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# The Role of Stress Factors in Skeletal Muscle Development, Growth, and Metabolism; Impacts of Temporally Spaced Repetition on Long-Term Information Retention in an Anatomy and Physiology Course

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# THE ROLE OF STRESS FACTORS IN SKELETAL MUSCLE DEVELOPMENT, GROWTH AND METABOLISM; IMPACTS OF TEMPORALLY SPACED REPETITION ON LONG-TERM INFORMATION RETENTION IN AN ANATOMY AND PHYSIOLOGY COURSE

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# THE ROLE OF STRESS FACTORS IN SKELETAL MUSCLE DEVELOPMENT, GROWTH AND METABOLISM; IMPACTS OF TEMPORALLY SPACED REPETITION ON LONG-TERM INFORMATION RETENTION IN AN ANATOMY AND PHYSIOLOGY COURSE

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We sought to identify the effect of sustained maternal inflammation at mid-gestation on fetal mortality, skeletal muscle growth, and development at term. Fetal rats had reduced total mass that coincided with impaired myoblast function, as myoD was reduced and myogenin expression was increased in hindlimb muscle. Fetuses also had increased circulating TNFα at term after maternal inflammation, which decreased TNFR and IL6R mRNA in muscle, reducing sensitivity to cytokines. These findings indicate that sustained maternal inflammation at mid-gestation impairs fetal skeletal muscle growth near-term due to changes in myoblast responsiveness to inflammatory regulation.

To evaluate the acute impact of inflammatory and adrenergic factors on muscle metabolism, basil and insulin-stimulated glucose uptake and oxidation was measured in primary rat soleus muscle incubated with TNFα, IL-6, β1 adrenergic agonist, or β2 adrenergic agonist with or without insulin. Beta 2 adrenergic agonist increased basal glucose oxidation, decreased basal glucose uptake, and synergistically increased insulin-stimulated glucose oxidation and Akt phosphorylation, but β1 agonist had no effect. Inflammatory cytokines antagonized insulin signaling but simultaneously increased glucose oxidation via MAPK. Together, our findings show acute exposure to

these stress factors increases glucose oxidation independently of glucose uptake and insulin signaling.

A final study identified the effects of timing of retrieval practice on long-term information retention. Students were given online retrieval practices one or five days after introduction of material. Short-term information retention was measured at one week and long-term information retention at semester's end. Timing of repetition had no discernable effects on short-term retention at one week. Average-performing students (B to D- overall) exhibited better long-term retention on three of four evaluated topics when retrieval practice occurred at five days. High-performing students (B+ or greater overall) exhibited better long-term retention in, one of four topics when retrieval practice occurred at five days. Increasing the interval between introduction to material and first retrieval practice enhances long-term information retention.

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# Chapter 1

# Literature Review

## Introduction

Intrauterine growth restriction (IUGR) predisposes offspring to metabolic dysfunction later in life. Poor prenatal growth can be a result of various maternal stressors including obesity, malnutrition, prolonged illness, substance abuse, and more (Valsamakis et al., 2006). Numerous epidemiological studies (Godfrey and Barker, 2000; Newsome et al., 2003; Barker, 2006) have linked poor intrauterine growth to lifelong deficiencies in skeletal muscle mass and an increased incidence of metabolic health problems. Growth restriction is most commonly due to a lack of sufficient nutrient transfer to the fetus due to placental insufficiency (Baschat, 2004; Cox and Marton, 2009). These fetuses experience chronic hypoxia, hypoglycemia, hypertension, and increased lactate (Thorn et al., 2011). In order to survive, growth restricted fetuses with placental insufficiency adapt to the poor intrauterine environment by repartitioning nutrients to vital organs at the expense of skeletal muscle, which impairs skeletal muscle growth, glucose metabolism, and insulin regulation. These adaptations allow the fetus to survive in utero, but, the consequences of metabolic adaptations are lifelong. The “thrifty phenotype hypothesis,” first linked prenatal adaptations to increased risk for metabolic diseases after birth (Hales et al., 1991). There has been a fair amount of investigation into the etiologies of these adaptations, but less is known about the role of stress factors in IUGR adaptive skeletal muscle development, growth, and metabolism.

## Skeletal Muscle Growth and Development

Skeletal muscle makes up about 40% of total body mass, predominantly in the hindlimbs, head and trunk area (DeFronzo et al., 1981b; Brown, 2014). Each muscle fiber is made up of multiple myoblasts that have fused during embryonic and early fetal development, yielding a fiber with hundreds of peripherally located nuclei (Yablonka-Reuveni et al., 1999), a characteristic not seen in cardiac or smooth muscle. Skeletal muscle is striated in appearance due to actin and myosin filaments that contract in order to move the skeleton. It is a highly metabolic organ that metabolizes glucose and fatty acids for energy. Skeletal muscle is constantly undergoing growth and repair; however, crucial muscle structure is established in utero.

### *Myogenesis*

Skeletal muscle is one of the main organs involved in metabolic homeostasis and therefore adequate development and growth is crucial for metabolic health (DeFronzo et al., 1981a; Brown, 2014). The development of skeletal muscle occurs through a multi-step process known as myogenesis. This process first involves migration of progenitor cells from the somite which then become committed to myoblasts. These myoblasts will proliferate, differentiate and eventually fuse to form multinucleated myofibers (Mitchell et al., 2002). The initial development of primary myofibers form the scaffolding of the muscles halfway through the first trimester. Toward the end of the first trimester, secondary myotubes begin to form around the existing primary fibers, but may not run the full length of the muscle (Wilson et al., 1992). A final wave of tertiary myotubes form around the existing fibers to complete myogenesis early in the third trimester. Thus, fiber number is static before birth and any postnatal skeletal muscle growth occurs by hypertrophy, which requires fusion of quiescent myoblasts with existing fibers to increase nuclear protein synthesis capacity.

*Myogenic regulatory factors (MRFs)*

Each step of myogenesis is carefully-regulated by a series of myogenic regulatory factors (MRFs) that stimulate or inhibit these processes. MRF regulation begins in the embryonic stage, when signals from the neural tube and notochord induce myogenesis with the expression of two paired-box (Pax) transcription factors, Pax3 and Pax7, that will initiate activation of the precursor cells originating from somites (Sambasivan and Tajbakhsh, 2007; Bentzinger et al., 2012).

Terminal differentiation of the myoblasts is dependent on the sequential expression of addition MRF transcription factors belonging to the basic-Helix-Loop-Helix (bHLH) family (Figure 1). They are myogenic determining factor 1 (myoD), myogenic factor 5 (myf5), and myogenin (Molkentin and Olson, 1996). After migration from the somites, high expression of myf5 followed by myoD mark these as active muscle cells, initiating the transformation from muscle stem cell to muscle progenitor cell (Rudnicki et al., 1993; Bentzinger et al., 2012). Expression of myf5 is the first indication that muscle progenitor cells are active and capable of proliferation and differentiation. It is typically co-expressed with Pax7, or shortly after (Bentzinger et al., 2012). Terminal differentiation coincides with the onset of myoD and myogenin expression, which signals the myoblasts to exit the cell cycle and fuse to form myotubes (Andrés and Walsh, 1996; Yablonka-Reuveni et al., 1999). The increase in myogenin initiates expression of Desmin, a type III intermediate filament, from differentiated myoblasts and formed myotubes during embryonic development that controls the structural development of the sarcomere. Desmin and myogenin are continually expressed throughout gestation as myoblasts fuse and form myofibers. These primary myofibers form the scaffolding of the muscles that run tendon-to-tendon and is present halfway through the first trimester.

In most mammals hyperplasia ends before birth and thus skeletal muscle must rely on satellite cells for growth and repair throughout postnatal life (Chiakulas and Pauly, 1965; Allen et al., 1979). Some myoblasts proliferate during fetal growth in the same organized manner as those being targeted for fiber formation; however, after primary fiber development a portion of these myoblasts do not fuse to form fibers but instead become quiescent satellite cells. These satellite cells are inactive stem cells that are stored adjacent to the muscle fiber between the sarcolemma and endomysium (Yablonka-Reuveni et al., 1999; Morgan and Partridge, 2003). During injury, skeletal muscle growth or cell death these satellite cells are activated to proliferate and differentiate into myoblast and then fuse with existing muscle fibers, thus adding nuclei and increasing protein synthesis (Anderson, 2006). Pools of satellite cells are maintained and stored within the muscle and are activated due to mechanical activities such as exercise, and even more so for severe injury (Anderson, 2006).

Quiescent myoblasts will continue to express Pax7 but those undergoing differentiation and fusion into myocytes, in some species, will have a decline in Pax7 coinciding with the increase in myoD expression (Yablonka-Reuveni et al., 1999). After myoblast proliferation, this group of mononucleated cells are able to upregulate myoD and exit the cell cycle, yet recover Pax7 expression. Differentiating myoblasts uphold myoD expression and upregulate myogenin as part of differentiation (Hawke and Garry, 2001; Day et al., 2009). This gives cells the ability to exit the cell cycle or allow for self-renewal to maintain stores of satellite cells available to act in the muscle without depleting the muscle stem cell pool. Throughout postnatal life, when muscle fibers need to be repaired, satellite cells are activated by upregulating myoD and undergoing proliferation, differentiation and then fusing with to existing muscle fibers (Zammit et al., 2004). The number of satellite cells within storage pools is highest in the postnatal

period and decreases with age (Morgan and Partridge, 2003). Any interruption in fetal myogensis can lead to impairment of this process and negative consequences for life-long skeletal muscle growth.

*Muscle fiber types and characteristics*

Skeletal muscle in most species is composed of heterogeneous proportions of three different fiber types (with rodents and pigs having four) each with their own unique characteristics. Fiber types are characterized by differing metabolic phenotypes related to twitch speed and strength of contraction. Moreover they are able to change over time based on change in activity and metabolic demand within the body (Rubinstein and Kelly, 1981). Type 1 muscle fibers contain myosin heavy chain Iβ and appear red as a result of higher concentrations of myoglobin, capillary content, and mitochondria (Scott et al., 2001). These features give type 1 fibers greater oxidative capacity, allowing them to use oxidative phosphorylation as their primary metabolic pathway and resulting in slower shortening and but greater duration due to a resistance to fatigue. Type 2a fibers are intermediate between type 1 and type 2x. They are red, fast-twitch fibers that utilize both oxidative and glycolytic pathways for energy (Ciciliot et al., 2013). Lastly, type 2x fibers are white (due to much less myoglobin and capillary content) fast-twitch fibers that use anaerobic glycolysis as their main metabolic pathway. They are more powerful but also more susceptible to fatigue (Schiaffino et al., 1989).

**Skeletal Muscle Glucose Metabolism**

Skeletal muscle makes up approximately 40% of total body mass, is responsible for 65% of total glucose metabolism, and account for greater than 85% of insulin-stimulated glucose metabolism (DeFronzo et al., 1981a; Brown, 2014). Glucose is the primary substrate for energy production within the body, and maintaining homeostatic

levels is primarily facilitated by skeletal muscle. Whole-body glucose utilization is mainly regulated by the production and release of insulin, as an increase in circulating insulin is associated with greater glucose consumption rates in the muscle (Rizkalla et al., 2004). High blood glucose concentrations stimulate the production and release of insulin. Once released, insulin inhibits production of glucose in the liver and stimulates uptake of glucose into cells for storage or metabolism (Saltiel and Kahn, 2001).

*Insulin & IGF-1 regulated muscle metabolism*

Insulin is the main hormone responsible for regulating blood glucose concentrations throughout the body. It is a protein hormone secreted by the β cells of the islet of Langerhans within the endocrine pancreas (Ionescu-Tirgoviste et al., 2014). Release of insulin is stimulated by blood glucose concentrations exceeding homeostatic levels. Tissues such as skeletal muscle and adipose tissue utilize glucose as energy or store it as glycogen (Pessin and Saltiel, 2000; Fritsche et al., 2008). The binding of insulin to its tyrosine kinase insulin receptor (IRβ) on the plasma membrane of cells leads to recruitment of insulin receptor substrate 1 (IRS-1) which can activate multiple pathways (Figure 2). One target protein, phosphatidylinositol-3-kinase (PI3K), activates protein kinase B (Akt/PKB) and protein kinase C (PKC) cascades (Wojtaszewski et al., 2000). Akt inhibits protein degradation by activating FoxO and stimulates protein synthesis via mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β) (Schiaffino and Mammucari, 2011). Furthermore, Akt stimulates the translocation of Glut4 transporters to the plasma membrane to increase glucose uptake (Pirola et al., 2004).

Insulin-like growth factor 1 (IGF-1) in combination with growth hormone is primarily responsible for tissue growth. Yet, studies show IGF-1 can have differing

tissue-specific effects (Baker et al., 1993). Schiaffino et. al. described the process whereby IGF-1 bound to its receptor functions through the same pathway as insulin to induce protein synthesis or degradation (Schiaffino and Mammucari, 2011). Binding of IGF-1 activates IRS 1/2 via tyrosine phosphorylation leading to activation of PI3K. This generates phosphoinositide-3,4,5,-triphosphate (PIP3) that creates a docking site for phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate and activate Akt. This process can stimulate protein cycling in the same manner as insulin via recruitment and activation of FoxO or mTOR, respectively (Schiaffino and Mammucari, 2011). Normal signal transduction of these two pathways is crucial for both skeletal muscle growth and metabolism.

*Metabolic fates of glucose*

When blood glucose is above homeostatic levels, insulin is released to stimulate uptake of glucose by skeletal muscle. Once in the cell, glucose can be metabolized multiple different ways (Figure 3), depending upon fiber type composition of the muscle. After entering the cell and being converted to glucose-6-phosphate, glucose cannot leave un-metabolized (Jensen et al., 2011). First, glucose-6-phosphate can be converted into glycogen and stored for later use. Metabolized glucose initially goes through the process of anaerobic glycolysis, which converts glucose into pyruvate and generates small amounts of ATP and NADH (Nelson et al., 2008). Pyruvate then enters the mitochondria where it is used by the TCA cycle to generate NADH and $FADH_2$. These reducing agents then continue to the electron transport chain to be used for oxidative phosphorylation (Nelson et al., 2008). Oxidative phosphorylation is a slower process, but is the most efficient use of glucose, as it produces much more energy than glycolysis alone (~36 ATP compared to ~2 ATP, respectively) (Lodish et al., 2000). When nutrient supply is low, as is the case in the nutrient restricted fetus, pyruvate can be metabolized

to lactate which can then leave the muscle cell and be sent to the liver to reform glucose (gluconeogenesis) in a process known as the Cori cycle. This switch from glucose oxidation to glycolytic lactate production is commonly associated with increased adrenergic activity (Kusaka and Ui, 1977; Limesand et al., 2007a).

## Cytokine Regulation of Skeletal Muscle

### *Resident macrophage phenotypes*

Cytokines are small signaling proteins that are released in greatest amounts by white blood cells, although many other tissues are capable, including muscle, fat and connective tissue. In skeletal muscle cytokines originate primarily from resident macrophages within the skeletal muscle (Wijesundera et al., 2014). Macrophages are heterogenic and express both pro and anti-inflammatory phenotypes (Figure 4) (Arnold et al., 2007). Classical activation results in the M1 phenotype that is pro-inflammatory, and thus produces inflammatory cytokines (Chazaud et al., 2009). This type of activation typically takes place after injury or an infectious event. Once the inflammatory response and has occurred, M1 macrophages begin to transition to the M2 phenotype through a polarization process facilitated by the interferon regulatory factors and signal transducer and activator of transcription factors (Barros et al., 2013; Wang et al., 2015). The M2 phenotype promotes tissue repair and recovery via production and secretion of growth factors, such as transforming growth factor β1 (TGF-β1) that suppresses inflammatory cytokines (Serhan and Savill, 2005).

### *Inflammatory cytokines*

The interleukin and tumor necrosis factor families of cytokines have been well-characterized. Two of the most commonly-studied inflammatory cytokines are

interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) (Dinarello, 2000). The release of these and other inflammatory cytokines mediates the inflammatory response and recruits white blood cells. This response can be suppressed by anti-inflammatory cytokines and other chemical signals such as suppressor of cytokine signaling (SOCS) and I-kB (O'Shea and Murray, 2008).

TNFα has two plasma membrane bound receptors, TNFR1 and TNFR2, but TNFR1 is the predominant isoform found in most tissues throughout the body, including skeletal muscle (Locksley et al., 2001). Binding of TNFα to TNFR1 receptor stimulates most of TNFα's physiologic activities (Chen and Goeddel, 2002). Once activated, TNFR1 recruits multiple adapter proteins that activate key enzymes such as IKK, which triggers the NF-κB signaling pathway (Figure 5). When inactivate, NF-κB is held in the cytosol and complexed to IκBα, which inhibits its activity. During inflammation IKK phosphorylates the IκBα protein, causing its ubiquitination and subsequent degradation, facilitating the release of NF-κB (Perkins, 2007). Activated NF-kB translocates to the nucleus and mediates the transcription of proteins involved in multiple processes of inflammation. NF-κB is known to increase the production and release of cytokines from macrophages and is therefore a common target in the search for drugs to treat inflammatory diseases (Hanada and Yoshimura, 2002).

IL-6 has a type-1 cytokine plasma membrane receptor (IL6R) composed of a ligand binding protein and a signal-transducing glycoprotein (Jones and Rose-John, 2002). Binding of I-L6 to its receptor activates janus kinase-3 (JAK3) and in turn transcription 3 (STAT3). Phosphorylation of STAT3 leads to its translocation to the nucleus where it mediates transcription of different inflammatory genes. Activated JAK/STAT commonly interacts with other pathways such as those initiated by receptor tyrosine kinase binding and the MAPK pathway (Rawlings et al., 2004). The signaling of

IL-6 is inhibited by the presence of SOCS and protein inhibitor of activated STAT (PIAS) proteins (Guo et al., 2012).

*Cytokine impact on insulin action*

Exposure to inflammatory cytokines inhibits insulin-stimulated translocation of Glut4 transporters to the plasma membrane but not the amount of Glut4 within the cell (Lorenzo et al., 2008). However, multiple studies suggest that by activating a non-canonical stress pathway, TNFα and IL-6 acutely stimulate glucose uptake independently of insulin (Carey et al., 2006a; Lorenzo et al., 2008; Vraskou et al., 2011; Saini et al., 2014b). The mechanism by which cytokines use to cause insulin resistance is not well-understood, however it is hypothesized that they inhibit activation of downstream targets of insulin (Lumeng and Saltiel, 2011). Cytokines reduce tyrosine autophosphorylation of the insulin receptor and inhibit Akt phosphorylation, a key facilitator of insulin action (Plomgaard et al., 2005; Bach et al., 2013). The conflicting results regarding whether inflammatory cytokines stimulate or inhibit glucose metabolism may be due to length of exposure. Prolonged exposure to TNFα and IL-6 has been linked to the development of insulin resistance in skeletal muscle (Marette et al., 2014). In individuals that are obese, these cytokines are highly produced in adipose tissue and elicit effects systemically (De Alvaro et al., 2004). Understanding this mechanism of action may be key in combating metabolic disease, as the development of insulin resistance is an important precursor to type II diabetes mellitus.

**Adrenergic Regulation of Skeletal Muscle**

*Adrenergic receptors and signaling pathways*

The catecholamines epinephrine and norepinephrine are released from the adrenal medulla into circulation and bind to a family of 7-transmemebrane g-protein

coupled adrenergic receptors expressed by tissues throughout the body (Table 1) (Neves et al., 2002). G proteins are a family of proteins that bind to GTP or GDP to elicit a response. In skeletal muscle, β2 receptors are the most prevalent isoform, but β1 and β3 are also present and are thought to function similarly to each other (Kim et al., 1991b). Variations in adrenergic profiles occur among different fiber types (Williams et al., 1984; Martin et al., 1989). β receptors work primarily through Gαs proteins to recruit adenylyl cyclase, which is responsible for the production of cAMP (Figure 6). Increased intracellular cAMP activates protein kinase A (PKA), subsequently phosphorylating regulatory proteins leading to a multitude of different responses, such as vasodilation, bronchodilation, smooth muscle relaxation and glucose regulation (Johnson, 1998). The β2 receptor can also be coupled to Gαi protein which activates an alternative signaling pathway that leads to the activation of p38 mitogen activated protein kinase (MAPK) (Daaka et al., 1997). Gαq proteins are stimulatory and commonly activated by hormones involved in calcium mobilization. Activation of these proteins leads to the activation of phospholipase C and the recruitment of IP3 and DAG (Neves et al., 2002).

*Acute actions of catecholamines in metabolism*

Norepinephrine and epinephrine alter homeostatic glucose and fat metabolism (Nonogaki, 2000). β adrenergic receptor stimulation has been shown to alter skeletal muscle glucose oxidation, energy expenditure, and glucose transport (Nevzorova et al., 2002; Pearen et al., 2009). Catecholamines alter normal glucose metabolism in order to re-direct available glucose to critical tissues. This is achieved, in part, by inhibition of insulin secretion via α2 adrenergic receptors on pancreatic beta cells and increasing glucagon secretion (Limesand et al., 2006; Yates et al., 2011a). Glucagon is released from the pancreas when insulin concentration is low in order to mobilize glucose stores. This activity inhibits glucose uptake by skeletal muscle, leaving more glucose available

for the brain and heart (Ngala et al., 2013) . Catecholamines and glucagon can further increase blood glucose concentrations by stimulating gluconeogenesis in fasting states (Shimazu, 1996). Catecholamines can also activate glycogenolysis in the muscle and liver in fed states to increase blood glucose concentration (Perseghin et al., 1997). Since skeletal muscle cannot regenerate glucose, the glycogen will be converted to lactate and shuttled to the liver for gluconeogenesis or the heart for metabolism (Nonogaki, 2000).

