on each gel.

Statistical analysis

Data were analyzed for main effects using a two-way ANOVA for repeated measures to evaluate the differences between young and aged mares and in response to exercise for *HSP70* and *HSP90* expression in whole blood and skeletal muscle, and HSP70 and HSP90 protein levels in skeletal muscle (SigmaStat 3.1, Systat Software, San Jose, CA). Post-hoc comparisons of means were conducted using Student-Neuman-Keuls for pair wise multiple comparisons where appropriate. The null hypothesis was rejected when $P \leq 0.05$.

Results

Whole blood HSP expression

Figure 3-1 displays *HSP70* and *HSP90* expression in whole blood of young and aged mares undergoing acute submaximal exercise or standing control treatments. Young mares had increased expression of *HSP70* at 2 (P=0.049) and 4 (P=0.019) hours post-exercise, while aged mares had increased expression of *HSP70* at 2 hours post-exercise ($P<0.001$). The increased expression of *HSP70* in young mares represented a 94-fold increase at 2 hours post-exercise and a 40-fold increase at 4 hours post-exercise as compared to resting *HSP70* expression. Aged mares had a 24-fold increase in *HSP70* expression at 2 hours post-exercise as compared to resting *HSP70* expression. Young mares had a 3-fold greater increase in *HSP70* expression at 2 hours post-exercise than aged mares (P=0.006). There were no differences within or between young and aged standing control samples ($P>0.05$) (**Figure 3-1**).

Exercise significantly increased *HSP90* expression in blood of young mares at 2 hours post-exercise ($P<0.001$), which represented an 8-fold change of HSP90 expression as compared to resting HSP90 expression. There were no significant changes due to exercise in aged mares at

any time post-exercise (*P*>0.005). There were no differences between or within young and aged standing control samples (*P*>0.005) (**Figure 3-1**).

Skeletal muscle HSP expression

Figure 3-2 displays *HSP70* and *HSP90* expression in skeletal muscle of young and aged mares undergoing acute submaximal exercise and standing control treatments. *HSP70* expression was significantly elevated in skeletal muscle of young horses at 4 hours post-exercise (*P*<0.001). Mean *HSP70* expression was increased 12-fold in young horses at 4 hours post-exercise as compared to rest. There was also a significant effect of exercise on *HSP70* expression in aged horses at 0 (*P*=0.001) and 4 hours (*P*=0.002) post-exercise. *HSP70* expression was increased 4-fold at 0 and 4 hours post-exercise versus *HSP70* expression in resting aged mares. There were no differences in *HSP70* expression in skeletal muscle between young and aged horses at any time post-exercise (*P*>0.05). There were no differences in *HSP70* expression within young (*P*>0.05) or aged (*P*>0.05) standing controls. However aged mares had at least 2-fold greater *HSP70* expression than young mares at all control timepoints (*P*=0.001) (**Figure 3-2**).

HSP90 expression was increased at 4 hours post-exercise in skeletal muscle of both young (*P*<0.001) and aged (*P*=0.003) mares. Changes in *HSP90* expression in both young and aged mares post-exercise versus resting *HSP90* expression represented a 1.8- and 1.3-fold change, respectively. There was no significant effect of age on *HSP90* expression post-exercise, however (*P*>0.05). There were no differences within or between young and aged standing control samples (P>0.05) (**Figure 3-2**).

Western immunoblotting

Total HSP70 protein concentration in skeletal muscle samples from young and aged mares was determined in both exercised and control horses. Western immunoblotting and densitometric analysis revealed no effect of age or acute submaximal exercise on HSP70 protein concentration in gluteus medius skeletal muscle ($P>0.05$) (**Figure 3-3**).

Additionally, total HSP90 protein concentration in the same skeletal muscle samples from young and aged mares undergoing either exercise or standing control treatments was determined. Western immunoblotting and signal quantification revealed no effect of age or acute submaximal exercise on HSP90 protein concentration in these samples ($P>0.05$) (**Figure 3-4**).

Figure 3-1. *HSP70* and *HSP90* expression in whole blood in response to acute submaximal exercise. Mean ± s.e. relative quantity values for expression of *HSP70* (3-1.A) and *HSP90* (3-1.B) in whole blood in response to acute submaximal exercise or standing control. Data points represented are those collected from horses before exercise or control treatments (-30 and -1 min), and at 0, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 3-1

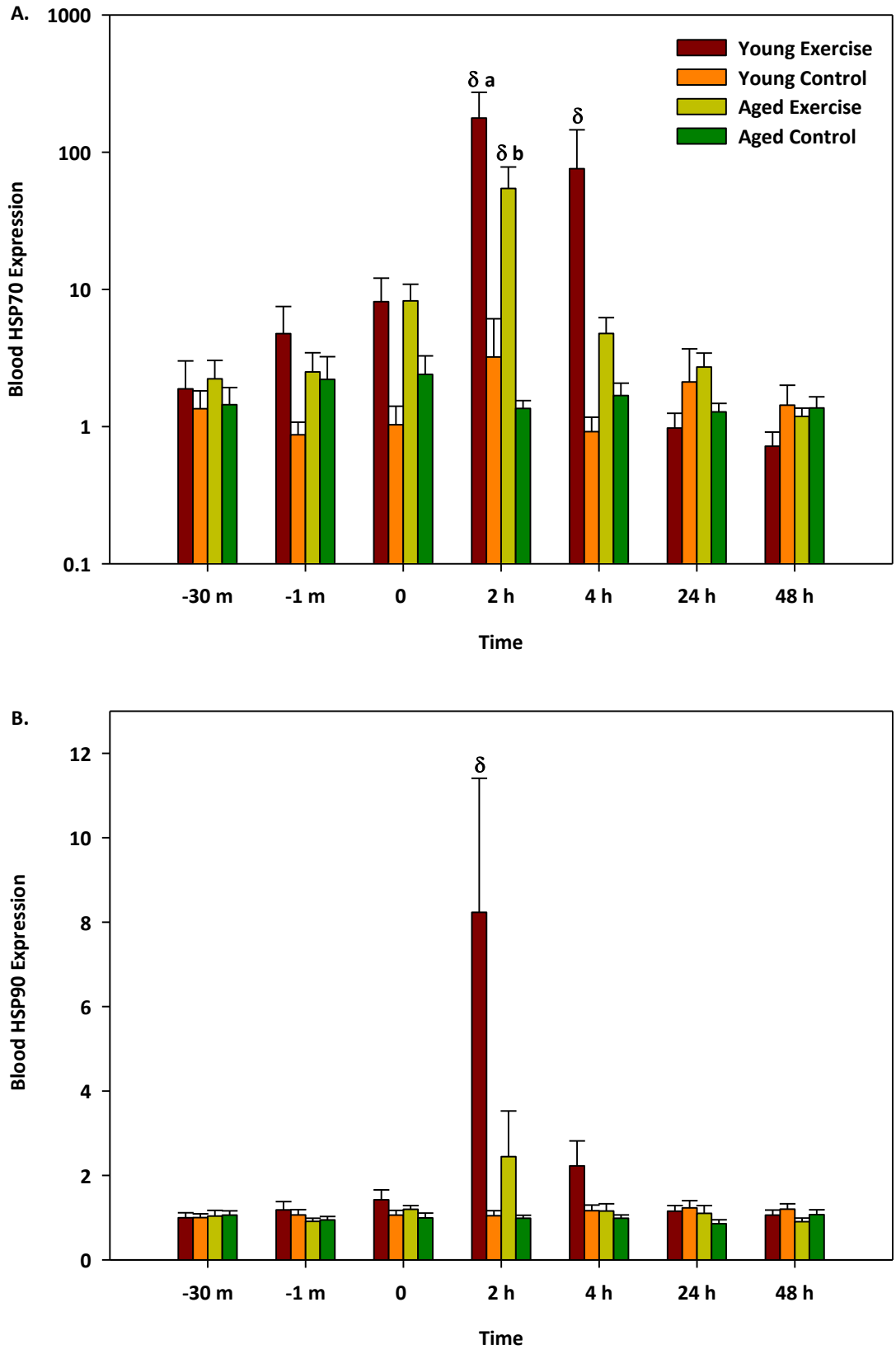

A.
Blood HSP70 Expression
1000
100
10
1
0.1
Young Exercise
Young Control
Aged Exercise
Aged Control
δ a
δ b
δ
-30 m
-1 m
0
2 h
4 h
24 h
48 h
Time
B.
Blood HSP90 Expression
12
10
8
6
4
2
0
δ
-30 m
-1 m
0
2 h
4 h
24 h
48 h
Time

Figure 3-2. *HSP70* and *HSP90* expression in skeletal muscle in response to acute submaximal exercise. Mean ± s.e. relative quantity values for expression of *HSP70* (3-2.A) and *HSP90* (3-2.B) in gluteus medius in response to acute submaximal exercise or standing control. Data points represented are those collected from horses before exercise or control treatments (-30 and -1 min), and at 0, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 3-2

