

## EXERCISE-INDUCED ANGIOGENESIS-RELATED GROWTH AND TRANSCRIPTION FACTORS IN SKELETAL MUSCLE, AND THEIR MODIFICATION IN MUSCLE PATHOLOGY

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### 1. ABSTRACT

Angiogenesis is the process of formation of new blood vessels; it is generally a rare occurrence in the adult, although it is a common adaptive response to exercise training in skeletal muscle. Current thinking is that angiogenesis is mediated by diffusible angiogenic factors and that the angiogenic activity is regulated through the balance between stimulatory and inhibitory factors. Recent studies have shown that up-regulation of angiogenic factors occurs in response to increased muscle activity in skeletal muscle. The major putative angiogenic factor, vascular endothelial growth factor (VEGF), seems to increase to a greater extent and more consistently than other measured angiogenic factors, such as fibroblast growth factor-2 (FGF-2) and transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ). While the regulating mechanisms in this response are not clear, present data indicate reduced oxygen tension and/or related metabolic alterations in the skeletal muscle as possible stimuli. Data on other angiogenic growth factors are limited, but an increase in endothelial cell-stimulating angiogenic growth factor (ESAF) has been observed in response to increased blood flow and muscle stretching. Therefore, different exercise associated stimuli may all contribute to exercise-induced angiogenesis in skeletal muscle, but possibly through differing angiogenic factors and mechanisms. Understanding these processes is important for the elucidation of mechanisms mediating exercise responsiveness in skeletal muscle, but also for the potential that such understanding might bring to the treatment and prevention of human diseases such as intermittent claudication.

### 2. INTRODUCTION

The adaptation of skeletal muscle to different types of exercise is well characterized. It includes increases in the number and size of mitochondria and in the activity of enzymes controlling oxidative metabolism and

capillarization (1-3). In this review, we summarize current knowledge of the mechanisms underlying the increases in vascularity that occur with exercise conditioning. These include angiogenic factors induced in response to increased muscle activity or endurance exercise. We also discuss how these factors are coordinated with increases in oxidative capacity, and how they might be altered in pathophysiological states.

### 3. CAPILLARIZATION: ITS IMPORTANCE AND CHANGE IN RESPONSE TO INCREASED MUSCLE ACTIVITY

#### 3.1. Oxygen uptake and metabolic exchange

The weight of evidence supports the concept that the major determinant of exercise tolerance during exercise using large muscle masses is through central cardiovascular hemodynamic factors, such as cardiac output (4-5). However, the ability to realize maximum performance will also depend on efficient peripheral mechanisms for oxygen uptake and utilization for example, peripheral oxygen delivery, distribution, extraction and mitochondrial respiration. Increased peak oxygen uptake, during constant oxygen delivery to skeletal muscle, after training, and a corresponding decrease in peak oxygen uptake after immobilization, are observations that support a role for limitations in peripheral factors as determinants of skeletal muscle oxygen uptake and performance during exercise (6-7). One-legged endurance training further demonstrates the limitation conferred by peripheral factors. For example, a higher peak oxygen uptake is observed with exercise with the trained leg when compared to maximal exercise with the contralateral untrained leg (8). Since one-legged exercise does not stress the capacity of central hemodynamics to perfuse the exercising leg (4,9), the increased capacity demonstrated with the trained leg is

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likely to be related to peripheral adaptations attributable to increases in oxygen extraction and utilization, such as increased capillarity and/or oxidative capacity.

Endurance training increases both capillarity and oxidative activity. It has been suggested that increases in peripheral oxygen uptake and utilization are more likely due to increases in oxidative capacity than capillarity (10-11). However, observations from exercise under hypoxic and hyperoxic conditions indicate that tissue O<sub>2</sub> diffusion capacity is an important factor determining peripheral oxygen uptake (12-14). Increases in capillarity results in a larger surface area available for diffusion, and decreased diffusion distance. It also leads to a greater cross-sectional area for oxygen flux, reduced blood flow velocity and increased transient time for metabolic exchange. An increased capacity to extract blood-borne substrates may contribute to the increased exercise performance capacity seen after endurance training, especially during longer submaximal exercise bouts when the dependence on substrate delivery to muscle becomes important. Another intriguing observation is that an increase in capillarity seems to precede increases in mitochondrial enzyme activity in several models of disease and exercise. Therefore, one can speculate that improvements in oxygen delivery, through increases in vascularity, may be necessary and sufficient for adaptive increases in aerobic-oxidative potential mediated by exercise in skeletal muscle (15-16). This hypothesis is supported by the observation that increased blood vessel formation is of great importance for tissue regeneration and remodeling in fully developed organs (17).

While exercise training increases exercise performance, it also directly (and favorably) influences multiple cardiovascular risk factors. It is well established that increased levels of physical activity result in improvements in total serum lipoprotein levels, serum low-density lipoprotein concentrations, lipid handling and in total body fat content (18), and in improvements in insulin sensitivity, glucose disposal and clinical glucose intolerance (19). It also induces improvements in blood pressure control in those with borderline or overt hypertension (20-22), and in favorable effects on systemic hemostatic parameters that are associated with the presence and progression of atherosclerotic disease (23). The basic biological mechanisms that mediate these beneficial adaptations of regular exercise are poorly understood. However, there is increasing evidence that the positive effects of regular exercise in humans, such as improved cardiovascular health, are mediated through chronic adaptations in the skeletal muscle to habitual exercise. It can be argued that many of the metabolic pathways that mediate favorable or unfavorable risk factor levels in humans take place on the surface membrane of capillary endothelial cells in skeletal muscle (24). For example, several independent studies support the theory that an increased endothelial surface area will increase glucose uptake, lipoprotein metabolism and augmentation of insulin sensitivity (25-28). Such arguments support the proposal that increases in capillarity are an important mechanism whereby exercise training leads to improvements in cardiovascular health.

