Howard Wright Stallings, III. VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION AT REST AND AFTER ACUTE EXERCISE IN OBESE AND LEAN SEDENTARY MALES. (Under the direction of Timothy P. Gavin, Ph.D.) Department of Exercise and Sport Science, November 2004

Obesity is the second leading cause of unnecessary deaths in the United States. At present, approximately 65% of Americans are overweight, and 31% are obese. Lower skeletal muscle capillarization, lower insulin sensitivity, and larger muscle fibers have been observed in obese compared to lean subjects. Vascular endothelial growth factor (VEGF), the most potent angiogenic growth factor, is increased by exercise and is important for capillary supply maintenance. Therefore, to determine if VEGF expression is lower in obesity, muscle biopsies of the vastus lateralis of eight obese (OB) and eight lean (LN) young sedentary men were taken prior to and 2 hours after a 1-hour systemic exercise bout. No differences were found in VEGF mRNA between LN and OB, while acute systemic exercise produced 10-fold increases in each group. Histochemistry revealed OB had lower capillary density (CD), lower capillary-to-fiber area ratio (CFA), and greater fiber cross-sectional area in both Type I and II fibers compared to LN ($P<0.05$), but no difference in capillary contacts per fiber. The homeostatic model assessment (HOMA), an index of insulin sensitivity, was significantly greater in OB (3.49 ± 0.63) than LN (1.24 ± 0.38; $P<0.05$). In this study, VEGF expression could not explain the differences found in CD and CFA between LN and OB. Contradictory to a resistance-training model, it is possible that a dysregulation exists in obesity between increasing skeletal muscle capillarization with increasing muscle fiber size.

VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION AT REST AND AFTER ACUTE EXERCISE IN OBESE AND LEAN SEDENTARY MALES

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DEDICATION

This thesis is dedicated in honor of my mother, Betty Jean Varnell Stallings, and in loving memory of my father, Howard (Ward) Wright Stallings, Jr.

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INTRODUCTION

Obesity is a major health concern worldwide and about 300,000 deaths each year in the United States are attributable to obesity (CDC, 2004). Using the National Institutes of Health's guidelines, 61 percent of Americans were overweight or obese in 1999. The accumulation of fatty acids and reduced rates of fatty acid oxidation associated with obesity can lead to insulin resistance, diabetes mellitus, heart disease, and ultimately death. Although its etiology is complex and not yet fully elucidated, possessing a lower percentage of slow-twitch, oxidative Type I skeletal muscle fibers may partially cause obesity. Another theory suggests that reductions in capillarization, known as capillary rarefaction, and oxidative capacity reduce fatty acid diffusion across capillaries into the mitochondria for oxidation. Capillary rarefaction and reductions in Type I oxidative fibers along with increases in Type IIb glycolytic fibers have been observed in obesity.

Insulin resistance is a detrimental characteristic of obesity because it predisposes individuals to diabetes and cardiovascular disease. Skeletal muscle fiber composition plays an important part in insulin sensitivity. Type I oxidative fibers are the most insulin-sensitive fibers while Type IIb glycolytic fibers are the least insulin-sensitive. The percentage of Type I muscle fibers is generally considered to be genetically predetermined, but fiber type conversions from Type IIb glycolytic fibers to Type IIa oxidative-glycolytic intermediate fibers may result from an appropriate

stimulus such as endurance exercise training. While the percentage of insulin-sensitive Type I oxidative fibers may be unalterable, insulin sensitivity can be enhanced through angiogenesis, or the formation of new capillaries. Increased capillarization allows for greater substrate flux from the blood into the muscle cells by reducing the diffusion distance from the capillary wall to the sarcolemma (Lillioja et al., 1987).

Given the appropriate intensity and duration, endurance exercise training has been repeatedly shown to promote angiogenesis. Although the complete mechanism of new capillary formation is not fully known, growth factors are known to play an integral part. Vascular endothelial growth factor (VEGF) is considered to be the putative growth factor responsible for new capillary formation. VEGF is a 45-kDa homodimeric glycoprotein that is a specific endothelial cell mitogen known to regulate angiogenesis in wound healing, reproductive functions, tumor growth, and skeletal muscle (Leung et al., 1989).

Because capillary rarefaction is associated with obesity and VEGF is an important growth factor in angiogenesis, it is plausible that aberrations in VEGF regulation could contribute to the capillary rarefaction observed in obesity. Therefore, the purpose of this investigation is to determine if VEGF mRNA levels are lower in obese than in lean subjects at rest. We will also determine the relationship between skeletal muscle capillarization, fiber-type, and obesity.

Statement of Problem

- Is the lower skeletal muscle capillarization in obesity associated with a lower resting level of Vascular Endothelial Growth Factor (VEGF) mRNA?
- Is there an attenuated exercise-induced response of VEGF mRNA to an acute exercise bout in sedentary obese subjects that may indicate a potential abnormality in the ability to increase VEGF?

Research Hypotheses

1. The capillary rarefaction observed in obesity is associated with reduced VEGF.
2. Obese subjects will have an attenuated exercise-induced VEGF mRNA response as compared to lean subjects after a single bout of acute exercise at 50% $\dot{V}O_{2MAX}$.

Delimitations

- This investigation will attempt to recruit lean and obese subjects who are non-smokers, normotensive, and sedentary. They will have no diagnosed metabolic or cardiovascular disease and will not be taking medication to treat any metabolic or cardiovascular condition.
- VEGF mRNA will be assessed via Northern blot analysis on muscle samples taken from the vastus lateralis of the participants.
- Sedentary subjects will be utilized and sedentary status will be classified as not having participated in regular physical activity designed to increase heart rate

for 30 minutes a session for more than 3 times per week over a period of the previous 3 months.

- Lean and obese subjects will be used. Body Mass Index (BMI) will be used to determine a subject's status. BMI $\geq$ 30 kg/m^2 will be considered obese, and BMI $\leq$ 25 kg/m^2 will be considered lean.

Limitations

- Sedentary status will be based on the subject's self-reported physical activity level in response to the criteria listed above.
- Maximal aerobic capacity ($\dot{V}O_2MAX$) will also be assessed. The $\dot{V}O_2MAX$ will be performed on a Lode electronically-braked cycle ergometer. This value will be assumed to be an accurate measure of the subject's aerobic capacity.
- The subjects will be asked to exercise for one hour at 50% of their $\dot{V}O_2MAX$. This will serve as the stimulus necessary to induce the VEGF mRNA response.

Definition of Terms

1. Angiogenesis: the formation of new capillaries from pre-existing capillaries.
2. Body Mass Index (BMI): a measure used in determining body composition; found by dividing a person's weight in kilograms by their height in meters squared (kg/m^2)
3. Capillary Density (C/D): the amount of capillaries per muscle fiber area
4. Capillary-to-fiber perimeter exchange index (CFPE index): a new measure of capillarity which assesses capillaries per individual fiber and has been shown to

better correlate with changes in oxygen consumption than capillary density (Hepple, 1997)

5. Capillary rarefaction: a reduction of functional capillaries
6. Capillary: a microscopic blood vessel connecting arterioles and venules where oxygen and other nutrients are exchanged between the blood and tissues
7. Hydrostatic weighing: a method of determining percent body fat from body density by weighing a person underwater
8. Insulin Resistance: an abnormal physiological level of insulin needed to maintain glucose homeostasis
9. Maximal aerobic capacity ($\dot{V}O_2MAX$): a measure of oxygen consumption by attempting to achieve one's cardiorespiratory system's highest level of work
10. Muscle biopsy: surgical removal of a piece of muscle tissue for examination
11. Myosin ATPase fiber typing: a muscle biopsy staining method used to determine and evaluate muscle fiber composition
12. Northern blot analysis: an electrophoretic method for identifying the presence or absence of particular mRNA molecules using nucleic acid hybridization
13. Obesity: an abnormal increase in the proportion of fat cells, mainly in the viscera or subcutaneous tissues of the body
14. Residual Volume: the amount of air left in the lungs after a forced maximal exhalation

15. Rosenblatt capillary staining procedure: a method of staining muscle biopsy samples to view and quantify the capillaries surrounding muscle fibers
16. Type I muscle fibers: slow-twitch, oxidative, muscle fibers
17. Type II Diabetes (NIDDM): an excess of glucose in the blood associated with a an inability of insulin in the body to maintain glucose homeostasis
18. Type IIa muscle fibers: fast-twitch, intermediate glycolytic/oxidative fibers
19. Type IIb muscle fibers: fast-twitch, glycolytic muscle fibers
20. Vascular Endothelial Growth Factor (VEGF): a 45-kDa homodimeric glycoprotein that is a potent and specific mitogen for vascular endothelial cells
21. Waist-to-Hip Ratio: the minimum waist circumference divided by the maximum hip circumference

REVIEW OF LITERATURE

Obesity and Insulin Resistance

Obesity is a multifactorial disease characterized by having an abnormally high proportion of body fat (Bray, 1987), and is a major health problem in the United States and other industrialized countries. Body mass index, or BMI, is a generally accepted tool for quantifying obesity in normal populations. BMI is computed by dividing body mass in kilograms by height in meters squared. In 1998, the National Institutes of Health established guidelines for the determination of obesity. Using BMI as criteria, they declared values less than 25.0 kg/m^2 as lean, 25.0 to 29.9 kg/m^2 as overweight, 30.0 to 34.9 kg/m^2 as Class I obesity, 35.0 to 39.9 kg/m^2 as Class II obesity, and greater than 40.0 kg/m^2 as Class III obesity (NIH, 1998). Although BMI is not a measure of percent body fat, a strong positive correlation (≈ 0.8) exists between BMI and percent body fat (Benn, 1970).

Obesity is a major risk factor for a number of conditions such as hyperinsulinemia, insulin resistance, diabetes, hypertension, stroke, cardiovascular disease, cancer, hyperlipidemia, osteoarthritis, and psychological stress (Nieman, 1998). The prevalence of obesity has steadily increased over the last three decades and continues to increase dramatically. According to the Centers for Disease Control, (CDC), approximately 46% of all adults in the United States were overweight or obese in the late 1970's, but that number had increased to 55% by the early 1990's, with the

rates in teens almost doubling (Jacobson, 2000). In 1999, the prevalence of persons overweight and obese was estimated at 61 percent for Americans over the age of 20 years old, and 13 percent for children and adolescents. Approximately 300,000 Americans die each year from conditions related to being overweight and obese, which is second only to cigarette smoking as the highest cause of preventable disease and death (CDC, 2004).

As stated previously, obesity is a multifactorial disease and its etiology is complex. However, a reduced ability to oxidize fats for fuel and a propensity to store them in adipose tissue and skeletal muscle are major characteristics of obesity. During fasting conditions, obese skeletal muscle esterifies more fatty acids and has lower fatty acid oxidation compared to lean people, which could lead to accumulation of triglycerides in muscle cells (Kelley et al., 1999).

Hyperinsulinemia, insulin resistance, Type II diabetes mellitus, and hypertension are associated with and may result from obesity. Over 80% of diabetics are either obese or hypertensive. Also, approximately 80% of obese subjects are insulin resistant and hypertensive (Bassett, 1994). Therefore, obesity has the potential to be partly responsible for these pathological conditions. "Syndrome X" is the term given to the cluster of risk factors for coronary artery disease that are usually found in obesity. They consist of insulin resistance, hypertension, hyperinsulinemia, glucose intolerance, high triglyceride level, and decreased high-density lipoprotein cholesterol level (Reaven, 1988). The etiology of Syndrome X is still unknown because of the

complex and multifactorial nature of the factors involved. There is considerable debate over which factor or factors cause the others. Some contend that obesity-induced insulin resistance results in hyperinsulinemia and ultimately hypertension, while some argue that the changes in skeletal muscle structure due to essential hypertension induce insulin resistance (Julius et al., 1992). Before causality can be established, more research is required to elucidate the underlying mechanisms of the risk factors. However, this will be difficult due to numerous confounding variables that may be impossible to eliminate.

