UNIVERSITY OF CALIFORNIA, SAN DIEGO

Is Myocyte-derived VEGF in Adult Mice Required for Normal Skeletal Muscle Structure and Function?

A dissertation submitted in partial satisfaction of the requirements for the Doctor of Philosophy

in

Biomedical Sciences

by

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2009

The Dissertation of Amy Elizabeth Knapp is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2009

DEDICATION

I dedicate this dissertation in loving memory of my grandfather, Myron L. Bloom, who exemplified loyalty, commitment, and courage. The pillar of our family, he is dearly missed.

And to Derek, my love and my best friend. He encourages me to challenge myself and inspires me to be better.

And finally, to Leah and Owen, the two who have shown me what is truly important.

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ABSTRACT OF THE DISSERTATION

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Impaired exercise capacity in patients with chronic disease, such as chronic obstructive pulmonary disease (COPD), is likely due in part to skeletal muscle dysfunction. One possible contributing factor is altered regulation of angiogenesis leading to reduced skeletal muscle capillarity. Capillaries are a critical component of oxygen transport to mitochondria, and sufficient capillarity is thus important to endurance exercise. Expression of vascular endothelial growth factor (VEGF), an important regulator of skeletal muscle angiogenesis, is reduced in skeletal muscle in COPD. Recently, we showed that reduction in VEGF across all skeletal muscle cell

types in adult mice causes extensive (~60%) and permanent capillary loss. We also showed that capillary loss of this magnitude was associated with severe exercise limitation. Furthermore, VEGF is cytoprotective, and apoptosis from lack of VEGF could lead to muscle inflammation and/or atrophy that could compromise muscle integrity and result in contractile dysfunction. By far the most VEGF expressed in muscle comes from the myocytes themselves. Accordingly, this thesis tests the hypothesis that *myocyte*-specific VEGF (as opposed to VEGF from other cells in muscle tissue, such as satellite and endothelial cells) is critical for maintaining skeletal muscle capillarity, muscle function, and exercise capacity in adult cage-confined mice. To test this hypothesis, we developed a novel, inducible skeletal myocyte-specific VEGF knockout mouse. We found that these mice exhibit >90% reduction in whole muscle VEGF protein levels (similar to our prior models), yet only a modest impairment of exercise capacity and no reduction in capillarity or impairment of contractile function. This suggests that in adult mice, VEGF from other cells within muscle must be more important for capillary maintenance and muscle contraction.

CHAPTER 1

INTRODUCTION

1.1 Significance

Vascular endothelial growth factor (VEGF) is a potent regulator of angiogenesis (Ferrara 1999), the process of new capillary growth. VEGF has been studied widely in the pathogenesis of cancer (Hicklin and Ellis 2005) and certain retinopathies (Penn, Madan et al. 2008) and also regulates physiologic angiogenesis in skeletal muscle in response to training and hypoxia (Amaral, Papanek et al. 2001; Deveci, Marshall et al. 2001). We have recently shown that VEGF is also required for the maintenance and development of the adult skeletal muscle microvasculature and that lifelong reduction in muscle VEGF greatly reduces endurance exercise capacity in transgenic mice (Tang, Breen et al. 2004; Olfert, Howlett et al. 2009).

VEGF is an intriguing molecule as either aberrant increases or decreases of VEGF are implicated in the etiology of many diseases. Increases in VEGF have been shown to increase capillary growth surrounding tumors (Gannon, Mandriota et al. 2002) and decreased VEGF expression is associated with capillary loss in chronic disease (Jobin, Maltais et al. 1998; Barreiro, Schols et al. 2008; Doi, Noiri et al. 2010). Our laboratory is interested in VEGF's role in the regulation of skeletal muscle capillarity and its responses to acute exercise and training. More recently we have questioned how this molecule may be involved in the etiology of muscle dysfunction seen in patients with chronic obstructive pulmonary disease (COPD).

COPD initiates systemic hypoxia, oxidative stress and inflammation that likely contribute to lung, heart and skeletal muscle dysfunction. Due to this multiple organ involvement, COPD patients experience decreased exercise capacity and a decline in their quality of life. Peripheral skeletal muscle dysfunction contributes, in part, to the reduction in exercise capacity seen in these patients but the mechanisms underlying the dysfunction are not clear. Up to 25% of COPD patients exhibit muscle wasting or cachexia with unknown cause (Schols 2002). Circulating TNFα has been shown to cause muscle atrophy and decreased skeletal muscle force production in mice (Langen, Schols et al. 2006; Hardin, Campbell et al. 2008). Early studies in COPD have reported increased systemic TNFα in cachectic patients (Di Francia, Barbier et al. 1994; Takabatake, Nakamura et al. 2000; Eid, Ionescu et al. 2001) although more recent evidence disputes this claim, finding no difference in TNFα levels between cachectic and noncachectic groups (Broekhuizen, Grimble et al. 2005; Van Helvoort, Heijdra et al. 2006). This is likely due to advances in the sensitivity of the methods used to measure TNFα. These findings, however, do not eliminate TNFα as a possible contributor to cachexia. Since measurements of TNFα are made long after cachexia has set in, it is unknown whether TNFα may initiate the process of muscle wasting and contribute to peripheral muscle dysfunction.

Other possible causes of skeletal muscle dysfunction in COPD are increased oxidative stress (Rabinovich, Ardite et al. 2001; Rabinovich, Ardite et al. 2006), decreased oxidative capacity due to an increased proportion of Type II fibers compared to Type I fibers (Whittom, Jobin et al. 1998; Gosker, van Mameren et al.

2002), reduced mitochondrial density (Gosker, Hesselink et al. 2007), and decreased oxidative enzyme capacity (Jakobsson, Jorfeldt et al. 1995; Maltais, Simard et al. 1996). Decreased capillarity has also been observed in some COPD patients (Jobin, Maltais et al. 1998) but again, the mechanisms underlying this change are not known.

Evidence from our laboratory and others suggests that COPD patients have a dysregulation of VEGF in peripheral skeletal muscles. We have preliminary data showing resting and exercise-stimulated VEGF mRNA is reduced by 34% and 43%, respectively in the vastus lateralis muscle of COPD patients compared to age matched controls (Fig. 1.1). These findings were recently supported in a study by Barreiro et al. which reported significantly lower VEGF protein in resting skeletal muscles from COPD patients compared to controls (Barreiro, Schols et al. 2008). Furthermore, we have shown VEGF to be an important cytoprotective molecule in mouse skeletal muscle as well as a critical factor in the maintenance of adult skeletal muscle capillarity (Tang, Breen et al. 2004).

Many cell types in skeletal muscle produce VEGF. These include but are not limited to the mature myocytes (Birot, Koulmann et al. 2003), satellite cells (Christov, Chretien et al. 2007), endothelial cells (Lee, Chen et al. 2007), pericytes (Darland, Massingham et al. 2003), fibroblasts (Coppe, Kauser et al. 2006) and macrophages (Hirose, Maeda et al. 2008). We have recently shown that the myocyte is the major source of VEGF in skeletal muscle, as deletion of the VEGF gene specific to this cell type results in 90% reduction in VEGF protein in whole skeletal muscle (Olfert, Howlett et al. 2009). The contribution of VEGF from the remaining cell types in

muscle must be small since it only accounts for ~10% of total VEGF. This has been demonstrated in the heart where VEGF deleted from only the endothelial cells resulted in normal tissue levels of VEGF (Lee, Chen et al. 2007). Therefore, we hypothesize that deletion of VEGF from adult skeletal myocytes will result in decreased capillarity, muscle function and exercise performance.

Using a skeletal *myocyte*-specific, inducible VEGF knockout model, this project attempts to determine the importance of VEGF that is generated in the myocytes per se (rather than in other muscle tissue cells such as endothelial cells, satellite cells, fibroblasts, macrophages and pericytes) in protecting adult skeletal muscle against capillary regression and muscle weakness. It is expected that this information will provide insight into the mechanisms that may underlie the muscle dysfunction seen in COPD patients.

1.2 Vascular Endothelial Growth Factor-A (VEGF)

1.2.1 VEGF overview

VEGF is part of the platelet-derived growth factor (PDGF) family consisting of placenta growth factor (PIGF) and VEGF A, B, C, D and E. VEGF A is the most widely expressed and the most biologically active form with effects specifically on the vasculature and on angiogenesis. Therefore, VEGF-A will be the focus of this project and, herein, referred to as VEGF. There are at least 3 murine isoforms of VEGF, ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$) which are produced through alternative splicing of a single gene (Shima, Kuroki et al. 1996). The 3 isoforms differ in their heparin binding affinities: VEGF120 is freely diffusible into the extracellular milieu, VEGF

188 has a high affinity for heparin sulfate and is sequestered in the extracellular matrix, while VEGF 164 is found in both soluble form and bound to the extracellular matrix (Houck, Leung et al. 1992; Park, Keller et al. 1993).

VEGF was first recognized for its role in vascular permeability and originally named vascular permeability factor (VPF) (Senger, Galli et al. 1983; Dvorak, Brown et al. 1995). It was then discovered to have mitogenic activity on endothelial cells thus giving it its final name, vascular endothelial growth factor (Ferrara and Henzel 1989). VEGF was later shown to regulate survival, migration and differentiation of these cells (Eichmann, Corbel et al. 1997; Gerber, Dixit et al. 1998; Gerber, McMurtrey et al. 1998; Ferrara 1999) all of which support new capillary growth, a process known as angiogenesis. VEGF has been shown to regulate angiogenesis in vitro and in vivo (Connolly, Heuvelman et al. 1989; Leung, Cachianes et al. 1989; Wilting, Christ et al. 1992; Wilting, Christ et al. 1993) and has been studied most widely in pathological angiogenesis where local tissue hypoxia either in large tumors or in the retina stimulates the expression of VEGF mRNA through the transcription factor, hypoxia inducible factor 1α (HIF1α) (Plate, Breier et al. 1992; Shweiki, Itin et al. 1992; Levy, Levy et al. 1996; Pierce, Foley et al. 1996; Maxwell, Dachs et al. 1997). VEGF is not only involved in angiogenesis, it is critical to the process of vasculogenesis as deletion of even a single allele is embryonically lethal due to abnormal blood vessel development (Carmeliet, Ferreira et al. 1996; Ferrara, Carver-Moore et al. 1996).

1.2.2 VEGF receptors

VEGF has its biological effects through binding of two tyrosine kinase receptors; flt1 (VEGFR1) and flk1 (VEGFR2). These receptors appear to regulate different processes during angiogenesis but are both important for the development of functional blood vessels as deletion of either receptor results in embryonic lethality (Fong, Rossant et al. 1995; Shalaby, Rossant et al. 1995). Flk 1 regulates endothelial cell proliferation, migration and differentiation and is thought to mediate angiogenesis and vasculogenesis (Shalaby, Rossant et al. 1995; Eichmann, Corbel et al. 1997). The functions of flt 1 are less clear and given its high affinity for VEGF and weak tyrosine kinase activity may act as a VEGF inhibitor and a negative regulator of angiogenesis (Ferrara 1999). VEGF also binds neuropilin-1, a non-tyrosine kinase receptor, that is thought to enhance the angiogenic responses of flk 1 but is not sufficient to mediate angiogenesis alone (Hicklin and Ellis 2005).

Although VEGF has classically been considered to have effects solely on endothelial cells, it is now proving to regulate important processes in other cell populations. VEGF receptors are expressed on vascular smooth muscle cells (Ishida, Murray et al. 2001), cardiac myocytes (Takahashi, Seko et al. 1999), osteoblasts (Deckers, Karperien et al. 2000), neurons (Sondell, Lundborg et al. 1999), regenerating myocytes (Rissanen, Vajanto et al. 2002) and myogenic satellite cells (Germani, Di Carlo et al. 2003; Bryan, Walshe et al. 2007). VEGF regulates survival and differentiation in these cell populations in vitro but it remains to be seen how important VEGF is on these processes in vivo.

1.3 VEGF and Skeletal Muscle

1.3.1 Source of VEGF in skeletal muscle

Using *in situ* hybridization, two studies have demonstrated the expression of VEGF within muscle fibers after a bout of exercise (Breen, Johnson et al. 1996; Brutsaert, Gavin et al. 2002). This was later confirmed with the measurement of VEGF mRNA expression in pure isolated single muscle fibers (Birot, Koulmann et al. 2003). These studies suggest that muscle cells are the primary source of VEGF in skeletal muscle and that VEGF may act in a paracrine fashion to stimulate and promote the survival of endothelial cells. In support of this, we have shown that mice with a life-long, myocyte-specific VEGF gene deletion have a 90% decrease in VEGF protein and a 48% decrease in capillary to fiber ratio in whole skeletal muscle (Olfert, Howlett et al. 2009). Similarly, mice with a cardiac myocyte-specific VEGF gene deletion have a 6 fold reduction in VEGF mRNA expression in the heart accompanied by coronary hypovascularization and contractile dysfunction (Giordano, Gerber et al. 2001).

There also exist fiber type differences in the distribution of VEGF. Slow twitch muscles are more vascularized than fast twitch muscles as evidenced by a higher capillary to fiber ratio in normal resting muscle (Deveci, Marshall et al. 2001). Given VEGF's role in regulating angiogenesis, it is not surprising then that at basal levels, VEGF protein is more abundant in slow twitch, oxidative muscles (i.e. soleus) than in fast twitch glycolytic muscles (i.e. tibialis anterior) (Annex, Torgan et al. 1998). Based on this fiber type distribution of VEGF, it is unknown whether the

withdrawal of VEGF from myocytes will differentially affect slow and fast twitch skeletal muscles with regards to capillarity and muscle function.

1.3.2 Not just an endothelial cell growth factor

In addition to having a role in endothelial cell signaling, recent studies have shown that VEGF and its receptors, flt1 and flk1, are expressed in differentiated and regenerating muscle fibers (Kosmidou, Xagorari et al. 2001; Rissanen, Vajanto et al. 2002; Birot, Koulmann et al. 2003; Arsic, Zacchigna et al. 2004) and in muscle satellite cells (Germani, Di Carlo et al. 2003; Arsic, Zacchigna et al. 2004; Bryan, Walshe et al. 2007; Christov, Chretien et al. 2007). Satellite cells are quiescent cells that lie between the basal lamina and sarcolemma of muscle fibers. They are activated by mechanical stretch and ischemic injury whereupon they enter into the cell cycle, proliferate, migrate and fuse to form new fibers and repair damaged ones (Tatsumi, Sheehan et al. 2001; Hill, Wernig et al. 2003). Recent evidence suggests that VEGF plays a role in this modulation of satellite cell function by promoting cell survival and migration during muscle regeneration (Germani, Di Carlo et al. 2003; Arsic, Zacchigna et al. 2004). Indirect evidence for this role of VEGF in vivo comes from a study in mice lacking skeletal muscle hypoxia inducible factor 1 α (HIF1α), a transcription factor that regulates VEGF expression. These mice showed a failure to increase VEGF after a single bout of exercise and increased muscle damage after 4 days of endurance running implicating that VEGF could have a protective role in regenerating muscle (Mason, Howlett et al. 2004).