*β agonist use in livestock*

β agonists and antagonists have been used in the pharmaceutical industry for some time. The impact β adrenergic stimulation has on lean muscle growth has led to the use of dietary β adrenergic agonists for growth promotion in livestock including cattle, pigs, and sheep (Beermann, 2002). These adrenergic agonists are isoform specific and have been shown to stimulate skeletal muscle accretion and decrease fat mass, thus improving feed efficiency (Mersmann, 1998). This increase in skeletal muscle growth is suspected to be the result of increased protein synthesis, decreased protein degradation, or both (Kim and Sainz, 1992). The two β agonists currently FDA approved in livestock are ractopamine HCl (β1 specific) and zilpaterol HCl (β2 specific) (Scramlin et al., 2010). Both improve muscle growth through increased protein synthesis, but studies show zilpaterol also decreases protein degradation (Crome et al., 1996). These β agonists have been beneficial in enhancing muscle growth in animals for meat production despite the physiologic mechanism that leads to more efficient growth still being unknown.

**Placental Function**

Normal fetal growth is dependent on adequate transfer of nutrients between the dam and the developing fetus, and the placenta fulfills this critical role. Nutrients and

fetal wastes are transported across the placental barrier by simple diffusion (oxygen and carbon dioxide), facilitated diffusion (glucose and lactate) and active transport (amino acids) (Bauer et al., 1998). The placenta is an important hormone-producing tissue that plays a role in the growth, development, and metabolism of the fetus. Furthermore, it protects the fetus from pathogens, and the maternal immune system (Regnault et al., 2002). The efficiency of nutrient transfer is dependent on normal placental growth and vasculature development. The placenta is the junction where fetal and maternal blood supplies maintain intimate contact, and fetal and placental weights are affected when the materno-fetal interface is reduced (Knight et al., 1977; Reynolds and Redmer, 1995).

*Fetal growth & development*

Throughout gestation, fetal growth increases exponentially and must be accommodated by increased nutrient supply. Fetal size increases most dramatically in late gestation, yet maximum placental size is reached about midway through gestation (Reynolds and Redmer, 1995). However, the vascularity of the placenta and thus its nutrient transport capacity continues to develop until term. During the period of peak fetal growth, the uterine blood flow increases three to four-fold (Rosenfeld et al., 1974). This change in uterine blood flow coincides with an increase in umbilical blood flow as well. Vascular growth in the placenta is regulated by angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) that appear to originate predominantly from the maternal side of the placenta (Burgess and Maciag, 1989; Sharkey et al., 1993). During the period of peak placental growth (mid-gestation), many forms of chronic maternal stress can stunt placental growth. A placenta that is not able to meet the nutrient demands of the fetus results in a condition known as placental insufficiency, which is the most common cause of intrauterine growth restriction (IUGR) (Reynolds et al., 2009).

## Fetal Adaptations to Stress

*Placental insufficiency-induced intrauterine growth restriction (PI-IUGR)*

Disruption in the normal flow of nutrients from the dam to fetus due to placental insufficiency impairs fetal growth and alters development. The chronic lack of fetal nutrients in late gestation causes fetal adaptations resulting in a phenotype known as intrauterine growth restriction (IUGR), which can have both perinatal and postnatal consequences (Figure 7). IUGR affects 10-15% of the population in the United States, 25% worldwide, and is a major cause of fetal and infant morbidity and mortality (Saleem et al., 2011). IUGR is caused by numerous different maternal factors such as prolonged illness, obesity, malnutrition, and environmental stressors that stunt the placenta. In later gestation as fetal growth increases rapidly, the stunted placenta is unable to meet the nutrient demands of the fetus (Yates et al., 2012a).

In response to a lack of adequate nutrients, the fetus adapts by repartitioning nutrients for organs that are vital to survival such as the brain, heart, and liver at the expense of skeletal muscle and fat, which causes asymmetric growth restriction (Hales and Barker, 1992; Long et al., 2009). After birth nutrients are abundant, but the growth and metabolic adaptations that occur due to nutrient sparing activity in utero persist throughout postnatal life. The thrifty phenotype hypothesis states that prenatal adaptations in response to poor nutrition make IUGR-born individuals about 18-fold more susceptible to developing metabolic syndrome later in life compared to their average-sized counterparts (Hales and Barker, 2001). Barker's hypothesis has lead to a new area of research called Developmental Origins of Health and Disease (DOHaD), which attempts to identify these programming events that result in postnatal metabolic dysfunction.

*Characteristics of the IUGR fetus*

The delivery of oxygen to the developing fetus is crucial for normal growth and metabolic function. The demand for oxygen and other nutrients continues to increase throughout gestation and peaks near term (Godfrey and Barker, 2000). For this reason, uteroplacental blood flow must increase throughout gestation to maintain nutrient requirements for the fetus. Oxygen transport can be affected by multiple factors such as umbilical and uterine blood flow, placental surface area and permeability, and hemoglobin content and binding affinity (Carter, 1989). Normal umbilical and uterine blood flow are the most critical component of oxygen delivery but under nutrient deficient conditions, vasculature develops incorrectly. Studies using Doppler velocimetry on PI-IUGR fetuses have demonstrated that development of placental vasculature is deficient, thus preventing the increase in nutrient transfer in later gestation and causing progressively worsening fetal hypoxemia throughout the third trimester (Regnault et al., 2003; Galan et al., 2005). A decrease in placental permeability also creates a hypertensive phenotype in PI-IUGR fetuses, as restricted diffusion leads to an increase heart rate to maintain adequate nutrient delivery to the brain, heart and adrenal glands (Galan et al., 2005). Near term, PI-IUGR sheep and human fetuses have blood oxygen levels 30-50% lower than normal fetuses (Barry et al., 2008; Macko et al., 2016).

When fetuses are hypoxic, epinephrine and norepinephrine secretion from the adrenal medulla is dramatically increased (Yates et al., 2014). This increase in catecholamine release stimulates an increase in heart rate and blood pressure to maintain adequate oxygen delivery to critical organs. In addition, insulin secretion is inhibited, which decreases glucose uptake in insulin-sensitive skeletal (Jellyman et al., 2005). Since an insufficient placenta can fulfill normal nutrient requirements until the fetus outgrows it, the increase in catecholamines does not occur until late gestation

(Macko et al., 2013; Andrews et al., 2014). Studies in IUGR humans and rats have shown up to a 5-fold increase in catecholamine concentration in plasma (Hiraoka et al., 1991). High concentrations of catecholamines in the blood together with decreased insulin appears to shift the metabolic fate of glucose to a proportional increase in anaerobic glycolysis and a proportional decrease in oxidative phosphorylation. Anaerobic glycolysis produces large quantities of lactate, which subsequently causes hyperlactatemia and acidosis in the developing fetus (Yates et al., 2012a).

Hypoglycemia is a hallmark of PI-IUGR, even when maternal glucose concentrations are at normal levels. Glucose homeostasis can initially be maintained throughout the first part of gestation when the fetus is small, but when peak growth takes place, the fetus's initial response is to mobilize glucose stores (Baschat, 2004). However, the ability of the fetus to store glucose is limited and the fetus becomes hypoglycemic by the start of the third trimester (Gagnon, 2003). In order for glucose to get to the fetus, it must pass through the placenta via facilitated diffusion (Illsley, 2000). In IUGR fetuses where placental vasculature development is impaired, transporting adequate glucose across the placenta becomes increasingly difficult (Economides and Nicolaides, 1989). Oxygen and glucose are directly correlated to fetal growth and in late gestation, when nutrient demand is at an all-time high and not able to be met, growth can be stunted up to 50% (Baschat, 2004; Barry et al., 2008).

*Skeletal muscle adaptations*

The tissue most affected by repartitioning of nutrients is skeletal muscle. If nutrient deficiency occurs during early gestation, fiber number can be dimished, as this is the period when primary fibers are forming (Zhu et al., 2004). Conversely, if hypoxemia and hypoglycemia occur in late gestation, muscle hypertrophy rather than

number will be impaired as the majority of fibers are already formed (Greenwood et al., 2000; Yates et al., 2016). Recent studies have found that PI-IUGR fetuses exhibit smaller fiber diameters but not fewer fibers when compared to controls due to impaired proliferation and differentiation of myoblasts (Yates et al., 2014). Moreover, the proportions of fiber types are altered in IUGR fetuses. We recently found that highly oxidative fibers are reduced in the hindlimb of IUGR fetal sheep but glycolytic fibers were not different, likely due to differences in sensitivity to catecholamines (Yates et al., 2016). This altered distribution of fiber types likely decreases the oxidative capacity of IUGR muscle, shifting metabolism to anaerobic glycolysis (Jensen et al., 2007). The decrease in growth capacity and metabolic capacity may explain the deficit of lean mass and more marked asymmetric growth.

*Adaptations in insulin regulation*

Skeletal muscle is the primary site for insulin-stimulated glucose utilization and thus is sensitive to changes in circulating insulin. IUGR fetuses demonstrate a decrease in circulating insulin and IGF-1 concentrations (Harper et al., 1987; Thorn et al., 2009). IGF-1 stimulates myogenesis and the reduction in IGF-1 may contribute to reduced muscle mass. Fetal growth is also dependent on insulin and a well-functioning β cell population. In response to low levels of glucose, β cells increase their sensitivity to glucose (Chen et al., 2014b). However, elevated catecholamines have also been shown to cause up to a 76% reduction in beta cell mass accompanied by decreased plasma insulin concentrations (Limesand et al., 2005). Chronic exposure to catecholamines decreases insulin levels, in order to suppress nutrient-expensive muscle growth in an environment that does not favor it (Butler et al., 2003).

In a hypoxic environment, catecholamines are released from the fetal adrenal medulla (Yates et al., 2012b) and bind to α2- adrenergic receptors on beta cells in the pancreas to suppress insulin secretion while concurrently stimulating α cells to release glucagon (Leos et al., 2010a). The hypoinsulinemic states redistributes glucose to critical organs and skeletal muscle compensates by increasing its use of fatty acid oxidation. Fatty acids are converted to lactate which is then used in the Cori cycle hepatic gluconeogenesis. This system is active in the PI-IUGR fetus unlike normal fetuses and allows lactate to be turned into glucose by the liver (Limesand et al., 2007a).

*Postnatal impacts of IUGR fetal adaptations*

The adaptations that occur before birth predispose IUGR-born individuals to greater risk for the development of obesity, diabetes, and insulin resistance during childhood and later in life (Hales and Barker, 2001; McMillen and Robinson, 2005; Thorn et al., 2011). The underlying mechanisms for these programming events are unknown, but nutrient-sparing adaptations cause the IUGR individuals to be characterized by reduced muscle mass and muscle growth capacity (DeFronzo et al., 1981a; Yates et al., 2014). These adaptations persist throughout life and disrupt normal muscle growth and metabolism. Adaptive muscle development also impairs protein synthesis (Limesand et al., 2009) and fat mobilization (Desai and Ross, 2011), resulting in a higher fat-to-muscle ratio at term. However, increased fat deposition is independent of the decrease in skeletal muscle. These adaptations lead to catch-up growth postnatally, increasing susceptibility to obesity, type-2 diabetes, insulin resistance and cardiovascular disease (Kensara et al., 2005). IUGR adaptations result in permanent alterations of tissue structure and functionality. The shift from glucose oxidation to fat oxidation in skeletal muscle has been proposed as the main cause of decreased muscle mass and increased

fat (Dulloo, 2008). Reduced muscle mass and thus insulin-stimulated glucose utilization likely contribute to whole body insulin resistance in adulthood (Yates et al., 2014).

## Conclusion

Much is known about how the different physiological components develop under normal intrauterine circumstances, but adaptive fetal programming in response to chronic fetal stress and the associated postnatal outcomes remain a necessary topic of investigation. The objective of the following studies was to help fill the gap in knowledge about how inflammatory and adrenergic components affect skeletal muscle growth, development, and metabolism. Metabolic syndrome affects 35% of all adults in the United States and is increasing (Eckel et al., 2005). Understanding the physiological mechanisms of these diseases and their origins will provide important information that could lead to pre and post-natal interventions.

**Table 1.** Adrenergic receptor sub-type characteristics

| Sub-type | Type of G-Protein Receptor | Predominant Location |
|---|---|---|
| α1 | Gαq | smooth muscle<br>vascular smooth muscle |
| α2 | Gαi | presynaptic neurons |
| β1 | Gαs | heart |
| β2 | Gαs, Gαi | smooth muscle<br>skeletal muscle<br>vascular smooth muscle |
| β3 | Gαs | adipose tissue |

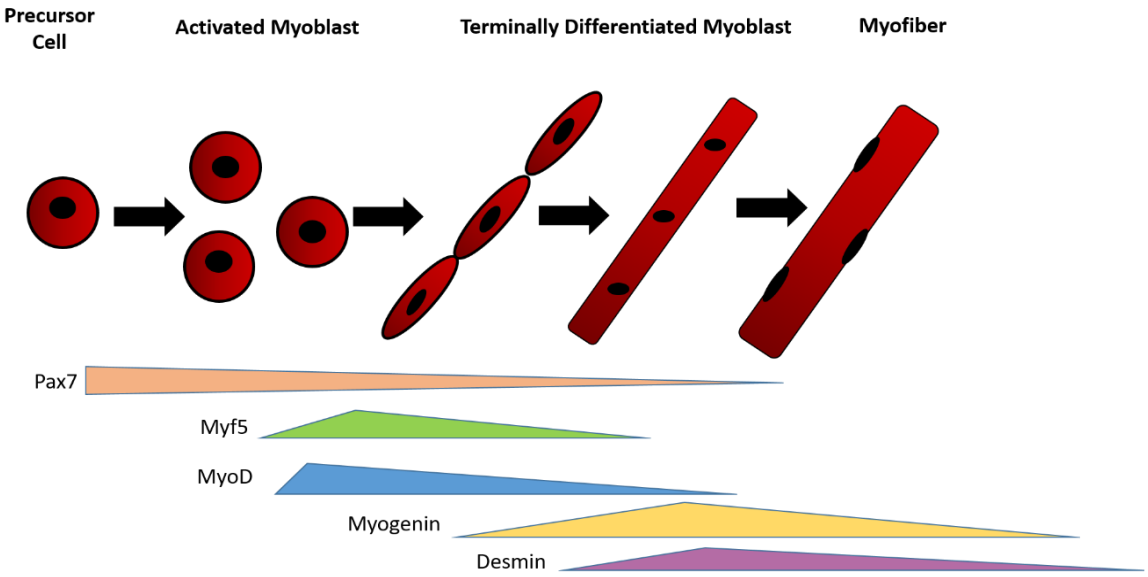


**Figure 1.** Myogenic progression from precursor cells to mature muscle fibers.

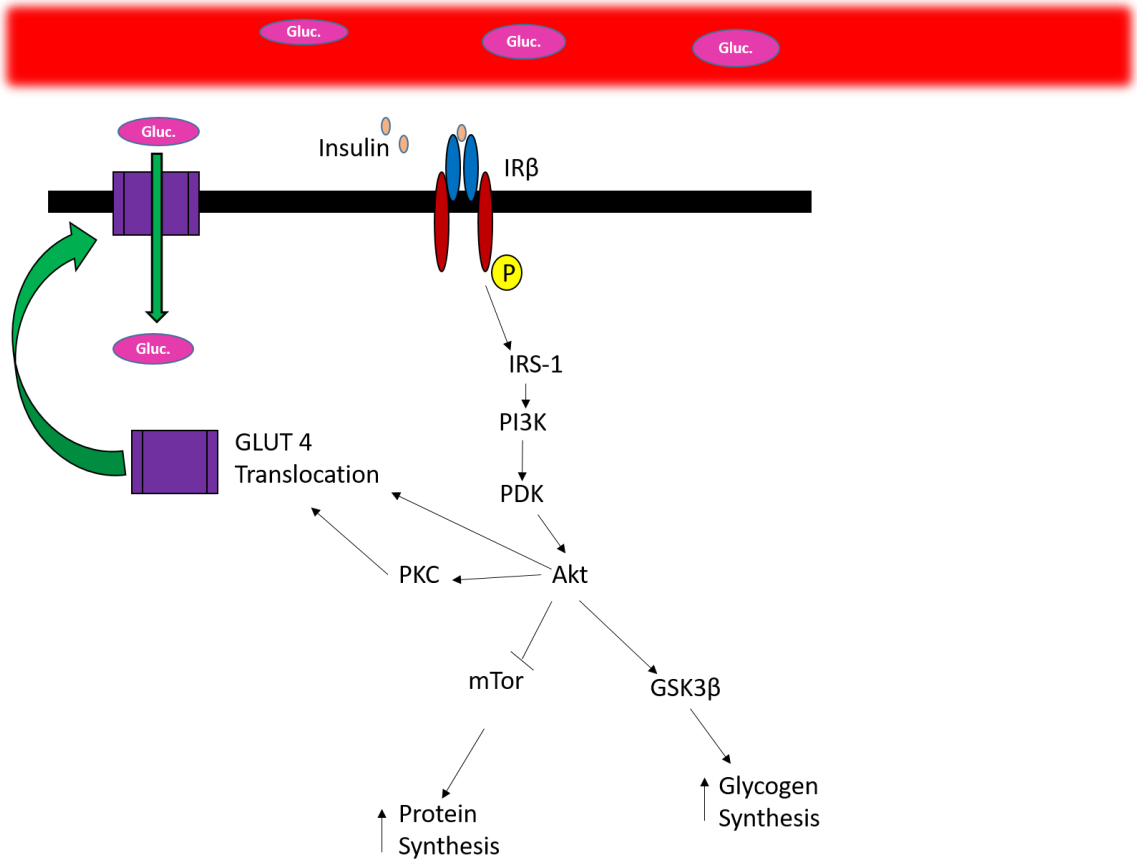


**Figure 2.** Schematic illustrating the major pathways by which insulin controls glucose transport and metabolism in skeletal muscle.