Hyperinsulinemia is defined as supraphysiological levels of insulin released in the body in an attempt to regulate blood glucose level. Hyperinsulinemia can ultimately lead to insulin resistance, or the condition in which abnormally high physiological levels of insulin are chronically needed to maintain glucose homeostasis. Insulin resistance can lead to Type II diabetes mellitus in susceptible people. Approximately 15.7 million Americans have diabetes mellitus, and it is estimated that 798,000 new cases are diagnosed in the United States every year (NIDDK, 1999). Ninety to ninety-five percent of these cases are Type II diabetes, formerly known as adult-onset diabetes mellitus or non-insulin dependent diabetes mellitus (NIDDM) (NIDDK, 1999). Impaired fasting glucose, clinically defined as a fasting glucose level of 110 to 125 mg/dL, affects an estimated 13.4 million Americans and can eventually lead to diabetes (NIDDK, 1999).

Skeletal Muscle Fiber-type and Obesity

Skeletal muscle is the largest single tissue mass in the human body comprising about 40% of total body weight (Tikkanen et al., 1996). According to DeFronzo et al. (1981), skeletal muscle disposes of more than 80% of glucose during hyperinsulinemic conditions of a euglycemic clamp. Skeletal muscle is the primary site of insulin-mediated glucose uptake and, thus, is considered the primary site of insulin resistance (Hernandez et al., 2001; Lillioja et al., 1987). There are three types of skeletal muscle fibers: slow-twitch, oxidative Type I fibers; fast-twitch, oxidative-glycolytic Type IIa fibers; and fast-twitch, glycolytic Type IIb fibers (Saltin, 1983). Slow-twitch Type I fibers have the highest oxidative capacity because they have the highest number of mitochondria, oxidative enzymes, and capillaries associated with them. Type IIa fibers have fewer capillaries than Type I fibers and are less oxidative, and Type IIb fibers have the lowest oxidative capacity (Saltin, 1983). Muscle fiber composition can vary considerably between different muscles and individuals. For example, the most commonly used muscle in biopsy studies, the vastus lateralis, can have as high as 96% Type I oxidative fibers or as low as 13% with the average being 50% (Saltin, 1983).

The percentage of Type I fibers is thought to be genetically predetermined, however, Type IIb glycolytic fibers may convert to more oxidative Type IIa fibers with exercise (Saltin, 1983). Since Type IIb fibers have much lower oxidative capacity than Type I fibers, high percentages of Type IIb fibers and/or low percentages in Type I fibers have been considered as a possible etiology of obesity (Hickey et al., 1995;

Kriketos et al., 1997; Kriketos et al., 1996; Lillioja et al., 1987; Wade et al., 1990). Wade et al. studied muscle fiber composition from the vastus lateralis of eleven healthy, sedentary men and found a significant inverse relationship ($r = -0.677$, $P < 0.05$) between the percentage of Type I fibers and percent body fat as determined by the skinfold method (Wade et al., 1990). Lillioja et al. studied skeletal muscle fiber type, capillary density, and *in vivo* insulin action in 23 Caucasian and 41 Pima Indian nondiabetic men. Waist-to-thigh ratio, used as a measure of body fat distribution, moderately correlated with percent Type IIb fibers ($r = 0.40$, $P < 0.002$) (Lillioja et al., 1987). Hickey et al. studied rectus abdominus muscle in 12 nondiabetic control subjects, 12 obese nondiabetics, and 10 obese non-insulin dependent Type II diabetics. They found a significant negative correlation, ($r = -0.50$, $P < 0.01$), between percent Type I oxidative fibers and BMI (Hickey et al., 1995). These data support the hypothesis that reductions in Type I fibers and relative increases in Type IIb fibers contribute to the inability to oxidize fats and may predispose one to obesity.

Skeletal Muscle Capillarization and Obesity

Capillaries are the smallest vessels of the vascular system that connect the arterial and venous systems. They are composed of a single layer of endothelial cells and a basement membrane. In the body, capillaries create a total surface area of about 1,000 m^2, roughly equal to the size of a tennis court (Pugsley & Tabrizchi, 2000). This large surface area is important in the diffusion of substrates and nutrients. Movement of nutrients, substrates, and water from the blood into surrounding tissues is usually

achieved through simple diffusion due to concentration gradients and hydrostatic and osmotic pressures (Pugsley & Tabrizchi, 2000). Substrates may then enter the mitochondria where they can be used to make energy to power the biological processes of life.

Capillarization is related to skeletal muscle fiber composition and generally exists on a continuum. As previously stated, there are more capillaries around slow-twitch, oxidative Type I muscle fibers, fewer around fast-twitch, oxidative-glycolytic Type IIa fibers, and the fewest number of capillaries surrounding fast-twitch, glycolytic Type IIb fibers (Saltin, 1983). Increased capillarization enhances substrate and nutrient diffusion into cells by allowing more surface area and more capillary-to-fiber contact. The barrier offering most resistance for diffusion of substrates from capillaries to mitochondria is considered to be the sarcolemma (Vock et al., 1996). Increased capillarization enhances substrate flux by increasing the available surface area for diffusion and by decreasing the diffusion distance between the capillary and the sarcolemma (Ivy, 1997).

Increased capillarization is a positive adaptation that results from endurance exercise training and enhances glucose and insulin delivery from the blood to the mitochondria (Ivy, 1997). Conversely, decreased capillarization, also known as capillary rarefaction, is an undesirable phenomenon exhibited in several pathological states such as hypertension, insulin resistance, diabetes, and obesity (Isaksson et al., 1993; Julius et al., 1992; Lillioja et al., 1987; Serne et al., 1999). The loss of functional

capillaries reduces the total capillary surface area and capillary-to-fiber contacts which increases diffusional distances and increases the resistance to substrate and oxygen diffusion from the blood to the mitochondria. The cause of capillary rarefaction is controversial, primarily because rarefaction is seen in conditions such as hypertension, obesity, and diabetes that often exist concomitantly. It is plausible that hypertension and insulin resistance in obesity result from increased diffusion distances due to capillary rarefaction; however, these relationships have yet to be proven (Serne et al., 1999). Insulin resistance in obesity, hypertension, and Type II diabetes reduces the vasodilatory effects of insulin such that skeletal muscle blood flow is not increased. Capillary rarefaction associated with insulin resistance impairs the delivery of insulin, other hormones, and substrates to the tissues (Pinkney et al., 1997).

Although capillarization is muscle fiber type dependent, increases in the number of capillaries around all fibers can be achieved through exercise training (Hepple et al., 1997). Capillarization has been shown to better correlate with insulin sensitivity than the presence of any specific muscle fiber type (Lillioja et al., 1987). While the percentage of oxidative Type I fibers has been shown to moderately correlate with insulin sensitivity (Hickey et al., 1995; Lillioja et al., 1987), measures of capillarization tend to better correlate to insulin sensitivity. Lillioja et al. used the euglycemic clamp method for measuring insulin sensitivity in 23 Caucasians and 41 Pima Indian nondiabetic men and found correlations with percent Type I fibers ($r = 0.29$, $P < 0.02$) and with capillary density ($r = 0.63$, $P \leq 0.0001$) (Lillioja et al., 1987).

Both capillarization and insulin sensitivity are known to increase with endurance exercise training (Ivy, 1997). These data demonstrate an important association between the skeletal muscle capillarization and homeostatic glucose maintenance.

Angiogenesis and Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is a required process for normal organ development and differentiation in embryogenesis, wound healing, and reproductive functions (Ferrara & Davis-Smyth, 1997). Angiogenesis is thought to be a rare natural occurrence in the adult, but it is known to occur in response to endurance exercise training (Gustafsson & Kraus, 2001). Although the regulation of angiogenesis is not fully known, current data suggest that angiogenesis is triggered by diffusible growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor-β_1 (TGF- β_1), and vascular endothelial growth factor (VEGF) (Gustafsson & Kraus, 2001). However, VEGF is considered the most putative growth factor for new capillaries because it is "potent, diffusible, and specific for vascular endothelial cells" (Ferrara, 1999).

VEGF is a 45-kDa heparin-binding homodimeric glycoprotein (Ferrara & Henzel, 1989). $VEGF_{165}$ is the predominant molecular species, but other isoforms, such as $VEGF_{121}$, $VEGF_{189}$, and $VEGF_{206}$, can be produced via alternative exon splicing of a single VEGF gene (Houck et al., 1991).

VEGF regulation in skeletal muscle is not currently fully understood. However, several factors have been shown to regulate exercise-induced VEGF expression. A single bout of hypoxic exercise resulted in a greater increase in VEGF

mRNA than normoxic exercise in rats (Breen et al., 1996). The VEGF increase observed during exercise is thought to be regulated partly by hypoxia-inducible factor-1 beta (HIF-1β). Gustafsson et al. (1999) used a single-leg knee extension exercise model under conditions of nonrestricted and restricted blood flow to look at exercise-induced expression of VEGF, FGF-2, and HIF-1 mRNA. The 15 to 20% decrease in blood flow in the restricted condition was used to observe changes in the growth factors during different degrees of oxygen delivery. The main findings of the study revealed that a single 45-minute bout of human knee extensor exercise increased VEGF mRNA by 178% when the two conditions are pooled. They also found strong positive correlations between the exercise-induced changes of VEGF mRNA and HIF-1α and HIF-1β mRNA; however, no significant differences between nonrestricted and restricted blood flow were found (Gustafsson et al., 1999). No significant differences were found in FGF-2 mRNA due to exercise (Gustafsson et al., 1999). Reduced oxygen tension, which results in an increase in HIF-1β and VEGF mRNA, could be the necessary stimulus resulting from exercise that causes increases in VEGF gene expression (Gustafsson & Kraus, 2001).

Richardson et al. (1999) also used a 30-minute bout of single-leg knee extension in humans during normoxic and hypoxic conditions to look at the role intracellular P_{O_2} plays in exercise-induced growth factor expression. They found that VEGF mRNA significantly increased over resting values after a single bout of knee-extensor exercise in normoxia and hypoxia; however, the normoxic and hypoxic

exercise values were not statistically different from each other (Richardson et al., 1999). Richardson et al. suggested the existence of an intracellular P_{O_2} threshold such that P_{O_2} values below this threshold would not result in a greater angiogenic stimulus. They believed that the level of hypoxia used in their study (~ 12% oxygen) was below this threshold resulting in no further increase (Richardson et al., 1999).

A recent study by Amaral et al. (2001) illustrates the importance of VEGF in exercise-induced angiogenesis. The investigators injected three doses of monoclonal VEGF-neutralizing antibody (0.6 mg/100 g) into 12 rats intraperitoneally 1 day before and the following 2 days of the exercise protocol to block endogenous VEGF. Exercising animals ran on a treadmill at 20 m/min, 5% grade, for 1 h/day for 3 consecutive days. After the last training period, the animals were anesthetized within 2 hours of exercising and the hindlimb muscles were removed and used to determine vessel density. The increase in the microvessel densities of the tibialis anterior and the gastrocnemius muscles due to 3 days of exercise training was completely blocked by the VEGF-neutralizing antibody (Amaral et al., 2001). The inhibition of exercise-induced angiogenesis as a result of VEGF inhibition demonstrates the importance of VEGF in the angiogenic adaptive response to short-term exercise.