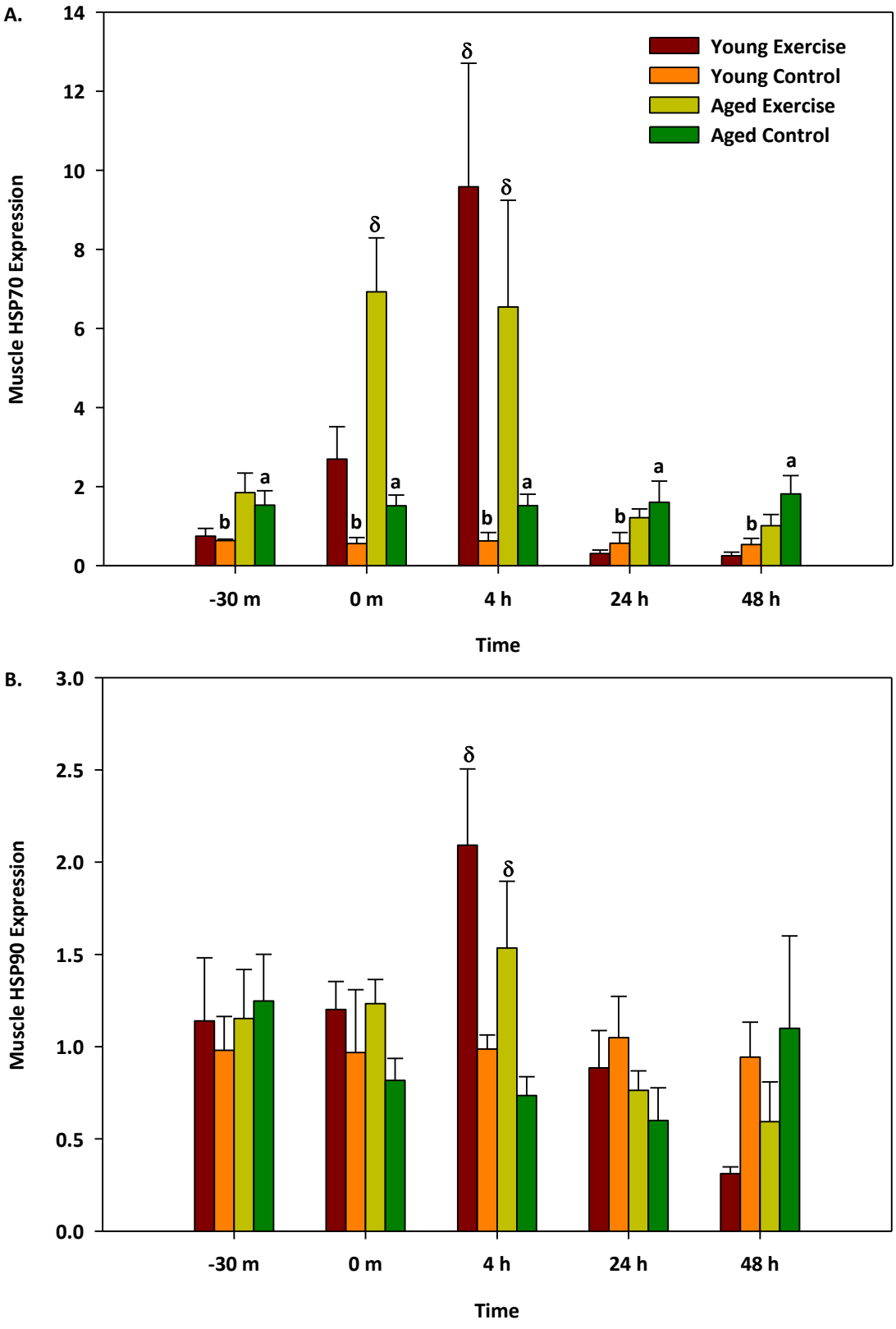

A.
Muscle HSP70 Expression
14
12
10
8
6
4
2
0
Young Exercise
Young Control
Aged Exercise
Aged Control
δ
δ
δ
b
b
b
b
b
a
a
a
a
a
-30 m
0 m
4 h
24 h
48 h
Time
B.
Muscle HSP90 Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
δ
δ
-30 m
0 m
4 h
24 h
48 h
Time

Figure 3-3

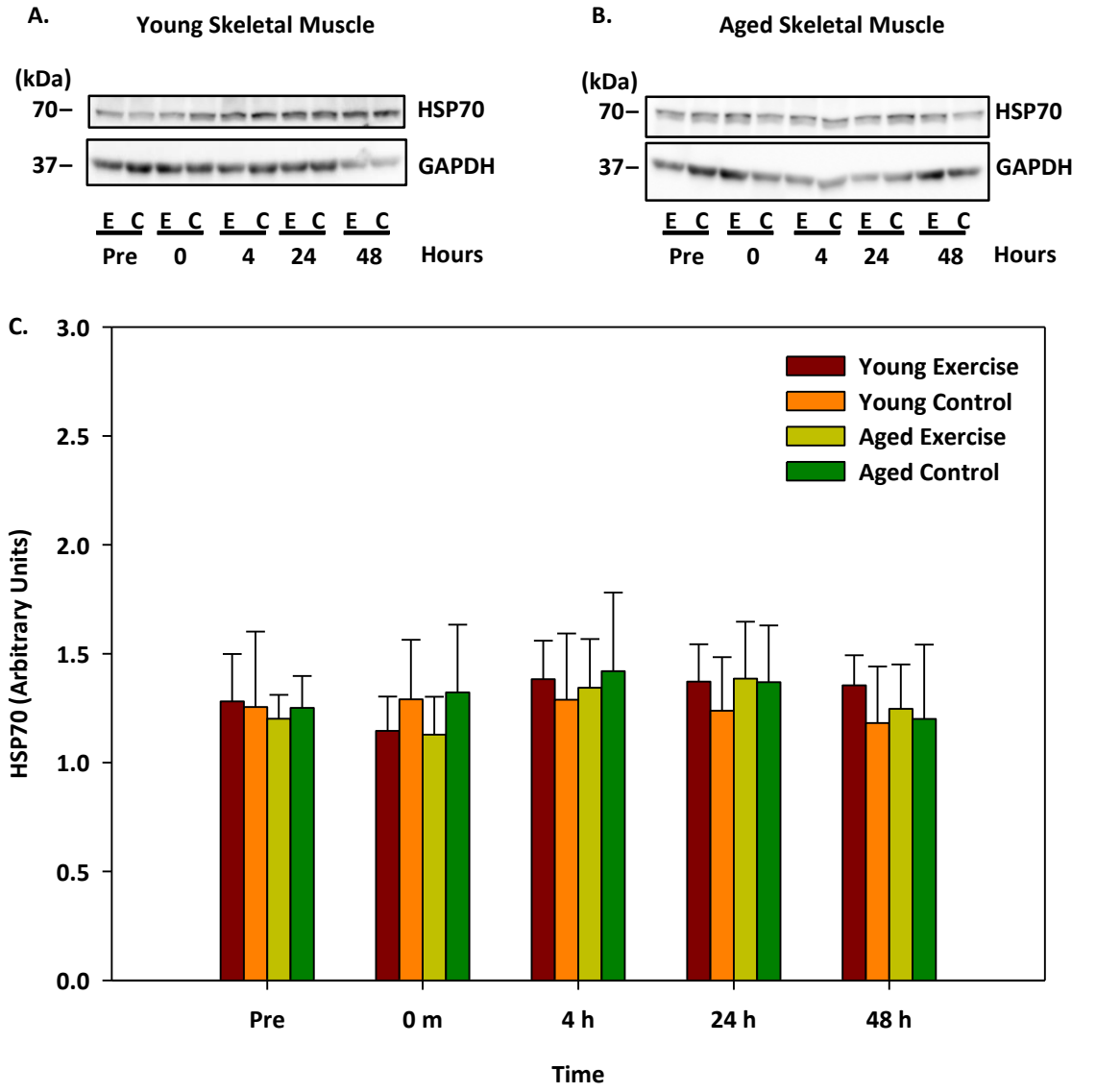


Figure 3-3. HSP70 protein content in equine skeletal muscle in response to acute submaximal exercise. Example of typical western immunoblotting results in skeletal muscle samples from young mares (n=6; 3-3.A) and aged mares (n=6; 3-3.B). Samples were taken before exercise (Pre), and at 0, 4, 24 and 48 hours post exercise. Each exercised horse (E) had time-matched standing control (C) samples taken on separate testing days. Chemiluminescence signals at 70 kDa correspond with HSP70, while signals at 37 kDa correspond with the loading control GAPDH. Chemiluminescence signals were quantified via densitometry, with values normalized to a control sample run on each gel. Values are reported as mean ± s.e. arbitrary units (3-3.C) Band quantification showed no change in HSP70 content due to either age or acute submaximal exercise.

Figure 3-4

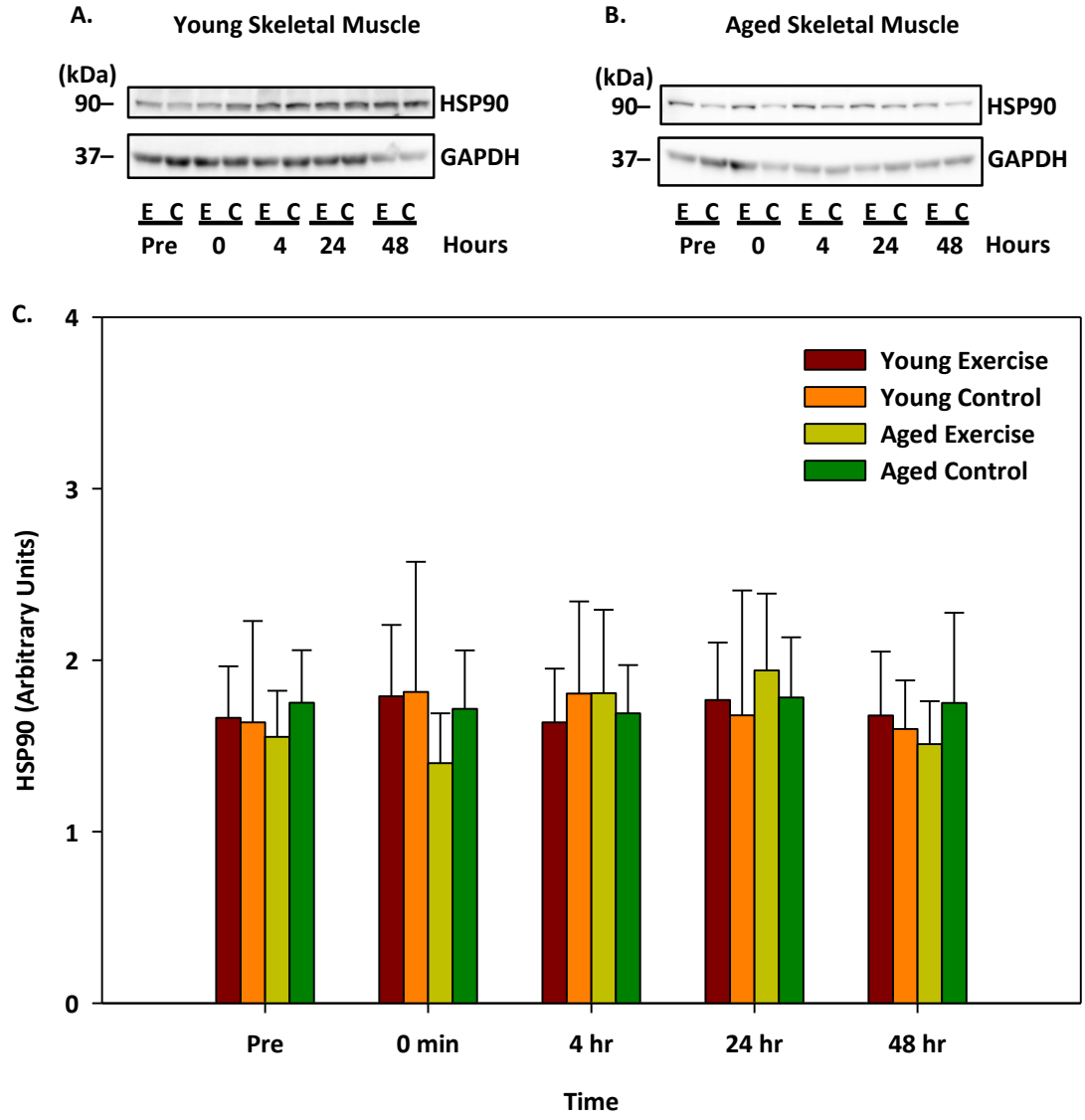


Figure 3-4. HSP90 protein content in equine skeletal muscle in response to acute submaximal exercise. Example of typical western immunoblotting results in skeletal muscle samples from young mares (n=6; 3-4.A) and aged mares (n=6; 3-4.B). Samples were taken before exercise (Pre), and at 0, 4, 24 and 48 hours post exercise. Each exercised horse (E) had time-matched standing control (C) samples taken on separate testing days. Chemiluminescence signals at 90 kDa correspond with HSP90, while signals at 37 kDa correspond with the loading control GAPDH. Chemiluminescence signals were quantified via densitometry, with values normalized to a control sample run on each gel. Values are reported as mean ± s.e. arbitrary units (3-4.C) Band quantification showed no change in HSP90 content due to either age or acute submaximal exercise.