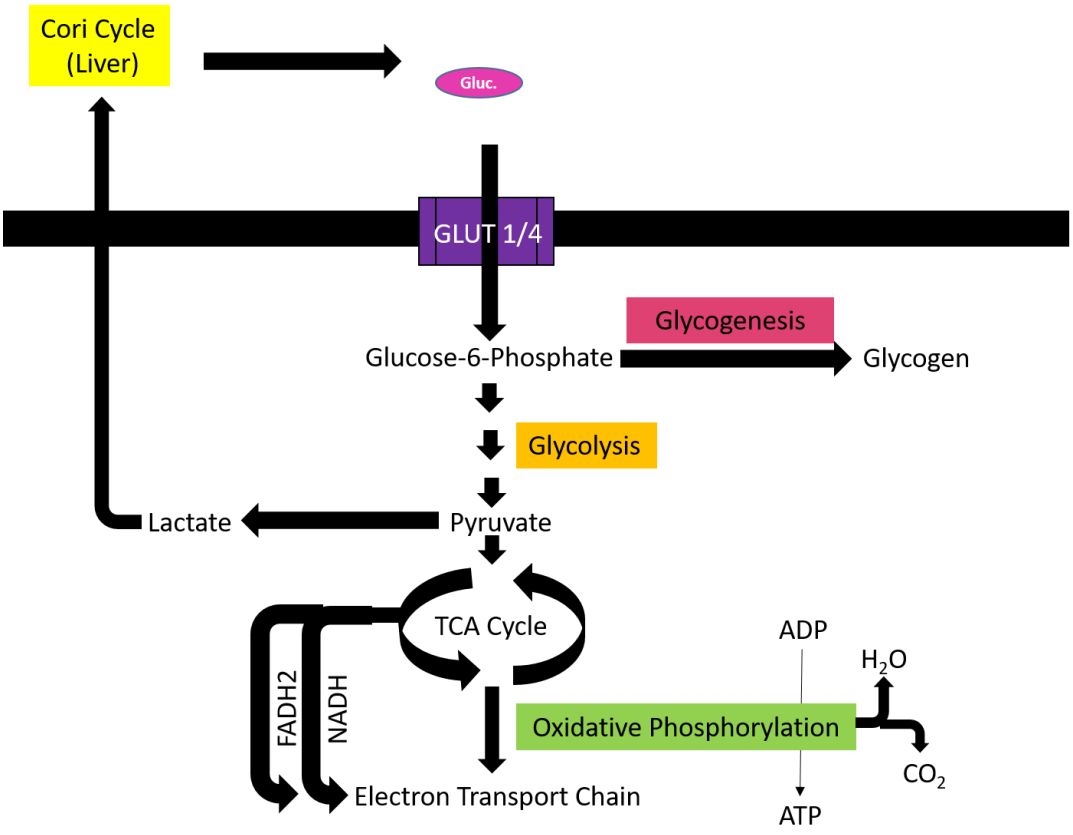


**Figure 3.** Primary metabolic fates of glucose within skeletal muscle.

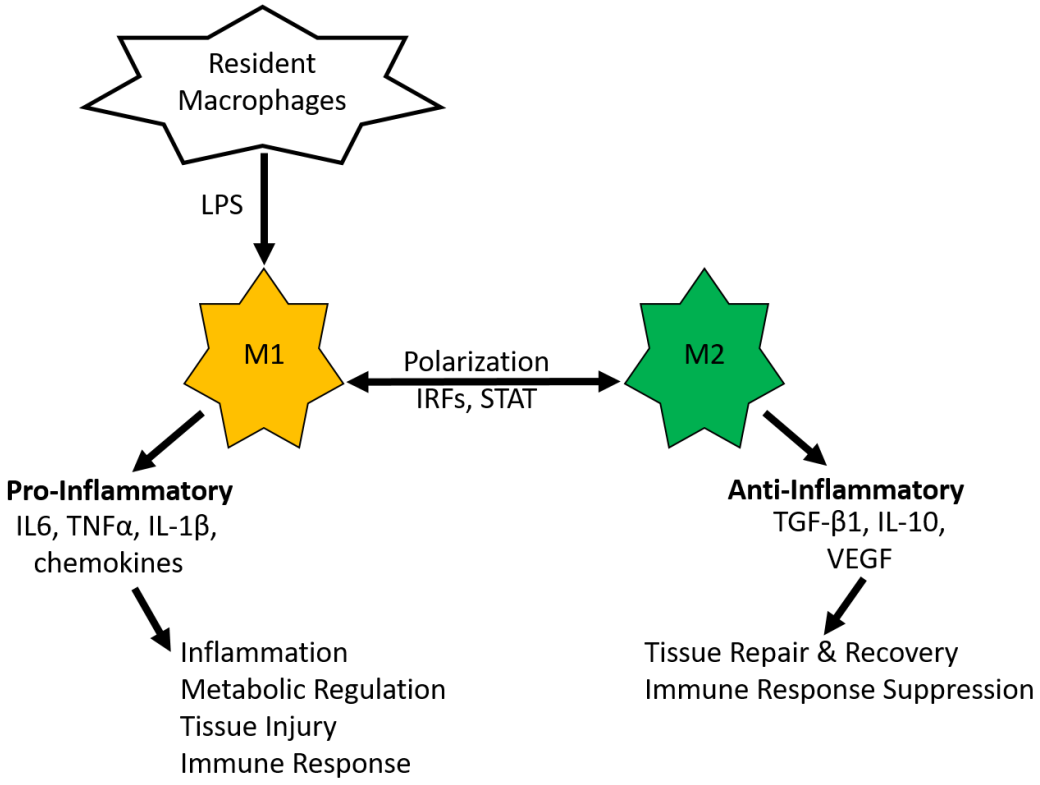


**Figure 4.** Illustration of the different properties of pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages.

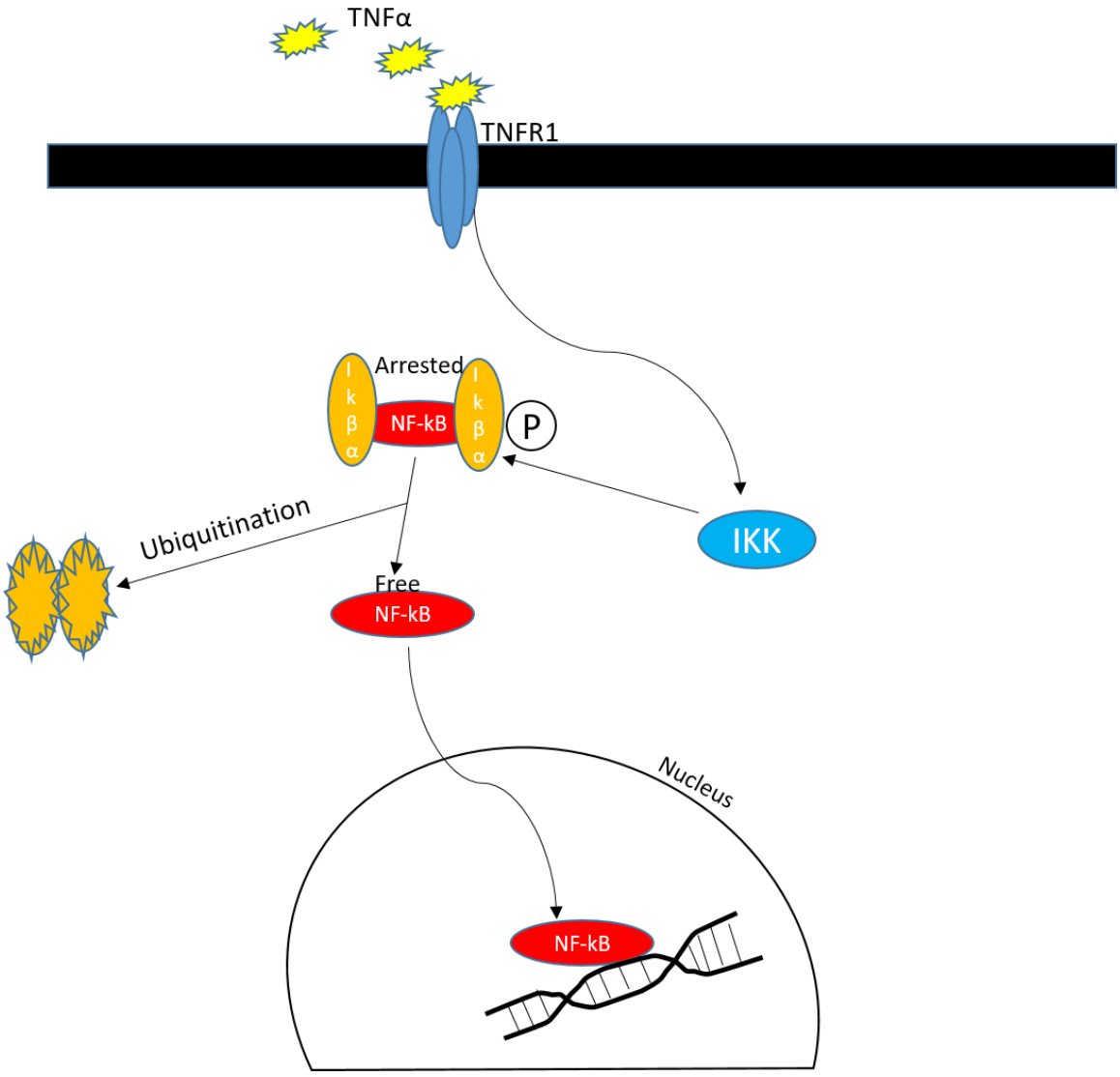


**Figure 5.** Canonical activation of the NF-kB signaling pathway by activation of TNFR1

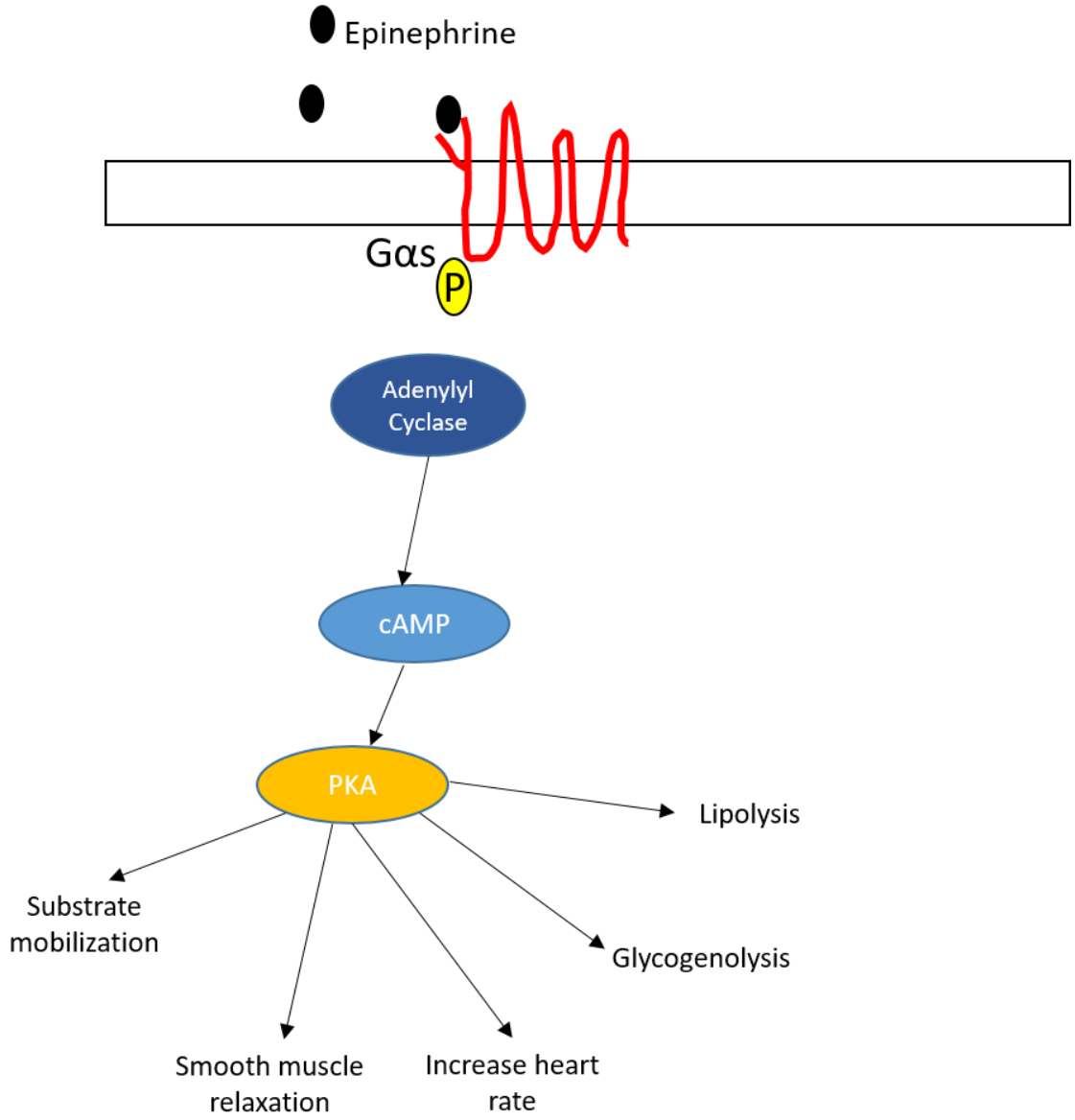


**Figure 6.** β1-3 adrenergic receptor signaling pathway through Gαs

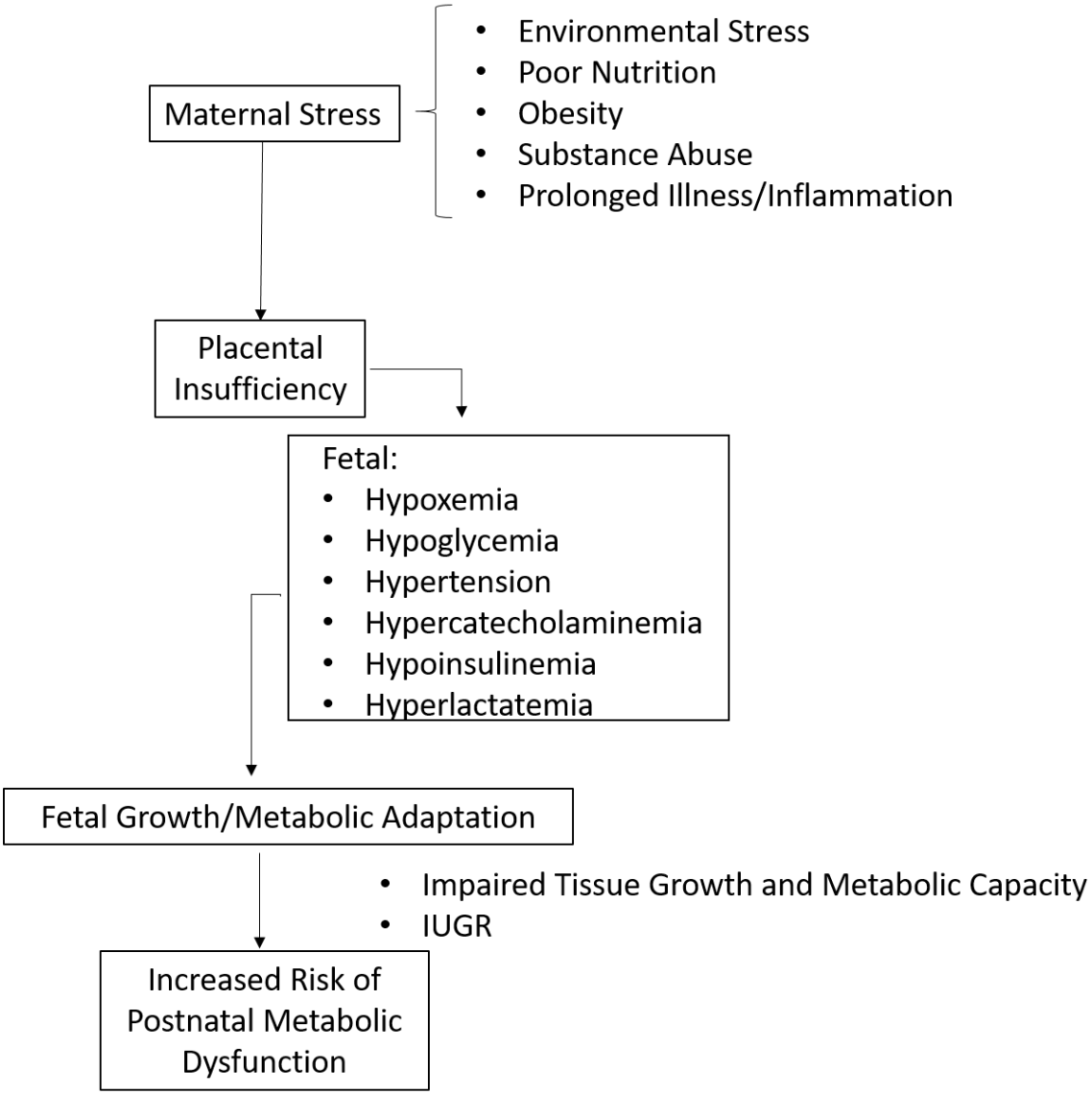


**Figure 7.** Outline of events leading to intra-uterine growth restriction and increased incidence of postnatal metabolic dysfunction.

# Chapter 2

## Maternal inflammation at mid-gestation in pregnant rats impairs fetal muscle growth & development at term


### Abstract

Intrauterine growth restriction (IUGR) is linked to lifelong decreases in skeletal muscle mass and an increased risk for metabolic dysfunction. The poor intrauterine environment resulting from maternal stress increases fetal loss and causes growth-restricting adaptations in skeletal muscle. Therefore, the objective of this study was to determine the effects of sustained maternal inflammation at mid-gestation on fetal mortality, skeletal muscle growth, and metabolic parameters at term. Timed-pregnant Sprague-Dawley rats were treated daily with bacterial endotoxin (LPS) on days 9-11 of gestation to induce maternal inflammation. On day 20, the number of fetuses did not differ between groups, but total fetal mass was lower ($P < 0.05$) in LPS-treated rats. Fetal plasma TNFα tended to be greater ($P < 0.10$) and fetal skeletal muscle TNFR1 and IL6R mRNA tended ($P < 0.10$) to be decreased in LPS-treated rats compared to controls. RNA markers of total macrophages (CD68) and M2 macrophages (CD163) tended ($P < 0.10$) to be decreased in LPS fetal muscle compared to controls. CD68-positive nuclei were decreased in LPS fetal muscle ($P < 0.05$) but CD163-positive nuclei were not different. Moreover, there was a tendency ($P < 0.10$) for decreased myoD-positive cells and increased ($P < 0.10$) myogenin-positive cells in LPS-treated fetal hindlimb compared to controls. Decreased total macrophages combined with increased plasma TNFα indicate that fetal macrophages in LPS-treated rats are more productive despite reduced prevalence in skeletal muscle. Moreover, the decrease in muscle cytokine receptor expression is likely a compensatory response to higher concentrations of circulating cytokines that reduces skeletal muscle cytokine sensitivity. Lastly, the

reduction in myoD-positive nuclei and increased in myogenin positive nuclei indicates impaired myogenesis, which is likely responsible for decreased fetal mass. Together, our findings demonstrate that maternal inflammation at mid-gestation causes fetal adaptations that impair subsequent muscle development and growth.

**Introduction**

Intrauterine growth restriction (IUGR) is a leading cause of perinatal morbidity and mortality. Low birth weight resulting from preterm birth and/or IUGR is an underlying factor in 60-80% of perinatal death worldwide, particularly in underdeveloped countries (UNICEF., 2008). Furthermore, studies have linked IUGR due to poor fetal nutrition to increased incidence of metabolic syndrome in adult life (Barker et al., 1993; Godfrey and Barker, 2000; Simmons et al., 2001; Barker, 2006). The thrifty phenotype hypothesis developed by David Barker (Hales et al., 1991) explains how IUGR-associated fetal malnutrition forces the fetus to spare nutrients by altering tissue-specific metabolism in order to survive. Postnatal nutrient availability may be abundant for IUGR-born individuals, yet these fetal adaptations persist and put individuals at greater risk for metabolic dysfunction (Limesand et al., 2007a). In utero, adaptive changes in glucose metabolism have a large effect on skeletal muscle development, growth, and metabolism (Yates et al., 2016). Skeletal muscle is responsible for the majority of insulin-stimulated glucose utilization, and adaptive changes in skeletal muscle growth capacity result in lifelong deficits in muscle mass and metabolic homeostasis (Brown and Hay, 2016). Skeletal muscle growth requires proliferation, differentiation, and fusion of myoblast into new muscle fibers early in gestation and fusion with existing fibers in the third trimester of pregnancy and after birth (Zhu et al., 2004). This process can be impaired by inflammation from resident macrophages within skeletal muscle. Classically-activated M1 macrophages are pro-inflammatory and produce TNFα and IL-6 initially, but polarize to an anti-inflammatory M2 phenotype that inhibits production of

inflammatory cytokines and stimulates tissue repair via production of growth factors (Mantovani et al., 2004; Kharraz et al., 2013). The acute effects of inflammation on myoblast function have been investigated in vitro (Frost et al., 1997; Guttridge et al., 2000) and, we postulate that inflammatory stress may have similar effects on fetal myoblasts in utero. Impaired myogensis and the resulting decrease in skeletal muscle mass affect long-term metabolic health. Therefore, the objective of this study was to determine the effects of sustained maternal inflammation at mid-gestation on fetal mortality, skeletal muscle growth, and metabolic phenotype at term.

**Materials & Methods**

*Animals and experimental design.* Animal use and care was approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex, which is accredited by the American Association for Accreditation of Laboratory animal care.

Time-mated adult Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN). From day 9 to 11 of gestation, dams received daily intraperitoneal injections of saline (n=6) or lipopolysaccharide (LPS, n=7) from *E. coli* O57:B7 (Sigma) at a concentration of 100μg/kg BW (Figure 1). Maternal whole blood was collected and rectal temperature recorded throughout the three-day treatment period. Maternal body weight was recorded daily for the entirety of the study. On day 20 of gestation, dams were euthanized by decapitation under heavy isoflurane anesthesia. Maternal body weight, fetal mass, and fetal number were recorded and maternal and fetal blood samples were collected. Whole fetal hindlimbs were collected from three randomly-selected fetuses per rat. For each fetus one hindlimb was fixed in 4% paraformaldehyde and the other was snap-frozen. Whole fetus, placenta, umbilical cord, and fetal heart,

gastrointestinal tract, liver, and kidney were collected and fixed for future analysis. Myoblasts were isolated from the remaining fetal skeletal muscle.

*Blood sample analysis.* Maternal and fetal blood glucose concentrations were determined at necropsy with a blood glucometer (Bayer, Mishawaka, IN). Plasma was isolated by centrifugation (14xg, 2 min) and TNFα concentrations were determined by Quantikine ELISA kit (R&D Systems, Minneapolis, MN) as previously described (hui Seo et al., 2017). Intra and inter-assay CV was less than 10%. After centrifugation, blood cellular fractions were lysed by the addition of TRI Reagent (Sigma-Aldrich, St. Louis, MO) at three times the original blood volume, vortexed, and stored at -80°C for future gene expression analysis.

*Gene expression.* RNA was extracted from ground flash-frozen fetal hindlimb (30 mg) using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Extracted RNA was quantified by Nanodrop spectrophotometry (NanoDrop Technologies, Inc.) and subsequently reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). Primers (Table 1) were developed for digital droplet PCR (ddPCR) analysis. Each reaction well contained ddPCR Evagreen Supermix (Bio-Rad Laboratories, Richmond, CA), 10μM of each primer, and 1 μl of cDNA template. Droplets were generated in a QX200 Droplet Generator (Bio-Rad Laboratories) with Droplet Generator Oil for Evagreen (Bio-Rad Laboratories). Droplets were transferred to a PCR plate and sealed with a Pierceable Foil Heat Seal at 180°C by the PX1 PCR Plate Sealer (Bio-Rad Laboratories). The plate was then placed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories). Samples were activated (95°C for 5 min), denatured for 40 cycles (95°C for 30 s), annealed and extended for 40 cycles (60°C for 1 min), and the signal was stabilized (4°C for 5 min and 90°C for 5 min). Finally, droplets were read on the QX200 Droplet Reader (Bio-Rad Laboratories) and analyzed in QuantaSoft Software (Bio-Rad Laboratories) to obtain copies/μl for genes of interest. Results were normalized to the

ubiquitously expressed Ywhaz gene, which was shown to be stable across treatment groups.

Expression of CD68 and CD163 was also measured using qPCR by the UNL Veterinary Diagnostic Center. Relative mRNA expression was determined using Power SYBR Green PCR Master Mix kits (Applied Biosystems, Foster City, CA) with the Fast 7500 real-time PCR System (Applied Biosystems). Samples were initially denatured (10 min at 95°C), followed by 40 cycles of 95°C for 15s, and an annealing and extension phase at 60°C for 1 min. mRNA expression was determined in triplicate from cDNA and normalized to the concentration of the housekeeping gene β-actin.

*Immunohistochemistry*. Fixed fetal hindlimbs were embedded in OCT Compound (Fischer Health Care, Gardena, CA) and 8μm cross sections were cut and mounted on glass microscope slides. Slides were first dried at 37°C for 30 min and then thrice washed in PBS containing 0.5% Triton-X100 (PBS+). Antigen retrieval was performed by boiling slides in 10 mM Citric Acid for 20 minutes. To block non-specific binding, slides were incubated with 0.5% NEN blocking buffer (Perkin-Elmer, Waltham, MA) at room temperature for 1 hr. Slides were then incubated overnight at 4°C with primary antibodies diluted in PBS + 1% bovine serum albumin. Negative controls were incubated without primary antibody. Sections were stained with rabbit antibody against myf5 (1:100;Santa Cruz, Dallas, TX), and mouse antibodies against myoD (1:200, Dako, Santa Clara, CA) and myogenin (1:250, Abcam) to identify nuclei expressing these myogenic factors. Macrophage profiles were determined by co-staining for total macrophages (CD68, 1:50: Abcam, Cambridge, MA) and M2 macrophages (CD163, 1:100,:Abcam). All nuclei were identified by counterstaining with DAPI (1:2000, Sigma). Immunocomplexes were detected with Alexa Flour 594 (1:2000, Cell Signaling, Danver MA) or Alexa Flour 488 (1:1000, Cell Signaling). Staining was visualized on an Olympus IX73 (Center Valley, PA) and digital micrographs were captured with a DP80 microscope

camera (Olympus). Images were analyzed with CellsSens Dimension software (Olympus) to determine proportions of positive-staining nuclei within fetal skeletal muscle sections. Animal identifications and treatments were encoded to eliminate bias.