Glucose regulation influences VEGF expression as varying levels of glucose and insulin have been shown to regulate VEGF expression *in vivo* and *in vitro*. Hyperinsulinemia (Bermont et al., 2001), hyperglycemia (Goalstone et al., 1998; Natarajan et al., 1997; Sone et al., 1996), and hypoglycemia (Dantz et al., 2002; Sone

et al., 1996) have all been shown to increase VEGF expression. Obesity is an insulin-resistant state and hyperinsulinemia ultimately results from increased insulin secretion in an attempt to maintain glucose homeostasis (Reaven, 1988). If glucose homeostasis cannot be maintained through endogenous hyperinsulinemia, hyperglycemia and diabetes mellitus will ensue (Reaven, 1988). Goalstone et al. (1998) found increased VEGF expression as a result of 24-hour preincubation of rat vascular smooth muscle cells in hyperinsulinemic medium (10 nM) (Goalstone et al., 1998). Bermont et al. (2001) also found increases in VEGF mRNA and protein in endometrial adenocarcinoma cells after 24-hour incubation in hyperinsulinemic (10 nM) medium (Bermont et al., 2001).

Diabetics and subjects with impaired fasting glucose are hyperglycemic, which may potentially affect VEGF expression. Natarajan et al. (1997) demonstrated that chronic exposure of human and porcine vascular smooth muscle cells to hyperglycemia (25 mM) increased VEGF mRNA and protein (Natarajan et al., 1997). Sone et al. (1996) observed the effects of high glucose (16.5 mM) on cultured bovine retinal pigmented epithelial cells (BRPE) after 1, 3, and 10 days of incubation. VEGF protein concentration was significantly higher in the high glucose group than the control group (5 mM) after 10 days of incubation (231.0 ± 33.0 ng/g and 173.4 ± 36.7 ng/g, respectively, $P < 0.01$) (Sone et al., 1996).

Hypoglycemia has also been shown to upregulate VEGF. For example, Sone et al. (1996) also looked at VEGF expression in BRPE after an acute hypoglycemic event

in BRPE cells. After a 10-day adaptation period in a medium at a normal physiological glucose level (5 mM), the glucose concentration was reduced to either 2.75 mM or 0.5 mM and the cells incubated in this low glucose medium for 48 hours. VEGF concentrations were significantly higher in the 2.75 mM and 0.5 mM groups than the control group at 5.5 mM (258.7 ± 41.5, 249.1 ± 46.9, and 164.8 ± 24.1, respectively, $P < 0.01$) (Sone et al., 1996).

Hypoglycemia has been shown to upregulate VEGF expression *in vivo*. Dantz et al. (2002) used a hypoglycemic clamp on 16 lean, young, healthy men to investigate the effects of low blood glucose on VEGF, several other hormones, and alteration in cognitive function. Serum VEGF levels increased from 86.1 ± 13.4 to 211.6 ± 40.8 pg/mL ($P = 0.002$) from baseline to the end of the clamp (Dantz et al., 2002). The researchers also used a hyperinsulinemic euglycemic clamp to determine the effects of insulin on VEGF. In contrast to other findings previously mentioned, the researchers did not find an increase in serum VEGF due to increased insulin levels. They suggested that the higher insulin levels did not sufficiently stimulate VEGF production in the periphery. They postulated that the source of the observed increase in serum VEGF was the hypothalamus-pituitary system, which responded as a neuroprotective mechanism to preserve cognitive function because the brain was hypoglycemic in the technique. The researchers did not believe that the increased serum VEGF originated from the skeletal muscle because it was not hypoglycemic in their study (Dantz et al., 2002).

To our knowledge, no investigation has been performed to determine if the capillary rarefaction commonly seen in obesity is related to VEGF mRNA levels at rest or after an acute exercise bout. However, unpublished data from a study by Gavin et al. suggests that the maximum VEGF mRNA level after a single, one-hour bout of cycle ergometry at 50% $\dot{V}O_2MAX$ in young sedentary males is negatively correlated with percent body fat ($r = -0.66$, $P = 0.07$, unpublished data) (Gavin, Robinson et al., 2004). Thus, we hypothesize that obese subjects will have lower resting and exercise-induced VEGF mRNA levels. If VEGF is lower in obese subjects at rest and after exercise, this may signify a lower capacity to promote new capillary formation and could further contribute to their inability to move substrates and hormones into skeletal muscle.

METHODOLOGY

Experimental Design

Prior to testing, the Institutional Review Board at East Carolina University reviewed the methodology of the study and granted approval to use human subjects. Potential subjects were asked to describe their activity levels to determine their eligibility for the study. If body mass index and sedentary status criteria were met, potential subjects were given the choice to participate. Each subject carefully read and signed an informed consent form explaining the risks and benefits associated with the muscle biopsy, hydrostatic weighing, maximal aerobic capacity test, and exercise procedures involved. They were given the opportunity to ask questions about the protocol.

Subjects

The purpose of this study was to determine if resting VEGF mRNA and the VEGF mRNA response to acute systemic exercise are lower in obese compared to lean subjects. Eight sedentary lean (BMI $\leq$ 25 kg/m^2; LN) and eight sedentary obese (BMI $\geq$ 30 kg/m^2; OB) young males participated in this study. Subjects were considered sedentary if they had not participated in more than thirty minutes of physical activity for more than three times per week over the previous three months. None of the subjects who participated in the study were smokers, diabetics, or had known cardiovascular disease.

Consenting subjects reported to the Human Performance Laboratory on one occasion for fasting blood draw, anthropometric measurements (height and weight), hydrostatic weighing, and determination of maximal oxygen consumption ($\dot{V}O_2MAX$). Two vials of fasting blood were drawn and centrifuged for 10 minutes. The supernatant was removed from the vials and placed into 2 cryogenic vials and stored at -80°C. A YSI 2300 STAT PLUS Glucose and Lactate Analyzer (Yellow Springs Instrument, Yellow Springs, OH) was used to determine fasting glucose and lactate. Fasting insulin was analyzed by a Beckman-Coulter Access® 2 Immunoassay system. All samples were analyzed on the same day.

Hydrostatic densitometry

The hydrostatic weighing procedure was performed to determine percent body fat. Prior to the $\dot{V}O_2MAX$ test, subjects were instructed to sit in a chair attached to a scale in a tank of water, and then maximally expire all of the air in their lungs while completely submerged. A technician read the scale to obtain the subject's underwater weight after the subject had completely emptied their lungs of air. This process was repeated at least six times so that six values are obtained within 100 grams of each other. The chair weight, water temperature, the subject's dry weight, average underwater weight, and residual volume were recorded for later determination of body density and percent body fat via computer software (BodyComp32, version 2.1.17).

Residual volume was measured using gas dilution by nitrogen washout prior to the hydrostatic weighing procedure and the $\dot{V}O_2MAX$ test (Wilmore et al., 1980). A 5-L

bag was filled with 100% oxygen and the subject wore nose clips to prevent breathing through the nose. The subject was instructed to form a tight seal around the mouthpiece on the bag to avoid air leakage from the sides of the mouth. A valve on the mouthpiece allowed airflow either through the mouthpiece to ambient air or into the bag of oxygen. The subject was instructed to inhale and exhale two normal breaths of room air, and then inhale a third time before maximal expiration. They were asked to raise their hand when they could get no more air out. The valve was then turned so that subjects were breathing 100% oxygen from the bag. The subject inhaled and exhaled slowly eight times to allow adequate mixing of the air in the bag with the air in their lungs. On the ninth breath, the subject expired maximally to get as much of the air in their lungs into the bag, and then they raised their hand to signal that they have done so. The valve was turned so that the bag was closed. An oxygen and carbon dioxide analyzer, (AEI Technologies), was used to determine the percentages of oxygen and carbon dioxide in the sample.

Body density was determined from the subject's residual volume, dry weight, suit weight, average underwater weight, water temperature, and weight of the chair. These values were entered into a computer program (BodyComp32, version 2.1.17) and body density and percent body fat were determined. For the calculation of body density in Caucasians and Asian Americans, the Siri formula was used (Siri, 1961), while the Schutte equation was used (Schutte et al., 1984) for African-Americans.

Maximal Oxygen Consumption

An incremental exercise test to exhaustion was performed on a Lode™ electrically-braked cycle ergometer to determine $\dot{V}O_2MAX$. The subject wore headgear and a mouthpiece connected to a TrueMax 2400 metabolic measurement system (Parvo Medics, Salt Lake City, Utah). The TrueMax 2400 was calibrated according to the manufacturer's guidelines. All subjects used the same protocol, which started at a workload of 75 watts for the first five minutes and then increased 25 watts every minute thereafter until the subject reached exhaustion and could not continue. Blood pressure and rating of perceived exertion (RPE) were monitored and recorded throughout the test. Heart rate was recorded every minute (Polar Heart Rate Monitor™). Gases were continuously measured and their $\dot{V}O_2MAX$ was averaged and recorded every twenty seconds throughout the maximal test. The criteria for a maximal test was reached if three of the following four markers were achieved: a respiratory exchange ratio (RER) value greater than or equal to 1.10, the subject's maximal heart rate within ± 10 bpm of his age-predicted maximum heart rate, the subject's rating of perceived exertion (RPE) exceeded 17 on the Borg scale (6-20), and the subject's $\dot{V}O_2$ did not increase 150 mL/min with an increase in workload. The subject's $\dot{V}O_2MAX$ and maximum workload achieved were recorded.

Muscle biopsies and 50% $\dot{V}O_2MAX$ exercise

Subjects reported to The Brody School of Medicine on a second occasion no earlier than three days after their $\dot{V}O_2MAX$ test. Subjects were instructed to fast for 10

hours the night before the experiment and were given a Slim-Fast shake to consume between 30 and 60 minutes prior to arrival at Brody. This allowed subjects a standardized breakfast to have energy for the exercise bout while not confounding the study. Prior to exercise, a single muscle biopsy of the vastus lateralis was performed (Bergstrom, 1962). A resting blood draw was performed along with the resting muscle biopsy to measure glucose, insulin, and lactate before the experiment. Subjects exercised on a Lode cycle ergometer for one hour at 50% of $\dot{V}O_2MAX$ as determined during the maximal oxygen consumption test. An exercise blood draw to measure glucose, insulin, and lactate after exercise was taken from the subject while on the bicycle ergometer two minutes before cessation of exercise. A second muscle biopsy of the vastus lateralis of the leg opposite to the pre-exercise biopsy was performed 2 hours after completion of exercise.

Muscle biopsy procedure

The site of the biopsy was determined; the area was clean-shaven, and sterilized with povidone-iodine. A sterile fenestrated drape was placed over the biopsy site. Ethyl chloride (Gebauer Pharmaceutical Preparations, Cleveland, OH) was sprayed onto the site to numb the skin before injecting 5 cc of 1% lidocaine subcutaneously (Abbott Laboratories, Chicago, IL). A 0.5-1.0 cm incision was made into the skin and the fascia of the muscle using a #11 sterile stainless steel surgical blade. A sterilized biopsy needle was inserted through the incision and a small piece of muscle was obtained. Slight pressure was applied on the site for ten minutes without ice and then

ten minutes with an ice pack or until the bleeding ceased. The site was cleaned and closed with a Steri-Strip and Band-Aids, and a pressure-wrap was applied.

A 10 to 20 mg piece of muscle was mounted in an OCT-tragacanth gum mixture, frozen in liquid nitrogen-cooled isopentane, and stored at -80°C. The remainder of the muscle sample was used in Northern blot analysis to determine levels of VEGF mRNA.