Discussion

The objective of this study was to characterize *HSP70* and *HSP90* gene expression in whole blood and skeletal muscle, and to characterize HSP70 and HSP90 protein concentrations in skeletal muscle of young and aged Standardbred mares. RT-PCR of whole blood revealed that while both young and aged mares had increased expression of *HSP70* following exercise, young mares had significantly higher *HSP70* expression than aged mares, and the exercise-induced increased *HSP70* expression had a longer duration in young mares. *HSP70* in skeletal muscle was increased immediately following exercise in old mares, and remained elevated until 4 hours post-exercise, while in skeletal muscle of young mares, *HSP70* expression was increased only at 4 hours post exercise. *HSP90* expression in whole blood increased in response to exercise only in young mares, while *HSP90* expression in skeletal muscle was increased at 4 hours post-exercise in young and aged mares.

Interestingly, *HSP70* expression in skeletal muscle from aged standing control mares was greater than expression in young standing control mares. This could be partially related to increased reactive oxidative species present in aged tissue (Halliwell, 1989; Papa and Skulachev, 1997; Fulle et al., 2004; Genova et al., 2004; Liochev, 2013) since it has been suggested that redox imbalance can lead to increased HSP70 transcription (Madamanchi et al., 2001; Ahn and Thiele, 2003), and that HSP70 can counteract cellular damage caused by increased reactive oxidative species (Currie et al., 1988; Mocanu et al., 1993). However, oxidative stress was not assessed in the skeletal muscle samples taken from the current study, so no direct correlation between antioxidant status and *HSP70* expression in resting, aged muscle can be claimed.

Western immunoblotting did not reveal changes in protein concentration of HSP70 or HSP90 in skeletal muscle from young or aged horses. The observation of changes in HSP gene expression

without concurrent changes in protein is not without precedent in the literature. Increased expression of *HSP70* in whole blood and skeletal muscle has been seen following acute exercise in humans, with no related increase in HSP70 protein observed (Puntschart et al., 1996; Febbraio and Koukoulas, 2000; Walsh et al., 2001; Milne and Noble, 2002; Febbraio et al., 2004). Other studies have shown increases in HSP70 protein content occurring from 24 hours to 7 days following acute non-damaging exercise (Khassaf et al., 2001; Morton et al., 2006; Paulsen et al., 2007). Regarding differences in HSP protein content due to age, skeletal muscle HSP70 content in young and aged sedentary rats were not different following acute exercise (Naito et al., 2001b). The study described here and previous investigations into changes in HSP protein concentrations have typically relied on semi-quantitative methods, and have typically reported high individual variation in the HSP response to disturbances in homeostasis. These factors may explain the sometimes conflicting results found in the literature regarding HSP protein in tissues following exercise or exposure to other stressors.

Rodent (Blake et al., 1991a; Kregel et al., 1995; Locke and Tanguay, 1996; Heydari et al., 2000), human (Deguchi et al., 1988; Rao et al., 1999; Njemini et al., 2002; Njemini et al., 2003; Marotta et al., 2007) and cellular (Liu et al., 1989) models of aging have shown that HSP70 and HSP90 gene expression and protein production is reduced in various tissues after heat stress. Given that HSPs are important mediators of cellular function and survival, their decline likely plays a large part in age-related changes in endocrine, immune and tissue function. Since exercise is a proven inducer of HSP expression, the regulation and function of HSPs in skeletal muscle is a logical course of investigation. Overexpression of *HSP70* in adult and aged mice contributed to enhanced skeletal muscle recovery from damaging exercise (McArdle et al., 2004a), and reduced accumulation of oxidation products and maintenance of cellular survival signaling following non-damaging contraction (Broome et al., 2006). Evidence from rodent and human studies suggests

that increased HSP70 production can modulate post-exercise cytokine production (Asea et al., 2000; Hung et al., 2005; Paulsen et al., 2007). Exercise conditioning increases HSP70 protein content in skeletal muscle of young and aged rats, but aged rats had significantly lower HSP70 content in fast-twitch muscles (Naito et al., 2001b).

The HSP90 response to exercise has not been as extensively studied as HSP70. It is known that HSP90 contributes to refolding of denatured proteins (Nathan et al., 1997) and signal transduction for tyrosine and serine/threonine kinases (Aligue et al., 1994; Strepanova et al., 1996; van der Straten et al., 1997) following disturbances in homeostasis. HSP90 is crucial for proper activity of, and signaling through, the glucocorticoids receptor (Picard et al., 1990; Bohen, 1995; Eckl and Richter, 2013). Decreased expression of HSP90 in aged tissue has been documented in humans (Faassen et al., 1989; Hunter and Poon, 1997; Rao et al., 1999) and rats (Zhang et al., 2002). Interestingly, impaired recovery from heat shock has been shown to result from HSP90 inhibition, suggesting that interactions among the different members of the HSP family are required for proper cellular recovery and protection from stress (Duncan, 2005). It is worth recalling that HSP70 and HSP90 are involved in auto-regulation of the HSP response through binding of HSF1, and that by inhibiting the response of HSP90 one may reduce HSF1 action on the genome. Therefore, the roles of HSP90 and its decline during aging warrant further investigation, especially in relation to changes in its production in response to acute and chronic homeostatic perturbations, its relation to glucocorticoid signaling, and its influence on expression of other HSPs.

Exercise conditioning changes the magnitude and timing of the HSP response to acute exercise in rodents and humans (Fehrenbach et al., 2000; Gonzalez et al., 2000; McClung et al., 2008; Morton et al., 2008). Evidence presented here suggests that aging also alters the magnitude and

timing of *HSP70* and *HSP90* expression in whole blood and skeletal muscle. HSP70 protein content in human skeletal muscle is influenced by muscle fiber phenotype and exercise conditioning background (Folkesson et al., 2013). It should be noted that fast twitch, oxidative muscle fibers decrease with age in rats (Singh and Kanungo, 1968) and man (Larsson et al., 1978) and that aged horses also experience a loss of oxidative fiber types (Lehnhard et al., 2004; Kim et al., 2005). The altered magnitude and timing of *HSP70* and *HSP90* expression in skeletal muscle from aged horses may therefore be understood as a result of shifts in muscle fiber types that occur with aging, and possible changes in the stimulus threshold required to prompt HSP expression.

It has been suggested that HSPs, especially HSP70, aid in the restoration of insulin signaling by counteracting chronically activated inflammatory pathways (McCarty, 2006; Geiger and Gupte, 2011). There is evidence that HSPs may modulate the effects of pro-inflammatory cytokines (van Eden et al., 2005; Yamada et al., 2007; Noble and Shen, 2012). Manipulating the HSP response has attenuated insulin resistance in rodent (Atalay et al., 2004; Chung et al., 2008; Gupte et al., 2010; Geiger and Gupte, 2011; Kondo et al., 2012) and cellular (Drew et al., 2013) models. There has been a well-documented reduction of HSP expression in humans (Kurucz et al., 2002; Bruce et al., 2003; Burkart et al., 2008; Chung et al., 2008) and rats (Atalay et al., 2004; Ooie et al., 2005; Gupte et al., 2008) with insulin resistance. Inhibition of the HSP response has been associated with increased inflammatory mediators (Dai et al., 2000; Heydari et al., 2000; Park and Liu, 2001; Wang et al., 2006a; Marotta et al., 2007; Chung et al., 2008; Hooper and Hooper, 2009; Geiger and Gupte, 2011). To date, there have been no investigations relating inflammation, insulin resistance and heat shock proteins in horses.

The HSP response to exercise in horses has received limited study thus far. *HSP70* expression was increased following acute submaximal exercise in gluteus medius samples from trained horses (Poso et al., 2002). In a second study, muscle biopsies were taken before exercise and four hours post-exercise in trained horses, and were analyzed for changes in HSP70 and HSP90. HSP70 and HSP90 were not altered at 4 hours post-exercise, however, markers of oxidative stress were elevated (Kinnunen et al., 2005). In a third study, trained Standardbreds were used to study the effect of α-lipoic acid, a known antioxidant, supplementation on HSP72 expression (Kinnunen et al., 2009). Trained horses underwent acute submaximal exercise tests before and after 5 weeks of antioxidant supplementation. Muscle biopsies were taken at the time of each exercise test; specifically at rest, and at 6, 24 and 48 hours post-exercise. Each horse served as its own control (Kinnunen et al., 2009). At 24 hours post-exercise, skeletal muscle HSP70 was elevated in response to treatment as compared to control samples. HSP90 expression was not altered by either acute exercise or antioxidant supplementation (Kinnunen et al., 2009).

Conclusions

This study indicates that expression of HSPs in whole blood can be used as a biomarker for disturbances in homeostasis, especially exercise, and the effects of aging in horses. Caution should be used before making inferences on tissue-specific HSP expression, however. Also, changes in mRNA may not directly translate into changes in protein. The results presented here also support previous findings that *HSP70* is more active than *HSP90* following acute exercise in whole blood and skeletal muscle. Changes in HSP70 and HSP90 content may contribute to loss of functionality, but it remains to be seen if exercise conditioning changes HSP expression and protein in aged horses, and how those changes would compare to adaptation in young horses. Finally, development of tools to better quantify HSP protein in tissue is needed. Most studies to

date have employed semi-quantitative methods to measure HSP protein response to exercise or other stressors, such as western immunoblotting, immunohistochemistry or flow cytometry. This may explain lack of observed changes in HSP protein following acute exercise in several published papers and in this study. Understanding HSPs in the adaptive response to stressors will allow for a better understanding of how exercise conditioning and environmental management can be optimized for the betterment of equine athletes and aging horses.

Chapter 4.

Glucose-insulin homeostasis and characterization of proteins involved in glucose uptake signaling in equine skeletal muscle

Abstract

This study tested the hypothesis that glucose-insulin homeostasis, and activation of proteins involved in skeletal muscle glucose uptake in horses are altered by age and acute, submaximal exercise. Unconditioned young (n=6; 5.5 ± 2.8 years) and aged (n=6; 22.6 ± 2.25 years) Standardbred mares were assessed for insulin sensitivity via frequently sampled intravenous glucose tolerance test (FSIGT). All mares then underwent a single bout of submaximal exercise. Plasma insulin and glucose concentrations were measured via radioimmunoassay and enzyme-electrode interface, respectively. Gluteus medius biopsies were taken pre- and post-exercise, and were analyzed via western immunoblotting for changes in activation of AMPK, Akt and AS160. Minimal model analysis of FSIGT and repeated measures ANOVA were utilized to analyze data. The null hypothesis was rejected when $P \leq 0.05$. There was no difference between young and aged mares for minimal model parameters ($P>0.05$). In response to acute exercise, young mares had an elevated insulin concentration at 2 ($P=0.009$) and 4 ($P=0.007$) hours while aged mares had elevated insulin at 30 ($P<0.001$) and 60 ($P=0.001$) minutes post-exercise. Neither age nor exercise altered insulin area under the curve (AUCi) ($P>0.05$). Glucose concentration was elevated from 0 minutes to 2 hours post-exercise in young mares ($P<0.001$), while in aged mares glucose remained elevated only until 60 minutes post-exercise ($P=0.037$). Plasma glucose concentration in young mares was significantly greater than in aged mares at 0 ($P=0.008$), 30 ($P=0.009$), 60 minutes ($P=0.041$) and 2 hours ($P=0.017$) post-exercise. Exercise caused an increase in glucose area under the curve (AUCg) in young ($P=0.007$) and aged ($P=0.031$) mares, however there was no age effect on AUCg ($P>0.05$). Neither age nor exercise altered activation of AMPK, Akt or AS160 ($P>0.05$). In conclusion, age alone is not sufficient to alter insulin sensitivity in horses. Also, neither age nor a single bout of submaximal exercise was sufficient to alter activation of proteins involved in glucose uptake in muscle.