1.4 VEGF and Skeletal Muscle Angiogenesis

The adult skeletal muscle microvasculature exhibits remarkable plasticity and unlike the capillary networks in most adult tissues, responds quickly to physiologic stimuli. Endurance training (Amaral, Papanek et al. 2001), chronic electrical stimulation (Brown, Cotter et al. 1976; Brown, Hudlicka et al. 1995) and chronic hypoxia (Deveci, Marshall et al. 2001) have all been shown to increase skeletal muscle capillarity while immobilization of hindlimb muscles causes capillary regression (Desplanches, Mayet et al. 1987; Fujino, Kohzuki et al. 2005); both processes occurring within days or weeks.

Increasing evidence links the expression of VEGF with capillary proliferation in skeletal muscle. VEGF is increased in skeletal muscle of different species following electrically stimulated contractions (Hang, Kong et al. 1995; Annex, Torgan et al. 1998; Hudlicka, Milkiewicz et al. 2002), acute bouts of running exercise (Breen, Johnson et al. 1996; Richardson, Wagner et al. 1999; Choi, Liu et al. 2005) and exposure to systemic hypoxia (Tang, Breen et al. 2004). Flt 1 and flk 1 are also upregulated with muscle activity (Olfert, Breen et al. 2001; Hudlicka, Milkiewicz et al. 2002; Birot, Koulmann et al. 2003) and acute hypoxia (Cherwek, Hopkins et al. 2000; Milkiewicz, Hudlicka et al. 2003; Tang, Breen et al. 2004) and flt 1 protein levels correlated with increased capillary proliferation after muscle activity (Milkiewicz, Hudlicka et al. 2003).

The increased levels of VEGF mRNA and protein following muscle activity have been shown to occur prior to or concurrent with an increase in capillarity, lending

support to the hypothesis that VEGF plays a major role in triggering skeletal muscle angiogenesis (Hang, Kong et al. 1995; Annex, Torgan et al. 1998; Skorjanc, Jaschinski et al. 1998; Amaral, Linderman et al. 2001; Amaral, Papanek et al. 2001; Hudlicka, Milkiewicz et al. 2002; Lloyd, Prior et al. 2003; Milkiewicz, Hudlicka et al. 2005). Further support for this comes from studies in which increasing levels of VEGF through viral gene delivery caused an increase in skeletal muscle capillarity in ischemic muscle (Shimpo, Ikeda et al. 2002; Gounis, Spiga et al. 2005). Moreover, VEGF inhibitors administered during exercise training prevented increases in skeletal muscle angiogenesis (Amaral, Papanek et al. 2001; Lloyd, Prior et al. 2005).

Results from our laboratory show a direct link between VEGF and the control of skeletal muscle angiogenesis during development and in the adult. We demonstrated that deleting the VEGF gene in mice during late gestation by conditional knockout targeted to heart and skeletal muscle causes a dramatic decrease in the capillary to fiber ratio and capillary density of gastrocnemius muscle (-48% and -39% respectively) (Olfert, Howlett et al. 2009). When these VEGF myocyte-deficient mice were endurance trained as adults, they were not able to increase gastrocnemius capillarity as compared to control mice (Olfert et al., unpublished) demonstrating that myocyte-derived VEGF is an important paracrine mediator of skeletal muscle angiogenesis.

1.5 VEGF and the Adult Skeletal Muscle Microvasculature

As evidenced above, there is little doubt that VEGF plays an important role in new capillary growth in skeletal muscle. However, there is still question as to

VEGF's role in maintaining the adult microvasculature, in particular that of skeletal muscle.

1.5.1 Insufficient VEGF leads to apoptosis

Inactivation of VEGF in various cell types in vivo causes apoptosis (Gerber, Hillan et al. 1999; Tang, Breen et al. 2004; Tang, Rossiter et al. 2004; Lee, Chen et al. 2007). VEGF's antiapoptotic effects are attributed to increases in Bcl-2, A1 and Akt signaling transduced through the flk-1 receptor (Gerber, Dixit et al. 1998; Gerber, McMurtrey et al. 1998; Nor, Christensen et al. 1999). Consistent with this, administration of VEGF has been reported to prevent apoptosis in hypertrophied and ischemic hearts (Friehs, Barillas et al. 2006; Ruixing, Jiaquan et al. 2006) and in retinal vessels formed as a result of retinopathy of prematurity (Alon, Hemo et al. 1995).

1.5.2 VEGF inhibitor studies

VEGF inhibitor studies in adult tissues provide conflicting results as to the dependence of adult capillary networks on VEGF. Using a mFlt(1-3)-IgG, Gerber et al. found that endothelial cells lose their dependence on VEGF as a survival factor after the fourth postnatal week while Kamba et al. demonstrated that inhibition of VEGF signaling with either a small-molecule VEGFR tyrosine kinase inhibitor, VEGF trap or VEGF decoy receptors caused capillary regression in some adult organs (Gerber, Hillan et al. 1999; Kamba, Tam et al. 2006). However, in the tongue, a representative skeletal muscle, VEGF inhibition failed to decrease capillary density (Kamba, Tam et al. 2006).

Despite the capillary regression observed in some organs, Kamba saw few physiologic consequences and attributed this to the ability of the existing vasculature to meet oxygen and metabolic demands of these organs (Kamba, Tam et al. 2006). Muscle is very different in that it must meet the increased oxygen and metabolic demands presented during exercise. Therefore, capillary regression in peripheral skeletal muscles could have negative consequences to proper muscle function. Studies investigating the effects of decreased VEGF signaling on the maintenance of the adult skeletal muscle microvasculature have only just recently been undertaken.

1.5.3 Decreased VEGF signaling in adult skeletal muscle

Most VEGF gene deletion studies inactivate the VEGF gene during development of the animal, which precludes them from giving us insight into VEGF's role in maintaining adult capillary networks. Tang et al. have employed an AAV*cre*/*loxP* strategy that successfully deleted VEGF in all cell types in a small region of adult mouse gastrocnemius muscle. They observed a 64% and 67% decrease in capillary to fiber ratio 4 and 8 weeks after infection with AAV*cre*, respectively, that was accompanied by increased myocyte and endothelial cell apoptosis in the areas lacking VEGF (Tang, Breen et al. 2004). This study demonstrates the importance of VEGF in the survival of adult capillaries in skeletal muscle, however, this model does not allow us to determine the physiological effects associated with capillary loss since only a small area of a single muscle was affected, representing only about 30-70 individual fibers.

1.5.4 Decreased VEGF signaling in endothelial cells

Mice with a lifelong, global deletion of VEGF in endothelial cells ($VEGF^{ECKO}$) exhibit organ failure between 40 and 50 weeks of age due to vascular abnormalities and evaluation of different organs revealed increases in endothelial cell apoptosis (Lee, Chen et al. 2007). Interestingly, plasma and tissue VEGF levels were not different from control mice, indicating that the endothelial cells are not a significant source of VEGF and that paracrine sources of VEGF could not rescue the endothelial cell dysfunction. Moreover, isolation of these VEGF deficient endothelial cells demonstrated a decrease in their survival rate when stressed that could not be rescued with exogenous VEGF. Furthermore, these cells do not lose their ability to form new vessels on matrigel plugs when stimulated with exogenous VEGF. These results suggest different roles of VEGF on the endothelial cell depending on its source; a potential autocrine role in the endothelial cell that confers survival and a paracrine function that regulates angiogenesis.

1.6 Potential Consequences of Decreased VEGF on Muscle Function and Exercise Capacity

1.6.1 Excessive apoptosis causes atrophy, inflammation and contractile dysfunction

Apoptosis, or programmed cell death, is a natural reparative process in which cell surface receptors on damaged cells signal their immediate removal by phagocytes. This is an efficient process in vivo that maintains tissue homeostasis while preventing damaged cells from releasing cytoplasmic contents into tissues causing an

inflammatory response. However, if a tissue is overloaded with apoptotic cells that are not cleared quickly and efficiently, more apoptosis, atrophy, secondary necrosis, and inflammation could result. This has been demonstrated in the lung where lung tissue that was instilled with either labeled apoptotic cells or apoptotic macrophages resulted in increased lung cell apoptosis, TGFβ, TNFα and collagen accumulation (Wang, Antonini et al. 2003; Wang, Scabilloni et al. 2006).

Given VEGF's antiapoptotic properties, lack of VEGF signaling in skeletal muscle could result in excessive apoptosis and inflammation. Circulating cytokines, in particular TNFα, are implicated in the cause of the muscle wasting and contractile dysfunction seen in some chronic diseases (Spate and Schulze 2004). In support of this, overexpression of TNFα in the lung results in increased circulating TNFα and skeletal muscle wasting (Langen, Schols et al. 2006). Furthermore, a single intraperitoneal injection of TNFα depressed specific force production in the soleus and diaphragm (Adams, Mangner et al. 2008; Hardin, Campbell et al. 2008). Reactive oxygen species (ROS) production may mediate the TNFα induced muscle weakness since treatment with antioxidants restored normal muscle function (Hardin, Campbell et al. 2008). These studies demonstrate a causal role for TNFα in increasing muscle atrophy and decreasing muscle force and could be the ultimate result of VEGF withdrawal from skeletal muscle.

Excessive myonuclear apoptosis could also result in muscle atrophy and loss of muscle function. This is evidenced by studies in the aging population where apoptotic myofibers are associated with muscle loss and weakness (Marzetti and Leeuwenburgh

2006). In addition, caspase-3, which is activated by apoptotic stimuli, has been shown to increase proteolysis in skeletal muscle (Du, Wang et al. 2004) and to decrease diaphragmatic force production and cardiac function due to degradation of contractile proteins (Ruetten, Badorff et al. 2001; Communal, Sumandea et al. 2002; Supinski and Callahan 2006). These effects occurred independent of apoptosis and were mitigated with a caspase-3 inhibitor (Ruetten, Badorff et al. 2001; Supinski and Callahan 2006). Deletion of VEGF from the myocyte may set forth a pro-apoptotic chain of events that would decrease bcl-2 and increase caspase-3 that could lead to contractile protein degradation and muscle dysfunction.

1.6.2 Decreased capillarity can limit O_2 availability which can decrease exercise endurance

The regulation of muscle capillarity is important since it is a critical component of the oxygen transport system which determines maximal endurance capacity (Wagner 2001). The oxygen transport system depends on the integrated contribution of several components including the lungs, heart, vasculature, blood (hemoglobin-O_2 capacity) and the muscle structure, *each* of which affects overall oxygen delivery (Wagner 1996). A critical determinant of maximal oxygen utilization is O_2 conductance to the muscle (Roca, Hogan et al. 1989) and evidence suggests this is determined by the number of capillaries that interface with each muscle fiber (Bebout, Hogan et al. 1993; Hepple, Hogan et al. 2000). Endurance training causes an increase in skeletal muscle capillarity, which increases oxygen availability to the muscle by 1) increasing capillary surface area for diffusion 2) decreasing the average oxygen diffusion path length and 3) increasing red cell transit

time through skeletal muscle. Decreased skeletal muscle capillarity could limit O_2 availability to the mitochondria, which could limit oxidative metabolism and contribute to muscle fatigue (Roca, Hogan et al. 1989; Zhang, Bruton et al. 2006).

Lending support to this hypothesis, Olfert et al. has demonstrated that mice with a lifelong deletion of VEGF in skeletal and heart muscle, have a 48% reduction in capillary to fiber ratio in skeletal muscle and a 61% reduction in capillary density in the heart accompanied by a 34% reduction in maximum running speed and an 81% reduction in endurance running time compared to controls (Olfert, Howlett et al. 2009). This model demonstrates the importance of the muscle microvasculature in maintaining exercise endurance, however, it does not allow us to determine the contribution of skeletal muscle capillarity alone in contributing to the decreased exercise capacity.

1.7 Hypothesis and Approach

The purpose of this study was to test the hypothesis that in skeletal muscle of ***adult*** cage-confined mice, ***myocyte***-derived VEGF is necessary to maintain exercise capacity, capillarity and muscle function. In order to test this hypothesis we have generated a tamoxifen-inducible, skeletal myocyte-specific VEGF knockout mouse (skeletal actin-driven Cre crossbred with VEGF*loxP*) with greatly diminished VEGF in all skeletal muscles. In particular, we crossed VEGF*loxP* mice x HSA-Cre-ER^{T2} mice, in which cre-recombinase expression is under control of the human skeletal actin (HSA) promoter and cre-recombinase activity is tamoxifen-inducible (Schuler,

Ali et al. 2005). Therefore, the VEGF gene is deleted only in skeletal myocytes of adult mice, and only following administration of tamoxifen.

This novel, inducible, skeletal *myocyte*-specific VEGF gene deletion model complements our previous work with the localized (30-70 fiber) VEGF deletion model of Tang (Tang, Breen et al. 2004) and the lifelong skeletal and cardiac VEGF deletion model of Olfert (Olfert, Howlett et al. 2009). The Tang model allows for deletion of VEGF in all cell types in a small region of gastrocnemius muscle in the adult mouse but because the area of gene deletion is limited to 30-70 fibers, does not allow overall muscle function and exercise capacity to be measured. The Olfert model allows for muscle-wide deletion of myocyte (cardiac and skeletal) derived VEGF, which unlike the Tang model, allows muscle function and exercise capacity to be measured but the deletion is lifelong and is not confined to skeletal muscle. This new model allows for muscle-wide deletion of VEGF in only the ***skeletal myocytes*** and as with the Olfert model allows for functional measurements to be made. As with the Tang model, the deletion can be initiated at any chosen age so that effects of VEGF deletion in the ***adult*** can be studied.

The results obtained upon completion of this project will significantly enhance our understanding of the importance of skeletal myocyte VEGF in regulating adult skeletal muscle capillarity and in maintaining muscle contractile function. Loss of either could lead to impaired exercise capacity as seen in patients with chronic disease.