Capillary staining procedure

The Rosenblatt capillary staining procedure (Rosenblatt et al., 1987) was utilized to simultaneously determine capillarization and fiber type in the muscle samples. The muscle sample was placed in the cryostat at -20°C one hour prior to sectioning. Ten μm sections were cut and placed on slides and stored in a Columbia jar inside the cryostat. The Columbia jar was placed in the 4°C refrigerator for 30 minutes prior to beginning the staining process. The jar was allowed to sit at room temperature for 2 minutes before adding the Guth and Samaha fixative. One hundred mL of fixative consisted of 86 mL of distilled water, 2.305 g of sodium cacodylate, 0.9998 g of calcium chloride, 11.501 g of sucrose, and 13 mL of 37% formaldehyde. The pH was adjusted to 7.60 and the temperature was maintained between 18 and 19.5°C to ensure adequate staining. The slides were fixed for 5 minutes at room temperature with the Guth and Samaha fixative and then rinsed 15 times with distilled water, taking care not to disrupt the muscle sections on the slides. The slides were added to an incubation medium at 37°C for 60 minutes. Seventy-two mL of incubation medium

consisted of 0.450 g of Gelatin, 30 mL of 0.1 M Tris-maleic acid buffer, 30 mL of distilled water, 4.50 mL of 2% lead nitrate, and 7.5 mL of 1% $CaCl_2$. The pH of the incubation medium was adjusted to 7.20 prior to adding the 1% $CaCl_2$. The solution was heated to 37°C and 37.5 mg of ATP (Sigma 2383) was added. After a 60-minute incubation period in the 37°C water bath, the slides were rinsed 15 times with distilled water. The slides developed in a 2% ammonium sulfide solution for 2 minutes in the fume hood. After 2 minutes, the slides were rinsed 15 times with distilled water and allowed to air dry. Permount and coverslips were used to protect the samples.

Quantification of fiber type and capillarization

After the slides were dry, they were viewed under a microscope and a digital image of the muscle section was taken for quantification of capillarization. Type I muscle fibers stain dark, while Type II fibers stain light. The viewing area of the digital images was calibrated (SigmaScan 5.0) to convert the number of pixels in the image to μm for the determination of muscle fiber area (FA) and perimeter (P). Manual quantification of capillary number was performed on each fiber to estimate capillary contacts, (CC, the number of capillaries touching an individual muscle fiber), capillary-to-fiber ratio on an individual fiber basis ($C:F_i$), and sharing factor, (SF, the number of fibers sharing each capillary). Capillary density (CD) was measured on an individual fiber basis calculated with the fiber as reference. The CFPE index was calculated using the following equation:

$$\text{CFPE index} = (C:F_i)/P \quad (1)$$

$C:F_i$ was computed by the summation of each CC divided by its SF. For example, an individual muscle fiber having 5 CC with SF of 3 and 1 CC with SF of 2 would yield the following: $C:F_i = (5/3) + (1/2) = 2.17$ (Hepple, 1997). The resulting value was then divided by the perimeter of the individual fiber in question to yield that fiber's CFPE index. This measurement of capillarity has been shown to better correlate with changes in oxygen consumption and, according to Hepple, it "allows quantitation of the capillary supply relative to the region of greatest resistance to oxygen flux, namely the capillary-to-fiber surface" (Hepple et al., 1997).

Northern blot analysis

VEGF mRNA was measured using quantitative Northern blot analysis. The total cellular RNA from the vastus lateralis muscle samples was isolated using TRIzol® reagent (Life Technologies, Inc.). The tissue samples were placed in a basket in a container of liquid nitrogen. The tissue homogenizer was cleaned two times each with bleach, ethanol, distilled water, and TRIzol®. Approximately 50 mg of tissue was placed in the homogenizer along with 1 mL of TRIzol®. The actual weight of the tissue was recorded. The homogenizer was used to grind the tissue samples into solution by alternating 3 times between 30 seconds of grinding at medium speed and 30 seconds of cooling on ice. TRIzol® was used to clean the homogenizer between samples to avoid sample contamination. The homogenized solution was decanted into a fresh microcentrifuge tube and clearly labeled. The solution was allowed to come to room temperature for five minutes before 200 μL of Chloroform was added. The tube

was inverted several times before incubation for 3 minutes at room temperature and microcentrifuged for 10 minutes. The clear supernatant was decanted into a fresh microcentrifuge tube and 700 μL of isopropanol was added. The tube was vortexed, incubated at room temperature for 10 minutes, and then centrifuged for 10 minutes at 4°C. The isopropanol was removed from the tube without disturbing the pellet. The pellet was washed with 500 μL of 75% ethanol diluted with DEPC-treated water by vortexing and microcentrifuging for 5 minutes so that the pellet was dislodged. Excess ethanol was carefully removed and the pellet was allowed to dry for 5 minutes before being resuspended in 100 μL of DEPC-treated water, followed by incubation at 65°C for 5 minutes. Ten μL of the RNA isolate was added to 990 μL of DEPC-treated water for spectroscopy at 260 nanometers. DEPC-treated water was used as a blank for spectroscopy and to clean between samples. RNA preparations were quantitated by absorbance at 260 nm, and intactness was assessed by ethidium bromide staining after separation by electrophoresis in 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to Zeta probe membrane. RNA was cross-linked to the membrane by ultraviolet irradiation for 1 min and stored at 4°C. The blots were probed with oligolabeled [α-^{32}P] deoxycytidine triphosphate cDNA for VEGF. The human VEGF probe is a 0.93-kb cDNA fragment isolated from the *EcoR* I site of pUC-derived plasmid. Prehybridization and hybridizations were performed in 50% formamide, 5 x SSC (20 x SSC is 0.3 M sodium chloride, 0.3 M sodium citrate), 10 x Denhardt's solution (100 x Denhardt's solution is 2% Ficoll, 2% polyvinyl

pyrrolidone), 50 mM sodium phosphate (pH 6.5), 1% sodium dodecyl sulfate (SDS), and 250 μg/ml salmon sperm DNA at 42°C. Blots were washed with 2 x SSC and 0.1% SDS at room temperature and 0.1 x SSC and 0.1 x SDS at 65°C for the VEGF mRNA. Blots were exposed to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) by use of a Cronex Lightning Plus screen at -80°C. Autoradiographs were quantitated by densitometry (Gel Pro Analyzer) within the linear range of signals and normalized to β-actin mRNA levels.

Statistical Analysis

A two-way (group × exercise level) analysis of variance (ANOVA) was used to determine differences in VEGF mRNA. Bonferroni's test was used to determine significance between conditions. Student's *t*-test was used to test for significant differences between group means of morphometric and anthropometric measurements. Pearson product moment correlations were utilized to determine relationships between variables. Significance was established at $P \leq 0.05$ for all statistics. Multivariate regression analysis was performed to determine which variable contributed most to the degree of obesity, (as determined by BMI and percent body fat), between capillarization, muscle fiber composition, and resting and exercise-induced VEGF mRNA levels.

RESULTS

Subject Characteristics

Mean BMI for LN and OB was 22.38 ± 1.15 and 36.13 ± 1.99 $kg \cdot m^{-2}$, respectively (Table 1). Age and height were not different between LN and OB (23.25 ± 1.35 versus 21.50 ± 0.57 years; 1.75 ± 0.02 versus 1.80 ± 0.03 m, respectively). As expected, LN had significantly lower weights and body fat percentages as determined by hydrostatic densitometry than OB (68.75 ± 3.24 versus 117.02 ± 8.11 kg; 17.26 ± 1.56 versus 31.40 ± 3.02 percent fat, respectively).

Fat free mass (FFM), as determined from hydrostatic densitometry, was significantly lower in LN (LN: 56.52 ± 2.18 kg; OB: 79.49 ± 3.68 kg, respectively). Mean Waist-to-Hip Ratio (WHR), a commonly used index of abdominal adipose distribution, was not significantly different between LN and OB (0.83 ± 0.02 versus 0.87 ± 0.03, respectively); however, the individual waist and hip circumferences (measured in centimeters) were significantly different between groups. Average maximum power output achieved on the bicycle ergometry test was not significantly different between LN (231.25 ± 11.01 Watts) and OB (256.25 ± 13.12 Watts).

VEGF mRNA

VEGF mRNA data at rest and in response to acute exercise are presented in Figure 1. VEGF mRNA was not different at rest between LN (1.00 ± 0.16 arbitrary units, or AU) and OB (0.75 ± 0.09 AU). Exercise-induced VEGF mRNA levels

increased significantly over resting levels within each group (LN: 10.69 ± 2.89 AU; OB: 8.23 ± 1.75 AU). There was no difference in the VEGF mRNA response to acute exercise between groups.

Glucose, Insulin, and HOMA

Obesity is associated with insulin resistance and obese individuals tend to have higher fasting values of glucose and insulin (Kriketos et al., 1996). Since VEGF has been shown to be regulated by changes in glucose and insulin, (Dantz et al., 2002; Natarajan, Bai, Lanting, Gonzales, & Nadler, 1997; Sone et al., 1996), we measured plasma glucose and insulin in an attempt to better explain any differences in VEGF we may have seen between the groups. Blood was drawn at three different timepoints for plasma extraction: after an overnight fast (fasting), immediately prior to the exercise bout (resting), and two minutes before the conclusion of the exercise bout (exercise; Table 2).

Average fasting glucose tended ($P = 0.057$) to be greater in OB (4.58 ± 0.17 mmol) than LN (5.16 ± 0.25 mmol). Fasting insulin values were significantly lower in LN (6.57 ± 2.61 $\mu U \cdot ml^{-1}$) than OB (14.62 ± 2.38 $\mu U \cdot ml^{-1}$; $P < 0.05$). HOMA was significantly lower in LN (1.24 ± 0.40) than OB (3.49 ± 0.68). Resting and exercise glucose values were not significantly different between the groups. Resting insulin values were significantly greater in OB than LN (OB: 26.31 ± 4.60 $\mu U \cdot ml^{-1}$; LN: 10.04 ± 2.60 $\mu U \cdot ml^{-1}$). Exercise insulin values were also significantly greater in OB than LN (LN: 3.24 ± 1.16 $\mu U \cdot ml^{-1}$; OB: 7.98 ± 1.98 $\mu U \cdot ml^{-1}$).

Muscle Morphology

Muscle fiber type percentage, fiber size, and capillarization values are presented in Table 3. OB had significantly larger muscle fiber area than LN (6693.55 ± 455.34 and 4621.83 ± 232.96 μm^2, respectively). Fiber perimeter was also significantly larger in OB than LN (345.68 ± 13.37 and 286.75 ± 8.08 microns, respectively). The number of capillaries per individual fiber, (C/F_i), was not significantly different between the groups when comparing fiber types individually or together. Overall capillary density was significantly lower in OB vastus lateralis (206.79 ± 19.21) than LN (265.24 ± 10.51). Capillary density was lower in obese skeletal muscle when Type I and Type II fibers were considered individually. The capillary-to-fiber-perimeter-exchange (CFPE) index, which is the quotient of C/F_i and perimeter, was not significantly different. Both CD ($r = 0.828$) and CFPE ($r = 0.733$) correlated highly with relative $\dot{V}O_2$ per fat free mass (ml $O_2 \cdot kg\ FFM^{-1} \cdot min^{-1}$) (Figure 2). Representative photomicrographs of fiber type and capillarization determination of lean and obese skeletal muscle can be found in Figure 3.