Introduction

Changes in glucose-insulin homeostasis have been reported to occur with aging in horses (Malinowski et al., 2002; Nielsen et al., 2010; Liburt et al., 2012), with reduced insulin sensitivity (Malinowski et al., 2002), and reduced insulin secretion and β-cell responsiveness reported in horses over the age of 20 (Liburt et al., 2012). Insulin resistance has also been reported in aged humans (Boirie et al., 2001; Scheen, 2005) and rats (Carvalho et al., 1996; Qiang et al., 2007). Previously reported findings implicate loss of insulin sensitivity, insulin resistance and equine metabolic syndrome in several equine pathologies, including impaired reproductive function (Vick et al., 2006), hepatic lipidosis (Johnson et al., 2012), osetochondrosis (Ralston, 1996; Johnson et al., 2012) and laminitis (Coffman and Colles, 1983; Field and Jeffcott, 1989; Pass et al., 1998). The direct connection between insulin resistance and laminitis remains unclear, but most likely relates to changes in glucose availability, vasculature and the inflammatory milieu, all influencing the health and structure of hoof tissue (Treiber et al., 2006).

Intense exercise in aged horses results in greater plasma insulin concentrations as compared to young horses, possibly indicating a greater requirement for muscle glycogen repletion (Malinowski et al., 2002). Exercise training in aged horses moderately alters the insulin response to acute exercise and improves overall insulin sensitivity, however insulin dynamics remain significantly different in aged verses young horses (Malinowski et al., 2002; Liburt et al., 2012). The mechanisms behind changes in glucose-insulin homeostasis, and adaptive changes due to exercise training, have not been thoroughly investigated in equine tissue. Molecular signaling intermediaries may be the key to understanding and ameliorating changes in insulin-glucose homeostasis and loss of functionality in aged horses.

The comparative literature indicates that several molecular modulators of insulin signaling and glucose uptake are altered by exercise or age. One such mediator of cellular homeostasis is AMPK, a highly conserved enzyme that regulates energy homeostasis (O'Neill, 2013). There is ample evidence that AMPK is activated during exercise in skeletal muscle of humans and rodents (Winder and Hardie, 1996; Hutber et al., 1997; Rasmussen and Winder, 1997; Vavvas et al., 1997; Winder et al., 1997; Hayashi et al., 1998; Rasmussen et al., 1998; Ihlemann et al., 1999a; Fujii et al., 2000; Wojtaszewski et al., 2000; Chen et al., 2003). As demonstrated in mice cellular glucose uptake in response to exercise is moderated by AMPK, independent of insulin signaling (O'Neill et al., 2011). Decline of skeletal muscle AMPK activity has been associated with aging and insulin resistance in rats (Qiang et al., 2007; Paturi et al., 2010). AMPK activity has not been investigated in aged horses.

In response to insulin signaling, the PI3K pathway is responsible for translocation of GLUT4 to the cellular membrane in order to transport glucose into the cell (Quon et al., 1995; Kohn et al., 1996; Cong et al., 1997; Dugani and Klip, 2005; Ishiki and Klip, 2005; Thong et al., 2005). One member of the PI3K pathway is Akt, which has many functions when phosphorylated, including direct stimulation of GLUT4 translocation to the cellular membrane (Quon et al., 1995; Dugani and Klip, 2005; Ishiki and Klip, 2005; Thong et al., 2005). Inhibition of Akt has been associated with impaired insulin signaling and cellular glucose uptake in human tissue culture (Krook et al., 1998; Zeng et al., 2000; Gonzalez and McGraw, 2006; Wang et al., 2006b) and in human skeletal muscle. Decline of skeletal muscle Akt has been observed with age in rats (Paturi et al., 2010; Trott et al., 2013), however Akt activity remains to be studied in aged equine skeletal muscle.

The most distal signaling protein involved in the insulin- and contraction-dependent GLUT4 translocation is known as the Akt substrate protein of 160 kDa (AS160) (Kramer et al., 2006a;

Kramer et al., 2006b; Thyfault et al., 2007a; Sakamoto and Holman, 2008). In an unstimulated state, AS160 is unphosphorylated and acts to anchor GLUT4 within the cytoplasm. When AS160 is phosphorylated through Akt action, GLUT4 exocytosis occurs (Thyfault et al., 2007a; Sakamoto and Holman, 2008). Activation of AS160 via the PI3K/Akt pathway has been observed in both rat (Bruss et al., 2005; Arias et al., 2007b) and human skeletal muscle (Deshmukh et al., 2006; Karlsson et al., 2006). Reduced activation of AS160 has been observed in human (Karlsson et al., 2005) and rodent (Lessard et al., 2007; Sakamoto and Holman, 2008) models of insulin resistance, and has been shown to change with age in rat skeletal muscle (Gupte et al., 2008). AS160 may therefore represent a point at which to study the effects of age and exercise on the activity of the PI3K/Akt and AMPK signaling pathways in equine skeletal muscle.

The objective of this study was to evaluate insulin sensitivity in young and aged mares, and to evaluate the role of molecular signaling mediators related to glucose uptake in skeletal muscle of young and aged horses. This study tested the hypothesis that glucose-insulin homeostasis, and activation of AMP-activated protein kinase (AMPK), the protein kinase Akt, and the Akt substrate protein of 160 kDa (AS160) in equine skeletal muscle are altered by acute submaximal exercise and by aging.

Materials and methods

Animals

Twelve unconditioned Standardbred mares were separated into an aged group (n=6; average age = 22.6 ± 2.25 years) and a young group (n=6; average age = 5.5 ± 2.8 years). Standardbreds have low genetic diversity and high inbreeding coefficients (McCue et al., 2012; Petersen et al., 2013), therefore a random sampling of young and aged mares was deemed to be an appropriate model for this experiment. Mares were housed in two-acre drylots and given *ad libitum* hay,

water, and mineral blocks. Feed was removed before exercise and sample collection. All mares were previously acclimated to the equine exercise physiology laboratory and procedures. None of the mares exhibited phenotypic signs of pituitary pars intermedia dysfunction or equine metabolic syndrome, such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). All horses were of similar body weight and body condition. This experiment took place during the early spring, but mares did not exhibit any signs of estrus, indicating that the study took place within the seasonal anestrous period. All methods and procedures used in this experiment were approved by the Rutgers University Institutional Animal Care Review Board.

FSIGT

Three mares were randomly selected for modified frequently sampled intravenous glucose tolerance (FSIGT) testing each day for four consecutive days. Mares were brought into stalls the morning of the test at 07:00 hours and weighed on an electronic scale. Grass hay and water were available *ad libitum*. A jugular catheter was inserted percutaneously using sterile techniques and with lidocaine anesthesia.

Baseline blood samples were collected at 08:30 and 08:59. At approximately 09:00 the test began with infusion of a glucose bolus of 0.3 g/kg body weight (dextrose solution 50%, Phoenix Pharmaceutical, St. Joseph, MO) over two minutes. At 20 minutes following the glucose dose, an insulin bolus (Humulin R, Eli Lilly & Co, Indianapolis, IN) of 30 mU/kg body weight was administered. No mares exhibited signs of hypoglycemia during or after the test.

Blood samples were collected at -30, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min after glucose administration. Samples were placed in pre-chilled lithium heparin blood collection tubes (Vacutainer, Franklin Lakes, NJ) and centrifuged at 3,000xg at 4 °C for 10 min. Plasma for determination of insulin concentration

was frozen at -80 °C. Insulin concentration was determined via radioimmunoassay (RIA) (Coat-A-Count, Siemens, Los Angeles, CA) kits previously validated for use in horses (Freestone et al., 1991). Samples were counted for 1 min in a gamma counter (Packard Instrument Co, Meridan, CT). Within assay coefficient of variation for insulin was 2.66%. Plasma glucose concentration was determined via enzyme-electrode interface (ABL 800 Flex, Radiometer, Westlake, OH).

Acute exercise tests and sample collection

The exercise test in this study employed a cross-over design in which mares were randomly assigned to exercise (E) or control (C) groups, and were then crossed-over so that each horse served as its own control. Prior to undergoing an acute bout of submaximal exercise, all mares first underwent an incremental exercise test on a Sato-I high-speed treadmill to determine the velocity at which blood lactate reaches 4 mmol/L (V_{LA4}).

After completion of the incremental exercise test, each horse completed an acute submaximal exercise test which consisted of running at the velocity corresponding with V_{LA4} until fatigue. Every testing day three horses completed the exercise tests and three horses served as standing controls. Four weeks after the acute submaximal exercise tests horses were crossed-over so that previously exercised mares were used as standing controls, and previously standing control mares completed the acute submaximal exercise tests.

Whole blood was collected via indwelling catheter in lithium heparin blood collection tubes, with plasma separated for analysis of insulin and glucose at -30 and -1 min prior to exercise, and 0, 30, 60 min, 2, 4, 24 and 48 hours post-exercise. Time matched blood samples were collected from control horses. Plasma insulin concentrations were determined via RIA as described above. Within assay coefficient of variation for insulin was 6.86%. Plasma glucose concentration was determined via enzyme-electrode interface at the same timepoints, as described above.

Percutaneous skeletal muscle biopsies of the gluteus medius were obtained in a time-matched manner from exercised and control horses via Bergström biopsy needle at a site one-third the distance along a line running from the tuber coxae to the root of the tail (Lindholm and Piehl, 1974). Biopsies were taken at 30 minutes prior to the bout of acute exercise, immediately following exercise, and at 4, 24 and 48 hours post-exercise. Biopsies were obtained from a depth of 8 cm, and samples were immediately frozen in liquid nitrogen and stored at -80° C until analyzed via western immunoblotting.

Western Immunoblotting

Skeletal muscle samples were suspended in lysis buffer (1% Triton-X 100, 50 mM HEPES, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 0.1% SDS, supplemented with 0.1 mM PMSF, 1 µg/ml each of aprotinin, leupeptin, and trypsin inhibitor, 10 mM NaF and 2 mM NaO) and homogenized via mechanical disruption (BeadBug Microtube Homogenizer, Benchmark, Edison, NJ). Samples were incubated at 4 °C for one hour with gentle agitation then centrifuged 10,000xg for 10 min. The resulting supernatants were assayed for protein with the BioRad Protein Assay (BioRad, Hercules, Ca). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 10% gels for AMPK and Akt or 12.5% gels for AS160. Proteins were transferred to PVDF (0.45 mM; Millipore, Bedford, MA) membranes. Equal sample loading and successful protein transfer was verified by Ponceau-S stain (G-Biosciences, St. Louis, MO). Membranes were blocked for 1 h at room temperature in Tris–buffered saline+0.05% Tween-20 (v/v) (TBS-T) and 5% non-fat dried milk (w/v), and were incubated with primary antisera at 4°C overnight with gentle agitation. Primary antibodies for P-AMPK and AMPK (Santa Cruz Biotechnology, Dallas, TX), P-Akt and Akt (Cell Signaling Technology, Danvers, MA), and P-AS160 and AS160 (Millipore, Billerica, MA) predicted to work in equine samples were used to detect

proteins of interest. A polyclonal primary antibody for GAPDH (Imgenex, San Diego, CA) was used to confirm equal loading and for signal quantification. Membranes were then washed in TBS-T and incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibodies. Peroxidase activity was detected with ECL Prime (GE, Pittsburgh, PA). Band intensity for proteins of interest was quantified via densitometry (FluorChem, ProteinSimple, Santa Clara, CA), with values made relative to a standard sample. Quantification of phosphorylated proteins was made relative to total protein signals, while total protein quantification was made relative to GAPDH signal.