### 3.2. Changes in capillarity in response to physical activity

Vanotti and Magiday (1934) were the first to document increased capillarity after muscle activation alone (29). After direct transcutaneous electrical muscle stimulation for five minutes per day for four to six weeks, they observed a doubling in the capillary-to-fiber ratio in stimulated muscle compared with control muscle. Later

studies have confirmed the potential of electrical muscle stimulation to induce increased capillarity in skeletal muscle (30-32). Furthermore, exercise training studies in different animal species have demonstrated increases in capillarity in response to increases in voluntary muscle activity (33-34).

In human skeletal muscle, the percutaneous biopsy technique has made it possible to estimate the total number of capillaries and their relation to area and specific fiber-type in cross-sections of the muscle. In 1970, the first human studies were published showing an increase in capillarity in response to increased physical activity (35-38). It was also observed that cessation of exercise training rapidly induced a regression in capillarity (39-40). Conflicting data exist, however, about the relationship between the increases in muscle activity and capillarity. For example, there is a lack of information in humans about the extent to which increased capillarity depends upon training intensity and duration. Low intensity training regimes often result in unchanged capillarity. Moreover, studies on short duration, high-intensity exercises, such as sprint and strength training, fail to demonstrate an consistent adaptation in capillarity (41-42). Some disagreement between different exercise studies regarding capillary adaptive responses may be related to the two most commonly used variables, capillaries per square millimeter and the number of capillaries found around each fiber. These are both influenced by fiber size, which itself changes with physical activity (1,37-40). Measured capillary density is also influenced by artifactual changes in fiber type areas, produced through preparative techniques. The most frequently used variable for measurements of capillary growth in skeletal muscle is the number of capillaries per fiber, in which artifactual errors due to technique and physiological changes in fiber size are eliminated (1,37). An additional problem with capillary measurement, unrelated to measured variables, is that histochemical and immunohistochemical techniques have different sensitivities, and may therefore provide different estimates of the number of capillaries identified in a given section (43-45).

Nevertheless, increases in capillarity in skeletal muscle, measured as capillary per fiber, in response to both long-term electrical stimulation and endurance exercise, have been confirmed in numerous studies (1,3). Furthermore, during the last decade animal preparations using other techniques, such as electron microscopy and measurements of activated endothelial cells labeled with bromodeoxyuridine, have also demonstrated angiogenic responses to increased muscle activity. These studies have provided more mechanistic information about capillary growth in response to various exercise stimuli (46-48).

## 4. ANGIOGENESIS AND ANGIOGENIC GROWTH FACTORS

### 4.1. Angiogenesis

Increases in capillarity in response to increased physical activity, measured as the number of capillaries per fiber, is due to capillary growth or angiogenesis. Angiogenesis is the formation of new blood vessels and is generally a rare occurrence in the adult. However, it is a common adaptive response to exercise training of skeletal muscle. It is also observed in the uterus during the myometrial proliferative phase of the ovarian cycle and in wound healing (49-50). Angiogenesis also seems to be a central and important process in various diseases such as hemangiomas and malignant cancers (50). Understanding the importance of angiogenesis in tumor progression has led to intensive research on the basic

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mechanisms underlying angiogenesis and its regulation. The goal of this work has been primarily to discover mechanisms for the prevention of tumor growth through inhibition of angiogenesis (52- 53). In contrast, efforts have also been made to find ways to stimulate angiogenesis (also known as therapeutic angiogenesis) for the treatment of individuals with pathological states characterized by reduced blood flow to peripheral tissues, such as ischemic heart disease and peripheral vascular insufficiency (54-55).

Angiogenesis is a very complex process. Current thinking is that angiogenesis is mediated by diffusible angiogenic factors, either through increased synthesis or through release from intracellular and extracellular storage sites, and that the angiogenic activity is regulated through the balance between stimulatory and inhibitory factors (50,56-58). The development of *in vivo* bioassays, *in vitro* analysis techniques and murine transgenic models have made it possible to identify and characterize several angiogenic factors (59). From these studies, it has become apparent that such factors mediate angiogenesis, either directly or indirectly through other secondary factors. However, it is not clear whether similar mechanisms or factors mediate angiogenesis under normal and pathologic conditions. For example, involvement of other physiological systems, such as the coagulation and fibrinolytic pathways, seem to be important during development of the cardiovascular system and in some pathophysiological conditions, such as arteriosclerosis (60). Additionally, angiogenesis clearly involves an inflammatory component during wound-healing (61). Physiological angiogenesis in skeletal muscle that occurs in response to stimuli generated by muscle activity may therefore also be due to a combination of independent systems (46-48).

### 4.2. Angiogenic factors

To understand the angiogenic response to exercise, it is important to understand the current state of knowledge regarding different factors possessing angiogenic properties. Therefore, a brief summary about the most well-known angiogenic factors is presented here.

#### 4.2.1. Vascular endothelial growth factor (VEGF)

VEGF is regarded as the major putative angiogenic factor. It is a 34-45 kD homodimeric glycoprotein and is a highly specific mitogen for vascular endothelial cells (62-63). At least five different isoforms, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub> and VEGF<sub>201</sub>, are generated through alternative splicing (64). VEGF is expressed in spatial and temporal association with physiological events during angiogenesis, and has been found to play an important role in tumor angiogenesis, development of the cardiovascular system, wound healing and also in diseases with pathologic angiogenic responses (50,65-66).

Three different VEGF receptors have been described, all with tyrosine kinase activity: VEGF-R1 (also known as Flt-1), VEGF-R2 (KDR), and VEGF-R3 (Flt-4). VEGF-R1 and R2 are high-affinity receptors for VEGF (67). As these receptors are confined to endothelial cells (68), this ensures that VEGF activity is also localized there. Activation of the two receptors induces different biological effects (69-70). However, a homozygous knockout of the VEGF-R1 and VEGF-R2 genes leads to embryonic death at day 8.5 and day 7 in mice, in both cases from an underdeveloped cardiovascular system (71-72).