*Statistical analysis*. All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary NC) to determine treatment effects. Dam was the experimental unit. Skeletal muscle mRNA concentrations from ddPCR were normalized to the Ywhaz housekeeping gene and expressed as copies per copy of Ywhaz. Macrophage mRNA concentrations analyzed by qPCR were normalized to β-actin and expressed as the amount relative to the controls. All data are expressed as means ± standard error. Proportions of nuclei positive for myogenic factors and macrophage markers were determined from an average of 250 and 850 positive nuclei, respectively, counted across 18 fields of view.

**Results**

*Morphometrics and blood analysis.* The number of fetuses per litter did not differ between control and LPS-treated rats, but total fetal mass was reduced ($P < 0.05$) in LPS fetuses (Table 2). Maternal blood glucose concentration was reduced ($P < 0.05$) in LPS-treated rats 12 hours after the first daily injection but did not differ from controls otherwise (Figure 2). Maternal plasma TNFα was greater ($P < 0.05$) in LPS-treated rats six hours after the first daily injection but did not differ from controls otherwise. Fetal blood glucose concentration at necropsy did not differ between control and LPS rats (2.88mM±6.8 and 2.83mM±3.3, respectively), but LPS rats tended to have increased ($P < 0.10$) fetal plasma TNFα concentrations compared to controls (0.02±0.26 and 0.83±0.29 pg/ml, respectively).

*Skeletal muscle gene expression.* When measured by qPCR CD68 mRNA expression was decreased ($P < 0.05$) in LPS fetal hindlimb compared to controls but CD163 mRNA concentrations did not differ between treatments (Figure 3). When

measured by ddPCR, CD68 mRNA concentrations tended to be decreased ($P < 0.10$) and CD163 concentrations were decreased ($P < 0.05$) in muscle from LPS fetuses when compared to controls. TNFR1 and IL6R mRNA tended to be reduced ($P < 0.10$, Figure 4) in LPS fetal skeletal muscle. β adrenergic receptor (Adrβ1, Adrβ2, and Adrβ3) mRNA in fetal skeletal muscle was not different between control and LPS fetuses (data not shown). Additionally, insulin receptor and Fn14, the receptor for the inflammatory cytokine TWEAK, did not differ (data not shown).

*Skeletal muscle immunohistochemistry.* CD68-positive nuclei/nm$^2$ tended to be decreased ($P < 0.10$) in LPS fetal skeletal muscle compared to controls but CD163-positive nuclei/nm$^2$ were not different (Figure 5). The proportion of CD68-positive nuclei–to-CD163-postive nuclei did not differ between LPS fetal skeletal muscle and controls (0.94±0.20 vs. 1.24±0.24, respectively). MyoD-positive nuclei/nm$^2$ in fetal hindlimb muscle was decreased ($P < 0.05$, Figure 6) in LPS fetal skeletal muscle but myf5-postive nuclei/nm$^2$ did not differ between control and LPS rats (128.4±28.5 vs. 92.0±24.1, respectively). Myogenin-positive nuclei tended to be greater ($P < 0.10$, Figure 7) in LPS rats compared to controls.

**Discussion**

In the present study, we show that reduced total fetal mass at term is a consequence of maternal inflammation at mid-gestation. This decrease in fetal mass appears to be the result of restricted skeletal muscle growth, as reduced myoD and increased myogenin in hindlimb muscle was indicative of impaired myoblast function. Fetuses from LPS-treated dams had higher concentrations of circulating TNFα near term, which indicates that greater inflammation may be responsible for reductions in myoblast-induced fetal skeletal muscle growth. Additionally, increased circulating cytokines were accompanied by decreases in skeletal muscle TNFR and IL6R mRNA, which together with decreased myoD and increased myogenin indicate a compensatory

decrease in cytokine sensitivity due to greater circulating inflammatory cytokines. These findings indicate that sustained maternal inflammation at mid-gestation impairs fetal skeletal muscle growth near term due to changes in myoblast responsiveness to critical cytokine regulation.

Total fetal mass was decreased late in gestation following sustained maternal inflammation. This was most likely due to decreases in skeletal muscle mass, which makes up almost half of total body mass (DeFronzo et al., 1981b). We attribute decreased skeletal muscle growth to impaired myoblast function, as myoD was decreased and myogenin was increased in fetuses from LPS-treated dams. myoD is a crucial transcription factor that helps to drive myoblasts out of the proliferation stage and into terminal differentiation (Bentzinger et al., 2012). The absence of myoD results in impaired myoblast function, as myoblasts will continue to proliferate rather than exiting the cell cycle and fusing with preformed fibers (Rudnicki et al., 1993). The onset of myogenin expression marks the entry into the differentiated state and stimulates fusion of differentiated myoblasts to existing fibers (Andrés and Walsh, 1996). The combination of decreased myoD and increased myogenin indicates a greater percentage of differentiated myoblasts but a reduced percentage of active proliferating myoblasts. This imbalance in MRF expression represents decreases in the total myogenic cell population and thus, muscle growth capacity. Additionally, maternal inflammation resulted in higher concentrations of TNFα in fetal plasma, well after treatment ended. TNFα has been shown to impede differentiation and fusion of myoblasts at high concentrations (Miller et al., 1988). High fetal plasma concentrations of inflammatory cytokines appeared to reduce TNFR and IL6R in skeletal muscle indicating a reduced sensitivity to these important regulators of skeletal muscle. Reduced sensitivity impedes the ability of cytokines to elicit a regulatory effect on myoblasts and causes an imbalance between proliferation and differentiation by allowing precocious differentiation.

Interestingly, increased circulating TNFα concentrations and decreased skeletal muscle cytokine sensitivity was not the result of greater numbers of resident macrophages, as mRNA and protein markers showed decreased total macrophage populations in fetal hindlimb after maternal inflammation. M1 macrophages are pro-inflammatory and produce inflammatory cytokines as part of the immune response, but they also polarize to an anti-inflammatory M2 phenotype that stimulates tissue repair (Mantovani et al., 2004; Kharraz et al., 2013). Our data show normal M2 macrophage population but decreased total macrophages. However, these fetal macrophages are still producing greater amounts of inflammatory cytokines 10 days after maternal inflammation had subsided. Although it is not certain whether these inflammatory cytokines are from placental or fetal origin, the temporal spacing between induction of maternal inflammation and fetal plasma TNFα response suggest they are not of maternal origin. Moreover, since fetal TNFα is greater despite decreased macrophage number, it appears that fetal M1 macrophages have increased activity. It should be noted that, although macrophages are the primary producers of inflammatory cytokines under most conditions, other white blood cells and tissues are also capable of producing them to varying degrees. As a compensatory action for chronic exposure to inflammatory cytokines in LPS fetuses, the major cytokine receptors were decreased in skeletal muscle. Inflammatory cytokines bind to these receptors and activate the NF-κB signaling pathway to upregulate gene expression of additional inflammatory cytokines, chemokines, immunoreceptors, and other inflammatory factors (Pahl, 1999). Activation of this pathway is responsible for the inhibitory effect of cytokines on myoblast differentiation and thus reduced sensitivity at the level of the receptor may explain the increase in myoblast differentiation and concurrent reduction in proliferating myoblast.

Fetal inflammation may be a direct response to maternal inflammation or may be indirectly caused by placental insufficiency. Maternal TNFα was acutely elevated in

response to LPS treatment but did not differ from controls at day 20 of gestation when fetuses were collected, and in fact was not different on the second and third days of LPS administration. Fetal circulating TNFα was increased at term, suggesting fetal inflammation is most likely due to placental insufficiency. Additionally, previous studies found that inflammatory cytokines do not typically pass the placental barrier (Aaltonen et al., 2005), indicating that all cytokines within the fetus and amniotic fluid are of conceptus origin. Together, these findings indicate that the fetal inflammatory response observed in this study is most likely due to placental insufficiency.

In conclusion, our findings show maternal inflammation at mid-gestation results in decreased fetal mass due to impaired myogenesis that is still apparent at term. We demonstrate that impaired myogenesis is due to myoblast dysfunction as evidenced by decreased myoD and increased myogenin. Moreover, myoblast dysfunction is the likely result of decreased sensitivity to these inflammatory cytokines. The inflammatory response may be due to amplified activity of M1 macrophages, as macrophage number was actually decreased in fetal skeletal muscle after maternal inflammation. Together, our findings show that maternal inflammation induces fetal adaptive responses that interrupt myoblast regulation, causing myoblast dysfunction and impaired skeletal muscle development and growth.

**Table 1.** *Primers for PCR.*

| Gene | Primer Sequence | Product Size | Accession Number |
|---|---|---|---|
| CD68[1] | ATCATTGGCCTGGTCCTCCTG<br>GGGCTGGTAGGTTGATTGTCG | 186 | NM_001031638.1 |
| CD163[1] | GGGGTGTCCAAATGTTCAGGAAG<br>CTCGCAGGAGATACTGTCAAGCC | 198 | NM_001107887.1 |
| TNFR1 | TTGTAGGATTCAGCTCCTGTC<br>CTCTTACAGGTGGCACGAAGTT | 109 | NM_013091 |
| Fn14 | CACTGATCCAGTGAGGAGCA<br>GGCAATTAGACACCCTGGAA | 88 | NM_181086 |
| IL6R | CACGAGCCATCATGAAGAGA<br>GCCAAGGTGCTTGGATTTTA | 96 | NM_017020 |
| Adrb1 | GGGAGTACGGCTCCTTCTTC<br>CGTCTACCGAAGTCCAGAGC | 45 | NM_012701 |
| Adrb2 | AGCCACCTACGGTCTCTGAA<br>GTCCCGTTCCTGAGTGATGT | 208 | NM_012492.2 |
| Adrb3 | TTGCCTCCAATATGCCCTAC<br>AAGGAGACGGAGGAGGAGAG | 46 | NM_013108 |
| Ywhaz | CCGAGCTGTCTAACGAGGAG<br>GAGACGACCCTCCAAGATGA | 88 | NM_013011 |

[1]Used for qPCR and ddPCR

**Table 2.** *Morphometric data.*[1]

| Variable | Control (n = 6) | LPS-treated[2] (n = 7) | *P* Value |
|---|---|---|---|
| Number of Live Fetuses | 14.7 ± 0.9 | 13.7 ± 0.8 | NS[3] |
| Total Fetal Mass, g | 71.4 ± 2.8 | 60.8 ± 2.8 | 0.02 |

[1] Measured at day 20 of gestation (term 21 days).
[2] Daily maternal injections (IP) of 100ng/kg BW lipopolysaccharide from *E. Coli* 057:h7.
[3] Not significant

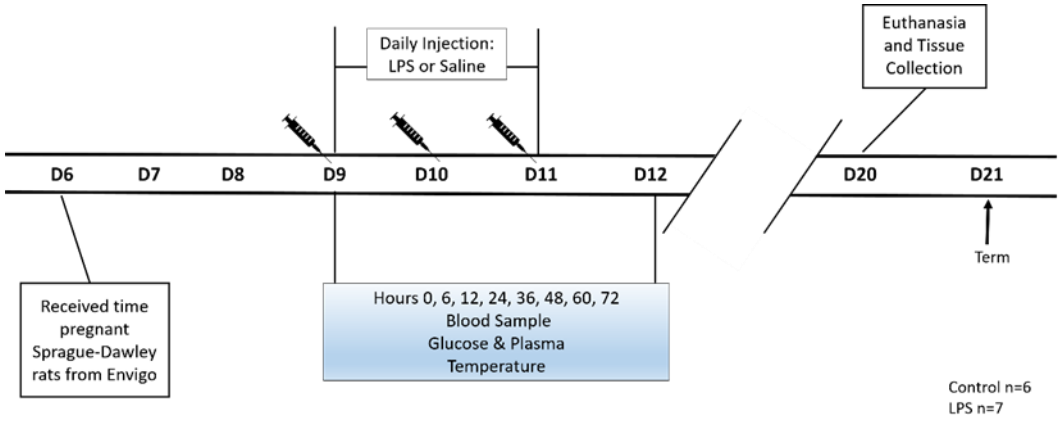


**Figure 1.** Timeline for experimental procedures. D=day of gestation.

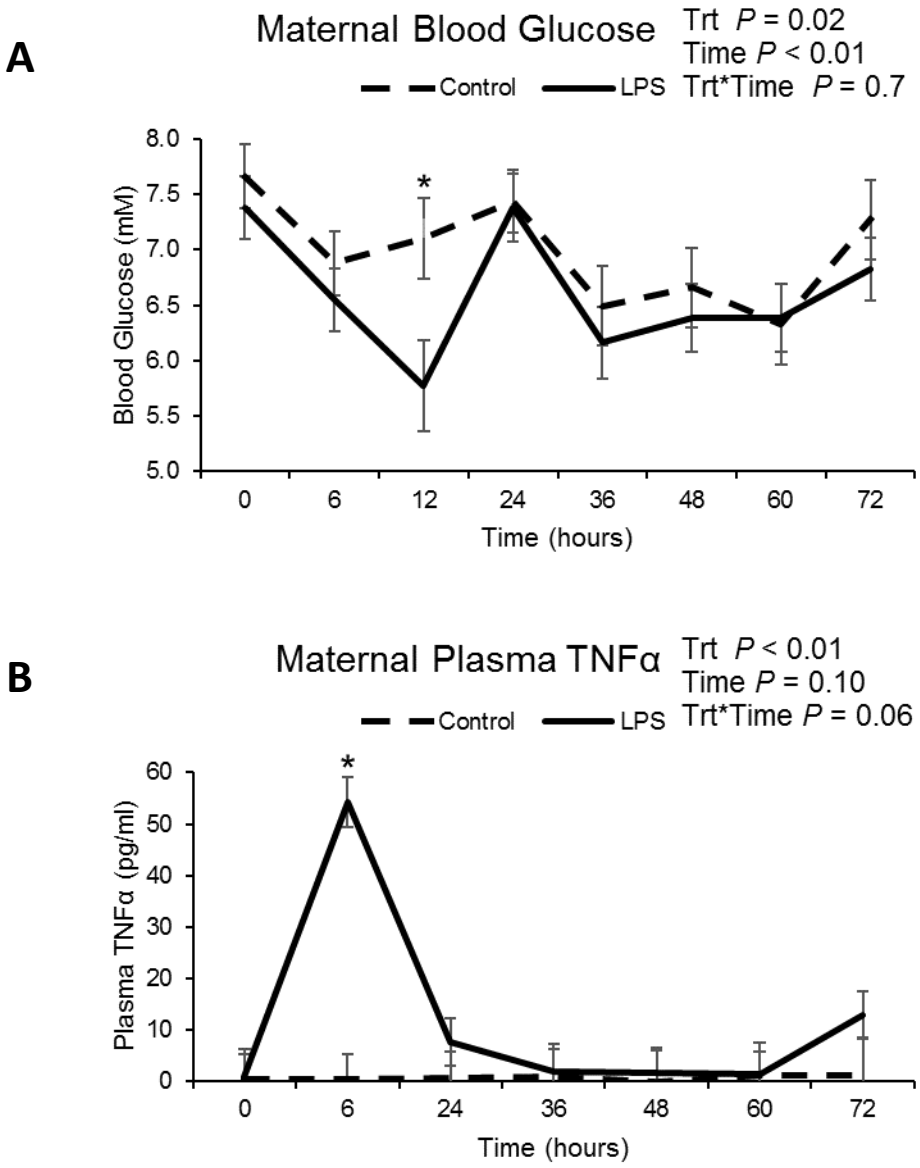


**Figure 2**. Maternal blood glucose concentration (A) and plasma TNFα concentration (B) measured during the treatment period. * means differed ($P < 0.05$) between control and treatment dams. # means tended to differ ($P < 0.10$) between control and treatment dams.

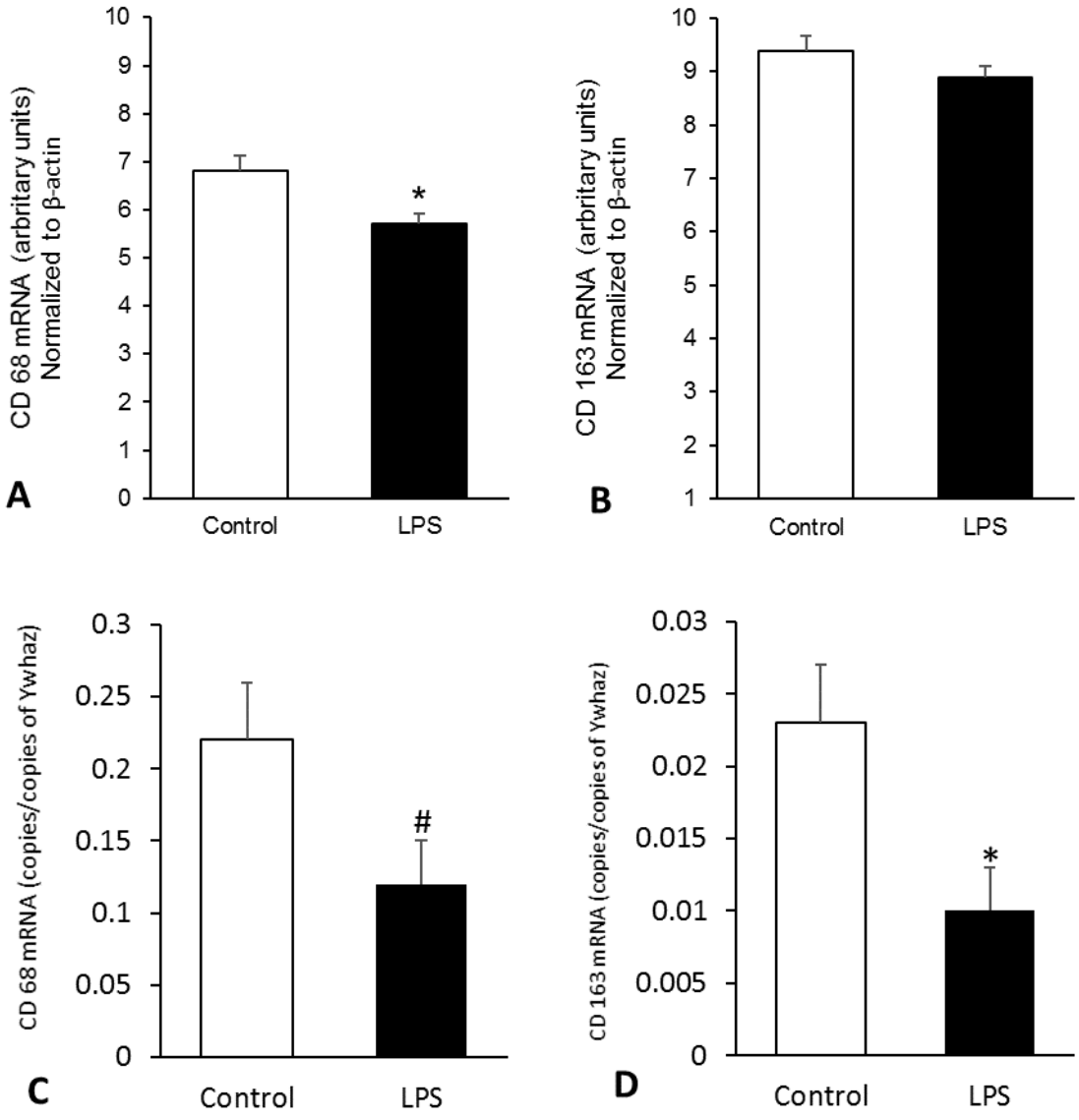


**Figure 3.** Gene expression analysis for total (A&C) and M2 (B&D) macrophage markers in fetal hindlimb as measured by qPCR (A&B) and ddPCR (C&D) * means differed ($P < 0.05$) between control and LPS fetuses. # means tended to differ ($P < 0.10$) between control and LPS fetuses.

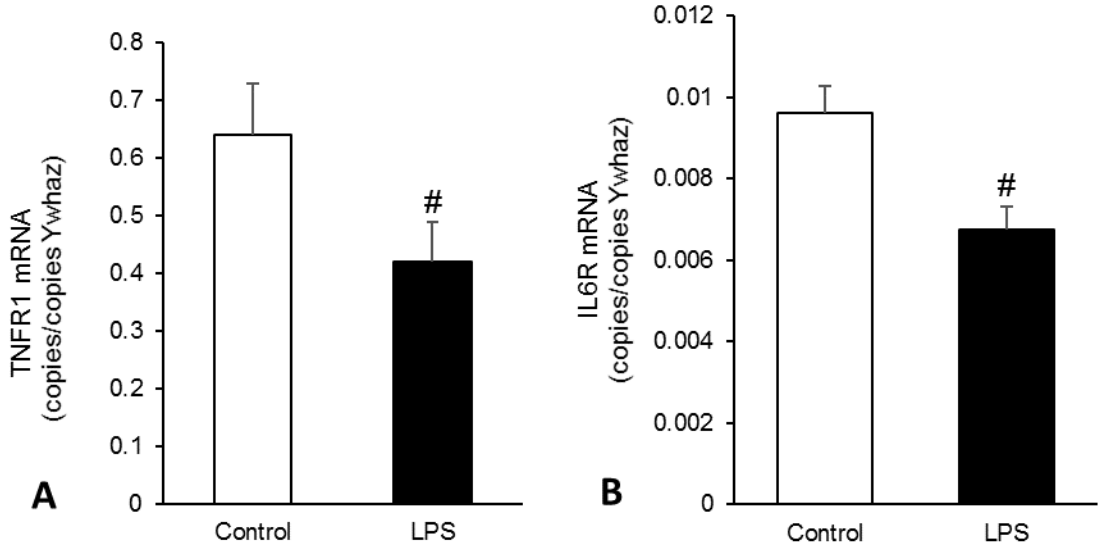


**Figure 4.** Gene expression analysis (ddPCR) of TNFR1 (A) and IL6R (B) in fetal hindlimb after maternal inflammation. # means tended to differ ($P < 0.10$) between control and LPS fetuses.