Statistical analysis

Data were analyzed for main effects using a two-way ANOVA for repeated measures to evaluate the differences between young and aged mares in respect to insulin and glucose during the FSIGTT. A two-way ANOVA for repeated measures was used to evaluate the effects of age and exercise for plasma insulin and glucose, and P-AMPK, AMPK, P-Akt, Akt, P-AS160 and AS160 in skeletal muscle (SigmaStat 3.1, Systat Software, San Jose, CA). Areas under the curve were calculated for insulin (AUCi) and glucose (AUCg) (SigmaPlot 9.0, Systat Software, San Jose, CA). Post-hoc comparisons of means were conducted using Student-Neuman-Keuls for pair wise multiple comparisons where appropriate. Linear regression analysis was used to determine correlations between factors, where appropriate. The null hypothesis was rejected when $P \leq 0.05$.

Results

Frequently sampled intravenous glucose tolerance test

Insulin sensitivity (SI) – the capacity of insulin to promote glucose disposal; glucose effectiveness (SG) – the capacity of glucose to mediate its own disposal without change in plasma insulin; acute insulin response to glucose (AIRg) – quantification of endogenous insulin secretion in response to the glucose dose; and disposition index (DI) – the index that describes β-cell responsiveness while accounting for both endogenous insulin secretion (AIRg) and SI were determined in each mare by FSIGT testing and Minimal Model analysis of data (Hoffman et al., 2003). Minimal Model analysis revealed no difference between the means of young and aged mares for SI, SG, AIRg or DI (P>0.05) (**Table 4-1**). Area under the curve for both insulin (AUCi) and glucose (AUCg) were not different between young and aged mares (P>0.05) (**Figure 4-1**). Also, there were no differences in baseline insulin or glucose between young and aged mares (P>0.05).

Table 4-1

	Young	**Aged**
Insulin sensitivity (SI) (l/mU/min)	2.32 ± 0.96	2.46 ± 1.06
Glucose effectiveness ($min^{-1)}$	0.020 ± 0.003	0.025 ± 0.003
Acute insulin response to glucose (AIRg) (mU x min/l)	70.74 ± 30.67	162.14 ± 38.94
Disposition index (AIRg x SI)	101.61 ± 48.54	424.63 ± 216.44

Table 4-1. FSIGT results. Comparison of minimal model data for young (n=6) and aged (n=6) mares. Data are expressed as mean ± s.e. There was no difference between young and aged horses for insulin sensitivity, glucose effectiveness, acute insulin response to glucose or disposition index (P>0.05).

Figure 4-1

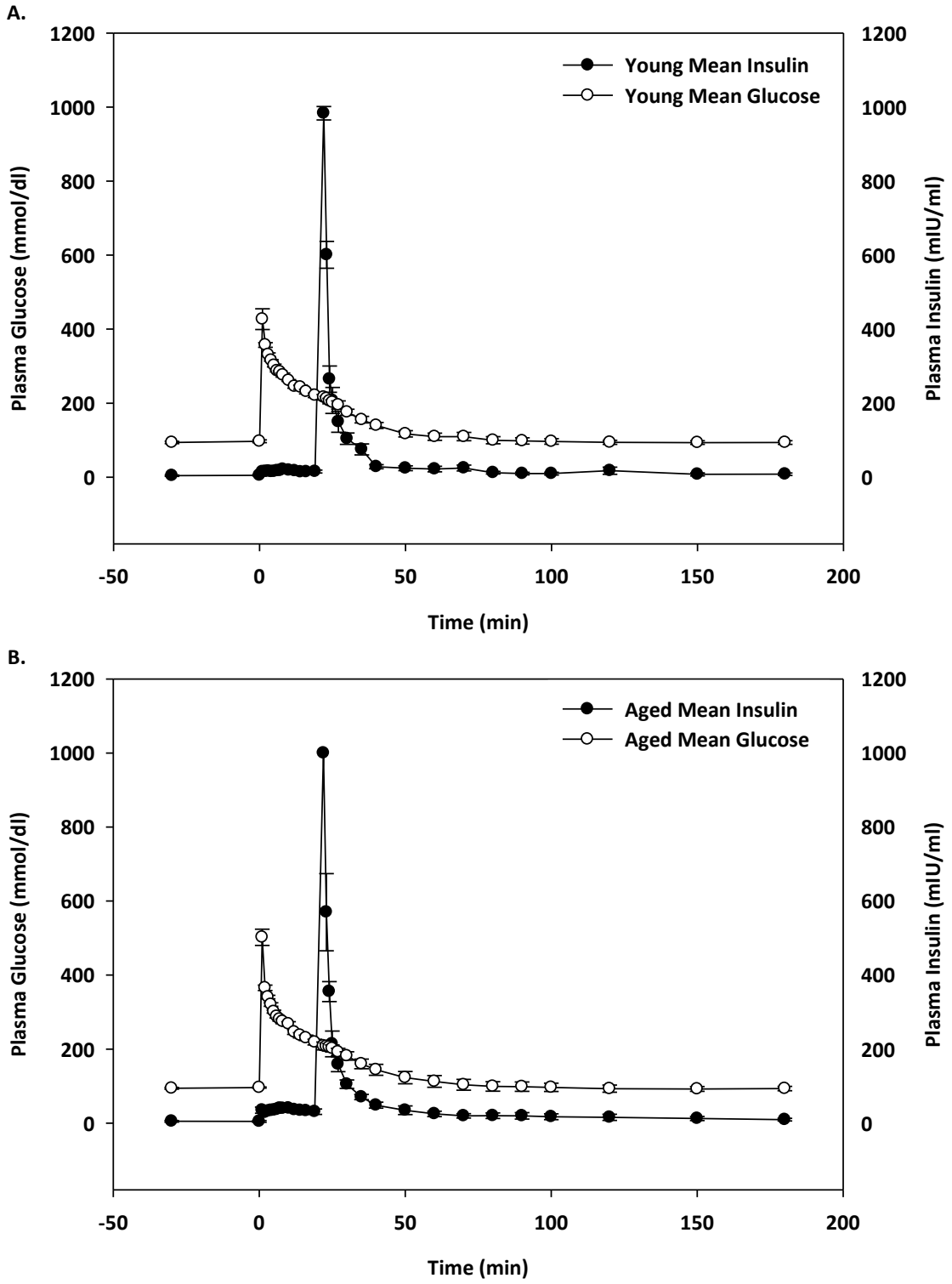


Figure 4-1. Insulin and glucose during the course of a frequently sampled intravenous glucose tolerance (FSIGT) test. Mean ± s.e. plasma insulin and glucose concentrations in young mares (4-1.A) and aged mares (4-1.B) during FSIGT testing from -30 to 180 minutes.

Plasma insulin and glucose in response to exercise

RIA determination of plasma insulin concentration in response to acute submaximal exercise showed that young mares had an elevated insulin concentration at 2 (P=0.009) and 4 (P=0.007) hours while aged mares had elevated insulin at 30 (P<0.001) and 60 (P=0.001) minutes post-exercise, however there was no significant difference between age groups at these times (P>0.05) (**Figure 4-2**). Neither age nor exercise caused a significant change in AUCi (P>0.05).

Glucose concentration in response to acute exercise, as determined via enzyme-electrode interface, revealed that young mares had elevated plasma glucose concentrations at 0 (P<0.001), 30 (P<0.001), 60 minutes (P<0.001) and 2 hours post-exercise (P<0.001), while in aged mares glucose remained elevated only at 0 (P<0.001), 30 (P=0.006) and 60 minutes (P=0.037) post-exercise. Plasma glucose concentration in young mares was significantly greater than in aged mares at 0 (P=0.008), 30 (P=0.009) and 60 minutes (P=0.041) post-exercise (**Figure 4-2**). Exercise caused an increase in AUCg in young (P=0.007) and aged (P=0.031) mares; however there was no difference in AUCg due to age (P>0.05). There was no difference in plasma glucose concentration or AUCg between young and aged standing control mares (P>0.05).

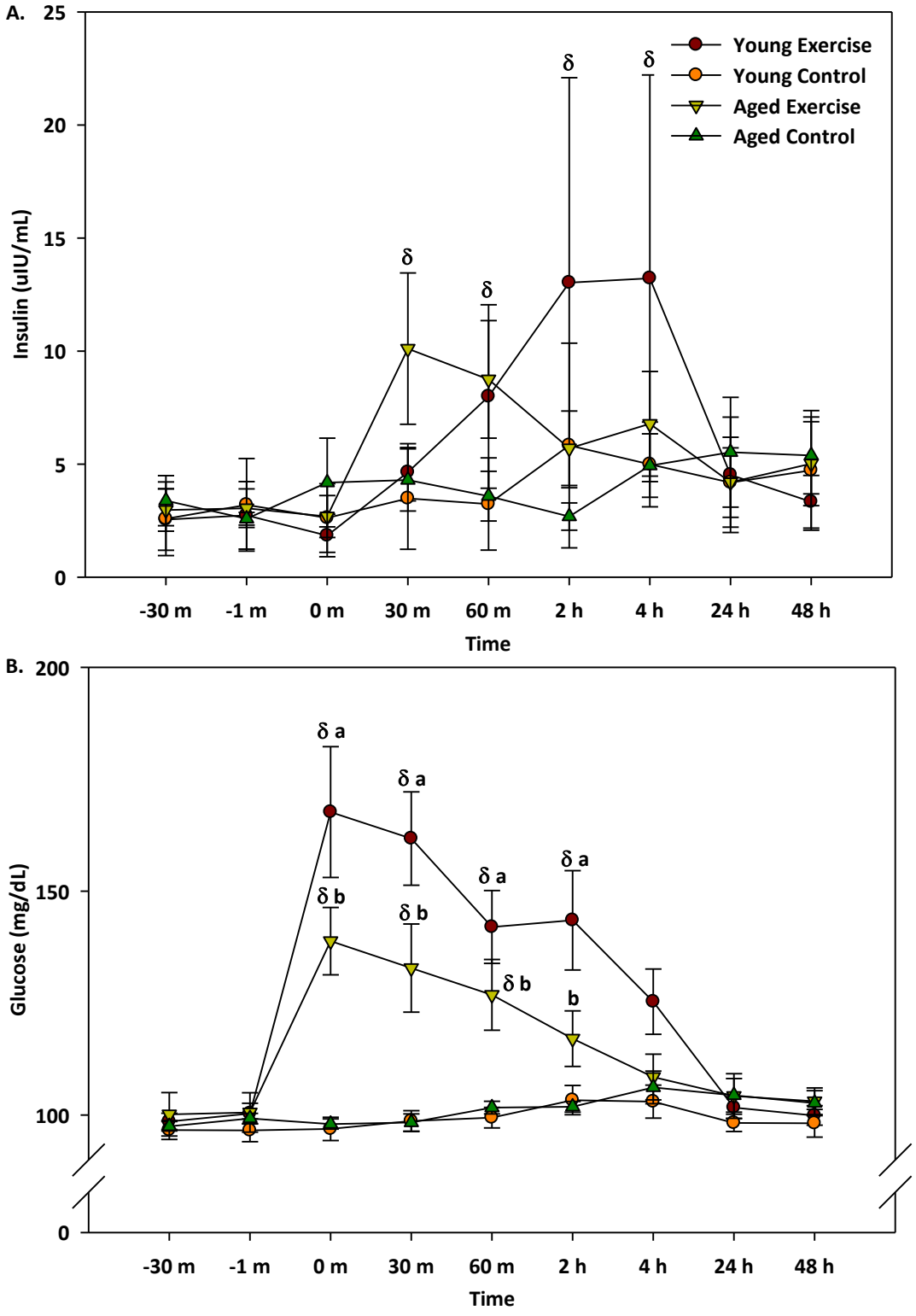
Finally, there were no differences in baseline insulin and glucose between young and aged mares (P>0.05).