Several factors, including cytokines, growth factors, and tumor suppressor factors, seem to be involved in the regulation of VEGF production (62-63). Hypoxia and

hypoglycemia are other well-described stimulators of VEGF gene expression (73-74). Hypoxia-induced transcription of VEGF seems at least to be regulated partly by the transcription factor hypoxia inducible factor-1 (HIF-1), but other mechanisms, such as mRNA stabilization, may also be involved in the increased VEGF mRNA level occurring in response to hypoxia *in vitro* (75-79). Furthermore, factors involved in blood flow regulation, such as nitric oxide (NO), adenosine and prostaglandins, have been shown to induce VEGF expression *in vitro* (80-82). However, the effect of NO on VEGF expression is controversial. Some studies have observed a stimulating effect, while others have shown an inhibitory effect (82-85). VEGF is a potent stimulator of vasodilation and vessel-permeability, which seems to be mediated through NO and prostacyclins (86). NO has also been suggested to act as a downstream mediator of VEGF-stimulated angiogenesis (87).

Other growth factors have been identified in the VEGF family such as VEGF B and VEGF C. These two growth factors are not as well characterized as VEGF, but both seem to have some angiogenic potential (88). In contrast to VEGF, neither of these factors has been shown to be regulated by hypoxia (89).

#### 4.2.2. Fibroblast growth factors (FGF)

The angiogenic properties of growth factors of the FGF family, have been extensively studied. The best-characterized members are the 15-17 kD anionic mitogen fibroblast growth factor 1 (FGF-1; acidic FGF; aFGF) and the 18-23 kD cationic mitogen FGF-2 (basic FGF; bFGF) (89-90). FGF-1 and 2 stimulate angiogenesis *in vitro* as well as *in vivo*, and the biological effects are mediated through at least four different receptors, all with tyrosine kinase activity (54,92-93). The biological effects of FGF-1 and 2 are less spatially restricted than those of VEGF (90-92). Therefore, these FGFs could have other roles beside angiogenesis in skeletal muscle adaptation to exercise.

Both FGF-1 and FGF-2 lack conventional leader sequences for cellular secretion, and therefore it has been questioned whether secretion via the endoplasmic reticulum occurs *in vivo*. Other mechanisms have been proposed to be responsible for FGF release and activation of an angiogenic response, such as cell damage, tissue stretching and subsequent release from extra-cellular binding to heparin (94-95). Whether hypoxia induces FGF activation is unclear. No hypoxic element has been found in the promoter region of FGF-1 or FGF-2, and hypoxic exposure *in vitro* has failed to induce FGF-2 expression (96). However, increased concentrations of FGF-2 have been found in ischemic tissues, as for example in cases of lower limb ischemia. (97-98).

FGF-1 and FGF-2 are thought to induce vasodilation through NO, in a fashion similar to VEGF (99). FGF-2 and VEGF also have synergistic stimulatory effects on angiogenesis *in vivo*, although they act through different mechanisms (100-101). Additionally, FGF-2 seems to up-regulate VEGF gene expression, for example, in smooth muscle cells (102).

#### 4.2.3. Angiopoietins

A close relationship has recently been observed between a new group of factors, angiopoietins, and VEGF during development and angiogenesis in adult tissue. The angiopoietins include a receptor agonist, angiopoietin 1 (Ang-1), and a receptor antagonist, angiopoietin 2 (Ang-2) (103). Similar to VEGF, the angiopoietins are specific for endothelial cells because of the restricted location of their receptors (104-105). Ang-1 acts via the tyrosine kinase

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receptor, Tie-2, and seems to maintain and stabilize mature vessels by promoting interaction between the endothelial cells and surrounding cells. Ang-2 is thought to block the Tie-2 receptor, and in the absence of VEGF leads to vessel regression. However, in the presence of high VEGF levels Ang-2 facilitates an angiogenic response (106).

### 4.2.4. Transforming Growth Factors (TGF- $\beta$ ) and Platelet Derived Growth Factors (PDGF-BB)

In contrast to VEGF and FGF, which are direct-acting angiogenic factors, the angiogenic effects of TGF- $\beta$  and PDGF-BB are indirectly mediated (58). Mice lacking TGF- $\beta$  or PDGF-BB die *in utero* from defective vascular maturation (107-108). The genes of both factors have a shear stress element in their promoter regions. These promoter elements mediate up-regulation of these factors in endothelial cells in response to increased shear stress *in vitro* (109).

### 4.2.5. Integrins

Stretching induced by different mechanisms may influence the communication between endothelial cells and the surrounding tissue (extracellular matrix: ECM). Integrins are heterodimeric cell-surface receptors that link the cytoskeleton to the ECM, and have been proposed to be involved in the regulation of angiogenesis (110). For example, one member of the integrin family, integrin  $\alpha_v\beta_3$ , is expressed on the surface of newly formed cells but is barely detectable in mature vessels (111). Furthermore, interfering with integrin  $\alpha_v\beta_3$  induces programmed cell death (apoptosis) in proliferating endothelial cells, which suggests its importance for the angiogenic process (111-112).

## 5. THE RESPONSE OF ANGIOGENIC FACTORS AND RELATED TRANSCRIPTION FACTORS TO EXERCISE STIMULI

### 5.1. Expression of angiogenic factors in response to increased muscle activity or endurance type of exercise

Numerous chemical and hormonal factors that change with muscle activity have been explored for possible angiogenic activity (3). The first study to investigate a specific angiogenic factor in response to increased activity in skeletal muscle was performed by Morrow *et al* (1989) (113, Table 1). In this study, increases in mitogen activity were observed as early as day 3 of motor nerve stimulation of skeletal muscle, and the activity increased with continued stimulation. The increase in mitogen activity was reflected by increases in FGF-1 and FGF-2 protein. Long-term direct electrical stimulation of skeletal muscle in rats has not however been shown to increase FGF-2 at the mRNA level (114, Table 1). Changes in FGF-1 mRNA in response to electrical stimulation have not been studied.