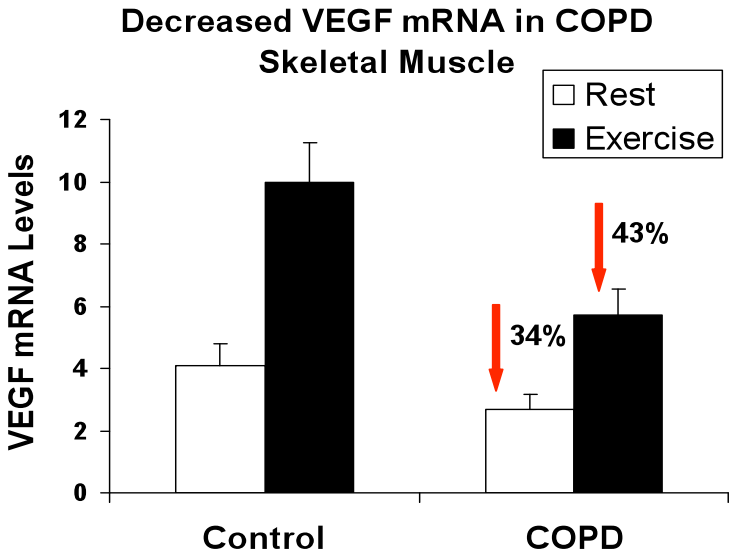

Figure 1.1. Decreased VEGF mRNA in COPD Skeletal Muscle. VEGF mRNA quantitated from RT-PCR data obtained from muscle biopsies from the Vastus Medialis muscle at rest and immediately post exercise (single-leg kicking for 30 min at 50% of VO2max (n=5 each group).

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CHAPTER 2

METHODS

2.1 Introduction

This chapter describes the methods and assays used in order to test the hypothesis that myocyte-dervied VEGF is necessary to maintain capillarity, muscle function and exercise capacity. We first describe the generation of a model of temporally controlled VEGF deletion in adult skeletal myocytes. Genotyping was performed by PCR analysis of the cre-recombinase (Cre) and VEGF*loxP* genes from tail DNA. Animals identified with both VEGF*loxP* +/+ and Cre +/- were designated myoVEGF-/- and littermates that were identified with VEGF*loxP* +/+ and Cre -/- were used as controls (VEGF +/+). Intraperitoneal injection of tamoxifen given to both myoVEGF-/- and VEGF+/+ was used to induce VEGF gene deletion in the myoVEGF-/- mice but had no effect on the VEGF+/+ mice. Since a mutated estrogen receptor is responsible for the activation of Cre in this model, we only used male mice in order to ensure that activation of Cre was only induced with tamoxifen and not with endogenous estrogen produced by female mice.

In order to verify that we had a working model of skeletal myocyte-specific VEGF gene deletion, VEGF protein levels were measured in gastrocnemius, soleus and EDL muscles. The gastrocnemius muscle was chosen as it is a major muscle recruited during treadmill running in rodents (Laughlin and Armstrong 1982). The

soleus and EDL muscles were chosen to represent muscles with different fiber type compositions. The EDL is a fast, glycolytic muscle, containing ~100% type II fibers (Jeneson, de Snoo et al. 2007), while the soleus is a slow, oxidative muscle, containing ~60% type I fibers and ~40% type IIa fibers (Gavin, Westerkamp et al. 2006). In order to confirm that there was no VEGF deletion in organs other than skeletal muscle, we evaluated VEGF protein levels in the heart, lung, kidney and brain. These organs contribute to exercise performance, and since deletion of VEGF in these tissues could affect capillarity and functionality of the organ (Giordano, Gerber et al. 2001; Oosthuyse, Moons et al. 2001; Tang, Rossiter et al. 2004; Kamba, Tam et al. 2006; Olfert, Howlett et al. 2009), this would confound any conclusions made in determining the contribution of skeletal myocyte-derived VEGF on exercise capacity.

After tamoxifen induction the animals were subjected to exercise testing on a treadmill either 2 or 7 weeks after the last tamoxifen injection. Aerobic capacity was assessed using a maximal speed test. Maximal oxygen consumption (VO_2 max) is a more direct way of measuring aerobic capacity, however, this can only be measured in one animal at a time. Since VEGF deletion is temporally controlled in our mice and we needed to test a large number of animals immediately before and after tamoxifen injections, it would not have been feasible to get VO_2 measurements on all of our mice in a timely manner. It has been shown that there is a tight correlation between maximum running speed and VO_2 max in mice (Hoydal, Wisloff et al. 2007), therefore, we used the former to estimate aerobic capacity.

Determinants of endurance capacity include oxygen delivery to the muscle as well as proper muscle function. Deletion of VEGF in skeletal myocytes, which we hypothesized could lead to decreased capillarity (oxygen supply) and muscle function, could contribute to a loss of endurance capacity. We assessed endurance capacity by measuring time to exhaustion on a treadmill set at a constant speed. Both groups of animals (myoVEGF -/- and VEGF +/+) ran at the same speed that was determined from preliminary studies indicating a small decrease in maximum running speed in myoVEGF-/- mice. We chose the endurance run speed to be ~70% of the average maximum speed from the myoVEGF-/- group in order to ensure that the degree of difficulty was not too great for the myoVEGF -/- mice. This allowed us to evaluate endurance capacity based on absolute workload.

In order to determine the contributing factors to a possible decrease in exercise capacity, capillarity and in vitro muscle function were evaluated. Measurement of capillary to fiber ratio was used to evaluate capillarity in the skeletal muscle. The number of capillaries that interface with the muscle fibers is one of the main determinants of oxygen conductance to skeletal muscle that in turn contributes to maximal endurance capacity (Roca, Hogan et al. 1989; Bebout, Hogan et al. 1993; Hepple, Hogan et al. 2000). Decreased force production from the muscle cells caused by either impaired excitation-contraction coupling (contractility) or increased fatigue due to metabolite production (e.g. H^+ and P_i) could also contribute to a decline in exercise endurance (Fitts 1994; Allen, Lannergren et al. 1995). We used an in vitro, muscle stimulation protocol where the muscle was perfused with a hyperoxic gas

mixture in order to assess muscle function independent of capillarity. The soleus and EDL muscles were chosen to represent muscles with different fiber type compositions and for their small size, which helps prevent oxygen diffusion limitations in the in vitro protocol. We measured peak force and the force-frequency relationship in order to assess contractility and measured time to fatigue in order to test fatigue resistance of the muscles.

In order to evaluate the cellular factors that could contribute to a decline in exercise capacity due to skeletal myocyte VEGF gene deletion, we assessed apoptosis, muscle damage and cytokine production in gastrocnemius and soleus muscle. These measurements were made 24 hours after the endurance run to exhaustion. Since the maximum speed test and run to exhaustion were performed on consecutive days, 24 hours apart, we chose this time point to determine if there was any indication of muscle damage after the first running bout that could contribute to the decline in exercise capacity on the second day. Other groups have shown that apoptosis and inflammation are present 24 hours after a bout of exhaustive exercise (Sandri, Carraro et al. 1995; Aoi, Naito et al. 2004; Kocturk, Kayatekin et al. 2008) and we hypothesized that this could be worsened by the deletion of VEGF in skeletal muscle.

2.2 Experimental Methods and Assays

2.2.1 Generation of inducible, skeletal myocyte-specific VEGF knockout mice

Homozygous VEGF/*loxP* mice, containing 34-bp *loxP* sequences flanking the exon 3 region in the VEGF gene (kindly provided by Dr. Napoleone Ferrara,

Genentech, Inc., San Francisco, CA) were crossed with a heterozygous HSA-Cre-ER^{T2} mouse line (kindly provided by Dr. Pierre Chambon, IGBMZ, France) where Cre-ER^{T2} is selectively expressed in skeletal myonuclei (controlled by the human skeletal muscle α-actin promoter). Cre recombinase activity is dependent on tamoxifen (Schuler, Ali et al. 2005). Thus, only after administration of tamoxifen is Cre activated (see below) and VEGF deleted. Mice resulting from this cross are homozygous for VEGF/*loxP* and either heterozygous for HSA-Cre-ER^{T2} (myoVEGF -/- after tamoxifen) or null for HSA-Cre-ER^{T2} (VEGF +/+). In VEGF/*loxP* mice, the exon 3 region of the gene (flanked with *loxP* sites) provides critical coding common to all murine VEGF isoforms (i.e. $VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$) (Robinson and Stringer 2001; Ferrara, Gerber et al. 2003), thus, the VEGF gene mutation in our mouse model results in loss of all VEGF-A isoforms in skeletal muscle. Subsequent breeding pairs were sibling-sibling matings.

2.2.2 DNA analysis

Genotyping for identification and selection of breeding pairs was performed on DNA extracted from mouse tail sections (DNeasy Tissue Kit, Qiagen Inc., Valencia, CA). Later, DNA obtained from the gastrocnemius muscle was also used to verify animal genotype. Genotype determination was made by PCR analysis using TaqPro™ Red Complete DNA Polymerase Master Mix (Denville Scientific, Inc., Metuchen, NJ) using the following primers: for VEGF/*loxP*, forward primer 5'-TCCGTACGACGCATTTCTAG-3' and reverse primer 5'-CCTGGCCCTCAAGTACACCTT-3'; for Cre recombinase, forward primer

5’-CTAGAGCCTGTTTTGCACGTTC-3’ and reverse primer

5’-TGCAAGTTGAATAACCGGAAA-3’. PCR analysis was performed on a Robocycle Gradient 40 (Stratagene Inc., La Jolla, CA) with the following conditions for both VEGF/*loxP* and Cre: one 2-minute incubation at 95°C (polymerase activation), followed by 30 cycles of 75 sec at 94°C (denaturation), 100 sec of 53°C (annealing), and 170 sec at 72°c (extending), followed by one 10-minute period at 72°C. PCR product from each animal, along with positive and negative controls, was run on 2% ethidium bromide stained agarose gel in 1X TAE buffer.

2.2.3 Tamoxifen injections

MyoVEGF -/- and VEGF +/+ mice were given a 100µl intraperitoneal injection of tamoxifen (Sigma-Aldrich, St. Louis, MO) diluted in autoclaved olive oil (final concentration 10mg/ml) daily, for 5 consecutive days (Schuler, Ali et al. 2005).

2.2.4 Exercise tests

All mice were familiarized to a small animal treadmill (model CL-4, Omnitech, Columbus, OH) for 10 minutes and performed a warm-up at 15 m/min for 1 minute prior to performing the exercise tests. Maximal running speed was determined using the following treadmill protocol: 20 m/min, 10˚ incline for 1min, after which running speed was increased by 2 m/min every minute, until the mouse was no longer able to keep pace with the treadmill and/or maintain its normal running position on the treadmill. Endurance capacity was determined by measuring the duration of a sub-maximal run to exhaustion. The treadmill was set at 20 m/min, 10˚ incline and was maintained at this speed and incline until the mice reached exhaustion. Exhaustion was

defined as the point at which the mouse was no longer able to maintain normal running position on the treadmill and/or were unwilling to run, as indicated by the frequent contact with (touching the shock grid with each stride) or sitting on an electrical shock grid (≤ 0.2 milliamps) at the rear of the treadmill.

2.2.5 Contractile function: Isolated whole muscle stimulation protocol

Mice were anesthetized (sodium pentobarbital 50 mg/kg) and the soleus and EDL muscles were dissected with both tendons in tact. Muscles were immediately put in Tyrode's solution (121 mM NaCl, 5 mM KCl, 1.8mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 24 mM $NaHCO_3$, 5.5 mM Glucose, 0.1 mM EGTA) until it was time for each experiment. For each test, the muscle was mounted in a chamber that was continuously perfused with an oxygenated (95% O_2, 5% CO_2) Tyrode's solution at room temperature to ensure no unstirred layers around the muscle, sufficient oxygenation to the core of the muscle, and stable pH (7.4). One tendon was tied with silk thread to a force transducer and the other tendon tied to an adjustable tube at the opposite end of the chamber, allowing muscle length to be changed incrementally to determine optimal muscle length (L_o), the length at which maximal tetanic force is produced. Muscles were stimulated at supramaximal voltage using platinum electrodes placed on either side of the muscle. Following 15 minutes of equilibration, the muscles were tested for contractile function and fatigueability.

In order to assess contractile function the force frequency relationship was established for each muscle. Maximum isometric tetanic force was measured by recording force output at stimulation frequencies of 1, 15, 30, 50, 80, 100, and 120 Hz

(500ms train duration, .2 ms pulse duration) for soleus and 1, 10, 15, 30, 45, 60, 75, 90, 120, 150 (300 ms train duration, .2 ms pulse duration) for EDL. The muscles were stimulated to contract once every 2 minutes to prevent fatigue. Stability of the muscle was checked by periodically stimulating the muscle at maximal frequency (100 Hz for soleus and 150 Hz for EDL) throughout the course of the experiment.

Five minutes after the last contraction, fatigueability was assessed in each muscle. To measure fatigue, isometric tetanic contractions were elicited with 500 ms (solues) or 300 ms (EDL) train duration and .2 ms pulse duration at 50 Hz (soleus) or 70 Hz (EDL). Stimulation frequency was increased every 2 min (soleus) or 1 min (EDL) in a progressive manner (from a contraction every 8 to 4 to 3 to 2 to 1 sec) and terminated when the developed force had fallen to 50% of the initial maximal developed force.

When all testing was finished L_o was measured using a reticle with a surgical microscope (Zeiss OPMI, Thornwood, NY), tendons were removed and the muscles were blotted and weighed. Optimal fiber length (L_f) was calculated by multiplying L_o by the muscle length ratio (.44 for EDL and .71 for soleus) as previously determined in the literature (Brooks and Faulkner 1988). Cross sectional area (CSA) was calculated with the following equation (Del Prete, Musaro et al. 2008):

$$\text{CSA } (mm^2) = \frac{\text{muscle mass (mg)}}{L_f \text{ (mm)} \times 1.06 \text{ (mg/mm}^3\text{)}}$$

Forces were either expressed as specific force (N/mm^2) or relative force (% of maximum tetanic force).