Regression analysis

Linear regression analysis revealed a significant correlation between plasma insulin and plasma glucose concentrations following exercise in both aged (R=0.31; P=0.022) and young mares (R=0.376; P=0.008).

Figure 4-2. Mean ± s.e. plasma insulin and glucose concentrations in response to acute submaximal exercise. Plasma insulin (4-2.A) and glucose (4-2.B) concentrations in young (n=6) and aged (n=6) mares in response to acute submaximal exercise or standing control. Data points represented are those collected from before exercise or control treatments (-30 and -1 min), and at 0, 30, 60 min, 2, 4, 24 and 48 post-exercise or standing control. A delta (δ) symbol denotes an exercise related difference. Different superscripts (a, b) represent differences due to age.

Figure 4-2


A.
Insulin (uIU/mL)
25
20
15
10
5
0
Young Exercise
Young Control
Aged Exercise
Aged Control
δ
δ
δ
δ
-30 m
-1 m
0 m
30 m
60 m
2 h
4 h
24 h
48 h
Time
B.
Glucose (mg/dL)
200
150
100
0
δ a
δ a
δ a
δ a
δ b
δ b
δ b
b
-30 m
-1 m
0 m
30 m
60 m
2 h
4 h
24 h
48 h
Time

Western immunoblotting

Skeletal muscle content of several proteins believed to be involved with glucose uptake was quantified via western immunoblotting and densitometry in samples from young and aged mares that underwent acute submaximal exercise or standing control treatments. **Figures 4-3**, **4-4** and **4-5** illustrate western immunoblotting results for AMPK, Akt and AS160 in, respectively.

Phosphorylated AMPK (P-AMPK) was not altered by acute submaximal exercise in either age group ($P>0.05$), and was not different between young and aged standing controls ($P>0.05$). Total AMPK was not altered by acute exercise in either age group, and was not different between either young or aged standing control horses ($P>0.05$) (**Figure 4-3**).

Concentrations of phosphorylated Akt (P-Akt) were not altered by exercise in young and aged mares ($P>0.05$), and were not different between young and aged standing controls ($P>0.05$). Additionally, total Akt concentrations were not altered by age or acute submaximal exercise ($P>0.05$), and were not different between young or aged standing control horses (**Figure 4-4**).

Concentration of phosphorylated AS160 (P-AS160) was not altered in young or aged horses under either exercised or standing control conditions ($P>0.05$). Total AS160 protein concentrations also did not change in young or aged horses undergoing either acute submaximal exercise or standing control treatments ($P>0.05$) (**Figure 4-5**).

Figure 4-3. P-AMPK and AMPK protein content in equine skeletal muscle in response to acute submaximal exercise. Example of typical western immunoblotting results in skeletal muscle samples from young mares (n=6; 4-3.A) and aged mares (n=6; 4-3.B). Samples were taken before exercise (Pre), and at 0, 4, 24 and 48 hours post exercise. Each exercised horse (E) had time-matched standing control (C) samples taken on separate testing days. Chemiluminescence signals at 63 kDa correspond with P-AMPK and total AMPK, while signals at 37 kDa correspond with the loading control GAPDH. Chemiluminescence signals were quantified via densitometry, with values normalized to a control sample run on each gel. Values are reported as mean ± s.e. arbitrary units. Band quantification showed no change in either P-AMPK (4-3C) or total AMPK (4-3.D) content due to age or acute submaximal exercise.

Figure 4-3

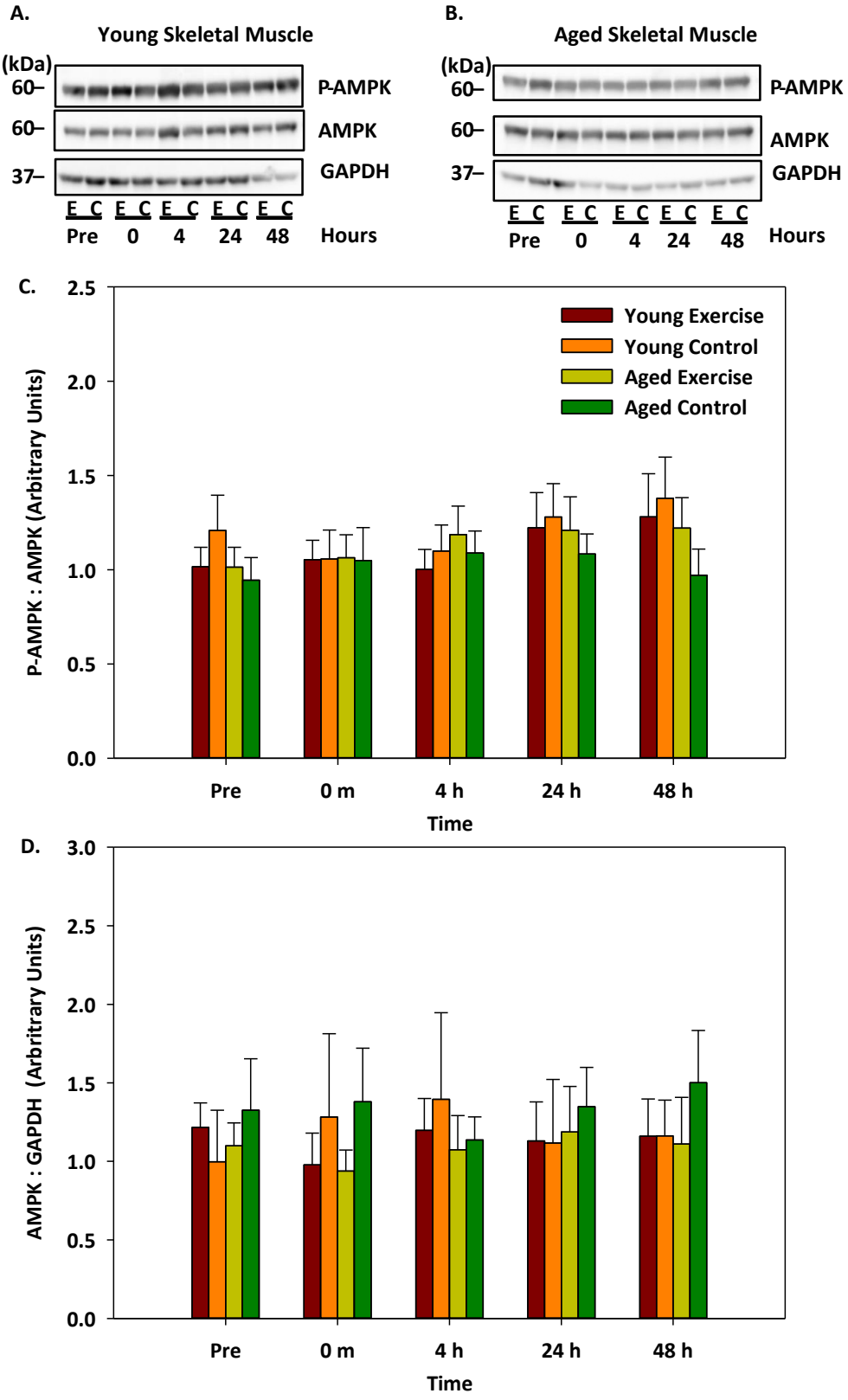

A.
Young Skeletal Muscle
(kDa)
60–
P-AMPK
60–
AMPK
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
B.
Aged Skeletal Muscle
(kDa)
60–
P-AMPK
60–
AMPK
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
C.
P-AMPK : AMPK (Arbitrary Units)
2.5
2.0
1.5
1.0
0.5
0.0
Young Exercise
Young Control
Aged Exercise
Aged Control
Pre
0 m
4 h
24 h
48 h
Time
D.
AMPK : GAPDH (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
0 m
4 h
24 h
48 h
Time

Figure 4-4. P-Akt and Akt protein content in equine skeletal muscle in response to acute submaximal exercise. Example of typical western immunoblotting results in skeletal muscle samples from young mares (n=6; 4-4.A) and aged mares (n=6; 4-4.B). Samples were taken before exercise (Pre), and at 0, 4, 24 and 48 hours post exercise. Each exercised horse (E) had time-matched standing control (C) samples taken on separate testing days. Chemiluminescence signals at 60 kDa correspond with P-Akt and total Akt, while signals at 37 kDa correspond with the loading control GAPDH. Chemiluminescence signals were quantified via densitometry, with values normalized to a control sample run on each gel. Values are reported as mean ± s.e. arbitrary units. Band quantification showed no change in either P-Akt (4-4.C) or total Akt (4-4.D) content due to age or acute submaximal exercise.