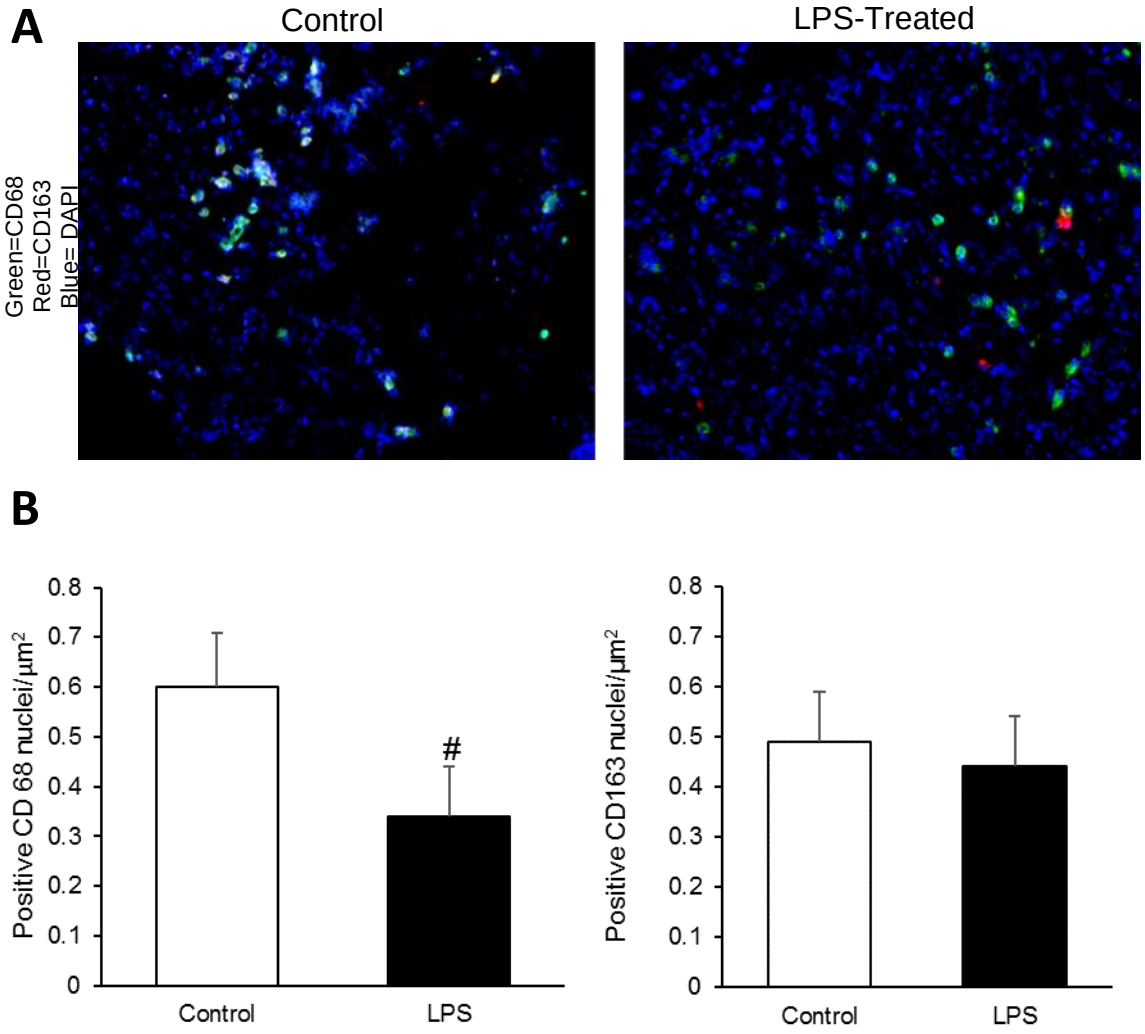


**Figure 5.** Immunostaining of markers for total (CD68) and M2 (CD163) macrophages in cross-sections of fetal hindlimb muscle after maternal inflammation. A. Representative micrographs are depicted for control and LPS fetal hindlimb cross sections (8μm). Sections were co-stained for total (green) and M2 (red) macrophages and counterstained with DAPI (blue). B. Analysis of positive total and M2 macrophages. # means differed ($P < 0.10$) between control and LPS fetuses.

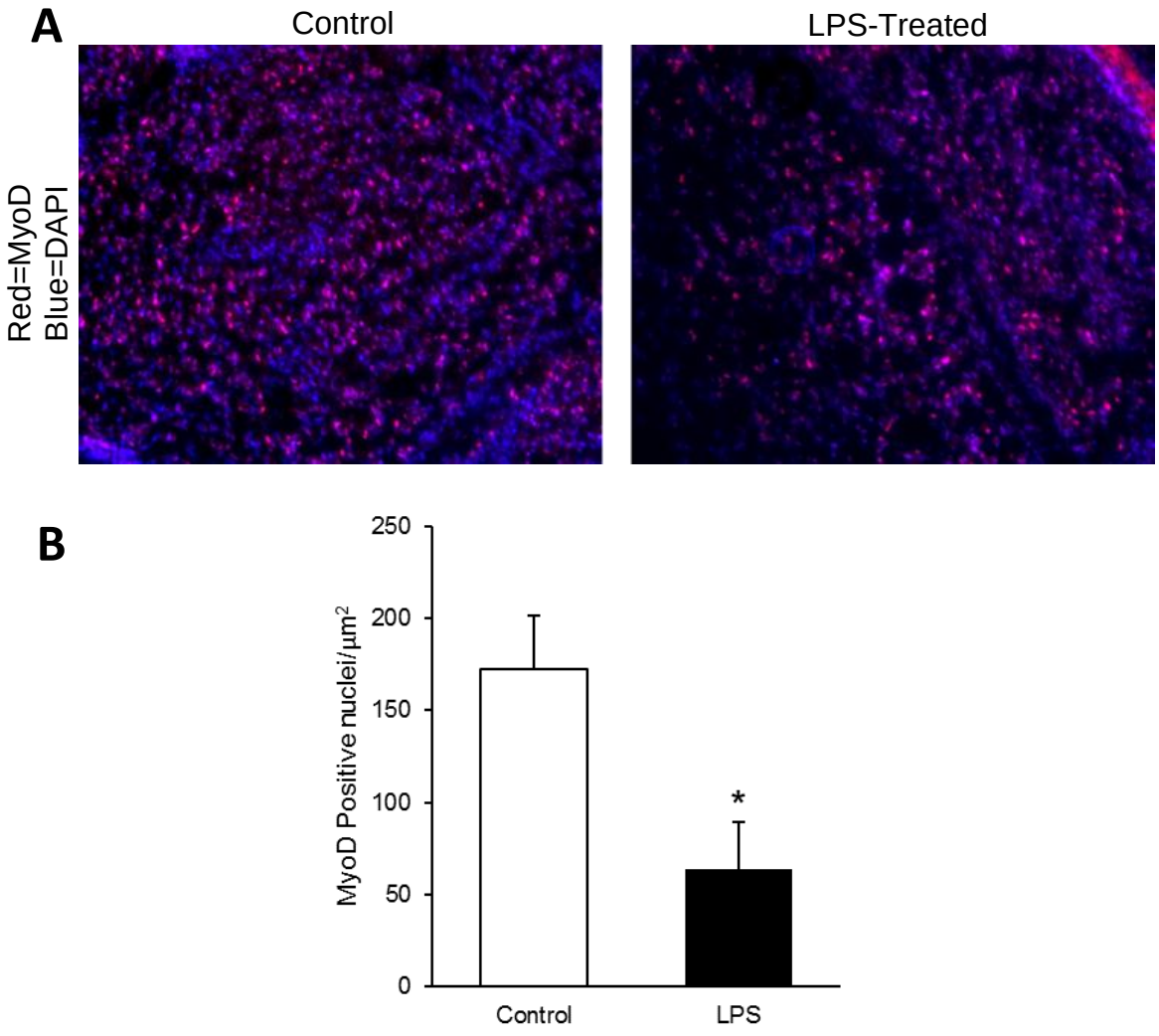


**Figure 6.** A. Immunostaining of myoD in fetal hindlimb muscles after maternal inflammation. Representative micrographs are depicted for control and LPS fetal hindlimb cross sections (8 µm). Sections were stained for myoD (red) and counterstained with DAPI (blue). B. Analysis of myoD positive nuclei. * means differed ($P < 0.05$) between control and LPS fetuses.

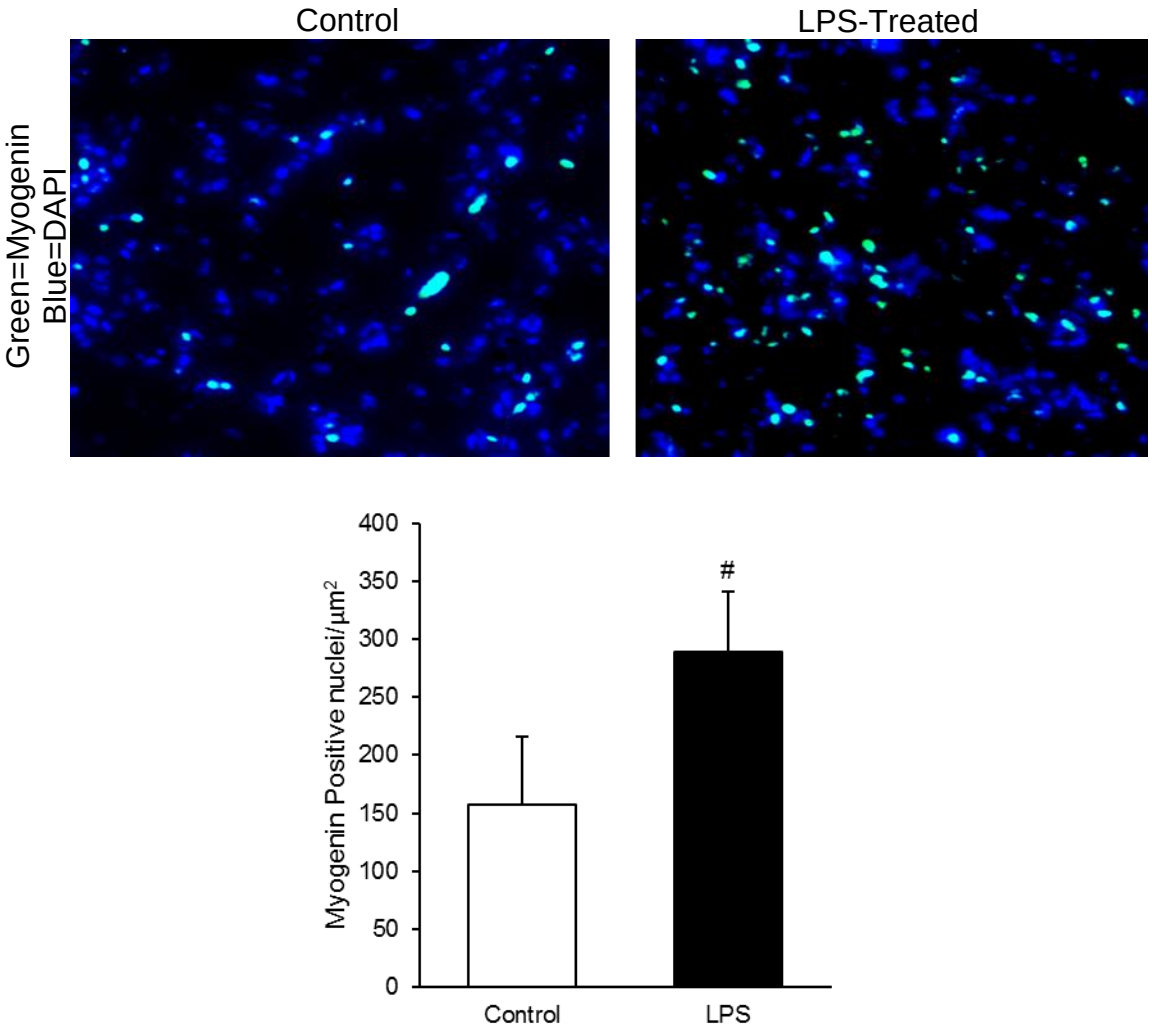


**Figure 7.** A. Immunostaining of myogenin in fetal hindlimb muscles after maternal inflammation. Representative micrographs are depicted for control and LPS fetal hindlimb cross sections (8 µm). Sections were stained for myogenin (green) and counterstained with DAPI (blue). B. Analysis of myogenin positive nuclei. # means tended to differ ($P < 0.10$) between control and LPS fetuses.

# Chapter 3

## Acute exposure of primary rat soleus muscle to zilpaterol HCl (β2 adrenergic agonist), TNFα, or IL-6 in culture increases glucose oxidation rates independent of the impact on insulin signaling or glucose uptake.


### Abstract

Recent studies show that adrenergic agonists and inflammatory cytokines can stimulate skeletal muscle glucose uptake, but it is unclear if glucose oxidation is similarly increased. Thus, the objective of this study was to determine the effects of ractopamine HCl (β1 agonist), zilpaterol HCl (β2 agonist), TNFα, and IL-6 on glucose uptake and oxidation rates in unstimulated and insulin-stimulated soleus muscle strips from adult Sprague-Dawley rats. Effects on phosphorylation of Akt (phospho-Akt), p38 MAPK (phospho-p38), and p44/42 MAPK (phospho-p44/42) was also determined. Incubation with insulin increased ($P < 0.05$) glucose uptake by ~47%, glucose oxidation by ~32%, and phospho-Akt by ~238%. Insulin also increased ($P < 0.05$) phospho-p38, but only after 2 hours in incubation. Muscle incubated with β2 agonist alone exhibited ~20% less ($P < 0.05$) glucose uptake but ~32% greater ($P < 0.05$) glucose oxidation than unstimulated muscle. Moreover, co-incubation with insulin + β2 agonist increased ($P < 0.05$) glucose oxidation and phospho-Akt compared to insulin alone. Conversely, β1 agonist did not appear to affect basal or insulin-stimulated glucose metabolism, and neither β agonist affected phospho-p44/42. TNFα and IL-6 increased ($P < 0.05$) glucose oxidation by ~23% and ~33%, respectively, in the absence of insulin. This coincided with increased ($P < 0.05$) phospho-p38 and phospho-p44/42 but not phospho-Akt. Furthermore, co-incubation of muscle with insulin + either cytokine yielded glucose oxidation rates that were similar to insulin alone, despite lower ($P < 0.05$) phospho-Akt.

Importantly, cytokine-mediated increases in glucose oxidation rates were not concomitant with greater glucose uptake. These results show that acute β2 adrenergic stimulation, but not β1 stimulation, directly increases fractional glucose oxidation in the absence of insulin and synergistically increases glucose oxidation when combined with insulin. The cytokines, TNFα and IL-6, likewise directly increased glucose oxidation in the absence of insulin, but were not additive in combination with insulin and in fact appeared to disrupt Akt-mediated insulin signaling. Rather, cytokines appear to be acting through MAPKs to elicit effects on glucose oxidation. Regardless, stimulation of glucose oxidation by these key stress factors did not rely upon greater glucose uptake, which may promote metabolic efficiency during acute stress by increasing fractional glucose oxidation without increasing total glucose consumption by muscle.

**Introduction**

Skeletal muscle comprises about 40% of total body mass in humans, yet it accounts for greater than 85% of the body's insulin-stimulated glucose utilization (DeFronzo et al., 1981b; Brown, 2014). The role of insulin in metabolic regulation is well-understood, but muscle metabolism can be influenced by additional factors including catecholamines and cytokines (Glund et al., 2007; Pillon et al., 2013; Fernandes et al., 2014). Catecholamines (i.e. epinephrine, norepinephrine) are released into circulation by the adrenal medulla and act by binding to a large class of G protein-coupled adrenergic receptors ($\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$, $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2D}$, β1, β2, and β3) (Stiles et al., 1984) located throughout the body. Thus, the impact that adrenergic stimulation has on a specific tissue is a function of the specific receptor type or types that it expresses. In skeletal muscle, β2 adrenergic receptors are the most highly-expressed isoform, but β1 receptors and to a lesser extent β3 and $\alpha_{1D}$ receptors are also present (Kim et al., 1991a; Shi et al., 2016). Growth studies in animals (Lopez-Carlos et al., 2012) have led

to the development of isoform-specific β adrenergic growth promoters that are used as feed additives in the livestock industry to increase meat yield per animal (Johnson et al., 2014). In addition, reports show that β agonists are commonly used by athletes to boost muscle growth and athletic performance, despite being restricted by the World Anti-Doping Agency (Collomp et al., 2010). However, far less is known about the effects of β agonists on muscle metabolism. The seemingly complex effects of inflammatory cytokines on metabolism are likewise only beginning to be understood. Inflammation is known to cause insulin resistance (Marette et al., 2014), yet recent studies show that two major inflammatory cytokines, TNFα and IL-6, may stimulate glucose metabolism in muscle independent of their actions on insulin signaling (Glund et al., 2007; Gray and Kamolrat, 2011; Remels et al., 2015). Insulin increases glucose uptake in skeletal muscle through a well-characterized signaling cascade that begins with binding of its transmembrane tyrosine kinase receptor followed by sequential activation of the downstream targets, IRS1, PI3K, and Akt via phosphorylation (Pirola et al., 2004). This canonical pathway ultimately stimulates translocation of glucose transporter, Glut4, to the cell membrane where it is imbedded and facilitates greater glucose passage into the muscle cell (James et al., 1988). Phosphorylation of Akt appears to be a critical step in most insulin-regulated events, and thus the ratio of phosphorylated Akt to total Akt is considered to be a reliable indicator of insulin signaling (Pajvani and Scherer, 2003). In addition to the canonical PI3K/Akt-mediated signal transduction pathway, mitogen-activated protein kinases (MAPKs) have also been identified as activators of Glut4-mediated glucose uptake (Sweeney et al., 1999; Somwar et al., 2000). Insulin-stimulated glucose oxidation rates have long been presumed to be proportional with glucose uptake rates (Hay and Meznarich, 1986), but we postulate that this relationship may not be maintained with the additional influence of adrenergic or inflammatory factors. Moreover, it is unclear whether these factors would regulate skeletal muscle glucose metabolism

through direct effects or by altering insulin signaling. Thus, the objective of this study was to determine the respective impacts of β1 and β2 adrenergic agonists, TNFα, and IL-6 on glucose uptake and oxidation rates in intact soleus muscle strips isolated from adult rats. Furthermore, we sought to determine whether these effects were insulin-associated or insulin-independent by incubating muscle strips with each factor alone or in combination with insulin.

## Materials and Methods

### *Animals and tissue isolation*

The following experiments were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Soleus muscles collected tendon-to-tendon from adult Sprague-Dawley rats (females 252.86 ± 14.93 g, males 378 ± 15.72 g) after decapitation under heavy isoflurane anesthesia were used to measure glucose uptake (n=10), glucose oxidation (n=9), and protein expression (n = 8). Males and females were spread evenly across groups. Isolated soleus muscles were washed in ice-cold phosphate buffered saline (PBS; pH 7.4), and each muscle was dissected longitudinally (tendon to tendon) into 25-45 mg strips. For all experiments, muscle strips were pre-incubated for 1 hour at 37°C in gassed (95% $O_2$, 5% $CO_2$) Krebs-Henseleit bicarbonate buffer (KHB, pH 7.4; 0.1% bovine serum albumin; Sigma-Aldrich, St. Louis, MO) spiked with respective treatment (Table 1) and 5 mM D-glucose (Sigma-Aldrich, St. Louis, MO). Muscle strips were then washed for 20 minutes in treatment-spiked KHB with no glucose. Pre-incubation and wash media for glucose uptake (but not glucose oxidation) experiments also contained

35 mM and 40 mM mannitol (Sigma-Aldrich), respectively. Glucose uptake and glucose oxidation rates were determined as described below. Protein analysis was performed in soleus strips from parallel incubations.

*Glucose uptake*

Glucose uptake rates were determined from intracellular accumulation of [$^{3}$H]2-deoxyglucose as previously described (Jacob et al., 1996), with some modifications. After being pre-incubated and washed, soleus strips were incubated at 37°C for 20 minutes in treatment-spiked KHB containing 1 mM [$^{3}$H]2-deoxyglucose (300 µCi/mmol) and 39 mM [1-$^{14}$C] mannitol (1.25 µCi/mmol). Muscle strips were then removed, thrice washed in ice-cold PBS, weighed, and lysed in 2M NaOH (Sigma-Aldrich) at 37°C for 1 hour. Lysates were vortexed and mixed with UltimaGold scintillation fluid, and specific activity of $^{3}$H and $^{14}$C was measured by liquid scintillation with a Beckman-Coulter 1900 TA LC counter (Brea, CA). Specific activity of the media was likewise determined in triplicate 10-µl aliquots mixed with 500 µl distilled water and scintillation fluid. Mannitol concentrations were used to estimate the amount of extracellular fluid in each lysate, and intracellular accumulation of 2-deoxyglucose was calculated as total 2-deoxyglucose in the lysate less the extracellular concentration. All radioactive compounds and scintillation fluids were purchased from Perkin-Elmer (Waltham, MA).

*Glucose oxidation*

Glucose oxidation rates were determined by oxidation of [$^{14}$C-U] D-glucose as previously described (Henriksen and Tischler, 1988), with some modifications. After being pre-incubated and washed, muscle strips were placed in one side of a sealed dual-well chamber and incubated at 37°C for 2 hours in treatment-spiked KHB containing 5 mM [$^{14}$C-U] D-glucose (0.25 µCi/mmol). NaOH (2M) was placed in the

adjacent well to capture $CO_2$. After 2 hours, chambers were cooled at -20°C for 2 minutes. HCl (2M; Sigma-Aldrich) was added to the media through a rubber seal on the top of the chamber, and the chambers were then incubated at 4°C for 1 hour to release bicarbonate-bound $CO_2$ from the media. Finally, chambers were unsealed and each muscle strip was washed and weighed. NaOH was collected from each chamber, mixed with UltimaGold scintillation fluid, and analyzed by liquid scintillation to determine specific activity of captured $^{14}CO_2$. Specific activity of media was determined as described above.