2.2.6 Whole muscle VEGF, TNFα and IL-6 protein levels

Gastrocnemius, solues and EDL muscles were evaluated for VEGF levels in order to confirm VEGF deletion in these tissues. VEGF levels in heart, lung, brain and kidney were also measured to confirm that there was no effect of VEGF gene deletion on these organs. Tissue was homogenized in a lysis buffer containing 50 mM Tris/Hcl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and protease inhibitors (Complete™ tablet, Roche Company, Mannheim, Germany). Homogenates were centrifuged at 4°C, 10,000 rpm, for 10 minutes and supernatants assayed for total protein concentration (Bio-Rad DC protein assay, Bio-Rad Laboratories, Hercules, CA). VEGF protein levels were measured using an enzyme-linked immunoassay kit for mouse designed for detecting $VEGF_{165}$ and $VEGF_{121}$ isoforms of VEGF-A (VEGF Mouse ELISA, R&D systems, La Jolla, CA) and normalized to total protein. MyoVEGF-/- mice that did not have >90% reduction in VEGF protein levels compared to control mice were excluded from further analysis. TNFα and IL-6 protein levels were measured in gastrocneimus using mouse ELISA kits (Endogen). Samples were assayed in duplicate using 50µl of muscle homogenate per well and normalized to total protein.

2.2.7 Skeletal muscle capillarity

The medial and lateral hindlimb muscles (i.e. gastrocnemius, soleus and plantaris muscles) were surgically removed, sliced in half transversely at the muscle

mid-belly, and quickly flash frozen with freezing medium gel (TBS Tissue Freezing Medium™, Triangle Biomedical Sciences, Durham, NC) in isopentane cooled liquid N_2. Muscle samples were stored at -80°C. Sections cut at 10μm were prepared for assessment of muscle capillarity using the alkaline phosphatase capillary staining method (Mrazkova, Grim et al. 1986). Sections were viewed by light microscopy and digitally imaged (25-40x magnificaiton). The acquired images were viewed using computer software (MATLAB version 7.0, The MathWorks, Inc., Natick, MA) for assessment of the number of capillaries and muscle fibers in the lateral, superficial and deep regions of the gastrocnemius and in the soleus and EDL muscles. Capillaries were counted from 700-2000 randomly selected fibers from each lateral section of gastrocnemius (including both deep and superficial regions), from greater than 300 fibers in the soleus and greater than 400 fibers in the EDL. Results were expressed as the capillary to fiber ratio.

2.2.8 Muscle atrophy

The wet weight of skeletal muscles normalized to body mass was used as an indicator of skeletal muscle atrophy. Tendons were removed and the muscles then blotted to absorb any surface fluids before weighing.

2.2.9 Active caspase -3 immunohistochemistry

Frozen hindlimb sections were cut at 10μm thickness and air dried onto slides. Slides were fixed in 4% paraformaldehyde on ice for 10 min and then washed twice for 5 min each in wash buffer (1X phosphate buffered saline, pH 7.6). Slides were then incubated in 3% H_2O_2 diluted in methanol (10 min) in order to quench

endogenous peroxidases. Following two more washes (5 min each) slides were blocked for 1 hr (1X phosphate buffered saline, 0.3% Triton-X 100, 5% normal goat serum) at room temperature. Slides were incubated overnight at 4° with anti-cleaved caspase-3 (Asp175) (Cell Signaling, Danvers, MA) diluted in blocking buffer (1:200) followed by anti-rabbit biotinylated secondary antibody for 1 hr at room temperature. Signal was detected using Vectastain ABC reagent (30 min) and DAB (brown) substrate (8 min).

2.2.10 Skeletal muscle morphology

Gastrocnemius and soleus muscle sections (10μm) from mice 24 hours after their run to exhaustion were stained with hematoxylin and eosin to evaluate skeletal muscle damage. Sections were air dried on slides and then immersed in hematoxylin for 2 min. Slides were rinsed repeatedly in distilled water and then immersed in tap water for 2 min. Slides were stained in eosin for 2 min and then washed in successive 95% ETOH until clear followed immediately by dehydration in 100% ETOH. Slides were viewed by light microscopy (40x magnification) and evaluated for necrotic fibers, phagocyte infiltration and fibers with central nuclei.

2.2.11 Statistical analysis

All data are presented as mean ± SEM. SEM was used in order to estimate how our sample means represent the population means opposed to using standard deviation which is a descriptive statistic used to describe the variability in the study sample. Separate repeated measures ANOVA 2 (group: VEGF +/+ or myoVEGF -/-) × 2 (time: pre- and post-tamoxifen) were conducted for the dependent variables;

maximum running speed and time to exhaustion. For the EDL muscle, a 2 (group: WT or KO) × 10 (frequency: 1, 10, 15, 30, 45, 60, 75, 90, 120 and 150Hz) mixed factorial ANOVA was performed with force as the dependent variable. Similarly for soleus muscle, a 2 (group: VEGF +/+ or myoVEGF -/-) × 7 (frequency: 1, 15, 30, 50, 80, 100 and 120 Hz) mixed factorial ANOVA was performed with force as the dependent variable. Pairwise comparisons for body and muscle weights, VEGF, TNFα and IL-6 protein levels, capillary to fiber ratios, peak force, and time to fatigue were made with the use of Student's t test. An alpha level at $p \leq 0.05$ was selected for all statistical comparisons. The analyses were conducted using the Statistical Package for the Social Sciences software (v. 16.0, SPSS Inc., Chicago, IL).

2.3 Study Design

2.3.1 Skeletal myocyte-specific VEGF gene deletion in adult mice

This study was approved by the University of California, San Diego, Animal Subjects Committee. Adult male (15 week old) myoVEGF-/- and VEGF+/+ mice were weighed and then tested for maximal running speed and the following day run at 20 m/min to determine the time to exhaustion. After 2 days rest mice were treated with tamoxifen for 5 consecutive days (D0 through D4). On days D21 (2 weeks post tamoxifen) and D63 (7 weeks post tamoxifen) separate groups of mice were re-tested for maximal running speed and on D22 and D64 were re-tested for the time to exhaustion. On D23 and D65 mice were weighed and euthanized. Lower limb skeletal muscles [soleus, gastrocnemius, plantaris and tibialis anterior (T.A.)] from one leg, and non-skeletal muscle organs (heart, lung, brain and kidney) were weighed

and collected to measure VEGF protein levels by ELISA. Half of the contralateral gastrocnemius, soleus and EDL were used for histochemical and immunohistochemical assays, and the other half was used to measure cytokines by ELISA. Separate mice were used for the isolated whole muscle stimulation protocol. In these mice, skeletal myocyte VEGF gene deletion was induced with tamoxifen as described above but the mice were not submitted to the running tests. Soleus and EDL muscles were isolated and used for the whole muscle stimulation protocol and then frozen for analysis of VEGF protein levels by ELISA.

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CHAPTER 3

RESULTS

This chapter presents the data collected from male, inducible, skeletal myocyte-specific VEGF deleted mice (myoVEGF -/-) and controls (VEGF +/+). Both VEGF +/+ and myoVEGF -/- mice were injected with tamoxifen for 5 consecutive days at approximately 15 weeks of age and then data was collected at 2 and 7 weeks after the last tamoxifen injection. All results are presented from the 2 and 7 week time points unless otherwise stated. All data are mean ± SE. Body mass, muscle weights and cross-sectional areas are presented first (Table 3.1, 3.2 & 3.3) followed by VEGF protein levels in skeletal muscles (Figure 3.1) and non-skeletal muscle organs (Figure 3.2). Maximum running speed and endurance capacity are shown both before tamoxifen injections and after tamoxifen injections (Figure 3.4 & 3.5). Skeletal muscle capillary to fiber ratio is reported for the gastrocnemius, EDL (only at the 7 week time point) and soleus (Figure 3.5). Peak force, time to fatigue and force-frequency curves are shown for the EDL and soleus (Figure 3.6, 3.7 & 3.8). Muscle damage is reported from gastrocnemius and soleus muscle analyzed 24 hours after a run to exhaustion (data not shown) and finally, activated caspase-3 and cytokine levels are reported at the 2 week time point, from resting (cage-confined) and exercised (24 hours post exercise) muscle (Figure 3.9 & 3.10).

3.1 Body mass, muscle weights and muscle cross-sectional areas

Body masses were not different between VEGF +/+ and myoVEGF -/- groups before tamoxifen injections or two and seven weeks after tamoxifen injections (Table3.1). Muscle weights (gastrocnemius, soleus and EDL) and cross-sectional areas (soleus and EDL) were also not different in the myoVEGF-/- mice compared to controls (Table 3.2 and 3.3).

3.2 VEGF protein levels

MyoVEGF-/- mice showed large reductions in skeletal muscle VEGF protein following myocyte-specific VEGF gene deletion. Two weeks following tamoxifen delivery, VEGF protein in the gastrocnemius, soleus and EDL muscles was reduced by 94, 97 and 91% respectively (Figure 3.1A). Similarly, at the seven week time point, VEGF protein in the gastrocnemius and soleus was reduced 92 and 97% respectively (Figure 3.1B). The difference in VEGF protein in the EDL muscle between myoVEGF -/- and controls at seven weeks could not be quantified due to limited sensitivity of the VEGF ELISA kit, where some myoVEGF-/- samples had absorbances below the lowest standard curve value. However, it is evident that the VEGF levels have been greatly reduced in these muscles compared to controls (Figure 3.1B). Deletion of the VEGF gene from skeletal myocytes had no effect on VEGF protein in non-skeletal muscle organs (Figure 3.2).

3.3 Exercise capacity

Two and seven weeks following tamoxifen injections, maximum running speed was significantly reduced (-10% and -6% respectively) in myoVEGF-/- mice

compared to controls (Figure 3.3). Two weeks following tamoxifen delivery myoVEGF -/- mice exhibited a significant 47% decrease ($p < .05$) in time to exhaustion while 7 weeks following tamoxifen, mice displayed a non-significant trend towards lower endurance capacity (Figure 3.4).

3.4 Capillarity

MyoVEGF -/- mice do not have reduced capillary to fiber ratios in the gastrocnemius, soleus or EDL muscles at either two or seven weeks after tamoxifen (Figure 3.5).

3.5 Muscle contractile function

At two weeks following tamoxifen, there were no differences in peak force (Figure 3.6), time to fatigue (Figure 3.7) or contractile function (Figure 3.8 A & B) in the soleus or EDL muscles between myoVEGF -/- and VEGF +/+ mice. Likewise, at seven weeks post tamoxifen, myoVEGF -/- mice did not exhibit impaired peak force (Figure 3.6) or increased fatigueability (Figure 3.7) in either the soleus or EDL and had no contractile dysfunction in the soleus (Figure 3.8C). However, the EDL force-frequency relationship revealed a slightly but significantly right-shifted curve ($p < .05$) that could indicate some impairment in contractile function in this muscle (Figure 3.8D).

3.6 Skeletal muscle atrophy, apoptosis, muscle damage and cytokines

There was no evidence of skeletal muscle atrophy in the myocyte-specific VEGF knockout mice as neither muscle weights nor cross-sectional fiber areas differed between the knockout animals and controls (Table 3.3). Apoptosis in the

gastrocnemius and soleus muscles from myocyte-specific VEGF gene deleted mice was assessed with cleaved caspase-3 immunohistochemistry. Figure 3.9 depicts examples of myocytes positive for cleaved caspase-3 in both a myoVEGF -/- and VEGF +/+ gastrocnemius muscle section. Two weeks post tamoxifen delivery, gastrocnemius and soleus muscle sections from myoVEGF -/- and VEGF +/+ mice had 0-2 active caspase-3 positive myocytes 24 hours following an endurance run to exhaustion. There were no differences noted between the two groups. Two and seven weeks following tamoxifen injections, H&E stained gastrocnemius and soleus muscles from VEGF +/+ and myoVEGF -/- mice revealed no difference in muscle damage 24 hours following an endurance run to exhaustion as would be indicated by an increased number of necrotic fibers, infiltrating phagocytes and regenerating fibers with central nuclei (data not shown). The cytokines, TNFα and IL-6, were also not different from control in gastrocnemius muscle from myoVEGF -/- mice. This was found both at rest and 24 hours following the endurance run to exhaustion (Figure 3.10).

Table 3.1. No effect of VEGF loss on body mass, g

	VEGF +/+	myoVEGF -/-	VEGF +/+	myoVEGF -/-
Weeks post tamoxifen	2	2	7	7
Pre-tamoxifen	23.9 ± .4	23.7 ± .5	23.5 ± .4	23.3 ± .4
Post-tamoxifen	24.8 ± .3	25.0 ± .3	26.4 ± .3	25.7 ± .4
N	16	9	14	15

Values are in grams, mean ± SE. N = number of mice per group.

Table 3.2. No effect of VEGF loss on skeletal muscle weights, mg

	VEGF +/+	myoVEGF -/-	VEGF +/+	myoVEGF -/-
Weeks post tamoxifen	2	2	7	7
Gastrocnemius	116.25 ± 3.16	115.65 ± 4.84	122.95 ± 3.15	117.45 ± 3.65
$N_{Gastrocnemius}$	10	6	8	8
Soleus	8.82 ± .24	8.74 ± .21	9.10 ± .40	8.97 ± .41
N_{Soleus}	6	5	7	7
EDL	10.45 ± .55	9.20 ± .35	10.41 ± .69	10.13 ± .34
N_{EDL}	6	5	7	7

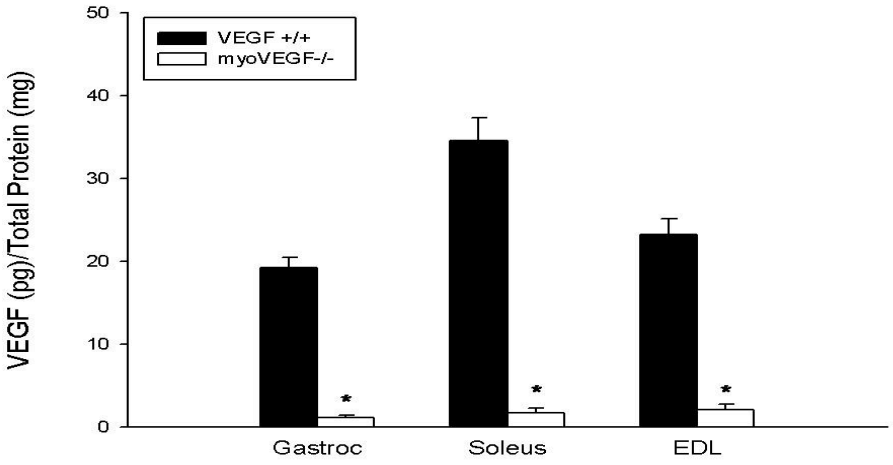
Values are means ± SE. N = number of muscles per group.

Table 3.3. No effect of VEGF loss on EDL and soleus cross-sectional areas, mm^2

	VEGF +/+	myoVEGF -/-	VEGF +/+	myoVEGF -/-
Weeks post tamoxifen	2	2	7	7
Soleus	1.06 ± .02	1.06 ± .01	1.04 ± .05	1.09 ± .07
N_{Soleus}	6	4	5	7
EDL	1.87 ± .10	1.63 ± .04	1.80 ± .15	1.79 ± .07
N_{EDL}	6	5	6	7

EDL: Extensor digitorum longus. Values are means ± SE. N = number of muscles per group.