Subsequent to the completion of this study, VEGF protein and VEGF receptor mRNA and protein levels were determined from the biopsies of the vastus lateralis muscle in LN and OB subjects. No significant differences were found between groups in VEGF receptor Flt-1 (Figure 4) and KDR (Figure 5) mRNA levels at rest or after exercise; however, there was a main effect for exercise ($P<0.05$). No significant differences in VEGF (Figure 6a), KDR (Figure 6b), and Flt-1 (Figure 6c) protein levels

($pg \cdot \mu g^{-1}$) were found between the groups at rest or after acute exercise. The ratio of KDR mRNA to Flt-1 mRNA (Figure 6d) was not different between the groups before or after acute exercise. There was no main effect for exercise in any of the preceding variables (Gavin, Stallings et al., 2004)

Table 1

Subject Characteristics

	Lean (n=8)	Obese (n=8)
BMI	22.38 ± 1.07	36.13 ± 1.86 *
Weight (kg)	68.75 ± 3.24	117.02 ± 8.11 *
Body Fat (%)	17.26 ± 1.46	31.40 ± 2.82 *
Age (years)	23.25 ± 1.26	21.50 ± 0.54
Height (cm)	175.50 ± 2.13	179.63 ± 3.00
Maximum power output (Watts)	231.25 ± 10.30	256.25 ± 12.28
$\dot{V}O_{2MAX}$ ($L \cdot min^{-1}$)	2.74 ± 0.09	3.44 ± 0.19 *
$\dot{V}O_{2MAX}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	40.24 ± 1.51	29.65 ± 1.50 *
Fat Free Mass (kg)	56.52 ± 2.18	79.49 ± 3.68 *
$\dot{V}O_{2MAX}$ ($ml \cdot kg^{-1} \cdot FFM \cdot min^{-1}$)	48.56 ± 1.16	43.45 ± 2.16 †
Relative exercise load ($Watts \cdot kg^{-1} \cdot FFM^{-1}$)	2.05 ± 0.07	1.62 ± 0.07 *

Note: Mean ± standard error

* Significantly different from lean.

† $P = 0.055$

Table 2

Insulin, Glucose, and HOMA

	Lean (n=8)	Obese (n=8)
Fasting Glucose ($mg \cdot dL^{-1}$)	82.53 ± 2.82	92.94 ± 4.16
Fasting Insulin ($\mu U \cdot mL^{-1}$)	6.57 ± 2.44	14.62 ± 2.23 *
HOMA	1.24 ± 0.38	3.49 ± 0.63 *
Resting Glucose ($mg \cdot dL^{-1}$)	89.99 ± 3.85	102.44 ± 6.04
Resting Insulin ($\mu U \cdot mL^{-1}$)	10.04 ± 2.60	26.31 ± 4.60 *
Exercise Glucose ($mg \cdot dL^{-1}$)	85.38 ± 5.66	89.05 ± 5.83
Exercise Insulin ($\mu U \cdot mL^{-1}$)	3.24 ± 1.16	7.98 ± 1.98 *

Note: Mean ± standard error

* Significantly different from lean.

Table 3

Skeletal Muscle Characteristics

	Lean (n=8)	Obese (n=8)
Overall fiber area (μm^2)	4621.83 ± 232.96	6693.55 ± 455.34 *
Type I fiber area (μm^2)	4273.34 ± 332.31	5935.77 ± 532.01 *
Type II fiber area (μm^2)	4924.54 ± 273.38	7181.66 ± 426.21 *
Overall fiber perimeter (μm)	286.75 ± 8.08	345.68 ± 13.37 *
Type I fiber perimeter (μm)	275.83 ± 12.26	324.77 ± 16.06 *
Type II fiber perimeter (μm)	295.83 ± 7.71	359.21 ± 12.25 *
Overall capillary density (per mm^2)	265.24 ± 10.51	206.79 ± 19.21 *
Type I capillary density (per mm^2)	277.36 ± 13.86	217.01 ± 18.98 *
Type II capillary density (per mm^2)	252.33 ± 12.28	198.83 ± 18.94 *

Note: Mean ± standard error

- Significantly different from lean.

Table 3

Skeletal Muscle Characteristics (cont.)

	Lean (n=8)	Obese (n=8)
Overall capillary contacts	3.12 ± 0.14	3.43 ± 0.30
Type I capillary contacts	3.01 ± 0.21	3.18 ± 0.26
Type II capillary contacts	3.16 ± 0.13	3.59 ± 0.34
Overall individual capillary-to-fiber ratio	1.19 ± 0.06	1.31 ± 0.12
Type I C/F_i ratio	1.14 ± 0.09	1.21 ± 0.09
Type II C/F_i ratio	1.20 ± 0.05	1.38 ± 0.14
Overall CFPE (capillaries×1,000 μm^{-1})	4.12 ± 0.15	3.80 ± 0.32
Type I CFPE	4.08 ± 0.20	3.75 ± 0.27
Type II CFPE	4.06 ± 0.15	3.82 ± 0.36
Overall CFA (capillaries×10,000 μm^{-2})	2.58 ± 0.10	1.99 ± 0.18 *
Type I CFA	2.67 ± 0.12	2.10 ± 0.18 *
Type II CFA	2.47 ± 0.12	1.93 ± 0.18 *
Type I fibers (%)	38.81 ± 5.05	37.70 ± 2.96

Note: Mean ± standard error

* Significantly different from lean.

Figure 1

Representative Northern blot analysis (A) and quantitative densitometry (B) of the ratio of VEGF mRNA to β-actin mRNA in vastus lateralis muscle of lean and obese men before and 2 hr after the completion of a 1 hr of cycle ergometer exercise.

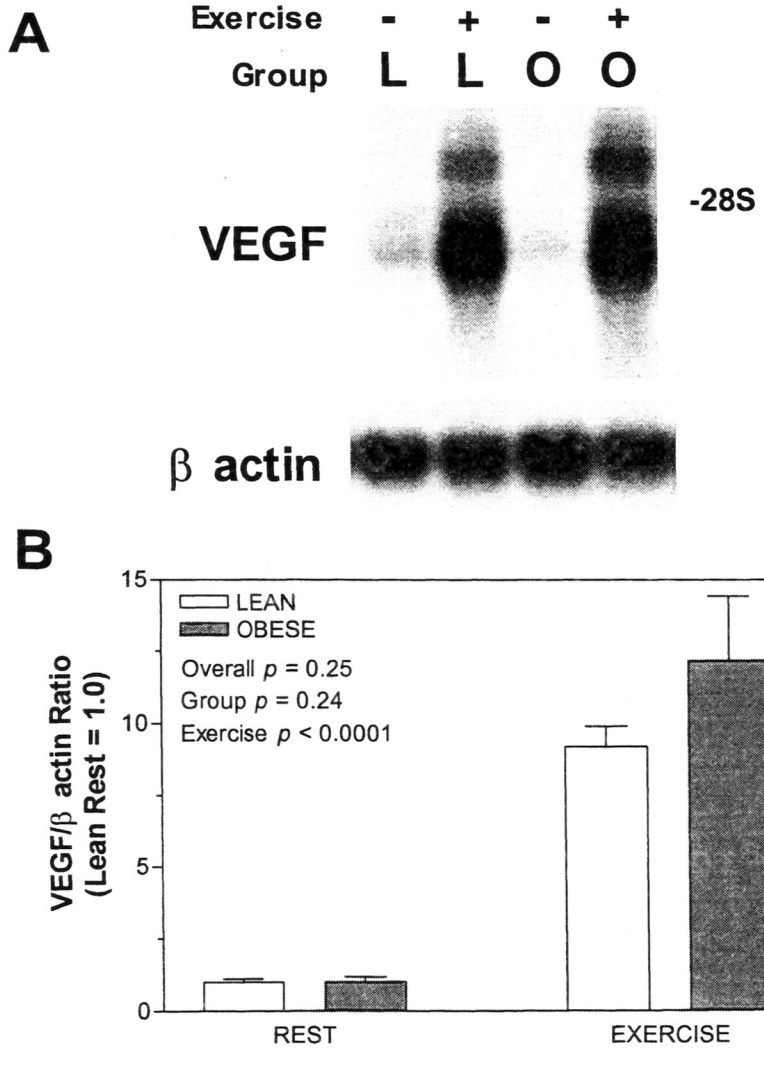


Figure 2

Linear regression between relative $\dot{V}O_{2MAX}$ per kg FFM and capillary density (A) and between relative $\dot{V}O_{2MAX}$ per kg FFM and CFPE (B). $N = 16$.

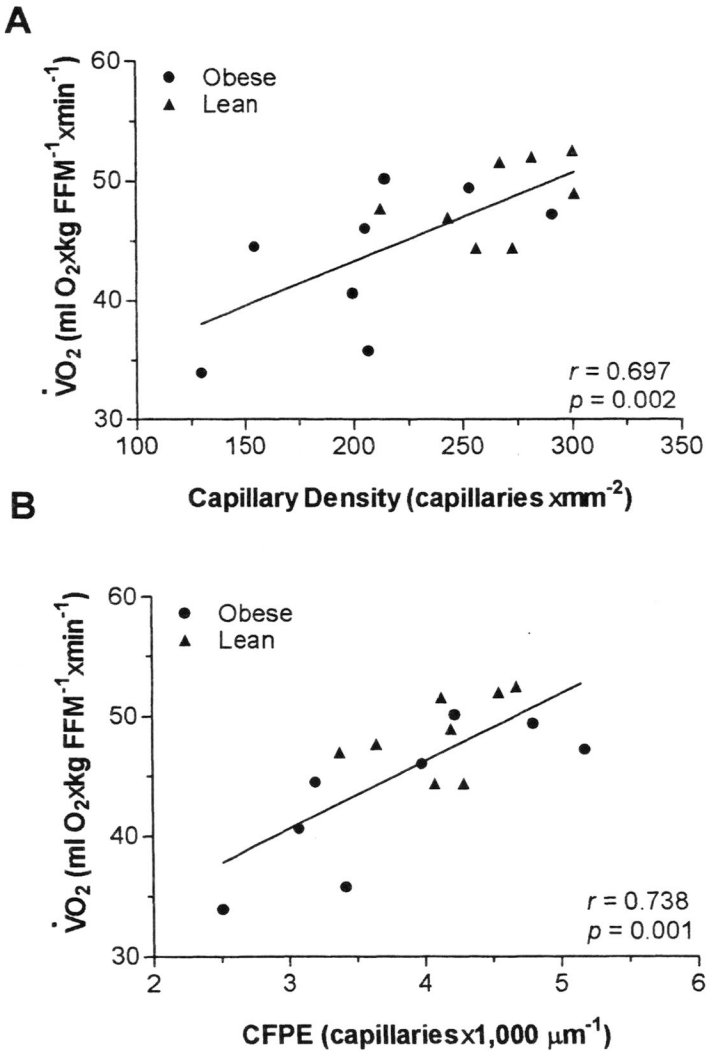
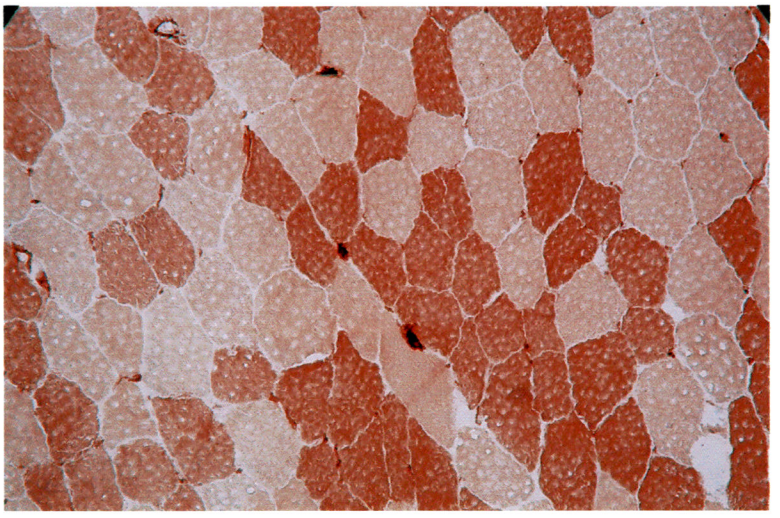


Figure 3

Rosenblatt stain for simultaneous determination of fiber type and capillarization in young lean (A) and obese (B) skeletal muscle. Capillaries are dark-stained regions between fibers. Type I fibers stain dark and Type II fibers stain light. Bar = 50 μm.