*Western immunoblot*

The respective activities of Akt and MAPKs were estimated by the proportions of phosphorylated target protein to total target protein as previously described (Martineau and Gardiner, 2001; Khamzina et al., 2005; Li et al., 2005; Morley et al., 2015), with minor modifications. Target protein concentrations were determined in soleus strips that were incubated in treatment-spiked KHB for 1 or 2 hours and then snap-frozen and stored at -80°C. Each muscle strip was thoroughly homogenized in 200 µl of radioimmunoprecipitation buffer containing manufacturer-recommended concentrations of Protease and Phosphatase Inhibitor (Thermo Fisher, Carlsbad, CA). Homogenates were then sonicated and centrifuged (14,000 x *g* for 5 minutes at 4ºC), and supernatant was collected. Total protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher). Protein samples (35 µg) were boiled for 5 minutes at 95°C in BioRad 4x Laemmli Sample Buffer (BioRad, Hercules, CA) and then separated by SDS-polyacrylamide. Gels were transferred to polyvinylidene fluoride low florescence membranes (BioRad), which were incubated in Odyssey block solution (Li-Cor Biosciences, Lincoln, NE) for 1 hour at room temperature and then washed with 1X TBS-T (20 mM Tris-HCL + 150 mM NaCl + 0.1% Tween 20). Membranes were subsequently

incubated overnight at 4ºC with one of the following rabbit antibodies diluted in Odyssey block solution + 0.05% Tween-20: anti-Akt (1:1,000), anti-phospho-Akt (1:2,000), p44/42 MAPK (1:2,000), phospho-p44/42 MAPK (1:1,000), p38 MAPK (1:1,000), or phospho-p38 MAPK (1:1,000) (Cell Signaling, Danvers, MA). An IR800 goat anti-rabbit IgG secondary antibody (1:10,000 for Akt; 1:5,000 for MAPKs; Li-Cor) diluted in Odyssey block solution with 0.05% Tween-20 and 0.01% SDS was applied for 1 hour at room temperature. Blots were scanned on an Odyssey Infrared Imaging System and analyzed with Image Studio Lite Software (Li-Cor). For each protein of interest, phosphorylation rates were estimated by the proportions of phosphorylated protein to total protein (phospho-Akt, phospho-p38, phospho-p44/42, respectively).

*Statistical analysis*

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). Each data point was derived from the average of 2 technical replicates (i.e. in each experiment, each condition was studied in 2 muscle strips from each rat). Four separate metabolic experiments were performed, each with its own non-stimulated and insulin-only conditions as well as its own group of rats: glucose uptake with adrenergic agonists (n = 10), glucose uptake with cytokines (n = 10), glucose oxidation with adrenergic agonists (n = 9), and glucose oxidation with cytokines (n = 9). Likewise, separate groups of rats were used to determine protein concentrations in soleus muscle strips after 1 hour (n = 8) or 2 hours (n = 4) in incubation. Data are presented as mean ± standard error.

**Results**

*Glucose uptake and oxidation rates*

*Adrenergic agonist stimulation.* Glucose uptake and oxidation rates were greater ($P < 0.05$) for muscle incubated in media spiked with insulin compared to muscle incubated in un-spiked (basal) media (Figure 1). When muscle was incubated in media spiked with β1 agonist alone, glucose uptake and oxidation rates did not differ from unstimulated muscle (incubated in basal media). Moreover, when muscle was co-incubated with insulin + β1 agonist, glucose uptake and oxidation did not differ from muscle incubated with insulin alone. Conversely, muscle incubated with β2 agonist alone exhibited decreased ($P < 0.05$) glucose uptake and increased ($P < 0.05$) glucose oxidation compared to unstimulated muscle. Moreover, glucose uptake rates did not differ between muscle incubated with insulin alone or with insulin + β2 agonist, but glucose oxidation rates were greater ($P < 0.05$) for muscle incubated with insulin + β2 agonist compared to muscle incubated with either insulin alone or β2 agonist alone.

*Inflammatory cytokine stimulation.* As with the adrenergic agonist studies, glucose uptake and oxidation rates were greater ($P < 0.05$) for muscle incubated with insulin compared to unstimulated muscle (Figure 2). Glucose uptake rates for muscle incubated with TNFα alone or IL-6 alone did not differ from rates in unstimulated muscle. Likewise, glucose uptake for muscle incubated with insulin + TNFα or insulin + IL-6 did not differ from muscle incubated with insulin alone. However, glucose oxidation rates for muscle incubated with TNFα alone or IL-6 alone were greater ($P < 0.05$) than for unstimulated muscle and, in fact, were similar to muscle incubated with insulin alone. Interestingly, glucose oxidation rates for muscle incubated with insulin + TNFα or insulin + IL-6 did not differ from muscle incubated with insulin alone, TNFα alone, or IL-6 alone.

*Akt phosphorylation*

Muscle incubated with insulin exhibited greater ($P < 0.05$) phospho-Akt than unstimulated muscle after 1 and 2 hours in incubation (Figure 3). Conversely, phospho-

Akt did not differ between unstimulated muscle and muscle incubated with β1 agonist, β2 agonist, TNFα, or IL-6 alone at either time point. After 1 hour, phospho-Akt did not differ between muscle incubated with insulin alone or insulin + β1 agonist but was greater ($P < 0.05$) in muscle incubated with insulin + β2 agonist. After 2 hours, phospho-Akt was greater ($P < 0.05$) in muscle incubated with either insulin + β1 agonist or insulin + β2 agonist than in unstimulated muscle but was less ($P < 0.05$) than in muscle incubated with insulin alone. Conversely, phospho-Akt was less ($P < 0.05$) in muscle incubated with insulin + TNFα or with insulin + IL-6 than in muscle incubated with insulin alone. In fact, phospho-Akt did not differ between unstimulated muscle and muscle incubated with insulin + IL-6.

*MAPK phosphorylation*

*p38 MAPK.* After 1 hour in incubation, phospho-p38 did not differ between unstimulated muscle and muscle incubated with insulin alone, β1 agonist alone, β2 agonist alone, insulin + β1 agonist, insulin + β2 agonist, or insulin + IL-6 (Figure 4). However, muscle incubated with TNFα alone, IL-6 alone, or insulin + TNFα exhibited greater ($P < 0.05$) phospho-p38 after 1 hour than unstimulated muscle or muscle incubated with insulin alone. After 2 hours, muscle incubated with insulin alone exhibited greater ($P < 0.05$) phospho-p38 than unstimulated muscle, but no other groups differed from unstimulated muscle.

*p44/42 MAPK.* After 1 hour, phospho-p44/42 did not differ between unstimulated muscle and muscle incubated with insulin alone, β1 agonist alone, β2 agonist alone, or insulin + β1 agonist (Figure 5). However, phospho-p44/42 was lower ($P < 0.05$) in muscle incubated with insulin + β2 agonist compared to unstimulated muscle. Conversely, muscle incubated with TNFα alone, IL-6 alone, insulin + TNFα, or insulin + IL-6 exhibited greater ($P < 0.05$) phospho-p44/42 than unstimulated muscle or muscle

incubated with insulin alone. No difference in phospho-p44/42 was observed among any groups after 2 hours in incubation.

**Discussion**

In this study, we show that acute stimulation of skeletal muscle with either β2 adrenergic agonist or inflammatory cytokines can increase glucose oxidation rate independent of insulin activity. Moreover, we show that these increases in glucose oxidation are not dependent upon concomitant increases in glucose uptake. Adrenergic stimulation of glucose metabolism was isoform-specific, as β2 agonist reduced basal glucose uptake, increased basal glucose oxidation, and synergistically enhanced insulin-stimulated glucose oxidation and Akt phosphorylation, but β1 agonist had no discernable effects on glucose metabolism or insulin signaling. Inflammatory cytokines antagonized insulin signaling but simultaneously stimulated skeletal muscle glucose oxidation, seemingly via MAPK-mediated signaling pathways. Together, these findings indicate that acute exposure to these common stress factors can increase glucose oxidation independently of changes in glucose uptake and insulin signaling.

Acute β2-specific adrenergic stimulation in the absence of insulin had contrasting effects on skeletal muscle glucose uptake and oxidation rates, as glucose uptake was reduced but glucose oxidation was improved. Previous studies in humans and animals have indicated that glucose oxidation rates are typically proportional to glucose utilization rates across a variety of physiological conditions (Hay et al., 1983; Peterson et al., 2015), yet our findings show that fractional glucose oxidation by soleus muscle grew substantially during acute β2 adrenergic stimulation, independent of the rate of glucose uptake. This may help to explain previously reported reductions in (whole-body) fractional glucose oxidation rates in growth-restricted fetal sheep (Limesand et al., 2007b), which exhibit reduced expression of β2 receptors in muscle, fat, and other

tissues (Chen et al., 2010; Leos et al., 2010b; Yates et al., 2012b; Chen et al., 2014a). In addition to increasing basal glucose oxidation rates, acute β2 adrenergic stimulation synergistically enhanced the effects of insulin on skeletal muscle glucose oxidation, as the two factors together elicited a greater combined impact than either factor individually. The additive effect of β2 adrenergic agonist and insulin appeared to be mechanistically facilitated by greater activation of the canonical signaling component, Akt, which is a key mediator for most of insulin's intracellular effects (Saltiel and Kahn, 2001). Conversely, β1 adrenergic stimulation produced no such additive effect with insulin for glucose uptake or oxidation and did not appear to have any direct impact on the metabolic outputs measured in this study. One possible explanation for the distinctly different impacts of the respective β agonists in the present study is the substantially lower expression of β1 adrenergic receptors in muscle relative to β2 receptors. Kim et al. (Kim et al., 1991a) showed that the β1 isoform comprises only about 15-21% of the total β receptor population expressed by the soleus muscle of the adult rat and that the β2 isoform can account for essentially all β receptors in some muscles. Similar β receptor populations were found in human muscle biopsies (Liggett et al., 1988; Elfellah et al., 1989). The present findings also help explain greater muscle growth efficiency in food animals supplemented with dietary β2 agonist compared to β1 agonist (Weber et al., 2012; Arp et al., 2014; Brown et al., 2014).

Acute exposure of muscle to the inflammatory cytokines, TNFα and IL-6, antagonized insulin signaling, as expected, but stimulated glucose oxidation directly. Inflammation has long been linked to insulin resistance (Heliövaara et al., 2005; Lazar, 2005; Yates et al., 2011b), and this effect was evident in our study by the reduction of insulin-stimulated Akt phosphorylation after 2-hour exposure to either TNFα or IL-6. Despite this apparent impairment of insulin signaling, glucose uptake and oxidation rates

were nonetheless similar between muscle stimulated with insulin alone or co-stimulated with insulin and cytokines, indicating direct stimulation by cytokines. Moreover, cytokines were able to stimulate glucose oxidation in the absence of insulin. Together, these results show that inflammatory cytokines increase glucose metabolism in skeletal muscle independently of their impact on canonical insulin signaling. Furthermore, we show that the direct effects on glucose oxidation are not dependent upon parallel changes in glucose uptake. Previous studies have shown that short-term exposure to IL-6 acutely increases glucose uptake in humans and in human muscle biopsies via AMPK-mediated signaling pathways rather than PI3K or Akt-mediated pathways (Carey et al., 2006b; Glund et al., 2007; Saini et al., 2014a). Acute exposure of culture-derived C2C12 myotubes to TNFα likewise increased glucose uptake, as well as glucose utilization (Remels et al., 2015). It is unclear why cytokines did not increase basal glucose uptakes rates in the present study, but one possible explanation is the relatively short exposure time, as other studies have found that similar durations of IL-6 stimulation required co-incubation with the IL-6 receptor in order to observe increased glucose uptake (Gray et al., 2009; Saini et al., 2014a). Nevertheless, it is noteworthy that greater glucose oxidation was independent of glucose uptake rates in the present study. Glund et al. (Glund et al., 2007) previously found a moderate increase in glucose oxidation rates in IL-6 stimulated human muscle biopsies that was attributed to a concomitant increase in glucose uptake. However, we observed an ~36% increase in glucose oxidation with no increase in glucose uptake. Cytokine-stimulated increases in glucose oxidation were paralleled by greater phosphorylation of p38 and p44/42 MAPKs. Greater MAPK activity has been associated with increased glucose oxidation in cardiac myocytes (Palomer et al., 2009) and may likewise represent an Akt-independent mechanism for increasing glucose oxidation in skeletal muscle, much the same way that greater AMPK activation

is thought to increase glucose uptake (Glund et al., 2007; Gray et al., 2009; Saini et al., 2014a).

**Conclusions**

Our findings show that acute exposure of adult rat soleus muscle to β2 adrenergic agonist or to inflammatory cytokines stimulates greater glucose oxidation. Furthermore, we found that this effect was independent of insulin action and was not contingent upon increases in glucose uptake. In fact, muscle stimulated with TNFα and IL-6 showed evidence of decreased insulin signaling but greater MAPK activity, indicating that MAPKs may be important mediators of cytokine-induced glucose oxidation. Conversely, β2 adrenergic stimulation enhanced basal and insulin-stimulated glucose oxidation without affecting Akt or MAPK activity. The increase in fractional glucose oxidation rates appears to indicate greater skeletal muscle metabolic efficiency at the onset of the stress response, which may help to improve outcomes of acute adverse events.

Table 1. Components of each treatment media.

| Media[1] | | Additive[2] | | | | |
|---|---|---|---|---|---|---|
| | | Humulin R (5mU/ml) | Ractopamine (10μM) | Zilpaterol (0.5μM) | hTNFα (20ng/ml) | rIL-6 (45ng/ml) |
| a. | Basil (un-spiked) | - | - | - | - | - |
| b. | Insulin | + | - | - | - | - |
| c. | β1 Agonist | - | + | - | - | - |
| d. | β2 Agonist | - | - | + | - | - |
| e. | Insulin + β1 Agonist | + | + | - | - | - |
| f. | Insulin + β2 Agonist | + | - | + | - | - |
| g. | TNFα | - | - | - | + | - |
| h. | IL-6 | - | - | - | - | + |
| i. | Insulin + TNFα | + | - | - | + | - |
| j. | Insulin + IL-6 | + | - | - | - | + |

[1]Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin, gassed with 95% $O_2$ + 5% $CO_2$.

[2]All additives were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of Humulin R, which was purchased from Eli Lilly (Indianapolis, IN).

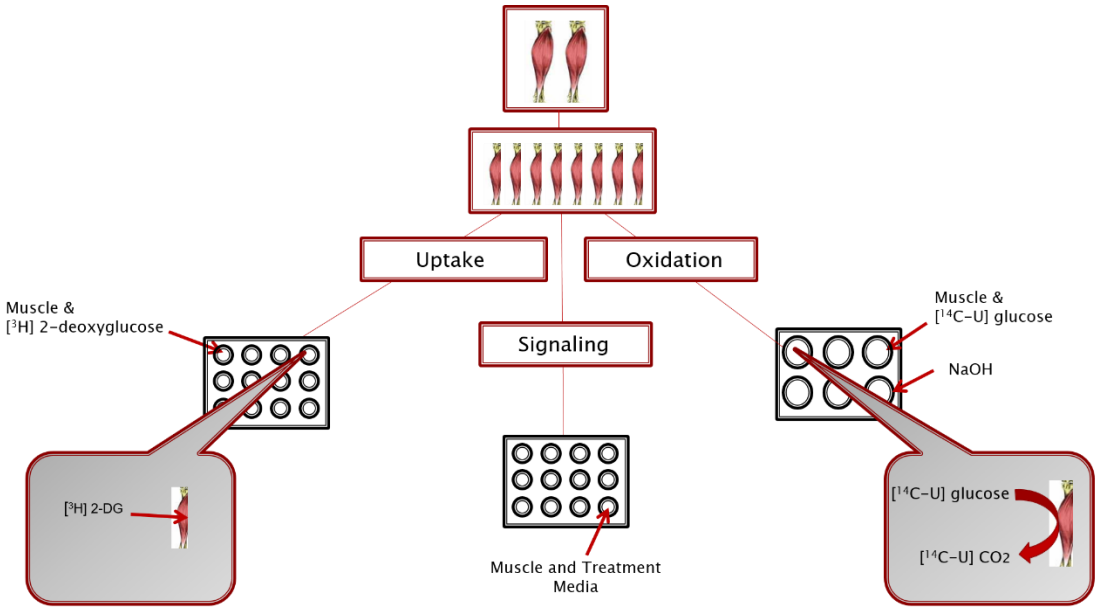


**Figure 1:** Schematic of metabolic studies performed using isolated adult rat soleus muscle.

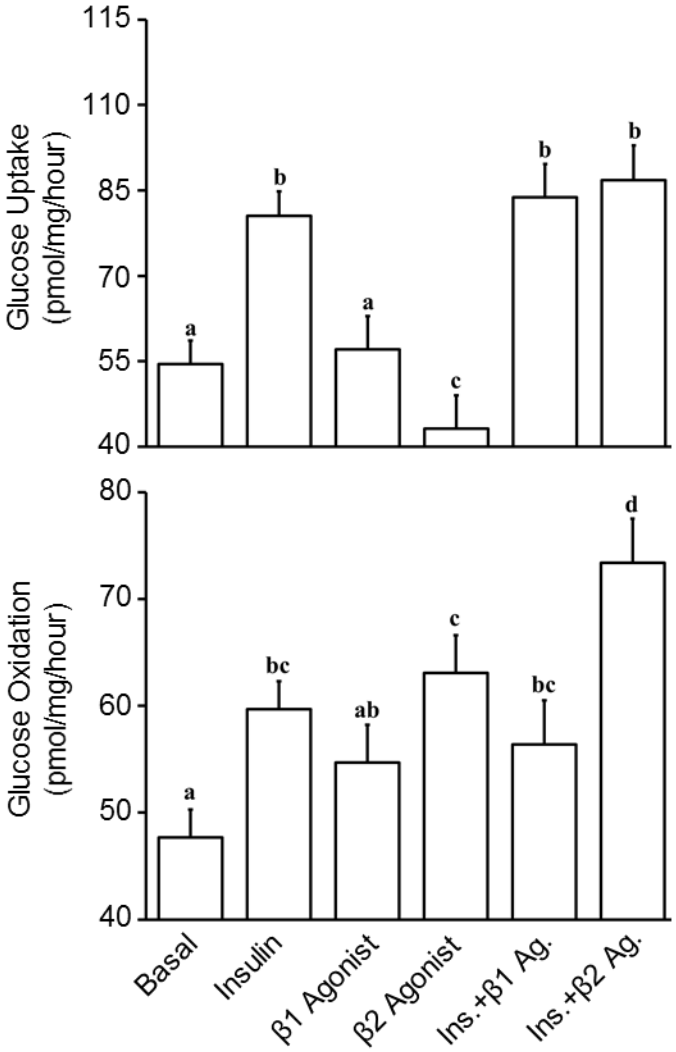


**Figure 2.** Glucose uptake and oxidation rates in primary rat soleus muscle during 20-minute (n = 10) or 2-hour incubation (n = 9), respectively, with isoform-specific adrenergic agonists. [a,b,c,d]means with different superscripts differ ($P < 0.05$).

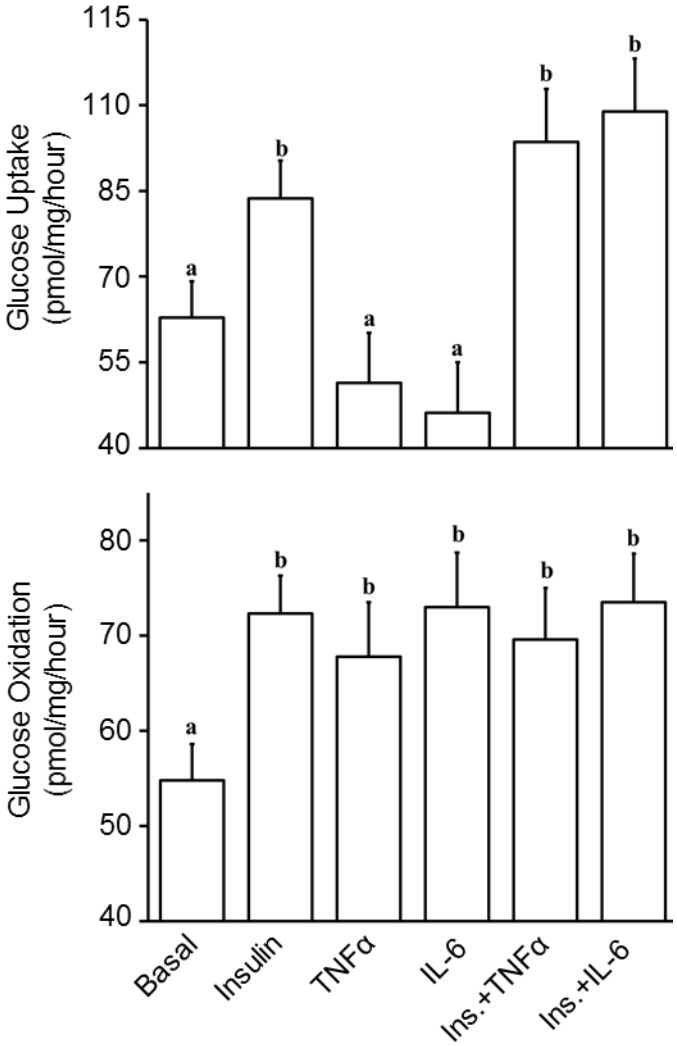


**Figure 3.** Glucose uptake and oxidation rates in primary rat soleus muscle during 20-minute (n = 10) or 2-hour incubation (n = 9), respectively, with inflammatory cytokines. [a,b]means with different superscripts differ ($P < 0.05$).