A.



B.

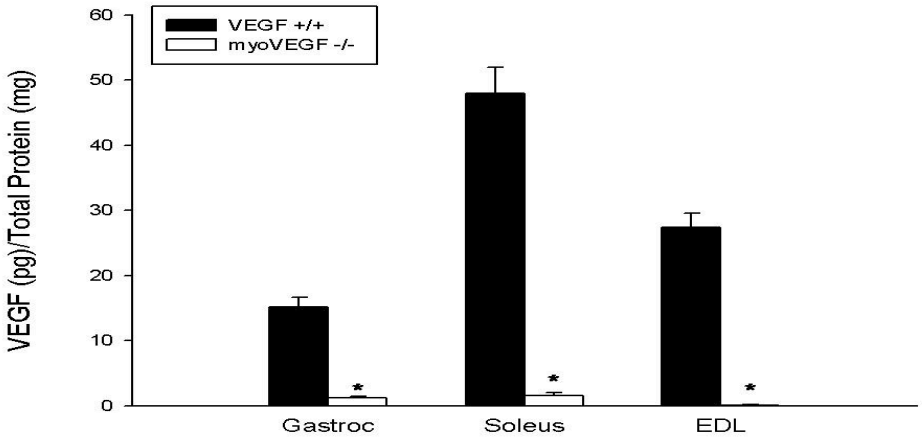


Figure 3.1. Greatly reduced post-tamoxifen VEGF skeletal muscle protein levels. A.) 2 weeks post tamoxifen. B.) 7 weeks post tamoxifen. Gastroc: gastrocnemius; EDL: extensor digitorum longus. Values are mean ± SE. *Significant difference ($p < .05$) compared to VEGF+/+ mice.

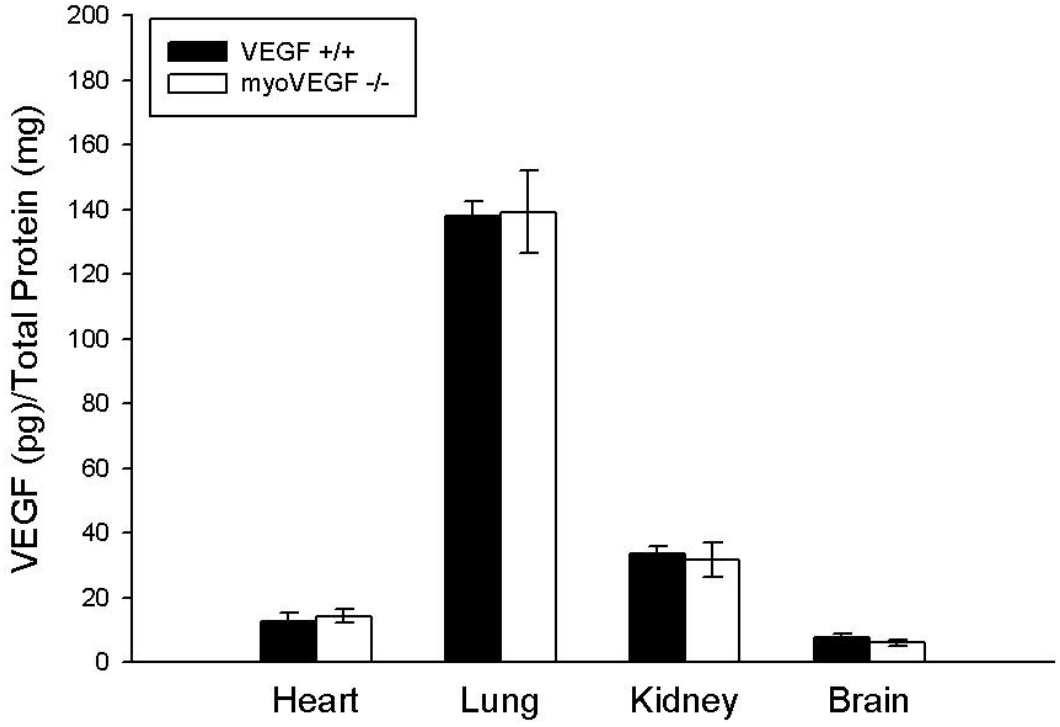
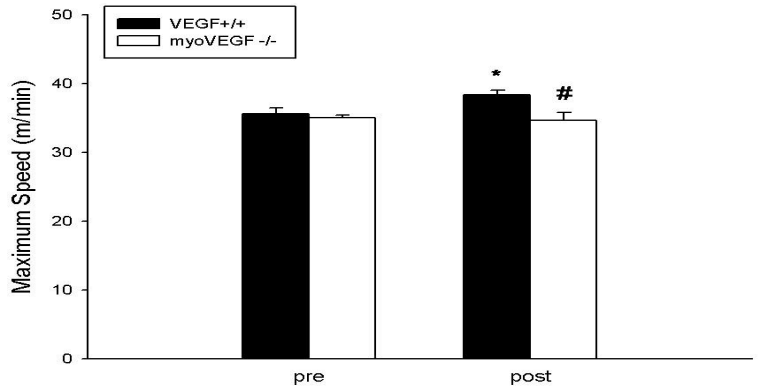


Figure 3.2. Normal post-tamoxifen VEGF protein levels in heart, lung and kidney. As expected, there were no differences between the two groups in heart, lung, kidney or brain. Values are mean ± SE.

A.



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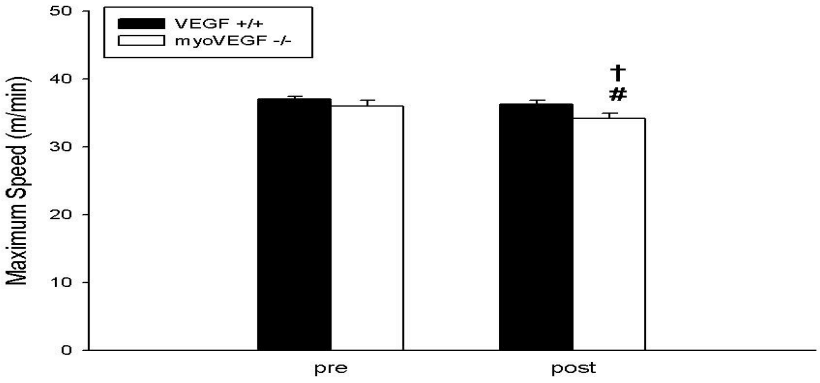
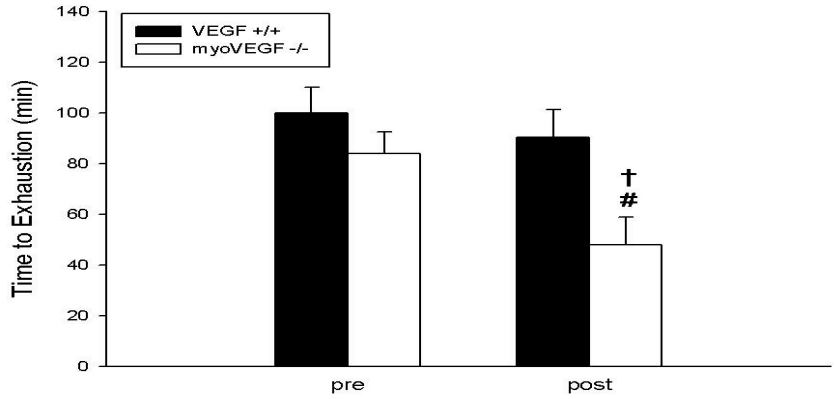


Figure 3.3. Pre and post-tamoxifen maximum running speeds, showing a small reduction after VEGF deletion. A.) 2 weeks following tamoxifen. B.) 7 weeks following tamoxifen. Values are mean ± SEM. *Significant difference ($p < .05$) compared to VEGF+/+ pre. † Significant difference ($p < .05$) compared to myoVEGF -/- pre. # Significant difference ($p < .05$) compared to VEGF+/+ post.

A.



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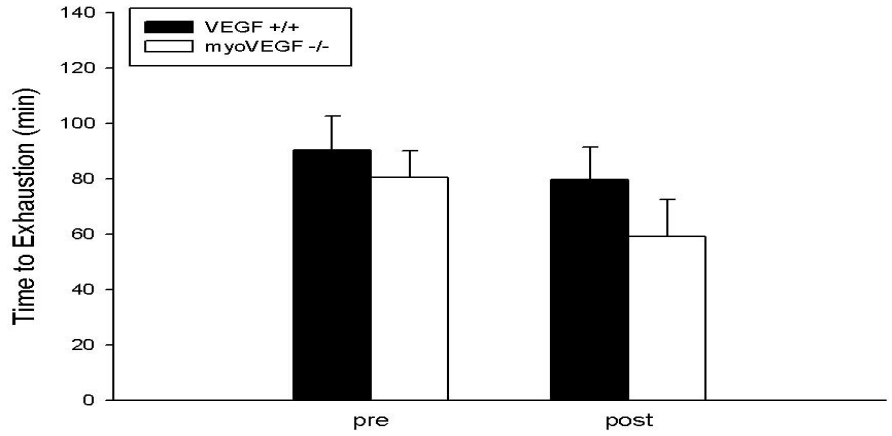
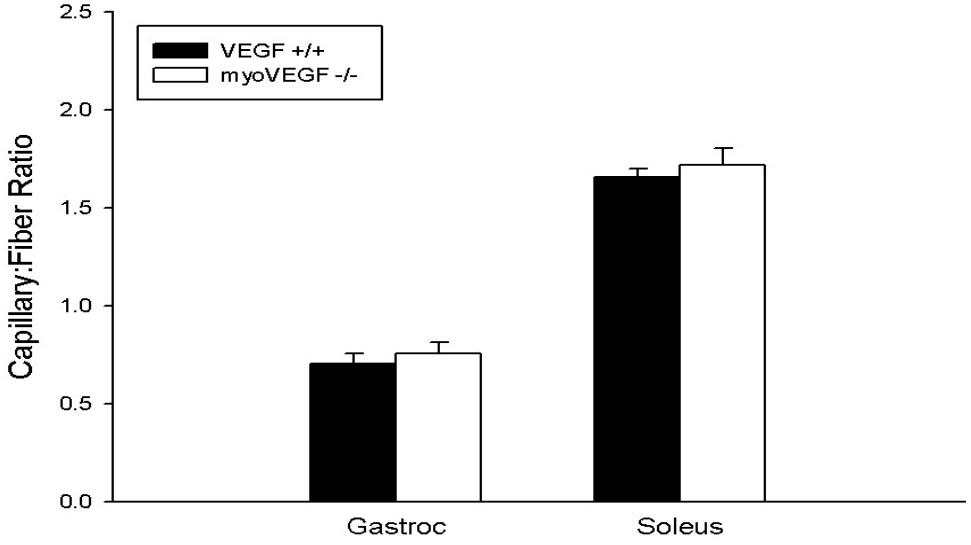


Figure 3.4. Pre and post-tamoxifen time to exhaustion. A.) 2 weeks following tamoxifen. B.) 7 weeks following tamoxifen. MyoVEGF-/- mice showed a significant 47% decrease in time to exhaustion compared to VEGF+/+ at 2 weeks, but at 7 weeks there was no significant effect of VEGF deletion. Values are mean ± SEM. † Significant difference ($p < .05$) compared to myoVEGF -/- pre. # Significant difference ($p < .05$) compared to VEGF+/+ post.

A.



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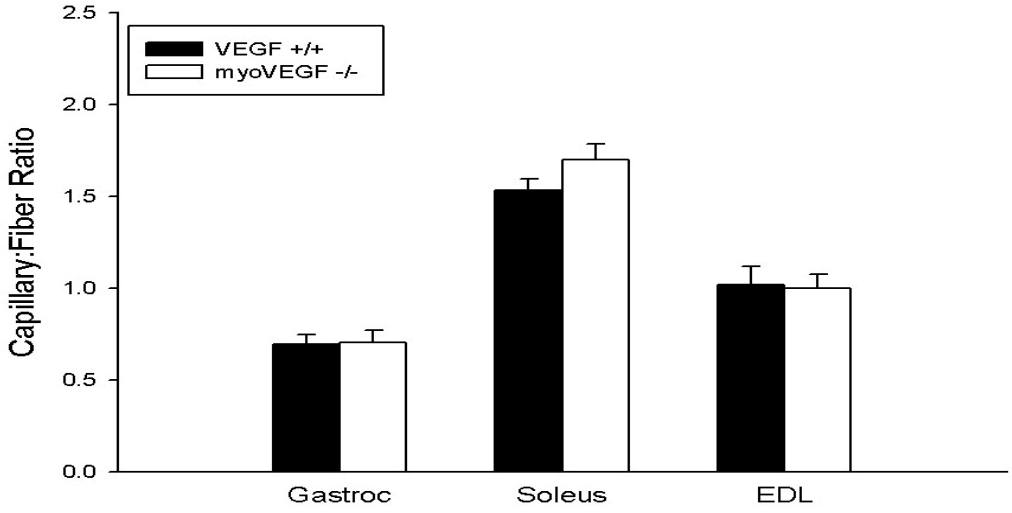
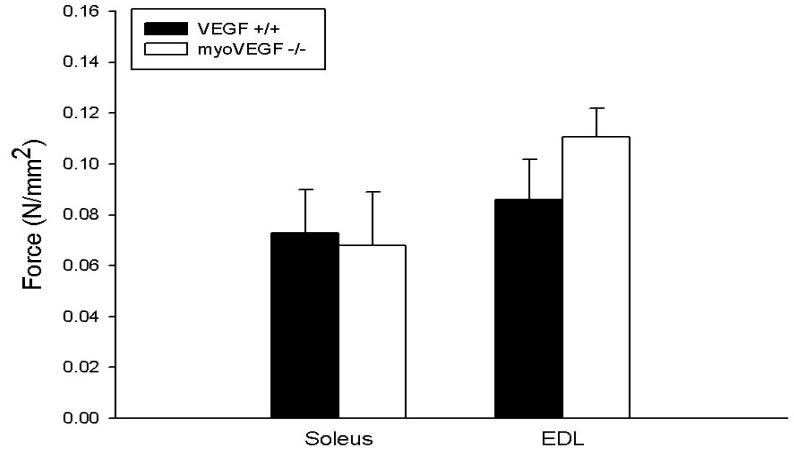


Figure 3.5. Capillary:fiber ratio is unaffected by VEGF reduction. A.) 2 weeks following tamoxifen. B.) 7 weeks following tamoxifen. N= 4-6 mice for each group. Gastroc: gastrocnemius; EDL: extensor digitorum longus. Values are mean ± SE.