A.



B.

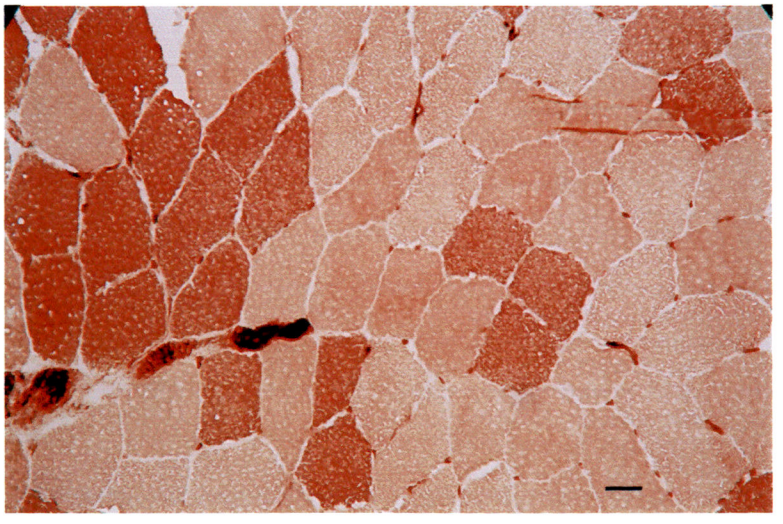


Figure 4

Representative Northern blot analysis (A) and quantitative densitometry (B) of the ratio of Flt-1 mRNA to β-actin mRNA in vastus lateralis muscle of lean and obese men before and 2 hr after the completion of a 1 hr of cycle ergometer exercise.

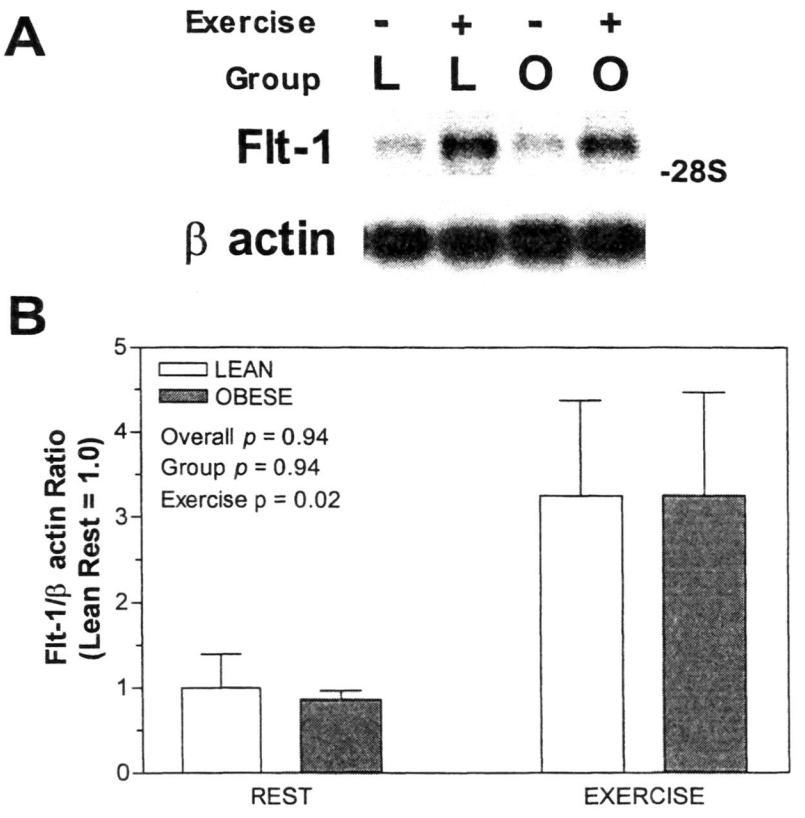


Figure 5

Representative Northern blot analysis (A) and quantitative densitometry (B) of the ratio of KDR mRNA to β-actin mRNA in vastus lateralis muscle of lean and obese men before and 2 hr after the completion of a 1 hr of cycle ergometer exercise.

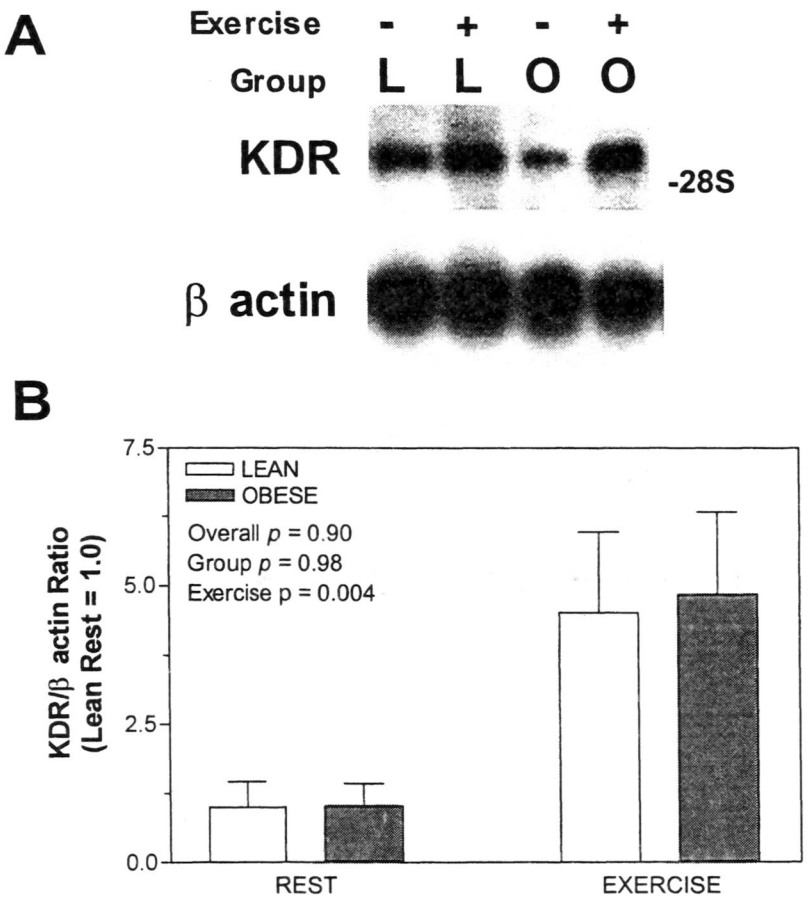
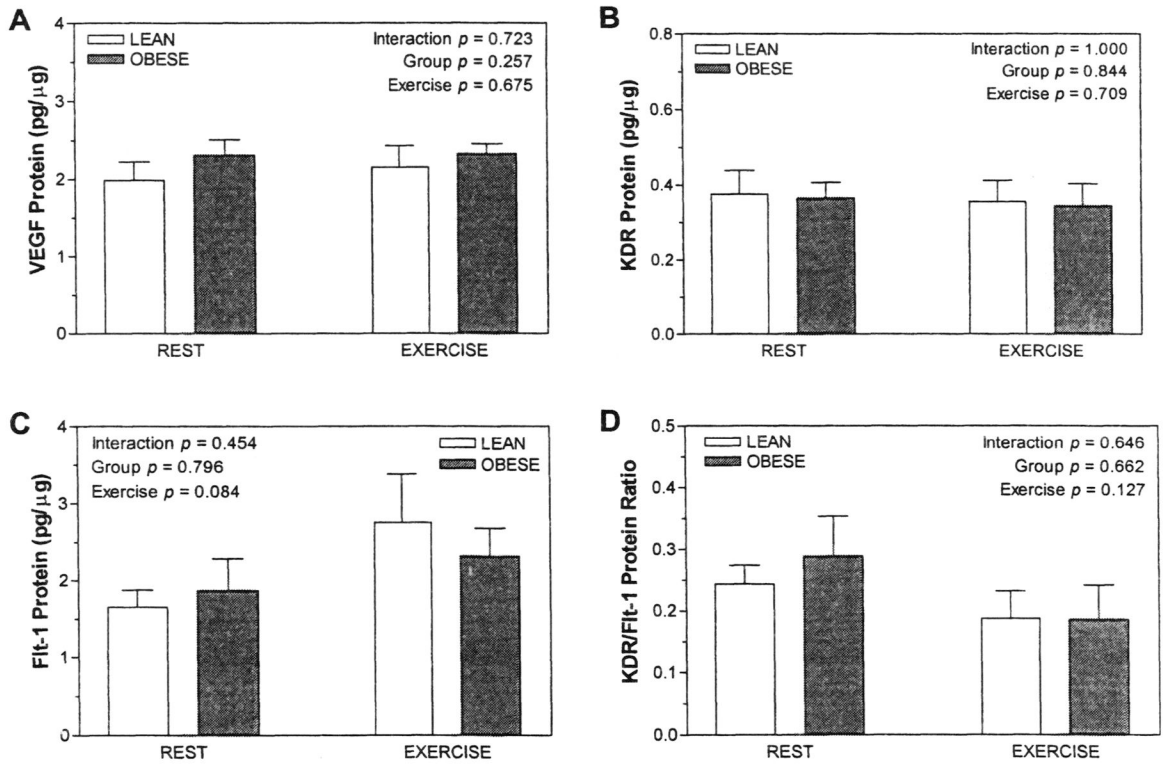


Figure 6

VEGF (A), KDR (B), Flt-1 (C), and KDR/Flt-1 ratio protein levels in obese and lean skeletal muscle before and 2hr after 1hr of cycle ergometer exercise.



DISCUSSION

The major findings in the current study are 1) resting VEGF mRNA is not different between young sedentary lean (LN) and obese (OB) men at rest or 2 hours after 1-hour of acute systemic bicycle exercise; 2) increases in exercise-induced VEGF mRNA are not different between LN and OB; 3) vastus lateralis muscle fiber area and perimeter are significantly greater in OB; 4) capillary density is lower in OB; and 5) insulin and HOMA at rest and during exercise are significantly greater in OB.

An earlier study from our lab established the time course of the exercise-induced increase in VEGF mRNA in humans in response to acute systemic exercise (Gavin, Robinson et al., 2004). The vastus lateralis of young sedentary males was biopsied before, and at 0, 2, and 4 hours after a 1-hour exercise bout at 50% VO_2max on a bicycle ergometer. Compared to resting VEGF mRNA, VEGF mRNA was significantly increased at 2 and 4 hours post-exercise (Gavin, Robinson et al., 2004). Although not significant, percent body fat negatively correlated with maximum VEGF mRNA response after exercise in this study ($r = -0.66$, $P = 0.07$, unpublished observation). Observations that obesity is associated with lower skeletal muscle capillarization, (Isaksson et al., 1993; Lillioja et al., 1987), along with the knowledge of the importance of VEGF in capillary maintenance (Ferrara, 1999; Tang et al., 2004), led us to hypothesize that obesity may be associated with lower resting and exercise-induced levels of VEGF mRNA when compared to lean counterparts.