In contrast to the FGF mRNA response, up-regulation of VEGF mRNA have been observed in response to electrical stimulation of skeletal muscle in both rats and rabbits (16,115). The VEGF mRNA expression seems to follow a bimodal time-course, with the first elevation as early as the first day of stimulation, followed by a second rise after six to eight days (16). This may indicate an initial VEGF mRNA stabilization followed by a later transcriptional activation. However, stimulation for more than ten days induces a decrease from the initial increase in VEGF mRNA expression (16,115).

Motor nerve stimulation of skeletal muscle has also been reported to increase the VEGF protein concentration as early as day 3 (15). These observations

suggest a parallel increase in VEGF mRNA and protein levels in response to the increased muscle activity induced by motor nerve stimulation. Furthermore, the change in VEGF mRNA and protein levels follows a similar time course to changes in vascular density, which precede any increases in mitochondrial enzyme activity (15-16). However, in the steady-state condition, the VEGF protein levels are observed to be proportionate to myoglobin protein in different skeletal muscles in rabbits, indicating a correlation between the VEGF protein levels and overall oxidative capacity (15).

Studies of one session of voluntary endurance-type exercise in rats and humans support the observations of nerve-stimulated skeletal muscle that increases in VEGF expression occur in response to increased muscle activity (116-118). One session of treadmill exercise in rats increases VEGF, FGF-2 and TGF- $\beta$  mRNA in skeletal muscle in relation to exercise intensity; mRNA remains elevated for up to four hours after exercise (116). This mRNA expression pattern suggests a close link between physiological events associated with exercise and mRNA production. VEGF mRNA also increases in response to a single session of one-legged endurance exercise in human skeletal muscle. In contrast to treadmill exercise in rats, in these studies there were no changes in the amounts of FGF-2 mRNA (117-118). Preliminary data from a study of 10 days of one-legged exercise training in humans support earlier findings from motor nerve stimulation in rats and rabbits, that an increase in VEGF mRNA is followed by a similar increase in VEGF protein (119). Furthermore, similar to a single session of acute exercise in humans, no changes were observed in FGF-2 mRNA levels, nor in protein levels (119). The finding of stable FGF-2 at the protein level in response to endurance training is also supported by earlier observations in rats (120). VEGF protein expression seems to be located between the muscle fibers (in the ECM) without any VEGF immunostaining in the myocytes (15). In contrast, the VEGF mRNA expression has been localized in the subsarcolemma of the muscle fiber, implying that skeletal muscle itself is the source of matrix VEGF (116). In addition, electrical muscle stimulation beyond the threshold for muscle contraction has been shown to induce VEGF mRNA expression in the skeletal muscle fiber with a subsequent increase protein secretion into the surrounding medium *in vitro* (121). This latter observation indicates, furthermore, that stimulation of the muscle itself could act as a stimulus for angiogenesis.

Endothelial cell-stimulating angiogenic growth factor (ESAF) is another factor with angiogenic properties in which increased activity in parallel with angiogenesis has been observed in response to electrical stimulation in the skeletal muscle (122). ESAF is not as well-characterized an angiogenic factor as VEGF or FGF, but it has been shown to activate various enzymes suggested to be important for the angiogenic process, such as progelatinase A, procollagenase and prostromelysin (123).

### 5.2. Angiogenesis and angiogenic factors response in skeletal muscle to local hypoxia/ ischemia and related metabolic alterations

Reduced oxygen tension and/or related metabolic alterations have been suggested as possible primary stimuli for the induction of angiogenesis in response to exercise (124). This theory is supported by numerous studies,

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**Table 1.** Response of angiogenic factors to muscle activity and exercise associated stimuli.