A.



B.

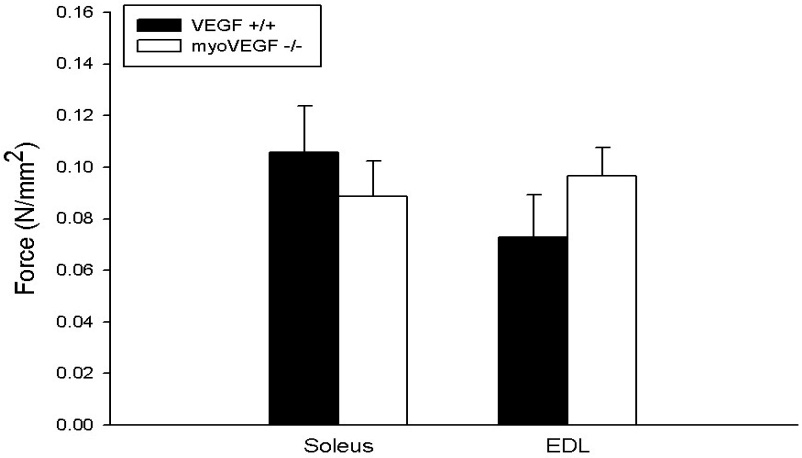
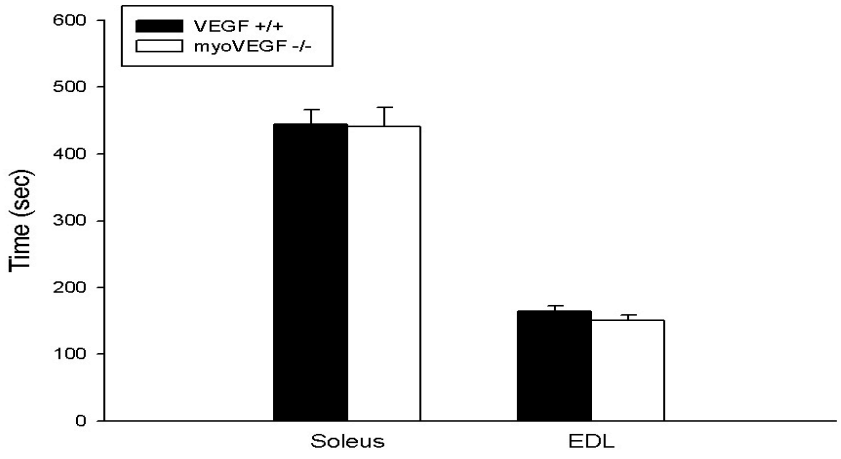


Figure 3.6. No significant effect of VEGF deletion on peak force in isolated muscles. EDL: extensor digitorum longus. A.) 2 weeks following tamoxifen (N = 4-6 muscles per group). B.) 7 weeks following tamoxifen (N = 5-7 muscles per group). Values are mean ± SE.

A.



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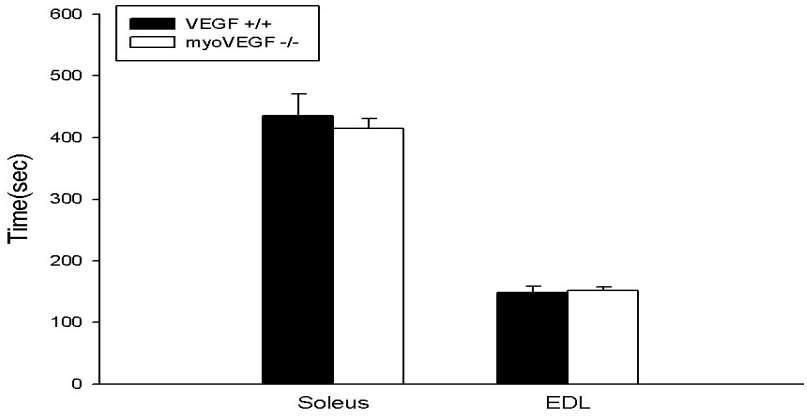


Figure 3.7. No significant effect of VEGF deletion on time to fatigue in isolated muscles. EDL: extensor digitorum longus. A.) 2 weeks following tamoxifen (N = 4-6 muscles per group). B.) 7 weeks following tamoxifen (N= 5-7 muscles per group). Values are mean ± SE.

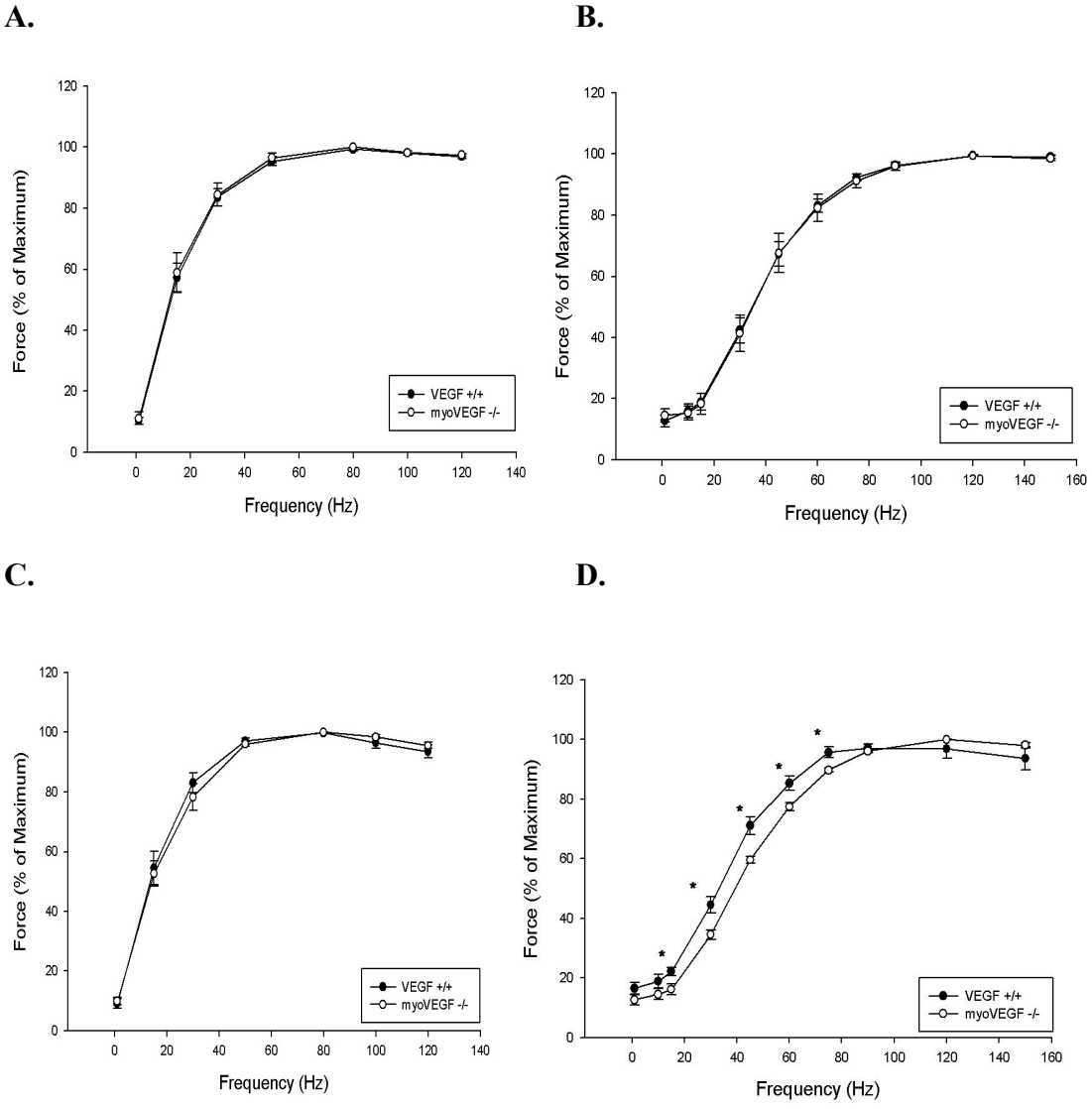
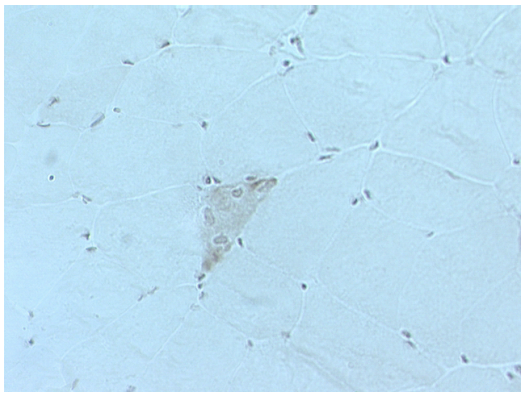


Figure 3.8. Force-frequency curves were mostly unaffected by VEGF reduction. A.) Soleus, 2 wks post-tamoxifen (N = 4-6 mice). B.) EDL, 2 wks post-tamoxifen (N=5-6). C.) Soleus, 7 wks post-tamoxifen (N=5-7). D.) EDL, 7 wks post-tamoxifen (N= 6-7). Only EDL at 7 weeks post-tamoxifen responded, showing a significant right shift. Values are mean ± SE. *Significant difference ($p < .05$) in myoVEGF -/- muscles compared to VEGF +/+.

A.



B.

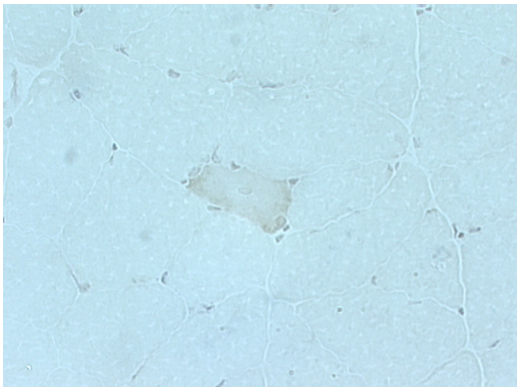
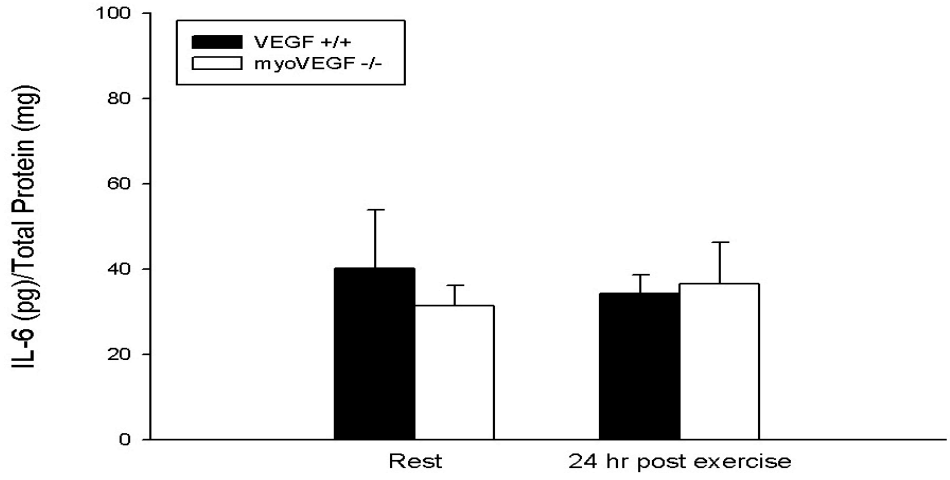


Figure 3.9. No difference in activated caspase-3 24 hours after a submaximal run to exhaustion, two weeks post-tamoxifen. The number of apoptotic myofibers did not differ between VEGF +/+ (A) and myoVEGF -/- (B) mice.

A.



B.

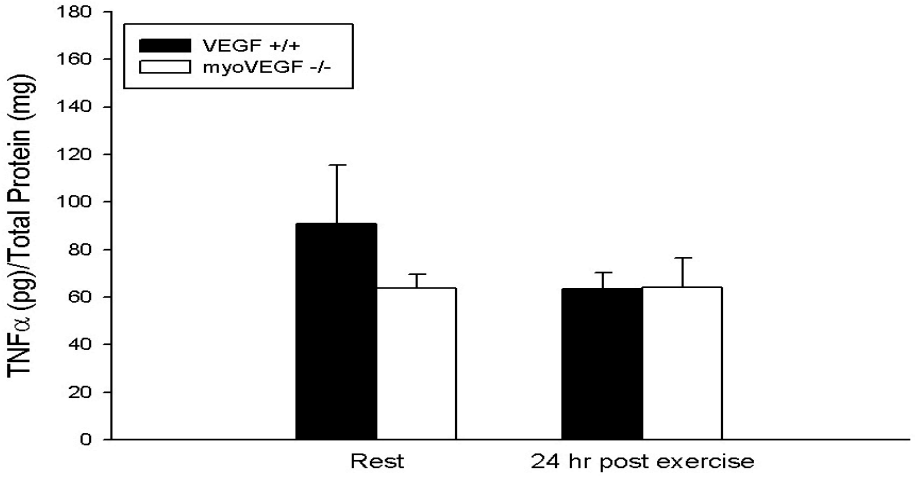


Figure 3.10. No effect of VEGF reduction on IL-6 and TNFα protein in gastrocnemius muscle two weeks post tamoxifen, either in cage-confined resting mice (rest) or mice 24 hours after a run to exhaustion (24 hr post exercise). A.) IL-6 (N = 5-6 mice). B.) TNFα (N = 4-6 mice). Values are mean ± SE.

CHAPTER 4

DISCUSSION

4.1 Inducible, Skeletal Myocyte-specific VEGF Knockout Model

In this study we sought to determine whether deletion of VEGF from adult skeletal muscle could affect skeletal muscle capillarity, muscle function and exercise performance. Previous models in our laboratory have demonstrated the critical nature of VEGF in regulating skeletal muscle capillarity but they have not allowed us to simultaneously evaluate the effect of VEGF deletion on maintaining the adult skeletal muscle microvasculature and on exercise performance and muscle function. We hypothesized that deletion of VEGF from adult skeletal myocytes would cause capillary regression and impairment in exercise capacity and muscle function.