Figure 4-4

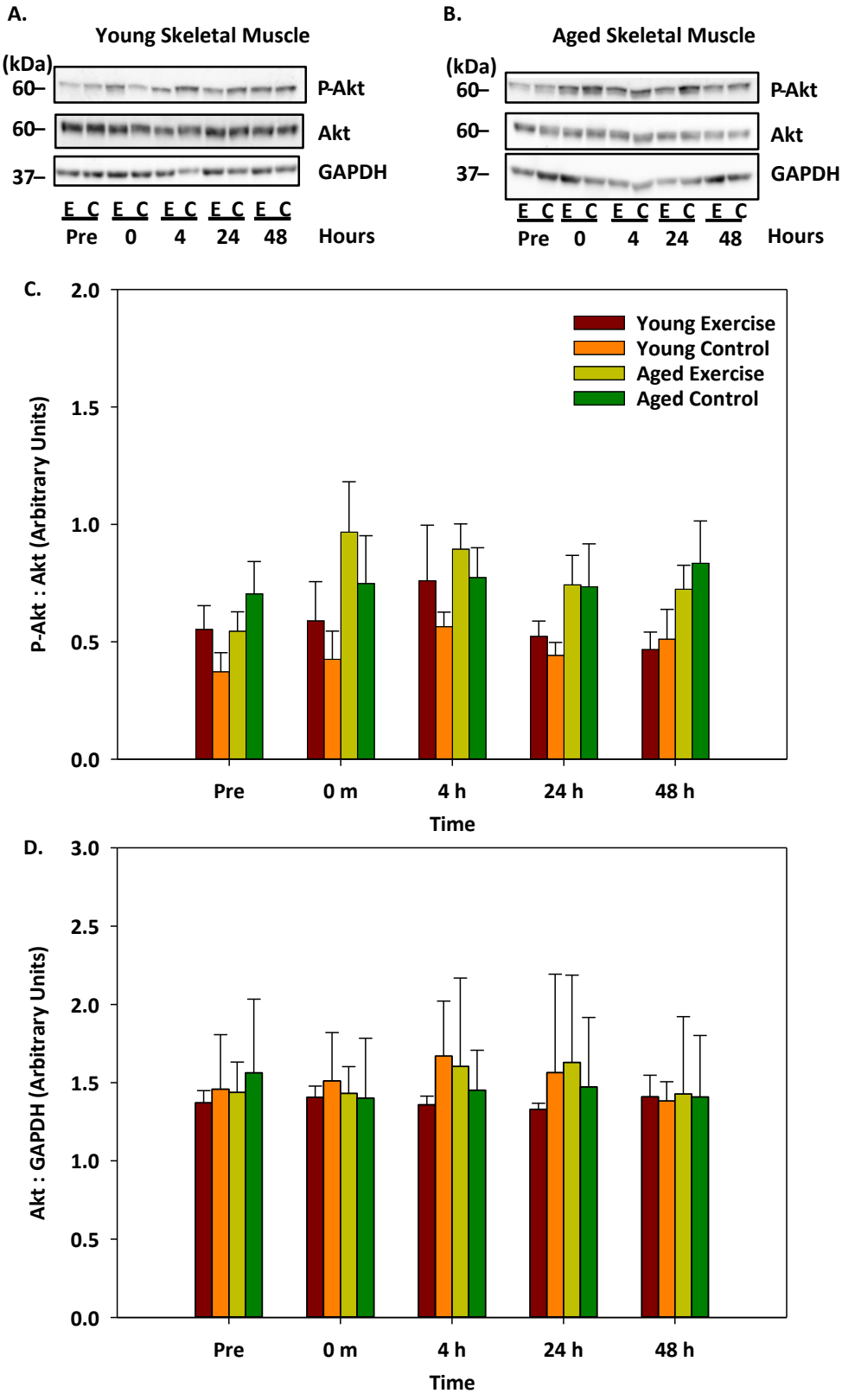
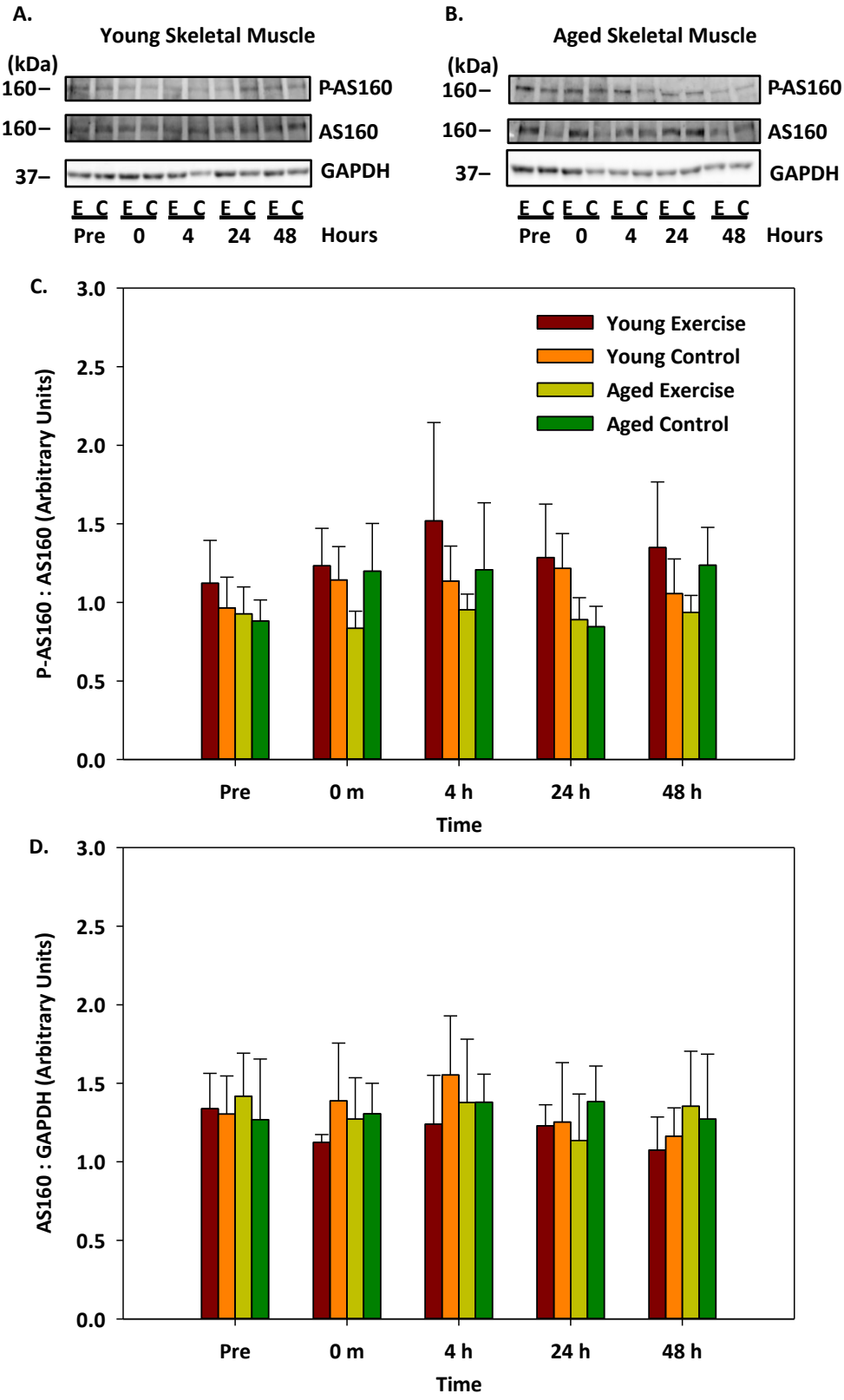

A.
Young Skeletal Muscle
(kDa)
60–
P-Akt
60–
Akt
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
B.
Aged Skeletal Muscle
(kDa)
60–
P-Akt
60–
Akt
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
C.
Young Exercise
Young Control
Aged Exercise
Aged Control
P-Akt : Akt (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
Pre
0 m
4 h
24 h
48 h
Time
D.
Akt : GAPDH (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
0 m
4 h
24 h
48 h
Time

Figure 4-5. P-AS160 and AS160 protein content in equine skeletal muscle in response to acute submaximal exercise. Example of typical western immunoblotting results in skeletal muscle samples from young mares (n=6; 4-5.A) and aged mares (n=6; 4-5.B). Samples were taken before exercise (Pre), and at 0, 4, 24 and 48 hours post exercise. Each exercised horse (E) had time-matched standing control (C) samples taken on separate testing days. Chemiluminescence signals at 160 kDa correspond with P-AS160 and total AS160, while signals at 37 kDa correspond with the loading control GAPDH. Chemiluminescence signals were quantified via densitometry, with values normalized to a control sample run on each gel. Values are reported as mean ± s.e. arbitrary units. Band quantification showed no change in either P-AS150 (4-5.C) or total AS160 (4-5.D) content due to age or acute submaximal exercise.

Figure 4-5


A.
Young Skeletal Muscle
(kDa)
160–
P-AS160
160–
AS160
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
B.
Aged Skeletal Muscle
(kDa)
160–
P-AS160
160–
AS160
37–
GAPDH
E C E C E C E C E C
Pre 0 4 24 48 Hours
C.
P-AS160 : AS160 (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Young Exercise
Young Control
Aged Exercise
Aged Control
Pre
0 m
4 h
24 h
48 h
Time
D.
AS160 : GAPDH (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
0 m
4 h
24 h
48 h
Time

Discussion

Insulin sensitivity, glucose-insulin homeostasis and β-cell function were assessed in young and aged mares via FSIGT and minimal model analysis. The data indicate that there was no difference in insulin sensitivity, glucose effectiveness, acute insulin response to glucose or disposition index between unfit young and aged Standardbred mares. This indicates that the horses used in this study had no overt signs of equine metabolic syndrome or other endocrine dysfunction. Previous work from our lab has indicated that mares over the age of 20 had significantly altered insulin sensitivity and β-cell function (Malinowski et al., 2002; Liburt et al., 2012). The difference from previous work to that reported here may be due to the observation that all mares used in the current study were of similar weight and body condition score. The literature indicates that decreased insulin sensitivity and development of insulin resistance in horses are primarily caused by weight gain and obesity, increased chronic inflammation, and diets high in starch and sugar (Hoffman et al., 2003; Frank et al., 2006; Vick et al., 2007; Vick et al., 2008; Adams et al., 2009; Carter et al., 2009). Dietary restriction and weight loss have proven effective at improving insulin sensitivity (McGowan et al., 2013) and reducing systemic inflammation (Adams et al., 2009). While exercise training can alter insulin sensitivity in young, mature and aged horses (Malinowski et al., 2002; Stewart-Hunt et al., 2006; Liburt et al., 2012), exercise training without dietary restriction will not reverse insulin resistance in obese horses (Carter et al., 2010). Advanced age may therefore only exacerbate reduced insulin sensitivity occurring primarily due to obesity and systemic inflammation. More pronounced differences in this study may have been observed through the inclusion of young lean, young obese, aged lean and aged obese groups.

Data reported here indicate that changes in plasma insulin and glucose concentrations in response to acute submaximal exercise are not altered by age. However, the timing of the

insulin response to exercise was different between young and aged mares. Young mares had peak plasma insulin concentration at 2 hours post-exercise as compared to peak plasma insulin concentration at 30 minutes post-exercise in aged mares. Plasma glucose concentrations are elevated in both age groups at the completion of exercise, and decline through 60 minutes post-exercise in aged mares, and 2 hours post-exercise in young mares. Differences in plasma insulin and glucose dynamics between young and old mares are most likely related to altered cortisol levels in aged mares. Cortisol typically peaks at 30 minutes post-exercise in horses, and will remain elevated through 1 hour post-exercise (Gordon et al., 2007; McKeever et al., 2014). Aged horses have a decreased cortisol response to acute exercise (Horohov et al., 1999; Malinowski et al., 2006). Cortisol and insulin have opposing roles concerning energy homeostasis: cortisol is responsible for energy substrate mobilization, while insulin mediates cellular glucose uptake (McKeever et al., 2014). An altered cortisol response to acute exercise in aged horses may explain the earlier rise in plasma insulin concentration and the blunted concentration of plasma glucose seen here. Additionally, cortisol signaling through glucocorticoid receptors may be impaired in aged horses, further diminishing its effectiveness as a mediator of energy homeostasis. Inhibition of glucocorticoid receptors with age has been suggested in the comparative literature (Roth and Livingston, 1976; Kalimi, 1984; Peiffer et al., 1991; Perlman et al., 2007), but no known studies have examined their activity in horses.

Cellular uptake of glucose during and in response to acute exercise relies on contraction-induced GLUT4 translocation to the cellular membrane (Douen et al., 1990a; Douen et al., 1990b; Holloszy and Hansen, 1996; Hayashi et al., 1997; Goodyear and Kahn, 1998). Contraction-mediated skeletal muscle glucose uptake is believed to be due to activation of and signaling through AMPK (Hutber et al., 1997; Rasmussen and Winder, 1997; Vavvas et al., 1997; Hayashi et al., 1998; Rasmussen et al., 1998; Bergeron et al., 1999; Ihlemann et al., 1999b; Kurth-Kraczek

et al., 1999; Hayashi et al., 2000). Greater insulin-independent glucose uptake mechanisms may be present in tissue from young animals, allowing for a delayed increase in plasma insulin concentration while still replenishing cellular energy stores. Reduced ability of contraction-induced glucose uptake mechanisms in aged animal tissue may explain the earlier rise in plasma insulin concentration seen here. This may also explain why it has been previously reported that aged mares had greater plasma insulin concentrations following acute incremental exercise to fatigue (Malinowski et al., 2002).