VEGF mRNA in lean and obese at rest and after acute exercise

In the current study, sedentary lean and obese college-aged males were recruited to investigate if VEGF mRNA levels at rest and after exercise were altered in obese skeletal muscle. Muscle biopsies of the vastus lateralis were taken immediately before and two hours after a one-hour exercise bout on a bicycle ergometer. The two-hour muscle biopsy time-point was selected because in the study by Gavin et al (2004), it yielded the first significant increase in VEGF mRNA over resting levels and because the 2-hour and 4-hour VEGF mRNA values were not statistically different from each other. Systemic bicycle exercise was utilized in the current study because this modality significantly upregulated VEGF mRNA in the Gavin et al (2004) study, and because this is a traditional exercise method (Gavin, Robinson et al., 2004). Other studies have used non-traditional, single-leg knee extensor (KE) exercise to elicit increases in skeletal muscle VEGF mRNA (Gustafsson et al., 1999; Richardson et al., 2000). The systemic bicycle exercise was deemed more appropriate because it is an exercise mode of everyday life.

The current study revealed no significant differences between the groups in VEGF mRNA at rest or after acute exercise. Compared to resting levels, VEGF mRNA was significantly higher at the two-hour post-exercise timepoint as a result of the acute exercise bout at 50% VO_2max. Exercise-induced VEGF mRNA increased more in the current study (~ 10-fold) than the former study (~ 4.5-fold). It is unclear

why VEGF mRNA had a higher fold increase in the current study as compared to Gavin et al (2004) because the relative workload was the same. Sample size was greater in the current study, but mean age, height, mass, and percent body fat were similar. Overall relative VO_2max was lower in the current study, mainly due to the fact that OB relative VO_2max was 25% lower than LN, while LN was not different from the previous study. Mean maximum power output was ~ 25% higher in the current study (243 ± 9 compared to 193 ± 6 Watts). A methodological difference between the studies is the use of a Monarck cycle ergometer versus a Lode electronically-braked cycle ergometer in the current study. While this may not explain the discrepancy between the results, it is important to note that in order for a subject to maintain the correct workload on the Monarck cycle ergometer, he had to maintain a specified pedaling rate. Therefore, the workload in the former study was not as controlled as in the current study since the Lode electronically-braked cycle ergometer adjusts the resistance to maintain the correct workload at any pedaling rate the subject chooses.

Subject characteristics

Study participants were selected based on body mass index (BMI) criteria as defined by the National Institutes of Health (BMI $\leq$ 25 is lean, while BMI $\geq$ 30 is obese) (Flegal et al., 1998). Subjects had to be sedentary, (no regular physical activity for the previous 3 months), non-smokers, and have had no known cardiovascular disease. The mean values for age and height were not significantly different between the lean (LN) and obese (OB) groups. As expected, BMI, weight, and percent body fat

were significantly different, as well as markers of abdominal adiposity such as sagittal diameter and umbilicus, waist, and hip circumferences.

Subjects in the current study cannot easily be compared to subjects in other obesity-related literature because, to date, few experimental studies on obesity exist in young, college-aged males. Most of the literature investigating human obesity is in older, more middle-aged (age 30 to 50) females and males, most likely because the prevalence and risks associated with obesity increase considerably within this age range. At the present time, a quick literature search of human studies on NIH's PubMed website using the search terms "obese college male" revealed only 1056 hits, while the terms "obese male" using age limit criteria of greater than 19 years old resulted in 21,248 hits (*http://www.pubmed.com*). According to the most recent National Health And Nutrition Examination Survery (NHANES III), which was conducted between 1988 and 1994, 30.6% of young males (between 20 and 29 years of age) were classified as overweight ($25.0 \leq BMI \leq 29.9$), while 42.5% of middle-aged males (between 30 and 59 years of age) were in this range. Furthermore, only 8.4% of young males were classified as obese ($30.0 \leq BMI \leq 34.9$), while this was seen in 16.9% of middle-aged males. College-aged males were used in the current study to determine if differences in VEGF mRNA exist between young, lean and obese, but otherwise healthy males. Since other comorbidities such as diabetes, hypertension, and/or cardiovascular disease are more frequently observed with increasing age and

obesity levels, and since they may have confounded the study, older males were not recruited.

Skeletal muscle fiber size and capillarization in obesity

Skeletal muscle fiber area and perimeter in obesity have been shown to be significantly larger than that of lean counterparts (Lillioja & Bogardus, 1988), as was the case in the current study. Measures of capillarization per fiber area have also been shown to be significantly lower in obese than lean due to the significantly larger fiber area, as was the case here. This relative method of quantifying capillarization reveals a simple, but important point: each individual capillary adjacent to obese skeletal muscle is responsible for supplying a larger muscle area than capillaries found in lean muscle. As a result, diffusional distances are greater, which may negatively affect delivery of oxygen and nutrients, such as insulin.

Several different techniques of quantifying skeletal muscle capillarization were utilized in the current study. It is necessary to point out that while the number of capillaries per individual fiber area was significantly different between LN and OB, the capillary-to-fiber ratio (on an individual fiber basis), the number of capillaries around a fiber (capillary contacts), and sharing factor (the number of fibers sharing each capillary) were not significantly different. Capillary-to-fiber perimeter exchange (CFPE) index was also not different between LN and OB. CFPE index is a relative measure of capillarization that takes fiber perimeter into account, and according to Hepple et al, estimates capillary-to-fiber surface area, which represents the greatest

resistance to oxygen flux (Hepple et al., 1997). These data support the notion that capillarization is important for oxygen delivery to working skeletal muscle. More capillaries supplying a muscle fiber surface area could increase oxygen diffusion, thus allowing for more physical work to be performed and, higher VO_2 values to be achieved.

The lower capillary densities observed in obesity may result from the inability or lack of VEGF to produce ample capillaries to maintain the same capillary densities observed in lean muscle as the obese muscle fiber cross-sectional area increases. It is well known that obese muscle fiber area is significantly larger than lean counterparts (Lillioja & Bogardus, 1988), as was the case in the current study. It has been reported that obese muscle has significantly lower capillarization when considering number of capillaries per individual fiber area (Lillioja et al., 1987), which the current study demonstrated. In obesity, muscle fiber size increases, yet capillarization does not increase to account for increased fiber area. In contrast, resistance training exercise models usually result in increased muscle fiber cross-sectional areas in conjunction with a simultaneous increase in capillary number, which is generally sufficient to maintain the capillary density compared to that of untrained muscle. McCall et al. studied muscle hypertrophy and capillarization in muscle biopsies of college men before and after 12 weeks of intensive resistance training (1996). Significant increases in muscle fiber area of both type I and type II fibers and the number of capillaries per fiber were observed (McCall et al., 1996). This suggests that the resistance-training

stimulus increased capillarization to accommodate the increased muscle fiber area to at least maintain, if not improve, the subjects' previous capillary density ratio. However, this maintenance of the capillary network does not exist in untrained obese skeletal muscle as it hypertrophies. Perhaps the chronic overload due to increased fat mass on obese skeletal muscle is not a sufficient stimulus to upregulate VEGF for the production of new capillaries to maintain capillary density.

The fact that we did not find significant differences between VEGF mRNA at rest or after acute exercise in our lean and obese groups may have had to do with our study design. We used the National Institutes of Health BMI guidelines for determination of lean and obese, which may have not allowed us to truly see differences between the groups. BMI was used as a determination for inclusion in the current study because it is a quick-and-easy measure that uses height and weight to estimate percent body fat (McArdle, 1992). High BMI generally means high percent body fat, but this is not always true. For example, football players and weightlifters often have high BMI values but may not have high percent body fat. In the current study, BMI correlated well with percent body fat ($r = 0.903$; $P < 0.0001$). However, one subject in the High OB group was a former football player and had a BMI of 35.13 and a percent body fat of 22.65.

Our findings of lower capillary density and increased fiber size in obesity are consistent with previous findings (Kriketos et al., 1996; Lillioja & Bogardus, 1988). Since VEGF is the most potent angiogenic growth factor known to regulate capillaries,

we hypothesized lower VEGF mRNA at rest in the obese group and an attenuated exercise-induced VEGF increase compared to lean counterparts as a possible explanation for this discrepancy. Contrary to our hypothesis, both groups showed similar resting VEGF mRNA levels and exercise-induced increases. These findings suggest that obese individuals have ample VEGF at rest and the capacity to produce VEGF in response to a stimulus such as exercise to maintain capillarization like that of their lean counterparts. However, the fact remains that capillary density is lower in obese subjects when compared to lean. The number of capillaries per fiber was not different between the groups in the current study. This finding suggests a defect in capillary regulation in obesity. As muscle fiber sizes increase with increasing obesity, the number of capillaries per fiber does not increase in order to compensate for the change in fiber area.

In conclusion, the current study did not reveal differences in VEGF mRNA between lean and obese at rest or after exercise. Absolute measures of capillarization, such as the number of capillaries per fiber and capillary contacts, were not different between the groups; however, relative measures were significantly lower in obese due to larger cross-sectional areas in these subjects. A future study comparing normal weight to extremely obese subjects (BMI $\geq$ 35) may reveal that obesity is associated with lower exercise-induced increases in VEGF mRNA compared to lean, thus resulting in a reduced capillary network. As for now, the mechanisms for the capillary rarefaction observed in obesity remain a mystery.

REFERENCES

Amaral, S. L., Papanek, P. E., & Greene, A. S. (2001). Angiotensin II and VEGF are involved in angiogenesis induced by short- term exercise training. *American Journal of Physiology Heart Circulatory Physiology, 281*(3), H1163-1169.

Bassett, D. R., Jr. (1994). Skeletal muscle characteristics: relationships to cardiovascular risk factors. *Medicine and Science in Sports and Exercise, 26*(8), 957-966.

Benn, R. T. (1970). Indices of height and weight as measures of obesity. *British Journal of Preventive Social Medicine, 24*(1), 64.

Bergstrom, J. (1962). Muscle electrolytes in man: determination by neutron activation analysis in needle biopsy specimens: a study in normal subjects, kidney patients, and patients with chronic diarrhoea. *Scandinavian Journal of Clinical Laboratory Investigation, 14*, 1-10.

Bermont, L., Lamielle, F., Lorchel, F., Fauconnet, S., Esumi, H., Weisz, A., & Adessi, G. L. (2001). Insulin up-regulates vascular endothelial growth factor and stabilizes its messengers in endometrial adenocarcinoma cells. *The Journal of Clinical Endocrinology & Metabolism, 86*(1), 363-368.

Bray, G. (1987). Overweight is Risking Fate. In R. a. J. Wurtman (Ed.), *Human Obesity* (Vol. 499, pp. 349). New York: Annals of the New York Academy of Sciences.

Breen, E. C., Johnson, E. C., Wagner, H., Tseng, H. M., Sung, L. A., & Wagner, P. D. (1996). Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *Journal of Applied Physiology, 81*(1), 355-361.

CDC. (2004). *The Surgeon General's Call To Action To Prevent and Decrease Overweight and Obesity; Overweight and Obesity: What You Can Do*, from http://www.cdc.gov

Dantz, D., Bewersdorf, J., Fruehwald-Schultes, B., Kern, W., Jelkmann, W., Born, J., Fehm, H. L., & Peters, A. (2002). Vascular endothelial growth factor: a novel endocrine defensive response to hypoglycemia. *The Journal of Clinical Endocrinology & Metabolism, 87*(2), 835-840.

Ferrara, N. (1999). Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney International, 56*(3), 794-814.

Ferrara, N., & Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocrine Reviews, 18*(1), 4-25.