Factor	Species	Stimuli	Response	Comments	Reference
VEGF	Rat	Electrical stimulation (10 Hz, 300 $\mu$ s pulses, 21 days)	mRNA $\uparrow$	- After 10 days stimulation the VEGF mRNA levels started to decrease.	115
	Rabbit	Electrical stimulation (10 Hz, 12 h/day, 1 h on-1 h off, 50 days)	mRNA $\uparrow$	- The VEGF mRNA expression followed a bimodal time pattern, first elevation day 1 of stimulation with a second rise after 6-8 days of stimulation	16
	Rabbit	Electrical stimulation (6-10 Hz, continuously, 56 days)	Protein $\uparrow$	- The changes in VEGF mRNA followed a similar time pattern as the changes in capillary density, which both preceded the increase in oxidative capacity. - The changes in VEGF mRNA followed a similar time course as the changes in capillary density, which both preceded the increase in oxidative capacity. - In the steady state condition the VEGF protein levels were proportionate to the myoglobin protein levels. -The VEGF proteins were found to be located between the muscle fibers (in the extracellular matrix) without any VEGF immunostaining in the myocytes.	17
	Rat	Treadmill exercise A) normoxic (1 h, 15 or 20 m/min, 10° ) B) hypoxic (12% O <sub>2</sub> , 1 h 15 m/min, 10° )	mRNA: A) 15 m/min $\uparrow$ 20 m/min $\uparrow\uparrow$ B) $\uparrow\uparrow$	- VEGF mRNA expression was observed to be located in the subsarcolemma of the myocytes itself. - Hypoxic exercise increased VEGF mRNA levels to a greater extent than normoxic exercise.	116
	Dog	A) Passive hyperperfusion B) El stim (50 Hz, 1 contraction/s for 3 min)	mRNA: A) — B) $\uparrow$	- Increased blood flow to similar level as induced by electrical muscle stimulation increased VEGF gene expression to a less extent than electrical stimulation.	143
	Rat	Infusion; NP, Ach, PGE <sub>1</sub> , PGE <sub>2</sub> , PGI <sub>2</sub>	mRNA: NP, Ach $\uparrow$ PGE <sub>1</sub> , PGE <sub>2</sub> — PGI <sub>2</sub> $\downarrow$	- NO, PGE <sub>1</sub> , PGE <sub>2</sub> and PGI <sub>2</sub> , adenosine and acetylcholine, all failed to increase the VEGF mRNA to the levels observed with electrical stimulation or a single session of exercise.	144
	Human	One-leg knee extension (45 min.), A) non-restricted blood flow B) restricted blood flow (15-20% lower)	mRNA: A) non-restricted $\uparrow$ B) restricted $\uparrow$	- A trend of increased VEGF mRNA expression was observed with exercise under restricted blood flow compared to exercise under non-restricted blood flow. - Exercise-induced lactate levels in skeletal muscle correlated with the exercise-induced increase in VEGF mRNA.	117
	Human	One-leg knee extension (30 min) A) normoxia B) hypoxia (12% O <sub>2</sub> )	mRNA: A) normoxia $\uparrow$ B) hypoxia $\uparrow$	- In contrast to hypoxic exercise in rats no further increase in VEGF mRNA levels was observed during hypoxic exercise in humans.	118
	Human	One leg knee extension training (three times per week, 8 weeks) A) basal levels B) response to one acute bout of exercise after compared to before training	mRNA: A) basal level — B) acute response to exercise $\downarrow$	- Training resulted in a 35% increase in muscle oxygen consumption and an 18% increase in number of capillaries per muscle fiber	132
	Rabbit	Electrical stimulation (10 Hz continuously)	Protein $\uparrow\uparrow$	- The increase in mitogen activity was reflected by an increase in FGF-1 protein levels.	113
FGF-2	Rabbit	Electrical stimulation (10 Hz continuously)	Protein $\uparrow$	- The increase in mitogen activity was reflected by an increase in FGF-2 protein levels. FGF-1 increased to a greater extent than FGF-2	113
	Rat	Infusion; NP, Ach, PGE <sub>1</sub> , PGE <sub>2</sub> , PGI <sub>2</sub>	mRNA: NP, Ach, --- PGE <sub>2</sub> PGE <sub>1</sub> --- PGI <sub>2</sub> $\downarrow$	- The most consistent observation was a slightly reduced level of FGF-2 mRNA in response to PGI <sub>2</sub>	144
	Dog	A) Passive hyperperfusion B) Electrical stimulation (50 Hz, 1 contraction/s for 3 min)	mRNA: A) --- B) ---	- Neither passive hyperperfusion nor electrical stimulation changed the FGF-2 mRNA levels.	143
	Rat	Stretch overload (2 weeks)	mRNA ---	- No increase in FGF-2 mRNA levels was observed even though angiogenesis was observed.	142
	Rat	Treadmill exercise A) normoxic (1 h, 15 or 20 m/min, 10° ) B) hypoxic (12% O <sub>2</sub> , 1 h 15 m/min, 10° )	mRNA: A) 15 m/min $\uparrow$ 20 m/min $\uparrow\uparrow$ B) $\uparrow\uparrow$	- No further increase in FGF-2 mRNA levels was observed with hypoxic exercise.	116

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**Table 1.** continued

Factor	Species	Stimuli	Response	Comments	Reference
	Rat	Treadmill exercise training (20 m/min until exhaustion, 4/ day, 7 days) A) trained-ligated femoral artery B) sedentary- ligated femoral artery	Protein A) --- B) ---	- No increase in FGF-2 protein level was observed even though angiogenesis was observed in the trained ligated group.	120
	Human	One-leg knee extension (30 min) A) normoxia B) hypoxia (12% O <sub>2</sub> )	mRNA: A) normoxia --- B) hypoxia ---	- In contrast to hypoxic exercise in rats no further increase in FGF-2 mRNA levels was observed during hypoxic exercise in humans.	117
	Human	One-leg knee extension (45 min) A) non-restricted blood flow B) restricted blood flow (15-20% lower)	mRNA: A) nonrestricted --- B) restricted ---	- In contrast to hypoxic exercise in rats no further increase in VEGF mRNA levels was observed during exercise with restricted blood flow in humans.	118
	Human	One leg knee extension training (three times per week, 8 weeks) B) basal levels B) response to one acute bout of exercise after training compared to before training	mRNA: A) basal level – B) acute response to exercise—	- Training resulted in a 35% increase in muscle oxygen consumption and an 18% increase in number of capillaries per muscle fiber	132
TGF-β <sub>1</sub>	Rat	Infusion; NP, Ach, PGE <sub>1</sub> , PGE <sub>2</sub> , PGI <sub>2</sub>	mRNA: NP, Ach --- PGI <sub>2</sub> PGE <sub>1</sub> --- PGE <sub>2</sub> ↓	- Decreased TGF-β <sub>1</sub> mRNA levels in response to PGE <sub>1</sub> was the most consistent observation.	144
	Dog	A) Passive hyperperfusion B) Electrical muscle stimulation (50 Hz, 1 contraction/s for 3 min)	mRNA: A) ↑ B) ---	- Passive hyperperfusion increased the TGF-β <sub>1</sub> mRNA levels, but a similar increase in blood flow in combination (and induced by) by electrical muscle stimulation failed to increase the TGF-β <sub>1</sub> mRNA levels.	143
	Rat	Treadmill; A) normoxic (1 h, 15 or 20 m/min, 10° ), B) hypoxic (12% O <sub>2</sub> , 1 h 15 m/min, 10° )	mRNA: A) 15 m/min -- 20 m/min ↑ B) ↑	- Hypoxic exercise no further increase in TGF-β <sub>1</sub> mRNA levels.	116
ESAF	Rat	Electrical muscle stimulation (10 Hz, 8 h/day 7 days)	Activity ↑	- ESAF was inversely related to vascular supply in the control muscle. In response to electrical stimulation a parallel increase was observed in ESAF activity and angiogenesis	122
	Rat	Stretch overload (2 weeks)	Activity ↑	- ESAF increased after 2 weeks in parallel with angiogenesis.	142

VEGF, Vascular endothelial growth factor; FGF-1, Fibroblast growth factor 1; NP, nitroprusside; Ach, acetylcholine; PGE<sub>1</sub>, PGE<sub>2</sub>, PGI<sub>2</sub>, prostaglandins E<sub>1</sub>, E<sub>2</sub>, I<sub>2</sub>; ESAF, endothelial cell-stimulating angiogenic factor; TGF-β<sub>1</sub> transforming growth factor beta 1

showing an augmented increase in exercise-induced angiogenesis with reduced blood flow to the exercising leg in both animals (125-127) and healthy humans (128).