Information concerning AMPK activation in equine skeletal muscle is lacking, but extensive work has been done investigating AMPK activity in regards to exercise and aging. Activation of AMPK was decreased in skeletal muscle from aged rats, although total AMPK protein content and *AMPK* expression was not diminished with age (Qiang et al., 2007). Altered expression and activity of AMPK in skeletal muscle occur in conjunction with diet-induced insulin resistance in rats (Kraegen et al., 2006; Liu et al., 2006). Chemical induction of AMPK activity is typically accomplished by treatment with 5-amino-imidazole-4-carboxamide ribonucleoside (AICAR) (McCarty, 2004). AICAR treatment and acute exercise normally activated AMPK in induced AMPK expression in skeletal muscle of both normal and diabetic humans, with no differences between groups (Musi et al., 2001). AICAR treatment (Reznick et al., 2007a) and acute exercise (Reznick et al., 2007b; Ljubicic and Hood, 2009) activated AMPK in skeletal muscle of young rats, but aged rats displayed no change in AMPK activity in response to either stimulus.

The results reported here indicate that skeletal muscle AMPK activation is not different between young and aged horses in response to acute submaximal exercise. Previous work has shown that AMPK activity is exercise intensity-dependent, with activation occurring when muscle glycogen levels are low (Derave et al., 2000; Wojtaszewski et al., 2000; Wojtaszewski et al., 2002; Chen et

al., 2003; Barnes et al., 2005). Therefore differential activation of AMPK may occur in horses undergoing strenuous exercise.

This study also investigated skeletal muscle Akt activity in response to acute exercise in young and aged horses. Insulin-stimulated glucose uptake in skeletal muscle is believed to occur approximately 3 – 4 hours post-exercise (Hansen et al., 1998), which coincides with skeletal muscle Akt phosphorylation occurring 0 -3 hours following exercise (Katta et al., 2009). Evidence suggests that Akt is activated by *in vitro* skeletal muscle contraction (Sakamoto et al., 2002; Sakamoto et al., 2003; Bruss et al., 2005; Sakamoto et al., 2006; Arias et al., 2007a). Evidence for exercise-induced Akt activation *in vivo* is mixed, with some studies reported increased Akt phosphorylation following skeletal muscle contraction (Nader and Esser, 2001; Sakamoto et al., 2003; Katta et al., 2009), while others report no changes in Akt activity (Markuns et al., 1999; Wojtaszewski et al., 1999; Wadley et al., 2004). Contraction-induced activation of Akt *in vitro* is reduced in aged skeletal muscle (Funai et al., 2006; Haddad and Adams, 2006), and post-exercise Akt phosphorylation is blunted in skeletal muscle of aged humans (Fry et al., 2011). The data reported here indicate that Akt is not activated in skeletal muscle following acute submaximal exercise, and that aging does not affect Akt phosphorylation.

AS160 represents the convergence of insulin- and exercise-induced glucose uptake by skeletal muscle. The role of AS160 in GLUT4 vessicle exocytosis was first identified in adipocytes (Sano et al., 2003; Zeigerer et al., 2004). In skeletal muscle, AS160 becomes activated through phosphorylation in response to insulin or contractile activity *in vitro* (Bruss et al., 2005), in mice (Kramer et al., 2006a; Kramer et al., 2006c) and in humans (Bruss et al., 2005; Treebak et al., 2007; Dreyer et al., 2008). AMPK likely stimulates GLUT4 translocation through phosphorylation of AS160 (Bruss et al., 2005; Kramer et al., 2006a; Treebak et al., 2007), and Akt is responsible

for AS160 phosphorylation in response to insulin (Gonzalez and McGraw, 2006; Sequea et al., 2012). Age-related decreases in phosphorylation of Akt and AS160 were observed following stimulation with insulin (Gupte et al., 2008), and AS160 activity is reduced in skeletal muscle of insulin resistant humans (Karlsson et al., 2005) and rodents (Lessard et al., 2007; Thyfault et al., 2007b). Arias and colleagues sought to elucidate the exercise-induced AS160 activation pathway. Concerning contraction-mediated glucose uptake by skeletal muscle, AMPK, Akt and AS160 were all found to be phosphorylated post-exercise, but it was concluded that exercise-induced AS160 activation was due to AMPK activity alone (Arias et al., 2007a).

Data on AS160 phosphorylation in equine tissue is limited, and indicate that in an unstimulated state there is no difference in levels of phosphorylated AS160 in skeletal muscle (Waller et al., 2011a) or adipose tissue (Waller et al., 2011b) between normal or insulin resistant horses. However, these studies did not utilize acute exercise or other form of homeostatic perturbation. To our knowledge, this was the first study to attempt characterization of AS160 activity in skeletal muscle of young and aged horses following acute exercise. Our findings indicate AS160 activation was not altered by either age or acute submaximal exercise.

In conclusion, age alters the insulin and glucose response to acute submaximal exercise in horses; however age alone is not sufficient to alter insulin sensitivity in clinically normal mares. Although the findings presented here do not indicate changes in AMPK, Akt or AS160 activity in skeletal muscle, this does not preclude alterations in their activation in aged tissue. Exercise intensity determines activation of AMPK and Akt, therefore the exercise test used may not have been sufficient to provoke changes in their activation. Furthermore, western immunoblotting is a semi-quantitative method of protein analysis; therefore a more nuanced approach may be required for quantification of these proteins in equine tissue.

Chapter 5.

Conclusions and future directions

Important Findings

The major findings of this study were: 1) acute submaximal exercise was a significant physiological challenge in young and aged mares; 2) peak post-exercise plasma cortisol concentrations were similar in young and aged mares, but young mares had elevated plasma cortisol concentrations for a longer period post-exercise; 3) acute submaximal exercise prompted a change in *IL-1β* expression in blood, with young mares having greater changes in expression; 4) *TNF-α* and *IL-6* were not altered in blood following acute submaximal exercise, and no changes in cytokine expression were observed in muscle post-exercise; 5) *HSP7*0 expression in blood was altered post-exercise in both young and aged mares, but young mares had a greater change in and longer duration of HSP70 expression following exercise; 6) in blood, *HSP90* expression was altered following acute submaximal exercise in young horses only; 7) in skeletal muscle, *HSP70* expression increased immediately following exercise in aged mares, and was increased at 4 hours post-exercise in both young and aged mares; 8) HSP90 expression in skeletal muscle was increased at 4 hours post-exercise in both young and aged mares; 9) skeletal muscle HSP70 and HSP90 protein content were not altered by either age or acute submaximal exercise; 10) timing and magnitude of plasma insulin and glucose concentrations post-exercise were altered by age; and 11) there were no detectible changes due to age or acute submaximal exercise for total or phosphorylated AMPK, Akt and AS160 in skeletal muscle of horses.

Limitations

While this experiment elucidated some of the mechanisms of cellular adaptation to exercise, there were limitations to the study design and execution. First, a limited number of animals were available for this study. A greater number of young and aged horses, as well as inclusion of

lean and obese young and aged mares, may have created enough power to observe statistical differences in several of the parameters investigated. Additionally, this experiment used only female horses. Inclusion of both intact and castrated male horses could have provided clues for determination of sex-related differences in cortisol, inflammatory cytokines, heat shock proteins and mediators of GLUT4 translocation and energy homeostasis following acute exercise.

Second, while young and aged horses were significantly different in terms of chronological age, the aged mares used in this study may have been phenotypically similar to middle-aged horses. This observation is based on similar values for VO_{2max} between young and aged horses as stated in Chapter 2, similar peak cortisol concentrations between young and aged horses following exercise as stated in Chapter 2, and similar insulin sensitivity between young and aged horses as stated in Chapter 4. Selection of aged mares for inclusion in this experiment was limited to clinically healthy, sound mares from the resident heard of the New Jersey Agricultural Experiment Station. Future work should include a greater number of phenotypically aged horses.

Finally, quantification of skeletal muscle proteins of interest, such as HSP70, HSP90, AMPK, Akt and AS160, was hindered by lack of adequately sensitive methods. As mentioned previously, western immunoblotting is a semi-quantitative method, best suited for qualitative experiments. There are currently limited methods for tissue protein quantification. However, the results presented here demonstrate that the proteins of interest are highly conserved among mammalian species, therefore validation of new protein quantification methods in equine tissue is eminently feasible. Additionally, since AMPK and Akt are known transcription factors, quantification of gene expression downstream of AMPK or Akt activation may provide an indirect measure of their activity in young and aged equine tissue.

Summary and Conclusions

The data from this study suggest that cortisol and insulin concentrations following a single bout of exercise are altered in aged horses. This can have implications for energy substrate utilization and recovery of skeletal muscle. The alteration in post-exercise plasma glucose concentrations due to age reported here indicate that glucose mobilization and uptake in response to exercise may be impaired in aged horses.

The changes in post-exercise *HSP70* and *HSP90* expression in whole blood due to age may indicate an impaired ability to respond and adapt to acute disturbances in homeostasis. Additionally, earlier increased expression of *HSP70* in aged skeletal muscle may indicate that older horses have a lower threshold for the heat shock protein response.

Finally, no changes were observed for skeletal muscle protein content of HSP70 or HSP90, or for total or phosphorylated AMPK, Akt and AS160. These proteins are believed to be important for the recovery from and adaptation to exercise. The lack of observable changes in this study is most likely due to the semi-quantitative nature of western immunoblotting, and is not without precedence in the literature.

Implications for future research

Several steps can be taken to build on the findings presented here. First, validation of multiplex protein assays may be necessary to assess changes in serum and tissue content of the proteins of interest investigated here. With proper quantitative tools future investigations can also expand to other members of the heat shock protein super family, inflammatory signaling mediators such as JNK, and other members of the insulin signaling pathway in equine tissue. The

highly conserved nature of these proteins makes validation of new techniques possible, and will further establish horses as proper models for aging and exercise physiology.

While this study focused on a single bout of submaximal exercise, the effects of exercise conditioning on molecular mediators of inflammation, insulin signaling and adaptation to homeostatic disturbances should be investigated in response to exercise training. In particular, acute exercise and exercise conditioning can be used to determine the functionality of the heat shock protein response in young, aged and obese horses. These findings would have translatable information for horse trainers working with aging horses or with horses coming out of extended periods of inactivity.

Finally, HSP expression in whole blood may be a useful biomarker for the post-exercise adaptive response in horses. This study was limited in the number of skeletal muscle biopsies compared to the number of whole blood samples. In whole blood HSP expression seemed to be most altered at 2 hours post-exercise. While HSP expression in skeletal muscle was altered at 4 hours post-exercise, caution should be used when drawing direct comparisons between whole blood and skeletal muscle HSP expression. With better time-matched sampling of whole blood and skeletal muscle post-exercise, it can be determined if HSP expression of whole blood can be a suitable biomarker for the adaptive response to exercise in horses. This can be a useful tool for assessment of adaptation to exercise in horses undergoing exercise conditioning. Also, the potential of HSP expression as a biomarker can be useful for researchers studying insulin resistance and aging in horses.

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