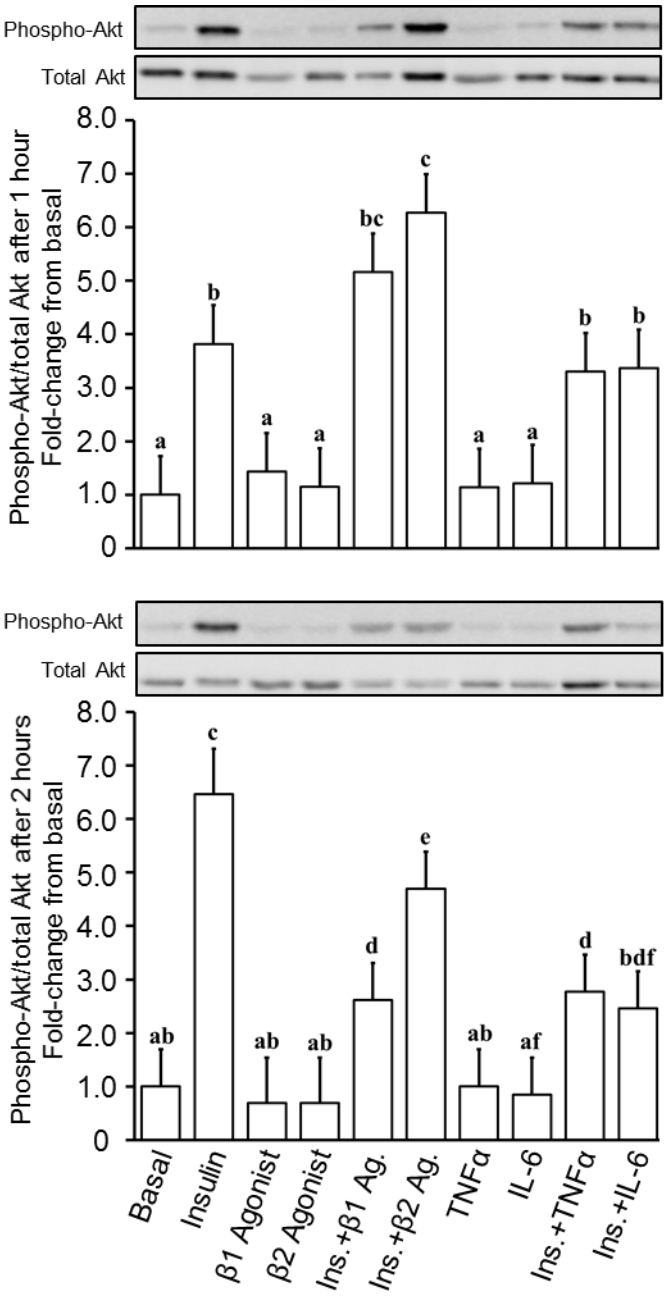


**Figure 4.** Akt phosphorylation in primary rat soleus muscle after 1 hour (n = 8) or 2 hours (n = 4) in incubation with isoform-specific adrenergic agonists or cytokines. $^{a,b,c,d,e,f}$means with different superscripts differ ($P < 0.05$).

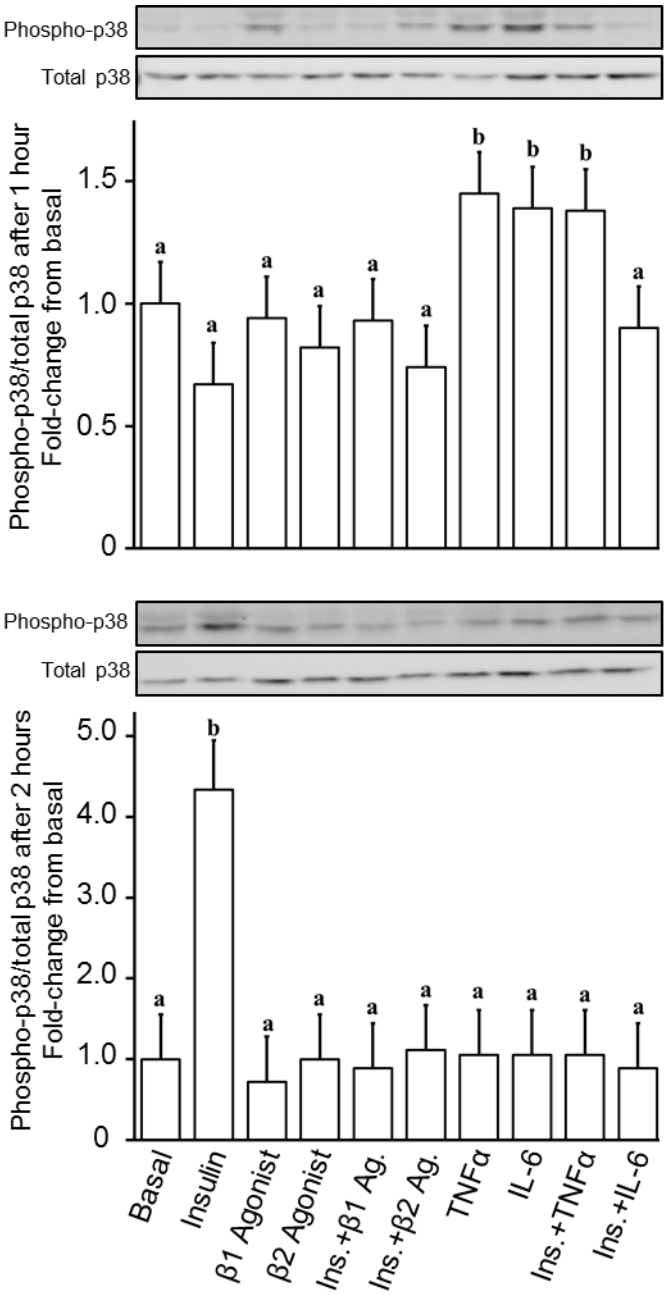


**Figure 5.** p38 MAPK phosphorylation in primary rat soleus muscle after 1 hour (n = 8) or 2 hours (n = 4) in incubation with isoform-specific adrenergic agonists or cytokines. [a,b]means with different superscripts differ ($P < 0.05$).

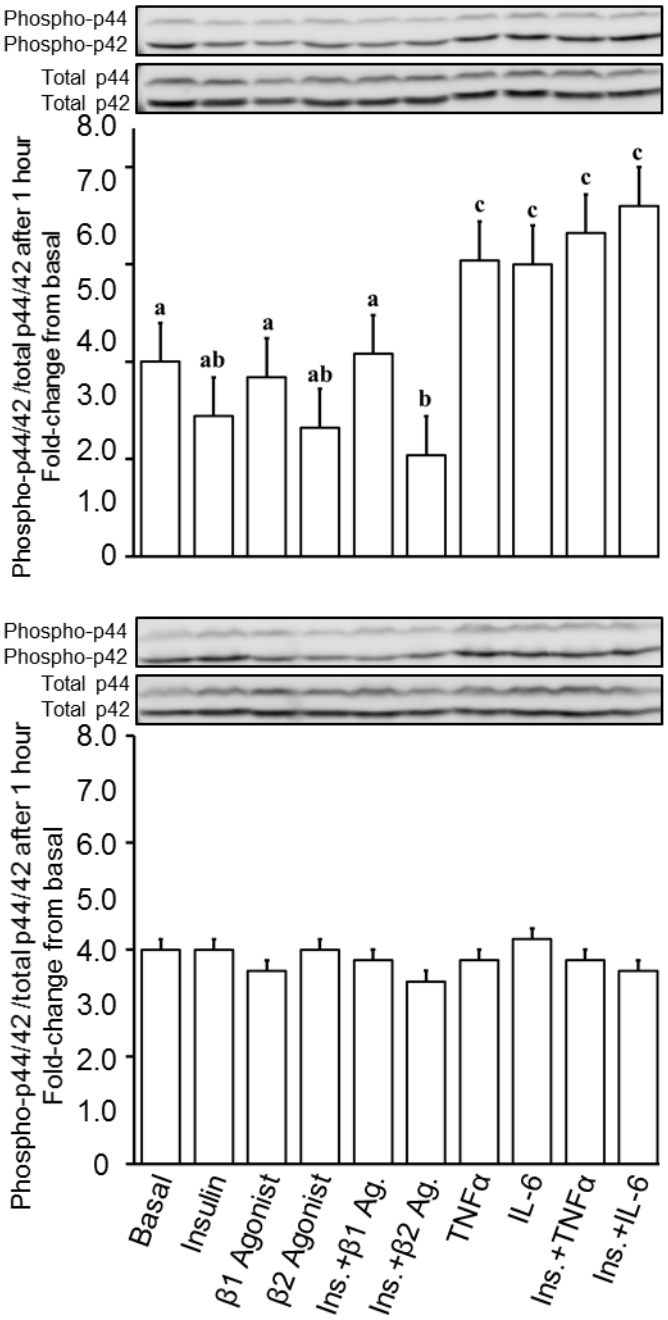


**Figure 6.** p44/42 MAPK phosphorylation in primary rat soleus muscle after 1 hour (n = 8) or 2 hours (n = 4) in incubation with isoform-specific adrenergic agonists or cytokines. $^{a,b,c}$means with different superscripts differ ($P$ < 0.05).

# Chapter 4

## Homework as a retrieval practice is more beneficial to long-term information retention when given 5 days after introducing new material compared to 1 day afterward.


### Abstract

In previous studies, temporally-spaced repetition of previously-presented material (practice) increased student retention of information. Our objective was to determine the effect of assigning online homework one or five days after introduction of material on short-term and long-term retention. Seventy-two students in an undergraduate Anatomy and Physiology course were presented new material in weekly 2-hr lab sections. Students then received homework via the Blackboard on-line portal either one or five days after the laboratory section. In-class quizzes were given seven days later, during the next laboratory section, to assess short-term retention. Long-term retention was determined from a comprehensive practical exam administered at semester's end. Corresponding assessment questions on respective practices and assessments targeted the same concepts, followed the same format, and were similarly-phrased. Students were grouped by overall course performance into High-performing and Average-performing categories, and data were collected for four hallmark Anatomy and Physiology topics: Cells and Tissues, Muscular System, Immune System, and Digestive System. Short-term retention of information did not differ due to timing of homework for High or Average-performing students. However, homework completed five days after introduction of material benefited long-term retention more than the same homework completed one day afterward, as average-performing students performed better ($P < 0.10$) on the practical for three of the four topics when homework on those topics were completed at five days. Performance on the fourth topic was not different. High-performing students performed similarly on three out of four topics, but performance on

the immune system was greater ($P < 0.10$) for High-performing students who completed the homework at five days. Overall, these findings indicate that the longer period of time between introduction of material and first retrieval practice was more beneficial to long-term retention but had no effect on short-term retention. Furthermore, the less profound effect observed in high-performing students could be explained by greater academic abilities or better study habits that overshadowed the difference due to timing of homework.

**Introduction**

Beginning in the 1990s, there has been a push to improve undergraduate education in science, technology, engineering and mathematics (STEM) education. The goal of this initiative is to develop new methods to improve student learning, assesment and other considerations for how STEM subjects are taught (Fairweather, 2008). These new teaching strategies seek to turn classrooms into student centered, peer-driven environments rather than the traditional lecture environments most commonly employed (Labov et al., 2009). Over the past couple of decades, one strategy widely explored is the use of assessments to evaluate student learning and instructor effectiveness. Most commonly, assessments such as quizzes and exams are used as mechanisms to estimate how much a student has learned. However, recent studies demonstrate a use for assessments as a study tool to increase learning and retention for later assessments (Roediger and Karpicke, 2006a). The benefit that repetition of previously-presented material has on information retention has been demonstrated in academia and is commonly used as a study tactic by students (Karpicke and Roediger, 2007). The concept of retrieval practice was developed many years ago, but, it is not widely used today and further research regarding its effectiveness has been sporadic. The use of assessments as a form of retrieval practice requires students to retrieve previously-

presented material from memory rather than mass practice, just as rereading or cramming. This method, known as the Testing Effect, has been shown to produce more durable learning (Roediger and Butler, 2011). The Testing Effect is dependent on the retrieval period, which may be classified as equal spacing or expanded spacing. Equal intervals between spaced retrieval practices refers to giving assessments on multiple occasions with a consistent interval of time between each assessment. Expanded intervals use multiple assessments, but the interval of time increases after each assessment (Logan and Balota, 2008). However, there is a gap in the knowledge regarding the impact of the length of the interval between initial introduction and first assessment.

Many of the previous studies evaluated K-through-12 students and used relatively short periods of time before assessing information retention. We postulate that retrieval practice is beneficial to college students as well. Moreover, although any practice assessment is more beneficial than no practice, we hypothesize that the period of time between introduction of material and the first retrieval practice can impact long-term retention. Therefore, the objective of this study was to identify whether 1 or 5 days between introduction of material and first retrieval practice is more beneficial for short-term as well as long-term information retention in college students enrolled in a sophomore-level Anatomy and Physiology course.

## Methods

### *Informed consent of participants*

All procedures for data collection in this study were approved by the Human Subjects Institutional Review Board at the University of Nebraska-Lincoln. Prior to data collection, students were informed of their option to participate in this study by an independent party without instructors or teaching assistants present. Students were not informed of the objectives of the study to ensure a double-blind design. All students were

required to complete the assignments and assessments as part of the course. However, only data from consenting individuals were included in analyses. Students that agreed to participate received a 1.5% bonus on their overall course grade as compensation for their participation. The same bonus was offered to non-consenting students for an equal alternative effort. Of the 75 students enrolled in the course, 72 consented to participate. Consent forms are included in the appendix of this thesis.

*Class structure and student demographics*

The sixty-five students who successfully completed the Anatomy and Physiology of Domestic Animals course in the Department of Animal Science at the University of Nebraska-Lincoln (UNL) were assessed in this study. Enrollment consisted primarily of sophomore (52%) and junior (28%) undergraduate students pursuing Bachelor of Science degrees in Animal Science (57%), Veterinary Science (9%), Fisheries and Wildlife (22%) or a closely related field. The class consisted of 30% male and 70% female students with interests in animal-based careers after graduation.

Anatomy and Physiology of Domestic Animals is a 16-week course consisting of three 1-hour lecture periods and one 2-hour laboratory period each week. It is required for all Animal Science, Veterinary Science, and Fisheries and Wildlife students. The course is designed to cover the function of all major systems of the mammalian body and the anatomical features that comprise each. Topics assessed for this study were: Cells and Tissues, The Muscular System, The Digestive System, and The Immune System (Figure 1). Laboratory activities corresponded to discussions presented in lecture. The weekly laboratory sessions were organized by physiological system and began with a 10-to-15 minute presentation consisting of an overview of the main concepts for the week's topic. Following the presentation, the remainder of the laboratory period was devoted to active learning activities.

*In-class activities*

For Cells and Tissues, students were presented with a brief presentation and video that explained the process of gene transcription and translation. Students were then given a worksheet (in appendix) that directed them to use the information in the video and identify optimal concentrations of different components involved in producing proteins. Afterward, microscopes were set up to display various tissue types. Students worked with a partner to examine the slides and identify the type of tissue, including compact bone, cardiac muscle, smooth muscle, skeletal muscle, adipose tissue, and different types of epithelial tissue. This exercise familiarized students with using microscopes and introduced them to the tissues within the major systems that are discussed throughout the semester.

The Muscular System lab began with a short presentation discussing the different characteristics of the three muscle types:  skeletal, cardiac, and smooth. The main activity of the lab was exploring the major muscle groups of animals by dissecting them in fetal pigs. The lab coordinator performed the dissection with an overhead projector and students working in pairs concurrently performed the dissection on their own pigs. Students were provided a worksheet, with questions about location and orientation of specific muscles, how muscles are named, the process of myogenesis, and a diagram that they labeled to demonstrate their understanding of muscle anatomy.

The presentation for the Digestive System involved a description of digestion in ruminants, non-ruminants, hindgut-fermenters, and avian species. To explore digestive anatomy, fetal pigs were utilized for dissection of the entire digestive tract led by the lab coordinator. Multiple preserved rumens were displayed to demonstrate the differences between the non-ruminant and ruminant stomach. The urinary system was also discussed during this lab and included a short presentation on kidney function and dissection of fresh pig kidneys. The worksheet (in appendix) contained questions about the anatomical features of the digestive tract and the urinary tract.

Lastly, the Immune System lab explored different animal pathologies associated with the previously-discussed systems covered throughout the semester. The presentation provided material explaining the different types of immunity, components of the immune system, and signs and symptoms of immune responses. Students worked in small groups on case studies prepared by the lab coordinator. Each group was asked to identify the clinical symptoms, suggest diagnostic tests, interpret the results, and discuss their diagnosis, treatment, and prognosis for the case. Students then presented their case to the class for further discussion.

*Retrieval practice and assessments*

The active learning activities were designed to demonstrate practical applications for the topics of interest. Using information from the lab presentation and the active learning activity, students completed laboratory assignments (in appendix) in class. The laboratory assignments consisted of 2 to 5-page packets with questions designed to guide students through the activity and highlight the important points of the topic. Questions were a combination of short answer, true/false, fill-in-the-blank, and matching. Students also filled out diagrams or created their own diagram to demonstrate their understanding of the topic. In general, students were encouraged to work on the assignment in pairs or small groups and to utilize their notes, textbook, and internet and to ask questions of the lab coordinator or teaching assistants. The assignment was collected at the end of the laboratory period, graded, and returned to the students the following week.

Students were randomly assigned to receive online homework (in appendix) through the Blackboard Learn portal either 1 (day 1 group) or 5 (day 5 group) days after their laboratory period (Figure 2). Online homework assignments were the same for all students and consisted of an open-note 10-question assessment that focused on the key concepts of the week's lab. The questions were a combination of multiple choice, short

answer, fill in the blank and true/false. Students had thirty-six hours from the time they were notified via email of the homework availability to complete the quiz. Each student received homework on d1 for two topics and d5 for two topics of the four evaluated.

At the beginning of the following week's lab, seven days later, short-term information retention was evaluated by in-class quizzes that covered the previous week's material. The in-class quizzes were closed-note and focused on the concepts emphasized in the homework. Each consisted of 10 short-answer questions phrased similarly to those included in the online homework.

During the last week of the semester, a cumulative final practical exam (in appendix) was administered to all students to evaluate long-term information retention. Students had two hours to complete the written exam which required them to demonstrate their knowledge of the course topics using information from laboratory activities they had performed during the semester (ie. dissection, tissue histology identification, identification of specific bones on a skeleton etc.). Performance on questions pertaining to four topics were evaluated. These questions were similarly phrased and contained fill in the blank, multiple choice and short answer questions. All laboratory presentations, assignments, online homework, in-class quizzes, and final practical were written by the lab coordinator and approved by the instructor of the course. All assignments were graded by the lab coordinator or teaching assistants. Students had the right to appeal any grade to the lab coordinator and then instructor.

*Statistical analysis*

Data collected in the laboratory portion of the class were combined with lecture scores, and students were grouped based on overall course performance. Students classified as High-performing received a B+ or greater in the course (n=14). Students classified as Average-performing received between a B and D- in the course (n=51). Students not successfully completing the course were not included. Performance on

homework, in-class quiz, and practical exam was recorded as the percentage of total points earned. Data for performance of assessments were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). High-performing and Low-performing groups were analyzed separately. Data are presented as means ± standard error.

**Results**

*Short-term information retention.* Performance on in-class assignments did not differ between High-performing students in the day 1 or day 5 group for any topic (Figure 3). Average-performing students in the day 1 group had greater ($P < 0.10$) performance on the Muscular System homework than Average-performing students in the day 5 group. The Average-performing day 1 group also performed better ($P = 0.10$) on the Digestive System homework, but performance did not differ for the other two topics. Performance on in-class quizzes administered seven days after introduction to material did not differ between the day 1 group and day 5 group in High-performing or Average-performing students for any topic (Figure 4).

*Long-term information retention.* The Average-performing day 5 group, performed better ($P < 0.10$) than the day 1 group on the Cells and Tissues, the Digestive System, and the Immune System portions of the practical exam (Figure 5). Performance for the Muscular System portion did not differ between the Average-performing day 1 and day 5 group.

In High-performing students, performance on Cells and Tissue, the Digestive System, and the Muscular System portions of the practical exam did not differ between the day 1 group and day 5 group. However, High-performing students in the day 5 group performed better ($P < 0.10$) on the Immune System portion than the day 1 group.

**Discussion**

In this study, we show that an interval lasting several days between introduction to material and first retrieval practice benefited long-term information retention in college students more so than an interval lasting only one day. Interestingly, both interval lengths benefitted short-term information retention equally. We also show that these effects differ depending on the performance level of the student, as the benefit of the larger interval was profound in average-performing students but was diminished in high-performing students. This is likely due to better study habits already being utilized in High-performing students. Together, these findings show that it is beneficial to have several days between introduction of material and first retrieval practice for long-term retention rather than a single-day interval, but, these effects may be less effective in high-performing students.

Increasing the interval between a student's first exposure to new information and their first retrieval practice appears to increase retention of the information. Homework assessments administered five days after the students' laboratory period increased their performance on the final practical exam despite having no effect on performance at one week. Previous studies found that repeated study habits, such as re-reading or re-writing information improves short-term retention, but repeated assessment increases long-term retention (Roediger and Karpicke, 2006b; Butler and Roediger III, 2007). These previous studies measured long-term retention at one week and one month, respectively, but our study indicates that these improvements in information retention are maintained for a longer period of time. In the previous studies, enhanced retention was observed when repeated testing occurred within 24 hours after presentation of material, but our study showed no such effect on short-term or long-term information retention when the assessment occurred at 24 hours. However, by utilizing the Testing Effect five days after introduction of material instead of one day, student information retention was improved when assessed 4 to 13 weeks later. The greater benefit of the longer interval may be

explained by the rate at which students forget material (Wheeler et al., 2003; Pashler et al., 2007). Based on these rates, students who are asked to retrieve information 24-hours after initial introduction to material have not had ample time to forget information and thus can rely on short-term memory to regurgitate facts without retaining concepts in long-term memory. This may also explain why students who received the homework 24-hours after the lab section scored higher on some homework. At five days after introduction of material, students must retrieve information that they have likely forgotten, asking them to re-learn the material creates more neural pathways to enhance long-term retention (Willis, 2007).