Exercise under hypoxic conditions in rats further increases VEGF mRNA expression when compared to exercise under normoxic conditions. There is no similar induction of FGF-2 or TGF-? mRNA with hypoxia (116). This suggests that local reductions in PO<sub>2</sub> and/or related metabolic alterations could be important for the induction of VEGF mRNA expression in response to exercise and also supports these stimuli as important in the angiogenic response to exercise . In contrast, human studies have failed to show any

further increase in VEGF mRNA expression during one-legged exercise with reduced oxygen delivery, achieved either by flow restriction or hypoxic breathing, compared with exercise under 'normal' conditions (117-118). However, blood flow-restriction or hypoxic breathing induces a smaller further reduction in oxygen tension compared with the reduction occurring during the rest-to-exercise transition exercise under 'normal' conditions. Therefore, it is not possible to exclude an oxygen tension related angiogenic response.

It is known that oxygen regulation of VEGF is due, in part, to hypoxia-inducible factor 1 (HIF-1) activity (75-77). Skeletal muscle oxygen tension during exercise is low enough,

under both normal exercise and exercise with restricted blood flow or hypoxic breathing, to activate HIF-1 $\alpha$  (129). In resting human skeletal muscle, VEGF mRNA levels correlate with those of the two HIF-1 subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ , at the mRNA level (130). Similar correlations between exercise-induced VEGF mRNA expression and mRNA concentrations of the two subunits of HIF-1 are found in skeletal muscle (117). However, oxygen-regulation of HIF-1 seems to be mediated primarily through protein stabilization and translocation of the HIF-1 $\alpha$  subunit from the cytoplasm into the nucleus, (131) and not from events occurring at the level of gene expression. This seems also to hold true for the response to a single session of exercise in human skeletal muscle (H. Ameln, Karolinska Institutet, Stockholm, unpublished data). The finding that HIF-1 is up-regulated in skeletal muscle supports the hypothesis that exercise-induced reduction in oxygen tension in skeletal muscle is one possible stimulus for increases in VEGF gene-expression in response to muscle activity. An interesting additional observation is that exercise-induced lactate levels in skeletal muscle correlate with exercise-induced increases in VEGF mRNA expression (117). Also, the highest VEGF mRNA levels in skeletal muscle are observed during the earliest stages of electrical stimulation in rabbits and rats (16,115). At this point, the muscles also have the highest lactate levels as they attempt to adapt to the sudden increases in contractile work.

Recently, a study reported an attenuated increase in VEGF mRNA to one acute exercise bout in humans, after a eight week of single leg knee-extension training, compared to the increase in VEGF mRNA in response to a single bout of exercise observed before the training program. The training program increased the number of capillaries per fiber and oxygen uptake and it may therefore be hypothesized that the training adaptations including increased local O<sub>2</sub> tension, resulted in a reduced angiogenic response to acute exercise (132). An additional observation, which supports the involvement of low oxygen tension and/or related metabolic alterations in regulation of VEGF, is that the VEGF protein levels increase in parallel with angiogenesis in ischemic rabbit hind limbs (Table 2, 98). Regulation through tissue oxygen tension also provides a theoretical physiological feedback mechanism for angiogenesis, whereby insufficient tissue oxygenation stimulates new vessel formation, which in turn reduces the stimulus for further blood vessel growth or recruitment.

Some researchers have argued that lowered oxygen tension and/or related metabolic alterations cannot serve as a stimulus for angiogenesis in skeletal muscle as there is no augmentation of an angiogenic response during electrical stimulation under restricted blood flow conditions (133). Interestingly, low intensity electrical muscle stimulation during restricted blood flow conditions induces angiogenesis, but high intensity electrical stimulation induces muscle damage without any changes in capillary numbers per fiber (134). This may indicate that severe "metabolic stress" or too low oxygen tension will have deleterious effects, rather than the promotion of favorable adaptive mechanisms. This may be one reason for the observed discrepancy between the studies with voluntary muscle activity and those with electrical muscle stimulation. Another argument against the role of oxygen tension as an angiogenic stimulus in skeletal muscle is that, in contrast to voluntary exercise in humans and animals (135-136), oxygen tension measurements during electrical stimulation indicate that oxygen tension is not significantly reduced, although

there is a significant angiogenic response under these conditions (137).

### 5.3. Angiogenesis and angiogenic factors response in skeletal muscle to changes in blood flow and muscle stretching

Hudlicka *et al.* and others (3,138) have proposed that both exercise-induced increases in blood flow and tissue stretch, through shear stress, wall tension and basement membrane stretching, are important stimuli for angiogenesis in skeletal muscle (47-48,139-142). The importance of blood flow as an angiogenic stimulus is supported by the observation that non-perfused vessels regress during normal development of the cardiovascular system. *In vitro* studies indicate that mechanical factors, such as shear stress and tissue stretching, induce endothelial cell proliferation, changes in cell-cell and cell-matrix interaction and up-regulation and release of angiogenic factors. In fact, shear stress response elements (SSREs) have been found in promoter regions of various growth factor genes, for example TGF- $\beta$  and in FGF-2 (109,138).