In order to test this we generated a skeletal *myocyte*-specific VEGF knockout model, in which myocyte VEGF deletion could be temporally controlled in the adult mouse. After VEGF gene deletion, these mice have greatly diminished VEGF protein (> 90%) in skeletal muscles (Figure 3.1), confirming the finding of Olfert et al that myocyte derived VEGF is the major source of VEGF in skeletal muscle (Olfert, Howlett et al. 2009). Although we cannot empirically confirm that VEGF was deleted solely from the myocytes, evidence in the literature supports this claim in our model. We crossed VEGF*loxP* mice with HSA-Cre-ER^{T2} mice in which the Cre-ER^{T2} gene was inserted into a large human skeletal actin genomic sequence that contained upstream and downstream regulatory regions so as to ensure that deletion of the VEGF

gene occurred only in skeletal muscle (Schuler, Ali et al. 2005). Schuler et al. report that in the HSA-CRE-ER^{T2} mouse line, immunohistochemical staining showed that ~50% of total skeletal muscle nuclei were positive for Cre expression. Moreover, they report tamoxifen dependent recombination in ~50% of floxed target gene alleles and since, on average, 50% of the nuclei in skeletal muscle are myonuclei (Schmalbruch and Hellhammer 1977), Schuler et al. concluded that recombination occurred in almost all myonuclei. It thus follows that almost all myonuclei in our model should be deleted of the VEGF gene. Furthermore, in a skeletal muscle specific knockout model that used the muscle creatine kinase (MCK) promoter to drive Cre expression, Bruning et al. also reported a 50% recombination efficiency in skeletal muscle. The fact that we see the same degree of VEGF protein loss as our lifelong muscle VEGF knockout model, which used an MCK*cre*, supports that we have deleted VEGF only in the myoctyes.

We chose two time points, 2 and 7 weeks after induction of VEGF deletion, at which to evaluate the effects of skeletal myocyte VEGF deletion. The two week time point was chosen in accordance with Schuler et al. who reported that 13 days after tamoxifen administration, using the same HSA-Cre-ER^{T2} mice, mRNA transcripts from their gene of interest were reduced greater than 10 fold and by 19 days post tamoxifen they were undetectable. This allowed us to evaluate the early effects of VEGF deletion in adult skeletal muscle. The 7 week time point was chosen in accordance with Tang et al. where they showed a decrease in skeletal muscle capillarity at 4 and 8 weeks after deletion of VEGF from adult skeletal muscle. We

hypothesized that at the 7 week time point we would also see decreased capillarity in skeletal muscle and that this would lead to decreased exercise performance and muscle function.

4.2 Summary of Results

Early after VEGF deletion (2 weeks), myoVEGF -/- mice exhibit reduced exercise capacity with no evidence of capillary regression or in-vitro muscle contractile dysfunction compared to littermate controls (VEGF +/+). At 2 weeks post-tamoxifen injections, myoVEGF -/- mice also have no evidence of ongoing skeletal muscle apoptosis, inflammation or damage. Body weights and muscle weights were also not affected indicating no muscle atrophy. Seven weeks after inducing VEGF deletion in skeletal muscle, myoVEGF-/- mice have an attenuated reduction in exercise capacity, no capillary regression and a small but significant decrease in contractile function in the EDL. This model highlights the importance of myocyte-derived VEGF in modestly affecting exercise endurance capacity in the adult mouse while not being critical to skeletal muscle capillary maintenance.

4.3 Myocyte VEGF and Capillary Maintenance

Our finding that deletion of VEGF exclusively from skeletal myocytes in cage-confined adult mice does not affect skeletal muscle capillarity (Figure 3.5) was surprising given the previous findings from our laboratory where VEGF deleted from all cells types within a small region of adult gastrocnemius muscle from VEGF*loxP* mice resulted in significant capillary loss (Tang, Breen et al. 2004). Four and eight weeks after infection with an AAV*cre*, we reported almost undetectable levels of

VEGF and 60% reduction in skeletal muscle capillary to fiber ratio in the areas of AAV*cre* infected muscle, demonstrating for the first time the dependence of adult skeletal muscle capillaries on VEGF expression (Tang, Breen et al. 2004).

The differences between the Tang model and the current model likely highlight the cell-specific importance of VEGF production on capillary maintenance. We do not see 100% reduction in whole skeletal muscle VEGF protein in our myoVEGF -/- mice (Figure 3.1). There is still 5-8% VEGF still in the muscle that could be the result of VEGF production from other cell types within muscle (e.g. endothelial cells, satellite cells, fibroblasts and macrophages). Our current results taken together with the results from the Tang model leads us to conclude that VEGF produced from other cell-types in muscle may be enough to maintain the adult capillary network.

Our finding that >90% of VEGF protein was lost in skeletal muscle of myoVEGF-/- mice, mirrors the finding in the lifelong myocyte VEGF deleted mice (Olfert, Howlett et al. 2009). In the lifelong model, however, the mice experienced a large reduction in skeletal muscle capillarity that we did not see in the myoVEGF-/- mice (Figure 3.5). This is likely explained by the timing of VEGF deletion. In the Olfert model, deletion of VEGF from skeletal muscle in late gestation likely affected the development of the mature skeletal muscle microvasculature leading to reduced capillarity in the adult that could not be compensated by VEGF produced from other cell types in muscle. This is in agreement with Gerber et al., who found that inhibition of VEGF in early postnatal life caused a reduction in the number of capillary vessels in the kidney, heart and liver (Gerber, Hillan et al. 1999). In contrast to the lifelong

myocyte VEGF deletion model, in the current model, the adult capillary network was already formed when VEGF deletion was induced and, therefore, VEGF from cells other than the myocytes may have been able to compensate for the loss of VEGF and maintain capillarity.

In support of this, a recent study by Lee et al. found that lifelong deletion of VEGF in only the endothelial cells caused these cells to undergo apoptosis in vivo and in vitro. Interestingly, tissue levels of VEGF were normal indicating that the contribution of endothelial cell-derived VEGF is small (Lee, Chen et al. 2007). It also implies that paracrine sources of VEGF could not rescue the VEGF deficient endothelial cells. In contrast, the VEGF deficient endothelial cells were still able to respond to paracrine VEGF signaling as they retained the ability to form new vessels when exposed to exogenous VEGF. Although this study did not look at effects on skeletal muscle, they found unaltered VEGF levels in the heart and no change in myocardial vessel density. The authors concluded that autocrine signaling of VEGF in the endothelial cell may be responsible for maintaining their survival, although the lack of capillary regression seen in the heart argues either for a role of paracrine sources of VEGF in maintaining capillarity or for no role for VEGF at all. In our skeletal myocyte-specific VEGF deletion model, VEGF production by the endothelial cells should not be affected and, therefore, could support the survival of these cells and be the reason we do not see capillary regression.

Another important cell-type in muscle that produces and secretes VEGF is the skeletal muscle satellite cell (Chazaud, Sonnet et al. 2003; Christov, Chretien et al.

2007). We cannot confirm that VEGF production in the satellite cells in our mouse model is unaffected; however, evidence in the literature suggests that quiescent skeletal muscle satellite cells do not express skeletal actin, the promoter regulating *cre*-recombinase in our model (Gunning, Hardeman et al. 1987; Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997; Sabourin, Girgis-Gabardo et al. 1999). Studying satellite cells in their quiescent state is very difficult since isolation of the cells from skeletal muscle serves to activate them. It is unknown whether in the quiescent state these cells produce VEGF. Nonetheless, VEGF levels could be normal in some populations of satellite cells compared to the large reductions in VEGF that we see from the mature myoctyes. Christov et al. observed a close spatial relationship between muscle satellite cells and endothelial cells and demonstrated reciprocal signaling between the two cell types during angiogenesis and myogenesis (Christov, Chretien et al. 2007) suggesting that VEGF produced by the satellite cells could support the survival of the endothelial cells and prevent capillary regression.

Pericytes are a third cell-type present in skeletal muscle that produce VEGF and may serve to stabilize the mature skeletal muscle capillaries. Pericytes are important in the process of angiogenesis as they cover newly formed endothelial cell sprouts providing strength and stabilization of mature vessels (Carmeliet 2005). In addition, pericytes are a source of VEGF in the retina (Darland, Massingham et al. 2003) and in Lewis lung carcinomas (Sennino, Kuhnert et al. 2009). Interestingly, pericytes may be activated by a decrease in VEGF signaling. A recent study showed that tumors expressing high levels of VEGF have leaky and tortuous vessels while

tumors lacking VEGF have normal vessels and increased pericyte coverage (Greenberg, Shields et al. 2008). Greenberg et al. also showed that VEGF is a negative regulator of perictye proliferation and migration during PDGF mediated angiogenesis and that these effects were mediated through VEGFR2. This suggests that deletion of VEGF could actually serve to stabilize vessels by preserving pericyte coverage. However, these findings may not be relevant to the adult microvasculature since Kamba et al. found that pericyte coverage did not protect certain mature capillary beds from regression in response to VEGF inhibition (Kamba, Tam et al. 2006).

It is improbable that a single cell type in skeletal muscle is responsible for stabilizing the mature capillary network. It is more likely that a combination of cells producing VEGF are able to confer survival of the endothelial cells and that other mural cells such as pericytes may be responsible for stabilizing the mature vessels. We have only looked in cage-confined mice that have not been stressed. It is likely that paracrine signaling of VEGF from the adult myocyte is more important for regulating an angiogenic response in muscle as a result of injury or training. This is supported by studies that have sequestered circulating VEGF with decoy receptors or VEGF-neutralizing antibodies and have blocked the angiogenic response due to overload or training (Amaral, Papanek et al. 2001; Williams, Cartland et al. 2006).

An important finding that may be relevant to our results suggests that as vessels mature they lose their dependence on VEGF and do not regress in response to VEGF withdrawal (Gerber, Hillan et al. 1999). Kamba et al. challenged this notion by

demonstrating capillary loss in some normal adult mouse organs including pancreatic islets, thyroid and small-intestinal villi in response to various VEGF inhibitors (Kamba, Tam et al. 2006). VEGF induces fenestrations in endothelial cells (Roberts and Palade 1995) and Kamba et al. found that there were more fenestrations in the organs that experienced capillary loss as a result of VEGF blockade. Skeletal muscle has a continuous endothelium with no fenestrations (Roberts and Palade 1995) and in agreement with our results, Kamba et al. did not see capillary regression in the tongue, a representative skeletal muscle, in response to the VEGF inhibitors. The mice in these studies were treated with VEGF inhibitors for 1-3 weeks while in our myocyte-specific VEGF deletion model we looked at the effects of VEGF withdrawal over a 7-week time period. According to Tang et al., 7 weeks should have been sufficient time to see a decrease in capillarity in response to VEGF deletion in skeletal muscle. Since in the Tang model the VEGF deletion was more severe (i.e. VEGF was deleted from all cell types in muscle) than in our myocyte-specific VEGF deletion model, it remains to be seen if longer periods of decreased VEGF signaling could result in capillary regression in skeletal muscle in the current mouse model.

4.4 Myocyte VEGF and Decreased Exercise Capacity

4.4.1 Effects 2 weeks post-tamoxifen

Endurance exercise performance is determined by a number of factors, namely, the supply of oxygen and nutrients to the myocytes, contractile function of the myocytes, and metabolic demand of the muscle. Our skeletal myocyte VEGF gene deleted mice showed decreased exercise capacity (Figure 3.3 & 3.4) only two weeks

following VEGF gene deletion with no evidence of muscle dysfunction (Figure 3.6, 3.7 & 3.8) or decreased capillarity (Figure 3.5). The lifelong myocyte VEGF deletion model of Olfert et al. also showed decreased maximum running speed and time to exhaustion, although to a much greater degree than in our mice. In this model, however, the mice also experienced a dramatic decrease (60%) in skeletal muscle capillary to fiber ratio. Since a major determinant of exercise endurance capacity is the number of capillaries that interface with the muscle fibers (Roca, Hogan et al. 1989; Bebout, Hogan et al. 1993; Hepple, Hogan et al. 2000), it is likely that this is the reason for their deficit in exercise performance. Olfert et al. also report VEGF deletion in the heart along with decreased capillary density, which cannot be ruled out as additional causes for the decreased running performance.

Early after VEGF deletion in skeletal muscle, apoptosis and inflammation could lead to impaired exercise performance due to muscle atrophy (Marzetti and Leeuwenburgh 2006) or decreased muscle contractile function (Adams, Mangner et al. 2008; Hardin, Campbell et al. 2008). However, we did not find any evidence of increased myocyte apoptosis in the myoVEGF -/- mice compared to controls (Figure 3.9) and no muscle damage (data not shown) or increases in inflammatory cytokines (Figure 3.10). In agreement with this, delivery of a VEGF-R antagonist did not cause skeletal muscle damage in sedentary or exercise trained rats (Lloyd, Prior et al. 2005). Furthermore, in the heart, myocyte VEGF deletion did not result in ongoing cardiomyocyte apoptosis or necrosis nor were TNFα levels elevated compared to control mice (Giordano, Gerber et al. 2001). This leaves us with a question as to what

caused the decreased maximal running speed and time to exhaustion in our skeletal myocyte VEGF knockout mice.

Although we did not measure fiber type composition of the muscles or oxidative and glycolytic enzyme activities, it is unlikely that fiber type changes or metabolic limitations lead to the decreased endurance capacity seen in our myocyte VEGF deficient mice. This is supported by prior work showing that lifelong myocyte VEGF deficient mice had increased, not decreased, oxidative and glycolytic enzyme activities. Moreover, there were no fiber type differences compared to control mice (Olfert, Howlett et al. 2009).

4.4.2 Possible causes of decreased exercise capacity at 2 weeks post-tamoxifen

Reduced NO bioavailability could be a direct result of VEGF withdrawal from muscle. VEGF upregulates the expression of endothelial nitric oxide synthase (eNOS) mRNA and protein and induces NO release from endothelial cells (van der Zee, Murohara et al. 1997; Hood, Meininger et al. 1998). Several groups have shown that this is mediated through Flk-1/VEGFR2 (Kroll and Waltenberger 1998; Kroll and Waltenberger 1999; Shen, Lee et al. 1999; Ahmad, Hewett et al. 2006). Decreases in VEGF signaling could downregulate eNOS expression and NO release from endothelial cells that would directly affect dilation of blood vessels. Lending support to this hypothesis, preeclampsia, a condition in pregnancy, characterized by increased levels of soluble VEGFR2 (sFlt-1), which is responsible for decreasing circulating levels of VEGF, leads to NO dependent hypertension (Lam, Lim et al. 2005)

Interestingly, one of the major side effects of anti-VEGF therapy is also hypertension thought to be caused by decreased release of NO and prostacylin (Kamba and McDonald 2007; Verheul and Pinedo 2007).