Although longer spacing of the online homework increased long-term information retention, it had no benefit on short-term information retention. At one week, retrieval practice had only occurred once in the form of the online homework, and the absence of a difference in short-term retention may be explained by the type of practice. The open-note, unrestricted-resource design of the online quiz may have functioned more as recognition than recall, imitating re-studying rather than re-testing. Multiple studies (Mandler and Rabinowitz, 1981; McDaniel et al., 1989; Karpicke and Roediger, 2007; McDaniel et al., 2007) have compared the effectiveness of re-studying versus re-testing and have found that re-testing resulted in better information retention. However, the in-class quiz was closed-note and required students to recall information from memory in order to answer the questions and thus was itself a retrieval practice that contributed to the testing effect and better long-term information retention. The recall-versus-recognition argument can also be applied to the types of questions on the retrieval practices. Tests or quizzes that are short answer require more thorough recall than multiple-choice assessments which require primarily recognition (McDaniel et al., 2007). However, since both the online and in-class quizzes incorporated combinations of

multiple choice, short answer, and fill-in-the-blank questions this would likely not apply to the effects seen in this study.

For High-performing students, interval effects were less profound. Students who received online homework five days after introduction to material performed better in only one of the four evaluated topics on the final practical. The decreased benefit may be explained by the very nature of this category of students as High-performing students were those that received a B+ or higher in the course and may have already developed effective individual study techniques that overshadow the benefit of the interval length. Although the online homeworks and in-class quizzes may be beneficial to their learning of material, they will presumably perform well regardless of when repetition occurs.

**Implications**

Our findings demonstrate that the timing of the first retrieval practice is important in enhancing long-term information retention. Periods lasting several days between introduction to material and retrieval are more beneficial to retention than shorter periods, which could be considered mass practice. Although, these effects were minimal in students who perform well with their own study habits, this knowledge may be easily implemented in the classroom. Instructors can strategically place assessments throughout courses that will act as methods of increasing students' retention rather than for final evaluation of student learning. This simple adjustment could improve student outcomes by enhancing durable learning and individual STEM teaching strategies.

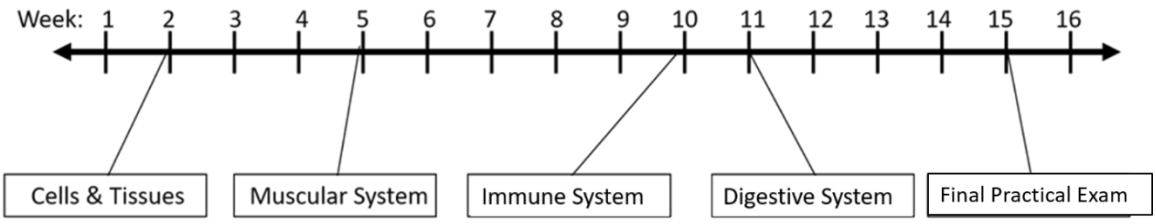


**Figure 1.** Schematic showing weekly lab topics and practical exam.

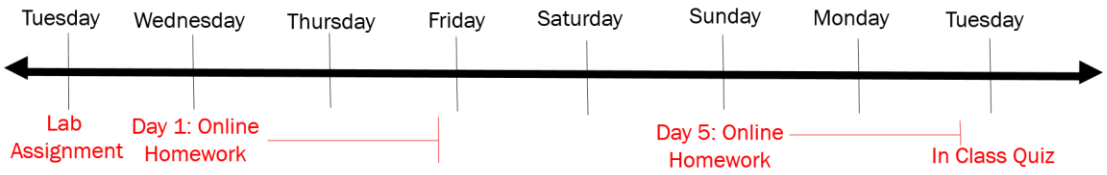


**Figure 2.** Schematic showing administration times for assignments, homework and quizzes each week.

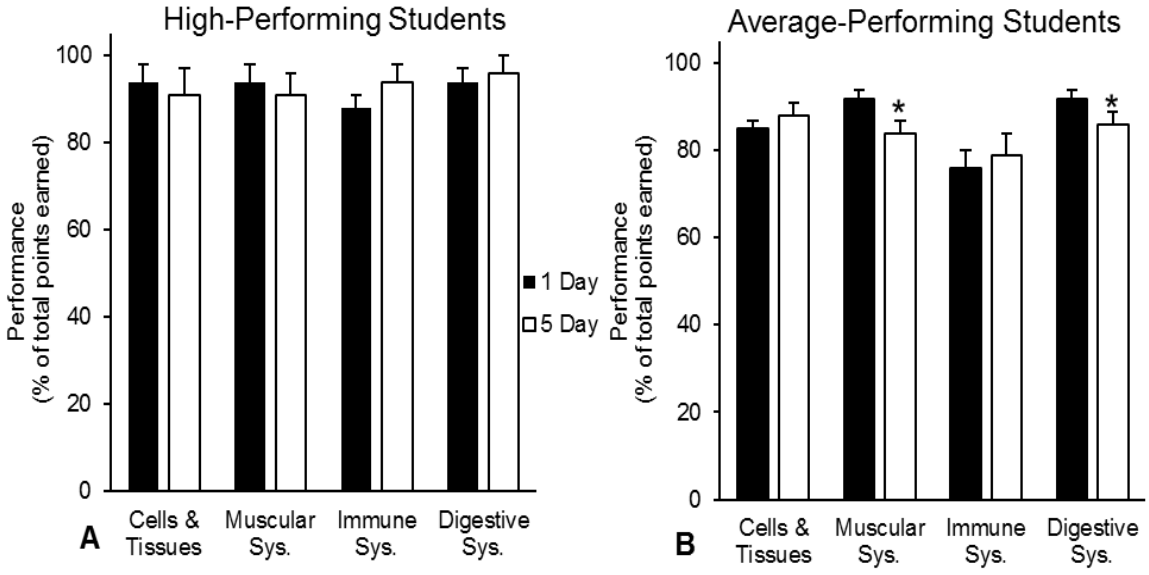


**Figure 3.** Performance on online homeworks in High-performing (A) and Average-performing (B) students given one day or five days after introduction of material. * Means differ ($P < 0.10$) within topic.

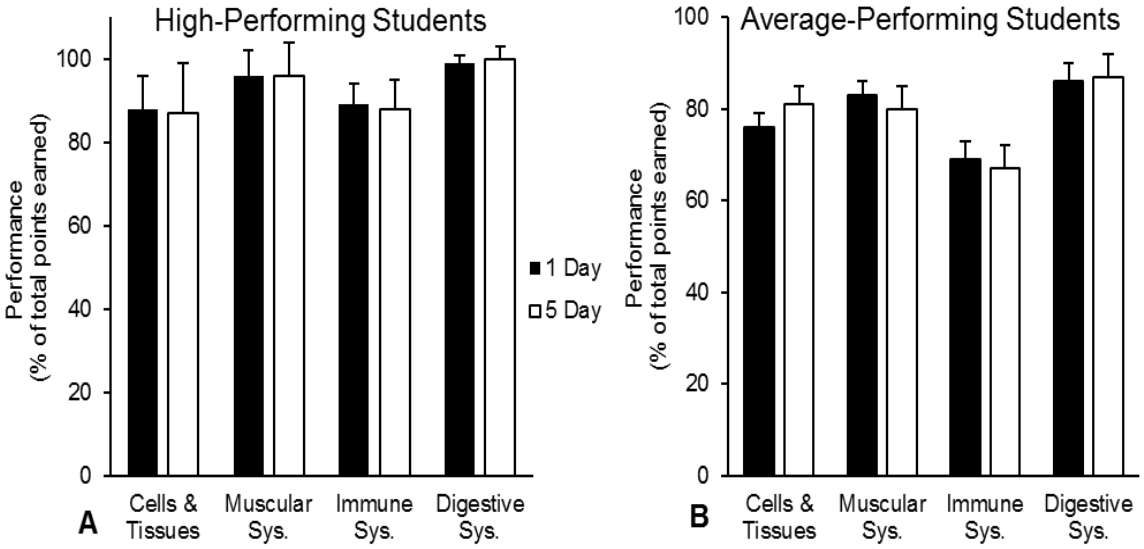


**Figure 4.** Performance on in-class quizzes in High-performing (A) and Average-performing (B) students given one day or five days after introduction of material. * Means differ ($P < 0.10$) within topic.

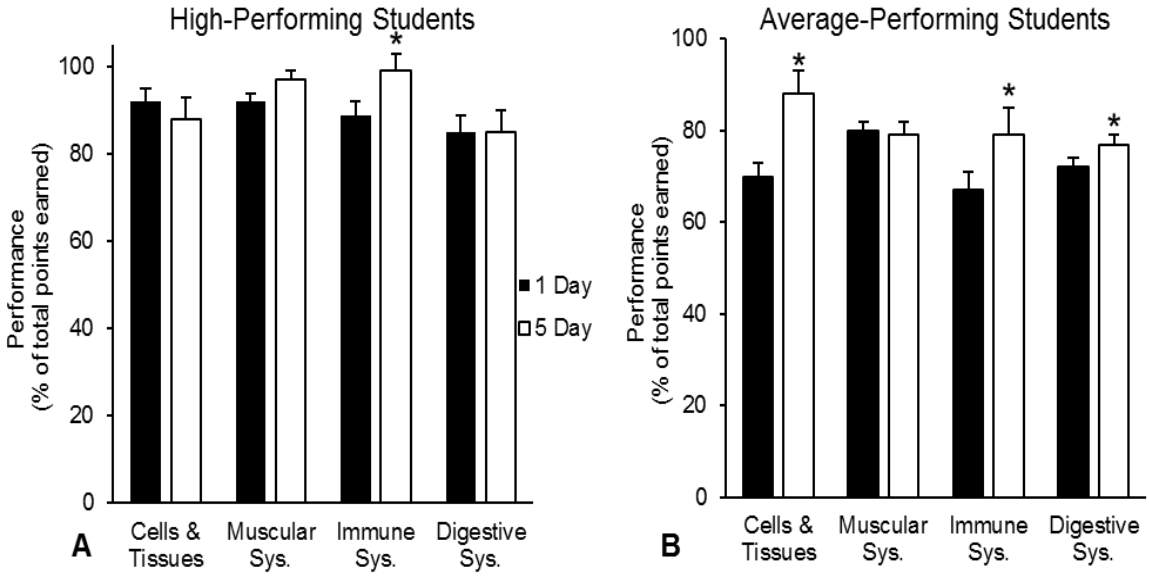


**Figure 5.** Performance on the final practical exam in High-performing (A) and Average-performing (B) students given one day or five days after introduction of material. * Means differ ($P < 0.10$) within topic.

**Appendix**

University of Nebraska Lincoln

IRB# 20150915557 EX
Date Approved: 09/09/2015
Valid Until: 09/09/2020

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
DEPARTMENT OF ANIMAL SCIENCE

*Participant Informed Consent Form*

IRB#15557

**Title:** Assessment of the effectiveness of laboratory teaching methods in ASCI 240

**Purpose:**
This research project will aim to determine the effectiveness of some of the instruction techniques we use in this course. You are invited to participate in this study because you are a UNL student and are enrolled in ASCI 240.

**Procedures:**
You will be asked to perform in class as usual. The methods that we are evaluating are long-standing in this course and are due for evaluation. The techniques we use to evaluate them will not influence the information or impact the learning experience of this course. Moreover, it will not impact your grade. The procedures will last for the duration of the semester, and will be conducted in class and on Blackboard. You will not be asked to complete any additional work that is not already required in this course. Overall student performance data (general student information, grades and assignments) will be collected. Furthermore, all data collected will be kept confidential and only used for research purposes.

**Benefits:**
There are no direct benefits/detriments to you as a research participant. However, the data collected should improve the class structure going forward.

**Risks and/or Discomforts:**
There are no known risks or discomforts associated with this research.

**Confidentiality:**
Any information obtained during this study which could identify you will be kept strictly confidential using the same standards as grade confidentiality. The data will be stored in a locked cabinet in the investigator's office and will only be seen by the investigator. Any identifiable records will be destroyed at the semester's end. The information obtained in this study may be published in scientific journals or presented at scientific meetings but the data will be reported as aggregated data (not specific to any student).

**Compensation:**
You will receive a 1.5% bonus on your final course grade for participating in this project. Students choosing not to participate will be able to receive an equal bonus by meeting with Dr. Yates.

**Opportunity to Ask Questions:**
You may ask any questions concerning this research and have those questions answered before agreeing to participate in or during the study. Or you may contact the investigator(s) at the phone numbers below. Please contact the University of Nebraska-Lincoln Institutional Review Board at (402) 472-6965 to voice concerns about the research or if you have any questions about your rights as a research participant.

**Freedom to Withdraw:**
Participation in this study is voluntary. You can refuse to participate or withdraw at any time without harming your relationship with the researchers or the University of Nebraska-Lincoln, or in any other way receive a penalty or loss of benefits to which you are otherwise entitled.

**Consent, Right to Receive a Copy:**
You are voluntarily making a decision whether or not to participate in this research study. Your signature certifies that you have decided to participate having read and understood the information presented. You will be given a copy of this consent form to keep.

**Signature of Participant:**

Signature of Research Participant _____ Date _____

**Name and Phone number of investigator(s)**

| | | |
|---|---|---|
| Dustin Yates, PhD Principal Investigator | Office: (402) 472-6305 | dustin.yates@unl.edu |
| Caitlin Cadaret, Secondary Investigator | Phone: (530) 917-0086 | caitlin.cadaret@gmail.com |

**Cells and Tissues: Online Quiz**

1. True/False: Epithelial tissues that have multiple layers are called stratified

2. Multiple Choice: What are the amino acids that make up protein attached to during translation?

tRNA rRNA mRNA DNA

3. Short Answer: What type of tissue contains axons?

4. Multiple Choice: What type of muscle lines arteries?

Smooth cardiac
Skeletal none of the above

5. Multiple Choice: What enzyme transcribes DNA into different types of RNA?

RNA Polymerase negative transcription factors
Positive Transcription Factors RNAse

6. Short Answer: What type of epithelial cell is within the air sacs of the lung and allows oxygen to diffuse easily?

7. Short Answer: What type of epithelial cell is tall and skinny and only has one layer?

8. Multiple Choice: The overall goal of transcription and translation is to turn ____ into _____.

DNA into Protein mRNA into tRNA
mRNA into protein rRNA into a ribosome

9. Multiple Choice: Translation occurs on the...

Ribosomes of the Rough ER Rough ER
Smooth ER Nucleus

10. Multiple Choice: Transcription transcribes _____ into _____.

DNA into mRNA mRNA into tRNA
DNA into protein DNA into protein

**Cells and Tissues: In-Class Quiz**

1. What is RNA in relation to DNA?

2. Where in the cell does transcription and translation occur?

3. What is the role of RNA polymerase in transcription?

4. List the 4 nucleotides of DNA. Include which nucleotides pair with each other:

5. _____ are the building blocks of proteins.

6. True or False: Prophase is the stage of mitosis in which chromosomes condense

7. True or False: Metaphase is the stage of mitosis in which cytokinesis begins

8. How do the following factors influence protein concentrations?
    a. Transcription factors

    b. Transcription factor affinity

    c. Polymerase affinity

**Muscular System Online Quiz**

1.What type of muscle has a fast speed of action?
skeletal and cardiac  smooth and skeletal
smooth and cardiac  none of the above

2. Multiple Choice: What is the end of muscle that is attached to the bone to be moved called?
insertion  belly
origin  fascia

3. Multiple Choice: how are fibers arranged on a multipennate shaped muscle?
tendons branched within muscles  fibers on one side of the tendon
fibers on both sides of the tendon  fibers parallel to the muscle axis

4. Multiple Choice: _____ muscles bring bone closer to the midline of the body.
adductor  supinator
abductor  pronator

5. Multiple Choice: What abductor muscle is located on the back of the trunk near the shoulder blades?
latissimus dorsi  pectoralis
trapezius  abdominals

6. Multiple Choice: The front of the thigh is a collection of muscles that make up the_____. While the back of the thigh is a group of muscles that make up the_____.
quadriceps, hamstring  soleus, gastrocnemius
hamstring, quadriceps  gastrocnemius, soleus

7. Multiple Choice: What is an example of a flexor muscle?
bicep  pectoralis
tricep  scapularis

8. Multiple Choice: What muscle is on the top of the shoulder and rotates the arm?
deltoid  tricep
bicep  flexors and extensors

9. Multiple Choice: What is the most common orientation of muscles?
parallel  pennate
convergent  sphincter

10. Multiple Choice: What type of muscle contains intercalated disks?
cardiac  smooth
skeletal  all of the above

**Muscular System: In-Class Quiz**

1. Draw a line from the muscle type to its matching characteristics.

| | |
|---|---|
| Smooth | Multinucleated, peripheral located nuclei and striated |
| Skeletal | striated, centrally located nuclei and has intercalated disks |
| Cardiac | not striated, centrally located nuclei and spindle shaped |

2. Cardiac muscle is under _____ control and has a _____ speed of action.

3. What are four ways muscles can be oriented?

4. What two muscles (that we dissected) make up the calf?

5. Where are 3 places smooth muscle is located? What is its role in these locations?

Bonus: What is the most common method used to prevent white muscle disease in young cattle?

**Digestive System Online Quiz**

Which of the following locations would have a sphincter muscle?
all of the above esophagus
stomach urethra

2. Multiple Choice: Salivary amylase begins breakdown of what in the mouth?
carbohydrates lipids
protein none of the above

3. Multiple Choice: What is considered the "true stomach" of ruminant animals?
Abomasum rumen
Omasum reticulum

4. Multiple Choice: What accessory organ produces bile?
liver pancreas
gall bladder small intestine

5. Multiple Choice: What is considered the "true stomach" in chickens?
proventriculus gizzard
ventriculus vent

6. Multiple Choice: What structure within the kidneys acts as a funnel for urine into the ureter?
pelvis medulla
cortex pyramids

7. Multiple Choice: What is the functional unit of the kidney?
nephron cortex
medulla calcyes

8. Multiple Choice: What blood vessel brings waste-filled blood to the kidney for filtering?
renal artery efferent arteriole
renal vein afferent arteriole

9. Multiple Choice: Which of the following is not a function of the kidney?
returns bile to the blood secretes waste
removes metabolic waste from blood
regulates blood composition

10. Multiple Choice: Where is urine excreted from the body?
urethra ureter
bladder kidney

**Digestive & Urinary System: In-Class Quiz**

What is the "true stomach" of a:

Ruminant:

Chicken:

The small intestine can be broken down into what three structures?

What accessory organ secretes bile?

Give two examples of non-ruminant animals.

What is the functional unit of the kidney?

The renal pelvis drains into what muscular tubes that brings urine to the bladder?

Urine leaves the body through what structure?

What are two functions of the urinary system?

In order, explain how food travels through the ruminant stomach.

Bonus: Explain what an NG tube is used for in the treatment of equine colic.

**Immune System Online Quiz**

Which of the following leukocytes are not secretory cells?

macrophages eosinophils
basophils natural killer cells

2. True/False: adaptive immunity is antigen specific.

3. True/False: adult animals are more susceptible to diseases than younger animals.

4. True/False: pregnant animals are immunocompromised.

5. Multiple Choice: Which leukocyte makes up 50-60% of total leukocyte population?

neutrophils monocyte
macrophage basophils

6. Multiple Choice: T Cells are produced in the_____ and mature in the_____.

bone marrow, thymus bone marrow, bone marrow
thymus, bone marrow thymus, thymus

7. Short Answer: What are 3 indicators that an animal is not feeling well?

8. Short Answer: What are some environmental factors that can contribute to contraction of an illness?

9. Short Answer: what cells produce antibodies?

10. Multiple Choice: Which of the following vaccines is an example of a toxoid vaccine?

tetanus measles
flu rabies

**Immune System: In-Class Quiz**

1. What is an erythrocyte?

2. What is an antigen?

3. What are the three common types of vaccines used in animals?

4. What substance makes pus?

5. What cell is the largest leukocyte in a blood smear and becomes a macrophage when it enters tissue?

6. Describe the process of phagocytosis (2 points)

7. What substance in an inflammatory response makes blood vessels more "leaky?”

8. What cells produce and secrete antibodies?

9. What is the scientific name for a platelet?

Bonus: What were the “sick” sheep from last week’s lab infected with?

**ASCI 240 Final Lab Practicum-Questions from topics evaluated**

**Muscular**

Station 3. What muscles are being shown on the pig?

1. 2. 3.

These three muscles collectively make up what?

Station 4. Demonstrate your knowledge of the main muscle groups by correctly labeling the given figure.

A. B. C. D.

**Immune**

Station 7. Explain the process of phagocytosis in at least 4 steps.

What is the major difference between passive and active immunity?

Station 8. What type of white blood cell in shown on this microscope?

What are two examples of external defenses against pathogens?

**Urinary/Renal**

Station 9. For filtration, what is the functional unit of the kidney?

Identify the structures on the model of the kidney.

A.

B.

C.

Station 10. What structure allows urine to move from the kidneys to the urinary bladder?

Urine exits the body, from the bladder, through what structure?

**Digestive**

Station 11. Identify the correct compartments of the stomach in the ruminant animal.

A. B. C. D.

Of the structures you listed, which compartment catches large particles that will be regurgitated and re-chewed?

Station 12. Which accessory organ produces bile? Which accessory organ stores and secretes bile?

(Circle the correct words) A Cow/Pig is a ruminant/non-ruminant animal and can therefore digest cellulose.

**Cells & Tissues**

Station 17. What type of muscle is this slide showing? How do you know?

Where would you expect to find this type of muscle within the body?

Station 18. This is lung. Alveoli are lined with what type of tissue/cells?

Why is this tissue type ideal for gas exchange?

Station 19. Transcription creates_____from _____ and takes places in the_____.

Translation creates_____from a _____sequence and occurs in the_____.

What are the optimal conditions for producing large amounts of protein at a rapid rate?

| | Concentration | Affinity |
|---|---|---|
| Positive Transcription Factor | Lowest---------- Highest | Lowest---------- Highest |
| Negative Transcription Factor | Lowest---------- Highest | Lowest---------- Highest |

| | | |
|---|---|---|
| RNase | Lowest----------Highest | Constant |
| RNA Polymerase | Constant | Lowest----------Highest |
| Protein-Degrading Enzymes | Lowest----------Highest | |

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