However, administration of vasodilatory factors or isolated increases in blood flow do not increase in mRNA expression of VEGF, FGF-2 or TGF- $\beta$  (143). Without any increase in blood flow, administration of NO, prostaglandins (PGE<sub>1</sub>, PGE<sub>2</sub> and PGI<sub>2</sub>), adenosine and acetylcholine, fail to increase the mRNA expression of FGF-2 and TGF- $\beta$  or VEGF mRNA to the levels seen with electrical stimulation or exercise (143); this despite the fact that NO, prostaglandins and adenosine have been shown to stimulate increases in angiogenic factors *in vitro* (80,81,82). Furthermore, increases in blood flow without muscle contraction (passive hyperperfusion) do not change VEGF or FGF-2 gene expression *in vivo* (144). In contrast, electrical muscle stimulation in combination with similar increases in blood flow observed with pharmacological agents, results in increases in VEGF mRNA. Therefore, it appears that for blood flow to have an effect on angiogenic responses, it must be coupled with changes in oxygen tension, local metabolites or increases in contractile activity. It should, however, be noted that VEGF mRNA expression in the two studies cited (143-144) were measured in whole muscles, which includes a mixed population of cell types. It may be that isolated changes in blood flow to exercising muscle only influence the expression of angiogenic factors in endothelial cells. Exercise results in both an increases in both blood flow and in muscle activity. The increase of angiogenic factors expression observed with exercise may therefore occur both in muscle fibers and in endothelial cells.

Although stretching has been observed to up-regulate FGF at both the mRNA and protein levels *in vivo* (145), no increases in FGF mRNA or protein expression are observed in parallel with angiogenesis when FGF-2 is measured during stretch-induced angiogenesis in skeletal muscle (142). In contrast to FGF-2, increased activities of ESAs are seen to occur in parallel with angiogenesis not only after skeletal muscle electrical stimulation (122) but also after skeletal muscle stretching and following increased blood flow (142,146).

Other factors with angiogenic properties may play a role in exercise-induced angiogenesis. Many factors (such as PDGF-BB and integrins) that have been shown to be responsive *in vitro* to stimuli that occur during exercise, such as increased shear stress and muscle stretch, have not, however, been studied in response to muscle activation *in vivo*.

## Exercise induced angiogenic growth factors

**Table 2.** Angiogenesis and angiogenic factors in models of muscle pathophysiology

Factor	Species	Pathology associated stimuli	Response	Comments	Reference
VEGF	Rabbits	Ischemic hindlimb (0, 1, 5, 21 days) A) m. soleus SOL (slow) B) m. tibialis anterior TA (fast)	Protein A/ VEGF 121↑ VEGF 165↑ B/ VEGF 121↑ VEGF 165↑	- In the SOL muscle VEGF 121+165 increased in all time points and was greater than the levels observed in the TA muscle at days 5 and 21. -The initial isoform specific contribution was the VEGF121 in the SOL muscle and in the TA muscle the VEGF 165. The capillary density was increased in both the TA and SOL muscles after 21 days. - The proteins were located in the periphery of the muscle fibers and in non-muscle cells.	98
	Mouse	Ischemic hindlimb (0, 4, 7, 14, 21 35 days) A) APO -/- mice B) Wild type mice	Protein ↑	- In the APO -/- mice, reduced basal level of VEGF protein levels was observed in the skeletal muscle compared to the control mice. Development of the peak VEGF protein levels were both delayed and reduced in the APO -/- mice compared to the control mice.	148
	Rabbit/ Mouse	Ischemic rabbit/mouse hindlimb (0, 7,14,21 days)  A) young animal B) old animal	mRNA Mice: young > old  Protein Mice/Rabbit: young > old	- In the old mice, VEGF mRNA was markedly reduced compared to the young mice and this was especially apparent days 7 and 14 after ligation. - The VEGF protein levels were significantly lowered in old animals, compared to young animal but both increased compared to non-ischemic legs. Immunostaining confirmed the results of Western blot analysis by showing a lower level of VEGF protein in the old mice. - Capillary density in mice and rabbits were both significantly reduced in the old animals compared to the young animals.	152
	Human	One leg knee extension training (three times per week, 8 weeks) in individuals with chronic congestive heart failure NYHA II-III	mRNA ↑  Protein ↑	- Training increased citrate synthase activity as well as one legged VO <sub>2</sub> .	161
FGF-2	Mouse	Ischemic hindlimb (0, 3, 7, 14 days) A) NOD mice B) C 57 mice (control)	mRNA C 57 > NOD  Protein C 57 > NOD	- The VEGF mRNA levels were significantly reduced in the NOD mice compared to C 57 mice, especially at day 7 and 14 after ligation. - The VEGF protein levels were significantly reduced in the NOD mice compared to C 57 mice, from day 3 to day 14 day after ligation. - Immunostaining confirmed the results of Western blot analysis by showing a lower level of VEGF protein in the NOD mice.	147
	Rats	Ischemic hindlimb ( 0, 1, 3 weeks) A) adductor muscle B) calf muscle	Protein ↑	- The FGF-2 protein levels were markedly increased 1 and 3 weeks after ligation in both muscle groups compared to the muscle from the contralateral legs.	97
	Rabbit	Ischemic hindlimb (0, 1, 5, 21 days) A) m. soleus SOL (slow) B) m. tibialis anterior TA (fast)	Protein Sol↑ TA ---	- The FGF-2 protein levels were increased after 21 days but only in the SOL muscle.	98

VEGF,Vascular endothelial growth factor; FGF-2, Fibroblast growth factor 2; SOL=soleus; TA=tibialis anterior; NOD=Non Obese Diabetic

## 6. ANGIOGENESIS AND ANGIOGENIC FACTORS IN MUSCLE PATHOPHYSIOLOGY

### 6.1 Metabolic disorders and diabetes mellitus

As previously stated, there are several disease states in which the capillarity of skeletal muscle is altered. Insulin sensitivity and transport mediate glucose uptake and transport into skeletal muscle across the endothelial surface. In addition, lipoprotein lipase, responsible for the conversion of triglycerides to HDL cholesterol and for uptake of fatty acids into skeletal muscle for energy supply, resides on the capillary wall and is plentiful in skeletal muscle. There is evidence that capillarity in skeletal muscle

is altered in diseases such as diabetes mellitus (26). Retarded neovascularization and reduced levels of VEGF have been observed in non-obese diabetic (NOD) and in hypercholesterolemic mice (147-148). Thus, an understanding of how chronic exercise exposure leads to increases in capillarity may lead to an understanding of how exercise mediates its beneficial effects on health in humans.