Increased skeletal muscle blood flow during exercise is necessary to ensure adequate supply of oxygen and nutrients to the working muscle. Insufficient blood flow to skeletal muscle during exercise due to impaired eNOS expression or activity could cause decreased oxygen delivery to the muscle (Hogan, Bebout et al. 1993) and be a possible mechanism for the decreased exercise capacity in our mouse model. In support of this, mice treated with $N^{\text{omega-nitro-L-arginine}}$ (LNA), a universal nitric oxide synthase inhibitor, experienced decreases in maximal oxygen consumption and aerobic work. Administration of microspheres during the exercise test also revealed a reduction in limb blood flow compared to control mice (Maxwell, Schauble et al. 1998). In addition, eNOS knockout mice subjected to 8 weeks of voluntary wheel running displayed decreased distance/day, decreased maximum running speed and decreased distance/run compared to control animals (Momken, Lechene et al. 2004). These studies indicate that loss of NO bioavailability could be a cause of decreased oxygen delivery to skeletal muscle resulting in decreased exercise performance. However, NO is not the only mediator of vasodilaton and blood flow during exercise. Other factors such as prostaglandins and endothelium-derived hyperpolarizing factor (EDHF) may compensate for the loss of NO and maintain exercising blood flow. This has been demonstrated in skeletal muscle of male eNOS -/- mice (Sun, Huang et al.

1999; Huang, Sun et al. 2000) and could be why we see a smaller decline in exercise capacity at the 7 week time point.

We do not know whether diaphragm weakness could contribute to the decline in exercise capacity in the myoVEGF -/- mice. It is not possible to evaluate diaphragm function independent of peripheral muscle function in vivo and in this study we did not look at the effects of myocyte VEGF deletion on isolated diaphragm muscle. Studies in mice have shown that the diaphragm and peripheral skeletal muscles can respond differently to the same genetic knockout. In particular, diaphragm muscle from mdx mice, deficient in dystrophin, are more susceptible to the effects of fiber necrosis and show decreased force production compared to hindlimb muscles (Dupont-Versteegden and McCarter 1992). Furthermore, 4 weeks of treadmill running in K^{ATP} channel-deficient mice caused more fiber damage in the diaphragm than in the hindlimb muscles (Thabet, Miki et al. 2005). That diaphragm muscles are constantly active or in a "trained" state may have implications for how VEGF is regulated in this muscle. Patients with COPD have decreased levels of VEGF in peripheral skeletal muscles but have increased levels in the diaphragm likely due to their increased work of breathing (Alexopoulou, Mitrouska et al. 2005; Barreiro, Schols et al. 2008). A rhythmically active muscle with high levels of VEGF expression may be undergoing constant remodeling and be more sensitive to a loss of VEGF than sedentary peripheral muscles (Amaral, Papanek et al. 2001; Lloyd, Prior et al. 2003). Furthermore, we have shown that the diaphragm has a higher expression of VEGFR2 than the gastrocnemius (Olfert, Howlett et al. 2009) and Kamba et al. found

that organs with high expression of VEGFR2 were more susceptible to capillary regression due to VEGF inhibition (Kamba, Tam et al. 2006). If there is diaphragmatic weakness in our myoVEGF -/- mice due to either decreased capillarity or decreased muscle strength, this would lead to respiratory fatigue resulting in decreased exercise capacity.

4.4.3 Effects 7 weeks post-tamoxifen

At seven weeks we saw only a 6% decrease in maximal running speed (Figure 3.3) and a non-significant trend towards decreased time to exhaustion (Figure 3.4) with no decrease in capillarity (Figure 3.5) and only a small (but significant) decrease in contractile function in the EDL (Figure 3.8). The presence of a right shifted force frequency curve in the EDL of myoVEGF -/- mice demonstrates less force production at any stimulation frequency, suggesting contractile dysfunction in this muscle. However, this finding is puzzling given that the exercise response at 7 weeks (6% decreased maximum running speed and 25% decreased time to exhaustion compared to controls) was less abnormal than the exercise response observed at two weeks (10% decreased maximum running speed and 47% decreased time to exhaustion compared to controls) when the force-frequency curve was normal. This indicates that the minimal contractile dysfunction seen in the EDL does not contribute to a decline in whole body exercise capacity.

Two explanations could account for this discrepancy. The first is that although the significantly right-shifted force frequency curve is laboratory evidence of contractile dysfunction, it may not be physiologically significant (i.e. the muscle may

still be in a normal functioning range) and, therefore, may not contribute to a deficit in running performance. Unlike the contractile results in our mice, studies that have disrupted calcium homeostasis in skeletal muscle by deletion of genes that regulate either calcium release into the SR (FKBP12) or calcium reuptake (ATP2A1) have reported dramatic right shifted force frequency curves in the EDL and diaphragm, respectively, accompanied by decreased peak force (Pan, Zvaritch et al. 2003; Tang, Ingalls et al. 2004). Our EDL muscle has a small right shift in the force-frequency curve (Figure 3.8) without a decrease in peak force (Figure 3.6) underscoring that the contractile dysfunction in the EDL of our mice is likely small. However, the right-shifted force frequency relationship could be indicative of early muscle dysfunction in fast skeletal muscles that may worsen over time and eventually result in reduced exercise performance.

The second possible explanation is that since we did not test any other fast, glycolytic muscles we cannot assume that they are all affected in the same way as the EDL. The EDL is a very small muscle, and although it is recruited during treadmill exercise (Jeneson, de Snoo et al. 2007), the small amount of contractile dysfunction that is present in this muscle is unlikely to affect running exercise capacity.

4.4.4 Impaired contractility in the EDL 7 weeks post-tamoxifen

An explanation for why there is a right-shifted force frequency curve in the EDL of myoVEGF-/- mice is less clear. Deletion of VEGF from cardiomyocytes results in decreased coronary vascularization along with decreased cardiac contractility (Giordano, Gerber et al. 2001). Interestingly, hearts subject to HIF1α

gene deletion in cardiomyocytes exhibit decreased expression of VEGF mRNA and decreased cardiac contractility with only mild hypovascularity. Contractile studies on isolated HIF1α-null cardiomyoctyes revealed intrinsic contractile dysfuncion with prolonged calcium reuptake and a decrease in sarcoplasmic reticulum Ca^{2+} ATPase isoform 2 (SERCA2) expression, the primary sarcoplasmic reticulum isoform in heart and slow-twitch muscles (Huang, Hickey et al. 2004). Huang et al. also report that although there are putative HIF1α binding sites on the SERCA2 gene, in vitro studies showed that HIF1α only minimally induced transcription of SERCA2. The authors, therefore, concluded that the decrease in HIF1α was not likely the direct cause of decreased SERCA2 in their model.

The Huang study provides a link between decreased VEGF levels and single cell contractile dysfunction that could be caused by decreased expression of sarcoplasmic reticulum isoforms and impaired maintenance of calcium stores. SERCA1 is the predominant sarcoplasmic reticulum isoform expressed in fast-twitch skeletal muscles, such as the EDL. SERCA1 is required for calcium homeostasis and is responsible for maintaining SR calcium stores needed for proper excitation contraction coupling (Prasad, Okunade et al. 2004). Moreover, disruption of this gene causes a severe right shifted force frequency relationship in the diaphragm (Pan, Zvaritch et al. 2003).

We did not measure SERCA1 levels in the EDL so we cannot confirm that this is the cause of contractile dysfunction in this muscle. Moreover, we cannot explain

why we did not see any contractile dysfunction in the soleus (Figure 3.6 & 3.8). The soleus in the mouse is a mixed muscle with ~60% type 1 (slow-twitch) fibers and ~40% type IIa (fast-twitch) fibers (Gavin, Westerkamp et al. 2006). Presumably this muscle would be affected by either a decrease in SERCA1 or SERCA2 but we do not see any decreases in contractility of this muscle. Mice with a deficiency in FKBP12, a protein that binds and regulates the skeletal muscle Ca^{2+} release channel, show a rightward shift in the force-frequency relationship in the EDL without any change in the contractile properties of the soleus indicating that the soleus may be better able to adapt to disturbances in calcium homeostasis (Tang, Ingalls et al. 2004).

Future studies that look at longer term VEGF deletion from adult skeletal myocytes would be needed in order to determine if the contractile dysfunction in the EDL persists or worsens over time. Determining the cause of the dysfunction may be more challenging but single cell analysis of calcium homeostasis during contractions and measuring myosin heavy chain composition would be the first steps in this process.

4.5 Conclusion

Using a novel inducible, skeletal myocyte VEGF knockout mouse this study has revealed that in adult, cage-confined mice, myocyte derived VEGF is not critical in maintaining skeletal muscle capillarity but does temporarily impact exercise endurance capacity. Given VEGF's cytoprotective attributes and its importance in regulating skeletal muscle capillarity, it is striking that deletion of greater than 90% of skeletal muscle VEGF protein results in no capillary loss and minimal and temporary

effects on exercise performance and muscle function. This study suggests that other cells within muscle are critical to skeletal muscle capillary maintenance and that myocyte VEGF may be more important in protecting the muscle when it is stressed as with training or injury. It is also possible that myocyte VEGF regulates NO bioavailability and that loss of VEGF from the myoctye could lead to endothelial dysfunction culminating in exercise limitation.

4.6 Future Directions

In light of the findings from this study, some important questions arise: 1) Which cell types in muscle produce VEGF necessary to maintain skeletal muscle capillarity? 2) Is myocyte derived VEGF necessary for paracrine signaling to the endothelial cells during angiogenesis? 3)What are the mechanism(s) by which myocyte derived VEGF affects exercise endurance capacity?

In order to address the first question, systematic deletion of VEGF from different cells within skeletal muscle would have to be undertaken and then measurements of skeletal muscle capillarity made as a result of each deletion. A series of studies have demonstrated this approach in nervous tissue where deletion of mutant SOD1 was undertaken in different cell types in a model of amyotrophic lateral sclerosis (ALS). These authors found that deletion of mutant SOD1 from motor neurons delayed disease initiation (Boillee, Yamanaka et al. 2006), while deletion from microglia (Boillee, Yamanaka et al. 2006) and astrocytes (Yamanaka, Chun et al. 2008) slowed disease progression. They further showed that deletion from muscle cells had no effect on disease onset or progression (Miller, Kim et al. 2006) and

deletion from Schwann cells accelerated disease progression (Lobsiger, Boillee et al. 2009). These studies are an example of how this technique is able to identify each cell type as having unique effects on the whole tissue in response to the same gene deletion. If after deletion of VEGF from every cell type, one at a time, there was still no effect on capillaries this would imply that there is compensation from other cell types and that more than one cell type has to be depleted of VEGF in order to affect capillarity.

The second question, which addresses the effects of myocyte derived VEGF on the process of angiogenesis could be evaluated through a training study. It is known that endurance training increases skeletal muscle capillarity and we have shown this in our laboratory with a 7-day voluntary wheel running protocol (Olfert et al, unpublished). The myoVEGF-/- mice in this study are unique in that they have normal capillarity and minimal differences in maximum running speed and endurance exercise capacity 7 weeks post-tamoxifen despite greater than 90% reduction in VEGF protein levels in skeletal muscle. Therefore, at this time point our myocyte VEGF deleted mice could potentially train at the same absolute intensity as the control mice and should unequivocally tell us if myocyte-derived VEGF is necessary in the process of de novo capillary growth.

The third question is what caused the decrease in endurance capacity at 2 weeks post-tamoxifen in our myoVEGF -/-? We hypothesize that decreased NO bioavailability due to dysregulation of eNOS could cause a decrease in exercise capacity by decreasing blood flow and oxygen availability to the working muscles. In

order to test this hypothesis, the following measurements could be made in response to an acute exercise bout on a treadmill. Measurement of blood flow to exercising hindlimb muscles during the exercise bout would give an indication if oxygen delivery were impaired in the myoVEGF -/- mice. This method has been demonstrated in mice using microspheres in order to determine blood flow as a percentage of cardiac output (Maxwell, Schauble et al. 1998). Measurement of hypoxia responsive gene expression in the gastrocnemius muscle in response to acute exercise would indicate if the muscles were hypoxic during the exercise bout and finally measurement of eNOS mRNA and protein expression and activity in response to acute exercise would indicate if there was impairment in the regulation of this gene. For all of these measurements careful consideration of the intensity and duration of the exercise bout and the timing at which measurements are made would be critical in making accurate conclusions from these tests. If measurements are taken at the end of a maximal run to exhaustion, we may see no differences in the above parameters, since at maximal exercise both mice are running at the same relative intensity.

4.7 Implications for COPD

Patients with COPD experience moderate decreases in VEGF levels in peripheral skeletal muscles (compared to the > 90% reduction in this study) with decreased capillarity. Our results taken together with the Tang study suggest that the source of VEGF may be important in determining its effect on capillarity. The source of VEGF loss in COPD is unknown although given the moderate reductions in VEGF levels found in peripheral muscles from COPD patients, the results from this study

indicate that decreased VEGF levels alone are unlikely to be responsible for the reductions in capillarity seen in this patient population. Perhaps the decrease in VEGF seen in COPD is secondary to a loss of capillaries and to fiber type switching from type I to type II. The reasons for these changes are still unknown and need further investigation.

In light of the findings from this study we have hypothesized that myocyte VEGF regulates eNOS and NO release from endothelial cells and that loss of eNOS function secondary to a loss of VEGF could decrease exercising blood flow to skeletal muscle and cause decreased exercise capacity. Indeed endothelial dysfunction and loss of NO bioavailability is thought to contribute to the exercise deficits seen in patients with chronic heart failure (CHF) (Drexler and Hornig 1996). Loss of NO bioavailability in peripheral muscles of COPD has not been observed, although a study in non-COPD smokers found decreased eNOS expression in vastus lateralis muscle (Montes de Oca, Loeb et al. 2008) and we have demonstrated decreased VEGF expression in the soleus muscle from mice exposed to 8 weeks of cigarette smoke (Tang et al., unpublished). Given that smoking is the major cause of COPD, these alterations in VEGF and eNOS may be early contributors to the decreased peripheral muscle function seen in COPD. Future studies investigating the effects of VEGF depletion in skeletal muscles on NO availability may lead to therapeutic strategies that restore VEGF levels in peripheral muscles of COPD patients in order to improve endothelial function and in turn exercise capacity.

4.8 References

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