Ferrara, N., & Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical and Biophysical Research Communications, 161*(2), 851-858.

Flegal, K. M., Carroll, M. D., Kuczmarski, R. J., & Johnson, C. L. (1998). Overweight and obesity in the United States: prevalence and trends, 1960-1994. *International Journal of Obesity and Related Metabolic Disorders, 22*(1), 39-47.

Gavin, T. P., Robinson, C. B., Yeager, R. C., England, J. A., Nifong, L. W., & Hickner, R. C. (2004). Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *J Appl Physiol, 96*(1), 19-24.

Gavin, T. P., Stallings, III. H.W., Zwestloot, K. A., Westerkamp, L. M., Ryan, N. A., Moore, R. A., Pofahl, W. E., & Hickner, R. C. (2004). Lower capillary density, but no difference in VEGF expression in obese versus lean young skeletal muscle in humans. *J Appl Physiol.*

Goalstone, M. L., Natarajan, R., Standley, P. R., Walsh, M. F., Leitner, J. W., Carel, K., Scott, S., Nadler, J., Sowers, J. R., & Draznin, B. (1998). Insulin potentiates platelet-derived growth factor action in vascular smooth muscle cells. *Endocrinology, 139*(10), 4067-4072.

Gustafsson, T., & Kraus, W. E. (2001). Exercise-induced angiogenesis-related growth and transcription factors in skeletal muscle, and their modification in muscle pathology. *Frontiers in Bioscience, 6*, D75-89.

Gustafsson, T., Puntschart, A., Kaijser, L., Jansson, E., & Sundberg, C. J. (1999). Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. *American Journal of Physiology, 276*(2 Pt 2), H679-685.

Hepple, R. T. (1997). A new measurement of tissue capillarity: the capillary-to-fibre perimeter exchange index. *Canadian Journal of Applied Physiology, 22*(1), 11-22.

Hepple, R. T., Mackinnon, S. L., Goodman, J. M., Thomas, S. G., & Plyley, M. J. (1997). Resistance and aerobic training in older men: effects on VO2peak and the capillary supply to skeletal muscle. *Journal of Applied Physiology, 82*(4), 1305-1310.

Hernandez, N., Torres, S. H., Vera, O., De Sanctis, J. B., & Flores, E. (2001). Muscle fiber composition and capillarization in relation to metabolic alterations in hypertensive men. *Journal of Medicine, 32*(1-2), 67-82.

Hickey, M. S., Carey, J. O., Azevedo, J. L., Houmard, J. A., Pories, W. J., Israel, R. G., & Dohm, G. L. (1995). Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. *American Journal of Physiology, 268*(3 Pt 1), E453-457.

Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., & Leung, D. W. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Molecular Endocrinology, 5*(12), 1806-1814.

Isaksson, H., Cederholm, T., Jansson, E., Nygren, A., & Ostergren, J. (1993). Therapy-resistant hypertension associated with central obesity, insulin resistance, and large muscle fibre area. *Blood Pressure, 2*(1), 46-52.

Ivy, J. L. (1997). Role of exercise training in the prevention and treatment of insulin resistance and non-insulin-dependent diabetes mellitus. *Sports Medicine, 24*(5), 321-336.

Jacobson, M. (2000, March 2000). Obesity in America: Inevitable? *Nutrition Action Health Letter, 27*, 2.

Julius, S., Gudbrandsson, T., Jamerson, K., & Andersson, O. (1992). The interconnection between sympathetics, microcirculation, and insulin resistance in hypertension. *Blood Pressure, 1*(1), 9-19.

Kelley, D. E., Goodpaster, B., Wing, R. R., & Simoneau, J. A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *American Journal of Physiology, 277*(6 Pt 1), E1130-1141.

Kriketos, A. D., Baur, L. A., O'Connor, J., Carey, D., King, S., Caterson, I. D., & Storlien, L. H. (1997). Muscle fibre type composition in infant and adult populations and relationships with obesity. *International Journal of Obesity and Related Metabolic Disorders, 21*(9), 796-801.

Kriketos, A. D., Pan, D. A., Lillioja, S., Cooney, G. J., Baur, L. A., Milner, M. R., Sutton, J. R., Jenkins, A. B., Bogardus, C., & Storlien, L. H. (1996). Interrelationships between muscle morphology, insulin action, and adiposity. *American Journal of Physiology, 270*(6 Pt 2), R1332-1339.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., & Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science, 246*(4935), 1306-1309.

Lillioja, S., & Bogardus, C. (1988). Obesity and insulin resistance: lessons learned from the Pima Indians. *Diabetes Metab Rev, 4*(5), 517-540.

Lillioja, S., Young, A. A., Culter, C. L., Ivy, J. L., Abbott, W. G., Zawadzki, J. K., Yki-Jarvinen, H., Christin, L., Secomb, T. W., & Bogardus, C. (1987). Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *Journal of Clinical Investigation, 80*(2), 415-424.

McArdle, W. D., Katch, F.I., & Katch, V.L. (1992). *Exercise Physiology: Energy, Nutrition and Human Performance* (3rd ed.). Baltimore, MD: Williams and Wilkins.

McCall, G. E., Byrnes, W. C., Dickinson, A., Pattany, P. M., & Fleck, S. J. (1996). Muscle fiber hypertrophy, hyperplasia, and capillary density in college men after resistance training. *J Appl Physiol, 81*(5), 2004-2012.

Natarajan, R., Bai, W., Lanting, L., Gonzales, N., & Nadler, J. (1997). Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. *American Journal of Physiology Heart and Circulatory Physiology, 273*(5 Pt 2), H2224-2231.

NIDDK. (1999, September 1999). *Diabetes Statistics in the United States*, 1999, from http://www.niddk.nih.gov/health/diabetes/pubs/dmstats/dmstats.htm#what

Nieman, D. (1998). *The Exercise-Health Connection: How to Reduce Your Risk of Disease and Other Illnesses by Making Exercise Your Medicine.* Champaign, IL: Human Kinetics.

NIH. (1998). Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. National Institutes of Health. *Obesity Research, 6 Suppl 2*, 51S-209S.

Pinkney, J. H., Stehouwer, C. D., Coppack, S. W., & Yudkin, J. S. (1997). Endothelial dysfunction: cause of the insulin resistance syndrome. *Diabetes, 46 Suppl 2*, S9-13.

Pugsley, M. K., & Tabrizchi, R. (2000). The vascular system. An overview of structure and function. *Journal of Pharmacological and Toxicological Methods, 44*(2), 333-340.

Reaven, G. M. (1988). Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes, 37*(12), 1595-1607.

Richardson, R. S., Wagner, H., Mudaliar, S. R., Henry, R., Noyszewski, E. A., & Wagner, P. D. (1999). Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. *American Journal of Physiology, 277*(6 Pt 2), H2247-2252.

Richardson, R. S., Wagner, H., Mudaliar, S. R., Saucedo, E., Henry, R., & Wagner, P. D. (2000). Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *American Journal of Physiology - Heart Circulatory Physiology, 279*(2), H772-778.

Rosenblatt, J. D., Kuzon, W. M., Jr., Plyley, M. J., Pynn, B. R., & McKee, N. H. (1987). A histochemical method for the simultaneous demonstration of capillaries and fiber type in skeletal muscle. *Stain Technology, 62*(2), 85-92.

Saltin, B., and P.D. Gollnick. (1983). Skeletal muscle adaptability: significance for metabolism and performance. In *Handbook of Physiology. Skeletal Muscle.* (Vol. 10, pp. 555-631.). Bethesda, MD: American Physiological Society.

Schutte, J. E., Townsend, E. J., Hugg, J., Shoup, R. F., Malina, R. M., & Blomqvist, C. G. (1984). Density of lean body mass is greater in blacks than in whites. *Journal of Applied Physiology, 56*(6), 1647-1649.

Serne, E. H., Stehouwer, C. D., ter Maaten, J. C., ter Wee, P. M., Rauwerda, J. A., Donker, A. J., & Gans, R. O. (1999). Microvascular function relates to insulin sensitivity and blood pressure in normal subjects. *Circulation, 99*(7), 896-902.

Siri, W. E. (1961). Body composition from fluid spaces and density: Analysis of methods. In B. J. & H. A. (Eds.), *Techniques for measuring body composition* (pp. 223-244). Washington DC: National Academy of Sciences.

Sone, H., Kawakami, Y., Okuda, Y., Kondo, S., Hanatani, M., Suzuki, H., & Yamashita, K. (1996). Vascular endothelial growth factor is induced by long-term high glucose concentration and up-regulated by acute glucose deprivation in cultured bovine retinal pigmented epithelial cells. *Biochemical and Biophysical Research Communications, 221*(1), 193-198.

Tang, K., Breen, E. C., Gerber, H. P., Ferrara, N. M., & Wagner, P. D. (2004). Capillary regression in vascular endothelial growth factor deficient skeletal muscle. *Physiol Genomics*.

Tikkanen, H. O., Naveri, H., & Harkonen, M. (1996). Skeletal muscle fiber distribution influences serum high-density lipoprotein cholesterol level. *Atherosclerosis, 120*(1-2), 1-5.

Vock, R., Weibel, E. R., Hoppeler, H., Ordway, G., Weber, J. M., & Taylor, C. R. (1996). Design of the oxygen and substrate pathways. V. Structural basis of vascular substrate supply to muscle cells. *Journal of Experimental Biology, 199*(Pt 8), 1675-1688.

Wade, A. J., Marbut, M. M., & Round, J. M. (1990). Muscle fibre type and aetiology of obesity. *Lancet, 335*(8693), 805-808.

Wilmore, J. H., Vodak, P. A., Parr, R. B., Girandola, R. N., & Billing, J. E. (1980). Further simplification of a method for determination of residual lung volume. *Medicine and Science in Sports and Exercise, 12*(3), 216-218.

APPENDIX A

ECU INSTITUTIONAL REVIEW BOARD APPROVAL

University and Medical Center Institutional Review Board
East Carolina University
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Chair: Charles W. Daeschner, III, MD

TO: Thomas Gavin, PhD, Exercise and Sports Medicine, ECU, 363 Ward Sports Medicine Bldg.

FROM: Charles W. Daeschner, III, M.D., Chair, UMCIRB

DATE: January 24, 2003

RE: Full Committee Approval for Continuous Review of a Research Study

TITLE: "Effect of Obesity on Skeletal Muscle VEGF Expression"

UMCIRB #99-0538

The above referenced research study was initially reviewed and approved by the convened University and Medical Center Institutional Review Board (UMCIRB) on **2/27/02**. This research study has undergone a subsequent continuous review for approval on **1/22/03** by the convened UMCIRB. The UMCIRB deemed this **NC Institute of Nutrition** sponsored study **more than minimal risk** requiring a continuous review in **12 months**.

The above referenced research study has been given approval for the period of **1/22/03** to **1/21/04**. The following are the most currently approved items as they have been previously submitted:

- Protocol (no version date)
- Informed consent document (version 2, dated 4/8/02)
- Advertisement (no version date)
- Physical activity readiness questionnaire (PAR-Q) (no version date).

The following UMCIRB members abstained for reasons of potential for Conflict of Interest on this research study: None

The UMCIRB complies with 45 CFR 46, 21 CFR 50, 21 CFR 56, ICH Guidelines, UMCIRB standard operating procedures, institutional policies and other applicable federal regulations.

The UMCIRB recognizes the investigator and research team's commitment to comply with 45 CFR 46, the UMCIRB standard operating procedures and institutional policies in the conduct of all research. Investigator also must comply with 21 CFR 50, 21 CFR 56, ICH Guidelines, and all other applicable federal regulations in their research endeavors.