### 6.2. Peripheral vascular disease

In peripheral vascular disease, especially of the lower limb, chronic ischemia, due to large vessel occlusions, creates a setting in which capillary growth is



stimulated. Despite an increase in capacity of the capillary bed in these patients, the limitations in blood flow mediated by large vessel disease cause the clinical symptoms and limit the potential for exercise in this clinical population. Exercise training appears to alleviate some of the symptomatology in patients with intermittent claudication (149-150). This is probably mediated in part by angiogenesis and growth of larger capacitance vessels, and by increases in oxidative potential with subsequent increases in efficiency of each contractile element (myofiber) in the exercise-trained limb.

Increased basal VEGF and FGF-2 protein levels have been observed in animal models of lower limb ischemia (97-98). Interestingly, in old animals VEGF expression is reduced in lower limb ischemia compared to young animals. However, VEGF expression is still higher in the ischemic leg compared to the contralateral non-ischemic limb in both young and old animals (151). These findings suggest that not only the oxygen level but also some age-related mechanisms could be involved in the observed increase in VEGF expression in response to ischemia. In contrast, to our knowledge, no changes in angiogenic factor concentrations in response to exercise have been measured in skeletal muscle in patients with peripheral arterial diseases. Nevertheless, many groups are investigating therapeutic angiogenesis with angiogenic factors for the alleviation of pain and exercise intolerance in patients with peripheral vascular disease (54-55,152-153).

Understanding the mechanisms whereby exercise leads to vascular growth and increases in vascular capacitance in peripheral vascular disease can help to identify candidate therapeutic agents that may be used in those individuals who may not be able to obtain the beneficial effects of an exercise training program. Thus, this is an active area of investigation, and the one-legged ischemic training model provides one scientific setting for addressing these questions (128,154). However, some caution must be observed when using experimental models that reduce the oxygen delivery to 'normal' healthy tissues, as it is not clear whether similar mechanisms mediate angiogenesis in pathologic conditions.

### 6.3. Chronic congestive heart failure

Chronic congestive heart failure (CHF) is a clinical setting in which there are several and often severe abnormalities in peripheral skeletal muscle, which mediate exercise intolerance and other morbidities associated with this disease (155). However, exercise training reverses some of these skeletal muscle abnormalities, in parallel with improved exercise capacity and quality of life (156-158). In addition to an abnormality in skeletal muscle fiber type and metabolic (oxidative) potential in skeletal muscle, Duscha *et al.* have recently confirmed that capillary density is depressed in skeletal muscle of heart failure patients compared with matched control subjects (159). Increments in capillary density and mitochondrial volume density are likely to be responsible for improved exercise capacity in this patient group, through increased oxygen uptake, and thereby decreased "metabolic stress" during exercise (156-158). Thus, understanding the mechanisms whereby exercise training results in increases in capillarity in

skeletal muscle in normal subjects will also provide important information and possible clues to useful therapeutic agents for this population.

Preliminary data indicate that a lowered concentration of VEGF protein occurs in CHF compared with age-matched controls (160). The depressed capillary density and lowered VEGF concentration may be related to physical inactivity, but endothelial dysfunction with decreased production of NO could be another contributing factor. However, VEGF mRNA and protein concentrations increase in response to one-legged knee-extensor training program in patients with moderate heart failure, conducted three times a week for eight weeks (161). Thus, VEGF expression seems to parallel the skeletal muscle dysfunction with a depressed basal level but showing increases in response to exercise that are known to reverse skeletal muscle abnormalities. Interestingly, the more severe the exercise limitation in these patients (measured by lower peak  $\dot{V}O_2$ ), the less severe the deficiency in capillarity in skeletal muscle (159). It appears as if activities of daily living provide a more robust exercise stimulus (characterized as percentage of peak  $\dot{V}O_2$ ) in the more severely disabled subjects. It may be that, even if VEGF is lowered compared with age-matched controls, a relative increase in VEGF level occurs in response to increased "metabolic stress" during daily living in the more severely disabled subjects.

## 7. SUMMARY AND PERSPECTIVE

From these studies, it is apparent that up-regulation of angiogenic factors occurs in response to increased muscle activity in skeletal muscle. The major angiogenic factor, VEGF, seems to increase to a greater extent and more consistently than other measured angiogenic factors, such as FGF-2 or TGF- $\beta$ . While the regulatory mechanisms of this response are not clear, the present data suggest that reduced oxygen tension or related metabolic alterations in the skeletal muscle are possible stimuli. Furthermore, VEGF expression and angiogenesis seem to follow a similar time course in response to increases in contractile activity and both precede the change in skeletal muscle oxidative potential in electrical stimulation models. Available data indicate that VEGF mRNA increases in muscle fibers with the subsequent release of VEGF protein into surrounding media. In this scenario, the skeletal muscle fiber is one source for the production of angiogenic factors, and paracrine regulation of angiogenesis is suggested as one important mechanism in skeletal muscle. Data on other angiogenic growth factors are limited, and many factors that are responsive *in vitro* to stimuli that occur during exercise, such as increased shear stress, have not been studied in response to muscle activation or endurance exercise. However, an increase in ESAF activity has been observed in response to different exercise-associated stimuli, such as increased blood flow and muscle stretch. Therefore, reduced oxygen tensions and/or related metabolic alterations, increases in blood flow, muscle stretching and muscle activation may all contribute to exercise-induced angiogenesis, although through different angiogenic factors and mechanisms. These differences could mediate specificity in response to various physiological and pathophysiological stimuli in

skeletal muscle. Understanding these processes is important for the elucidation of mechanisms mediating exercise-responsiveness in skeletal muscle, but also for the potential that such understanding might bring to the treatment and prevention of human diseases such as intermittent claudication.

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## **Exercise induced angiogenic growth factors**

**Abbreviations:** VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; ESAF: endothelial cell-stimulating angiogenic growth factor; Ang: angiopoietin; TGF: transforming growth factor; PDGF: platelet derived growth factor; Ach: acetylcholine; NO: nitric oxide; NP: nitroprusside

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